**M.Sc. Previous Year**

**Zoology, Paper IV**

# POPULATON GENETICS, EVOLUTION AND GAMETE BIOLOGY

मध्यप्रदेश भोज (मुक्त) विश्वविद्यालय – भोपाल

**MADHYA PRADESH BHOJ (OPEN) UNIVERSITY - BHOPAL**

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# SYLLABI-BOOK MAPPING TABLE

## Populaton Genetics, Evolution and Gamete Biology

| Syllabi | Mapping in Book |
|---|---|
| **Unit I Population Genetivcs and Evolution - I**<br>1. Concepts of evolution and theories of organic evolution with an emphasis on Darwinism :<br>2. Neo - Darwinism : A. Hardy Weinberg law of genetic equilibrium. B. A detailed account of destabilizaing forces. (I) Natural Selection (II) Mutation (111) Genetic drift (IV) Migration (V) Meiotic drive.<br>3. Quantifying genetic Variability : A. Genetic Structure of natural populations. B. Phenotypic Variation. C. Models explaining changes in genetic structure of population. D. Factors affecting human disease frequency.<br>4. Molecular Population Genetics: A. Patterrns of changes in nucleotide and amino acid sequences. B. Ecological significance of molecular veriations. C. Emergence of Non-Darwinism Neutral Hypothesis.<br>5 Genetics of quantitative traits : A. Analysis of quantitative traits. B. Quantitative traits and Natural selection. C. Genotype environment interaction. | **Unit-1:** Population Genetics and Evolution - I **(Pages 3-62)** |
| **Unit II Population Genetics and Evolution - II**<br>1. Molecular evolution : A. Gene evolution. B. Evolution of Gene families. C. Assessment of Molecular Variation.<br>2. Origin of higher categories : A. Phylogenetic gradualism and punctuted equilibrium. B. Major trends in the origin of higher categories. C. Micro and Macro - evolution.<br>3. Molecular Phylogenetics : A. How to construct phylogenetic trees? B. Phylogenetic inference - Distance methods, Parsimony methods, Maximum likelihood method. C. Immunological techiques. D. Amino acid sequences and phyligeny. E. Nucleic acid phylogeny· DNA - DNA Hybridizations, Restriction Enzyme Sites, Nucleotide Sequence Comparisons and homologies. F. Molecular Clocks.<br>4. Origin and evolution of economically important microbes and animals.<br>5. Population genetics and ecology : A. Metapopulations. B. Monitoring natural populations. C. Why small populations become extinct? D. Loss of genetic Variations. E. Conservation of genetic resources in diverse taxa. | **Unit-2:** Population Genetics and Evolution - II **(Pages 63-107)** |
| **Unit III Gamete Biology - I**<br>1. Comparative account of differentiation of gonads in a mammal and an invertebrates.<br>2. Spermatogenesis: A. Morphological basis in Rodents. B. Morphological basis in any invertebrate. C. Gamete specific gene expression and genomics.<br>3. Biochemistry of Semen : A. Semen Composition and formation. B. Assessment of sperm functions. C. 'Y' specific probes.<br>4. Fertilization : A Pre fertilization events. B. Biochemistry of fertilization. C. Post-fertilization events.<br>5. Collection and cryopreservetion of gametes and embryos.<br>6. Ovarian follicular growth and differentiation : A Morphology. B. Endocrinology. C. Molecular Biology. D. Ovulation and ovum transport in mammals. | **Unit-3:** Gamete Biology - I **(Pages 109-156)** |

**Unit IV Gamete Biology - II**

1. **Biology of sex determination and sex differentiation a comparative account.**
2. **Multiple Ovulation and embryo transfer technology :** A. In vitro oocyte maturation. B. Superovulation. C. In vitro fertilization.
3. **Transgenic animals and knock - outs :** A. Production. B. Applications. C. Embryonic stems cells.
4. Care and breeding of experimental animals including bioethics.
5. Assisted reproduction technologies : A. Embryo Sexing and cloning. B. Screening for genetic disorders. C. ICSI, GIFT etc. D. Cloning of animals by nuclear transfer.
6. lmmunocontraception: A. Gamete specific antigens. B. Antibody mediated fertilization block and termination of gestation. C. Other contraceptive technologies. D. Surgical methods. E. Hormonal methods. F. Physical barriers. G. IUCD.

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# INTRODUCTION

NOTES

Genetics is the branch of science concerned with genes, heredity, and variation in living organisms. It seeks to understand the process of trait inheritance from parents to offspring, including the molecular structure and function of genes, gene behaviour in the context of a cell or organism, gene distribution, and variation and change in populations. Some traits are part of an organism's physical appearance; such as a person's eye-colour, height or weight. Other sorts of traits are not easily seen and include blood types or resistance to diseases. Some traits are inherited through our genes, so tall and thin people tend to have tall and thin children. Other traits come from interactions between our genes and the environment, so a child might inherit the tendency to be tall, but if they are poorly nourished, they will still be short. The way our genes and environment interact to produce a trait can be complicated.

Genes are made from a long molecule called DNA, which is copied and inherited across generations. DNA is made of simple units that line up in a particular order within this large molecule. The order of these units carries genetic information, similar to how the order of letters on a page carry information. The language used by DNA is called the genetic code, which lets organisms read the information in the genes. The information within a particular gene is not always exactly the same between one organism and another, so different copies of a gene do not always give exactly the same instructions. Each unique form of a single gene is called an allele. As an example, one allele for the gene for hair colour could instruct the body to produce a lot of pigment, producing black hair, while a different allele of the same gene might give garbled instructions that fail to produce any pigment, giving white hair. Mutations are random changes in genes, and can create new alleles. Mutations can also produce new traits, such as when mutations to an allele for black hair produce a new allele for white hair. This appearance of new traits is important in evolution.

A population of organisms evolves when an inherited trait becomes more common or less common over time. For instance, all the mice living on an island would be a single population of mice: some with white fur, some grey. If over generations, white mice became more frequent and grey mice less frequent, then the colour of the fur in this population of mice would be evolving. In terms of genetics, this is called an increase in allele frequency. Alleles become more or less common either by chance in a process called genetic drift, or by natural selection. In natural selection, if an allele makes it more likely for an organism to survive and reproduce, then over time this allele becomes more common. But if an allele is harmful, natural selection makes it less common.

The development of organisms also involves Gametes, which are an organism's reproductive cells. They are also referred to as sex cells. One of the gametes is frequently bigger and non-motile, as is customary. It's also called a female gamete, ovum, or egg cell. The other gamete cell is smaller in size and motile. It's also known as a sperm cell or a male gamete. Each human gamete has

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23 chromosomes, and when they fuse, a diploid zygote with 46 chromosomes is formed. These reproductive cells are formed in male and female gonads, or reproductive organs, in mammals. Male gametes are pollen in seed-bearing plants, whereas female gametes are encased in the plant's ovules. In plants, however, the gamete may or may not be a haploid cell.

This book, *Population Genetics, Evolution and Gamete Biology*, has been designed keeping in mind the Self-Instruction Mode (SIM) format and follows a simple pattern, wherein each unit of the book begins with the Introduction followed by the Objectives for the topic. The content is then presented in a simple and easy-to-understand manner, and is interspersed with Check Your Progress questions to reinforce the student's understanding of the topic. A list of Self-Assessment Questions and Exercises is also provided at the end of each unit. The Summary and Key Terms further act as useful tools for students and are meant for effective recapitulation of the text.

# UNIT 1 POPULATION GENETICS AND EVOLUTION - I

NOTES

**Structure**



## 1.0 INTRODUCTION

The origin of life on Earth is a set of paradoxes. In order for life to have gotten started, there must have been a genetic molecule—something like DNA or RNA—capable of passing along blueprints for making proteins, the workhorse molecules of life. But modern cells cannot copy DNA and RNA without the help of proteins themselves. To make matters more vexing, none of these molecules can do their jobs without fatty lipids, which provide the membranes that cells need to hold their contents inside. And in yet another chicken-and-egg complication, protein-based enzymes (encoded by genetic molecules) are needed to synthesize lipids. The origin of life on Earth is a scientific problem which is not yet solved. There are plenty of ideas, but few clear facts. It is generally agreed that all life today evolved by common descent from a single primitive life form. It is not known how this early form came about, but scientists think it was a natural process which

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took place perhaps 3,900 million years ago. This is in accord with the philosophy of naturalism: only natural causes are admitted. It is not known whether metabolism came first or genetics. The main hypothesis which supports genetics first is the RNA world hypothesis, and the one which supports metabolism first is the protein world hypothesis. Another big problem is how cells develop. All existing forms of life are built out of cells. Many religions teach that life did not evolve spontaneously, but was deliberately created by a god. Such theories are a part of creationism, which has 'Old Earth' and 'Young Earth' versions. Because of lack of evidence for such views, almost all scientists do not accept them. In this unit, we will discuss the concepts of evolution and theories of organic evolution, along with the Darwinism and Neo-Darwinism. It will also focus on the concepts of quantifying genetic variability, molecular population genetics, and genetics of quantitative traits.

## 1.1 OBJECTIVES

After going through this unit, you will be able to:

- Describe the concepts of evolution and theories of organic evolution
- Explain the Darwinism and Neo-Darwinism
- Discuss the concepts of quantifying genetic variability, molecular population genetics, and genetics of quantitative traits

## 1.2 CONCEPTS OF EVOLUTION AND THEORIES OF ORGANIC EVOLUTION

The origin of life on Earth is a set of paradoxes. In order for life to have gotten started, there must have been a genetic molecule—something like DNA or RNA—capable of passing along blueprints for making proteins, the workhorse molecules of life. But modern cells cannot copy DNA and RNA without the help of proteins themselves. To make matters more vexing, none of these molecules can do their jobs without fatty lipids, which provide the membranes that cells need to hold their contents inside. And in yet another chicken-and-egg complication, protein-based enzymes (encoded by genetic molecules) are needed to synthesize lipids.

The origin of life on Earth is a scientific problem which is not yet solved. There are plenty of ideas, but few clear facts. It is generally agreed that all life today evolved by common descent from a single primitive life form. It is not known how this early form came about, but scientists think it was a natural process which took place perhaps 3,900 million years ago. This is in accord with the philosophy of naturalism: only natural causes are admitted.

It is not known whether metabolism or genetics came first. The main hypothesis which supports genetics first is the RNA world hypothesis, and the one which supports metabolism first is the protein world hypothesis. Another big problem is how cells develop. All existing forms of life are built out of cells.

Melvin Calvin, recipient of the Nobel Prize in Chemistry, wrote a book on the subject, and so did Alexander Oparin. What links most of the early work on the origin of life is the idea that before life began there must have been a process of chemical change. Another question which has been discussed by J.D. Bernal and others is the origin of the cell membrane. By concentrating the chemicals in one place, the cell membrane performs a vital function.

Many religions teach that life did not evolve spontaneously, but was deliberately created by a god. Such theories are a part of creationism, which has 'Old Earth' and 'Young Earth' versions. Because of lack of evidence for such views, almost all scientists do not accept them.

## Origin of Life

How life originated on Earth has been the most complex and confusing question for all the evolutionary biologists of modern as well as old times. Molecular and chemical evolution from atoms and molecules to simple and then complex elements capable of replication and from these to even more complex forms leads to the origin of macromolecules like nucleic acids (DNA or RNA) and proteins followed by origin of life. Several theories and interpretations have been put forward explaining the origin of life. However, most of them lacking evolutionary evidences and significance are not accepted. Some of the important theories and hypothesis on origin of life are discussed in detail. Next important question in evolution is how the life that originated on Earth billions of years ago changed and evolved into variety of species and forms in time. There are several theories like theory of use and disuse of organs proposed by Lamarck, Theory of natural selection by Darwin and Neo-Darwinism also known as modern evolutionary theory. Concept of Neo-Darwinism explains the formation of new species by various factors like genetic variations, mutations, isolation of species, genetic drift and natural selection. Natural selection operates on large population by selecting beneficial alleles and fixing it in contrast to genetic drift which acts on small population and may or may not select advantageous allele.

Life can be defined as the ability of an organism to grow, reproduce and produce energy through chemical reactions by using natural resources like air, water, sunlight, etc. Evidences suggest that Earth came into being around 4.5-5 billion years ago. Initially Earth had only two components: solid mass (Lithosphere) and the surrounding gaseous envelope (Atmosphere). Gradually, the temperature of primitive Earth cooled down and when it reach below 1000ºC, liquid components known as hydrosphere started appearing.

The formed Earth consists of three different parts:

- **Bryosphere:** It is the central core of the Earth filled with molten magma having large amount of Nickel and Iron. It consists of two zones: Inner Core region (~800 miles radius) and Outer Core region (~1400 miles radius).
- **Pyrosphere:** It forms the middle part of the Earth, also called as Mantle. It is around 1800 miles in thickness and mainly consists of Manganese, Silica, and Magnesium.

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- **Lithosphere:** It is the outermost region of the Earth, also called as Crust. It is around 20-25 miles in thickness and mostly filled with Aluminum and Silica.

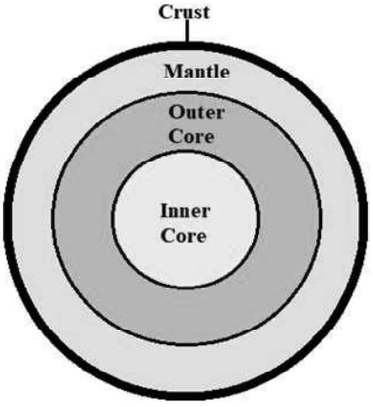


***Fig. 1.1*** *Layers of Earth: Crust, Mantle, Outer Core and Inner Core*

There were multiple conditions on Earth which might have help to originate and sustain the life on Earth:

- **Primitive Earth Had Very Little Or No Oxygen:** During that time Earth had a reducing environment because of predominance of Hydrogen and Hydrogen compounds with water (such as, Methane and Ammonia). Besides, these gases remain within the atmosphere due to gravitational forces. It is believed that the reducing environment of primitive Earth help proper interaction of inorganic substances which further leads to synthesis of organic compounds. These organic compounds are the raw materials for the formation or origination of life of Earth.
- **Energy Source:** The energy source on primitive Earth came from different sources, such as solar radiation, electric discharges, volcanic eruptions, heat, cosmic rays, and radioactive decays.
- **Long Time:** It has been estimated that it took almost around 1 billion year for the appearance of life on Earth after the formation. Such a long time is required for the chemical reactions to occur without any help of enzyme.

### 1.2.1 Theories Related to Origin of Life on Earth

Six major theories have been proposed to explain the origin of life on Earth:

1. **Theory of Special Creation:** This theory proposed that life on Earth is created by a supernatural power or vital spirit known as the 'God' that must be accepted on faith but cannot be studied scientifically. Most of the religions have similar beliefs and accepted this theory in one or the other way. According to the Christianity, god has created the universe first, then planet and followed by creation of animals, plants and human in six natural days. It was believed that on third day plants were created and on fifth day, fowl and fishes were created followed by animals and humans on sixth day (first man, Adam and then woman from clay with a soul). According to theory of special creation:

- All living organisms were created on same day, i.e., no difference in their appearance.
- They have been created in the present form which means no evolution have happened.
- Their bodies and organs were fully developed and had not undergone any adaptation to fulfil the requirement to run the life.

The objections to the theory of special creation include:

- This theory is purely a religious belief.
- No experimental and scientifically sound evidences to support these assumptions.
- Fossil evidences proved that living organism appear on Earth at different time frames and Earth was not inhabited by same animals and plants as we know now.

2. **Theory of Spontaneous Generation:** This theory is also known as Abiogenesis which assumes that non-living material give rise to life in a spontaneous manner. This concept of origin of life spontaneously from inanimate material was very common among people because of the observation of infected food with worms, flies and fungi whose life cycles and history were not known earlier. Several observations support this theory in following ways:
   - Development of horsehair worm, *Gordius* on horse tail hairs when dipped in water.
   - Appearance of fly larvae on rotten meat after few days.
   - Van Helmont claimed that he can produce mice from the dirty shirt and some wheat grains kept in dark cupboard within 3 weeks.

### Evidences against the Theory of Spontaneous Generation

This theory was criticized by Louis Pasteur, Lazzaro Spallanzani and Francisco Redi. These scientists have performed many laboratory experiments to disprove the theory of spontaneous generation:

**Redi's Experiment:** Francisco Redi did conclusive and well-designed experiment (Refer Figure 1.2) to disapprove the theory of spontaneous generation. He was the first person to provide a firm experimental proof for refutation spontaneous generation in his paper '*Esperienze intorno alla generazione deglinetti*'. He placed Meat or Fish (Eel) in 3 separate jars. First jar was left open while second and third jar were covered with gauze and the paper, respectively. The meat or fish started to decay in all the three jars and common flies started coming to the jars and laid eggs. In the first jar which was left open, flies entered and laid eggs which eventually hatched into maggots. While in the second and third jar, flies could not reach inside the jar, so no eggs were laid and no maggots were seen inside the jar. But flies laid eggs on gauze and paper and flies appeared there. With this experiment he concluded that maggots were not generated spontaneously but they arose from pre-existing flies. This simple experiment proved that organisms

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arise from the pre-existing organism only and not from non-living matter. Figure 1.2 illustrates the Redi's experiment to disprove the theory of Spontaneous Generation using Meat/Fish in three different jars and observation of emergence of Maggots.

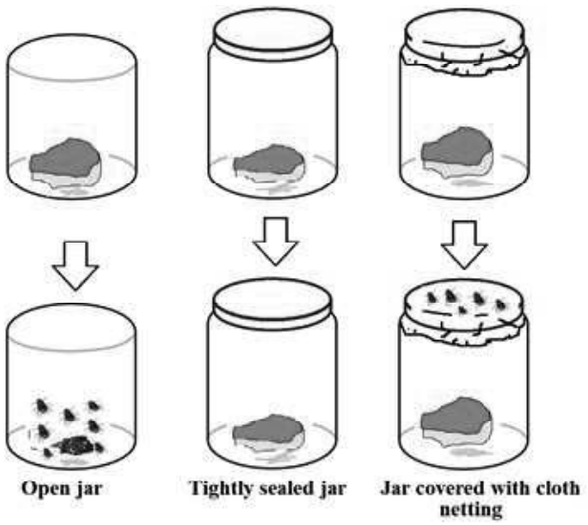


***Fig. 1.2*** *Redi's Experiment*

**Spallanzani's experiment:** After the discovery of microorganisms by Leeuwenhoek, it was thought that microbes arose spontaneously. Lazzaro Spallanzani tested spontaneous generation of microbes. In his experiment, he prepared flasks of vegetable or animal broth by boiling animal or vegetable samples for several hours and kept them open or sealed immediately. The broths remained free from microorganisms growing inside when sealed immediately. But, he observed the growth of microorganisms in broth when left open or when exposing the sealed broth. Therefore, he concluded that boiling at high temperature killed all microorganisms and in the absence of air, new microbes cannot be formed (Refer Figure 1.3). Other flask that was left open was in contact with the air and the spores of microbes which lead to the growth of microorganisms. However, his results were not accepted by few scientists as they believed that heating must have killed some 'vital force' which was required for spontaneous generation.

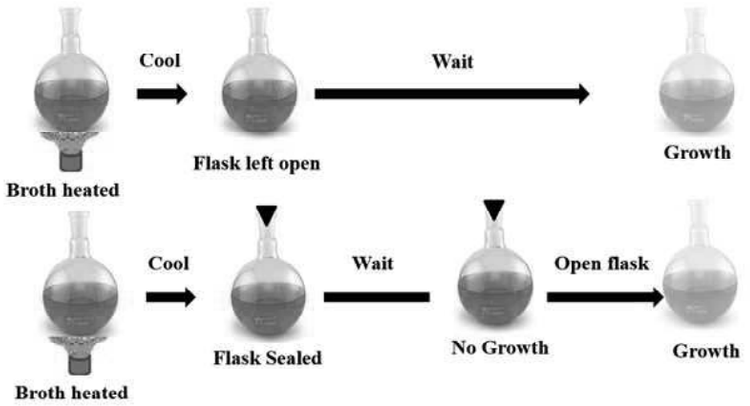


***Fig. 1.3*** *Spallanzani's Experiment to Challenge Theory of Spontaneous Generation of Microbes*

**Louis Pasteur Experiment:** Pasteur had performed an experiment in a swan-neck flask like an 'S' shaped tube. He had taken hay infusion/meat broth in the flask and boiled them for several hours. Then the flask was left unsealed for cooling, the steam condensed into the lower part of the S shaped tube which blocked the entry of microorganisms. No growth of microorganisms in the flask was seen till several days. But, there was appearance of microorganisms in the neck of the tube (bend) but the liquid broth remained sterile. But when the same flask was tilted so that the liquid comes in contact with the microorganisms, the growth of microorganisms in the flask started. Therefore, he disproved the theory of spontaneous generation (Refer Figure 1.4). His Experiment finally discard the theory of abiogenesis even for the microbes. He then established the theory of biogenesis.

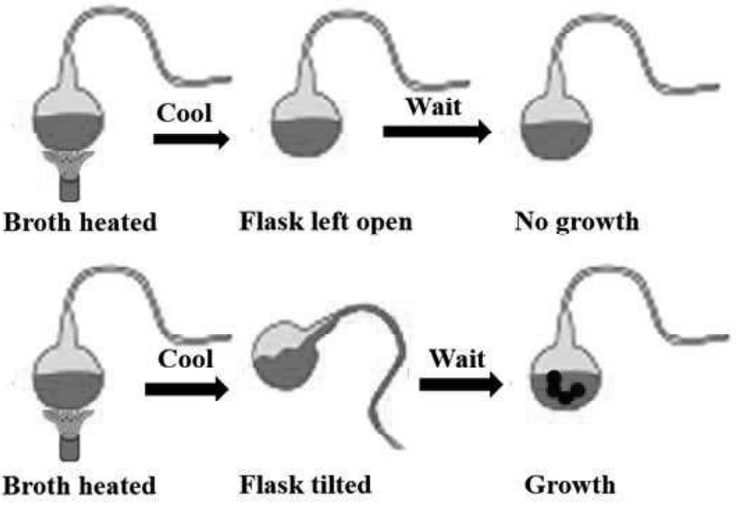


***Fig. 1.4*** *Louis Pasteur Experiment to Provide First Experiment Evidence for Refutation of Theory of Spontaneous Generation of Microbes*

3. **Theory of Catastrophism:** This is the extension of the theory of special creation. This theory assumes that life is originated because of special creation and it was followed by catastrophe due to geographical disturbances which killed the created life forms. Each catastrophe completely wiped out the previous life forms whereas each creation forms new life different from the previous ones. Therefore, each round of catastrophe/creation is responsible for existence of different types of organisms on Earth at different time frames. There was no scientific experiment to support the hypothesis and it was mostly based on imaginary concepts.
4. **Cosmozoic Theory:** This theory was given by Richter and strongly supported by Arrhenius. It proposed that life was present in the form of resistant spores in space throughout the universe and reached Earth accidently. Since Earth is the only planet which have conditions supporting the life, these spores grew, propagated and evolved into different organisms and existing life forms. This theory is also called as 'Theory of Panspermia or Spore Theory'. Initially, this theory was supported by the fact that fossils of microorganisms were found in meteorites in 1961. But no mechanism is

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known about the transfer and survival of these spores from space. Besides, the absence of life forms on any planet except Earth, no detail information about the origin and mechanism of transfer of spores across interplanetary space and reaching Earth and this theory is not so helpful in knowing the fundamental details about origin of life, the hypothesis did not receive much attention.

5. **Theory of Eternity of Life:** This theory proposed that life had neither beginning nor end. It assumes that life has ever been in existence and it will continue to be so in future as well. Therefore, it further believed that there is no question of 'Origin of Life' as it has no beginning or end. This theory is also called as 'Steady State Theory'. The main criticism comes from the evidences that initially Earth was formed and life appeared later on. And the question is– 'Where lives existed before the formation of Earth?'
6. **Modern Theory:** This theory is also known as 'Chemical Theory' or 'Theory of Primary Abiogenesis'. The modern theory proposed that non-living materials can give rise to life in primitive Earth conditions known as abiogenesis. The primitive Earth condition was totally different from the present conditions which do not allow abiogenesis. Later on, the concept of chemical theory was introduced by two famous scientists, A. I. Oparin and J. B. S Haldane. It consists of the following assumptions:
   - Spontaneous generation of life is not possible at the present environment.
   - Earth's atmosphere ~1 billion years have huge difference from the current conditions.
   - Primitive Earth's condition was reducing in nature.
   - Under these reducing conditions, the chemical inorganic molecules react with each other through a series of chain reactions and leads to formation of organic and other complex biomolecules.
   - The solar energy, heat and UV radiations act as sources of energy for the chemical reactions.

**Experimental Evidences in Support of Chemical Theory of Origin of Life**

The hypothesis proposed by Haldane was conclusively supported by the experiments of Miller and Urey where they mimicked the environment of primitive Earth. The experimental setup consists of a glass flask, a condenser, and a liquid flask interconnected with each other by the help of tubes and finally a source of electric spark is there to supply energy (Refer Figure 1.5). They introduced a mixture of Methane (CH4), Ammonia (NH3), and Hydrogen (H2) in the ratio of 2:2:1 and Water (H2O) vapour at 8000ºC into the set up. They allowed the whole contents to continuously circulate inside this closed glass apparatus for 18 days. During that time, they have also provided energy in the form of electric spark through two electrodes by providing around 75000 volts electricity. This electric sparks mimics lightening during primitive Earth atmosphere. The mixture was finally collected through the stop cock and analyzed chromatographically and also by calorimetric methods. The analysis indicates the presence of Amino Acids like

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Aspartic Acid, Alanine and Glycine; Nitrogen Bases like Adenine and simple Sugar Ribose. In addition, Hydrogen Cynide (HCN), Formaldehyde (HCHO) and other active intermediate compounds, such as Acetylene and Cyanoacetylene were also observed.

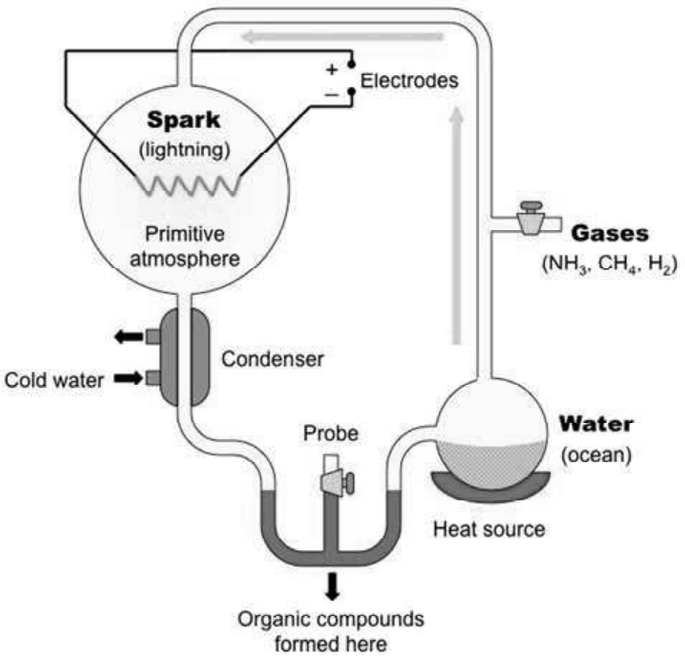


***Fig. 1.5*** *Experimental Set Up of Miller and Urey Mimicking Primitive Earth Environment*

The chemical reactions which might explain the formation of these compounds are as follows:

**Formation of HCN, HCHO, O and H2**

$CO_2 \rightarrow CO + [O]$ (Atomic Oxygen)

$CH_4 + 2[O] \rightarrow CH_2O + H_2O$

$CO + NH_3 \rightarrow HCN + H_2O$

$CH_4 + NH_3 \rightarrow HCN + 3H_2$

**Formation of Glycine:** The Formaldehyde, Ammonia, and HCN then react to form Glycine.

$$CH_2O + HCN + NH_3 \rightarrow NH_2\text{-}CH_2\text{-}CN + H_2O$$

$$NH_2\text{-}CH_2\text{-}CN + 2H_2O \rightarrow NH_3 + NH_2\text{-}CH_2\text{-}COOH$$

## 1.2.2 Proposed Steps for the Origin of Life

According to the chemical theory of origin of life, a series of chemical synthesis give rise to life. As per the hypothesis, origin of life has following four major steps:

1. **Formation of Inorganic Molecules:** Initially, the high temperature of primitive Earth did not allow the condensation of atoms to form inorganic

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molecules. As temperature cools down, condensation of different atoms give rise to simpler inorganic molecules. Hydrogen, Nitrogen, Oxygen and Carbon were the elements which was most abundant on the primitive Earth. These molecules react with each other and give rise to Methane, Ammonia, Carbon Dioxide and Water Vapour in presence of different sources of energy, such as Sunlight, Lighting, Volcanic Eruptions, etc.

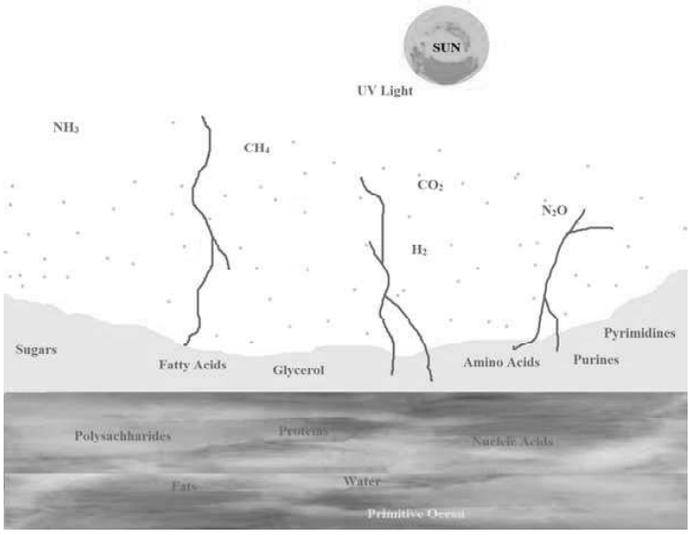


***Fig. 1.6*** *Condensation of Individuals Atoms to Form Simpler Inorganic Molecules, which Later form Complex Macromolecules of Life*

2. **Spontaneous Formation of Monomeric Organic Compounds:** Those inorganic molecules interact with each other to form simple monomeric organic compounds, such as Sugar, Fatty Acids, Glycerol, Amino Acids and Organic Bases (Purine/Pyrimidine). The reactions among the inorganic molecules to form simple organic molecules occur in reducing marine environment. The inorganic molecules were condensed along with rain as temperature of Earth cool down. Hence, both inorganic compounds and simple organic compounds were present in the primordial ocean.
3. **Spontaneous Formation of Complex Organic Compounds:** The simpler organic compounds again react with each other and form complex organic compounds, such as, Polypeptides, large sugar molecules from simple sugar, fat from combination of fatty acid and glycerol in presence of Sun as source of energy.
4. **Spontaneous Formation of Molecular Aggregates:** Large organic molecules aggregate spontaneously with each other and form large colloidal aggregates called as 'Coacervates'. Around the protein molecules in coacervates, a layer of water molecules form the covering. The membrane surrounding the molecules protects the molecule and also enhances the chemical reactions by bringing a high local concentration. These colloidal aggregates absorb protein and other molecules from the ocean and help in growing the coacervate as well as their internal complexity which results into division of coacervate into multiple small ones. These coacervates are

the preliminary organisms present in the ocean that leads to the formation of primary cells. The process of primary cell formation includes two steps.

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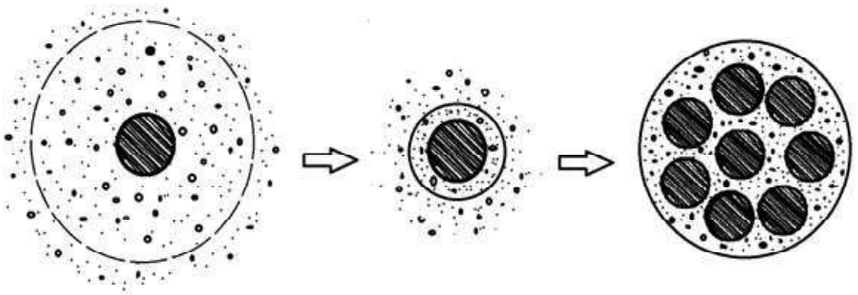

***Fig. 1.7*** *Formation of Coacervates by Removal of Water Molecules (shown as Dots) from Colloidal Particles*

5. **Formation of Eobionts or Protocells:** The coacervates have the capability to take up new molecules from surrounding to replenish the degraded molecules and maintain their internal size. Thus, coacervates have the basic property for a living system but it does not have complex molecules like enzymes, so the machinery behind acquiring new molecules was not regulated. Later on, nucleic acid becomes trapped inside the coacervates and thus, division process became precise. This form of coacervates having nucleic acid is known as Eobionts or Protocells.
6. **Formation of First Cells:** Presence of protein molecules and enzymes results into enhancement in the synthesis of several biomolecules like RNA and DNA inside eobionts. These biomolecules have taken over the task of protein synthesis. Following that the interaction of proteins with lipids allowed the formation of bio membrane which help primitive cell to improve its selectivity while taking up or excluding materials to surrounding. Further, mutation in DNA and selection of fast-growing cells by natural selection give rise to the origin of first primordial cell. The first cellular form appeared on Earth approximately 2000 Million Years Ago (MYA).

Thus, life was originated as primitive single cell which gradually lead to formation of multicellular organisms having well developed tissue and organ systems. Besides, individual organisms also acquire characters during their lifetime to better adapt themselves towards changing environmental conditions. This process of progressive advancement in the body organization of organisms is known as evolution. Therefore, the term evolution means change from one form to another. In order to understand the process of evolution, certain theories, such as Lamarckism, Darwinism, Neo-Darwinism, etc., have been put forward. Of all the theories of evolution, Neo-Darwinism is considered as the most relevant theory which is widely accepted.

## 1.2.3 Darwinism

Charles Darwin was born (12 February, 1809) in England sailed on H.M.S. Beagle for world exploration and visited some of islands of Atlantic ocean, coast of South

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America and some islands of South Pacific. During his voyage he noted the flora and fauna of many islands and collected numerous living and fossil specimens. Beagle also later sailed to the Galapagos Islands on the west coast of America. He observed great variations among the organisms that lived on these islands and exhibited radiations and modifications in form. He found giant tortoises distinctly different on each island. The common birds, finches were markedly different from the finches of main land. A.R. Wallace and C. Darwin both read Malthus's views on population, which state that population increases in a geometrical ratio, whereas food in arithmetical ratio. In 1859, these views were incorporated in the book, Origin of Species by Natural Selection or the preservation of favoured races in the struggle for life. The great scientist C. Darwin was died on 19 April, 1882.

The idea of natural selection is very simple, though its operation is highly complex and may be extremely subtle. Darwinism explains how evolution might have occurred in nature. Darwinism is based on three facts of nature can be summarized as follows:

- Organisms multiply in geometric ratio (Over-Production).
- Over production and number of survivors remains roughly constant (Struggle for existence).
- Struggle for existence and variations and heredity (Survival of the fittest and Natural Selection).
- Survival of the fittest and continued changes or adaptations in the organisms (Origin of new species).

### Over-Production (Prodigality or Fecundity)

According to Darwin, the driving force is provided by the tendency of living beings to increase their numbers rapidly. For examples, Fishes are noted for laying large numbers of eggs about 1,700,000 eggs. The similar prodigality of the salmon in egg production is common. One female toad may lay as many as 12,000 eggs. It has been calculated that one pair of houseflies breeding in April would have by August, if all eggs hatched and all resulting individuals lived to reproduce in their turn, 1.9x1020 descendants. According to Darwin the elephant is the slowest breeder of all known animals and have longer intervals between generations. It begins breeding when thirty years old, and goes on breeding till ninety years old, producing six offspring's in the interval and each surviving till one hundred years old. If this be so, after a period of from 740-750 years there would be nineteen million elephants alive descended from the first pair. Why, in fact, do we not find our lakes choked so with fish, our fields carpeted with toads, the earth overrun with elephants and so on? Because, for each species, there are certain checks or limiting factors, that opposes such an increase in number. The most important of these checks are limited food supply, predatory animals, diseases, space restrictions and inanimate environment.

### Struggle for Existence

It has been noticed that in each generation each species attempts to produce many more individuals that can hope to live to maturity under the prevailing limited factors.

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The result is a competition among the offspring for food, mates, and home territories and strive to survive the aggressions of predatory animals, disease, and the severities of inanimate nature. Darwin called this process 'the struggle for existence'. In this struggle those will succeed that have favourable or advantageous inheritable variations of structure, physiology and so on. Those individuals will fail that have no such variations or that have unfavourable or harmful variations. It is true that non inheritable and favourable variations will not make an individual to survive. Therefore such variations have no 'future' as regards improvement of the species concerned. The inheritable variations arise as new mutations and as new combination of genes originating in various ways. Darwin himself placed great stress on the importance of variations, including individual differences, and he recognized that they must be inheritable for the usefulness in evolution. He also knew that variation is universal, that 'No two individuals are alike'.

### Success in the Struggle for Existence

Darwin laid more stress upon survival. Individuals having favorable inheritable variations survive, while their less highly endowed individuals die. This phenomenon has been termed '**Survival of the Fittest'**. The 'fittest were thought of as those individuals that possess inheritable characteristics enabling them to succeed in the 'struggle for existence' in the particular circumstances and environment in which they live. Since they are the survivors, the fittest then become the parents of the next generation, whose members inherit the favorable characteristics from their parents. The most successful individuals or groups that contribute their genes in greatest number to the generation. So far as contribution to evolution is concerned, a living animal that does not reproduce is supposed to be dead, because he consumes food without making any contribution to the species in return. But individuals that live together in societies and do not reproduce, but provide essential services to the society of which they are a part, they also contribute their services to the next generation. Thus, be it on the individual or on the social level, success in the 'struggle for existence' means success in contributing to the next generation. Its examples are social insects as ants and bees.

### Nature of the Struggle for Existence

So far we have stressed competition between individuals in the same species for food, territory, etc., this is intraspecific competition. Interspecific competition also occurs and at times it is important evolution. Two closely related species (recently arisen from a common ancestral species, perhaps) may compete for the same food supply. If this competition is keen it may lead to changes in the two species so that competition will be lessened. Thus, two species of ground finch living on the same Galapagos Island may come to differ from each other in beak size, one specializes to feed on large seeds and the other on small seeds. In this case, one species may be so much more efficient than other in utilizing food supply that the less efficient species becomes extinct, at least in the territory originally shared by both.

The resistance to disease is highly important; ability to produce large numbers of viable offspring's is advantageous particularly in those species in which parents

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do not care for the young after hatching or birth; young ones that develop quickly have an advantage over those that slowly. When two competing strains differ in speed of that strain which produces mature offspring in less time will contribute more of its genes to further generations than that in which sexual maturity attained more slowly. Thus natural selection results from the cumulative action of all forces tending to ensure that individuals possessing one genetic constitution shall leave larger number of offspring than those individuals possessing some other constitution.

### Variation under Nature

The fact that no two organisms or parts of the organisms are exactly alike, no matter how closely related, is a commonly observed phenomenon. These differences are called variations. It is the basic prerequisite and progressive factor for evolution, because without variations, no change could occur and evolution would be impossible. But all the variations are not significant from evolutionary point of view. Some of them are changes occurred temporarily in the soma of the organisms and are not inherited to the offspring's. Only those variations which can be inherited can take part in the evolution of species. These variations are called heritable variations. Changes occurred in the genes, or the chromosomes of the germplasm are the only heritable variations. Some of the visible variation results from environment influences, particularly diet, and this play no direct part in the evolutionary process. Darwin observed that the various useful variations are selected by individuals and thus, evolution results. Darwin assumed variations as axiomatic without describing their real nature and origin, in plant and animals.

### Survival of the Fittest

Due to these various struggles for existence and useful heritable variations, only those individuals survive, which are best fitted to new conditions of life and the least fit are the first to perish. The well adapted individuals reach reproductive age and hand over their favourable characteristics to their offspring's, whereas less well-adapted individuals fail to do so. Nature select those individuals, which are sufficiently well-adapted and allows them to survive, and rejects those that are poorly adapted. The later usually perish before they reach sexual maturity. Darwin coined the term natural selection to describe this weeding out process. As environmental conditions are constantly changing, natural selection is forever favouring the emergence of new forms. The survival of the fittest is the result of natural selection which enforces adaptations

### Origin of Species

One mutation in a population causes usually a small change. Addition of one mutation to another probably accounts for many of the larger differences distinguishing separate species, genera and so on. In later generations a second mutation arises that is an improvement on, or addition to the first one. Natural selection now works on this second mutation until some generations later the whole population comes to possess it. Thus, step by step through the long expanses of geologic time greater and greater evolutionary change is produced by natural selection. The change as we have described will be in the nature of more perfect adaptation to

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the environment in which the animals are living, i.e., post-adaptation. (More and more perfect adaptation to a stable environment in which species is already living. This type of adaptation is called post-adaptation, since species has already entered the environment, and additional adaptation only perfects the animal for living under the prevailing conditions).

Suppose that the environment in which our hypothetical population live changes. This might result from geologic change in the inhabited region from climatic change, from biotic change or from that a portion of the population migrates into different region from the former region. As a result different mutations will prove advantageous in the struggle for existence, and the result will be that the population or a portion of it, gradually differ from the ancestral population. Eventually a descendent population may come to differ so much from the ancestral one that a biologist concludes that the two should be regarded as separate species. An ancestral population may give rise to two or more descendant populations. If these 'sister' populations become adapted to different environments they may in the course of time become sufficiently different from each other as to be considered separate races. If change continues they eventually become separate species

**Criticism (Objections) to Darwinism**

Some of the objections to the theory of natural selection, which Darwin explained vaguely, are the following:

- If species have descended as a result of gradations, there should be innumerable transitional stages and the species should not be as well-defined as we see them to be. Darwin's theory stresses upon small fluctuating variations, which are to a large extent non-heritable and can play no part in the evolution.
- How can natural selection bring about characters of no use like the tail of Giraffe of trifling importance? It does not explain the effect of use and disuse and presence of vestigial organs.
- It could not explain whether the instincts are acquired and modified through natural selection or not.
- He did not differentiate between somatic and germinal variations and considered all variations as heritable.
- How some species, when crossed, produce sterile offspring's, whereas when varieties are crossed they produce fertile offspring's?
- Darwin described the survival of the fittest, but not the arrival of the fittest.
- Over-specialization (some organs began to develop enormously in relation ton to size of body causing harm to individuals). For example, antlers of extinct Irish deer cause its extinction.
- Degeneration of organs.
- Darwin proposed artificial selection for improving races of domestic plants and animals, but these could never lead to definite or permanent specific variations.

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- Natural selection does not explain the evolution of land animals from aquatic ones.
- It appears somewhat absurd that variations tending in an infinitesimal degree should be preserved.
- Darwin's sexual theory involves passively on the part of male and an active choice on the part of female for a more beautiful, attractive and more powerful male. This theory was most criticized.

**Check Your Progress**

1. How can life be defined?
2. What were the components of Earth when it was found initially?
3. Name the components of Earth.
4. What are the factors on which Darwin's theory of natural selection based?

## 1.3 NEO - DARWINISM

Neo-Darwinism, also called the modern evolutionary synthesis, was introduced by Julian Huxley to generally designate the integration of Charles Darwin's theory of evolution by natural selection, Gregor Mendel's theory of genetics as the basis for biological inheritance, and mathematical population genetics. This theory generally approved important conclusion that the gradual process of evolution is because of genetic changes due to 'mutations', 'recombination' and 'genetic variations' which are further operated upon by natural selection who determines the evolutionary pathways.

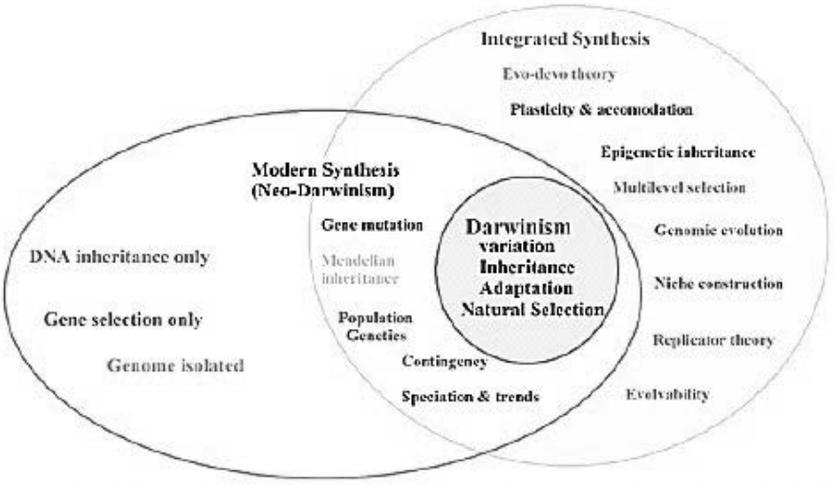


***Fig. 1.8*** *Various Factors and Interlinks between Darwinism and Neo-Darwinism*

Darwin's theory of natural selection was mostly accepted and strongly supported by Wallace, Huxley, Haeckel, and Weismann. Darwin's theory does not have any input of modern concepts of genetics and the whole machinery of how characters

appear and linger on in a population. Several experimental evidences turn out in favour of Darwinism. Thus, the theory was ultimately modified in the light of recent researches and advancements and generally called as Neo-Darwinism. The Synthetic theory is the merger of Darwinism and concepts of genetics, population dynamics, statistics, and heredity. This theory of evolution has been constantly improving since the beginning of 20th century by contributions from many scientists like R. A. Fischer, J. B. S. Haldane, Ernst Mayr, Julian Huxley, and G. G. Simpson who had worked on population dynamics. Besides, T. Dobzhansky, H. J. Muller, and H. De Vries, G. L. Stebbins further provides more information on genetics and mutation. G. H. Hardy, W. Weinberg, and Sewall Wright also did extensive study on population genetics and statistics, who further helped to clarify the machinery of heredity inheritance.

## 1.3.1 Hardy-Weinberg Law of Genetic Equilibrium

A population is said to be in genetic equilibrium when the allelic frequency in the gene pool remains constant through generations. In order to follow this, the population should have the following characteristics:

- The size of the population should be very large.
- There should be random mating among the individuals of the population.
- There should not be any migration.
- Any specific genotype should not be naturally selected.
- The males and females of the population should have the same allelic frequency.
- There should not be any mutation.

In the year 1908, G.H. Hardy from England and W. Weinberg from Germany, independently came up with a quantitative theory to define the genetic structure. The Hardy-Weinberg law basically explains the effect of reproduction on genotypic /allelic frequencies. The work remained unnoticed till Stern in the year 1943, recognized both papers and used their population formula.

According to Hardy-Weinberg Law: 'The allelic frequencies in a randomly mating large sized population remain constant from generation to generation if no evolutionary processes such as mutation, migration, natural selection, and genetic drift operates'.

### Population Genotypes

The Hardy-Weinberg law can be applied to individuals genes with two alleles, i.e., one dominant (A) and another recessive (a) allele. A population with two alleles will have three resulting genotypes AA, Aa, and aa. The frequency of each genotype will be represented by the number of individuals in a population with that genotype divided by the total number of individuals in the population (Refer Table 1.1).

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***Table 1.1*** *Genotype and Genotypic Frequency in a Population of 1000 Individuals*

| Genotypes | No. of Individuals | Genotype Frequency |
|---|---|---|
| **AA** | 360 | 0.36 |
| **Aa** | 480 | 0.48 |
| **Aa** | 160 | 0.16 |
| Total | 1000 | 1.0 |

Similarly, instead of genotypic frequency, we can also determine the allelic frequency. The allelic frequency represents the total number of copies of that allele in the population divided by a total number of copies of all the alleles of that gene in the population. Here, the allelic frequencies of both the alleles can be calculated as follows:

Genotype **AA**: 2 copies of **A** × 360 individuals = 720

Genotype **Aa**: 1 copy of **A** × 480 individuals = 480

Total number of copies of all the alleles (1000 individuals) = 2000

Therefore, the allelic frequency of **A** will be = 1200/2000 = 0.6

Similarly, the allelic frequency of **a** will be = 1.0 – 0.6 = 0.4

If the conditions are ideal and the frequency of two alleles A and a are p and q, respectively, then after one generation, the genotypic frequencies stabilizes in the proportions of $p^2$ (AA frequency), $2$pq (Aa frequency) and $q^2$ (aa frequency) (Refer Table 1.2).

***Table 1.2*** *Frequency of Offspring Genotype Post Random Mating*

| Possible Genotype of the Offspring | Allele from Mother | Allele from Father | Probability of Allelic Transmission from Mother | Probability of Allelic Transmission from Father | Probability of Allelic Transmission to the Offspring |
|---|---|---|---|---|---|
| **AA** | **A** | **A** | 0.6 | 0.6 | 0.6 x 0.6 =0.36 ($p^2$) |
| **Aa** | **A** | **a** | 0.6 | 0.4 | 0.6 x 0.4 =0.24 (pq) |
| **aA** | **a** | **A** | 0.4 | 0.6 | 0.6x 0.4 =0.24 (pq) |
| **Aa** | **a** | **a** | 0.4 | 0.4 | 0.4 x 0.4 =0.16 ($q^2$) |

The Hardy-Weinberg principal can be derived based on these results will be:

$(p + q)^2 = p^2 + 2pq + q^2$ (Refer Table 1.2).

Likewise, if the number of alleles is 3 (A1, A2 and A3) with frequencies p, q and r, then the frequencies of possible genotypes would be:

$(p + q + r)^2 = p^2 + q^2 + r^2 + 2pq + 2pr + 2qr$

It is a point to remember that this square expansion can be used to obtain the equilibrium genotypic frequencies for any number of alleles. Similarly, the sum of all genotypic frequencies must be 1. It means if there are two alleles, then their frequencies will be p + q = 1

And therefore, $(p + q)^2 = p^2 + 2pq + q^2 = 1$.

Likewise, if there are three alleles, then their frequencies will be:

$p + q + r = 1$, as well as, $(p + q + r)^2 = p^2 + q^2 + r^2 + 2pq + 2pr + 2qr = 1$.

Based on these concepts, we can determine that a population is in genetic equilibrium or not by simply testing it against the Hardy-Weinberg principle.

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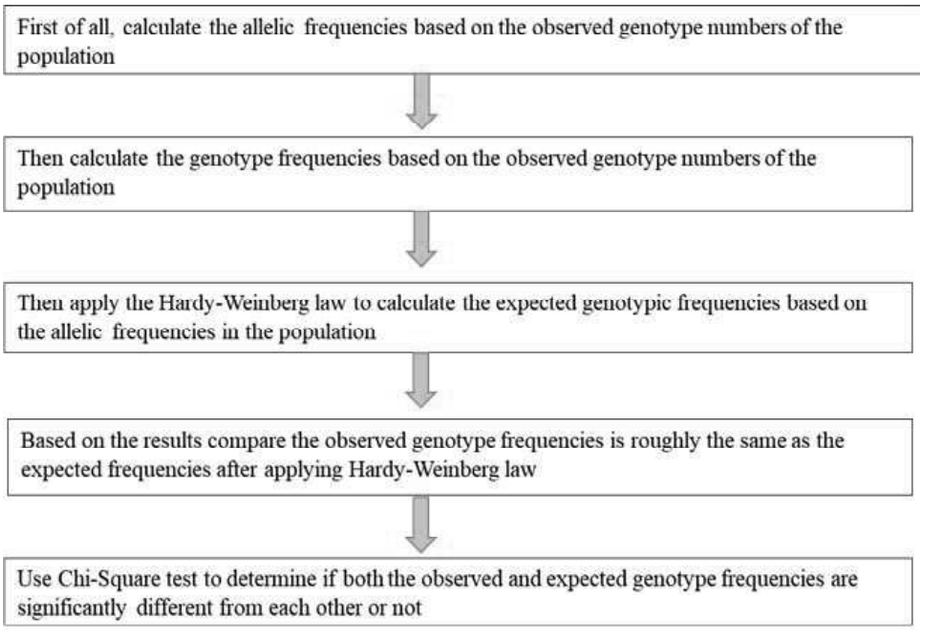


## Observed and expected genotypic frequencies and chi-square analysis

When we cross two individuals with already known genotype, we presume them to follow certain ratios based on the Mendelian Genetics. But, the observed genotype among the progeny may deviate from the expected genotype. As for example, the brown body colour (Y) is dominant over the yellow body colour (y) in German Cockroaches. We know if we will cross two brown heterozygous Cockroaches (Yy), the expected result should follow 3:1 ratio in the F1 generation. It means in a population of 50 Cockroaches, 37.5 should be brown bodied and 12.5 should be yellow bodied. But, in reality, we can get 40 brown and 10 yellow Cockroaches as F1 progeny. In order to evaluate the role of a change, we perform a statistical test known as the goodness of fit Chi-Square Test. This test determines how well the expected and observed values fit each other. This test basically measures the probability that the expected and observed genotype values arise by chance.

The Chi-Square Value can be calculated using the formula:

$$\chi^2 = \Sigma \frac{(\text{Observed-Expected})}{\text{Expected}}$$

Here $\Sigma$ means the total sum of all the squared differences between observed and expected divided by the expected values.

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| Genotype | Observed (O) | Expected (E) | $(O-E)^2$ | $(O-E)^2/E$ |
|---|---|---|---|---|
| Brown Color (YY/Yy) Cockroaches | 40 | 37.5 | 6.25 | 0.16 |
| Yellow Color (yy) Cockroaches | 10 | 12.5 | 6.25 | 0.50 |

To obtain the Chi-Square Value, we need to sum the total values of both the genotype, i.e., 0.16 + 0.5 = 0.66.

Here we also need to calculate the Degree of Freedom (*df*). Degree of Freedom determines the number of ways the observed frequency is free to vary. It can be calculated as $n - 1$, where $n$ represents the number of phenotypes. Here the number of phenotypes are two (brown and yellow), so the degree of freedom will be 2 – 1 = 1. Now use both the Chi-square and degree of freedom values and search it in Table 1.2 and see where it lies. At 1 degree of freedom and a Chi-Square Value of 0.66, our result lies between the values of 0.455 and 2.706. So the probability associated with our Chi-Square Value lies between 0.5 and 0.1. So there is less than 50% probability that this variation in genotypic ratio could be due to chance (Refer Table 1.3).

***Table 1.3*** *Values for Chi-Square Distribution*

| **Probability (*p*)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *df* | 0.995 | 0.975 | 0.9 | 0.5 | 0.1 | 0.05 | 0.025 | 0.01 | 0.005 |
| 1 | 0 | 0 | 0.016 | 0.455 | 2.706 | 3.841 | 5.024 | 6.635 | 7.879 |
| 2 | 0.01 | 0.051 | 0.211 | 1.386 | 4.605 | 5.991 | 7.378 | 9.21 | 10.597 |
| 3 | 0.072 | 0.216 | 0.584 | 2.366 | 6.251 | 7.815 | 9.348 | 11.345 | 12.838 |
| 4 | 0.207 | 0.484 | 1.064 | 3.357 | 7.779 | 9.488 | 11.143 | 13.277 | 14.86 |
| 5 | 0.412 | 0.831 | 1.61 | 4.351 | 9.236 | 11.07 | 12.832 | 15.086 | 16.75 |
| 6 | 0.676 | 1.237 | 2.204 | 5.348 | 10.645 | 12.592 | 14.449 | 16.812 | 18.548 |
| 7 | 0.989 | 1.69 | 2.833 | 6.346 | 12.017 | 14.067 | 16.013 | 18.475 | 20.278 |
| 8 | 1.344 | 2.18 | 3.49 | 7.344 | 13.362 | 15.507 | 17.535 | 20.09 | 21.955 |
| 9 | 1.735 | 2.7 | 4.168 | 8.343 | 14.684 | 16.919 | 19.023 | 21.666 | 23.589 |
| 10 | 2.156 | 3.247 | 4.865 | 9.342 | 15.987 | 18.307 | 20.483 | 23.209 | 25.188 |
| 11 | 2.603 | 3.816 | 5.578 | 10.341 | 17.275 | 19.675 | 21.92 | 24.725 | 26.757 |
| 12 | 3.074 | 4.404 | 6.304 | 11.34 | 18.549 | 21.026 | 23.337 | 26.217 | 28.3 |
| 13 | 3.565 | 5.009 | 7.042 | 12.34 | 19.812 | 22.362 | 24.736 | 27.688 | 29.819 |
| 14 | 4.075 | 5.629 | 7.79 | 13.339 | 21.064 | 23.685 | 26.119 | 29.141 | 31.319 |
| 15 | 4.601 | 6.262 | 8.547 | 14.339 | 22.307 | 24.996 | 27.488 | 30.578 | 32.801 |

Some points to remember are:

- In order to attain the equilibrium of genotypic frequencies, the population must be random mating and large sized.
- The sum of all genotypic frequencies must be always 1.
- In general, the time required to reach the equilibrium is one or at the most few generations, besides the population of individuals with one set of allelic frequencies mixes with another set through the process of random mating. Random mating can be defined as the mating between individuals where the choice of partner is without the influence of any genotype. Such population

is known as panmictic population and the process is called as panmixia or random mating.

- A population cannot evolve if it meets the criteria of Hardy-Weinberg law. As we know evolution requires a change in allelic frequencies and factors like mutation, natural selection, migration, genetic drift play an important role in evolution.
- When a population does not follow Hardy-Weinberg equilibrium, we have no basis to predict its genotypic frequencies, but even then allelic frequencies can be determined. Here it is important to note that we can determine allelic frequencies from the genotypic frequencies, but we cannot determine genotypic frequencies from allelic frequencies, it is possible only when the population attains the Hardy-Weinberg equilibrium.

**Applications**

Following are the applications:

- **Complete Dominance:** This law can also be implemented in case of complete co-dominance. If we consider two alleles A and a and A is dominant over a, then, in that case, the genotypes AA and Aa will have the same phenotype. As we know the frequency of aa genotype will be the square of the frequency of recessive alleles. Let us suppose q = 0.5, then q2 = (0.5)2=0.25. It means the phenotype of aa in the population will be 0.25 and thus the frequency of phenotypes AA 0.75.
- **Multiple Alleles:** The law can also be implemented to calculate the genotypic frequencies at loci with multiple alleles. For example, ABO blood group as there are three alleles IA, IB and IO with frequencies p, q, and r. As we know the sum of all genotypic frequencies must be 1 (p + q + r =1), therefore the genotype with random mating population will be (p + q + r)2.
- **Sex Linked Loci:** The Hardy-Weinberg law can also be applied to X-linked alleles. For example, in case of two X-linked alleles XA and Xa, the equilibrium frequencies for the female genotype will be (p + q)2 = p2 + 2pq + q2, Where the frequency of genotypes XA XA will be p2, frequency of XA Xa genotype will be 2pq and frequency of Xa Xa genotype will be q2. The male possesses only single X-linked allele, so the frequencies of will be p (frequency of XAY) and q (frequency of XaY). These proportion of genotype will be specific to male and female, rather than the entire population.

## 1.3.2 Detailed Account of Destabilizing Forces: Natural Selection, Mutation, Genetic Drift, Migration, and Meiotic Drive

The Hardy-Weinberg law suggests that the allelic frequencies do not change because of reproduction. But there are forces that increase and decreases the allelic frequency. The destabilizing forces that lead to change the allelic frequencies include mutation, genetic drift, migration, natural selection, and meiotic drive. In this section, we will try to understand all these evolutionary forces.

**Natural Selection:** The 'Natural Selection' can be defined as 'the differential reproduction of organisms as a function of heritable traits that influence the adaptation in the environment'. Based on the Darwin theory of Natural selection, if a species reproduce with an unchecked rate, the population will achieve exponential growth. However, this pattern of growth is seldom realized because not all organisms are able to reproduce. So, Darwin concluded that if the differential reproduction gets associated with environmental adaptation and vertically transmitted into subsequent generations, then the behavioral and physical trait will change with time in the direction of better adaptation by means of natural selection. The raw material for natural selection is genetic variation. If all the species are genetically identical, then the process of natural selection would not commence regardless of the fact that they can differentially reproduce. Natural selection can be depicted using reproductive fitness. It can be either absolute or relative. The absolute reproductive fitness can be measured by counting the raw number of gene copies transmitted to the subsequent generations. It can be expressed in terms of phenotype, genotype or individuals. The relative reproductive fitness can be calculated dividing the absolute reproductive fitness with the absolute fitness of reference individual.

**Mutation:** As discussed previously, the mutation is random and accidental changes in the nucleotide frequency mostly because of an error in copying the DNA, which can also create new alleles. However, the mutation rate is very low in most organisms. The environmental factors like ionizing radiation, chemicals and viruses constantly generates novel mutations in a genetic pool and thus destabilizes the genetic equilibrium. However, in multicellular organisms, not all the mutations affect the process of evolution, unless it affects the gamete-producing cells. Also, in eukaryotes, most of the DNA is junk and thus most of the mutations do not reach the gene pool. However, the somatic mutation can influence the physiology of a normal cell. It can induce abnormal cell growth ranging from a benign tumour to malignant carcinomas as thus can reduce the fitness of the organisms. On the other hand, the germinal mutations provide the raw material for evolution by introducing new genetic attribute. Based on contemporary evolution theory, it has been hypothesized that the germinal mutations are solely responsible for introducing new alleles into a species. For the organisms like bacteria and viruses where the reproduction is rapid and genome size is small, mutations affect directly and within a very short span of time. The amount of mutation can range from a single nucleotide to a whole chromosome. Regardless of this, all these genetic changes provide the raw material for natural selection.

**Genetic Drift:** Genetic drift can be defined as 'the change in allelic frequency of an existing gene variant in a population over time due to chance and chance alone'. In each generation, there is a chance that some individuals may leave behind a few more progenies than the other individuals. So the chances of accumulating the gene of more progenies in the gene pool will increase regardless of the fact that they are not necessarily fitter or better individuals. So, the genetic drift surely affects the allelic frequencies in a population but unlike natural selection, it follows a random process. Geneticists use the polymorphism in the junk DNA to study the phenomenon of genetic drift. In this case, the polymorphism can act as a

molecular clock that can assist in determining the rate of evolution because these genes are not influenced by natural selection.

**Migration:** Another factor that destabilizes the population is migration. Both immigration and emigration influence the allelic and genotypic frequencies. Migration tends to limit the divergence by generating continuous gene flow, which in turn increases the similarity between two populations. Migration has the potential to import beneficial alleles locally, but immigration from other environments may introduce alleles that are less fitted in the local habitat. This can ultimately lead to reduced fitness, also known as migration load. As we know, in natural selection the less fit immigrant genotype purges from a population, but stronger migration load may lead to persistent directional selection. For example, on rocky islands in Lake Erie, the water snakes exhibit a high frequency of high banded colour pattern, which is beneficial in the vegetated habitat of the mainland. But, the same phenotype is vulnerable to predators along the rocky island shores. Due to which, the island populations' exhibit persistent natural selection.

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**Meiotic drive:** The meiotic drive can be defined as 'the preferential transmission of an allele at a given locus during the process of meiosis'. In meiosis, the transmission probability of alleles from one to subsequent generations is fixed. However, the allele of one chromosome would increase in frequency, if instead of half the chromosome passes to all of the gametes. Such alleles cause meiotic drive and favour non-Mendelian transmission, leading to higher than expected allelic frequency. This process can even increase the allelic frequency of an allele conferring harmful trait on its carriers. Thus meiotic drive also acts as an evolutionary force capable of fixing a new gene into a population. The Segregation Distorter (SD) gene is an example of meiotic drive in *Drosophila melanogaster*. The SD is an autosomal meiotic drive gene, which induces the destabilizing of SD+ (wild type) gene during meiosis at the time of spermatogenesis. It leads to a population exclusively bearing SD over SD+ genotype.

All these processes– natural selection, mutation, genetic drift, migration, and meiotic drive act as destabilizing forces in any natural population. The evolutionary fixation of an allele of a given gene into a population may result from many evolutionary mechanisms acting simultaneously.

**Check Your Progress**

5. Why was Neo-Darwinism introduced?
6. When is population said to be in genetic equilibrium?
7. How can the Chi-Square Value be calculated?

## 1.4 QUANTIFYING GENETIC VARIABILITY

Genetic variation is vital for the long-term survival of species and supplies the raw material for all evolutionary changes in natural populations. The total genetic variation among the people in a specific group or environment is referred to as genetic diversity. It is possible to see an evolution in the understanding of genetic diversity

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quantification across time. Quantifying genetic variation means quantitative methods for measuring variation directly in DNA sequences to help decipher fundamental properties of populations and what they can tell us about evolution. It provides an overview of the evolutionary factors that contribute to genetic variation, like mutational input, effective population size, genetic drift, migration rate, and models of migration.

Specific processes influence genetic variability in small populations. In addition to the reduction in genetic variation due to simple selection (Bulmer effect), the combined effects of genetic drift and selection increase the risk of losing alleles at selected or unselected genes and promote inbreeding in the population by modifying the family structure. The three approaches to describing genetic variability yielded criteria for assessing this shift in genetic variability. The kinship and inbreeding coefficients, as well as the effective population size, can be employed at the genealogical level. The estimation of a trait's heritability is a useful measure of residual genetic variance at the trait level. The degree of genetic variation may be determined by looking at the polymorphism of known genetic markers at the genome level. These criteria will be included into particular instruments for genetic variability management.

### 1.4.1 Genetic Structure of Natural Populations

Amount and distribution of genetic variation amongst the population gives the genetic structure of a population. Basically, population genetic structure is the study of genetic variation in time and space. Usually, the valuations of the population genetic structure provides information about the dispersal of species, mating behaviours and the delimitation of species and population boundaries.

The subfield of genetics that relates with the genetic variances found across and between the populations is known as population genetics and is a part of evolutionary biology. Studies in this branch of biology examine phenomenon such as, adaptation, speciation, and population structure.

In the field of modern evolutionary advent, population genetics serves as a dynamic component. Initially it was, who also has shown their efforts for laying the foundation for related category of quantitative genetics. Primarily, modern population genetics, a highly mathematical discipline dealt with theoretical, laboratory, and field work.

Population genetic representations has shown their role in elucidating the statistical inferences from the available DNA sequence data and for agreement or disagreement of idea. Modern or current population genetics has constructed on this theory in many ways, most particularly by joining the theory with the statistical data from molecular biology.

Developments in the field of molecular biology have shaped a huge stock of data upon the genetic variations of real populations that has allowed a link to be made between the abstract population-genetic models and empirical data. Unlike conventional population-genetic analysis, which attempts for finding out how a given population will advance in the future, coalescent theory tries to rebuild the inherited state of a population by the present state, founded on the impression that

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the total number of genes present in the population will eventually originate from a mutual forefather. Coalescent theory supports modern research in population genetics.

Population genetics originated as an understanding of Mendelian inheritance and biostatic models. When population has the sufficient genetic differences in a population, it creates genetic variation and evolution. Blending inheritance was the only known common hypothesis before the foundation of Mendelian genetics was laid. Nevertheless, as know it is much clear that blending can never lead to the genetic variances found throughout the population and can never be the phenomenon behind evolution by natural or sexual selection.

The correct explanation of the variation conservation occurring in the population with Mendelian inheritance is given by Hardy–Weinberg principle, which states that the frequencies of alleles (variations in a gene) will be continued to be persistent in the non-appearance of selection, mutation, migration and genetic drift.

It was followed by the very important stage by effort of the Ronald Fisher, British biologist and statistician. From the sequence of papers initialised from 1918 and concluded in his book *The Genetical Theory of Natural Selection*, which was published in 1930, Fisher explained the reason behind the continuous variation. He suggested that the reason behind the presence of continuous variation as observed and calculated by the biometricians could be the collective deeds of various distinct genes, and is also the cause of variation found in allele frequencies in a population causing natural selection and evolution. Again in 1924, one more British geneticist, J. B. S. Haldane worked on the mathematics of allele frequency alteration on single gene locus under a wide-ranging surroundings. Haldane used statistical analysis too for the everyday instances of natural selection, such as peppered moth evolution and industrial melanism, and presented that selection coefficients could be more than expected by Fisher, which provided the quick adaptive evolution like that seen in camouflage strategy resulting from increased pollution.

The American biologist Sewall Wright, having experience in animal breeding experiments, paid attention on mixtures of interrelating genes, and the effects of breeding might be there on small, relatively isolated populations that showed genetic drift. In 1932, Wright presented the idea behind adaptive landscape and claimed that by genetic drift and inbreeding, it can be possible that the small isolated sub-population may move away from their adaptive peak, which allowed natural selection to again move them towards different adaptive peaks.

The joint efforts of Fisher, Haldane and Wright laid the foundation of population genetics. This united natural selection with Mendelian genetics, which was the most crucial step in emerging of the unified theory of in what way evolution worked. John Maynard Smith was Haldane's pupil, whilst W. D. Hamilton was attracted by the literatures by Fisher. The American George R. Price worked together with both Hamilton and Maynard Smith. American Richard Lewontin and Japanese Motoo Kimura were highly influenced by Wright and Haldane.

Although the field faced too many disapprovals, it is the one that explains about the mechanism of evolution. For example, the most popularly known 'gene's

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eye' opinion of evolution, which was proposed by biologists like G.C. Williams, W.D. Hamilton and Richard Dawkins, comes straight from the population-genetic perceptive; definitely, most of the significant features of gene's eye model were even present in Fisher's writings before. Supporters for gene's eye opinion contend that genotypes and organisms are typically only the provisional displays but genes are the real heirs of the evolutionary process. Natural selection is the origin of the competition amid gene lineages for greater depiction at the gene pool; generating organisms with adaptive structures is a 'strategy' that genes have invented for the security of successors. In the last thirty years, the opinion about Gene's eye has revolutionised diverse areas of evolutionary biology, chiefly the field of animal behaviour; but it is nothing but like a colourful layer on the origin of evolution understood in the terms of population genetics.

The unique, modern day synthesis theory of population genetics accepts that mutations facilitate the raw material whose work is only to emphasize the variation in frequency of alleles in populations. Allele frequency in the population is influenced by natural selection, genetic drift, gene flow and recurrent mutation.

Fisher and Wright didn't agree mutually on the characters of selection and drift. But, the molecular data about the molecular evolution on entire genetic differences led the way for the formation of neutral theory of molecular evolution. So, in this perspective, numerous mutations have deleterious effect and some not so noticed are neutral, i.e., are not under assortment. As the chance of every neutral mutation port to accidental (genetic drift), the way of evolutionary change is determined by the type of mutations that has occurred, and thus cannot be taken by models of alteration in the frequency of (existing) alleles only.

The origin-fixation assessment for population genetics simplifies this method elsewhere neutral mutations, and gets the rate at which certain change occurred as the product of the mutation rate and the fixation probability.

## 1.4.2 Phenotypic Variation

Phenotype is the assemblage of observable traits. In "genetic" sense, the genotype describes the phenotype. Phenotypes are traits or characteristics of an organism that can be observed morphologically, such as, size, colour, shape, capabilities, behaviours, etc. Not every phenotype can actually be observed.

For example, blood types are phenotypes that can only be observed with laboratory practices. The genomic variation found on an individual level commutatively leads to the combination of different variation producing phenotypic variation observed across a population. Phenotypic variation is universal.

Phenotype = genotype + development (in a given environment).

As all organisms of a population carries an exclusive genetic information, this information is presented in the form of specific nucleotide order of their nucleic acids as their genetic material. The genetic biochemistry of nearly all the organisms is DNA (deoxyribonucleic acid), except for viruses and bacteria that have RNA as their hereditary material.

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The genotype content varies between individual genome due to the mutations that takes place when the genome content is transferred from one generation to the other because of lower speed of natural or spontaneous changes that are referred as mutation. It can even create entirely new alleles in a population. In fact, in a population in which all copies of a chromosome were undistinguishable, new genetic variants can arise by mutation. When the phenotype expression occurs by the genotype, its expression is flexible to some extent. It is the real or obvious appearance of the genetic material in relations to anatomy, behaviour, and biochemistry. The flexibility in the expression of genetic potential is due to the interference caused by the environmental conditions and other circumstances.

Among the factors which contribute to phenotype distinction in the population are mainly genetic and environmental. Environmental factors like diet, temperature, oxygen levels, humidity, light cycles, and the presence of mutagens can all influence the expression of a genes, which eventually affects the phenotype of an animal.

Dominance genetic variance is the phenotype variation caused due to the interactions between alternate alleles that affect one trait at one specific locus or the expression of an organism's genetic code, or its genotype. Variations are crucial for the survival of an individual. The natural selection of a population is based on the best fitted population in an environment which is based on the evolution caused by mutation which brings change in the genomic content of an individual and give rise to different traits to make the survival of a species easy in different circumstances. Phenotypic variation is desirable for natural selection and sexual selection because if deprived of it, there wouldn't be different traits, making the function of both selections invalid. Phenotypic variation is vital for evolution. In absence of a definite variance among individuals in a population there are no genetic selection pressures acting to modify the diversity and categories of alleles (forms of genes) existing in a population. Therefore, genetic mutations that are not able to produce phenotypic change are fundamentally disguised by evolutionary mechanisms.

Phenetic resemblance results when phenotypic alterations between individuals are minor. In such cases, it may take a noteworthy alteration in environmental conditions to produce significant selection pressure that results in more dramatic phenotypic variances. Phenotypic differences form modifications in fitness and mark adaptation.

### 1.4.3 Models Explaining Changes in Genetic Structure of Natural Populations

The change which occurs in any population's genetic composition over the period of time is defined as evolution by population geneticists. These changes are mainly brought up by four factors:

Natural selection, mutation, random genetic drift, and migration into or out of the population. The fifth factor is the due to the change of the mating pattern which causes the change in the genotype but not the allele frequencies; so, this factor is not categorised to count for evolutionary change by many theorists. The factors are outlined in details below.

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## Selection at One Locus

When a group of population of some genotypic variants have an advantage of survival or reproduction over other, it is favoured by natural selection more. When a population-genetic model of natural selection is considered, the simplest of them adopts one autosomal locus with two alleles, $A_1$ and $A_2$, as above. The three diploid genotypes $A_1A_1$, $A_1A_2$ and $A_2A_2$ have changed finesses, represented by $w_{11}$, $w_{12}$ and $w_{22}$ respectively. These fitness's are expected to be the same over generations. So, according to this, a genotype's fitness can be understood as the average number of positive gametes of the organism with the genetic constitution donates to next generation which again depends upon on how fine the organism survive, how many mating it attains, and how fertile it is. If $w_{11}$, $w_{12}$ and $w_{22}$ would all be equivalent, at that time the natural selection will happen, maybe making the genetic configuration of the population to alter.

For example, originally, i.e., previously when the selection has been functioned, zygote genotypes follow Hardy-Weinberg magnitudes and the occurrences of the $A_1$ and $A_2$ alleles are $p$ and $q$ correspondingly, where $p + q = 1$. The zygotes at that moment raise to adulthood and reproduce, producing novel generation of offspring zygotes. The job here is to place the frequencies of $A_1$ and $A_2$ at the second generation; lets represent these by $p2$ and $q2$ respectively, where $p2 + q2 = 1$. (Remember that in both generations, the gene frequencies are occupied at the zygotic stage; which differ from the adult gene frequencies if there is differential survivorship.)

At the zygotic stage of the foremost or first generation, the genotypic frequencies are $p^2$, $2pq$ and $q^2$ for $A_1A_1$, $A_1A_2$, $A_2A_2$ respectively according to the Hardy-Weinberg law. The three genotypes give positive gametes in part to their fitness, i.e., in the ratio $w_{11}$: $w_{12}$: $w_{22}$. The average fitness in the population is $w = p^2 w_{11} + 2pq\, w_{12} + q^2 w_{22}$, so the total number of successful gametes produced is $Nw$, where $N$ is the population size. Assuming there is no mutation, and that Mendel's law of segregation holds, then an $A_1A_1$ organism will give only $A_1$ gametes, an $A_2A_2$ organism will give only $A_2$ gametes, and an $A_1A_2$ organism will produce $A_1$ and $A_2$ gametes in equivalent proportion. Consequently, the amount of $A_1$ gametes, and therefore, the frequency of the $A_1$ allele in the second generation at the zygotic stage, is:

$$\begin{aligned} p' &= [N p^2 w_{11} + \tfrac{1}{2} (N\, 2pq\, w_{12})] / Nw \\ &= (p^2 w_{11} + pq\, w_{12}) / w \end{aligned} \quad (1)$$

Equation (1) is termed as 'recurrence' equation which expresses the frequency of the $A_1$ allele in the second generation in words of their frequency of first generation. The variation between frequencies of different generations can then be represented as:

$$\begin{aligned} \Delta p &= p' - p \\ &= (p^2 w_{11} + pq\, w_{12}) / w - p \\ &= pq\, [p\, (w_{11} - w_{12}) + q\, (w_{12} - w_{22})] / w \end{aligned} \quad (2)$$

If $\Delta p > 0$, so natural selection has directed the $A_1$ allele for rise in frequency;

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If $\Delta p < 0$ so selection has directed the $A_2$ allele for rise in frequency.

If $\Delta p = 0$ so no gene frequency difference has happened, in other terms, the arrangement follows allelic equilibrium. (Though the condition $\Delta p = 0$ does not propose the absence of natural selection and the situation for that is $w_{11} = w_{12} = w_{22}$. It is likely for natural selection to happen but to have no consequence on gene frequencies.

Equations (1) and (2) describe the effect a fitness differences between genotypes have on origin of evolutionary change which allows us to discover the significances of various dissimilar selective regimes.

Supposing firstly that $w_{11} > w_{12} > w_{22}$, i.e., the $A_1A_1$ homozygote is fitting than the $A_1A_2$ heterozygote, which in turn is fitting than the $A_2A_2$ homozygote. By examination of equation (2), $\Delta p$ must be positive can be stated (up to the point on which both p and $q$ are zero, $\Delta p = 0$).

Therefore, the frequency of the $A_1$ allele in every generation, would be having more value than in the preceding generation, till it finally come to the fixation point and the $A_2$ allele is removed as of the population.

Once the $A_1$ allele gets to fixation, i.e., $p = 1$ and $q = 0$, no additional evolutionary change will happen, for if $p = 1$ then $\Delta p = 0$.

This makes good sense instinctively: since the $A_1$ allele deliberates the benefit of fitness on the organisms carrying it, its relative frequency at population will rise from generation to generation until it is secured.

It is understandable that similar reasoning can be used in the situation where $w_{22} > w_{12} > w_{11}$. Equation (2) explains that $\Delta p$ should be having negative value then, so long as neither $p$ nor $q$ is zero, so the $A_2$ allele will curve to fixation, removing the $A_1$ allele.

It is more exciting condition to note when the heterozygote is higher in fitness to both of the homozygotes, i.e., $w_{12} > w_{11}$ and $w_{12} > w_{22}$—a phenomenon known as **heterosis**. Instinctively the result of such situation can be estimated: an equilibrium condition should be reached in which both alleles have their presence in the population.

Equation (2) confirms this intuition. It is easy to see that $\Delta p = 0$ if either allele has gone to fixation (i.e., if $p = 0$ or $q = 0$), or, thirdly, if the following condition obtains:

$$p\ (w_{11} - w_{12}) + q\ (w_{12} - w_{22}) = 0$$

Which reduces to

$$p = p^* = (w_{12} - w_{22}) / (w_{12} - w_{22}) + (w_{12} - w_{11})$$

(The asterisk sign indicates an equilibrium condition.)

As the value of p must be non-negative, this state can only be fulfilled in the presence of heterozygote advantage or heterozygote inferiority; it denotes the equilibrium state of the both alleles of the population. This equilibrium is termed as polymorphic.

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Monomorphic equilibrium is the state in which any one allele has undergone fixation. The likelihood for polymorphic equilibrium is rather noteworthy as it explains that natural selection will not always yield genetic homogeneity; in some conditions, selection conserves the genetic differences seen in a population.

Population genetic model is the foundation which is used to address and find solutions for various queries about natural selection. As, for example, rate of revolutionary change can be understood by considering a parameter that calculates the fitness variation between genotypes. Questions like just how much generations are desired for the choice of the increment found in the $A_1$ allele from 0.1 to 0.9? And if the expected deleterious allele is recessive, how lengthier it would be taken for the removal of this from the population than if it were dominant? Population geneticists have carried mathematical concepts for the theory of evolution, to a degree that would have seemed to be unbelievable in Darwin's day. Obviously, the mentioned one-locus model above is too simple for addressing many realistic populations, as it includes simplifying assumptions that are doubtful to hold true. Current studies like, selection is hardly the only evolutionary force in operation, genotypic fitness's are doubtful to be constant across generations. Mendelian segregation does not always hold precisely, and so-on.

Much research in population genetics contains in inventing more accurate evolutionary models, which depend on less modest assumptions and are thus more complex. But the one-locus model demonstrates the spirit of population-genetic perceptive, and the associated explanation of the evolutionary process that it brings.

### Selection-Mutation Balance

As it is well known that mutation is the reason of genetic variation found among the population and the force that prevents the genetically homogenous situation that would definitely be found among the population. On considering mutation, the above drawn conclusions need a modification. To maintain a situation of polymorphism, recurrent mutation ensures the expression of other alleles even at a low frequency which are comparatively inferior to the supreme alleles present. Population geneticists are also keen in exploring about what would occur if selection and mutation would act concurrently.

Going with our one-locus, two allele model, let' assume that the $A_1$ allele is comparatively dominant to $A_2$, but recurrent mutation from $A_1$ to $A_2$ avoids $A_1$ from dispersal to fixation. The mutation rate from $A_1$ to $A_2$ per generation, i.e., the $A_1$ allele's amount which mutate in each generation, is symbolized as $u$. (Empirical approximations of mutation rates are characteristically in the region of $10^{-6}$.) Back mutation from $A_2$ to $A_1$ can be discounted, because we are supposing that the $A_2$ allele is at a very low frequency in the population, thanks to natural selection.

What occurs to the gene frequency dynamics under these norms?

Remembrance of equation (1) stated before, that states the occurrence of the $A_1$ allele in relation of its frequency in the preceding generations. Subsequently some fraction ($u$) of the $A_1$ alleles must have mutated to $A_2$, the recurrence equation should have change like:

$p' = (p^2 w_{11} + pq\, w_{12})\,(1 - u) / w$

To take explanation for mutation.

Like stated initially, equilibrium is got when $p' = p$, i.e., $\Delta p = 0$. The case for equilibrium is so:

$$p = p^* = (p^2 w_{11} + pq\, w_{12})\,(1 - u) / w \qquad (3)$$

A useful explanation of equation (3) can be attained by having some expectations for the genotype fitness's, and having a new representation.

Let us assume that the $A_2$ allele is wholly recessive (which is frequently the situation for deleterious mutants). Which tells that the $A_1A_1$ and $A_1A_2$ genotypes have equal fitness.

So, genotypic fitness's can be represented as $w_{11} = 1$, $w_{12} = 1$, $w_{22} = 1 - s$, where $s$ means the alteration in fitness of the $A_2A_2$ homozygote from another two genotypes. ($s$ is termed as the selection co-efficient against $A_2A_2$). Meanwhile, as it is assumed that the $A_2$ allele is deleterious, it follows that $s > 0$. Replacing these genotype fitness's in equation (3) gives:

$p^* = p\,(1 - u) / p^2 + 2pq + q^2(1 - s)$

Which decreases to:

$p^* = 1 - (u/s)^{½}$

Or consistently (since $p + q = 1$):

$$q^* = (u/s)^{½} \qquad (4)$$

Equation (4) represents the equilibrium frequency of the $A_2$ allele, in the supposition that it is entirely receding. Remember that as $u$ has increment, $q^*$ also have. Which is extremely instinctive: the larger the mutation rate from $A_1$ to $A_2$, the larger the frequency of $A_2$ that can be kept at equilibrium, for a given value of $s$. On the other hand, as $s$ rises, $q^*$ declines. It's too instinctive: the tougher the selection is in contrast to the $A_2A_2$ homozygote, the lesser is the equilibrium frequency of $A_2$, for a given value of $u$.

It is not difficult to note why equation (4) is said to label selection-mutation balance—natural selection is repeatedly eliminating $A_2$ alleles from the population, though mutation is repeatedly reproducing them.

Equation (4) describe that the equilibrium frequency of $A_2$ which would be upheld, like an role of the rate of mutation from $A_1$ to $A_2$ and the scale of the chosen disadvantage acted by the $A_2A_2$ homozygote.

Notably, equation (4) was derivative of the supposition that the $A_2$ allele is totally receding, i.e., that the $A_1A_2$ heterozygote is phenotypically similar to the $A_1A_1$ homozygote. Though, it is upfront for the derivation of alike equations of situations where the $A_2$ allele is dominant, or partially dominant. If $A_2$ is dominant, or partially dominant, its equilibrium frequency would be lesser than if it is wholly recessive; for selection is much effective at eliminating it from the population. In the heterozygotes, the deleterious allele which is recessive, 'hides' itself and so, discharge the elimination power of selection, but a dominant allele cannot.

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One important matter of discussion before ending the topic is that as this explanation has focused completely on deleterious mutations, i.e., that decrease the fitness of its host organism. This may appear odd, given that helpful mutations play so vital a role in the evolutionary process. The reason lies in the fact that in the population genetics, the most important point is in understanding the reason behind the genetic diversity observed in biological populations. May if the gene is helpful, natural selection is probable to be the major cause of its equilibrium frequency; the rate of irregular mutation to that gene would have greatest minor role. This is the case in which the gene is deleterious that mutation plays a key role in preserving it in a population.

**Random Drift**

Random genetic drift can be understood as sampling error which comes to observation when there are chance variations in gene frequency that is found in finite populations.

For many evolutionary models, population is expected to be infinite, or very large, exactly in order to abstract away from chance variations. But though mathematically suitable, this assumption is frequently unrealistic. In real life, chance factors will unvaryingly play a role, mainly in small populations.

In broader sense, the term 'random drift is used to mention at all stochastic influences which can affect the gene frequencies in a population, counting for example accidental variations in survival and pairing accomplishment; and occasionally in a narrow sense, to refer to the chance sampling of gametes to make the offspring group (which grows because organisms yield much more gametes than would be created from it to the fertilized zygote). The wider sense is used here.

Nature of random drift can be understood using a simple example. A population with just ten organisms, five of type *A* and five of type *B*; the organisms reproduce asexually and produce offspring of the same type. Supposing that neither type of them is selectively superior to the other—both are similarly well-adapted to the environment.

Though, it can be said that these two types will be producing two different types of undistinguishable numbers of offspring, for which chance factors may play a role.

For example, if all the type *B*s in consideration may decease by accident before reproducing, so in this case, the frequency of *B* in the second generation will decrease to zero. In this condition, random drift is the cause of decline of the *B* type (and thus the spread of the *A* type). So, always it is the matter for excitement for the evolutionists to find the reason of frequency change of a given gene change if it is drift, selection, or some combination of the two. The label of 'random drift' is somewhat confusing. If it is considered that the spread of the *A* type is due to random drift, or chance, it cannot be inferred that cause can be found of its spread. In theory, it can apparently be discovered that the complete fundamental section about why every organism in the population left precisely the number of offspring that it did. For explaining the evolutionary variation as random drift, it cannot be denied that it's just the causal story to be told. But it says that the

spread of the *A* type is not because of its adaptive dominance over the *B* type. In other words, the *A* and the *B* types had the similar expected number of offspring, so were likewise fit; but the *A* types had a larger actual number of offspring. In a finite population, actual reproductive output will nearly at all times diverge from expectancy, leading to evolutionary alteration.

A comparison with coin tossing can brighten random drift phenomena. Assuming that a fair coin is tossed ten times. It can be calculated that the probability of heads for any one toss is ½, and so the expected frequency of heads in the order of ten is 50%. Nonetheless, the probability of really having half heads and half tails is only 242/1024, or about 23.6%. So even after having a fair coin, the probability of having equal proportions of heads and tails in an order of ten tosses is uncertain and some diversion from expectation is more likely than not. However, the *A* and *B* kinds are exactly fit in the example above, it is probable that some evolutionary change will happen. This analogy can also prove the role of population size. If the coin is tossed hundred times rather than ten, the proportion of heads would maybe very close to ½. Similarly, the bigger the population, the less significant is the effect of random drift on gene frequencies; in the infinite limit, drift has no effect.

Drift confuses the task of population geneticist greatly. As explained before, it is clearly unbearable to infer that the configuration of the population in the second generation from its arrangement in the first generation; at most, it can be hoped to deduce the likelihood distribution over all the likely configurations. If for once drift is taken into the account, no simple reappearance relation for gene frequencies, of the sort expressed in equation (1) above, can be derived. For analysing the evolutionary significances of drift, population geneticists practice a mathematical technique, known as diffusion modelling. Though, several of these consequences are quite instinctive, and can be understood without the mathematics.

One important result of random drift is to decline the degree of heterozygosity in a population over time. It happens because at a given time, any finite population will finally become homozygous through drift (though if the population is large, the method to homozygosity will be slow.) It is relaxed to see why this is—for gene frequencies of 0 and 1 are 'absorbing boundaries', denoting that once the boundary is touched, there is no way back from it (apart from mutation). So finally, a given allele will ultimately become static in a population, or go extinct, the latter being the more likely fate. Indeed, it is seen from the mathematical models that a neutral allele rising by mutation has a very low probability of being fixed in a population; the bigger the population, the lesser the probability of fixation.

Random drift is also very effecting in causing the different subpopulations of a species which deviate genetically from each other, as the chance gathering of alleles will probably continue differently in each, particularly if the alleles give slight selective advantage or disadvantage. By chance, one population may become static for allele $A_1$, while a second population becomes fixed for another allele $A_2$. It is very important to note that this possibility is important one, if not done so mistakenly it can be concluded that the $A_1$ allele must have advantage in the first population's environment and the $A_2$ allele in the environment of the second, i.e.

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that selection was accountable for the genetic variation. Such an explanation can be right but is not the only one—random drift provides an alternative.

There is question about who plays the major role in molecular evolution, drift or selection and this topic was even much debated in the 1960s and 1970s; as it laid the heated disagreement between the selectionists and neutralists. M. Kimura headed the neutralist camp and argued that maximum molecular variants had no effect on phenotype, so were not subject to natural selection and random drift was the main cause of their fortune. Kimura claimed that the apparently constant rate at which the amino acid sequences of proteins evolved, and the degree of genetic polymorphism found in natural populations, could best be explained by the neutralist hypothesis (Kimura 1977, 1994). Selectionists contradicted that natural selection was also accomplished of explaining the molecular data. Recently, the controversy has diminished somewhat, without a clear victory for either side. Most biologists believe that some molecular variants are certainly neutral, though fewer than were claimed by the unique neutralists.

## Migration

Migration occurring into or out of a population is the fourth and final factor which affects the genetic composition. It can be understood with a simple case in which genetically different immigrants are entering into a population with a different genetic composition, so it is very obvious that this will cause the alteration in population's genetic composition. Migration has an evolutionary importance as many species are formed of a number of different subpopulations, largely isolated from each other but associated by chance migration. An extreme example of population subdivision can be understood by imagining ant colonies. Migration between subpopulations allows gene flow, which act like a 'glue', limiting the degree to which subpopulations can deviate from each other genetically.

The simplest model for analysing migration adopts that a given population obtains a number of migrants each generation, but sends out no emigrants. Assuming the frequency of the $A_1$ allele in the resident population is $p$, and the frequency of the $A_1$ allele among the migrants arriving in the population is $p_m$. The number of migrants coming into the population each generation is $m$ (i.e., as a proportion of the resident population.) So post-migration, the frequency of the $A_1$ allele in the population is:

$$p' = (1 - m)\, p + mp_m$$

The change in gene frequency across generations is therefore:

$$\Delta p = p2 - p$$

$$= “\ m\ (p - p_m)$$

So, migration will increase the frequency of the $A_1$ allele if $p_m > p$, decline its frequency if $p > p_m$, and leave its frequency unaffected if $p = p_m$. It is then an upfront matter to derive an equation giving the gene frequency in generation $t$ as a function of its initial frequency and the rate of migration. The equation is:

$$p_t = p_m + (p_0 - p_m)\,(1 - m)^{\,t}$$

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Where $p_0$ is the initial frequency of the $A_1$ allele in the population, i.e., before any migration has taken place. Since the expression $(1 - m)^t$ tends towards zero as $t$ grows large, it is easy to understand that equilibrium is reached when $p_t = p_m$, i.e., when the gene frequency of the migrants equals the gene frequency of the resident population.

This simple model adopts that migration is the only factor affecting gene frequency at the locus, but this is not likely to be the case. So, it is necessary to reflect how migration will interrelate with selection, drift and mutation. A stability between migration and selection can lead to the upkeep of a deleterious allele in a population, in a manner closely similar to mutation-selection balance, discussed above. The collaboration between migration and drift is especially exciting. It is seen that drift can form separate subpopulations of a species to deviate genetically. But, migration opposes this trend—it is a homogenising force that inclines to make subpopulations more alike. Mathematical models advise that even a fairly small rate of migration will be adequate to prevent the subpopulations of a species from deviating genetically. Some theorists have used this to claim against the evolutionary importance of group selection, on the grounds that genetic differences between groups, which are vital for group selection to operate, are improbable to persevere in the face of migration.

### Non-Random Mating

As it is very well known, the Hardy-Weinberg law of population-genetic analysis, was the derivative under the assumption of random mating. But commonly the deviations are found from the random mating. Organisms may incline to choose mates who are alike to them phenotypically or genotypically—a mating system known as 'positive assortment'. Otherwise, organisms may choose mates dissimilar to them— 'negative assortment'. Additional type of departure from random mating is inbreeding, or specially mating with relatives.

Depicting the consequences non-random mating can have is quite complex, but some conclusions are fairly easily seen. Firstly, and most importantly, non-random mating does not in itself disturb gene frequencies (so is not an evolutionary 'force' on a par with selection, mutation, migration and drift); rather, it disturbs genotype frequencies. For appreciating it, note that the gene frequency of a population, at the zygotic stage, is equal to the gene frequency in the collection of successful gametes from which the zygotes are shaped. The design of mating simply controls the way in which haploid gametes are 'packaged' into diploid zygotes. Thus, if a random mating population rapidly starts to mate non-randomly, this will have no effect on gene frequencies.

Secondly, positive assortative mating will incline to reduce the number of heterozygotes in the population, thus increasing the genotypic variance. To see this, consider again a single locus with two alleles, $A_1$ and $A_2$, with frequencies $p$ and $q$ in a given population. Originally the population is at Hardy-Weinberg equilibrium, so the proportion of $A_1A_2$ heterozygotes is $2pq$. If the population then starts to mate totally assortative, i.e., mating only occurs between organisms of alike genotype, it is clear that the proportion of heterozygotes must

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reduce. For $A_1A_1 \times A_1A_1$ and $A_2A_2 \times A_2A_2$ mating no heterozygotes will be produced; and only half the progeny of $A_1A_2 \times A_1A_2$ mating's will be heterozygotic. So, the proportion of heterozygotes in the second generation must be fewer than $2pq$. Conversely, negative assortment will lean towards increasing the proportion of heterozygotes from what it would be under Hardy-Weinberg equilibrium.

In general, inbreeding will lean towards increasing the homozygosity of a population, like positive assortment. The reason for this is obvious—relatives tend to be more genotypically alike than randomly chosen members of the population. In the wide proportion of species, including the human species, inbreeding has negative effects on organismic fitness—a phenomenon known as 'inbreeding depression'. The clarification for this is that deleterious alleles frequently tend to be recessive, so have no phenotypic effect when found in heterozygotes. Inbreeding decreases the proportion of heterozygotes, making recessive alleles more probable to be found in homozygotes where their negative phenotypic properties become apparent. The converse phenomenon— 'hybrid vigour' resulting from outbreeding—is extensively utilised by animal and plant breeders.

## Two-Locus Models and Linkage

Up to now, the discussion mainly included the gene frequency change at a single locus, which is said to be the simplest sort of population-genetic analysis. But, in practice it is improbable that an organism's fitness will depend on its single-locus genotype, so there is a boundary on the degree to which single-locus models can light the evolutionary process. The purpose of two-locus (and more generally, multi-locus) models is to trail deviations in gene frequency at more than one locus concurrently. Such models are always more complicated that their single-locus counterparts, but attain greater practicality.

The simplest two-locus model undertakes two autosomal loci, $A$ and $B$, each with two alleles, $A_1$ and $A_2$, $B_1$ and $B_2$ respectively. Thus, there have four types of haploid gamete in the population—$A_1B_1$, $A_1B_2$, $A_2B_1$ and $A_2B_2$—whose frequencies can be denoted by $x_1$, $x_2$, $x_3$ and $x_4$ respectively. (Note that the $x_i$ are *not* allele frequencies; in the two-locus case, we cannot equate 'gamete frequency' with 'allelic frequency', as is likely for a single locus.) Diploid organisms are formed by the fusion of two gametes, as before. Thus, there are ten likely diploid genotypes in the population—found by taking each gamete type in mixture with every other.

In the one-locus case, as we have seen in large arbitrarily mating population, there is a simple connection between the occurrences of the gamete types and of the zygotic genotypes that they form. In the two-locus case, the same relationship holds. Thus, for example, the frequency of the $A_1B_1 / A_1B_1$ genotype will be $(x_1)^2$; the frequency of the $A_1B_1 / A_2B_1$ genotype will be $2x_1x_3$, and so-on. This can be recognized thoroughly with a dispute based on arbitrary sampling of gametes, similar to the argument used in the one-locus case. The first characteristic of the Hardy-Weinberg law—genotypic frequencies is specified by the square of the array of gametic frequencies—so moves neatly to the two-locus case. However, the second feature of the Hardy-Weinberg law is the stable genotypic frequencies

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after one round of random mating which does not really apply in the two-locus case which is illustrated below in more detail.

A key idea in two-locus population genetics is that of linkage, or absence of independence between the two loci. To recognize linkage, assume the set of gametes formed by an organism of the $A_1B_1 / A_2B_2$ genotype, i.e., a double heterozygote.

If the two loci are unlinked, then the arrangement of this set will be {¼ $A_1B_1$, ¼ $A_1B_2$, ¼ $A_2B_1$, ¼ $A_2B_2$}, i.e., all four gamete types will be equally signified assuming Mendel's first law holds at both loci. So unlinked loci are independent—which allele a gamete has at the *A* locus has to do nothing about the allele it has at the *B* locus. The opposite process is the perfect linkage. If the two loci are perfectly related, then the set of gametes formed by the $A_1B_1 / A_2B_2$ double heterozygote has the configuration {½ $A_1B_1$, ½ $A_2B_2$}; which tells that if a gamete obtains the $A_1$ allele at the *A* locus, it necessarily receives the $B_1$ allele at the *B* locus and vice versa.

In physical terms, perfect linkage means that the *A* and *B* loci are situated close together on the same chromosome and the alleles at the two loci are thus inherited as a single unit. Unlinked loci are either on different chromosomes, or on the same chromosome but divided by a significant distance, hence, likely to be fragmented up by recombination.

Wherever the loci are on the same chromosome, perfect linkage and whole lack of linkage are two ends of a range. The grade of linkage is measured by the recombination fraction r, where 0 d" *r* d" ½. The conformation of the set of gametes shaped by an organism of the $A_1B_1 / A_2B_2$ genotype can be written in terms of *r*, as follows:

$A_1B_1$ ½ (1 − *r*)

$A_1B_2$ ½ *r*

$A_2B_1$ ½ *r*

$A_2B_2$ ½ (1 − *r*)

It can easily be observed that $r = ½$ means that the loci are not linked and thus, all four gamete types are formed in equal proportion, while $r = 0$ means that they are perfectly linked.

In a two-locus model, the gametic (and therefore genotypic) frequencies need not be persistent across generations, even in the absence of selection, mutation, migration and drift, contrast in the one-locus case. Though allelic frequencies will be constant, in the non-appearance of any evolutionary forces. It is possible to pen recurrence equations for the gamete frequencies, as a function of their frequencies in the preceding generation plus the recombination fraction. The equations are:

$$x_1' = x_1 + r(x_2x_3 - x_1x_4)$$

$$x_2' = x_2 + r(x_2x_3 - x_1x_4)$$

$$x_3' = x_3 + r(x_2x_3 - x_1x_4)$$

$$x_4' = x_4 + r(x_2x_3 - x_1x_4)$$

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It can be easily observed form this recurrence equations that gametic (and thus genotypic) frequencies will be steady across generations, i.e., $x_i' = x_i$ for each $i$, under either of two conditions: (i) $r = 0$, or (ii) $x_2x_3 - x_1x_4 = 0$.

Condition (i) means that the two loci are perfectly allied, and thus in effect behaving as one locus and condition (ii) means that the two loci are in 'linkage equilibrium', which tells that the alleles at the *A*-locus are in random association with the alleles at the *B*-locus. More exactly, linkage equilibrium means that the population-wide frequency of the $A_iB_i$ gamete is equivalent to the frequency of the $A_i$ allele multiplied by the frequency of the $B_i$ allele.

An important consequence in two-locus theory shows that in a given random mating, the quantity $(x_2x_3 - x_1x_4)$ will decline in every generation till it reaches zero at which the genotype frequencies will be in equilibrium. So, a population originally in linkage disequilibrium will come to a linkage equilibrium over a number of generations. The rate of approach is dependent on the value of *r*, the recombination fraction. In contrast to the one-locus case, where just one round of random mating is adequate to bring the genotype frequencies into equilibrium.

## 1.4.4 Factors Affecting Human Disease Frequency

A group of people with some mutual characteristic, such as age, race, gender, or place of residence makes a population.

For the measurement of how commonly a disease occurs in a population, these three factors are always considered:

(1) The number of affected individuals or cases,

(2) The magnitude of the population from which the cases rise, and

(3) The total of time that this population is followed.

Disease frequency is mainly described using its two measures, incidence and prevalence. Incidence measures the incidence of new disease and so captures the transition from health to disease.

Cumulative incidence and incidence rate are the two main components of incidence measures. Cumulative incidence is well-defined as the amount of a candidate population that becomes diseased over a stated time period. It is a dimensionless proportion that measures the average risk of contracting a disease over a certain time period. Incidence rate is the incidence of new cases of disease that arise during person-time of remark, and so it is a true rate. Cumulative incidence and incidence rate are correlated mathematically. Both measures are most valuable for the evaluation of the effectiveness of disease prevention activities and for etiological studies of disease.

Prevalence measures prevailing disease and so emphases on the period when a person is ill. Prevalence measures the quantity of the total population that is diseased at a point in time or during a period of time. Its numerator contains the number of existing cases, and its denominator comprises the total population, including sick, healthy, at-risk, and immune individuals.

Point prevalence refers to a sole point in time and is like a snapshot. Period prevalence refers to an exact duration of time that may be derived from a sequence of snapshots. Prevalence is typically used for approximating the needs of medical amenities and for allocating resources for treating diseased individuals. The incidence rate and prevalence are mathematically correlated.

Many procedures of disease frequency are commonly used in public health, counting the crude, cause-specific, and age-specific mortality rates; morbidity rate; live birth rate; infant mortality rate; attack rate; case fatality rate; and survival rate.

**Check Your Progress**

8. Define genetic diversity.
9. Who were the primary founders of population genetics?
10. What do you understand by phenotype?
11. What is population genetics?

## 1.5 MOLECULAR POPULATION GENETICS

Molecular population genetics aims to explain genetic variation and molecular evolution from population genetics principles. The field was born 50 years ago with the first measures of genetic variation in allozyme loci, continued with the nucleotide sequencing era, and is currently in the era of population genomics.

### 1.5.1 Patterns of Changes in Nucleotide and Amino Acid Sequences

Molecular population genetics use population genetics principles as a foundation to explain the genetic variation and molecular evolution. The foundation of this discipline of study was laid 50 years ago with the calculations of genetic variation in allozyme loci, continued with the nucleotide sequencing era, and is presently in the era of population genomics.

Mutations arises with the growing population which helps in the natural selection form the diverse population and causes evolution of more stable ones. The comparison between the genomes of the model organism shows the high level of diversity between the organisms. These new prospects partially gave the molecular structure, function, and evolution of life. With the application of bioinformatics, allozyme and DNA diversity of both coding and non-coding genomic regions with helped in precise gene homologous alignment across taxa, the depiction of gene and genome structure, expression, function, regulation, evolution, and the possible resolution of the genetic basis of speciation and adaptation.

The first elucidation of genetic variation was done by applying the technique of protein gel electrophoresis to several allozyme loci in the species of *Drosophila pseudoobscura* and human. During this period, a very extensive and sophisticated

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theoretical foundation was built by population genetics by the integration of principles of Mendelian inheritance with forces affecting deviations in allele frequency in populations that required to validate the Darwinian view which states that biological evolution is a population process by which genetic variation between species is transformed into genetic variation between species.

With the publication of seminal articles explaining electrophoretically detectable variation—or allozymes (i.e., proteins differing in electrophoretic mobility as a result of allelic differences in the protein sequence, which eventually result from the presence of variation in the corresponding DNA sequence)—in D. pseudoobscura and also in humans, the population genetics entered the molecular age. A few dozen different soluble proteins were studied in 100s of species, mostly enzymes with well-understood metabolic roles.

There are two ways of measuring genetic diversity: the amount of average heterologous loci in an individual [heterozygosity or gene diversity (H)], and the average quantity of polymorphic loci in the population [gene polymorphism (P)]. The results of this electrophoretic surveys showed a huge quantity of genetic variation in most populations, much more than had been estimated, and appeared to unequivocally support the balance rather than the classical hypothesis. Precisely, 43% of polymorphic loci was found in Drosophila and H 12%. Also, the levels of genetic diversity were found to vary non-randomly among populations, species, higher taxa, and several ecological, demographic, and life-history parameters. For example, most invertebrates (including Drosophila) look as if to be highly polymorphic; whereas, reptiles, birds, and mammals are only about half as variable on average (e.g., in humans, P and H are about 28 and 7%, respectively), and fish and amphibians are middle in their variability. So, by this data, it can be concluded that the population size is a main parameter in population genetics and the neutral theory was given to account for molecular evolution. The (nearly) neutral theory as the paradigm, setting the stage for the long-lasting neutralist vs. the selectionists' debate.

As protein electrophoresis is the widely used technique for performing large-scale surveys of genetic diversity in a wide range of species, it has several limitations too.

First, allozyme polymorphisms can be detected if the DNA sequence of the amino acid sequence shows variation.

Second, as the basis of the technique is the separation on the basis of mobility, the amino acid which affects the mobility of a protein in a gel (mostly associated with charge changes) can only be detected by electrophoresis, which is only the one-fourth of all possible mutational changes which are due to amino acid substitution.

The charge-state model (or stepwise mutation model) by Ohta and Kimura in 1973 explain the results of electrophoretic studies while accommodating these limitations of allozyme markers, and this model was further shadowed by some extensions. As, in 1996, Barbadilla et al. showed that if charge is taken as identical to electrophoretic mobility, like in the charge-state model, then for every case it

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can be expected to get symmetrical bell-shaped spreading of mobilities where the high frequency charge classes have an intermediate mobility. So, it can be concluded that the pure basis of this observed frequency pattern of electrophoretic variants is due to the statistical relations and displays no evidence about the underlying evolutionary forces. Also, they displayed that the detection power of proteins variation and separating power of electrophoresis is a decreasing role of the number of segregating positions.

In conclusion of the protein electrophoresis technique to measure genetic variation and its given limitation, Lewontin in 1991 assessed this analysis technique as not only as the milestone of evolutionary genetics which represents the initial stage in a journey to survey genetic variation in the populations; but also as an annoying milestone because the boom of electrophoresis flooded the earlier diversity of empirical work in evolutionary genetics, and because of the lack of fit of empirical data to the evolutionary genetics theory. As it is clear that the information about the DNA variation is much important for gathering answers to the questions the population genetics had already raised. Lewontin in 1991 said, "Those of us who now study DNA sequence variation believe that at this level we will resolve the problems generated by electrophoretic studies and that finally, because the structure of the observation of DNA sequences is qualitatively different from observations of amino acid variation, that the ambiguities will disappear."

### The Nucleotide Sequence Era

The earlier studies of DNA sequence variation were based on the detection of variation in restriction sites by restriction enzymes. The practise was widely followed in Drosophila. It was the initial stage of representing genetic diversity on DNA sequences, including the nucleotide site diversity (p), the equivalent of H for nucleotide sites. Studies on Drosophila showed interesting pattern, regions of the genome with low recombination have very low levels of genetic variability.

The Milestone study was published by Begun and Aquadro in 1992 reporting one of the most important observations in molecular evolution which says that the local rate of recombination is strongly positively linked to the level of genetic variation. The explanation of the study is the automatous relationship between recombination and mutation. So, if the recombination is mutagenic, the regions of low recombination should also have a low mutation rate, and hereafter, lower interspecific divergence conferring to the neutral theory.

After 1980, the introduction of techniques like PCR amplification and automated Sanger sequencing for the study of DNA sequence variation was boon for the process. Various different phylogeographic studies were published for analysing one or numerous mitochondrial DNA (mtDNA) loci.

As the level of divergence was shown to be independent of local recombination rates, so the association between recombination rate and levels of polymorphism was credited to the fixation of advantageous mutations and the associated hitchhiking effect.

The lesser the recombination of a region, the higher the hitchhiking effect, and so is the reduction of linked neutral variation which explain for the observed

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correlation. This hitchhiking hypothesis seriously risked the Kimura's neutral theory of molecular evolution.

The study of nucleotide sequence variation was done primarily by resequencing or sequencing many copies of a whole contiguous region of the genome by Kreitman (1983) in the Adh gene region from the 11 independently isolated chromosomes of five natural populations of D. melanogaster.

As automated sequencing machines were not available at that course of time, the study used the difficult method of Maxam–Gilbert sequencing. 43 SNPs were exposed by Kreitman in 1983, of which only 1 was responsible for the two allozyme variants—fast (Adh-f) and slow (Adh-s)—found before in nearly all-natural populations, whereas, the other 42 were silent polymorphisms in Molecular Population Genetics 1007 either coding or noncoding regions which were not identified in protein electrophoresis before. With these SNP variants, four InDel (Insertion-Deletion) polymorphisms and two homo-polynucleotide runs were found outside the coding region of the gene.

These data put light on the undiscovered broad spectrum of various genetic variants distributed throughout the population, and also supported the view that most amino acid changes were selectively deleterious. After years of Kreitman's innovative study, the initiation of automated Sanger sequencing gave new variation data for dozens of genes in several species, including Drosophila. By these studies, it was concluded that the levels of variation at silent sites differ among different taxa by less than a factor of 10 (compared to allozymes, which vary by ,104) that SNPs be more than all kinds of structural variants, and that transposable element (TE) insertions separate as low-frequency polymorphisms.

Recently the genetic diversity levels are valued by the compilation of polymorphism data of 167 species in 14 phyla, which defined that the autosomal nucleotide diversity differs by the order of two to three magnitude only in compared to the population census (Nc, the actual number of individuals in a population), which possibly varies by a factor of 108–1010.

Reproductive strongly also affects greatly the genetic diversity of species with other various ecological factors and life-history traits.

In resequencing, haplotypes or independent sequences are sampled which contain the DNA of interest and are homologous. These haplotypes can be obtained directly for e.g., in *Drosophila melanogaster* the single chromosome can be extracted though they are essential to be inferred in other outbreeding organisms. Like the primary usage of allozyme data, this haplotype sequences are the important tool for construction of statistical sequences which permit the quantification of variation. The estimation of nucleotide diversity is done by considering each nucleotide site of a region like an independent unit (one-dimensional measures of variation).

The nucleotide sequences are not segregated independently but their haplotype structure is the result of recombination and various processes. So, during studies, taking the information merely about the frequency distribution of segregating sites is not complete. The vital component is the association these segregating

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sites make. The association of segregating units and the haplotype structure presents the complete information about a nucleotide region.

Nucleotides as explained, are not independent but are related to their neighbours. Studies has shown that alleles are grouped in blocks from 100–150 bp to 2 kb in the Drosophila genome and 100 kb in the human genome (1000 Genomes Project Consortium 2015).

Recombination between nucleotides, selective and demographic forces influence the haplotype structure which is depicted by the use of multi-dimensional measures of genetic variation, such as, estimators of Linkage Disequilibrium (LD).

Using such advanced methods like multi-dimensional; diversity methods provide the key inside to the fascinating information's about the history and evolution of a DNA region, counting the effective recombination rate r = 4Ner underlying the region (where Ne is the effective population size and r is the recombination rate per locus).

Haplotypic data give the high levels of genetic resolution to conclude about the evolutionary history and the underlying evolutionary process by using both the one-dimensional and multi-dimensional diversity components. This gives the whole explanation of sequence variation.

As very rich information about the population genetics statistics is now available, it is used by different software's applications to make inferences about population genetics. These software applications are supposed to provide the data analysis, including DnaSP and PAML, which are still extensively used software packages for population genetics.

The analysed data from various sequence have now been stored in these databases which make easier the study about the new genomes and to gather information about the exiting and already researched one for studies. As 30 years of surveys of nucleotide variation is already been done in either specific loci or in 100s of genomic regions at a time, very large numbers of sequences in many genes and species accumulated in the databases, and tools were established to make practice of these publicly available data to depict genetic diversity at a large scale. But, with the advancement, now also even the largest compilations of surveys of genetic diversity were incomplete because they showed genetic diversity in specific sampled regions of the genome rather than providing unbiased genome-wide measurements. It was clear that the next natural step to the description of genetic variation would be the re-sequencing of complete genomes.

## 1.5.2 Ecological Significance of Molecular Variations

Genetic divergence can also be the result of the changes found even at the microscale levels or due to the ecological heterogeneity which is also known as epigenetics.

Ecology conquers in genetic diversity and divergence of populations, which can be illustrated by the vivid level of observed polymorphism, heterozygosity, and gene diversity across the taxonomic and biotic borders. The distribution of genetic diversity is totally a non-random process which leads to the formation of

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distinct populations, species, life-zones, habitats, climates, and other biological characteristics across the ecosystem.

Genetic diversity is maintained in the level of natural population by the prime factor, Environmental heterogeneity. It maintains the diversity at all geographical scales - *global*, *regional*, and *local*. Particularly, the diversity is depicted at the microscale level as seen in the "Evolution Canyon" model, or where the microscales are exposed to sharp ecological inter-climates or inter-soils divergence supplemented by studies of biochemical networks, kinetics, and physiological function of protein variation. Like the DNA variation, mitochondrial and nuclear, protein variation too is highly the result of the natural selection.

The previous studies on allozymes and later in the DNA studies including SNPs have shown the pattern and correlation between the genetic diversity. It involved many unrelated species which are subdivided into different biotic regimes. They strongly involve natural selection in their genetic divergence of species. Natural selection can occur in several forms but the most likely of it is by the processes of spatiotemporally variable and altering stressful environments and epistasis during the life cycle stages of the organism, succeeds in guiding evolutionary dynamics of adaptation and speciation in nature.

Evolutionary forces also add to natural selection by mutation, migration, and genetic drift. These forces both directly or indirectly, interact with natural selection and thus contribute differentially conferring to conditions at the molecular level to the population divergence.

The natural selection of a population adapts the group to various altering conditions of habitat and environment exposed to them. Like in a population with the different genetics of individuals which is varying significantly possess the adaption differently from others. Some of the individuals in the group can possess traits that make them resistant to disease or cold, which increases the rate of survival of a group which when breed with the others. This genetic variation offers the increased rates of survival in different climates and environments to the organisms.

If the changes in the environment are random over time and comprises a variety of diseases and predators, some alterations among individuals maximises the chances of some individuals surviving to reproduce, while others do not. In disease resistance, genetic diversity is significant as a disease can destroy a homogeneous population in which all the individuals are equally vulnerable to the disease. The existence of genetic flaws is increased when a small, isolated population's individuals may be enforced to breed with close relatives. During inbreeding, some genetic weaknesses found in the parents can be increased in future generations.

The Darwin's theory of natural selection to the 21$^{st}$ century was again revived by Templeton in 2009. Quantitative tests for natural selection on individual genes or complete genomes emphasize selection analyses. For the complete knowledge on selection pattern, the role each evolutionary force play and the importance of each in correlation much be experimented. The complete study on the interactive patterns of these forces with other forces as well as the formation of direct cause-

effect relationships among abiotic, biotic, and genetic factors may need subtle detailed future experimentation at both the protein and DNA levels and their networks. Though, the first comprehensive estimation about it can be inferred only by the different field and laboratory experiments on various populations and species, contributory to a new science of whole genome structure, expression, and the influence of immense *noncoding* genomic regions in adaptation and speciation.

## 1.5.3 Emergence of Non-Darwinism-Neutral Hypothesis

With reference to the neural theory of molecular evolution, maximum evolutionally changes at the molecular level are due to the random genetic drift caused by selectively neutral nucleotide substitutions. The cohabitation of huge numbers of species in high-diversity communities, and the near-homogeneity of some long-range outlines of biodiversity laid a way for the formation of neutral theory which explained the process.

The characterization of these ecological procedures of drift, dispersal, and speciation and their impact explains the construction of communities. The neutral theory of molecular evolution opposes that most evolutionary changes occur by random drift. Numerous predictions of the neutral theory are available. Mainly significant is the one of relationship between evolutionary rate and neutral mutation rate which are both equal. Similarly, the frequency distribution of a mutant gene in a population is recognized. Founded on the predictions, attempts are being made to guess the types and intensities of natural selection by means of data on evolutionary changes and polymorphisms of genes and genomes.

The theory is clearly based on the neutrality. It is the primary component of the model. An ecological community is said to be neutral if its dynamics does not depend on species' labels. This explanation of neutral theory can be understood from two situations:

First, at the species level, the community dynamics are symmetric. Which means that demographic rates are equivalent for all species. This state is essential for the idea of neutrality and shaped Hubbell's original definition. But the definition formed on this understanding comprises of models in which diversity is preserved by powerful intraspecific negative density dependence – models that would be taken as essential representations by most ecologists (e.g., Volkov et al., 2005).

Therefore, a second condition comes to the picture according to which neutrality is intraspecific and density dependence is equivalent to interspecific density dependence. Which means that the absence of intrinsic 'rare species advantage' – a steadying mechanism which can distinguish the functioning of individuals on its place with the rare species taken from the enriched species area. In force, most neutral theories are symmetric in non-solitary at the stage of species, but also at the stage of individuals.

### Key Characteristics of Neutral Models

Though the definition of neutrality delivers an all-encompassing guiding principle, exact neutral models are desirable for the forecasts and exam of the theory, and

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the meaning lets the possibility for substantial difference amid these models. Most significant ecological processes which maximum neutral models comprise are stochastic drift, speciation, and dispersal. Properly described depiction of a neutral model in Figure 1.

**Stochastic Drift:** In characteristic neutral models, stochastic birth and death processes estimate the complex, multifaceted and characteristically random fine-scale processes that take place in actual ecosystems. The demographic stochasticity that rules the working of such models is also denoted to as drift. Drift is predicted to run the dynamics of species loads when stabilizing forces, characteristic of deterministic niche theories, are weak.

Nearly neutral models oblige the drift process by applying a zero-sum rule at the community level, whereas others do not.

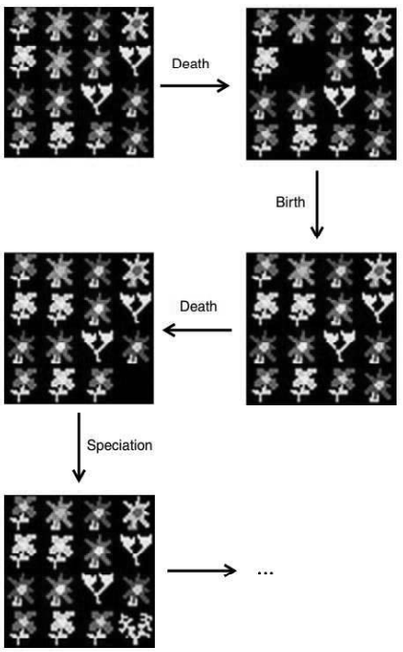


***Fig 1.9:*** *Representation of the procedures of birth, death, speciation and dispersal, shown on a spatially explicit lattice.*

The death of an individual unlocks a place on the lattice that is trailed with a birth event: the space is occupied by the offspring of an already existing organism. In spatially explicit models, dispersal restriction obliges the probability which state that the assumed organism's offspring would colonize the space positively, as a function of separation. The lattice structure here imposes a zero-sum restraint, so that the total number of individuals is fixed. Following models have relaxed this zero-sum constraint, so that the total number of individuals varies around a fixed average.

The current history of neutral theory has seen many advancements. Hubbell's original model has been widely tested, and maximum likelihood methods have supplemented a rigid backbone for approximation of neutral model parameters.

Similarly, the finding of new quantification tools is done for the simplification of the manner in which dispersal and speciation are used in the neutral models. Non-equilibrium field theory has given an outline of spatially explicit neutral theory in form of mathematical language of various-body physics. In count, most biologically accurate speciation styles have comprehensive Hubbell's original point specialization model, for example, random fission speciation copying from fragmentation philosophy.

**Check Your Progress**

12. What is the aim of molecular population genetics?
13. When were the first surveys of DNA sequence variation done?
14. Who revived the Darwin's theory of natural selection?

## 1.6 GENETICS OF QUANTITATIVE TRAITS

The phenotypic traits of the different organisms may be of two kinds, viz., qualitative and quantitative. The qualitative traits are the classical Mendelian traits of kinds such as form (e.g., round or wrinkle seeds of pea); structure (e.g., horned or hornless condition in cattle's); pigments (e.g., black or white coat of guinea pigs); and antigens and antibodies (e.g., blood group types of man) and so on. The quantitative traits, however, are economically important measurable phenotypic traits of degree such as height, weight, skin pigmentation, susceptibility to pathological diseases or intelligence in man; number of flowers, fruits, seeds, milk, meat or egg produced by plants or animals, etc.

### 1.6.1 Analysis of Quantitative Traits

Phenotypic traits from diverse organisms can be of two types, qualitative and quantitative.

The qualitative traits are basically the classical Mendelian traits of types like, form (e.g., round or wrinkle seeds of pea); structure (e.g., horned or hornless condition in cattle's); pigments (e.g., black or white coat of guinea pigs); and antigens and antibodies (e.g., blood group types of man) and so on.

The quantitative traits, though, are important quantifiable phenotypic traits which are of grade such as height, weight, skin pigmentation, susceptibility to pathological diseases or intelligence in human; number of flowers, fruits, seeds, milk, meat or egg produced by plants or animals, etc. The quantitative traits too are called metric traits.

They do not depict much differentiation among individuals and make a range of phenotypes that combine imperceptivity from one type to another to form continuous differences.

In distinction to qualitative traits, the quantitative traits may be altered diversely by the environmental conditions and are typically ruled be various factors or genes (maybe 10 or I00 or even extra), every factor adding a very less quantity

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of phenotype that its separate belongings can't be noticed by Mendelian methods but by only statistical methods. This type of genes that are non-allelic and result the phenotype of an only quantitative trait, are termed as polygenes or cumulative genes. Their inheritance is known as quantitative inheritance, multiple factor inheritance, multiple gene inheritance or polygenic inheritance. Certain characteristics of quantitative inheritance are mentioned below:

1. The division phenomenon happens on indeterminately high amounts of gene loci.
2. If a replacement of an allele occurs in a gene locus, then these allelic replacements have minor effects.
3. The genes of a multiple trait carry diverse biochemical purposes but alike phenotypic effects, so, the phenotypic effects of gene substitutions are substitutable.
4. Blocks of genes are destined together by overturns and conveyed as components from inversion heterozygotes to their progeny, but such blocks are fragmented up by crossing over in inversion homozygotes.
5. The polygenes carry pleiotropic effects; that is, one gene may adjust or overpower more than one phenotypic trait. A single allele may do only one thing chemically but may eventually affect many characters.
6. The environmental conditions carry significant effect on the phenotypic expression of poly genes for the quantitative traits. For example, height in various plants (e.g., corn, tomato, pea, and marigold) is genetically controlled quantitative trait, but some environmental factors, such as, soil, fertility, texture, water, temperature, duration and wavelength of incident light, occurrence of parasites, etc., also controls the height. Likewise, identical twins with identical genotypes, if cultivated in dissimilar kinds of environments, show different intelligence quotients.

Quantitative genetic variation is the substrate designed for phenotypic evolution in natural populations and for selective breeding of domestic crop and animal species. Quantitative genetic variation also stimulates susceptibility to common complex diseases and behavioural disorders in humans, as well as comebacks to pharmacological therapies.

Information of the genetic basis of variation for quantitative traits is thus important for addressing unanswered evolutionary questions about the keeping of genetic variation for quantitative traits within populations and the mechanisms of divergence of quantitative traits amid populations and species; for growing the rate of selective improvement of agriculturally important species; and for creating novel and more personalized therapeutic interventions to improve human health.

QTL mapping will recognize a genomic region comprising of one or more candidate genes affecting the trait. This is typically done in stages.

In the first stage, we plot Quantitative Trait Loci (QTLs) affecting the trait. QTLs are genomic regions in which one or more alleles disturbing the trait separate.

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In the second stage, we emphasis on to each QTL region to further slender the genomic breaks covering the gene or genes altering variation in the trait. The final stage is most stimulating: pinpointing the causal genes.

There are two basic approaches: linkage mapping and association mapping. Both of these approaches are based on the belief that QTLs can be followed via their genetic linkage to noticeable marker loci with genotypes that we can voluntarily classify.

Molecular markers are the most commonly used markers today such as Single Nucleotide Polymorphisms (SNPs), polymorphic insertions or deletions (InDels), or simple sequence repeats (also known as microsatellites). If a QTL is related to a marker locus, then most likely the individuals with different marker locus genotypes would be having a different mean value of the quantitative trait (Refer Figure 1.10).

Linkage mapping includes copying the linkage of a trait through a marker either by families in outbred populations (such as human populations), or by breeding experiments in which animal or plant strains that differ for the trait are crossed for several generations.

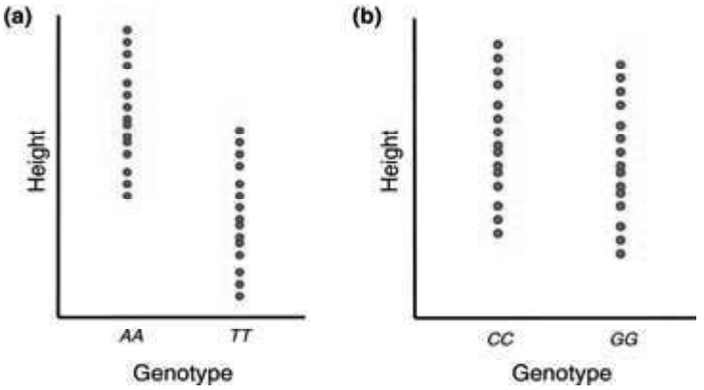


***Fig 1.10*** *Depiction of hypothetical data on height for 15 individuals at each of two marker loci, one with alleles A and T, the other with alleles C and G.*

In the above figure the individuals with the *AA* genotype are taller than those with the *TT* genotype. So, a QTL affecting height is linked to this marker locus.

There is no weighty difference in height between individuals with the *CC* and *GG* genotypes. Therefore, no QTLs affecting height are related to this marker locus.

In difference, association mapping aspects for relations between a marker and different values of a trait in unconnected individuals tested directly from a population. In both cases, we need to get quantities of the phenotype and know the marker locus genotypes for all individuals in the mapping population, at all marker loci. Then statistical method is adopted for determining if there are differences in the worth of the quantitative trait among individuals with different marker locus genotypes; if so, the QTL is related to the marker. This is repeated for every marker (or pair of adjacent markers) to achieve a genome scan for QTLs.

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Significance threshold is the horizontal line parallel to the *x*-axis and crossing the *y*-axis at the appropriate value. The significance threshold has been attuned to account for the number of autonomous tests performed, and was resolved by a permutation test. Indication for relation of a QTL with markers happens when the test for linkage produces a significance level that surpasses the permutation threshold. The best approximation of the QTL location is the position on the *x*-axis conforming to the greatest significance level.

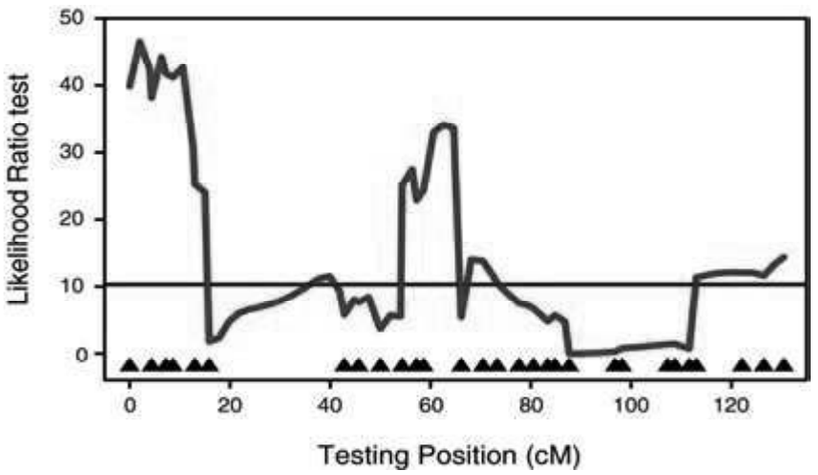


***Fig 1.11*** *The graphical representation depicts the results of a genome scan in which the positions of the markers are given on the x-axis (black triangles), and the result of the statistical test is specified on the y-axis (here a likelihood ratio test).*

## 1.6.2. Quantitative Traits and Natural Selection

Variation in the trait must be connected with fitness, should be genetically heritable and there should be no genetic constraints to evolution for natural selection to cause an evolutionary change in a trait. Fitness of individuals is related to the quantitative trait by the fitness function *f* in natural selection.

This function plays an important role in the prediction of fitness variances among individuals and in showing that whether an ideal is present within the series of phenotypes in the population or not. It may also be supposed of as recitation of the ecological environment in relation of the trait. Measurements of natural selection are helpful in studies of adaptation.

Current theoretical work in this area has effectively addressed two main goals:

First, for the development of coefficients of selection intensity for quantitative traits, unravelling direct from indirect effects and

Data of natural selection can also be implemented for a third purpose: for estimation of the selection surface or fitness function, the unknown function f connecting survival and/or reproductive achievement of individuals (JW) to the phenotypic character z in selection:

$W = f(z) + \text{random error}$

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As the fitness function is a complete description of selection pressures on individuals, it is of interest to study upon. For example, individuals survive differentially according to body size, in which case W is survival (1 or 0) and f (z) is the probability of existence as a function of size.

Natural selection is well-defined as inconsistency among phenotypes in fitness. Three types of univariate selection are commonly documented, on the basis of the form off over the array of phenotypes in the population.

Second, to guess evolutionary comeback to observed selection events.

**In directional selection**, individuals on one side of the population mean are favoured over those on the other. Directional selection may occur with other type as well, but in pure directional selection, fitness is either non-decreasing or non-increasing over the series of phenotypes (i.e., f is monotonic).

**In stabilizing selection**, intermediate phenotypes are favoured over extremes-the function has a mode, or optimum.

**In disruptive selection**, fitness of extreme individuals is greater than that of intermediates-the surface has a dip, or minimum.

The quantitative estimate of survival probabilities of individuals, the assessment of probabilities among individual's differing in size, and the calculation of whether an optimum body size occurs within the array of phenotypes present in the population by using the estimation.

When the knowledge about the broader range of phenotypes than is really found in any one population also primes to an approximation of the "adaptive landscape," describing mean fitness in the population WV as a function of z, the mean value of the phenotypic trait in selection.

When the trait is heritable and uncorrelated w with other traits under selection, then the direction of alteration in z leading to the extreme increase in mean fitness will typically be the track of evolutionary change in the population.

## 1.6.3 Genotype- Environment Interaction

Gene environment interaction (or genotype environment interaction or GxE or G×E) is termed as the process in which two different genotypes retort to environmental difference in diverse ways.

For the improvement of accuracy and precision in the assessment of both genetic and environmental influences, study about gene-environment interaction is important. A model of reaction is a graph that displays the association between genes and environmental factors when phenotypic changes are nonstop. It can provide assistance in the demonstration of GxE interactions. If the model of reaction is non parallel, as depicted in the figure beneath, it is because of the gene by environment interaction.

This shows that each genotype replies for the environmental difference in a unique style. Environmental difference can be physical, chemical, biological, behaviour patterns or life events.

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Gene–environment relations are focused for increasing the knowledge about various phenomena. In genetic epidemiology, gene–environment interactions are valuable for understanding some diseases. Every so often, the sensitivity to environmental risk factors for a disease are likely to be inherited in comparison to the disease itself getting inherited.

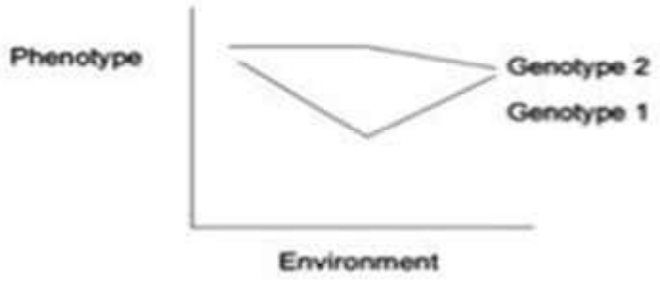


***Fig 1.12:*** *Model of reaction depict lines which are not parallel demonstrating a gene by environment interaction. Every genotype replies in the unique way to all the environmental variations.*

The exposure of similar environmental factors affects the individuals with the different genotypes differently. So, the gene–environment interactions can cause dissimilar disease phenotypes. For example, sunlight exposure has a stronger effect on skin cancer risk in fair-skinned humans in comparison to the individuals with darker skin.

These interactions are of specific attention to genetic epidemiologists for the prediction of disease rates and methods of inhibition with respect to public health. The term is also used between developmental psychobiologists to understand individual and evolutionary development in a better way.

The debate between the nature and nurture assume that variation in a trait is primarily due to either genetic variances or environmental variances. Though, the present scientific view holds that neither genetic differences nor environmental differences are only accountable for the production of phenotypic variation, and that virtually all traits are prejudiced by both genetic and environmental changes.

Statistical examination of the genetic and environmental differences causative to the phenotype can be studies to understand the gene–environment interactions. In developmental genetics, a fundamental collaboration is sufficient for assuring it as a gene–environment interactions.

1. **In Drosophila:** Gene–environment interaction can be seen in the classic experiment performed on *Drosophila* by Gupta and Lewontin in 1981. In which according to their demonstration the mean bristle number of *Drosophila* might differ according to the altering temperatures. Like it can be understood from the graph depicted below, the different genotypes react contrarily to the altering environment. Every line signifies a specified genotype, slope of the line imitates the altering phenotype (bristle number) with varying temperature.

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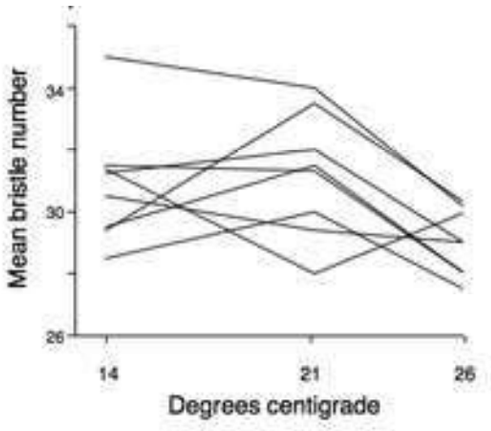


***Fig 1.13:*** *Mean bristle number by °C*

Of the studies groups, some of them may be having a rise in bristle number with rising temperature and some may have a shrill reduction in bristle number with reducing temperature. This presented that the models of reaction were non parallel for the studied flies, verifying that gene–environment interactions exist.

2. **In Plants**: One very exciting method of genotype and environment interaction is its use in the choice of sugarcane cultivars which are adapted to different environments. For example, after the analysis of twenty sugarcane genotypes grown-up at eight diverse locations completed, two crop cycles to recognize mega-environments correlated with advanced cane harvest, calculated as tons of cane per hectare (TCH) and percentage of sucrose (Pol% cane) by means of biplot diverse variety of GEI models. The authors then formed a new plan for studying both yield variables in a two-way joined plan even though the results presented a mean negative correlation. Through coinertia analysis, the finding of the best-fitted genotypes for both yield variables in all environments become easy.

   The use of these new plans like coinertia in GEI, showed the great match for analysis of AMMI and GGE, particularly when the harvest development suggests numerous yield variables. Seven genetically different yarrow plants were taken and three cuttings occupied from every plant.

   The section extracted from every genotype was grown with different elevations like low, medium, and high elevations, individually. Upon the maturation of plants, it was observed that no anyone genotype of them shown best results for all altitudes, and all of them showed coping for the elevations diversely. For example, if one genotype raised the tallest at the average elevation but reached only average height at the remaining two elevations. The finest growth at low had poor growth at high elevation and medium elevation. Of all the results, cumulatively, it was seen that

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the nastiest overall results was shown at the medium elevations, then also, it was able to give one tall and two medium-tall samples.

Elevation had a consequence on each genotype, not in the same order or in similar way. A sorghum bi-parental population was frequently grownup in seven varied geographic sites in a year. A collection of genotypes needs alike Growing Degree-Day (GDD) to flower in all different environments, though additional collection of genotypes requires fewer GDD in particular environments, but advanced GDD in different environments to flower. The multifaceted flowering time designs is credited to the collaboration of main flowering time genes ($Ma_1$, $Ma_6$, *FT*, *ELF3*) and an explicit environmental factor, Photo-Thermal Time (PTT) taking the correlation amid temperature and photoperiod.

**Check Your Progress**

15. What do you mean by the quantitative traits?
16. Mention any three characteristics of the quantitative inheritance?
17. What do you understand by gene environment interaction?

## 1.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Life can be defined as the ability of an organism to grow, reproduce and produce energy through chemical reactions by using natural resources like air, water, sunlight, etc.
2. When it was found initially Earth had only two components: Solid Mass (Lithosphere) and the surrounding Gaseous Envelope (Atmosphere).
3. The Earth consists of following three different parts:
   a. Bryosphere
   b. Pyrosphere
   c. Lithosphere
4. Darwin's theory of natural selection (Darwinism) is based on the three observations:
   a. Over production or prodigality of nature (organisms increase in geometric proportion).
   b. Constancy of number (size of the population remains more or less constant).
   c. Occurrence of variations (living organism exhibit variations).
5. Neo-Darwinism, also called the modern evolutionary synthesis, was introduced by Julian Huxley to generally designate the integration of Charles Darwin's theory of evolution by natural selection, Gregor Mendel's theory of genetics as the basis for biological inheritance, and mathematical population genetics.

6. A population is said to be in genetic equilibrium when the allelic frequency in the gene pool remains constant through generations.
7. The Chi-Square Value can be calculated using the formula:

$$\chi^2 = \Sigma \frac{(\text{Observed-Expected})}{\text{Expected}}$$

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8. The total genetic variation among the people in a specific group or environment is referred to as genetic diversity.
9. Population genetics was founded by Sewall Wright, J. B. S. Haldane and Ronald Fisher.
10. Phenotypes are traits or characteristics of an organism that can be observed morphologically, such as, size, colour, shape, capabilities, behaviours, etc.
11. The subfield of genetics that relates with the genetic variances found across and between the populations is known as population genetics.
12. Molecular population genetics aims to explain genetic variation and molecular evolution from population genetics principles.
13. The first surveys of DNA sequence variation were done in the 1980s using restriction enzymes to detect variation at restriction sites.
14. The Darwin's theory of natural selection to the 21st century was again revived by Templeton in 2009.
15. The quantitative traits, are economically important measurable phenotypic traits of degree such as height, weight, skin pigmentation, susceptibility to pathological diseases or intelligence in man; number of flowers, fruits, seeds, milk, meat or egg produced by plants or animals, etc.
16. The quantitative inheritance has following characteristics:
    a. The segregation phenomenon occurs at an indefinitely large number of gene loci.
    b. If a substitution of an allele occurs in a gene locus, then such allelic substitutions have trivial effects.
    c. The genes for a multiple trait have different biochemical functions but similar phenotypic effects, therefore, the phenotypic effects of gene substitutions are interchangeable.
17. Give environment interaction means different genotypes retort to environmental difference in diverse ways.

## 1.8 SUMMARY

- Life can be defined as the ability of an organism to grow, reproduce and produce energy through chemical reactions by using natural resources like air, water, sunlight, etc.
- Evidences suggest that Earth came into being around 4.5-5 billion years ago. Initially Earth had only two components: Solid Mass (Lithosphere) and the surrounding Gaseous Envelope (Atmosphere).

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- Bryosphere is the central core of the Earth filled with molten Magma having large amount of Nickel and Iron.
- Lithosphere is the outermost region of the Earth, also called as crust. It is around 20-25 miles in thickness and mostly filled with Aluminum and Silica
- Primitive Earth had very little or no Oxygen. During that time Earth had a reducing environment because of predominance of Hydrogen and Hydrogen compounds with Water (such as, Methane and Ammonia).
- Theory of Catastrophism is the extension of the theory of special creation. This theory assumes that life is originated because of special creation and it was followed by catastrophe due to geographical disturbances which killed the created life forms.
- Each catastrophe completely wiped out the previous life forms whereas each creation forms new life different from the previous ones.
- Cosmozoic Theory was given by Richter and strongly supported by Arrhenius. It proposed that life was present in the form of resistant spores in space throughout the universe and reached Earth accidently.
- Theory of Eternity of Life proposed that life had neither beginning nor end. It assumes that life has ever been in existence and it will continue to be so in future as well.
- Modern Theory is also known as 'Chemical Theory' or 'Theory of Primary Abiogenesis'.
- The modern theory proposed that non-living materials can give rise to life in primitive Earth conditions known as abiogenesis.
- Hydrogen, nitrogen, oxygen and carbon were the elements which was most abundant on the primitive Earth.
- Large organic molecules aggregate spontaneously with each other and form large colloidal aggregates called as 'Coacervates'.
- Charles Darwin was born (12 February, 1809) in England sailed on H.M.S. Beagle for world exploration and visited some of islands of Atlantic ocean, coast of South America and some islands of South Pacific.
- The 'fittest' were thought of as those individuals that possess inheritable characteristics enabling them to succeed in the 'struggle for existence' in the particular circumstances and environment in which they live.
- Darwin observed that the various useful variations are selected by individuals and thus, evolution results. Darwin assumed variations as axiomatic without describing their real nature and origin, in plant and animals.
- Natural selection favours only suitable (adaptive) variations and less-adapted variations are lost from the population with the death of organisms. These adaptive variations are accumulated in the gene pool of population and lead to speciation.
- Neo-Darwinism was introduced by Julian Huxley to generally designate the integration of Charles Darwin's theory of evolution by natural

selection, Gregor Mendel's theory of genetics as the basis for biological inheritance, and mathematical population genetics.

- The Hardy-Weinberg law suggests that the allelic frequencies do not change because of reproduction. But there are forces that increase and decreases the allelic frequency.
- The destabilizing forces that lead to change the allelic frequencies include mutation, genetic drift, migration, natural selection, and meiotic drive.
- The natural selection can be defined as 'the differential reproduction of organisms as a function of heritable traits that influence the adaptation in the environment'.
- The raw material for natural selection is genetic variation. If all the species are genetically identical, then the process of natural selection would not commence regardless of the fact that they can differentially reproduce.
- The environmental factors like ionizing radiation, chemicals and viruses constantly generates novel mutations in a genetic pool and thus destabilizes the genetic equilibrium.
- Migration tends to limit the divergence by generating continuous gene flow, which in turn increases the similarity between two populations.
- Migration has the potential to import beneficial alleles locally, but immigration from other environments may introduce alleles that are less fitted in the local habitat.
- The Segregation Distorter (SD) gene is an example of meiotic drive in Drosophila melanogaster.
- The SD is an autosomal meiotic drive gene, which induces the destabilizing of SD+ (wild type) gene during meiosis at the time of spermatogenesis. It leads to a population exclusively bearing SD over SD+ genotype.
- Genetic variation is vital for the long-term survival of species and supplies the raw material for all evolutionary changes in natural populations. The overall genetic variation among people in a group is referred to as genetic diversity.
- The original, modern synthesis view of population genetics assumes that mutations provide ample raw material, and focuses only on the change in frequency of alleles within populations.
- Phenotype is the constellation of observable traits. In a narrow 'genetic' sense, the genotype defines the phenotype. Phenotypes are traits or characteristics of an organism that we can observe, such as size, colour, shape, capabilities, behaviours, etc.
- Population geneticists usually define 'evolution' as any change in a population's genetic composition over time. The four factors that can bring about such a change are: natural selection, mutation, random genetic drift, and migration into or out of the population.

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- Natural selection occurs when some genotypic variants in a population enjoy a survival or reproduction advantage over others.
- Population geneticists have long been interested in exploring what happens when selection and mutation act simultaneously.
- Migration into or out of a population is the fourth and final factor that can affect its genetic composition. Obviously, if immigrants are genetically different from the population they are entering, this will cause the population's genetic composition to be altered.
- The aim of two-locus (and more generally, multi-locus) models is to track changes in gene frequency at more than one locus simultaneously. Such models are invariably more complicated that their single-locus counterparts, but achieve greater realism.
- Incidence and prevalence are the two basic measures of disease frequency. Incidence measures the occurrence of new disease and so captures the transition from health to disease.
- Bioinformatics analysed allozyme and DNA diversity at both coding and noncoding genomic regions permitting precise gene homologous alignment across taxa, the unravelling of gene and genome structure, expression, function, regulation, evolution, and the potential determination of the genetic basis of speciation and adaptation.
- Different levels of polymorphism, heterozygosity, and gene diversity cut across taxonomic and biotic borders, highlighting that ecology prevails in genetic diversity and divergence of populations.
- The development of neutral theory was motivated by the goals of explaining the apparent coexistence of large numbers of species in high-diversity communities, and the near-universality of certain large-scale patterns of biodiversity.
- Although the definition of neutrality provides an overarching guiding principle, specific neutral models are needed to generate predictions and test the theory, and the definition allows scope for substantial variation among these models.
- The phenotypic traits of the different organisms may be of two kinds, viz., qualitative and quantitative.
- For natural selection to cause evolutionary change in a trait, variation in the trait must be correlated with fitness and be genetically heritable and there must be no genetic constraints to evolution.
- Gene–environment interactions are studied to gain a better understanding of various phenomena. In genetic epidemiology, gene–environment interactions are useful for understanding some diseases.
- A classic example of gene–environment interaction was performed on Drosophila by Gupta and Lewontin in 1981. In their experiment they demonstrated that the mean bristle number on Drosophila could vary with changing temperatures.

## 1.9 KEY TERMS

- **Bryosphere:** It is the central core of the Earth filled with molten magma having large amount of Nickel and Iron.
- **Pyrosphere:** It forms the middle part of the Earth, also called as Mantle. It is around 1800 miles in thickness and mainly consists of Manganese, Silica, and Magnesium.
- **Natural Selection:** The natural selection can be defined as the differential reproduction of organisms as a function of heritable traits that influence the adaptation in the environment.
- **Allozymes:** These are different molecular forms of an enzyme that correspond to different alleles of a common gene (locus).

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## 1.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What are the proposed steps for the origin of life?
2. What are the applications of Hardy-Weinberg Law of Genetic Equilibrium?
3. Briefly explain the destabilizing forces which affect allelic frequencies.
4. Write a short note on two-locus models.
5. What are the fundamental ecological processes that most neutral models include?
6. Briefly explain the gene-environment interaction.

**Long Answer Questions**

1. Explain the major theories that have been proposed to explain the origin of life on Earth.
2. Discuss the calculation of population genotypes using Hardy-Weinberg Law of Genetic Equilibrium.
3. Explain the genetic structure of natural populations.
4. Describe the different single locus models of changes in genetic structure of population.
5. Discuss the patterns of changes in nucleotide and amino acid sequences.
6. Analyze the quantitative traits and its role in natural selection.

## 1.11 FURTHER READING

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# UNIT 2 POPULATION GENETICS AND EVOLUTION - II

NOTES

**Structure**



## 2.0 INTRODUCTION

Population genetics is a branch of genetics that studies genetic changes within and across populations. It is also a branch of evolutionary biology. Adaptation, speciation, and population structure are all studied in this discipline of biology. The emergence of the contemporary evolutionary synthesis required the use of population genetics. Sewall Wright, J. B. S. Haldane, and Ronald Fisher were the key pioneers. Modern population genetics covers theoretical, laboratory, and field study, and is traditionally a highly mathematical science. Population genetic models are utilised for both statistical inference and proof/disproof of concept using DNA sequence data. The emphasis on genetic processes like dominance, epistasis, and the degree to which genetic recombination breaks linkage disequilibrium distinguishes population genetics from newer, more phenotypic approaches to modelling

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evolution, such as evolutionary game theory and adaptive dynamics. In this unit, we will discuss the concepts of molecular evolution and origin of higher categories, along with the molecular phylogenetics. It will also focus on the origin and evolution of economically important microbes and animals, and the relationship between population genetics and ecology.

## 2.1 OBJECTIVES

After going through this unit, you will be able to:

- Describe the concepts of molecular evolution and origin of higher categories
- Explain the significance of molecular phylogenetics
- Discuss the origin and evolution of economically important microbes and animals
- Evaluate the relationship between population genetics and ecology

## 2.2 MOLECULAR EVOLUTION

Molecular evolution is a branch of evolutionary genetics that enables the study of process and pattern of evolution using molecular data. The tremendous advances in molecular genetics in recent years materialized into investigations of an evolutionary change directly by analyzing protein and nucleic acid sequences. The molecular data makes it convenient to study the path and trends of evolution. Until it became possible to examine genes directly, evolutionary biology was restricted to the study of time course related change of phenotypes of organisms. Under molecular evolution three main aspects are studied which are gene evolution, evolution of gene families and assessment of molecular variations as explained under.

### 2.2.1 Gene Evolution

New genes are the roots of new phenotypes or traits. How new genes originate and how they evolve is studied under the field called as gene evolution. It started in early 1940s with a group of scientists debating over which types of mutations are responsible for the significant differences observed between the lower or 'primordial' life forms such as amoeba and human. In the current genomic era, researchers are not only harbouring a database of the genomes of millions of species but also of different individuals within the same species. A detailed analysis and comparative studies of the protozoan, lower invertebrate and human genomes confirms the fact that the creation or evolution of new genes is of the most important sources of evolutionary change. Molecular processes responsible for gene evolution are:

**Gene Duplication:** New genes are created through a number of molecular events, acting either singly or in conjugation active at various levels in different genomes. Different classes of new genes are generated by unique molecular pathways, each with distinct molecular signatures (sequence of nucleotides) that well evident in

genomic sequence data. A complete set of techniques are available for pin pointing the exact time of the geological clock where the new gene originated, and it totally depends upon the class the new gene falls in that decides whether it might be easy and direct or is it impossible to determine which is the original copy gene (parental gene) and which copy is the new gene (offspring). Gene duplication is certainly established as one of the most prominent causes of evolutionary change and has several functional and evolutionary consequences. Duplication of sequences could be spanning a few nucleotides (base pairs) to involving the entire genome. A varying fraction of all genes-15-20% in some bacteria to 65% in the plant Arabidopsis has been found to be duplicated. Both homologous recombination or HR) and homology independent (non-homologous end joining or NHEJ) have been important in presenting copy number changes, i.e., duplication of genes.

**Inversions, Translocations, and Deletions:** Inversions, translocations, and deletions–all may create novel genes by reorienting and recombining the two sequences belonging previously to independent gene. For example, gene fusion, where two originally distinct genes are fused together in the same transcript giving rise to a novel protein. Gain-of-function mutations which exclusively are a consequence of gene fusions play an important role in many human cancers. Distinct gene sequences may also get joined via exon shuffling, which corresponds to recombination mediated rearrangement of exons originating from two or more different genes.

**Lateral Gene Transfer:** Lateral gene transfer also referred to as horizontal gene transfer is much common among certain prokaryotes, causing gain of new genes imparting several novel functions that contribute dramatically to their evolution. Horizontal (lateral) gene transfers can be identified from the organism's whole genome sequence data using numerous methods. A lateral gene transfer event generates anomalous or incongruent phylogenetic trees, whereby a particular gene may share the highest sequence similarity with a gene in a distantly related species. Laterally transferred genes can be identified in genomes when there are contigs (sequence reads) containing nucleotide stretches belonging to different genomes. Although prokaryote–prokaryote lateral gene transfers are considered to be fairly abundant, prokaryote–eukaryote (and eukaryote–prokaryote) are much rarer and eukaryote–eukaryote even more. Examples are several genes in eukaryotic nuclear genomes that came from mitochondrial and plastid genomes.

**De-novo Genes**: De novo genes refer to events, where a previously non-coding region is converted into a coding region via mutations or other molecular processes. De novo genes being quite rare, are said to be responsible for adding new exons (i.e., de novo exons). However, in 2006, Levine et al. reported the existence of five new genes in the D. melanogaster genome, all derived from noncoding DNA.

## 2.2.2 Evolution of Gene Families

Gene families have evolved mainly via concerted evolution. The birth of a gene family within a genome can occur in one of the several ways involving genome duplication, tandem (segment) duplication and duplicative transposition. Many gene families exist as clusters in the human genome which were formed by tandem gene

duplication. Duplication may include smaller or larger DNA segments that may not cover full gene and such duplications play vital role in acting as sources of genetic variability and also for acquiring new gene functions. Haemoglobin gene clusters of mammals are typical cases of tandem gene duplication. The primordial haemoglobin HBá gene duplicated to give rise to HBæ and HBè genes, and the human HBá cluster now contains four functional genes and three pseudo genes. Similarly, primordial HBâ gene duplication resulted in the present HBâ gene cluster. Mammalian Hox gene clusters are also good examples of tandem gene duplication.

Gene duplication is also attained by reverse transcription that occur in germ cell lineages. Templates for reverse transcription may be messenger RNA (mRNA), transfer RNA (tRNA), transcribed repetitive sequences such as Long Interspersed Nuclear Elements (LINEs) and Short Interspersed Nuclear Elements (SINEs) and retroviruses. Some of these reversely transcribed regions are called retro sequences and may become regulatory elements of adjacent genes. mRNA - derived retro-sequences may evolve into new members of the gene family to which they belong. The information flow is sometimes called homogenization. The resulting phenomenon wherein, the gene members display more homology (sequence identity) when compared among the same species than when compared between many species is called concerted evolution. Gene conversion and unequal crossing-over are the mechanism behind the concerted evolution.

There are many examples of gene families which have undergone massive evolution during the course of time, for example, Hox gene family that contains homeobox sequence of 180 nucleotides coding for 60- amino acid long DNA binding homeodomain functioning for transcriptional regulation. Human leukocyte antigen (HLA) that are present in major histocompatibility complex (MHC) region of human genome and harbour immunoglobulin domain belong to the immunoglobulin superfamily. The Class I and class II families of HLA which are involved in antigen presentation are best studied as gene family. MADS-box genes family has MADS-box sequence encoding 60 amino acids. This domain binds to DNA and is highly conserved. Major histocompatibility complex (MHC), another example region widely known because of extraordinary polymorphisms, has effect on disease susceptibility, tissue transplantation and graft rejection.

## 2.2.3 Assessment of Molecular Variations

The parameters are commonly used to measure the amount of variation in populations. The proportion of polymorphic loci is the proportion of examined loci in which more than one allele is present in a population. If we examined 50 different loci and found two or more alleles present at 25 of these loci, the percentage of polymorphic loci would be 25/50 = 0.5. The expected heterozygosity is the proportion of individuals that are expected to be heterozygous at a locus under the Hardy-Weinberg conditions, which is 2*pq* when there are two alleles present in the population. The molecular methods or techniques used for analysis of variations are grouped under two categories, viz. Non-PCR based (Restriction Fragment Length Polymorphism, i.e., RFLP, Ribotyping and Pulse Field Gel Electrophoresis, i.e., PFGE) and PCR based methods (e.g., Amplified Fragment Length Polymorphism, i.e., AFLP, Repetitive element PCR, i.e., Rep-PCR,

Random Amplified Polymorphic DNA, i.e., RAPD, VNTR and Multiple locus VNTR, i.e., MLVA, etc.). Some sequencing based methods like MLST (Multilocus Sequence Typing) and SNP (Single Nucleotide Polymorphism) are widely used in addition to the above. Now a days, PCR-methods combined with DNA Microarray are very popular for analyzing variations. Analysis of Molecular Variance (AMOVA) is a statistical tool inspired by the classical ANOVA (Analysis of Variance). AMOVA is applied to analyze variations among members of a single biological species. This test method was developed by Laurent Excoffier, Peter Smouse and Joseph Quattro in 1992.

**Check Your Progress**

1. How did gene evolution start as a field?
2. Who developed the AMOVA test?

## 2.3 ORIGIN OF HIGHER CATEGORIES

In taxonomy, higher categories are the classifications above the level of species, which are defined arbitrarily according to observed similarities among species, and which provide a useful hierarchical framework by which organisms may be described succinctly.

### 2.3.1 Phylogenetic Gradualism and Punctuated Equilibrium

Phyletic gradualism is a hypothesis about the pattern of evolution. It is a model of evolution which states that most speciation is slow, uniform and gradual. No clear line of demarcation exists between an ancestral species and a descendant species, except in cases where splitting occurs. Unlike the theory of punctuated equilibrium, it highlights the following points:

- Evolution occurs at a fairly constant rate.
- Gradual transformation of ancestral species gives rise to new species.
- The rate of evolution during the origin of new species is much like that at any other time.

According to this school of researchers even if a sudden appearance of a species is revealed by the fossil evidence with little signs of any transitional forms is owing to the incomplete fossil record. The crucial difference between punctuated equilibrium and phyletic gradualism concerns the rate at, and between, splitting events. Darwin himself was partly committed to gradualism because he credited gradualism for the evolution of adaptations, but not for the pattern of evolutionary rates.

**Punctuated Equilibrium**

Niles Eldredge and Stephen Jay Gould (1972) advocated the flash or abrupt appearance of new species or taxonomic forms and explained it with their theory of *Punctuated Equilibrium*. Although, the relative importance of punctuated and

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gradual patterns of evolution remains an issue of debate and scientific investigation. This theory says that species are generally stable, changing little for millions of years. This extremely slow rare or pace is interrupted or punctuated by a sudden burst of change resulting in a new species and this event leaves few fossils behind. As per this idea, the changes generating a novel species rarely occur in the mainstream population of an organism, where changes wouldn't endure due to extensive interbreeding among closely related species. Rather, speciation is more pronounced at the edge of a population, which usually is followed by a small group being easily separated geographically from the main body and acquiring variations/changes that provides a survival advantage and produces new, non-interbreeding species.

This theory opposes Darwin's more dynamic model of evolution. Punctuated equilibrium states that evolution only takes place in bursts of time that are rapid. It must be kept in mind that 'Rapid' in evolutionary terms could at times mean nearly as long as 500,000 years in some circumstances. Prior to the change which is often caused by an environmental factor, the life form's species or class lives in 'stasis' or an unchanged state for many, many years just as there is no need for change. Once the change takes place, quite quickly, the species re-enters a condition of stasis preserving its new evolutionary adaptation. Examples of punctuated equilibrium include:

- A marine species lives, breeds, and dies for thousands of years in stasis. All of a sudden, drastic changes in sea level creates a survival pressure and the animals must adapt. Their bodies develop in order to accommodate the environmental change, and from then on they diverge to become evolutionarily different from their ancestors.
- A species of birds exists in stasis for many thousands of years. Suddenly, their habitat is destroyed by, e.g., a bacterial attack causing their primary tree of shelter choice to die. Now, a pressure imposes itself upon the birds to adapt within the same environment to trees that are taller requiring more strong wings. Some birds die and the remaining birds which are fit enough adapt as per necessity and they return to a state of stasis.
- A worm species is living in the soil in a specific micro-environment (climate) and is in a state of stasis. Then changes occur in the climate causing the pH change of the soil. Some worms die as a result of this change, but those that survive adapt and reproduce with new ability to endure the altered pH of the soil. The species returns to stasis.

## 2.3.2 Major Trends in the Origin of Higher Categories

The origin of higher categories (taxa) and the underlying causes behind follow a complex pattern still we can see some specific trends in these events. The number of new categories at higher levels peak earlier than at lower levels (e.g., most phyla by end of Cambrian period). The time of appearance of a higher taxon will equal the oldest time of any contained taxon; higher taxa contain multiple lower taxa. For example, discovery of a fossil snail, clam, or squid would automatically

establish the origination (presence) of molluscs. As a rule, the time of appearance of a higher taxon will equal the oldest time of appearance of any contained taxon. Animals show a rapid increase in phylum diversity in the late pre-cambrian and Cambrian, with a slight to pronounced lag in the peak number of new taxa at successively lower levels. This is followed by time to time later peaks at various taxonomic levels. Protists, fungi, plants, etc., exhibit a more gradual increase in diversity but show similar lags and peaks. In conclusion, new higher taxa show appearance usually sooner than the lower taxa.

## 2.3.3 Micro and Macro Evolution

Micro-evolution is an alteration within a gene pool of a population over time that ends up in minor changes of an organism in the same species. In contrast, macro-evolution refers to those alterations or changes in organisms which gradually give rise to completely new species which are both genotypically as well as phenotypically apart from their ancestors. Macro-evolution differs from micro-evolution, as there are many observations of variation in case of micro-evolution and these variations do not require any statistically significant increase of functional genetic information; but in case of macro-evolution, the genetic change requires a statistically significant increment of functional genetic information, that is hard to attain. The main differences between micro and macro-evolution are listed in Table 2.1.

***Table 2.1*** *Differences between Micro and Macro-Evolution*

| **Micro-evolution** | **Macro-evolution** |
|---|---|
| 1. This type of evolution occurs on a small scale and within a single population. | 1. This evolution occurs on a large scale and goes beyond the level of the single species is macro evolution. |
| 2. It gives rise to changes in the gene pool, which results in a few changes in the same species also called Intra-species genetic change. | 2. New species originate as a result of Macro-evolution. |
| 3. The changes in micro evolution are acquired over short timescales. | 3. The changes seen in macro evolution take place over a long-time span. |
| 4. Genetic information gets altered or rearranged. | 4. There is alteration in genome resulting in the new species. |
| 5. Creationists support this type of evolution | 5. Due to insufficiently available experimental proof creationists don't support this theory of evolution. |
| Example: The peppered moth, new strains of flu viruses, Galapagos finches. | Example: Origin of different phyla, development of vertebrates from invertebrates, development of feathers. |

**Check Your Progress**

3. What do you mean by higher categories?
4. What does the phyletic gradualism emphasize?

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## 2.4 MOLECULAR PHYLOGENETICS

Molecular Phylogenetics is the field of evolutionary genetics dealing with analysis of genetic and hereditary differences at the molecular level. These differences are mainly in DNA and protein sequences which are utilized to gain information about the organism's evolutionary relationships. These analyses make it possible to ascertain the processes which eventually generated the diversity among species. The outcome of a phylogenetic analysis is represented in the form of a dendrogram or phylogenetic tree. Molecular phylogenetics is branch of molecular systematics playing a key role in modern taxonomy and bio-geographical research. Molecular phylogenetics has a deep correlation with molecular evolution. Molecular evolution is the process of selective changes (mutations) at a molecular level (genes, proteins, etc.) throughout various branches in the tree of life (evolution). Molecular phylogenetics draws conclusions about the evolutionary relationships that arise due to molecular evolution and results in the building of a phylogenetic tree. The figure displayed on the right depicts the phylogenetic tree of life as one of the first detailed trees, according to information known in the 1870s by Haeckel.

The early attempts at molecular systematics were also termed as chemotaxonomy and made use of proteins, enzymes, carbohydrates, and other molecules that were separated and characterized using techniques such as chromatography. These have been replaced in recent times largely by DNA sequencing, which produces the exact sequences of nucleotides or bases in either DNA or RNA segments extracted using different techniques. In general, these are considered superior for evolutionary studies, since the actions of evolution are ultimately reflected in the genetic sequences. At present, it is still a long and expensive process to sequence the entire DNA of an organism (its genome). However, it is quite feasible to determine the sequence of a defined area of a particular chromosome (conserved gene). Typical molecular systematic analyses require the sequencing of around 1000 base pairs. At any location within such a sequence, the bases found in a given position may vary between organisms. The particular sequence found in a given organism is referred to as its haplotypes. Principally, there are four base types, with 1000 base pairs, we could have $4^{1000}$ distinct haplotypes. However, for organisms within a particular species or in a group of related species, it has been found empirically that only a minority of sites shows any variation at all, and most of the variations that are found are correlated, so that the number of distinct haplophytes that are found is relatively small (Refer Figure 2.1)

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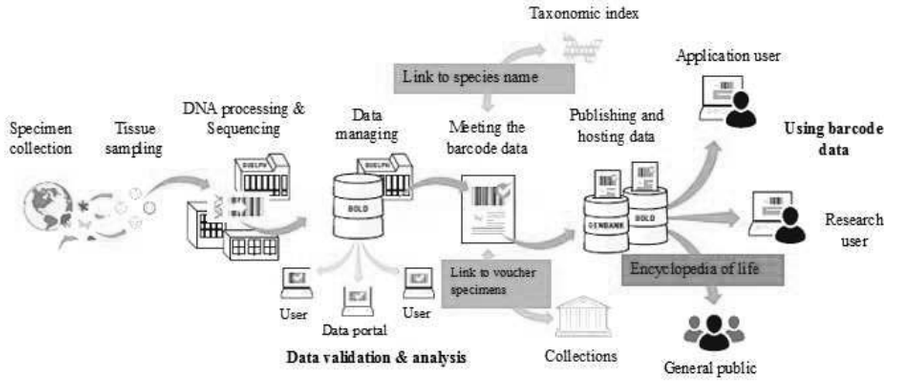


***Fig. 2.1*** *Outline for Tools and Techniques Employed in Molecular Phylogeny*

An older and superseded approach was to determine the divergences between the genotypes of individuals by DNA-DNA hybridization. The advantage claimed for using hybridization rather than gene sequencing was that it was based on the entire genotype, rather than on particular sections of DNA. Modern sequence comparison techniques overcome this objection by the use of multiple sequences. Once the divergences between all pairs of samples have been determined, the resulting matrix of differences is submitted to some form of statistical cluster analysis, and the resulting dendrogram is examined in order to see whether the samples cluster in the way that would be expected from current ideas about the taxonomy of the group. Any group of haplotypes that are all more similar to one another than any of them is to any other haplotype may be said to constitute a clade (Refer Figure 2.2).

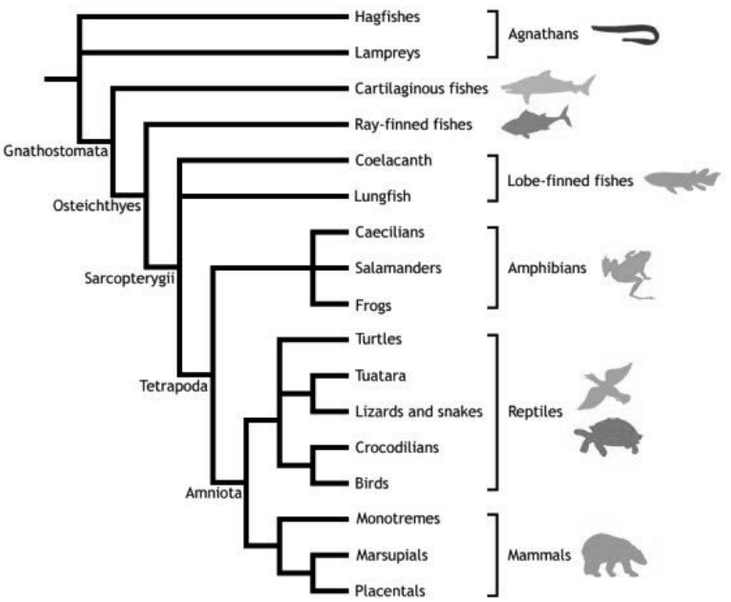


***Fig. 2.2*** *Cladogram of Different Vertebrates*

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## Applications and Techniques

Every living organism contains Deoxyribonucleic Acid (DNA), ribonucleic acid RNA, and proteins. In general, closely related organisms have a high degree of similarity in the molecular structure of these substances, while the molecules of organisms distantly related often show a pattern of dissimilarity. Conserved sequences, such as mitochondrial DNA, are expected to accumulate mutations over time, and assuming a constant rate of mutation, provide a molecular clock for dating divergence. Molecular phylogeny uses such data to build a 'relationship tree' that shows the probable evolution of various organisms. With the invention of Sanger sequencing, it became possible to isolate and identify these molecular structures. The most common approach is the comparison of homologous sequences for genes using sequence alignment techniques to identify similarity. Another application of molecular phylogeny is in DNA barcoding, wherein the species of an individual organism is identified using small sections of mitochondrial DNA or chloroplast DNA. Another application of the techniques that make this possible can be seen in the very limited field of human genetics, such as, the ever-more-popular use of genetic testing to determine a child's paternity, as well as the emergence of a new branch of criminal forensics focused on evidence known as genetic finger printing.

## Molecular Phylogenetic Analysis

There are several methods available for performing a molecular phylogenetic analysis. One method, including a comprehensive step-by-step protocol on constructing a phylogenetic tree, including DNA/Amino Acid contiguous sequence assembly, multiple sequence alignment, model-test (testing best-fitting substitution models), and phylogeny reconstruction using Maximum Likelihood and Bayesian Inference (Refer Figure 2.3).

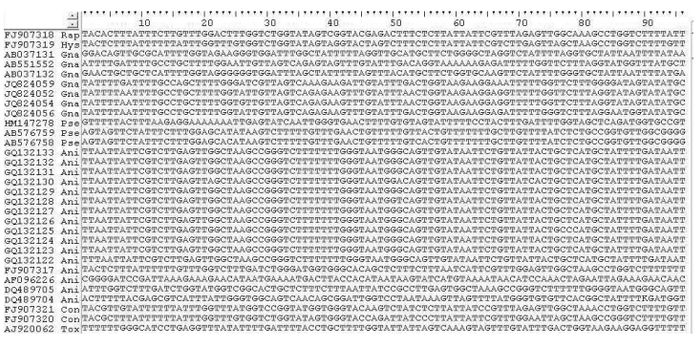

***Fig. 2.3*** *Multiple Sequence Alignment (MSA) by Software BioEdit*

A phylogenetic analysis typically consists of five major steps. The first stage comprises sequence acquisition. The following step consists of performing a multiple sequence alignment, which is the fundamental basis of constructing a phylogenetic tree. The third stage includes different models of DNA and amino acid substitution. Several models of substitution exist. A few examples include Hamming distance, the Jukes and Cantor one-parameter model, and the Kimura two-parameter model. The fourth stage consists of various methods of tree building, including distance-

based and character based methods. Common tree-building methods include Unweighted Pair Group Method using Arithmetic Mean (UPGMA) and Neighbour joining, which are distances based methods, Maximum parsimony, which is a character-based method, and Maximum likelihood estimation and Bayesian, which are character-based/model based methods. UPGMA is a simple method; however, it is less accurate than the neighbour-joining approach. Finally, the last step comprises evaluating the trees. This assessment of accuracy is composed of consistency, efficiency, and robustness (Refer Figure 2.4).


***Fig. 2.4*** *Five Stages of Molecular Phylogenetic Analysis*

MEGA (molecular evolutionary genetics analysis) is analysis software that is user-friendly and free to download and use. This software is capable of analyzing both distance-based and character-based tree methodologies. MEGA also contains several options one may choose to utilize, such as heuristic approaches and bootstrapping. Bootstrapping is an approach that is commonly used to measure the robustness of topology in a phylogenetic tree, which demonstrates the percentage each clade is supported after numerous replicates. In general, a value greater than 70% is considered significant (Refer Figure 2.5).

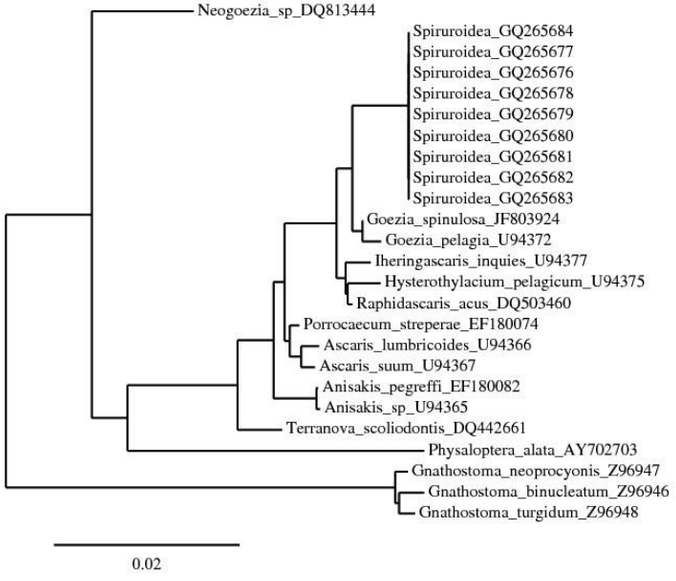


***Fig. 2.5*** *Neighbor Joining Tree Computed by Kimura's Two Parameter (K2P) Model by MEGA*

## Limitations of Molecular Phylogeny

Molecular systematics is an essentially cladistic approach: it assumes that classification must correspond to phylogenetic descent, and that all valid taxa must be monophyletic. This is a limitation when attempting to determine the optimal tree(s), which often involves bisecting and reconnecting portions of the phylogenetic

tree(s). The recent discovery of extensive horizontal gene transfer among organisms provides a significant complication to molecular systematics, indicating that different genes within the same organism can have different phylogenies. In addition, molecular phylogenies are sensitive to the assumptions and models that go into making them. They face issues such as long-branch attraction, saturation, and taxon sampling problems. This means that strikingly different results can be obtained by applying different models to the same dataset. Moreover, as previously mentioned, UPGMA is a simple approach in which the tree is always rooted. The algorithm assumes a constant molecular clock for sequences in the tree. This is associated with being a limitation in that if unequal substitution rates exist, the result may be an incorrect tree.

## 2.4.1 How to Construct Phylogenetic Trees

### What is Phylogeny Good For?

- To understand the process of molecular evaluation as objects typically are proteins or nucleic acid sequences.
- To identify what is most conserved important in some class of sequences.
- Tracing population history and multiple sequence alignment.
- To estimate the time of divergence between various species to understand lineage they last shared a common ancestor and human origin.

Phylogenetic trees are representation of phylogenetic information that shows relationships between species, it shares its most recent common ancestor with (Refer Figure 2.6).

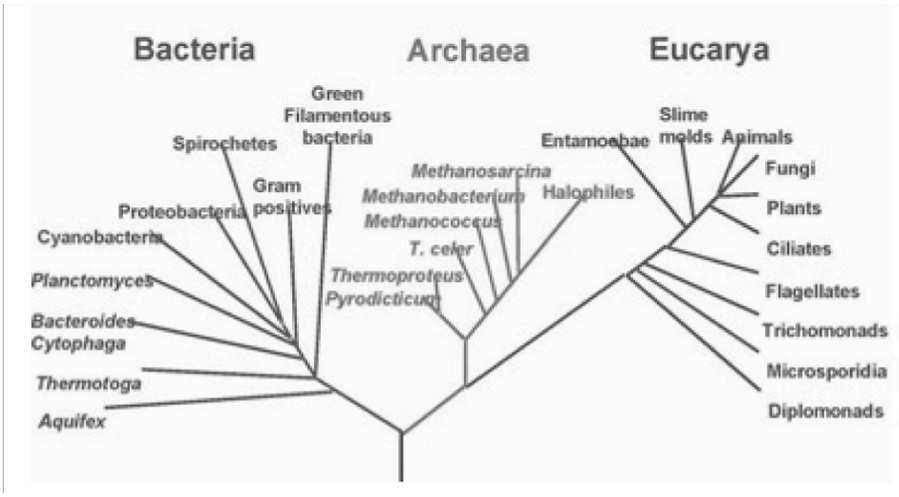


***Fig. 2.6 A** Phylogenetic Tree*

### Types of Phylogenetic Trees

These are significant in showing the evolutionary relationship among the biological species that are believed to have a common ancestor. Each node is a taxonomic unit. Each node with the descendent represents the most recent common ancestor

of the descendants. Edge lengths corresponds to time estimated and root is the common ancestor of the species under study (Refer Figure 2.7).

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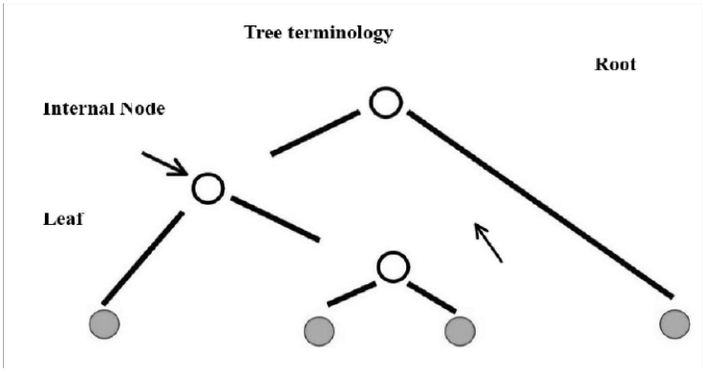


***Fig. 2.7** Phylogenic Tree Terminology*

**Rooted and Unrooted Phylogenetic Tree:** A rooted tree is a tree in which one of the nodes is stipulated to be the root, and the direction of ancestral relationship is determined. An unrooted tree, shows how close or distant the species are, and has no pre-determined root and induces no hierarchy. Rooting an unrooted tree involves inserting a new node, which will function as the root node. This can be done by introducing an uncontroversial out group, a species that is distant from all the species of interest. The proposed root will be the direct predecessor of the out group close enough to allow inference from sequence or trait data. It depicts only the relationship between organisms irrespective of the direction of the evolutionary time line. Unrooted trees can be generated from rooted trees by omitting the root indicating the origin of evolution.

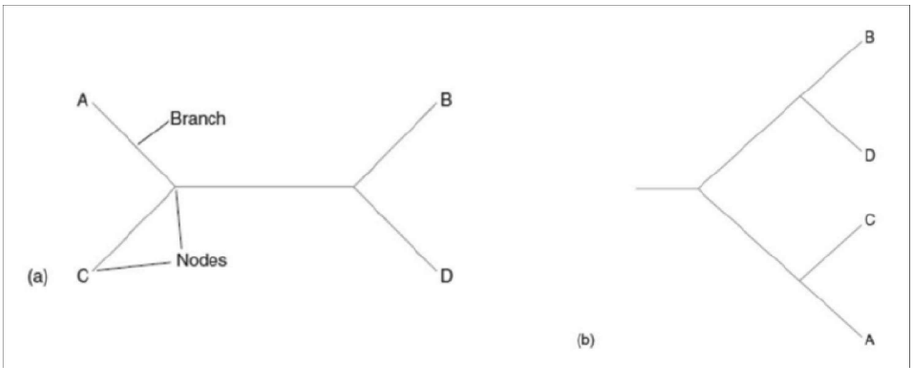


***Fig. 2.8** Unrooted Tree Representing the Relatedness of the Leaves without Assuming Ancestry*

An unrooted tree represents the relatedness of the leaves without assuming ancestry at all. Figure 2.8 (A); shows that A is more closely related to C than it is to either B or D, but does not specify the common ancestor for the four species or the direction of change. In contrast, the rooted tree Figure 2.8 (B), directed to a unique node does give a node that serves as the common ancestor and shows the development of the four species from this root.

The gradual change over time of the sequences of many rRNAs and proteins without destroying or severely altering their functions helps in determining their phylogenetic relationship. If sequences of similar molecules from two organisms differs, it means that they diverged very long time ago as there is an increase in changes with time.

The root of a tree signifies as the oldest point in the tree which represents the last common ancestor of all groups included in the tree unlike an unrooted tree that does not make any assumption about the common ancestor. Figure 2.9 illustrates phylogenetic tree. (a) un-rooted tree; (b) rooted tree and Figure 2.10 represents an example of unrooted phylogenetic tree.

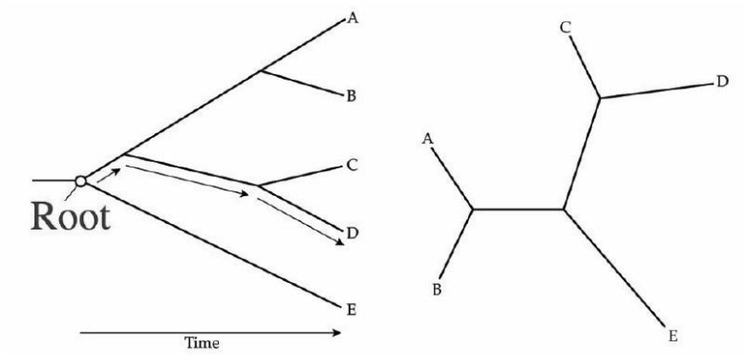


***Fig. 2.9*** *Phylogenetic Tree. (a) Unrooted Tree; (b) Rooted Tree*

In rooted tree there exists a particular node called the root from which the unique path leads to any other node. The direction of each path corresponds to evolutionary time and the root is the common ancestor of all taxonomic unit. In rooted evolutionary relationships are evident. In unrooted tree evolutionary relationships cannot be immediately assessed.

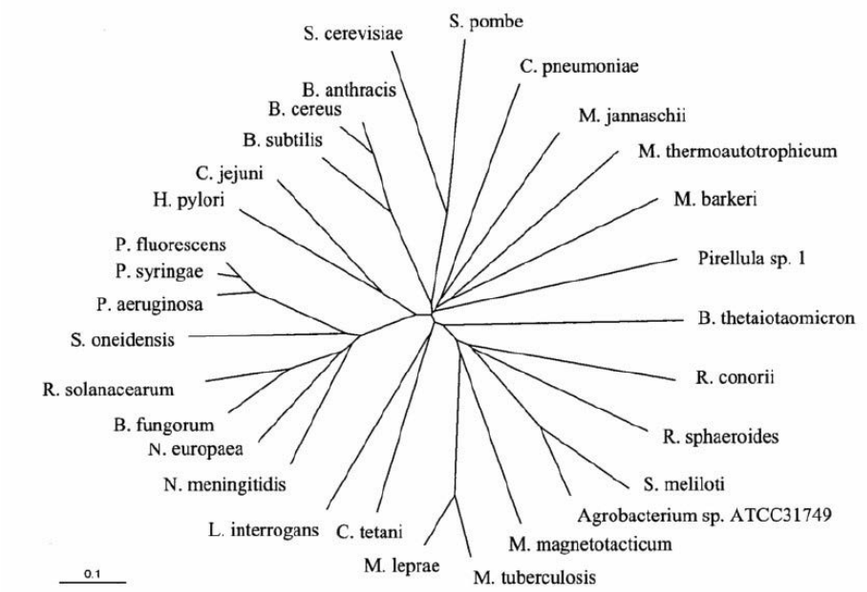


***Fig 2.10*** *Example of Unrooted Phylogenetic Tree*

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**Branch Length:** Branch length is the number of changes, for example nucleotide substitutions that have occurred along a branch. The total number of changes in a particular tree is called the tree length.

### How to Construct a Phylogenetic Tree?

The distance of the lines is used to determine how closely two organisms are related to one another or how long ago they may have had a common ancestor. The line that connect all the other lines is the representation of the common ancestor that is being looked at for comparison.

- **Step1:** Choosing a genome region to study.
- **Step 2:** Identifying and retrieving sets of homologous sequences from the same genome region of related individuals.
- **Step3:** Make a multiple alignment from base alignment or amino acid sequence (by using MUSCLE, BLAST, or other method).
- **Step 4:** Check the multiple alignment if it reflects the evolutionary process.
- **Step 5:** Choose the method to be used to determine the best fit nucleotide substitution model and calculate the distance or use the result depending on the method.
- **Step 6:** Verify the result statistically. Visualization and editing of trees.

A tree can show edge lengths, indicating the genetic distance between the connected nodes. To determine the constant pace of the evolutionary process molecular clock is a good indicator of their relatedness. According to this assertion informational macromolecules evolving at constant rates for different lineages, representing the root as the mid-point of the longest span across the tree. On such a basis phylogenetic tree can be produced theoretically that is distance preserving tree presented along a time-axis-assigning to each node the time in which occurred in the history of evolution.

However, using these sequences to construct phylogenetic tree, it is important to ensure that each nucleotide or amino acid in each sequence is compared only with the corresponding homologous nucleotides or amino acids in the other sequences. This is done by aligning the sequence to one another to obtain a discrete character matrix in which each row represents one of the sequences and each column is a set of homologous nucleotides or amino acids.

There are two types of data used for building phylogenetic trees:

**Character Based:** Seeks the tree that accounts for estimated evolutionary distances of each character separately, i.e., the individual substitutions among the sequences, to determine the most likely ancestral relationships. Each discrete character evolves independently from molecule sequences from individual data. The basic assumption is that characters at corresponding positions in a multiple sequence alignment are homologous among the sequences involved. Therefore the character states of the common ancestor can be traced from the dataset of a base in a specific position in a DNA sequence or amino acid in a protein. They are more rapid and less intensive than character based. But the actual characters are

NOTES

discarded once the distance matrix is arrived. On the other hand character based makes use of all known evolutionary information based on individual substitutions among the sequences and to determine the most likely ancestral relationships.

**Distance Based:** Seeks the trees that account for estimated evolutionary distances. True evolutionary distances between sequences can be calculated from observed distances. The evolutionary distances can be used to construct a matrix of distances between all individual pairs of taxa. A node is bifurcating binary or dichotomous, if has only immediate descendent lineages but multifurcating if it has more than two immediate descendent lineages. In a strictly bifurcating tree, each internal node is incident to exactly three branches, two derived and one ancestral (Refer Figure 2.11).

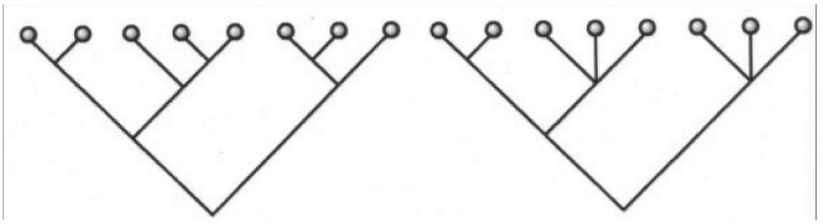


***Fig. 2.11*** *Distance Based Tree*

A bifurcation is always interpreted as a speciation event. There are two possible interpretation for a multifurcation in a tree. The polytomy represents two sequence of events whereby ancestral taxon gave rise to three or more decedent taxa simultaneously. The polytomy represents lack of resolution. The exact order of two or more bifurcation cannot be determined with the available data (Refer Figure 2.12).

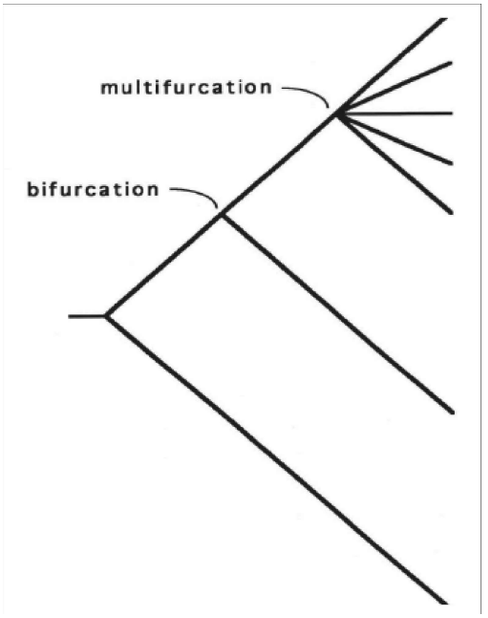



***Fig. 2.12*** *Multifurcation and Bifurcation*

NOTES

## Genetic Distance

Genetic distance is number of evolutionary events marked as mutations occurring between species since their divergence. In order to evaluate, we count the number of differences between two sequences. However, this may not always refer to the history of the sequence, as not all the events are recorded in current sequences. This could be replaced by the transition or transversion rate, along with different base frequencies between sites. All these models give similar results at low divergence.

The most popular methods can be classified into two main categories.

- Phenetic Methods (based on distance).
- Cladistic Methods (based on characters).

The former measures the pair-wise distance through dissimilarity between two genes. It helps in constructing tree from the resultant distance matrix. The latter evaluates all possible trees and seeks the one that optimizes the evolution.

## Limitations to the Use of Trees

Phylogenetic trees are meant to provide insight and not intended to represent an entire history of species. The output of the tree may be affected by several factors of gene transfer and protein function related to DNA degradation over time especially the evolutionary trees of the extinct organisms.

- **Polyphasic Taxonomy:** Studying phylogeny based on both genotypic and phenotypic information ranging from molecular characteristics to ecological characters.
- **Numerical Taxonomy:** Computer based approaches of grouping organisms is called numerical taxonomy which is based on presence or absence of selected characters in the group of organisms clusters based on relatedness. This method is used to express difference through evolutionary distance by estimating percent similarity to use measure of difference to create a tree. This method has great practical usefulness as well as being relatively unbiased in its approach. It has high degree of stability and predictability.

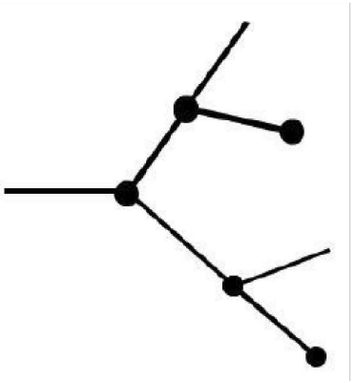
## The Phylogenetic Trees

- It seeks that either all internal nodes have degree 3, in which case T is called unrooted.
- In rooted T precisely one internal root node of degree 2.
- In a Binary T every internal node v has degree 3 except if T is rooted.

  Leave = Outer Branches

  Represent the taxa (sequences): Nodes = 1 2 3

**NOTES**



It represent the relationships among the taxa (sequences), for example, node 1 represent the ancestor sequence from which sequence A and sequence B is derived. The length of the branch represents the changes that occurred in the sequence prior to the next level of separation. External branches represents more recent diversions. Internal branches represents most ancient diversions.

There are two kinds of phylogenetic trees:

**Cladogram:** A group of species that shares its features from a common ancestor at (the root). There exists a particular root node. The path is from the root to the nodes. Root corresponds to the evolutionary time.

**Phenogram:** These are basically the unrooted tree. A phylogenetic tree where related descendants are not able to specify the common ancestor (root). The nodes of the tree are unable to indicate difference in the evolutionary time.

It is to be noted that a rooted tree is usually drawn with the root placed at the bottom, top or left of the figure.

- Rooted tree represents evolutionary connections over time.
- Unrooted trees depicts only the relationship between organisms.

Notice that the two trees are made from the same data.

- Leaf nodes are numbered 1-5.
- Internal nodes are numbered 6-8.
- Rooted tree has additional node 9, which is one root.

For example:

**First Question:** How many trees are possible to construct in a given 'n' species? If it's a reasonable number, a tree-construction algorithm is immediately obvious:

1. Enumerate every possible tree buildable from the n leaves.
2. Score each of them, returning the one with the best score.

**Solution:** Entering numbers into the above formulas, we get the following result:

| # Leaves (n) | # Unrooted Trees | # Rooted Trees |
|---|---|---|
| 4 | 3 | 15 |
| 5 | 15 | 105 |
| 6 | 105 | 945 |
| 8 | 10,395 | 135,135 |
| 10 | 2,027,025 | 34,459,425 |

In other words:

- This is an exponential function.
- It is not a reasonable number.
- We need to have a clever algorithm.

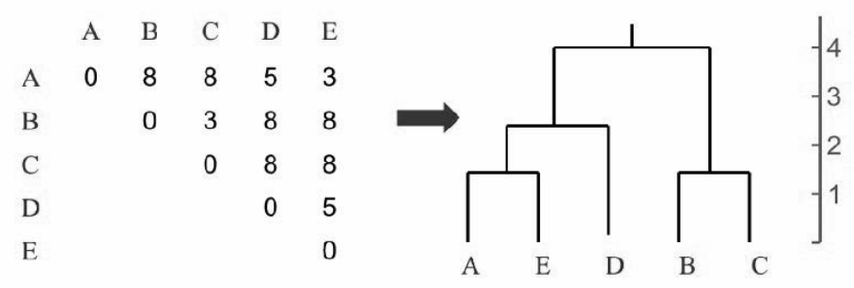
### Distance-Based Methods

We start by considering a couple of distance based methods: UPGMA and Neighbour Joining.

We define the problem as follows:

**Given:** An n × n matrix M in which Mij is the distance between objects i and j.

**Do:** Build an edge-weighted tree such that the distances between leaves i and j correspond to Mij.




The principle behind a distance based approach is that the vertical distance between any two leaves should correspond to the predefined distance.

The concept behind a distance based approach is that the vertical distance between any two leaves should correspond to an already defined distance.

### UPGMA

Construction of a distance tree using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Unweighted Pair Group Method using Arithmetic Averages:

- Primarily pick two leaves/cluster and merge them.

NOTES

- Create new node in tree for merged cluster.
- Distance dij between clusters Ci and Cj is defined as the average distance between pairs of elements from each cluster.

The algorithm runs as follows:

- Assign each sequence to its own cluster.
- Define one leaf for each sequence and place it at height 0.

For more than two clusters:

- Determine two clusters i, j with smallest dij.
- Define a new cluster Ck by merging Ci and Cj.
- Define a node k with children i and j; place it at height (½ dij).
- Replace clusters i and j with k.
- Compute distance between k and other clusters.

Join last two clusters, i and j, by root at height (½ dij).

Fortunately, there's a shortcut to calculating new distances:

- Given, a new cluster Ck formed by merging Ci and Cj, the distance to another cluster Cl is:

**UPGMA:** This is a simple distance method which stands for unweighted pair group method using arithmetic averages (Sokal & Michener 1958).

- Given a set of taxa X and a distance matrix D, UPGMA produces a rooted phylogenetic tree T with edge lengths.
- It operates by clustering the given taxa, at each stage merging two clusters and at the same time creating a new node in the tree.
- The tree is assembled bottom-up, first clustering pairs of leaves, then pairs of clustered leaves etc.
- Each node is given a height and the edge lengths are obtained as the difference of heights of its two end nodes.

Example X = f1; 2; 3; 4; 5g, distances given by distance in the plane.

**Neighbour Joining**

Neighbour joining is another distance-based method for reconstructing phylogenetic trees from distance data.

Like UPGMA, it constructs a tree by iteratively joining sub-trees.

Unlike UPGMA, it:

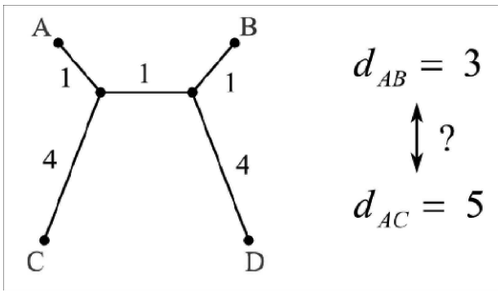
- Doesn't make molecular clock assumption.
- Produces unrooted trees.
- Assumes Additivity: The distance between pair of leaves is sum of lengths of edges connecting them.

NOTES

The two key differences are:

- How pair of subtrees to be merged is selected on each iteration.
- How distances are updated after each merge.

So far, each iteration we've chosen, the two clusters that are closest together but suppose the real tree (which is hidden from us) looks like this:




## Problem Based Learning

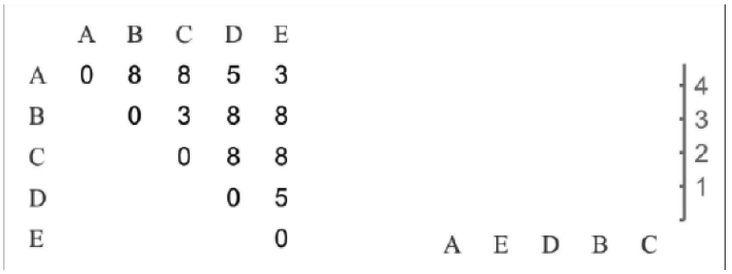
Let us say you are trying to create a phylogenetic tree for five species, labelled A to E. Their distances from each other are given by the following:

| | A | B | C | D | E |
|---|---|---|---|---|---|
| A | 0 | 8 | 8 | 5 | 3 |
| B | | 0 | 3 | 8 | 8 |
| C | | | 0 | 8 | 8 |
| D | | | | 0 | 5 |
| E | | | | | 0 |

Build the tree using UPGMA.

## Solution

Here's how the problem starts out.



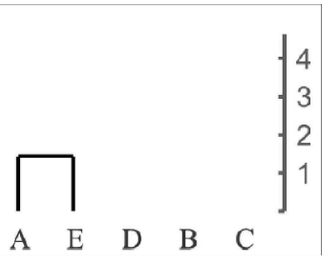

First we see which two leaves are closest to each other.

- There's a tie, so we're just going to pick A and E.
- We recalculate the distance matrix, so that AE is a member.
- Its distances are the averages of those of A and of E.
- In this case, that's trivial.

**NOTES**

Repeating, we chose to merge B and C.

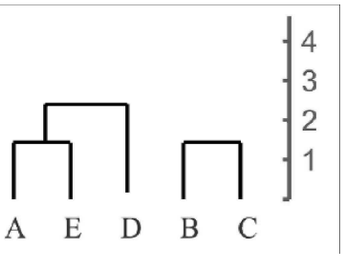
| | AE | B | C | D |
|---|---|---|---|---|
| AE | 0 | 8 | 8 | 5 |
| B | | 0 | 3 | 8 |
| C | | | 0 | 8 |
| D | | | | 0 |



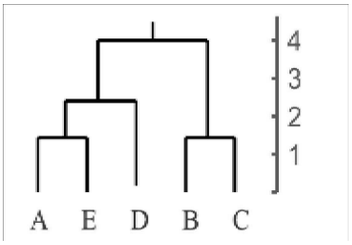

- Again, distances are easy to be recomputed.

Now AE is closest to D.

| | AED | BC |
|---|---|---|
| AED | 0 | 8 |
| BC | | 0 |




And merge the last two, making the tree's root.




You may have noticed that in this example, the distances were remarkably well-behaved.

- Averages were easy to calculate—too easy.
- Should be a tip-off that this is toy data.

Actually, this data was constructed using the molecular clock assumption.

- It is assumed that divergence of elements has occurred exactly at the same rate at all points on the tree.
- Evolution has taken place at a constant rate.
- This assumption however is not true most of the times because selection pressures acting at variable rates across:
- Time periods
- Organisms
- Genes within an organism
- Regions within a gene
- In other words, things evolve at different rates.

NOTES

- If this does hold, then the data is said to be ultra-metric. Ultra-metric data never really occurs in nature.
- Given ultra-metric data, UPGMA will reconstruct the tree T that is consistent with the data.

How can you tell if it is ultra-metric data?

- For any triplet of elements i, j, and k, one of two things will be true:
- The distances are all equal.
- Two are equal and the remaining one is smaller.
- For the matrix in the example, this was certainly true:

In general, for real data UPGMA is not guaranteed to return the correct tree.

| | A | B | C | D | E |
|---|---|---|---|---|---|
| A | 0 | 8 | 8 | 5 | 3 |
| B | | 0 | 3 | 8 | 8 |
| C | | | 0 | 8 | 8 |
| D | | | | 0 | 5 |
| E | | | | | 0 |

- Differing rates of evolution can confound it.
- Will do better with classic Darwinian evolution than with punctuated equilibrium.

However, it may still be a reasonable heuristic.

## 2.4.2 Phylogenetic Inference: Distance Methods, Parsimony Methods, Maximum Likelihood Method, and Immunological Techniques

Phylogenetic inference is drawn by reading the clues given by various methods of phylogenetic analysis which are namely, distance methods, parsimony methods and maximum likelihood methods.

### Distance Matrix Method

These involve a measuring of 'genetic distance' between the sequences being classified, are based a MSA (Multiple Sequence Alignment) step. Distance is often defined as the fraction of mismatches at aligned positions, with gaps either ignored or counted as mismatches. Distance methods work by building an all-to-all matrix from the sequence query set describing the distance between each sequence pair. The output is used for drawing a phylogenetic tree. Distance matrix methods include Neighbour Joining (NJ), Unweighted Pair Group Method with Arithmetic mean and Weighted Pair Group Method with Arithmetic Mean (UPGMA and WPGMA), Fitch-Margoliash method and method using out-groups.

NOTES

Neighbour-joining methods apply general data clustering techniques to sequence analysis using genetic distance as a clustering metric. The simple neighbour-joining method produces unrooted trees, but it does not assume a constant rate of evolution (i.e., a molecular clock) across lineages.

The UPGMA and WPGMA methods produce rooted trees and require a constant-rate assumption – that is, it gives a tree in which the distances from the root to every branch tip are equal.

The Fitch–Margoliash method uses a weighted least squares method for clustering based on genetic distance. Closely related sequences are given more weight in the tree construction. The least-squares criterion applied to these distances is more accurate but less efficient than the neighbour-joining methods. Another method used for phylogenetic tree construction is by using out-groups wherein distance-matrix methods also involve the inclusion of at least one out-group sequence known to be only distantly related to the sequences of interest in the query set. This usage can be seen as a type of experimental control. If the out-group has been appropriately chosen, it will have a much greater genetic distance and thus a longer branch length than any other sequence, and it will appear near the root of a rooted tree. A sequence moderately related to the sequences of interest; too close a relationship defeats the purpose of the out-group and too distant adds noise to the analysis.

### Parsimony Methods

Parsimony based methods follow character based approaches for solving the evolutionary relationship tree structure. Maximum parsimony method is the most widely used approach that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data. Here the shortest possible tree that explains the data is considered best. The foundation and basic work for these methods was done by James S. Farris in 1970 and Walter M. Fitch in 1971 along with many others. These methods operate by evaluating candidate phylogenetic trees according to an explicit optimality criterion; the tree with the most favourable score is taken as the best estimate of the phylogenetic relationships of the various taxa included in the study.

Evolutionary tree or trees are predicted in maximum parsimony method in a manner that minimizes the number of steps required to generate the observed variation in the sequences from common ancestral sequences. This method is also called as the minimum evolution method. A multiple sequence alignment (MSA) is required to predict the homologies. These positions will appear in vertical columns in the MSA. For each aligned position, phylogenetic trees that require the smallest number of evolutionary changes to produce the observed sequence changes from ancestral sequences are identified. This step is repeated for every position in the sequence alignment. The trees showing least number of changes overall for all sequence positions are chosen as the best and finally adopted. This method is best suited for highly similar sequences.

A maximum parsimony analysis runs in a very straightforward fashion. Trees are scored according to the degree to which they imply a parsimonious distribution

of the character data. The most parsimonious tree for the dataset represents the phylogenetic relationship.

### Maximum Likelihood Method

NOTES

Maximum likelihood method assesses a hypothesis about phylogeny based upon the probability that the proposed model and the hypothesized history would give rise to the observed data set. This method assumes that a history with a higher probability of reaching the observed state is preferred to a history with a lower probability. The method generates a phylogenetic tree exhibiting highest probability or likelihood. The Maximum Likelihood method of inference is available for both nucleic acid and protein data. Various web tools and programs are used for this analysis. For example, the following programs are available from the web:

- DNAML (DNA data only. By Joe Felsenstein in the Phylip package)
- FastDNAML (DNA data only. A faster algorithm applied by Garry Olsen applied to Joe Felsenstein's program DNAML)
- ProtML (DNA and protein. By Adachi and Hasegawa)
- Puzzle (DNA and protein. By Strimmer and von Haeseler). This program is much faster than PROTML

### Immunological Techniques

The amount of change between two antigens relative to a common ancestor is also used for phylogenetic inference. Nucleotide or amino acid sequences encoding antigenic determinants are analysed for patterns of change in order to reconstruct the phylogenetic relationships among species or individuals. Allelic variants are arranged into a phylogenetic pattern signifying evolutionary descent as a gene tree. Actually most of the times phylogeny describes the lineal history of antigenic variants irrespective of the processes which have resulted in that particular pattern of descent. Kuno et al. (1998) showed that many flavivirus clades identified by molecular phylogeny were similar to the antigenic classification by Calisher et al. (1989) based on the reactions with polyclonal sera.

## 2.4.3 Amino Acid Sequences and Phylogeny

The amino acid analysis is the analysis of amino acid sequences or protein coding sequences which is central to the study of molecular evolution. The construction of phylogenetic trees is the main tool for studying phylogeny, which are assembled based on algorithms that consider the numbers of mismatched amino acids or bases between aligned sequences. This strategy involves multiple comparisons which may be characterized by identical numbers of differences and are placed on the same evolutionary level. The resultant tree represents a hypothesis on the developmental paths that have led to the existing diversity. The reason for using differences in amino acid sequences for the evolutionary studies of proteins finds its roots in the mechanism of mutation, which causes altered bases in the coding sequence, consecutively resulting in changes in individual amino acids. However, natural selection–favours certain mutations over others.

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The phylogenetic analysis of proteins traditionally relies on the evaluation of amino acid sequences or coding sequences. Individual amino acids have measurable features that allow the translation from strings of letters (amino acids or bases) into strings of numbers (physicochemical properties). When the letters are converted to measurable properties, such numerical strings can be evaluated quantitatively with various tools of complex systems research. The investigation of protein evolution via quantitative study of the physicochemical characteristics pertaining to the amino acid building blocks broadens the spectrum of applicable research tools, accounts for mutation as well as selection, gives assess to multiple vistas depending on the property evaluated, discriminates more accurately among sequences, and renders the analysis more quantitative than utilizing strings of letters as starting points.

### 2.4.4 Nucleic Acid Phylogeny: DNA-DNA Hybridizations, Restrictions Enzymes Sites, Nucleotide Sequence Comparisons and Homologies.

All living forms possess Deoxyribonucleic Acid (DNA), Ribonucleic Acid (RNA), and proteins and closely related organisms have a high degree of similarity, while in the molecules of organisms distantly related a pattern of dissimilarity is encountered quite often. A molecular clock for dating divergence is provided using conserved sequences, such as, mitochondrial DNA that have accumulated mutations over time, and supposedly at constant rate. A relationship tree is built using such data which reflects the probable evolutionary pattern of diverse organisms. After the invention of di-deoxy-sequencing method in 1977, it became possible to isolate and identify these molecular structures. The transcriptome obtained by high-throughput sequencing, allows us to draw inference of phylogenetic relationships using an organism's transcriptome data.

The most popular way to identify similarity is the comparison of homologous sequences for genes using the sequence alignment techniques. DNA barcodes can be assigned by application of molecular phylogeny, in which the species of an individual organism is identified using small portions (segments) of mitochondrial DNA or chloroplast DNA sequence reads. Techniques of phylogeny based upon nucleic acid sequence and homology can also be applied in genetics for paternity testing and DNA fingerprinting.

#### DNA-DNA Hybridizations

DNA hybridization techniques are popularly used to infer the phylogenetic tree. With this analysis humans and chimpanzees have been placed as closest. The DNA-DNA Hybridization (DDH) techniques have been accepted and established as the gold standard for the genetic homology analyses of pair-wise sets of strains for classification purposes. The method has had an enormous relevance during the last half a century of classification of prokaryotes. Several different approaches have been developed to evaluate the degree of relatedness of two genomes and are mainly based on either measuring the degree of hybrid re-association or the

thermal stability of the hybrids. DDH has been often criticized as cumbersome and inaccurate, and for the inability to produce cumulative databases. For these reasons, and in light of the current developments of genome sequencing, DDH methods are called to be substituted by alternative approaches based on genome-to-genome sequence comparisons. However, until sequencing costs are reduced, DDH is still the method of choice to genomically circumscribe species. Here, three different approaches are presented in detail to facilitate the establishment of these techniques in microbial systematics laboratories.

### Restriction Enzyme Sites

The restriction enzyme sites can be used for phylogenetic inferences. The restriction enzyme site data can be analysed by maximum likelihood to estimate phylogenetic connections and links. There are two approaches for reconstruction of phylogenetic relationships using restriction patterns. The first is the cladistic approach, which reconstructs phylogenetic relationships much in the same way that inversions were used by Dobzhansky and his school. The second approach is the phenetic approach. In phenetic approach assumption of randomness must be made for evolutionary analyses of DNA restriction patterns. The estimation of phylogenetic relationships from restriction data by a phenetic approach involves the explicit or implicit assumption that an equilibrium exists where the nucleotides are completely randomly distributed throughout the genome.

### Nucleotide Sequence Comparisons and Homologies

Nucleotide sequences of organisms can be compared using various bioinformatics tools (eg. NCBI BLAST) and Sequence homologies between DNA, RNA, (or protein) sequences, can be defined in terms of shared ancestry in the evolutionary history of life. Two segments of DNA can have shared ancestry because of three phenomena: either a speciation event (orthologs), or a duplication event (paralogs), or else a horizontal (or lateral) gene transfer event (xenologs).

Nucleotide sequence similarity among DNA or RNA can be used to infer about homologies in their structure and function. Significant similarity is strong evidence that two sequences are related by evolutionary changes from a common ancestral sequence. Alignments of multiple sequences are used to indicate which regions of each sequence are homologous. The main programs used for alignment of nucleic acid sequences are BLAST and MEGA. The term "percent homology" is often used to mean "sequence similarity", that is the percentage of identical residues (*percent identity*), or the percentage of residues conserved with similar physicochemical properties (*percent similarity*),

Nucleotide homologies are utilized to detect orthologous and paralogous sequences among a group of organisms selected for phylogenetic analysis. Homologous sequences are orthologous if have descended from the same ancestral sequence separated by a speciation event. When a species diverges into two separate species, the copies of a single gene in the two resulting species are said to be orthologous. Orthologs, or orthologous genes, are genes in different species that originated by vertical descent from a single gene of the last common ancestor.

NOTES

The term "ortholog" was coined in 1970 by the molecular evolutionist Walter Fitch. Orthologs often, but not always, have the same function.

Orthologous sequences provide useful information in taxonomic classification and phylogenetic studies of organisms. The pattern of genetic divergence can be used to trace the relatedness of organisms.

Paralogous genes (sequences) are sequences originated via duplication events in the last common ancestor (LCA) of the species being compared. They result from the mutation of duplicated genes during separate speciation events. When descendants from the LCA share mutated homologs of the original duplicated genes then those genes are considered paralogs.

As an example, in the LCA, one gene (gene A) may get duplicated to make a separate similar gene (gene B), and both will be inherited to following generations. During speciation, one environment will favour a mutation in gene A (gene A1), producing a new species with genes A1 and B. Then in a separate speciation event, one environment will favour a mutation in gene B (gene B1) giving rise to a new species with genes A and B1. The descendants' genes A1 and B1 are paralogous to each other because they are homologs that are related via a duplication event in the last common ancestor of the two species.

## 2.4.5 Molecular Clocks

The molecular clock is a technique that uses the mutation rate of biomolecules to guess the time of divergence for two or more life forms in the evolutionary history. The amount of difference between the DNA of two species is a function of the time since their evolutionary separation. The nucleotide (DNA, RNA) or the amino acids sequences for proteins for the basis of such calculations. The reference points for determining the mutation rate are often fossil or archaeological record dates. The molecular clock was first tested in 1962 on the variants of haemoglobin in many animals, and is commonly used in molecular evolution to estimate times of speciation or radiation. It synonymous with an evolutionary clock or a gene clock. The name "molecular clock" was coined by Émile Zuckerkandl and Linus Pauling in 1962. They found that the number of amino acid differences in haemoglobin between different lineages changes roughly linearly with time, as estimated from fossil evidence. They postulated their observation in the form of molecular clock hypothesis that states that the rate of evolutionary change of any specified protein was approximately constant over time and over different lineages. It appears that the number of residue differences between a protein of any two species is mostly conditioned by the time elapsed since the lines of evolution leading to these two species originally diverged. Due to this the cytochrome C of mammals remains equally different from the cytochrome C of all birds. Since fish diverges from the main stem of vertebrate evolution earlier than either birds or mammals, the cytochrome c of both mammals and birds should be equally different from the cytochrome c of fish. Similarly, all vertebrate cytochrome c should be equally different from the yeast protein.

The discovery of the molecular clock advocating a relatively constant rate of molecular evolution provides an insight into the mechanisms of molecular

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evolution, and became one of the most useful new tools in biology. The unexpected constancy of rate of evolution can be explained assuming that most changes to genes (amino acid changes) are effectively neutral. Theory predicts several sources of variation in the rate of molecular evolution. However, even an approximate clock allows time estimates of events in evolutionary history, which provides a method for testing a wide range of biological hypotheses ranging from the origins of the animal kingdom to the emergence of new viral epidemics.

The molecular clock equips us with a universal tool which is capable of placing past evolutionary events in correct time order, but also depicts the mechanisms and processes through which evolution has taken place. The basic approach for estimating molecular dates is by measurement of the genetic distance between species, followed by use of a calibration rate (the number of genetic changes expected per unit time) to convert the genetic distance to time. Numerous methods are in practice, such as a simple division of genetic distance by a calibration rate to more advanced and sophisticated maximum likelihood 26, 66 or Bayesian approaches 57, 64, which estimate molecular dates along with other parameters of models of the DNA substitution process.

**Check Your Progress**

5. Define molecular phylogenetics.
6. What is the use of molecular evalutionary genetics analysis MEGA?
7. State any two uses of phylogeny?
8. What does neighbour joining mean?
9. What is the use of high-throughput sequencing?
10. What are the approaches for reconstruction of phylogenetic relationships using restriction patterns?
11. Who coined the term 'ortholog'?

## 2.5 ORIGIN AND EVOLUTION OF ECONOMICALLY IMPORTANT MICROBES AND ANIMALS

Existence of bacteria can be traced to early eras in the history of life on the earth. Fossilized bacteria were discovered in rocks which belong to as early as at least the Devonian period (419.2 million to 358.9 million years ago), although evidences exist supporting the presence of bacteria since early Pre-cambrian time, i.e., about 3.5 billion years ago. Bacteria were prevailing on the planet Earth at least since the later part of the Paleoproterozoic period roughly 1.8 billion years ago, when oxygen appeared in the atmosphere due to cyano-bacterial action. Bacteria have thus had plenty of time to adapt to their environments and to evolve and give rise to new species.

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The type of the original ancestor in the origin of life is a topic of research and debate. It is believed by many scientists the original living cell might have utilized RNA as its genetic material, since reports arrived about RNA molecules with catalytic functions. The bacteria and archaebacteria diverged from their common ancestor at a very early part of this time period. These two phyla of prokaryotes tend to inhabit different types of environments generating novel species at different rates. Many archaea are thermophiles and live in high temperature zones. The thermophiles bacteria by themselves constitute one major branch of the archaeal tree, whereas, many of the methanogens that contribute to another major branch can also grow at high temperatures. In contrast, no major eubacterial branch consists solely of thermophiles. Both bacteria and archaea contain members that are able to grow at very high temperatures, as well as other species that are able to grow at low temperatures. Another notable feature is that many eubacteria have largely adapted to aerobic conditions, whereas, most archaebacteria are obligate anaerobes. None of the archaea are obligate phototrophs. This implies that the archaea are a more primitive type of organism with an impaired genetic response to changing environmental conditions.

The archaebacteria are restricted to extreme or harsh habitats possibly due to their limited ability to adapt to new situations because in the adverse environments there is less competition. Organisms must evolve or adapt to changing environments, and mutations, which are changes in the sequence of nucleotides in an organism's DNA, occur not stop in all living creatures. The changes in DNA sequence often result in alterations in the amino acid sequence of the protein coded by that stretch of nucleotides or DNA bases. As a result, the altered protein might be either better-suited or less well-suited for function under the prevalent conditions. Although many nucleotide changes that can occur in DNA have no effect on the fitness of the cell, if the nucleotide change enhances the growth of that cell even by a small degree, then the mutant form would be able to increase its relative numbers in the population. If the nucleotide change retards the growth of the cell, however, then the mutant form would be outgrown by the other cells and lost.

The crucial factor in adaptation is the ability to transfer genetic information between organisms in the environment. Although, exchange of DNA is an essential part of the life cycle of higher eukaryotic organisms, it can occur in all eukaryotes. Genetic exchange is well known throughout the bacterial kingdom, and, although the amount of DNA that is transferred is small, this transfer can occur between distantly related genera. Here, the plasmid borne genes can get recombined with the bacterial chromosome and become a stable part of the inheritance. Many organisms especially bacteria possess mobile genetic elements called transposons capable of rearranging the order and presence of any genes on the chromosome. Transposons may play a role in helping to accelerate the rate of evolution.

**Check Your Progress**

12. What effect does the change in DNA sequences causes in an organism?
13. What will happen if the nucleotide change retards the growth of the cell?

## 2.6 POPULATION GENETICS AND ECOLOGY

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Population genetics is a branch of biology dealing with studies of the genetic composition of biological populations, as well as changes in genetic composition arising from the impact of various factors, including natural selection. Population geneticists pursue their goals by developing abstract mathematical models of gene frequency dynamics, trying to extract conclusions from those models about the likely patterns of genetic variation in actual populations, and testing the conclusions against empirical data. Population genetics bears intimate relationship with the study of evolution and natural selection, and is often termed as the theoretical cornerstone of neo Darwinism. This is because natural selection is one of the most important factors that can affect a population's genetic composition. Over reproduction or out reproduction is the mother of natural selection which occurs when some genetically varied individuals in a population out-reproduce other members which are without that mutation or variation. This happens when the mutated individuals or variants are better adapted to the environment, or prove fitter.

Presuming the fitness differences are at least partly due to genetic differences, this will cause the population's genetic makeup to be altered over time. The population genetics scientists permit the consequences of different evolutionary hypotheses to be explored in a quantitatively precise way by studying formal models of gene frequency change, and shed light on the course of evolution. The population genetics as a separate branch came into existence between late1920s and 1930s, mainly owing to experiments and investigations of R. A. Fisher, J. B. S. Haldane and Sewall Wright. Their achievement was to integrate the principles of Mendelian genetics, which had been re-discovered at the onset of 20th century, based upon the Darwinian theory of natural selection. Though the compatibility of Darwinism with Mendelian genetics is today taken for granted, in the early years of the twentieth century it was not. Darwin's gradualist account of evolution was not well acceptable to some of the early Mendelian followers, who believed that novel adaptations must arise in a single mutational step; whereas, many of the early Darwin supporters did not believe in Mendelian concept of inheritance, often because of the erroneous belief that it was incompatible with the process of evolutionary modification put forward and described by Charles Darwin.

Fisher, Haldane and Wright mathematically showed that Darwinism and Mendelism were not just compatible but excellent bed fellows having natural selection in common; this played a key part in the formation of the neo-Darwinian synthesis, and explains why population genetics came to occupy so pivotal a role in evolutionary theory. *Origin of Species*, published in 1859, propounded two main theses: firstly, that modern species were descended from common ancestors, and secondly that the process of natural selection was the major mechanism of evolutionary change. The first thesis quickly won acceptance in the scientific community, but the second did not. Many people found it difficult to accept that natural selection could play the explanatory role required of it by Darwin's theory.

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Darwin was perturbed very much by insufficient understanding of the inheritance mechanism, for it left him unable to counter one of the most crucial objections to his evolution theory. For a population to evolve by natural selection, the members of the population must vary if all organisms are identical, no selection can occur. So for selection to gradually modify a population over a long period of time, in the manner suggested by Darwin, a continual supply of variation is needed. This was the basis for Fleeming Jenkin's famous objection to Darwin, namely that the available variation would be used up too fast. Jenkins postulated via his reasoning, a blending theory of inheritance, i.e., an individual's phenotype traits are a blend of those present in its parents. (So for example, if a short and a tall organism mate, the height of the offspring will be intermediate between the two.) Jenkins argued that given blending inheritance, a sexually reproducing population will become phenotypically homogenous in just a few generations, far shorter than the number of generations needed for natural selection to produce complex adaptations. Fortunately for Darwin's theory, inheritance does not actually work the way Jenkins thought.

The Mendelian type of inheritance named after Gregor Mendel, is particulate rather than blending offspring inherit discrete hereditary particles (genes) from their parents, which means that sexual reproduction still preserves and does not diminish the heritable variation present in the population. However, this view took a long time to come mainly because of Mendel's contribution being overlooked by the scientific community for forty years and secondly, even after the re-discovery of Mendel's work at the turn of the twentieth century, it was widely believed that Darwinian evolution and Mendelian inheritance were incompatible. The role of natural selection in evolution was not accepted by early Mendelians, so were not agreed to the fact that Mendel had given Darwin's theory the lifeline it needed. The concepts of Darwinism and Mendelism which marked the birth of modern population genetics, were developed and achieved by a long and tortuous route. In his experiments on garden pea Mendel observed an unusual phenomenon. He began with two pure breeding lines, one producing plants with round seeds, the other wrinkled seeds. Upon crossing these to produce the first daughter generation (the F1 generation). He obtained F1 plants which all had round seeds the wrinkled trait (phenotype) had disappeared from the population. After that when F1plants were crossed with each other, in the F2 generation approximately one quarter of the F2 plants had wrinkled seeds. So the wrinkled trait had made a comeback, skipping a generation.

Obviously, our modern understanding of heredity is vastly more sophisticated than Mendel's, but the key elements of Mendel's theory discrete hereditary particles that come in different types, dominance and recessiveness, and the law of segregation turned out to be essentially correct. Mendel's factors are the genes of modern population genetics, and the alternative forms that a factor can take (e.g., R versus W in the pea plant example) are known as the alleles of a gene. The law of segregation is explained by the fact that during gametogenesis, each gamete (sex cell) receives only one of each chromosome pair from its parent organism. Other aspects of Mendel's theory have been modified in the light of later discoveries.

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Mendel thought that most phenotypic traits were controlled by a single pair of factors, like seed shape in his pea plants, but it is now known that most traits are affected by many pairs of genes, not just one. Mendel believed that the pairs of factors responsible for different traits (e.g., seed shape and flower colour) segregated independently of each other, but we now know that this need not be so. Despite these points, Mendel's theory marks a turning point in our understanding of inheritance.

Although Mendel's work was rediscovered in 1900, it did not make the scientific community to be converted to a supporter or believer of Mendelism with immediate effect. The dominant approach to the study of heredity at the time was biometry, reinforced by Karl Pearson in Great Britain, which involved statistical analysis of the phenotypic variation found in natural populations. Biometricians were mainly interested in continuously varying traits such as height, rather than the discrete traits such as seed shape that Mendel studied, and were generally believers in Darwinian gradualism. Opposed to the biometricians were the Mendelians, inspired by William Bateson, who strongly believed in discontinuous variation, and followed that major adaptive changes are a result of single mutational events, rather than by cumulative natural selection as per Darwin. A heated controversy between the biometricians and the Mendelians had arisen. As a result, Mendelian inheritance came to be associated with an anti-Darwinian view of evolution. Population genetics probably arose from the need to reconcile Mendel with Darwin, which turned out to be urgent as the empirical evidence for Mendelian inheritance was piled up. The first significant milestone was R. A. Fisher's 1918 paper, 'The Correlation between Relatives on the Supposition of Mendelian Inheritance', which showed how the biometrical and Mendelian research traditions could be unified. Fisher demonstrated that if a given continuous trait, e.g., height, was affected by a large number of Mendelian factors, each of which made a small difference to the trait, then the trait would show an approximately normal distribution in a population.

Since the Darwinian principles are believed to work best on continuously varying traits, showing that the distribution or segregation and independent assortment of such traits was compatible with Mendelism was an important step towards reconciling Darwin with Mendel. The full reconciliation could be materialized only in the second decade of twentieth century and early 30s, thanks to the mathematical work of Fisher, Haldane and Wright who individually developed formal models to explore the effect, extent and role of natural selection, mutation, and other evolutionary forces would modify the genetic composition of a Mendelian population over time. This work is a leap forward in understanding the process of evolution, since it enabled us to explore the consequences of various evolutionary hypotheses, quantitatively and not just qualitatively. Concrete mathematical arguments replaced the verbal arguments about the power and outcomes of natural selection or about the patterns of genetic variation to which it could give rise. The methodology of designing models to explain the process of evolution is still holds best in modern population genetics.

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## 2.6.1 Metapopulation

A metapopulation is a bunch of spatially separated populations of the same species that move together at some level. The term metapopulation was coined by Richard Levins in 1969 to explain a model of population dynamics of insect pests in agricultural fields, whereas, the thought is applicable to species in naturally or unnaturally fragmented habitats. In Levins' own words, it consists of 'a population of populations'. Populations, in most species are typically divided into smaller units because of geographic, ecological, or behavioural factors. As an example, the populations of fish in pools, trees on mountains or insects on host plants are subdivided because suitable habitat for these species is not continuous. Factors related to animal behaviour can also result into population subdivision Examples are troop formation in primates, territoriality in birds, and colony formation in social insects. A subdivided population can cause differences in the amounts of genetic connectedness among the parts of the population. Genetic connection is mainly guided by the quantity of gene flow and movement between groups that results in genetic exchange occurring among the subpopulations or subgroups. The genetic variation over the groups becomes homogenized when the amount of gene flow between groups is high. Under the conditions of gene flow being low, the genetic drift, natural selection, and even mutation in the separated populations can give rise to genetic differentiation. It is worthwhile at times to describe the population structure in a particular geographic framework. For instance within a watershed, there may be separated fish or plant groups containing a substantial amount of genetic exchange between them. On a bigger scale, possibilities exist for genetic exchange between neighbouring watersheds but in smaller amounts than among the groups within a watershed. On a fair larger scale, one could find populations in quite separated watersheds that presumably possess a tiny scale direct exchange but may share some genetic history, depending on the amount of gene flow among the adjacent groups or occasional long-distance gene flow.

This type of hierarchical illustration is of much help in describing the overall relationships of populations of an organism and in documenting the spatial pattern of genetic variation. In the recent past, there has been increasing interest in landscape and geographic approaches to estimating historical and contemporary gene flow. In addition, phylogeography, the joint use of phylogenetic techniques and geographic distributions, has been used to understand spatial relationships and distributions of populations within species or closely related species (Avise, 2000). Normally, while studying subdivision of populations, one assumes that the various subpopulations are always present. Another pool of researchers assumes that individual population subdivisions at explicit sites could become extinct and in the following years be recolonized from other subpopulations, resulting in a metapopulation. Because of the dynamics of extinction and recolonization a metapopulation can be quite different both ecologically and genetically from the traditional concept of a subdivided population.

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## 2.6.2 Why Small Populations become Extinct?

The effective population size, shows correlation with the phenomenon and extent of drift and the dimensions of inbreeding in the population. The genetic diversity of small populations tends to vanish quicker as compared to larger populations owing to stochastic sampling error (i.e., genetic drift). This happens since some versions of a gene might be lost due to random chance, and this is more likely to occur in small populations. Additionally, smaller population size means greater inbreeding or mating with close relatives. In closed populations, individuals will be more closely related to each other compared to individuals in the previous generation. For example, in a hypothetical population consisting of only four individuals, if two pairs each produced two offspring (meaning that four new individuals are present in the next generation), the offspring must either mate with a sibling, a parent, or an individual from the other pair. Assuming they choose the non-sibling/non-parent option, all of the offspring's in the third generation must mate with individuals that have the same grandparents or choose to forgo reproduction.

This example although is less likely due to the very small hypothetical population, the same patterns and forces could be present in larger albeit still small populations. The mechanisms of the loss of genetic diversity due to inbreeding and population drift although vary largely, their effects still remain the same and show similar impact on populations. The genetic diversity is reduced both due to inbreeding and population drift, which is associate in giving rise to increased risk of population extinction, reduced population growth rate, reduced potential for response to environmental change, and decreased disease resistance, which impacts the ability of released individuals to survive and reproduce in the wild.

## 2.6.3 Loss of Genetic Variations

Within interbreeding populations, non-random sexual reproduction can sometimes occur because one organism chooses to mate with another based on certain traits. In such cases, members in a population make specific behavioural choices, and these choices decide varied genetic combinations that arise in the next generations. In this condition, the mating patterns of that population are no longer left random. Non-Random mating can take place in two ways, with different consequences. One form of non-random mating is inbreeding, which occurs when individuals with similar genotypes are more likely to mate with each other rather than with individuals with different genotypes. The second form of non-random mating is called outbreeding, wherein there is an increased probability that individuals with a particular genotype will mate with individuals of another particular genotype. Inbreeding often leads to a reduction in genetic variation, while outbreeding may result in an increased amount of variation.

Random forces create genetic drift, and other changes such as random fluctuations in the numbers of alleles in a population. These changes in relative allele frequency, referred to as genetic drift, can either increase or decrease by chance over time. Typically, genetic drift occurs in small populations, where infrequently-occurring alleles face a greater chance of being lost. Once it begins, genetic drift will continue until the involved allele is either lost by a population or is

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the only allele present at a particular gene locus within a population. Both possibilities decrease the genetic diversity of a population. Genetic drift is common after a population experiences a population bottleneck. A population bottleneck arises when a significant mortality in a population and individuals are otherwise prevented from breeding, resulting in a drastic decrease in the population.

Genetic drift can be a source of the loss of rare alleles, and can decrease the size of the gene pool. Genetic drift usually makes a new population to be genetically distinct from its original population, budding out the hypothesis that genetic drift plays a role in the evolution of new species.

### 2.6.4 Conservation of Genetic Resources in Diverse Taxa

Plant Genetic Resources for Food and Agriculture (PGRFA) have been systematically collected and exchanged for some 500 years. The prime aim of conservation is to maintain the diversity of the full range of genetic variation within a particular species or taxa. Conservation of plant genetic resources can be accomplished by two methods; in-situ and ex-situ. The main motive behind conserving PGRFA is to ensure the future adaptability of cultivars and wild populations; to preserve data and traits that ensure sustainable agriculture; to promote the use of genetic resources in commerce and biotechnology and also to conserving the genetic diversity for cultural reasons. Ex situ conservation includes conservation of bio-diversity components external to their natural habitats. Gene banks are the main storage facilities used for such conservation; millions of accessions being stored in hundreds of gene banks around the world for conservation and utilization purposes.

In situ conservation means the maintenance and recovery of viable populations of species in their natural environment or surroundings and, in the case of domesticated or cultivated species, in the environments where the development of their distinctive traits has taken place. Common approaches for in-situ conservation are genetic reserve conservation and on-farm conservation. The Food and Agricultural Organization (FAO) has a lead role in strengthening the conservation of PGRFA via policy assistance, technical support and awareness raising. In collaboration with international, regional and national partners, they are involved in multiple projects to strengthen capacities in order to address technical and policy aspects and prepare gene bank standards and technical guidelines for crop specific conservation techniques and other publications. The adoption of the International Treaty on Plant Genetic Resources for Food and Agriculture in 2001 was a major milestone in towards uplifting the profile for plant genetic resources conservation and use. In 2004, FAO, in conjunction with Biodiversity International, acting on behalf of the international research organizations in this field, e.g., Consultative Group on International Agricultural Research (CGIAR), founded the Global Crop Diversity Trust to ensure the conservation and availability of crop diversity for the purpose of food security around the world. It is possible for the plant breeders to develop new and improved cultivars with desirable characteristics due to availability of diverse plant genetic resources (PGR) , which include both farmer-preferred

traits (yield potential and large seed, etc.) and breeders preferred traits (pest and disease resistance and photosensitivity, etc.). Since the very beginning of agriculture, agronomists have exploited natural genetic variability within crop species to meet subsistence food requirement, and now the focus it is on producing surplus food for the growing populations. In the middle of 1960s developing countries like India experienced the green revolution by meeting food demand with help of high-yielding and fertilizer responsive dwarf hybrids/varieties especially in wheat and rice. These prolonged activities that lead to the huge coverage of single genetic cultivars (boom) made situation again worse in other forms such as genetic erosion (loss of genetic diversity) and extinction of primitive and adaptive genes (loss of landraces). Currently after an advancement of agricultural and allied sciences and technology, we still are little sure whether we can meet the food requirements of the world in 2050; this question was recently raised at the world food prize event in 2014 and remains still unanswered since global population will exceed 9 billion in 2050. The per capita availability of food and water will become worse in the future coping with the undesirable climate change. Therefore, it becomes more important to explore the agriculture not only as a food-producing machine, but also as an important source of livelihood generation.

## 2.6.5 Monitoring Natural Populations

Genetic monitoring means the use of molecular markers to (i) identify individuals, species or populations, or (ii) to quantify changes in population genetic metrics (such as effective population size, genetic diversity and population size) over time. Changes in species abundance and/or diversity can be detected by genetic monitoring which has become an important tool in for conservation and livestock management. The types of molecular markers used to monitor populations are most commonly mitochondrial, microsatellites or single-nucleotide polymorphisms (SNP). Earlier studies used allozymes data to monitor these variations and changes in populations. Another biodiversity metric for implementation of the Convention on Biological Diversity is the species gene diversity.

### Types

Genetic monitoring can be successfully employed to detect many types of population changes such as population growth and decline, spread of pathogens, adaptation to environmental change, hybridization, introgression and habitat fragmentation events. Most of these changes are monitored using 'neutral' genetic markers (markers for which mutational changes do not change their adaptive fitness within a population). The markers that show adaptive responses to environmental change can be 'non-neutral' (e.g., mutational changes affect their relative fitness within a population) many a times.

There are two broad categories of genetic monitoring: Category I includes the use of genetic markers as identifiers of individuals (Category Ia), populations and species (Category Ib) for traditional population monitoring. Category II involves the use of genetic markers to study changes of population genetic parameters, which include estimators of effective population size ($N_e$), genetic variation, population inter-mixing, structure and migration. Examples include:

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**Estimating Abundance and Life History Parameters: Category Ia:** At the individual level, genetic identification can enable estimation of population abundance and population increase rates as per the mark-recapture models. The abundance of cryptic or elusive species that are difficult to monitor can be estimated by collecting non-invasive biological samples in the field (e.g. feathers, scat or fur) and using these to identify individuals through microsatellite or single-nucleotide polymorphism (SNP) genotyping. This census of individuals can then be used to estimate population abundance via mark-recapture analysis. For example, this technique has been used to monitor populations of grizzly bear, brush-tailed rock-wallaby, Bengal tiger and snow leopard. The rates of population recruitment and survival decide the overall population growth rates and can be judged conclusively by means of open mark-recapture models. For example, DNA from feathers shed by the eastern imperial eagle shows lower cumulative survival over time than seen for other long-lived raptors.

**Identifying Species–Category Ib:** Use of molecular genetic techniques to identify species can be useful for a number of reasons. Species identification in the wild can be used to detect changes in population ranges or site occupancy, rates of hybridization and the emergence and spread of pathogens and invasive species. Changes in population ranges were investigated in case of peninsula lynx and wolverine, whereas watching of west-slope cutthroat trout exposed large continuing ongoing hybridization with introduced rainbow trout and Canada lynx-bobcat hybrids. The evolution and spreading of pathogens can be tracked with the help of various molecular-diagnostic techniques, for example, identifying the unfold of West Nile virus among mosquitoes around in the jap US to spot or trace confirmed geographical origins of infection and the gene loci linked with parasite condition in the bighorn sheep. Genetic watching of invasive species is of conservation and economic interest, since invasions usually have an effect on the ecology and range of endemic species and shall also carry with them the risks of hybridization (e.g., for ducks, copepods, barred owl and hooter owl, and Lessepsian *Chimaera monstora*). Identification of species is also of paramount importance in monitoring of fisheries and wildlife trade, where standard visual identification of butchered or flensed products is troublesome or not possible. Watching of trade and consumption of species of conservation interest is done by means of molecular amplification and identification of meat or fish procured from markets. For instance, genetic market surveys are accustomed to trace protected species and populations of whale and dolphin species commonly seen in the markets. Many surveys of market trade have been centred on pinnipeds, hippocampus and sharks. Such surveys find much utility in providing the ongoing monitoring of the quantity and movement of fisheries and wildlife products via markets and check poaching or other illegal, unreported or unregulated (IUU) exploitation (e.g. IUU fishing). Though early applications centred on species identification and population assessments, market surveys additionally offer a chance for applying a battery of molecular ecology investigations that include the capture-recapture, assignment tests and population modelling. These developments are mostly in genetic monitoring of Category II.

**Monitoring Population Genetic Parameters – Category II:** Monitoring of population changes through genetic means is done retrospectively, through analysis

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of 'historical' DNA recovered from gene banks and repositories or of those archived in museums and comparison with latest or modern DNA of that species. It can also be used as a tool for evaluating in-progress changes in the status and persistence of current populations. Genetic measures of relative population changes include changes in diversity (e.g., heterozygosity and allelic abundance). Evaluation and studies of relative population changes through these metrics has been performed retrospectively for Beringian bison, Galapagos island turtle, houting, Atlantic salmon, northern pike, snapper, steelhead trout, large prairie chicken, Mauritius kestrel and Hector's dolphin and is the subject of several in progress studies, mainly involving the Danish and Swedish brown trout communities. Measuring absolute population changes (e.g., effective population size (Ne)) can be carried out by measuring changes in population allele frequencies ('$F_{temporal}$') or levels of linkage disequilibrium over time ('LDNe'), while changing patterns of gene flow between populations can also be judged by measuring differences in allele frequencies between populations over time. Such studies have been done mainly in grizzly bears, cod, red deer, leopard frogs and Barrel Medic.

Genetic monitoring had additionally been more and more utilized in studies to monitor environmental changes through changes in the frequency of adaptively selected markers.

As an instance, the genetically controlled photo-periodic response (hibernating time) of pitcher-plant mosquitos (Wyeomyia smithii) was altered in response to longer growing seasons for pitcher plants caused by warmer climate. In Experiments with wheat populations grown in contrasting environments for 12 generations it was found that changes in flowering time were closely associated with regulatory changes in single gene, pointing towards a pathway concerned with genetic adaptation to changing climate. Genetic monitoring is additionally useful in assessing the ongoing health of small, resettled populations. The best examples of this are found for New Zealand birds, several species of which were greatly affected by habitat destruction and the appearance of many predators in the 20th century and have now become part of relocation programs that transfer a few 'founder' individuals to predator-free offshore 'ecological' islands. For example, black robin and kakapo.

**Check Your Progress**

14. What happens in the second form of non-random mating?
15. What is the crucial factor in the adaptation?
16. What is population genetics?
17. What was R. A. Fisher's 1918 paper, 'The Correlation between Relatives on the Supposition of Mendelian Inheritance' all about?
18. Why is hierarchical representation of genetic relationship between sub-population useful?
19. Define metapopulation.
20. What are the types of population changes that can be detected by genetic monitoring?

## 2.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

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1. Gene evolution started as a field in early 1940s with a group of scientists debating over which types of mutations are responsible for the significant differences observed between the lower or 'primordial' life forms such as amoeba and human.
2. The AMOVA test method was developed by Laurent Excoffier, Peter Smouse and Joseph Quattro in 1992.
3. In taxonomy, higher categories are the classifications above the level of species, which are defined arbitrarily according to observed similarities among species, and which provide a useful hierarchical framework by which organisms may be described succinctly.
4. The Phyletic gradualism emphasises the following points:
   a. Evolution occurs at a fairly constant rate.
   b. Gradual transformation of ancestral species gives rise to new species.
   c. The rate of evolution during the origin of new species is much like that at any other time.
5. Molecular phylogenetics is the branch of phylogeny that analyzes genetic, hereditary molecular differences, predominately in DNA sequences, to gain information on an organism's evolutionary relationships.
6. MEGA (molecular evolutionary genetics analysis) is analysis software that is capable of analyzing both distance-based and character-based tree methodologies. MEGA also contains several options one may choose to utilize, such as heuristic approaches and bootstrapping.
7. Phylogeny is good for the reasons mentioned below:
   a. To identify what is most conserved important in some class of sequences
   b. Tracing population history and multiple sequence alignment
8. Neighbour joining is a distance-based method for reconstructing phylogenetic trees from distance data.
9. The transcriptome obtained by high-throughput sequencing, allows us to draw inference of phylogenetic relationships using an organism's transcriptome data.
10. There are two approaches for reconstruction of phylogenetic relationships using restriction patterns: Cladistic approach and the phenetic approach.
11. The term 'Ortholog' was coined in 1970 by the molecular evolutionist Walter Fitch.
12. The changes in DNA sequence often result in alterations in the amino acid sequence of the protein coded by that stretch of nucleotides or DNA bases.
13. If the nucleotide change retards the growth of the cell, then the mutant form would be outgrown by the other cells and lost.

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14. The second form of non-random mating is called outbreeding, wherein there is an increased probability that individuals with a particular genotype will mate with individuals of another particular genotype.
15. The crucial factor in the adaptation is the ability to transfer genetic information between organisms in the environment.
16. Population genetics is a field of biology that studies the genetic composition of biological populations, and the changes in genetic composition that result from the operation of various factors, including natural selection.
17. R. A. Fisher's 1918 paper, 'The Correlation between Relatives on the Supposition of Mendelian Inheritance' showed how the biometrical and Mendelian research traditions could be unified.
18. The hierarchical representation of genetic relationship between sub-population is useful in describing the overall relationships of populations of an organism and in documenting the spatial pattern of genetic variation.
19. A metapopulation consists of a group of spatially separated populations of the same species which interact at some level.
20. The types of population changes that can be detected by genetic monitoring include population growth and decline, spread of pathogens, adaptation to environmental change, hybridization, introgression and habitat fragmentation events.

## 2.8 SUMMARY

- Molecular evolution is a branch of evolutionary genetics that enables the study of process and pattern of evolution using molecular data
- Under molecular evolution three main aspects are studied which are gene evolution, evolution of gene families and assessment of molecular variations
- New genes are the roots of new phenotypes or traits. How new genes originate and how they evolve is studied under the field called as Gene evolution.
- Inversions, translocations, and deletions–all may create novel genes by reorienting and recombining the two sequences belonging previously to independent gene.
- De novo genes refer to events, where a previously noncoding region is converted into a coding region via mutations or other molecular processes.
- Gene families have evolved mainly via concerted evolution. The birth of a gene family within a genome can occur in one of the several ways involving genome duplication, tandem (segment) duplication and duplicative transposition.
- The parameters are commonly used to measure the amount of variation in populations. The proportion of polymorphic loci is the proportion of examined loci in which more than one allele is present in a population.

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- Phyletic gradualism is a hypothesis about the pattern of evolution. It is a model of Evolution which states that most speciation is slow, uniform and gradual.
- The origin of higher categories (taxa) and the underlying causes behind follow a complex pattern still we can see some specific trends in these events.
- Micro-evolution is an alteration within a gene pool of a population over time that ends up in minor changes of an organism in the same species.
- Phylogenetic trees are representation of phylogenetic information that shows relationships between species, it shares its most recent common ancestor with.
- There are significant in showing the evolutionary relationship among the biological species that are believed to have a common ancestor. Each node is a taxonomic unit.
- The distance of the lines is used to determine how closely two organisms are related to one another or how long ago they may have had a common ancestor.
- Molecular phylogenetics makes inferences of the evolutionary relationships that arise due to molecular evolution and results in the construction of a phylogenetic tree.
- Molecular systematics is an essentially cladistic approach: it assumes that classification must correspond to phylogenetic descent, and that all valid taxa must be monophyletic.
- A rooted tree is a tree in which one of the nodes is stipulated to be the root, and the direction of ancestral relationship is determined.
- An unrooted tree, shows how close or distant the species are, and has no pre-determined root and induces no hierarchy.
- In rooted tree there exists a particular node called the root from which the unique path leads to any other node. The direction of each path corresponds to evolutionary time and the root is the common ancestor of all taxonomic unit.
- In rooted evolutionary relationships are evident. In unrooted tree evolutionary relationships cannot be immediately assessed.
- Branch length is the number of changes, for example nucleotide substitutions that have occurred along a branch. The total number of changes in a particular tree is called the tree length.
- The evolutionary distances can be used to construct a matrix of distances between all individual pairs of taxa.
- A node is bifurcating binary or dichotomous if has only immediate descendent lineages but multifurcating if it has more than two immediate descendent lineages.

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- In a strictly bifurcating tree, each internal node is incident to exactly three branches, two derived and one ancestral.
- A bifurcation is always interpreted as a speciation event. Two possible interpretation for a multifurcation in a tree.
- The polytomy represents two sequence of events whereby ancestral taxon gave rise to three or more decedent taxa simultaneously.
- The output of the tree may be affected by several factors of gene transfer and protein function related to DNA degradation over time especially the evolutionary trees of the extinct organisms.
- Numerical taxonomy is a method to express difference through evolutionary distance by estimating percent similarity to use measure of difference to create a tree.
- Phylogentic inference is drawn by reading the clues given by various methods of phylogenetic analysis which are namely, distance methods, parsimony methods and maximum likelihood methods.
- Distance matrix method involves a measuring of 'genetic distance' between the sequences being classified, are based a MSA (Multiple Sequence Alignment) step.
- Parsimony based methods follow character based approaches for solving the evolutionary relationship tree structure.
- Maximum likelihood method assesses a hypothesis about phylogeny based upon the probability that the proposed model and the hypothesized history would give rise to the observed data set.
- The phylogenetic analysis of proteins traditionally relies on the evaluation of amino acid sequences or coding sequences.
- All living forms possess Deoxyribonucleic Acid (DNA), Ribonucleic Acid (RNA), and proteins and closely related organisms have a high degree of similarity, while in the molecules of organisms distantly related a pattern of dissimilarity is encountered quite often.
- DNA-DNA hybridization (DDH) techniques have been used as the gold standard for the genomic similarity analyses of pair-wise sets of strains for classification purposes.
- The restriction enzyme sites can be used for phylogenetic inferences. The restriction enzyme site data can be analysed by maximum likelihood to estimate phylogenetic connections and links.
- Sequence homologies between DNA and RNA (or protein) sequences can be defined in terms of shared ancestry in the evolutionary history of life.
- The molecular clock is a technique that uses the mutation rate of biomolecules to guess the time of divergence for two or more life forms in the evolutionary history.

- Bacteria were prevailing on the planet Earth at least since the later part of the Paleoproterozoic period roughly 1.8 billion years ago, when oxygen appeared in the atmosphere due to cyanobacterial action.
- Population genetics is a field of biology that studies the genetic composition of biological populations, and the changes in genetic composition that result from the operation of various factors, including natural selection.
- The rediscovery of Mendel's work in 1900 did not lead the scientific community to be converted to Mendelism overnight.
- Since the Darwinian process was widely believed to work best on continuously varying traits, showing that the distribution of such traits was compatible with Mendelism was an important step towards reconciling Darwin with Mendel.
- When a population is subdivided, the amounts of genetic connectedness among the parts of the population can differ.
- Population size, technically the effective population size, is related to the strength of drift and the likelihood of inbreeding in the population.
- Conservation focuses explicitly on maintaining the diversity of the full range of genetic variation within a particular species or taxa. Plant genetic resources can be conserved both in-situ and ex-situ.
- Genetic monitoring is the use of molecular markers to (i) identify individuals, species or populations, or (ii) to quantify changes in population genetic metrics (such as effective population size, genetic diversity and population size) over time.

## 2.9 KEY TERMS

- **Contig:** It is a set of overlapping DNA segments that together represent a consensus region of DNA.
- **Transcriptome:** It is the set of all RNA transcripts, including coding and non-coding, in an individual or a population of cells.
- **Thermophilic:** A thermophilic or thermophile is an organism that thrives at relatively high temperatures, between 41°C - 122 °C.
- **Phylogeography:** It is the study of the historical processes that may be responsible for the past to present geographic distributions of genealogical lineages.

## 2.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

### Short Answer Questions

1. Write a short note on phylogenetic gradualism and punctuated equilibrium.

2. What are the types of phylogenetic trees?
3. What is the significance and purpose of molecular clocks?
4. Why do small populations extinct?
5. Briefly explain the efforts put in the conservation of genetic resources in diverse taxa.

**Long Answer Questions**

1. Explain the molecular processes responsible for gene evolution.
2. Discuss the concept of molecular phylogenetics.
3. Evaluate the different methods of phylogenetic inference.
4. Describe the nucleic acid phylogeny in detail.
5. Discuss the meaning, types and significance of genetic monitoring with the use of examples.

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## 2.11 FURTHER READING

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# UNIT 3 GAMETE BIOLOGY - I

NOTES

**Structure**



## 3.0 INTRODUCTION

Developmental biology is the field of science that explores the potential of an unorganized fertilized cell to form a definite adult animal. Continuity of life through mysterious embryonic development is an organized process that begins with the formation of microscopic structures called as gametes. Gametes are the cells produced by parent generation though the process of gametogenesis and are laden with hereditary information. Gamete formation is a complex process which involves a structured division of diploid cell (Germ cell) into haploid gamete. Gametes in case of male are called as sperms and egg in case of females. As the name suggests, the process of sperm formation is known as spermatogenesis and that of egg

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formation is called as oogenesis. Both these processes undergo a series of events under the influence of specific hormones (different in male and female) to form gametes. These gametes from opposite sex fuse with each other to blend the genetic information through a complex process called as fertilization. This gives rise to the formation of a diploid structure called as zygote, which is the first diploid cell of the body. The sequence of diploid cell followed by haploid cell and the again diploid cell is the secret that maintains the chromosome number from generation to generation. In this unit, you will study spermatogenesis and biochemistry of semen. It will explain the process of fertilization and its types. In addition, it will explain ovarian follicular growth and differentiation. Also, it will explain the collection and cryopreservation of gametes and embryos. Besides, it gives the comparative account of gonads in a mammal and an invertebrate.

## 3.1 OBJECTIVES

After going through this unit, you will be able to:

- Describe the process of spermatogenesis
- Explain the biochemistry of semen
- Describe the process of fertilization
- Explain the collection and cryopreservation of gametes and embryos
- Discuss the ovarian follicular growth and differentiation
- Analyse the difference between gonads in a mammal and an invertebrate

## 3.2 SPERMATOGENESIS

For successful reproduction the utmost need is the production of functional gametes by the organism. Gametes are haploid reproductive cells which carries haploid set (in case of humans n=23) of chromosomes and are formed from diploid gonads (Testes in case of males and ovaries in case of females). Gametes are produced by specialized cell division known as reduction division or meiosis. Although, both spermatozoa and egg are morphologically distinct but both undergo similar stages of development. Gametes are the physical carriers of genetic information which transmit parental characters from one generation to next generation. In case of males the gamete is sperm where as in case of females the gamete is known as egg. Collectively the process of gamete formation is called as gametogenesis and is of two types:

- Spermatogenesis
- Oogenesis

Spermatogenesis is the developmental process from germ cell that ends at formation of haploid spermatid, which begins at the puberty and occurs in the seminiferous tubules of the testis. Once the process of spermatogenesis starts, it is a continuous process and occurs throughout the life of a male. The process of spermatogenesis is divided into three major phases: i. Proliferative phase or Multiplicative phase, ii.

Meiotic phase, and iii. Post Meiotic phase (Maturation phase) (Refer Figure 3.1 and 3.2).

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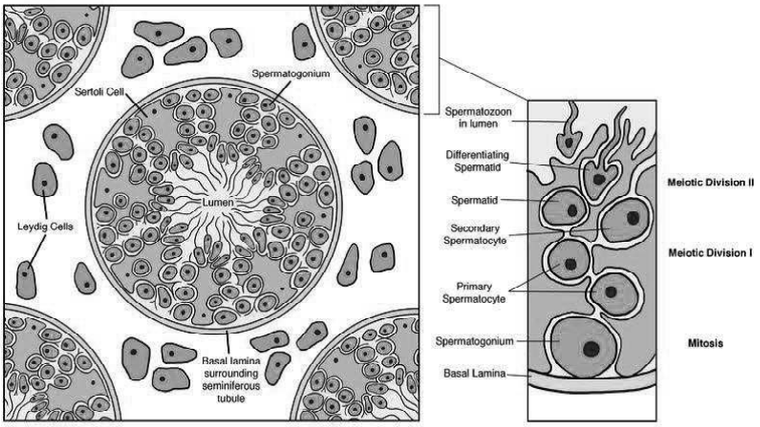


***Fig. 3.1*** *Seminiferous Tubule, Cross section of a Testis and Seminiferous Tubules*

## Proliferative Phase

It involves the rapid multiplications of primary germ cells which begin with the arrival of Primordial Germ Cell (PGCs) (also known as Gonocytes) at genital ridge of the male embryo. These gonocytes get incorporated into the sex cord which becomes seminiferous tubules with the course of development. The gonocytes gets differentiated into the numerous stem cells known as type A1 spermatogonia. Spermatogonia are the diploid cells with chromosome number 46 in case of human beings. These are true stem cells having the ability to reinitiate the process of spermatogenesis when transferred to mice whose sperm production is obliterated through injection of toxic chemicals. Spermatogonia resides in the stem cell niches at the junction of sertoli cells (the epithelium of seminiferous tubules), the interstitial cells (Leydig cells) and the testicular blood vessels. There are adhesion molecules that join the spermatogonia directly to the sertoli cells which provide nourishment to them throughout the process of development. This mitotic proliferation of stem cells amplifies the small population of gonocytes into a population of type A spermatogonia which can generate more than 1000 spermatids per second in adult human male.

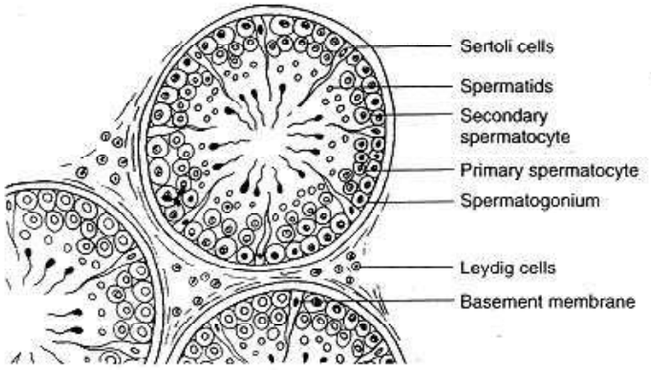


***Fig. 3.2*** *T.S. of Testis to Show Different Stages of Spermatogenesis*

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## Meiotic Phase

The undifferentiated type A Spermatogonia are the sperm stem cells and that generate another type A Spermatogonia and type A1 spermatogonium with the onset of puberty. Type A1 spermatogonia have high level of Stra8 transcription factor and are committed to a meiotic pathway. They undergo five mitotic divisions but keep cytoplasmic connection intact between themselves. These cells form a syncitum in which each cell communicates with others via cytoplasmic bridges about 1 micrometre in diameter. There are chains of 2-32 linked A1 spermatogonia in human males. These cells divide to produce type B spermatogonia which are the precursors of the spermatocytes and are the last cells of the line that undergo mitosis. They divide once to generate primary spermatocytes that enter into meiosis (Refer Figure 3.3).

The meiotic phase of spermatogenesis is regulated by numerous factors such as synthesis of BMPs by the spermatogonia and synthesis of retinoic acid by the sertoli cells during puberty. High concentration of the BMP8b is needed for the differentiation of the germ cells. Studies have showed that mice lacks BMP8b factor and thus is not able to induce spermatogenesis at the age of puberty.

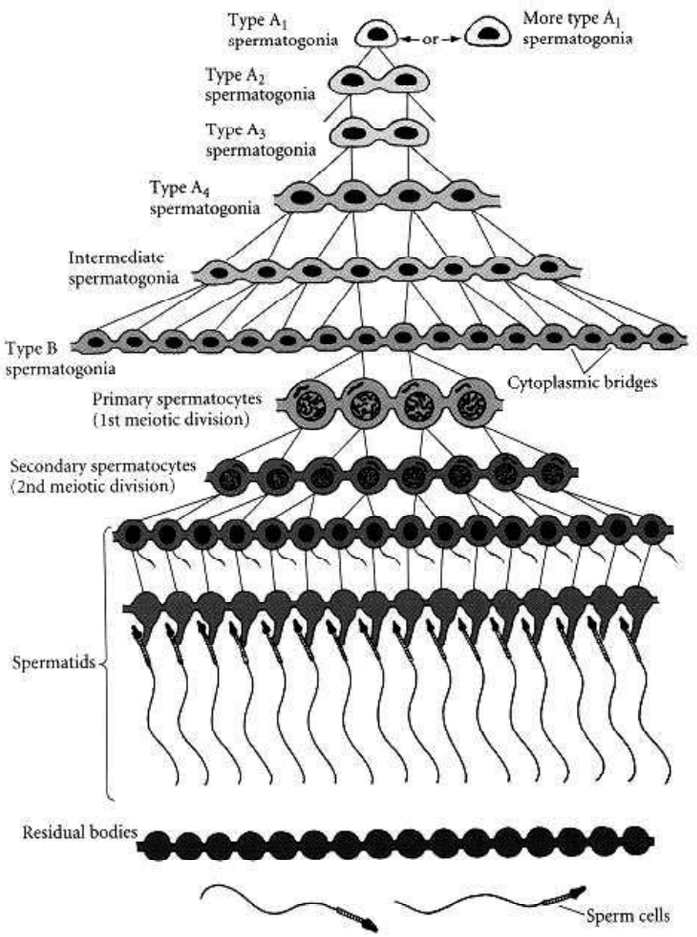


***Fig.3.3*** *Typical Spermatogenesis (Spermatocytogenesis and Spermiogenesis) in Vertebrates*

## Maturation Phase

In maturation phase primary spermatocytes undergoes the first meiotic division and produce secondary spermatocytes (chromosome number 23). During the first meiotic division homologous chromosomes and their non-sister chromatids undergo the process of crossing over which leads to introduction of variations in the secondary spermatocyte. Meanwhile, the secondary oocyte undergo the second meiotic division and produce haploid cells called as spermatids that remain connected to one another through their cytoplasmic bridges which get separated from each other during the process of spermiogenesis. The spermatids are having haploid nuclei but are functionally diploid. During the division from type A1 spermatogonia to spermatids the cells move farther and farther away from the basal lamina of the seminiferous tubules into the lumen of the seminiferous tubules. Thus, each type of cell can be found in a particular layer of the tubule. The spermatids are located at the border of the lumen where they lose their cytoplasmic connections and differentiate into spermatozoa. In human the progression from spermatogonial stem cells to mature spermatozoa take 65 days (Refer Figure 3.4).

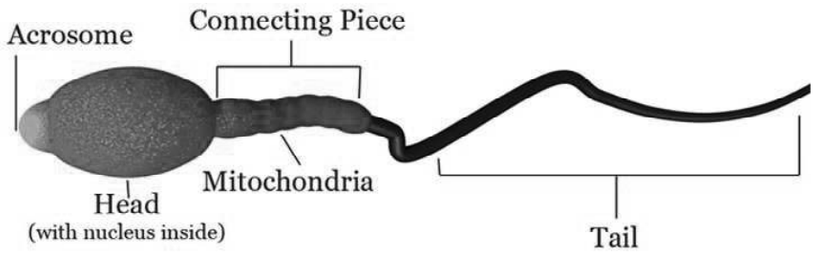


***Fig. 3.4*** *Mature Sperm Cell*

The transition between spermatogonia and spermatocytes is mediated by the factors release by the sertoli cells as follows:

- Glial cell lines- Derived Neurotropic Factor (GDNF)
- Stem Cell Factor (SCF)

GDNF levels determine whether the dividing spermatogonia last as the spermatogonia or enter the meiotic phase to become spermatocytes. Low level of GDNF is required for the differentiation of spermatogonia whereas high level of GDNF is needed for the self-renewal of stem cells. SCF promotes the transition to spermatogenesis. Both GDNF & SCF are up-regulated by FSH, so these two factors serves as link between sertoli cells and the endocrine system which provide the mechanism for FSH to increase the production of sperms.

## Post-Testicular Sperm Maturation

Studies have found that 'major maturation-associated' sperm membrane antigen (glycopeptides) appears on the surface of rat spermatozoa during post-testicular sperm maturation in the distal epididymis of rats. It has been found through in-situ transcript hybridization, molecular analyses of genomic DNA fragments and immunohistochemical staining that homologous counterparts are present in other mammals, including humans. In case of humans, this homologue is an abundant

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epididymal gene product that has been identified as lymphocyte surface antigen CD52. These Glycosyl-phosphatidyl Inositol (GPI)-anchored glycopeptides (absent in testis) are located within the epididymal epithelium that are transferred to sperm cell from epididymal through GPI-anchor; remains intact. Human TRPM 2/ clusterin, is another gene associated with sperm maturation.

## Hormonal Control of Spermatogenesis

Three hormones produced by male gonads directly or indirectly control the process of spermatogenesis. They are; testosterone, estradiol, and inhibin. Testosterone is secreted by Leydig cells which are present adjacent to seminiferous tubules. Estradiol and inhibin are secreted by sertoli cells or interstitial cells which are present inside the seminiferous tubules. The initiation of the process of spermatogenesis greatly depends on the activity of hypothalamus and adenohypohysis (Refer Figure 3.5).

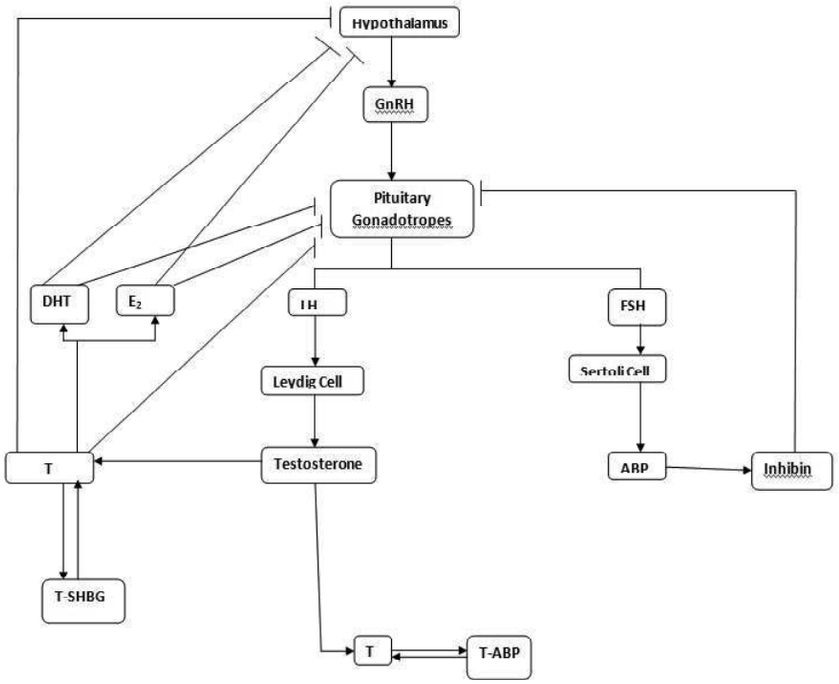


***Fig. 3.5:*** *Diagrammatic Representation of Hormonal control of Spermatogenesis.*

Deficiency of Fibroblast Growth Factor 2 (FGF-2) leads to abnormal spermatogenesis and altered sperm physiology. Earlier it was revealed that the presence of Fibroblast Growth Factor 2 (FGF-2) and its receptors (FGFRs) in human testis and sperms are involved in spermatogenesis and in motility regulations. Some studies were carried out to analyze the role of FGF-2 in the maintenance of sperm physiology using FGF-2 Knock Out (KO) mice experiment which showed that in Wild-Type (WT) animals, FGF-2 are expressed in germ cells of the seminiferous epithelium, in epithelial cells of the epididymis and in the flagellum and acrosomal region of epididymal sperm. This suggest that FGF-2 exerts a role in mammalian spermatogenesis and the lack of FGF-2 leads to deregulated sperm production as well as altered sperm morphology and function. FGF-2-deficient

mice constitute a model for the study of the complex mechanisms underlying mammalian spermatogenesis.

**Factors Affecting Spermatogenesis**

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Following are the factors that affect spermatogenesis:

- **Temperature:** Normal body temperature or above it, sensitize the seminiferous epithelium. So the optimal temperature (2 degree below body temperature) is maintained by the scrotum.
- **Scrotal Reflex:** Cremaster muscles and dartos smooth muscles in scrotum, position it towards or away from the heat of the body.
- **Others:** Deficiency of vitamin E, B and A; infectious diseases; X-ray exposure, metals like lead and cadmium, dioxin, alcohol, pesticides and anabolic steroids, and DNA damage due to oxidative stress.

## 3.2.1 Morphological Basis in Rodents

Spermatogonia (2n) → Spermatocyte (2n) → Spermatids (n) → Spermatozoa (n)

Spermatogenesis occurs in the seminiferous tubules of male sex organ, i.e., testes which vary in number from10 to 20 per testes in rat. It takes 52 days in rat and 35 days in mouse to accomplish the formation of mature spermatozoa from the stem cell spermatogonium. First step involves the development of primary spermatocytes from the primitive diploid stem cells called spermatogonia. The primary spermatocytes give rise to secondary spermatocytes which eventually give rise to spermatids. The spermatids undergo massive metamorphosis including the development of a tail and condensation of cytoplasm to produce the final reproductive call or gamete, i.e., spermatozoa in males. Spermatozoa are highly differentiated gametes. The process of spermatogenesis involves a few mitotic divisions of the spermatogonia, the final division giving rise to the spermatocyte. The spermatocyte undergoes a prolonged meiosis that starts with duplication of its DNA content during pre-leptotene, pairing and condensing of the chromosomes during zygotene and pachytene and finally undergoing two reductive divisions to give haploid spermatid. The life of a spermatid begins as a simple round cell which rapidly undergoes a series of complex morphological changes. This is followed by the condensation of the nuclear DNA making it highly condensed and elongated into a head region which is covered by a glycoprotein acrosome coat. The spermatid cytoplasm turns into a whip-like tail enclosing a flagellum and densely-packed mitochondria. The overall differentiation of the spermatid in rat comprises sequential 19 morphological steps (spermiogenesis). The cross section of a seminiferous tubule reveals the germ cells arranged in discrete layers. First layer on the basal lamina comprises of spermatogonia, then spermatocytes are arranged above them followed by one or two layers of spermatids further covers them from above. In any given normal tubule, four generations of cells develop simultaneously and in precise synchrony with each other. As and when each generation of these cells develops, it is pushed up through the epithelium, continuously supported by sertoli cells, until the mature sperms are released into the seminiferous tubule lumen, the process termed as spermiation.

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There are stage I-XIV in spermiogenesis (which are easily visible and distinguishable under light microscope) of the spermatogenic cycle. An acrosomic cap is formed during stages I - VIII on top of the round spermatids in the cycle of spermiogenesis. The early pachytene spermatocytes (EP) enlarge as they move into mid-pachytene (MP), and the intermediate spermatogonia (In) complete a number of mitotic divisions to become preleptotene spermatocytes. During stage VIII, the fully mature (step 19) elongated spermatids are released into the lumen. At this point a newly committed generation of spermatogonia (A) begin dividing and displace the newly formed preleptotene spermatocytes (PL) off the basal lamina. By stage IX, the round spermatid population has begun to elongate so that by stage XI there are step 11 spermatids that have an obvious elongated profile. The pachytene spermatocytes have become very large and enter late-pachytene (LP), and the preleptotene spermatocytes move into leptotene phase (L). During stage XIV the primary and secondary meiotic divisions take place and transform the large pachytene spermatocytes into new step 1 spermatids while zygotene spermatocytes enter early pachytene. It can be seen that the cellular makeup of the stage following meiotic division (stage I) is exactly the same as the cell association that the cycle began with, the difference being that one generation (of sperm) has been released and a new generation (of spermatogonia) has joined, and the rest of the cells are 14 days older and have moved up a layer.

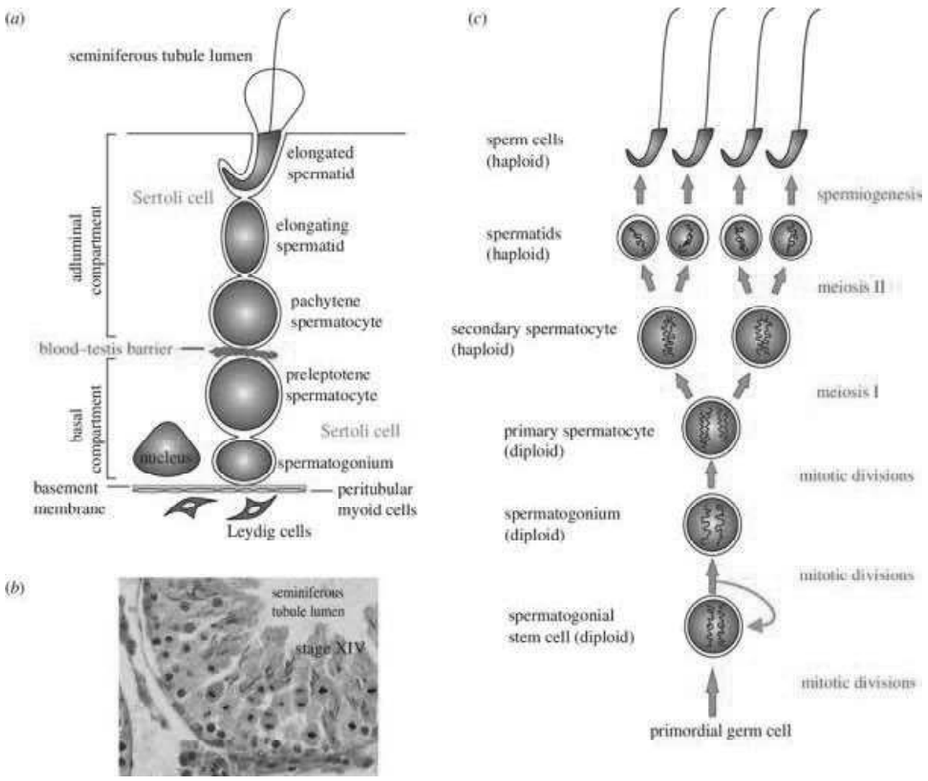


***Fig. 3.6*** *Stages in Spermatogenesis (Rat)*

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### 3.2.2 Morphological Basis in Any Invertebrate

Drosophila is a representative invertebrate in which gonad differentiation has been thoroughly studied. The drosophila testis is coiled tubular structure with blunt-ends and supports the production of sperm throughout the life. Spermatogenesis is accomplished by maintenance of a small number of stem cells that divide approximately once every 24 hours in a sustainable manner. Both germline and somatic stem cells are located at the apical tip of the testis, and under homeostatic conditions, stem cells divide asymmetrically to produce two cells: one cell maintains stem cell characteristics, while the other daughter cell initiates differentiation. In the germ line, the differentiating daughter cell is called a gonialblast (GB), and the GB undergoes mitotic amplification (transit amplifying; TA) divisions to generate a cyst of spermatogonia, which will differentiate into spermatocytes and, ultimately, mature sperm. There occur four rounds of mitotic divisions succeeded by two meiotic divisions in drosophila, yielding 64 haploid spermatids. The spermatids undergo elongation and maturation, i.e., spermiogenesis, the final stage of spermatogenesis, where after the sperm is released into the seminal vesicle.

Stem cells thrive in specialized microenvironments or 'niches' that are quite dynamic and provide structural and chemical support to the residing stem cells. One or more cell types and/or a basement membrane are integral components of the niche, providing structure and polarity to the system. These act as a source of growth factors for stem cell maintenance and survival. There are three highly interdependent cell types in drosophila testis niche: the hub, the Somatic Cyst Stem Cells (CySCs) and the Germline Stem Cells (GSCs). A number of ligands are secreted by the hub cells activating signalling molecules and transcription activator proteins that control the gene expression of germline stem cells and somatic cyst stem cells. CySCs also strongly influence GSC behaviour. Additional factors, such as, the Bone Morphogenetic Protein (BMP) and hedgehog (Hh) pathways, also regulate stem cell behaviour in the testis niche.

**Check Your Progress**

1. List the two types of gametogenesis.
2. What are the phases of the process of spermatogenesis?
3. In which phase do primary spermatocytes undergo the first meiotic division?

## 3.3 BIOCHEMISTRY OF SEMEN

The seminal fluid or semen, a liquid substance that is discharged from the male reproductive tract and that contains spermatozoa, which are necessary for fertilizing the female's eggs. Semen also possesses liquids that combines to form seminal plasma, which helps keep the sperm cells viable. It consists of several fluids including amino acids, citrate, enzymes, etc. In mature male, spermatozoa are produced by testes that contribute only ten per cent of the total semen.

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### 3.3.1 Semen Composition and Formation

In the reproductively mature human male, sperms production occurs in the testes (singular, testis); they constitute only about 2 to 5 per cent of the total semen volume. As sperm descend along the male reproductive tract, they are submerged in fluids produced and secreted by the various tubules and glands of the reproductive system. After release from the testes, spermatozoa are stored in the epididymis, in which secretions of potassium, sodium, and glycerylphosphorylcholine (an energy source for sperm) are contributed to the sperm cells. Maturation of sperm takes place in the epididymis. After this, they pass through a long tube, called vas deferens (ductus deferens), to the ampulla which is another storage site and secretes a yellowish fluid, i.e., ergothioneine, a substance that reduces (removes oxygen from) chemical compounds. The ampulla also secretes fructose, a sugar that nourishes the sperm. At the time of the process of ejaculation, liquids from the prostate gland and seminal vesicles are added, diluting the concentration of sperm in the seminal fluid and provide an optimum and suitable environment for them. Fluids contributed by the seminal vesicles are approximately 60 per cent of the total semen volume. Fructose, amino acids, citric acid, phosphorus, potassium, and hormones known as prostaglandins are main ingredients of these fluids. The prostate gland contributes about 30 per cent of the seminal fluid; the constituents of its secretions are mainly citric acid, acid phosphatase, calcium, sodium, zinc, potassium, protein-splitting enzymes, and fibrolysin (an enzyme that reduces blood and tissue fibres). A small amount of fluid is secreted by the bulbourethral and urethral glands; this is a thick, clear, lubricating protein commonly known as mucus.

### 3.3.2 Assessment of Sperm Functions

Sperm is the male reproductive cell and is derived from the Greek word *sperma* (meaning seed). In the types of sexual reproduction known as anisogamy and its subtype oogamy, there is a marked difference in the size of the gametes with the smaller one being termed the 'male' or sperm cell. A uniflagellar sperm cell that is motile is referred to as a spermatozoon, whereas, a non-motile sperm cell is referred to as a spermatium. Sperm cells cannot divide and have a limited life span, but after fusion with egg cells during fertilization, a new organism begins developing, starting as a totipotent zygote. The human sperm cell is haploid, so that its 23 chromosomes can join the 23 chromosomes of the female egg to form a diploid cell. In mammals, sperm develops in the testicles, is stored in the epididymis, and released from the penis. The main sperm function is to reach the ovum and fuse with it to deliver two sub-cellular structures:

- Male pro-nucleus that contains the genetic material
- Centrioles that are the structures that help organize the microtubule cytoskeleton

Related to sperm quality is sperm size, at least in some animals. For instance, the sperm of some species of fruit fly (*Drosophila melanogaster*) are up to 5.8 cm long -about 20 times as long as the fly itself. Longer sperm cells are better than their shorter counterparts at displacing competitors from the female's seminal

receptacle. The benefit to females is that only healthy males carry 'good' genes that can produce long sperm in sufficient quantities to outcompete their competitors (Refer Figure 3.7).

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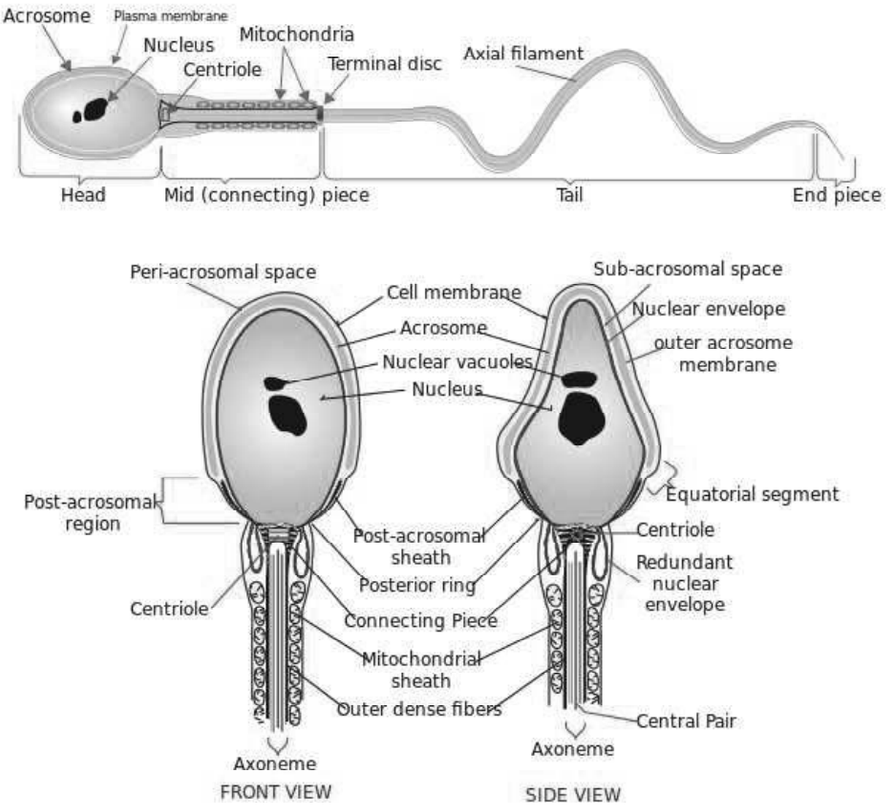


***Fig. 3.7** Human Sperm Cell*

## Physiological Maturation of Sperm

Haploid spermatid is round, unflagellated cells that undergo transformation, i.e., conversion of haploid, non-motile spermatid into motile elongated spermatozoa. Spermiogenesis is the process by which haploid round spermatid completes an extraordinary series of events to become streamlined motile spermatozoa. Spermiogenesis begins after spermatocytes complete two quick successive meiotic reductive divisions to produce haploid round spermatids. Further no cell division occurs as the spermatids undergo complex cytodifferentiation, over a period of 2–3 weeks in mice and rats to form mature elongated spermatids that are ultimately released from the seminiferous epithelium through a process known as spermiation. Main significance of this process is to increase the sperm motility and to make sperm more potential so that it could easily penetrate the ovum and process of fertilization could takes place. During spermiogenesis the developing sperms keep their heads embedded in the sertoli cells so that they could easily draw the nourishment from them (Refer Figure 3.8). There are following processes involved during changes and maturation of sperms:

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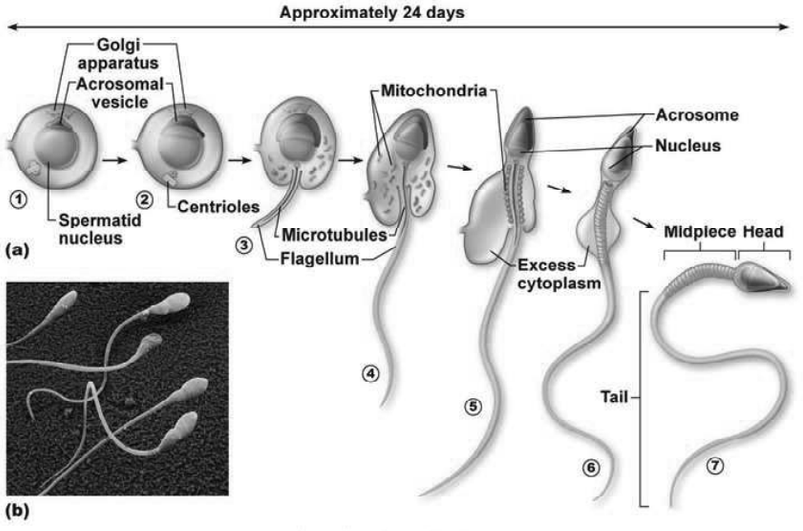


*Fig. 3.8 Representation of Changes during Spermiogenesis or Maturation of Sperms*

- **Changes in Nucleus:** Initially the change that takes place in the process of spermatogenesis is the progressive elongation and reduction in volume of the nucleus by losing water from the karyolymph. This step is essential, since it reduces the weight of the spermatozoa and enhances the sperm motility. Along with this condensation of DNA and reduction of RNA takes place. Chromatin fibres become closely packed and form uniformly dense mass. All these changes reduce the volume of nucleus to 0.5 %. In the compact chromatin, DNA is transcriptionally active therefore protein synthesis in spermiogenesis utilizes the stored mRNA molecule. This is an example of post transcriptional regulation of gene expression. During all these changes that take place, nucleus attains a characteristic shape and forms the sperm head. Shape of the nucleus is determined by the pattern of DNA-protein interaction during the condensation.
- **Changes in Golgi Complex:** Golgi apparatus of spermatid gathers anterior to the nucleus. Membrane bound pro-acrosomal vesicle develop in the golgi apparatus. These pro-acrosomal granules accumulate into a single large acrosomal vesicle having dense acrosomal granule in it. Acrosomal vesicle joins with nucleus and forms the future anterior end of the sperm. When the acrosomal vesicle attains its size, it assumes the final shape of mature acrosome.
- **Changes in Centrioles:** Spermatid has two types of nucleus, during the process of spermiogenesis both the centrioles change their position and lie behind the nucleus. Out of these two centrioles, one enters into depression developed in the posterior part of the sperm and latter one is called as the proximal centriole. The remaining centriole is known as distal centriole which will be present behind the proximal centriole and its axis coinciding with the longitudinal axis of the spermatozoa.

Functions of centriole are as follows:

i) Proximal centriole enters inside the ovum along with sperm and plays a role in the spindle formation.

ii) Distal centriole give rise to 9+2 axial filament of the flagellum and act as basal granule.

iii) In some mammals, proximal and distal centriole get disappears after organizing the connection between neck and middle piece.

- **Changes in Mitochondria:** Mitochondria play an important role in the process of spermiogenesis in the middle piece. It gets accumulated around the proximal part of the axial filament and distal centriole. Gradually, these mitochondria loses their individuality and fuse together forming two densely packed bodies, one on the either side of axial filament (mammals only) and this sheath is known as Nebenkern (spiral arrangement of sheath is visible only in mammals). In other animals, mitochondria are joined to form massive clumps known as mitochondrial bodies (Refer Figure 3.9).

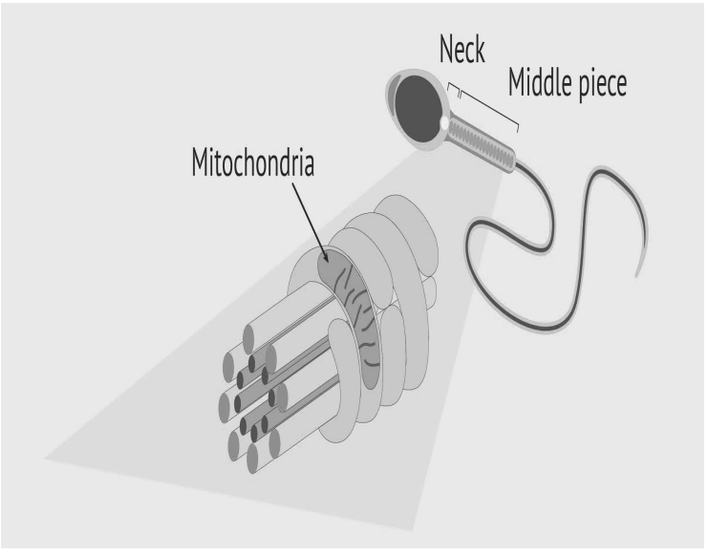


***Fig. 3.9*** *Mitochondria in a Sperm*

- **Changes in Cytoplasm:** Most of the cytoplasm in spermatid is lost and remaining cytoplasm forms a condensed layer in the peripheral region of the spermatid. This layer is known as Manchette band which surrounds the middle piece as well as the posterior part of sperm's head.
- **Changes in Plasma Membrane:** Plasma membrane extends to surround the acrosome, nucleus, middle piece and tail of the sperm. Initially, some proteins remain embedded over the surface of plasma membrane and acts as recognition or binding factors during the process of fertilization. Hence, above mentioned are all the changes that take place during the conversion of spermatid into spermatozoa.
- **Metamorphic Changes:** The different steps of spermiogenesis are distinguished by the morphological appearance of the developing acrosome

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and the change in the shape of the nucleus. During spermiogenesis, round spermatids having a spherical central nucleus, begin to assemble the acrosome and the axoneme structures required for the fertilization and motility respectively. Thus, the major event occurring during spermiogenesis is the assembly of the sperm flagellum. The central component of the flagellum, i.e., microtubule-based axoneme, is assembled soon after the completion of meiosis. As spermatids elongate, the accessory structures needed for flagella function (outer dense fibres, fibrous sheath, mitochondrial sheath) are assembled around the central axoneme. The final stage of spermiogenesis is known as spermiation and is the process by which the elongated spermatids undergo their final remodelling and get released from the seminiferous epithelium. Spermiation is a complex, multi-step process, which is particularly vulnerable to disruption. In case of mice, the last stage of sperm maturation is controlled by *Katnal1* gene. This is expressed in sertoli cells which supports and nurture the sperm at the site of spermatogenesis. Dysfunction of *Katnal1* gene leads to male infertility.

## Structure Sperms

Sperm, also called spermatozoon (plural spermatozoa), is a male reproductive cell produced by most of the animals (Refer Figure 3.10 and 3.11). It is flattened and has a whip tail with the exception of nematode worms, diplopods (for example, millipedes), decapods (for example, crayfish), and mites. In higher vertebrates, especially mammals, testes produce the sperm which unites (fertilizes) with an egg (ovum) of the female to form a new offspring. The sperm cells have half of the usual number of the chromosomes (n=23). When sperm cell unites with ovum, (also has 23 chromosomes), the resulting cell called Zygote (n equal to 46 chromosomes) determine the offspring's characteristic. Moreover, the sperm cell also carry X or Y chromosome which determines the sex of the young one. Sperm is 60 μm long, actively motile and is divided into 3 main regions:

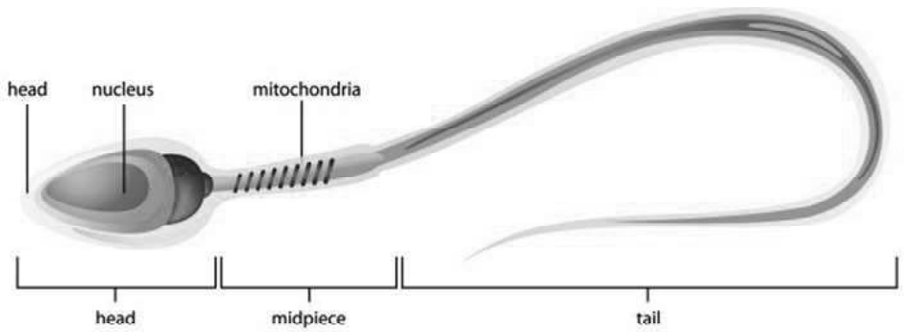


***Fig. 3.10*** *Sperm Cell Anatomy*

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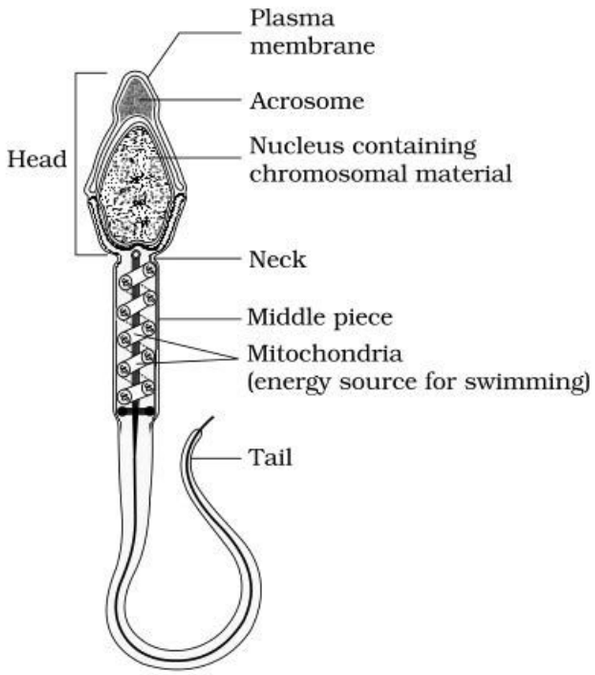


***Fig. 3.11*** *A Typical Structure of Mature Sperm*

- **Head:** The head of the sperm show variation in shape for different animal species. In humans it is flattened, almond-shaped, 4-5µm long and 2-3µm wide. The head portion is mainly a cell nucleus having genetic substances, known as chromosomes; that transmits specific characteristics of an individual, such as the colour of eyes, skin and hair. Each body cell of a healthy person has 46 chromosomes, which determine the individual's general physical makeup. Head of the sperm is covered by a cap called as acrosome that has enzymes which help sperm to enter an egg. An average ejaculation contains 300,000,000 to 400,000,000 sperm out of which only one enters the egg. Each egg and sperm produced shows slight difference in genetic information stored in the chromosomes which accounts for differences and similarities in between children of the same parents (Refer Figure 3.12).

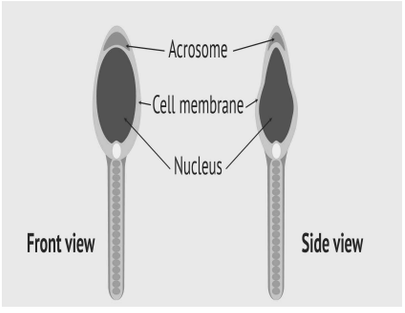


***Fig. 3.12*** *Head of Sperm*

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- **Neck and Middle Piece:** The neck is a constriction like structure of about 1µm length and is attached to basal plate, transverse oriented centriole, containing nine segmented columns of fibrous materials and continues as outer dense fibres in tail. The middle piece is 5µm long; consist of axoneme and dense fibres surrounded by mitochondria. The middle piece is divisible in two parts: principal piece and end piece. The principal piece is about 4.5µm long fibrous sheath interconnected by regularly spaced circumferential hoops, while the end piece is about 0.5µm long having axoneme, surrounded by small amount of cytoplasm and plasma membrane.
- **Tail:** It has three parts: a middle piece, principal piece, and end piece. A small middle portion of sperm contains mitochondria. The tail of sperm (sometimes called flagellum) is a slender, hair like bundle of filaments which connects with the head and middle portion. The tail is about 50µm long; having thickness of 1µm near the mitochondria which gradually diminishes to less than 0.5µm at the end of tail. The tail helps in sperm cell movement (whips and undulates) so that it can travel to the egg.

### Physiology of Sperm

After sperm deposition in the reproductive tract of female, tail movement is suppressed until the sperm reaches relatively short distance of the egg. This facilitates the sperm with increased chance of reaching to the egg before it exhausts its energy supplies. The activation of tail movements is a part of the process called as capacitation, in which the sperm undergoes a cascade of cellular changes to enables, its participation in the process of fertilization. Alkalinisation of sperm cytoplasm is the elementary change that occurs during capacitation where increase in pH levels takes place, mainly in the flagellum. This process takes place due to the rapid movement of protons out of cell via ion channels present on flagellum which leads to tail activation. Anandamide is the compound present in the female reproductive track (high concentration near the egg) plays an important role in opening the proton channels. When the sperm reaches the egg, the enzymes in the acrosome gets activated which all the sperm to penetrate the zona pellucid and the process is called as acrosome reaction, which leads to the fusion of the egg membrane with the sperm nucleus. The sperm that fail to reach the egg gets degenerated. Sperm cell survives in the human body up to 2 or 3 days after mating but can be stored for many months or years in frozen state with its viability intact.

### Anomalies in Spermatozoa

Certain studies have shown the following abnormalities in the ejaculated spermatozoa (Refer Figure 3.13):

- Spermatozoa with abnormal head, folded tail, connecting piece and sometimes missing tail.
- Immature spermatozoa having two or three tails of equal length, coiled and fused or/ along with round and small head; some time with wide head.
- Immature spermatozoa having two heads along with two fused tails; or one at each tip of the tail.

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- Spermatozoa with a triangular head and short, folded tail without an intermediate piece.
- Spermatozoa having no main piece but long intermediate piece along with short tail and rough head.
- Spermatozoa having a small, rounded, aberrant, bacillary and tapering head (Refer Figure 3.13).

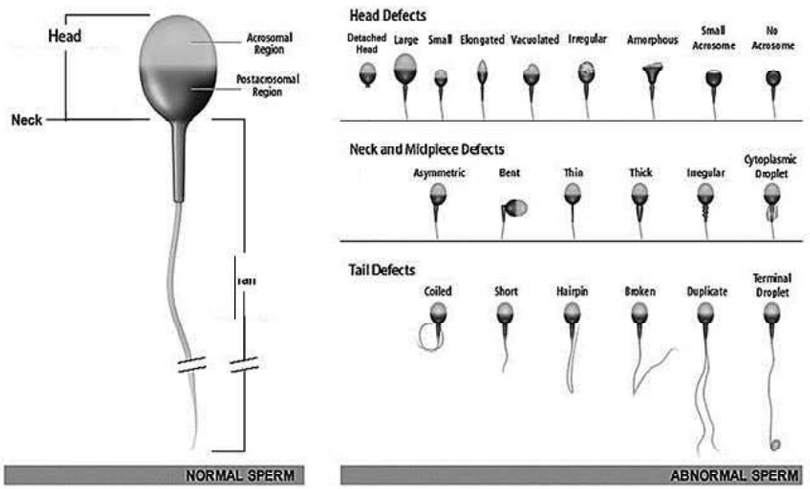


***Fig. 3.13*** *Diagrammatic Representation of Anomalies of Spermatozoa*

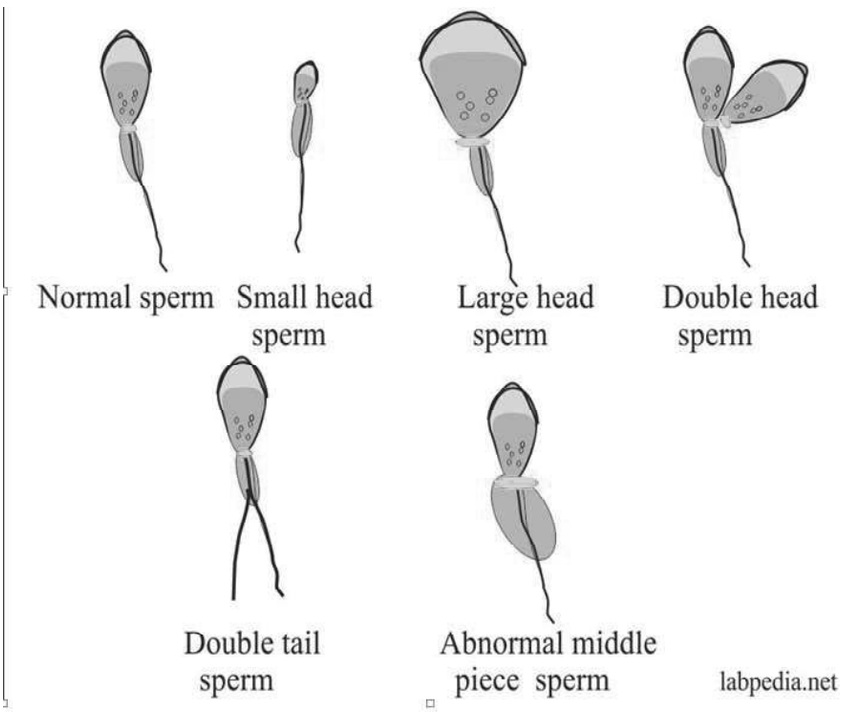


***Fig. 3.14*** *Sperm Morphology Abnormal Form*

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### 3.3.3 Gamete Specific Gene Expression and Genomics: 'Y' Specific Probes

In mammals which are diploid organisms, all somatic cells possess two matched sets of chromosomes, one inherited from each parent, i.e., the mother and the father. Thus, there are two copies of most of the genes in mammals. Normally both the maternal and paternal copy of each gene has the same potential to be active in any cell. Genomic imprinting is an epigenetic mechanism that changes this potential because it restricts the expression of a gene to one of the two parental chromosomes. Therefore, the cells that contain two parental copies of all genes will express only one parental copy of an imprinted gene and silence the other parental copy; whereas, non-imprinted genes are expressed from both gene copies (inherited from father and mother) in a diploid cell. Another phenomenon in gamete specific gene expression is of unequal parental genetic contribution include Y chromosome–linked genes present only in males, genes that escape X inactivation in females (producing a double dose of X-linked gene products compared with males), mitochondrial genes contributed mainly by the maternal parent, and messenger RNAs (mRNAs) and proteins present only in the sperm or egg cytoplasm. The group of genes exhibiting genomic imprinting usually code for factors regulating embryonic and neonatal growth. There are certain genes which are present only on one of the Y or the X chromosome. This can be explained as follows:

i) **Y-Specific Genes:** In dioecious organisms including mammals, there are at least 60 genes found only on the Y chromosome. The Y is the smallest chromosome in the human genome (<" 60 Mb) and represent around 2%–3% of a haploid genome. Chromosome banding experiments enabled the identification of different Y regions: the pseudo autosomal portion (divided into two regions: PAR1 and PAR2) and the euchromatic and heterochromatic regions. The most important gene present on the Y chromosome is SRY. In 1990, this gene responsible for testicular determination, named SRY (Sex-determining Region on the Y chromosome), was identified by Sinclair et al.

   One more important gene locus AZF present on the Y chromosome governs the fertility in males. The original AZF region was further sub-divided into three different non-overlapping sub-regions in Yq11 associated with male infertility, named AZFa, AZFb, and AZFc. Each one of these regions contains several genes proposed as candidate genes involved in male infertility. Although, there is no direct evidence for a role of Y in tumour progression, the Y chromosome loss and rearrangements have been associated with different types of cancer, such as bladder cancer. Both oncogenes and tumour suppressor genes are present on this chromosome, having a pathogenic significance mainly in male-specific organs such as, testis. One cancer predisposition locus has been assigned to this chromosome, the gonadoblastoma locus on the Y chromosome (GBY). The gonadoblastoma is a rare form of cancer that consists of aggregates of germ cells and sex cord elements.

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ii) **Dosage Compensation and X-linked Genes:** In females of higher vertebrates especially in mammals, dosage compensation of X-linked genes among males and females is shown clearly taking place via genetic inactivation of one of the two X chromosomes. The choice of X chromosome to be inactivated is random in somatic cells, i.e., either the paternally or maternally inherited X chromosome is inactivated in a given cell. The inactive X chromosome can be demarcated from the active X chromosome by the following characteristics: (i) overall transcriptional inactivation (apart from certain X-linked genes which escape inactivation and the Xist gene); (ii) heterochromatic condensation at interphase of the cell cycle, sometimes visible as the Barr body ; (iii) late replication during S phase ; (iv) DNA methylation of cytosine residues at CpG dinucleotides in the 59 region of X-linked genes ; (v) hypoacetylation of histone H4; and (vi) expression of the Xist (X inactive specific transcript) gene located at the X-inactivation centre. In case of X chromosome aneuploidy one X chromosome is always chosen to be active rather than different numbers of X chromosomes being chosen to be inactive. The X chromosome inactivation (XCI) occurs sequentially in development, coupled with cell differentiation. XCI first occurs in the trophectoderm and the primitive endoderm which are the first two cell lineages that differentiate. There is a single X chromosome active in male somatic cells. However, it is transcriptionally inactivated during spermatogenesis.

### X-Linked Recessive Inheritance

The X chromosome bears many genes required for growth and development as well as reproduction. The Y chromosome contains much fewer genes. Females have two X chromosomes (XX) and if one of the genes on an X chromosome has a mutational change, the normal gene on the other X chromosome can compensate for the changed copy. If this happens the female is usually a healthy carrier of the X linked condition.

### X Linked Dominant Inheritance

In spite of most of the X linked traits recessive, very rarely X linked conditions can be inherited in a dominant way. Although the female inherits one normal copy and one mutated copy of the gene, the changed gene will be enough to cause the disease or abnormal condition. If a male inherits a mutated X chromosome it is enough to cause the condition because males only have one X chromosome. An affected female has a 50% (1 in 2) chance of having affected children (sons and daughters). An affected male will have all daughters affected but all sons will be unaffected.

### Points to Remember about X linked Inheritance

- Female act as carriers of X linked mutations or traits and have 50% chance of passing on a changed gene to the offspring. If a mutated gene is inherited by a son from his mother, then he will be suffering from the disease condition. If a daughter inherits a changed gene she will just be a carrier.

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- A male with an X linked recessive genotype will always pass on the changed gene to his daughter, who will be a carrier. However, if he has an X linked dominant condition his daughter will suffer from the disease. A male will never pass on a changed X linked gene to his son.

**Check Your Progress**

4. What is spermatozoon?
5. Write the three parts of the tail of a sperm.
6. When does alkalinization of sperm cytoplasm occur?

## 3.4 FERTILIZATION: PRE FERTILIZATION EVENTS, BIOCHEMISTRY OF FERTILIZATION AND POST FERTILIZATION EVENTS

The idea of fertilization was known to Leeuwenhoek and according to him an ovum can develop into an animal only on its impregnation with sperm. In 1854, George Newport describes the entry of sperm into frog's egg. In 1875, Oscar Hertwig described the union of nuclei of both the gametes, i.e., sperm and ovum during fertilization in Sea urchin. Fertilization is defined as the process of union of two gametes, eggs and sperm. When mammalian eggs and sperm come into contact in the female oviduct, a series of steps is set in motion that can lead to fertilization and ultimately to development of new individuals. Eggs are non-motile, surrounded by protective egg coverings. These serve to recognize the sperm specifically and prevent fertilization by more than one sperm (polyspermy). The mammalian egg has zona pellucida layer around the plasma membrane beneath which cortical granules are present. The zona pellucida layer makes the egg impenetrable to more than one sperm. Sperms are highly motile cells consisting of nucleus and mitochondria to provide energy source and a flagellum for movement. The anterior end of the sperm is highly specialized which aids in penetration of the egg. Sperms are typically designed to activate the egg and to deliver their nuclei into the egg cytoplasm via seawater in marine forms, fresh water in fresh water forms and body fluid in viviparous animals. To increase the probability of fertilization, the number of sperms must exceed the number of eggs. Moreover the lifespan of gametes is limited; therefore fertilization must take place within a short duration of time. Eggs that are shed in water like that of most invertebrates, fishes and amphibians, have shorter life.

The egg and the sperm are optimized in opposite ways for the propagation of the genes they carry. The egg is non-motile and aids the survival of the maternal genes by providing large stocks of raw materials for growth and development, together with an effective protective wrapping. The sperm, by contrast, is optimized to propagate the paternal genes by exploiting this maternal investment; it is usually highly motile and streamlined for speed and efficiency in the task of fertilization. Competition between sperm is fierce and the vast majorities fail in their mission.

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Out of the billions of sperm released during the reproductive life of a human male, only a few ever manage to fertilize an egg.

Fertilization is a phenomenon in which mature male gamete penetrate the mature female gamete ovum and this penetration leads to the fusion of their pronuclei resulting in the formation of the diploid cellular structure called as Zygote. Fertilization is the second most important event in the process of sexual reproduction. There are four stages of fertilization:

- **Preparation**: It includes capacitation and acrosome reaction. The acrosomal vesicle fusion is the membrane fusion event of this stage.
- **Binding**: It is species-specific interaction of gametes.
- **Fusion:** Merging of sperm and egg plasma membranes is the membrane fusion event of this stage.
- **Activation**: It comprises cortical reaction (fusion of cortical vesicles with the egg plasma membrane) and pronuclear fusion.

The process of fertilization either taking place in surrounding medium outside of body or in side of body. It is a complicated process of sexual reproduction involves two gametes:

- **Eggs:** Eggs are large (~100µm), symmetrical and non-motile cells. Human eggs are arrested in metaphase of the second meiotic division and complete meiosis only upon fertilization. Their surface is covered by microvilli. Eggs are surrounded by zona pellucida which is a glycoprotein coat composed of three glycoproteins (ZPGP I-III). All three of the glycoproteins contain O- and N-linked oligosaccharides. The zona pellucida is not an osmotic barrier (in fact, even virus are capable of penetrating it), however it is a barrier to the sperm. The zona pellucida is the species specific barrier to fertilization as shown by the hamster experiment. Human sperm are incapable of fertilizing intact hamster eggs, but can fertilize hamster eggs stripped of their zona pellucida. This is used clinically to assess the fertilizing capacity of sperm (Refer Figure 3.15).

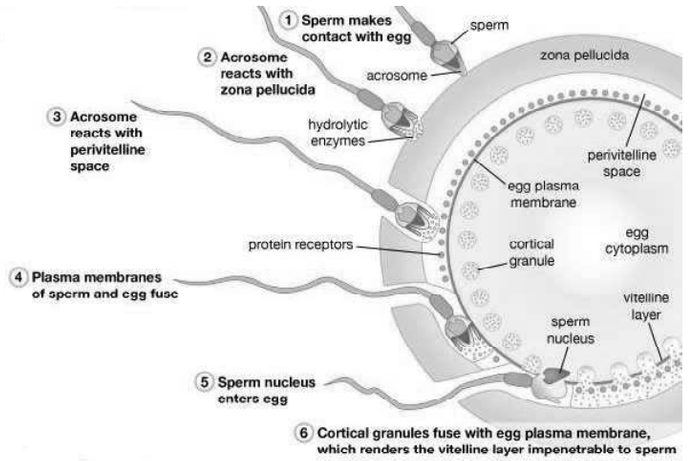


***Fig. 3.15*** *Diagrammatic Presentation of Fertilization*

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- **Sperms:** Sperms are small, asymmetrical and motile cells. They have components like:

  *1)* **Acrosome:** The acrosome is a lysosomal-like compartment derived from the Golgi. It has a low pH and contains soluble hydrolases (serine protease acrosin). In cross-section through the head of a sperm, one would cross four membranes in traversing from the plasma membranes to the nuclear membrane. During the acrosome reaction, fusion of the outer acrosomal membrane with the plasma membrane releases the contents of the acrosome and exposes the inner acrosomal membrane as the functional outer boundary of the sperm head.

  2) **Head:** It contains the spermatic haploid nucleus. Overlaying the head is a membrane bound vesicle, the acrosome. Sperm do not possess any organelles associated with protein synthesis (Golgi body, RER or lysosomes). The sperm plasma membrane is also highly differentiated and contains proteins localized in distinct regions. One of these, termed PH-30 or fertilin, is localized in the equatorial region of the sperm and is involved in sperm-egg plasma membrane fusion.

  3) **Middle Piece:** At the proximal portion of the tail. Mid piece contains a sheath of mitochondria, which produce the ATP necessary for the beating of the tail.

  4) **Tail:** Also referred to as the principal piece. The tail contains the flagellar apparatus, which is composed of '9+2' microtubules and accessory structures. The sliding of the microtubule is powered by the protein dynein. (Gibbons' movie of sliding microtubules)

## 3.4.1 Types of Fertilization

The fertilization process in animals can occur either internally or externally, a difference which is largely determined by the method of birth.

**Internal Fertilization:** Animals which use viviparous and ovoviviparous reproduction (embryos develop within the animal's body) and oviparous animals which lay hard shelled eggs, use internal fertilization. Internal fertilization involves the union of sperm and eggs within the body of the (usually female) parent. For internal fertilization to occur, the male must implant his sperm into the female reproductive tracts. Implantation can be achieved by either: Copulation, in which sperm transfer is performed by insertion of the penis or other male intromittent organ and ejaculation into the vagina, or cloaca,: or by a cloacal kiss, in which two birds press their cloacae together and sperm transfer takes place. Some animals, such as molluscs, arachnids, salamanders and certain insects, transfer a spermatophore, a bundle or capsule containing sperm, which is stored within the cloaca until oviposition takes place.

**External Fertilization:** Animals which are oviparous produce eggs which are lacking, or have thin egg membranes and reproduce by external fertilization. External fertilization is a reproductive strategy involving the joining of gametes outside of the body, either in a spawning event, where gametes from both sexes are rapidly released into an aquatic environment, or may occur when eggs are laid by a female on a substrate and are subsequently fertilized by a male. External fertilization holds certain benefits, such as reducing the chance of contracting sexually transmitted diseases, protection from violent behaviour between organisms and increasing the genetic variation within a population.

## 3.4.2 Mechanism of Fertilization

The fertilization is a complicated four steps process as discussed below (Refer Figure 3.16):

### Step-I Preparation of Sperm

Ejaculated sperms are not ready to fertilize an egg when they enter the vagina. In response to the dilution of semen in the vagina, they undergo several changes, which are collectively known as capacitation that includes:

- Intracellular Ca++ levels increases.
- Spermatic motility is activated and tail changes the beating frequency.
- Sperm cell surface antigens are lost. The loss of these proteins renders the sperm more receptive for binding to the egg.

### Step-II Sperm-Egg Binding

The process of sperm-egg binding was first studied and understood in invertebrates. In sea urchins, the sperm head binds directly to the egg's outer surface and this triggers the acrosome reaction. The acrosomal contents are released and there is a balanced Na+ influx and H+ efflux, causing an increase in pH. The increased pH triggers the dissociation of the profilactin complex (actin and profilin) and the released actin monomers polymerize to form a filament called the acrosomal process. This acrosomal process penetrates the egg coatings to allow fusion of the sperm and egg plasma membranes. In sea urchins then, the sperm literally skewers the egg. In humans the process of sperm-egg binding is not so simple. The complicating factor is the thick zona pellucida, which keeps sperm from binding close to the egg membrane.

### Sperm Receptor on Egg

Dr. Paul Wassarman used a competition assay to isolate and identify the factor in the zona pellucida that was involved in sperm egg binding. He incubated sperm with Zona Pellucida Glycoproteins (ZPGPs) which was isolated from unfertilized and fertilized eggs. He found that sperm pre-incubated with ZPGPs from unfertilized eggs were not able to fertilize eggs. When he pre-incubated sperm with ZPGPs isolated from fertilized eggs, known not to bind sperm, the sperm could still fertilize eggs. This showed that the isolated ZPGPs from unfertilized eggs contain a receptor for the sperm and that receptor is modified after fertilization.

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Dr. Barry Shur was studying a Golgi enzyme known as galactosyl transferase which catalyses the addition of galactosyl residues from a donor nucleotide sugar, UDP-galactose, to the terminal end of O-linked oligosaccharides. As in all enzymatic reactions, there are two stages in catalysis:

- The enzyme binds the substrates (in this case UDP-gal and O-linked oligosaccharide, and
- The enzyme catalyses the reaction and releases the products (in this case, UDP and the modified O-linked oligosaccharide with galactosyl residues on its ends).

It is important to understand that if one of the substrates is not present, the enzyme may be able to bind the available substrate, but will not be able to catalyse the reactions. This is important in sperm binding. Dr. Shur found that sperm, which have no Golgi apparatus, have galactosyl transferase on the surface of their plasma membrane. When sperms are ejaculated, they have oligosaccharides bound to the galactosyl transferase. During capacitation, these coating glycoproteins are removed, allowing the galactosyl transferase, to bind to other carbohydrates it may encounter, such as those attached to ZPGP III. However, there is no high energy UDP-galactose in the extracellular fluid surrounding the egg so catalysis does not occur and the sperm remains tightly bound to the egg zona pellucida. Many studies support a role of galactosyl transferase as a sperm protein involved in sperm-egg binding; however, other proteins may also be involved. A recent genetic knockout of galactosyl transferase in mice yielded mice that were completely fertile and showed normal sperm-egg binding.

### Acrosome Reaction

As a result of irreversible binding of the sperm to the egg, the zona pellucida triggers the acrosome reaction. The outer plasma membrane of the acrosome fuses at multiple sites with the plasma membrane and the contents of the acrosome are released. Two of the important components are acrosin, a serine protease and N-acetylglucoaminidase. Acrosin bores a hole in the zona pellucida so that the sperm can reach the egg itself. N acetylglucoaminidase hydrolyzes the O-linked oligosaccharides in ZPGP III to allow the sperm to detach. As a result of the membrane fusion, a new surface is exposed on the sperm (the inner acrosomal membrane) and this is thought to contain new binding sites for ZPGP II.

### Step-III Sperm-Egg Fusion

For many years the process by which the plasma membrane of the sperm and egg fused, was a black box. Recent studies by Dr. Judith White, Diana Miles, and Paul Primakoff and their colleagues, have now shed light on this process. Miles and Primakoff made an antibody to PH-30, a heterodimeric sperm membrane protein comprised of á and â subunits and showed that this antibody blocked fertilization but did not block binding of sperm to eggs stripped of their zona pellucida. This suggested that PH-30 was involved in sperm and egg fusion and it was given the name Fertilin.

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Cloning and sequencing of Fertilin revealed that á subunit had a hydrophobic domain that resembled with those on viral proteins that are known to be involved in membrane fusion. The â-subunit had a disintegrin domain. Disintegrins were first discovered in snake venom and act as competing ligands for integrins (for example, snake venom disintegrins, block platelet aggregation mediated by integrins). Fertilin was one of the first proteins of a family known as ADAMs proteins (for A Disintegrin and Metalloprotease containing protein) that are involved in cell-cell recognition and cell fusion events. Although the mechanism for how fertilin causes sperm-egg membrane fusion is not known, studies have supported its role in membrane fusion. For example, a peptide corresponding to the viral fusion peptide of á-fertilin is capable of fusing model membrane vesicles and the disintegrin domain of â-fertilin will block sperm-egg fusion. The egg integrin involved in sperm-egg fusion (the receptor for the â-subunit disintegrin) is known to be á6-â1. Once the sperm fuse with egg, the beating of tail stops immediately. The sperm instead is drawn into the egg by elongation and fusion of the egg's microvilli. As a result, the sperm nucleus and other organelles are incorporated into the egg cytoplasm. The sperm nucleus undergoes a series of changes, including chromatin decondensation and formation of a new nuclear envelope, to form a male pro-nucleus. The male pro-nucleus uses microtubules to migrate to the centre of the cell, where it fuses with the female pro-nucleus to reconstitute a diploid nucleus. Other sperm organelles (for example, mitochondria) persist during early cleavage stages of the embryo and it is conjectured that they may play a role in development.

### Step- IV Activation Response of Egg

The immediate events after fertilization include the egg's effort to prevent polyspermy. Polyspermy refers to the fertilization of the egg by more than one sperm, resulting in zygotes with greater than a diploid amount of DNA. This causes early embryonic defects and arrest of development. After sperm-egg fusion, the egg mounts the cortical reaction to prevent polyspermy. In all eggs, residing just under the plasma membrane there are membrane bound vesicles known as cortical granules. When a single sperm penetrates the egg, the cortical granules adjacent to the site are triggered to fuse with the plasma membrane, exocytosing their contents into the perivitelline space (the space between the plasma membrane and the zona pellucida). The cortical reaction is propagated over the surface of the egg by a wave of Ca++. This was shown by the aequorin experiment in which the photoprotein aequorin phosphoresced in a wave from the site of sperm penetration of the egg. As a result of the cortical reaction, two important enzymes are released into the perivitelline space:

- **Ovoperoxidase:** In sea urchins, ovoperoxidase catalyses the crosslinking of tyrosine residues in the extracellular matrix. This makes the extracellular matrix tough and insoluble (analogous to the tanning of leather) and a physical barrier is formed which prevents other sperm from fertilizing the egg. In mammals, ovoperoxidase does not catalyse tyrosine cross-linking to the point of insolubility. In mammals, its major effect is thought to be as a spermicidal agent.

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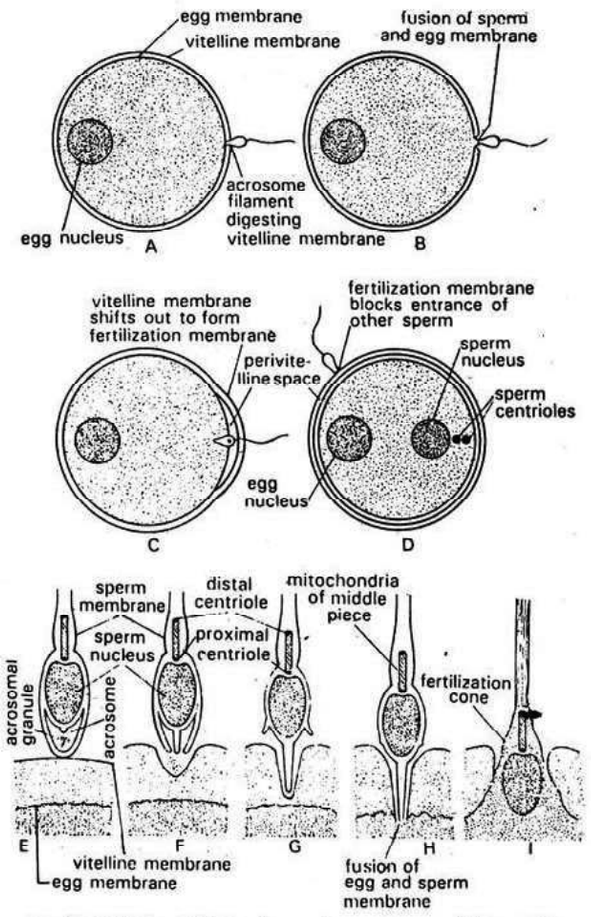


***Fig. 3.16*** *Schematic Representation of Fertilization*

- **Hydrolase:** Remember Wassarman's result showing that zona pellucida from fertilized eggs was incapable of blocking fertilization? Another cortical granule that is released is a specific hydrolase, which degrades O-linked oligosaccharides on ZPGP III. This renders the Zona Pellucida incapable of binding additional sperm, thus preventing polyspermy. Activation of the egg also includes the initiation of development of the new zygote. Protein synthesis and other metabolic processes are up-regulated to provide for the developing embryo.

### 3.4.3 Species Specific Fertilization by Fertilizin-Antifertilizin Reaction

One of the characteristic feature of fertilization is species specificity, i.e., spermatozoa of one species will only fertilize the ovum of that particular species only. Species specificity is of greatly biological importance as it helpful in maintaining the individuality of that species. This is achieved due to presence of specific chemical substances that are present on the surface of both the gametes. The Fertilizin secreted by the ovum and Anti-fertilizin secreted by sperm for the specific interaction which is essential for fertilization.

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Fertilizin is a sperm –agglutinating agent produced by an ovum and plays an important role in the preliminaries of fertilization. Chemically it is glycoproteinaceous in nature composed of carbohydrates (Glucose+ Fructose+ Galactose) and amino acids. Specific interaction of fertilizin takes place with other chemical substance secreted by sperm known as Anti-fertilizin that causes the sperm to adhere to egg and penetrate. Anti-fertilizin is composed of acidic amino acids such as Glutamic acid and Aspartic acid. The interaction between both these chemical substances makes the spermatozoa stick to egg surface therefore this adhesion of spermatozoa to egg of the same species through a chemical recognition is called as Agglutination (Refer Figure 3.17).

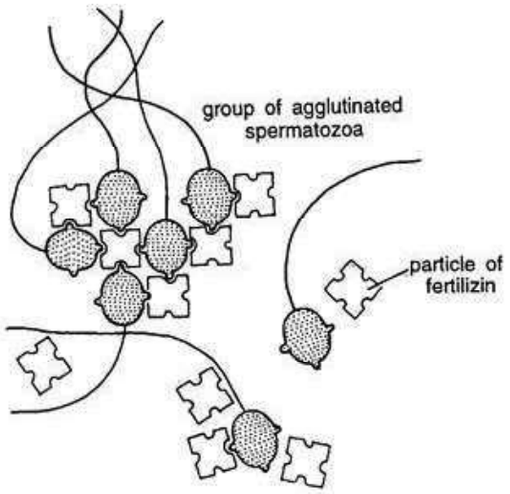


***Fig. 3.17*** *Agglutination of Sperm by Fertilizin and Anti-Fertilizing Reaction*

## Capacitation

After the ejaculation inside female genital tract mammalian sperm undergo a series of biochemical and physiological changes and are called as capacitation and these changes takes place in the female reproductive tract prior to the Acrosome Reaction (AR). Intracellular calcium plays an important role in bringing these changes in sperm. Ejaculated mammalian spermatozoa reside inside the female genital tract for several hours before acquiring the ability to fertilize the egg. In humans sperm must move out of the seminal plasma immediately after ejaculation and appear in the fallopian tube within minutes. These changes involve molecules absorbing on, or integrating into, the sperm plasma membrane during epididymal maturation. The removal or alteration of these molecules prepares the sperm toward successful binding to the egg and fertilization.

During mammalian fertilization, the capacitated spermatozoon penetrates the cumulus oophrous of the ovum, and then binds to the Zona Pellucida (ZP) with its plasma membrane intact. After binding to the egg ZP, the spermatozoon undergoes an exocytotic process called the Acrosome Reaction (AR). This event is required for fertilization, because it enables passage of the spermatozoon through the ZP and its subsequent fusion with the egg oolema.

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The capacitation includes multiple physiological and biochemical modifications. The biochemical changes associated with the capacitation process include:

- An efflux of cholesterol from the plasma membrane leading to an increase in membrane fluidity permeability gets increased to bicarbonate and calcium ions
- Hyperpolarization of the plasma membrane
- Changes in protein phosphorylation and protein kinase activity
- Increase in bicarbonate (HCO3–) concentration and intracellular pH
- Ca2+ and cyclic Adenosine Monophosphate (cAMP) levels also gets increased
- Generally the process lasts for 6 hours inside the female genital tract.

## 3.4.4 Monospermy and Polyspermy

As soon as sperm has entered the egg, the fusibility of the egg membrane, which was so necessary to get the sperm inside the egg, becomes a dangerous liability. In sea urchins, as in most animals studied, any sperm that enters the egg can provide a haploid nucleus and a centriole to the egg. In normal monospermy, in which only one sperm enters the egg, a haploid sperm nucleus and a haploid egg nucleus combine to form the diploid nucleus of the fertilized egg (zygote), thus restoring the chromosome number appropriate for the species. The centriole, which is provided by the sperm, will divide to form the two poles of the mitotic spindle during cleavage.

The entrance of multiple sperm, polyspermy leads to disastrous consequences in most organisms. In the sea urchin, fertilization by two sperm results in a triploid nucleus, in which each chromosome is represented three times rather than twice. Worse, since each sperm's centriole divides to form the two poles of a mitotic apparatus, instead of a bipolar mitotic spindle separating the chromosomes into two cells, the triploid chromosomes may be divided into as many as four cells. Because there is no mechanism to ensure that each of the four cells receives the proper number and type of chromosomes, the chromosomes would be apportioned unequally. Some cells receive extra copies of certain chromosomes and other cells lack them. Theodor Boveri demonstrated in 1902 that such cells either die or develop abnormally.

### Fast Block to Polyspermy

The fast block to polyspermy is achieved by changing the electric potential of the egg plasma membrane. This membrane provides a selective barrier between the egg cytoplasm and the outside environment and the ionic concentration of the egg differs greatly from that of its surroundings. This concentration difference is especially significant for sodium and potassium ions. Seawater has a particularly high sodium ion concentration, whereas the egg cytoplasm contains relatively little sodium. The reverse is the case with potassium ions. This condition is maintained by the plasma membrane, which steadfastly inhibits the entry of sodium ions into the oocyte and

prevents potassium ions from leaking out into the environment. If we insert an electrode into an egg and place a second electrode outside it, we can measure the constant difference in charge across the egg plasma membrane. This resting membrane potential is generally about 70 mV, usually expressed as –70 mV because the inside of the cell is negatively charged with respect to the exterior. Within 1–3 seconds after the binding of the first sperm, the membrane potential shifts to a positive level, about +20 mV. This change is caused by a small influx of sodium ions into the egg. Although sperm can fuse with membranes having a resting potential of –70 mV, they cannot fuse with membranes having a positive resting potential, so no more sperm can fuse to the egg. It is not known whether the increased sodium permeability is due to the binding of the first sperm or to the fusion of the first sperm with the egg.

### Slow Block to Polyspermy

The fast block to polyspermy is transient, since the membrane potential of the sea urchin egg remains positive for only about a minute. This brief potential shift is not sufficient to prevent polyspermy, which can still occur if the sperm bound to the vitelline envelope are not somehow removed. This removal is accomplished by the cortical granule reaction, a slower, mechanical block to polyspermy that becomes active about a minute after the first successful sperm-egg attachment.

Directly beneath the sea urchin egg plasma membrane is about 15,000 cortical granules, each cortical granule is about 1 μm in diameter. Upon sperm entry, these cortical granules fuse with the egg plasma membrane and release their contents into the space between the plasma membrane and the fibrous mat of vitelline envelope proteins. Several proteins are released by this cortical granule exocytosis. The first are proteases. These enzymes dissolve the protein posts that connect the vitelline envelope proteins to the cell membrane and they clip off the binding receptor and any sperm attached to mucopolysaccharides (released by the cortical granules) produce an osmotic gradient that causes water to rush into the space between the plasma membrane and the vitelline envelope, causing the envelope to expand and become the fertilization envelope. A third protein released by the cortical granules, a peroxidase enzyme, hardens the fertilization envelope by cross-linking tyrosine residues on adjacent proteins. The fertilization envelope starts to form at the site of sperm entry and continues its expansion around the egg. As it forms, bound sperm are released from the envelope. This process starts about 20 seconds after sperm attachment and is completed by the end of the first minute of fertilization. Finally, a fourth cortical granule protein, hyalin, forms a coating around the egg. The egg extends elongated microvilli whose tips are attached to this hyaline layer. This layer provides support for the blastomeres during cleavage.

## 3.4.5 Activation of Egg and Egg Metabolism

In Sea urchin the activation of egg and egg metabolism involves two mechanisms:

**Early responses:** The initial activation of all eggs is triggered by an increase in the concentration of free calcium ions inside cytoplasm of the egg. This increase is

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either due to calcium ions entering the egg from exterior or because of calcium ions released from the endoplasmic reticulum within the egg. Both processes vary in different species. In snails and worms, large amount of calcium probably enters the egg from outside, while in fishes, frogs, sea urchins, and mammals, most of the calcium ions probably come from the endoplasmic reticulum; however in both cases, a wave of calcium ions sweeps across the egg, beginning at the site of sperm-egg fusion. Presence of calcium ions is essential for activating the development of the embryo. Conversely, eggs can also get activated artificially in the absence of sperm by procedures that release free calcium into the oocyte. Steinhardt and Epel in (1974) found that injection of micro molar amounts of the calcium ionophore A23187 into a sea urchin egg elicits most of the responses characteristic of a normally fertilized egg. The elevation of the fertilization envelope, a rise of intracellular pH, a burst of oxygen utilization and increases in protein and DNA synthesis are all generated in their proper order. In most of these cases, development ceases before the first mitosis because the egg is still haploid and lacks the sperm centriole needed for the division. Calcium release activates a series of metabolic reactions. One of these is the activation of the enzyme NAD+ kinase, which converts NAD+ to NADP+. This change may have important consequences for lipid metabolism, since NADP+ (but not NAD+) can be used as a coenzyme for lipid biosynthesis. Thus, the conversion of NAD+ to NADP+ may be important in the construction of the many new cell membranes required during cleavage. Another effect of calcium release involves oxygen consumption. A burst of oxygen reduction (to hydrogen peroxide) is seen during fertilization and much of this 'respiratory burst' is used to crosslink the fertilization envelope. The enzyme responsible for this reduction of oxygen is also NADPH-dependent. Lastly, NADPH helps regenerate glutathione and ovothiols, which may be crucial for scavenging free radicals that could otherwise damage the DNA of the egg and early embryo.

**Late Responses:** Immediately after increase in the levels of calcium ion in sea urchin egg, its intracellular pH also increases and the rise in intracellular pH begins with a second influx of sodium ions, which causes a 1:1 exchange between sodium ions from the seawater and hydrogen ions from the egg. This loss of hydrogen ions is responsible for the rise in the intracellular pH. It is thought that the pH increase and the calcium ion elevation act together to stimulate new protein synthesis and DNA synthesis. If one experimentally elevates the pH of an unfertilized egg to a level similar to that of a fertilized egg, DNA synthesis and nuclear envelope breakdown ensue just as if the eggs were fertilized. The late responses of fertilization brought about by these ionic changes include the activation of DNA synthesis and protein synthesis. In sea urchins, a burst of protein synthesis usually occurs within several minutes after sperm entry. This protein synthesis does not depend on the synthesis of new messenger RNA; rather, it utilizes mRNAs already present in the oocyte cytoplasm. These messages include mRNAs encoding proteins such as histones, tubulins, actins, and morphogenetic factors that are utilized during early development. Such a burst of protein synthesis can be induced by artificially raising the pH of the cytoplasm using ammonium ions.

**Check Your Progress**

7. Name the four stages of fertilization.
8. Which two gametes are involved in the process of sexual reproduction?
9. What is the role of ovoperoxidase in sea urchins?

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## 3.5 COLLECTION AND CRYOPRESERVATION OF GAMETES AND EMBRYOS

Cryopreservation is an applied aspect of cryobiology which permits low temperature for the maintenance of diversity of the cells. However freezing process stands fatal to most living cells and organisms, basically due to formation of intra- and extracellular ice crystals resulting in changes to the chemical lattice structure of cell's biomolecules that lead to cellular mechanical constraints and injury (Refer Figure 3.18). The major hurdle for cells to overcome at low temperatures is the water-to-ice phase transition. Cell injury or frost injury when fast cooling rates are applied is because of intracellular ice formation, whereas slow cooling causes osmotic changes due to the effects of exposure to highly concentrated intra- and extracellular solutions or to mechanical interactions between cells and the extracellular ice. The application of cryoprotective agents and temperature control equipment has led to a gradual increase in successful cryopreservation of cells and tissues in recent years, with. The primary target of cryopreservation always remains reduce the damage of biological material which includes various cells and tissues such as bacterial, fungal, plant and mammalian cells at freezing and sub-freezing temperatures for the for storage. Technique of cryopreservation enables us find a source of preserving living tissues and viable cells for medical and biological research.

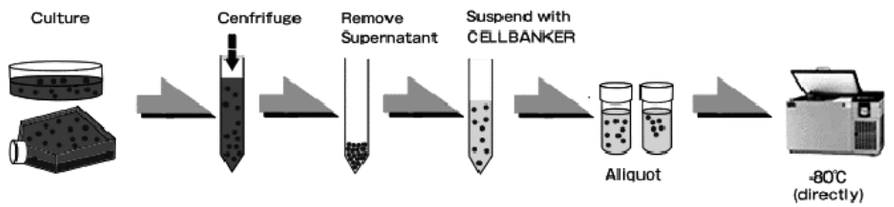


***Fig. 3.18*** *Schematic Presentation of Procedure of Cryopreservation of a Culture*

### 3.5.1 Cryoprotectants

In the process of cryopreservation, biological samples are usually maintains in a state of suspended animation at cryogenic temperature for any considerable period and is used to preserve the fine structure of cells. The freezing behaviour of the cells can be altered in the presence of a Cryo Protective Agent (CPA or Cryoprotectant), which affects the rates of water transport, nucleation and ice crystal growth. Different cryoprotectants which are widely used in the process of cryopreservation are as follow:

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- **DMSO (Dimethysulfoxide):** It was first synthesized by Alexander Zaytsex in 1866 and is commonly used for the cryopreservation of mammalian cells because of low toxicity levels. However use of DMSO is disadvantageous at certain point of time as it reduces the survival rate and the induction of cell differentiation which is caused by DNA methylation and histone alteration. So these negative effects of DMSO in cryopreservation create hindrances for its use in routine clinical applications.
- **Glycerol:** Glycerol is discovered as CPAs in 1949 and it is one of the most commonly used cryoprotectant because of its effectiveness. It is a non-electrolyte compound widely used for the cryopreservation of the bacterial cells and the spermatozoa. Both of these cryoprotectants act by reducing the electrolyte concentration in the residual unfrozen solution within in the cell at any given temperature.
- **Proteins:** In the process of cryopreservation proteins are also used as cryoprotectants and one such example is 'Sericin' which is a water soluble sticky protein, having a molecular weight of nearly 30KDa and isolated from the silkworm cocoon. It is a suited cryopreservative for the progenitor cells or the hepatocytes.
- **Cell Banker Series:** For rapid cell cryopreservation at –80°C and better survival rates of freezing and thawing, new different types of cryopreservation agent has been developed called as Cell Banker Series by Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan. Cell Banker Series contains 10% DMSO, glucose and prescribed high polymer and pH adjustors. Serum containing cell banker series used for the cryopreservation of the mammalian cells, whereas Cell Banker 3 or Stem Cell Banker contains 10% DMSO and other inorganic compound are suitable for the preservation of somatic cells and induced pluripotent stem cells.

## 3.5.2 Procedure of Cryopreservation

The process of cryopreservation and its procedure varies with different types of cells lines. Generally the process of cryopreservation involves following steps:

- Slow freezing
- Vitrification
- Subzero non-freezing storage
- Preservation in dry state.

Generally, the storage of mammalian cells in the dry state is not readily possible because of difficulties in introducing the disaccharide trehalose (disaccharide of glucose, 342Da ) and amino acids (used as preservatives in plants) into the intracellular region. The major steps in cryopreservation are:

- The mixing of CPAs with cells or tissues before cooling\Cooling of the cells or tissues to a low temperature and its storage
- Warming of the cells or tissues
- Removal of CPAs from the cells or tissues after thawing

- The appropriate use of CPAs is therefore important to improve the viability of the sample that to be cryopreserved.

Cryopreservation can be achieved by two methods either by Slow Freezing or either by Vitrification. Major distinction between these two methods is the concentration of CPAs and the cooling rates used during cryopreservation. Theoretically, if the cooling rate is low than cells could efflux the intracellular water, there is enough time to eliminate the super cooling which proves advantageous to prevent intracellular ice formation. That's why there are differences present in the capacity of different cells to move water across plasma membrane and thus cooling rates varies with different cell types.

- **Slow Freezing:** It encompasses the water within the cytoplasm with CPAs which reduces the cell damage and adjusts the cooling rate in accordance to the permeability of cell membrane. Cooling rate for this is about 1°C/min in the presence of CPA whose concentration is less than 1.0 M along with the use of Bench top portable freezing container. The biggest advantage of slow freezing is the reduction in the rate of the Contamination throughout the procedure. However the disadvantage of slow freezing is that it has high risk of the freeze injury due to the formation of extracellular ice.
- **Vitrification:** It is the process that involves the direct transformation of cell suspension from the aqueous phase to a glass state after the direct exposure to the liquid nitrogen. Simultaneously, the process requires the cooling of cells or tissues to a deep cryogenic temperature after treating with the CPAs with subsequent cooling to prevent ice nucleation. Process of vitrification is largely depends upon 3 factors: i. Viscosity of the sample, ii. Cooling and warming rates, and iii. Sample balance. Therefore the balance should be maintained between all the above mentioned factors to ensure the success of vitrification. Further the vitrification has 2 methods: Equilibrium vitrification and Non-equilibrium vitrification. Equilibrium vitrification requires the formulation of multimolar cryoprotectant mixtures and their injection into the cell suspension. Whereas the non-equilibrium vitrification which is further divided into carrier-based system includes the former plastic straws, quartz micro capillaries and cryoloops for obtaining a minimum drop volume and carrier-free systems involves the use of an extremely high freezing rate along with lower concentrations of the CPA mixture. Major advantage of vitrification is the low risk of freeze injury, thereby ensuring a sufficiently high cell survival rate. However, the high potential of contamination with pathogenic agents is present and therefore the technique requires good manipulation skills.

## 3.5.3 Advantages of Cryopreservation

Following are the advantages of cryopreservation:

- Possible banking of cells for human leukocyte antigen typing for organ transplantation
- The allowance of sufficient time for transport of cells and tissues among different medical centers

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- The provision of research sources for identifying unknown transmissible diseases or pathogens.
- The long-term storage of stem cells is highly beneficial in tissue engineering, which leads to regeneration of soft tissue esthetic function and for the treatment of known diseases having no current cure.

## 3.5.4 Applications of Cryopreservation

Cryopreservation finds many applications given as under:

- Cryopreservation of cells or organs
- Cryosurgery
- Biochemistry and molecular biology
- Food sciences
- Ecology and plant physiology
- Used in medical field for blood transfusion units to preserve blood components, in onco-therapy, e.g., bone marrow transplantation, artificial insemination and *in vitro* fertilization, semen and ovum preservation.

**Examples of cryopreservation**

Following are the few examples of cryopreservation:

- **Oocytes and Embryo:** Cryopreservation of the embryo was done for the first time in 1996, with the application in IVF cycle prior to chemotherapy in a woman diagnosed with breast cancer. Cryopreservation of mature oocytes is a proven technique for preserving the reproductive capacity of the infertile or diseased women.
- **Sperm, Semen and Testicular Tissue:** Cryopreservation is used as a first-line means for preserving fertility in men who have undergone vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiotherapy, or surgery.
- **Hepatocytes:** Isolated hepatocytes have wide range of applications in science and medicine over the past 40 years in areas such as physiological studies, investigations on liver metabolism, organ preservation &drug detoxification and experimental &clinical transplantation. In addition to this current interest is increasing in the applications of liver progenitor cells across a range of scientific areas, including both regenerative medicine and biotechnology, which raises the need for cryobanking.
- **Islets:** Development of islet cryopreservation methods has been ongoing, but results are still suboptimal, with a survival rate of less than the cryopreservation of the primary neuronal cells and cardiomyocytes as they are routinely used in the neuroscience and cardiology research.

## 3.5.5 Limitations of Cryopreservation

Despite of the numerous advantages of the cryopreservation technique both in basic and clinical research, there are some limitations also. Cells that perform

metabolic activities at low temperatures as low as "196°C (i.e., in liquid nitrogen), have inevitable side effects, including a genetic drift toward biological variations of cell-associated changes in biomolecules, e.g., lipids and proteins that cause substantial impairment of cellular activity and structure. If there were no limit to the amount of CPA that could be used, cells would be preserved perfectly. In conventional settings, however, CPAs themselves can be damaging to cells, especially when used in high concentrations. For example, there is a possibility that DMSO may alter chromosome stability, which can lead to a risk of tumour formation. Apart from endogenous changes in cells, the possible infection or contamination with cells such as tumorous ones should be prevented.

**Check Your Progress**

10. What does cryopreservation permit?
11. How can cryopreservation be achieved?
12. Name the factors that affect the process of vitrification.

## 3.6 OVARIAN FOLLICULAR GROWTH AND DIFFERENTIATION

Let us study the different aspects of ovarian follicular growth. The human ovary can be differentiated into an inner medulla and outer cortex with unclear boundaries. Blood vessels and nerves are found in medulla, while the cortex is filled by developing follicles. A cross-section of an ovary will reveal follicles in various stages of development. The histological features of each stage of follicular development and the major functional changes in the cells that compose follicles are important to study. Stages in the follicular development are summarized as follows:

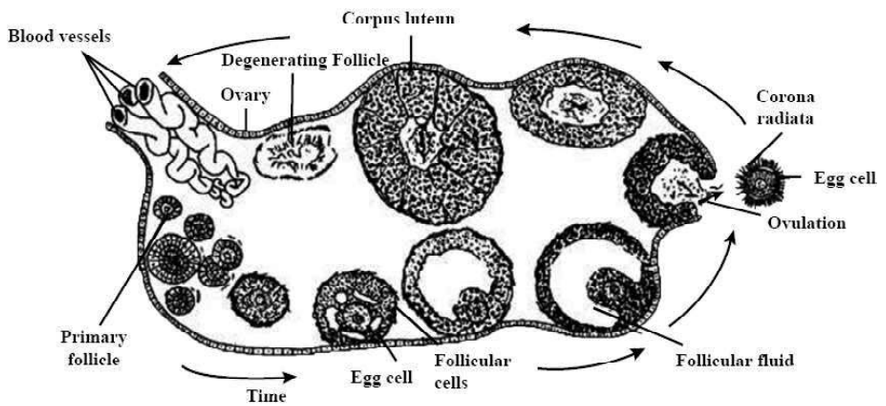


***Fig. 3.19*** *T.S. of Ovary*

### 3.6.1 Morphology

**(i) Primordial Follicle:** An ovarian follicle progresses through several distinct phases before it releases its ovum. During the first five months of

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development, a finite number of primordial follicles form in the foetal ovary. These follicles consist of oocytes surrounded by a single layer of squamous follicular cells. These primordial follicles remain in the process of the first meiotic division. At puberty, they begin to develop further and become primary follicles

(ii) **Early primary Follicle:** A limited number of primordial follicles at the beginning of each menstrual cycle are triggered to develop. The early primary follicle which is the first histological stage, consists of a central oocyte surrounded by a single layer of follicular cells which have become cuboidal. The zona pellucida is a thin band of glycoproteins that separates the oocyte and follicular cells. Proteins on the surface of sperm will bind to specific glycoproteins in the zona pellucida.

(iii) **Late primary Follicle:** In this late primary follicle stage the follicular cells proliferate into a stratified epithelium known as the zona granulosa. The enlargement of zona pellucida occurs and can be seen even more clearly in this stage.

(iv) **Secondary Follicle:** The appearance of a follicular antrum within the granulosa layer is a characteristic feature that separates a secondary follicle a from primary follicle. Fluid rich in hyaluronan and proteoglycans is filled inside the antrum. One can easily notice the increase in cell layers of the zone granulosa, the thicker zone pellucida, and larger oocyte. At this stage, a layer of cells outside the follicle become evident. These cells make the theca interna and produce oestrogens. It is important to mention that the production of oestrogen requires both the cells of the theca interna and granulosa cells. Oestrogens, like all steroid hormones, are produced from cholesterol through a multi-step process that employs many different enzymes. Neither the cells of the theca interna nor the granulosa cells contain all of the enzymes necessary to convert cholesterol into oestrogens. Theca cells contain enzymes that catalyze the initial conversion of cholesterol into androgens but lack aromatase that carries out the final steps of converting androgens into oestrogens. Consequently, androgens produced by theca cells diffuse into the granulosa cells which contain aromatase but lack the enzymes for the initial steps in oestrogen synthesis. The theca cells are in a better position to catalyze the initial steps in oestrogen synthesis because they are closer to blood vessels and can take up LDL to obtain cholesterol.

(v) **Graafian Follicle:** The Graafian follicle is the stage after the first meiotic division has completed but before ovulation. The oocyte is now a 2N haploid. The follicle is characterized by a large follicular antrum that makes up most of the follicle. The secondary oocyte, having undergone the first meiotic division, is located eccentrically. It is surrounded by the zona pellucida and a layer of several cells known as the corona radiata. When released from the Graafian follicle and into the oviduct, the ovum will consist of three structures: oocyte, zona pellucida and corona radiata.

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(vi) **Corpus Luteum:** Corpus Luteum After release of the ovum, the remaining cells of the granulosa and theca interna form the corpus luteum. The centre contains the remains of the blood clot that formed after ovulation. Surrounding the clot are granulosa lutein cells and on the outside theca lutein cells. Two substances, viz, the hormone Progesterone and to a lesser extent cholesterol are secreted by these cells. The granulosa lutein cells contain a pale yellow cytoplasm (due to lipid droplets) and have an appearance characteristic of steroid-producing cells. Theca lutein cells are smaller and more deeply stained. The region of the granulosa lutein cells has lots of blood vessels penetrated allowing them to take up cholesterol needed for progesterone synthesis. The activity of the cells of the corpus luteum is sustained by leutenizing hormone. Upon ovum fertilization and its subsequent implantation in the uterine wall, human chorionic gonadotropin replaces leutenizing hormone to sustain the activity of the cells in the corpus luteum.

(vii) **Corpus Albicans:** If the absence of fertilization, the cells of the corpus luteum remain active for roughly 14 days until the levels of LH fall and the corpus luteum involutes to form the corpus albicans. The secretory cells of the corpus luteum degenerate, are phagocytized by macrophages and replaced by fibrous material. Each menstrual cycle, several primordial follicles are stimulated to continue development but only one follicles completes development to release an ovum. The other follicles degenerate through a process called atresia which can occur at any stage of development. During atresia, granulosa cells undergo apoptosis and are replaced by fibrous material. The oocyte degenerates and the basement that separated the oocyte from granulosa cells thickens to become the glassy membrane.

## 3.6.2 Endocrinology

**Ultrastructure of Endocrine Cells in Ovary:** Many steroid hormones are secreted by the theca and granulosa cells. Theca cells remain steroid genic all through their life-span; whereas, granulosa cells become active and secrete steroids in the antral stage of development. The ultrastructure of steroid genic cells is characterized by morphologic adaptations that are related to the steroid genic function of the cells. The vesicular cristae are likely related to the expression of the cholesterol side-chain, cytochrome P450 (CYP11A) and the abundance of cholesterol in the inner mitochondrial membrane in contrast to the lamellar cristae which are reservoir of non-steroid genic cells. The smooth ER executes the expression of steroid genic enzymes. In non-steroid genic cells, the ER appears granular because of the abundance of ribosomes involved in protein synthesis. The lipid vesicles are a storage site for the cholesterol esters that can serve as precursor for steroid hormone biosynthesis. Because high concentrations of cholesterol are toxic to cells, they are isolated from the cytosol by encapsulation in membrane-bound vesicles.

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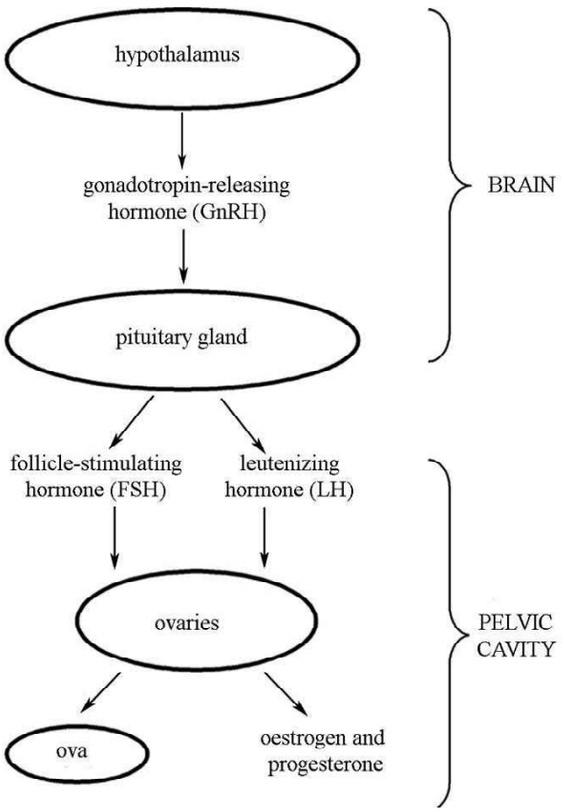


***Fig. 3.20*** *Flow Chart of Hormonal Control of Female Reproductive System*

Under normal conditions, women produce a single dominant follicle that ovulates a single oocyte each menstrual cycle. The process begins when a cohort of primordial follicles is recruited to initiate growth. Once a primordial follicle initiates growth, the theca and granulosa cells and the oocyte begin to express a developmental program in which a precise sequence and temporal pattern of gene activity occurs. If the entire developmental program is carried out, the follicle ovulates and luteinizes; if there are alterations or errors in this program, the follicle is destroyed by atresia. In women, the dominant follicle in each cycle originates from a primordial follicle that was recruited to grow about 1 year earlier. It takes approximately 300 d for a primordial follicle to progress through the pre-antral stages of development. Pre-antral follicle development can occur in the absence of gonadotropin stimulation and is therefore referred to as the gonadotropin-independent phase of development. There is recent evidence, however, indicating that the rate of follicle growth can be somewhat accelerated in the presence of high concentrations of Follicle-Stimulating Hormone (FSH). The rate of follicle growth accelerates markedly when the follicle enters the antral stages of development at approximately 2 to 3 mm in diameter. From this point onward, follicle development is highly dependent on FSH stimulation. At the end of the luteal phase of the menstrual cycle, there is a small increase in circulating FSH concentrations that is believed to stimulate the development of the cohort of small antral follicles from which one follicle will be selected to ovulate during the next

menstrual cycle. Withdrawal of FSH support is sufficient to cause the follicle to die by the process of atresia that is mediated by apoptosis. Although there is a rapid rate of proliferation of granulosa cells during antral follicle growth, the principal factor causing the tremendous expansion of the follicular diameter is the production of follicular fluid. A 4-mm follicle contains approximately 30 L of fluid, whereas a pre-ovulatory follicle contains up to 6.5 mL of antral fluid. Progression through the antral stages of development occurs in 40 50 d, equivalent to just over two menstrual cycles. The dominant follicle produces large quantities of estradiol which is one of its most important endocrine functions. The developmental state of follicle is communicated to the hypothalamus and pituitary through estradiol concentrations such that the mid-cycle ovulatory surge of LH is timed appropriately. A major function of hormone estradiol is to prepare the endometrium for embryo implantation. Both the theca interna and the granulosa compartments of the ovarian follicle are required for estradiol production. In addition, both LH and FSH stimulation are required for estradiol synthesis. These observations have been confirmed many times in a variety of mammalian species, and the molecular basis for the two-cell, two-gonadotropin concept for follicle oestrogen biosynthesis has been established. Alterations in the concentrations of the various steroid genic enzymes expressed in the cells is the second means for regulating steroid hormone biosynthesis to control cellular differentiation. The signal initiating granulosa cell growth and differentiation has not been fully defined. It is clear that gonadotropins are not involved because the granulosa cells in primordial follicles do not express FSH or LH receptors. The granulosa and theca cells are transformed into luteal cells by the process of luteinisation. Luteinisation is initiated by the surge of LH at mid-cycle, once the granulosa cells have acquired receptors for LH, and does not necessarily signify that ovulation has occurred. The LH flush causes large scale morphological changes in the follicle that converting it into corpus luteum. These include acquisition by the granulosa cells of the capacity of de novo synthesis of steroids (mainly progesterone and oestrogen) and invasion of the previously avascular granulosa cell layer by a vascular supply.

### 3.6.3 Molecular Biology

The abilities which are acquired by oocytes during their early follicular stage in vivo are explored via the study of in-vitro development of oocytes. Recently it was demonstrated that the inherently high variation in follicle numbers during follicular waves is also associated with significant alterations in intra-follicular estradiol production, which is the hallmark for follicular function, and expression of key genes important for differentiation, function, and survival of thecal, granulosa, and cumulus cells in the largest three follicles growing during follicular waves. The production of hormones and growth of oocytes capable of being fertilized are two major functions of ovarian follicle. The antral follicles perform these functions. Antral follicles possess an inner wall of granulosa cells supported by a distinct basal lamina. This specialized extracellular matrix separates the epithelial layer from the connective tissue and affects proliferation and differentiation of the granulosa cells. The mammalian oocytes develop and attain ovulatory maturity within these follicles. A follicle is made up by the oocyte covered by pre-granulosa

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or granulosa cells. Ovarian development starts in the embryo between 3 to 6 weeks post-conception, during which period a number of cellular events take place, such as massive colonization of the ovary with mesonephric cells, one of the precursors of the follicle cells, migration of the primordial germ cells into the genital ridge, gonadal sex differentiation, mitosis, and apoptosis of the germ-cells. The oocyte is known to play central role in follicular organization during the processes resulting in ovulation. It is assumed that the oocyte controls the proliferation of granulosa cells and later their differentiation into steroids and protein-secreting cells. Whereas, granulosa cells are indispensable for oocyte growth, differentiation, meiosis, cytoplasmic maturation, and control of transcriptional activity within the oocyte. The oocyte after it reaches a certain size threshold, secretes factors that destroy the capability of granulosa cells to promote the growth of the oocyte. This shows that the oocyte determines follicle growth of the follicle as well as its own growth, indirectly. The assembly of primordial follicles takes place during foetal life in the human and bovine species. It begins with an oocyte incompletely surrounded by flattened cells, called pre-granulosa cells. In bovine and porcine species the capacity to undergo meiosis is acquired more gradually after the formation of the antrum than in mouse. Smaller bovine follicles (< 2mm in diameter) display a lower rate of maturation and greater susceptibility to fertilization anomalies as compared to than bigger sized follicles. The *in vitro* development of blastocysts is lower when oocytes are obtained from follicles smaller than 6mm. A similar study comparing follicles of different size (3mm, 3.5mm, and 5mm) confirmed the direct relationship between the size of a follicle and the viability of the oocyte *in vitro*. It is possible that the oocyte acquires its capacity for development during the late follicular growth and that this capacity is not influenced by the beginning of follicular atresia. So the oocytes obtained from atretic follicles possess a capability of development *in vitro* just as in case of developing follicles. Primary oocytes, present in the primordial follicles in meiotic prophase I, result from consecutive mitotic division of oogonia throughout the foetal life. In developing oocytes, meiosis resumption and nuclear maturation in response to gonadotropin stimulation *in vivo* or *in vitro*, is characterized by chromosome condensation, progress from metaphase I to anaphase with extrusion of the first polar body and arrest at metaphase II. In the bovine, prophase of the first meiotic division begins at 70-80 days of foetal life. Primary follicles are made up of the primary oocyte and the surrounding single layer of flat to cuboidal granulosa cells. These cells, precursors of the GC, arrest the oocytes in the dictiyotene stage and prevent the continuation of meiosis. Immature oocytes that have not progressed through meiosis to MII are not able to be successfully fertilized.

### 3.6.4 Ovulation and Ovum Transport in Mammals

The process of setting free of mature ova from the ovaries is called ovulation. It occurs by different methods in different vertebrates. Egg fertilization involves a complex sequence of events that starts with the release of a mature egg from the follicle, continues with the appearance of the two pronuclei after sperm entry, and is completed with the first mitotic division. Understanding the complexities of this process in mammals has been limited to a large extent by ethical constraints.

However, with the advent of assisted reproductive technologies (ARTs), understanding of the various mechanisms involved in successful fertilization has been greatly enhanced.

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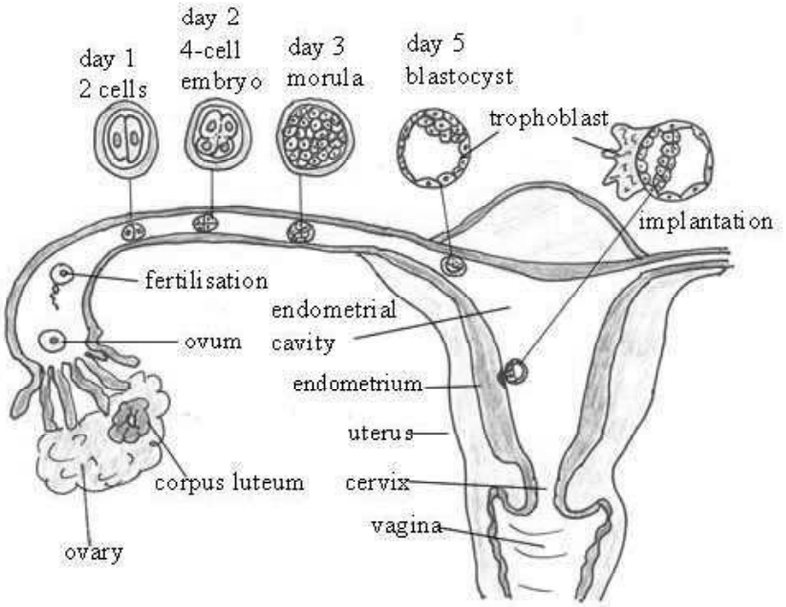


***Fig. 3.21*** *Ovum Transport*

Egg transport refers to the movement of the oocyte from the moment of expulsion from the ovarian follicle to entry into the distal segment of the fallopian tube before fertilization takes place. Once fertilized in the ampullary segment of the fallopian tube, the embryo spends about 5 days traveling into the remaining anatomical oviductal districts and arrives into the uterine cavity at the blastocyst stage. For purposes of clarity and accuracy, the term "egg transport" covers post-ovulation and pre-fertilization stages (i.e., the haploid life span of the ovulated oocyte). A subsequent section provides details concerning transport of the fertilized diploid oocyte (i.e., zygote) and pre-implantation embryo. The anatomy and physiology of the fallopian tube play an important role in egg transport and fertilization. The fallopian tube is a muscular tube with an average length of about 11–12 cm and is composed of four regions. The most distal portion is called the infundibulum, it is approximately 1 cm in length, and it includes the finger-like fimbria. The epithelial lining of the fimbria is densely ciliated and highly convoluted. This structure, along with the muscle-controlled movements of the fimbria, is thought to be important for capture of the cumulus-oocyte complex. The next portion of the oviduct is called the ampulla. This segment averages 5–8 cm in length. It is within this highly ciliated portion of the oviduct that fertilization and early embryo development occur. The ampulla is most often also the site for ectopic implantation (ectopic pregnancy). The next region, approximately 2–3 cm in length, is the isthmus. Like the ampulla, it too is ciliated yet less densely so. The isthmus is thought to regulate sperm and embryo transport. The last segment of the fallopian tube is called the intramural segment; it is the link between the isthmus of the oviduct and uterine cavity

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The ciliated and non-ciliated cells of the fallopian tube undergo cyclic changes with the menstrual cycle similar to those occurring in the endometrium. Further, each portion of the fallopian tube appears to be preferentially regulated by hormones that cause a distinct regionalization of activities depending on the day in the female reproductive cycle. For example, in the early follicular phase (day 4), propulsive forces operate throughout the length of the fallopian tube in the direction of the uterus. At day 8 (mid-follicular phase), the ampulla has alternating propulsive forces towards and away from the uterus. At the time of ovulation (around cycle day 14), ipsilateral transport to the ovary increases with increasing follicular diameter. It has been observed that pregnancy rates after intercourse are higher in those women who demonstrate ipsilateral transport, as opposed to those who fail to show lateralization. The fallopian tube function is critical for the early stages of fertilization.

At the time of ovulation, the oocyte is surrounded by a mass of specialized granulosa cells called the cumulus oophorus. Together, the oocyte and granulosa cells are called the cumulus-oocyte complex (COC). The innermost cell layers of the cumulus immediately overlying the zona pellucida of the oocyte are called the coronal cells. After cumulus maturation, the same cells are called the corona radiata because of their "sunburst" appearance. These cells have processes that extend through the acellular glycoprotein matrix of the zona to contact the oocyte plasma membrane for a rich metabolic exchange of nutrients via the so-called transzonal projections. The cumulus of the mature COC is sticky and is thought to facilitate the adherence of the COC to the surface of the fimbriae once it is expelled from the follicle at ovulation

After ovulation, the fertilizable life span of the mammalian oocyte is estimated to be about 24 hours. In contrast, the fertilizable life span of the spermatozoon is around 72 hours. Sperm motility can persist for much longer and has been documented in vivo for up to 5 days, but fertilizing ability is lost before motility. Sperm deposited in the proximal vagina can be found in the fallopian tube within 5 minutes. A number of sperm-related events must occur for successful fertilization. The first factor is that a sufficient number of mature, viable spermatozoa must be present in the ejaculate. Second, the morphology of the sperm must be such that the cervical mucus will allow passage into the uterus. Third, it is essential that a good percentage of the sperm have forwardly progressive motion to propel them through the cervical mucus into the uterine cavity and the fallopian tube for ultimate encounter with the COC. Fourth, sperm must undergo the acrosome reaction and hyperactivation during sperm transport into the female reproductive tract (vagina, uterus and tubes) to be enabled for cumulus cell penetration and zona pellucida binding

The term capacitation derives from the observation that sperm must spend time in the female reproductive tract in order to acquire the capacity or ability to fertilize an oocyte. Sperm can also undergo capacitation in vitro when they are incubated in media containing serum albumin as well as energy substrates and electrolytes. Capacitation begins as sperm swim through the cervical mucus. Proteins absorbed in the plasma membrane are removed and sperm surface molecules are

modified. An efflux of cholesterol from the sperm plasma membrane may be the initiating event for capacitation.

Fertilization occurs in the ampullary segment of the fallopian tube. Transit time of the zygote from the ampulla to the ampulla-isthmic junction is approximately 30 hours, after which the zygote remains in the isthmus another 30 hours before resuming transit through the isthmus. It is not until the 5th or 6th day after fertilization that the pre-implantation embryo arrives into the uterine cavity. During the time frame from fertilization to deposition of the embryo in the uterus, the propulsive forces in the fallopian tube are towards the uterus. The fallopian tube and its microenvironment are ideal for early embryo development.

**Check Your Progress**

13. When does the late primary follicle stage reach?
14. What is the characteristic feature that differentiates secondary follicles from primary follicles?
15. Define luteinisation.

## 3.7 COMPARATIVE ACCOUNT OF DIFFERENTIATION OF GONADS IN A MAMMAL AND AN INVERTEBRATE

Let us study the difference between the gonads in mammals and invertebrates in detail.

**Differentiation of Gonads in Human /Mammals**: Sex determination is the result of the translation of genetic sex into gonadal sex .If masculinizing hormonal signals are not secreted or not active, the embryonic gonad will differentiate into ovary. The H-Y antigen is known to play a pivotal role in gonadal differentiation. The presence or absence of this particular cell surface antigen determines the fate of a mammal of developing either into a male or a female

The most defining moment in our embryogenesis is fertilization, the point at which a mammal inherits either an X or a Y chromosome from the father. This genetic coin toss is very essential. These events begin early in the course of foetal development, when the Y-chromosomal *Sry* ("sex-determining region Y") gene is activated in males and acts as a switch that diverts the fate of the undifferentiated gonadal primordia, the genital ridges, towards testis development. This sex-determining event initiates a series of morphological changes, gene regulation, and molecular interactions which route or guide the differentiation of male characteristics. In the absence or having a non- functional *Sry,* alternative molecular cascades and cellular events force the genital ridges toward ovary development. Once the gonadal fate is decided, i.e., testes or ovary differentiation has occurred, sexual identity is further reinforced by means of action of sex-specific gonadal hormones. The processes of differentiation, migration, proliferation, and cell-cell communication are driving forces that distinguish testis and ovary during foetal development, and

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causing changes in gene regulation that govern these two alternate pathways. The research and understanding of complex mechanisms by which these organ systems develop, form the etiology, diagnosis, and management of disorders of sexual development.

The gonads begin to differentiate forming ovaries or testes after a considerable period of sexual ambiguity. All secondary sexual characters/ dimorphisms are a result of the differentiation of the gonads and their acquisition of endocrine function.

**Invertebrate Gonad Differentiation:** The invertebrates include millions of taxa which include both dioecious as well as hermaphrodite groups (species). In dioecious there are two types of chromosomal sex determination have been found, viz; heterogametic males (e.g., Hemipteran and Orthopteran insects which has XX/ XO type) and heterogametic females (e.g., *Drosophila* sp. and *Coccinia indica*). The gonads in some lower invertebrates are temporary organs, in higher forms they are permanent. In some invertebrates such as oligochaete worms and leeches, both male and female gonads exist in a single organism. Sponges do not have discrete gonads and the reproductive cells are formed by the aggregates of amoebocytes in the body wall. In Echinoderms the gonads are usually suspended from the radiating arms directly into the sea. Some invertebrates like molluscs, arachnids, millipedes and winged insects etc. also possess intromittent organs used to deliver sperms into the female body, which are a hallmark of internal fertilization. In most of the vertebrates and several invertebrates' sex steroids, including oestrogens, androgens and progestogens, play a vital role in reproduction. It has been proposed that the echinoderm sea cucumbers (*Holothuria* scabra) have the same sex steroids as vertebrates but the steroidogenic pathway in the sea cucumbers is still unclear. The presence of these sex steroids has been well demonstrated by using liquid chromatography-tandem mass spectrometry (LC–MS/MS) in H. scabra tissues both in the gonadal as well as neural tissues. Analysis of transcriptome data *In-silico* of the sea cucumber revealed 26 steroidogenesis-related genes expressed differentially in male and female eg. The expression of the HscCYP3A is high at the early stages of ovarian development, but not at other later stages in ovaries, however it remains low in testes. Steroids do have a role here in gonad maturation as well as reproduction. These are commonly referred to vertebrate-type steroids. Vertebrate-type steroids are also common in some species of starfish and sea urchins.

**Check Your Progress**

16. What will happen if masculinizing hormonal signals are not secreted or not active?
17. Which antigen plays a vital role in gonadal differentiation?
18. Name one invertebrate in which both male and female gonads exist in a single organism.

## 3.8 ANSWERS TO 'CHECK YOUR PROGRESS'

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1. The process of gamete formation is called as gametogenesis and is of two types, namely spermatogenesis and oogenesis.
2. The process of spermatogenesis is divided into three major phases: i. Proliferative phase or Multiplicative phase, ii. Meiotic phase, and iii. Post Meiotic phase (Maturation phase).
3. In maturation phase, primary spermatocytes undergoes the first meiotic division and produce secondary spermatocytes (chromosome number 23).
4. A uniflagellar sperm cell that is motile is referred to as a spermatozoon.
5. The tail of the sperm has three parts: a middle piece, principal piece, and end piece.
6. Alkalinisation of sperm cytoplasm is the elementary change that occurs during capacitation where increase in pH levels takes place, mainly in the flagellum.
7. The four stages of fertilization are preparation, binding, fusion and activation.
8. The process of sexual reproduction involves two gametes, namely egg and sperm.
9. In sea urchins, ovoperoxidase catalyzes the crosslinking of tyrosine residues in the extracellular matrix.
10. Cryopreservation is an applied aspect of cryobiology which permits low temperature for the maintenance of diversity of the cells.
11. Cryopreservation can be achieved by two methods either by Slow Freezing or either by vitrification.
12. The process of vitrification is largely depends upon 3 factors— viscosity of the sample, vooling and warming rates, and sample balance.
13. The late primary follicle stage is reached when the follicular cells proliferate into a stratified epithelium known as the zona granulosa.
14. The characteristic feature that distinguishes secondary from primary follicles is the appearance of a follicular antrum within the granulosa layer.
15. Luteinization is the process that transforms the granulosa and theca cells into luteal cells.
16. If masculinizing hormonal signals are not secreted or not active, the embryonic gonad will differentiate into ovary.
17. The H-Y antigen is known to play a pivotal role in gonadal differentiation.
18. In some invertebrates such as oligochaete worms and leeches, both male and female gonads exist in a single organism.

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## 3.9 SUMMARY

- Gametes are the cells produced by parent generation though the process of gametogenesis and are laden with hereditary information.
- Gametes are haploid reproductive cells which carries haploid set (in case of humans n = 23) of chromosomes and are formed from diploid gonads.
- The process of gamete formation is called as gametogenesis and is of two types, spermatogenesis and oogenesis.
- The process of spermatogenesis is divided into three major phases: i. Proliferative phase or Multiplicative phase, ii. Meiotic phase, and iii. Post Meiotic phase (Maturation phase).
- Thethree hormones produced by male gonads directly or indirectly control the process of spermatogenesis. They are testosterone, estradiol and inhibin.
- Spermatogenesis occurs in the seminiferous tubules of male sex organ, i.e., testes which vary in number from10 to 20 per testes in rat.
- Semen, also known as seminal fluid, is a fluid that is emitted from the male reproductive tract. It contains sperm cells that have the ability to fertilize female's eggs.
- Sperm is the male reproductive cell and is derived from the Greek word *sperma* (meaning seed).
- Sperm cells cannot divide and have a limited life span, but after fusion with egg cells during fertilization, a new organism begins developing, starting as a totipotent zygote.
- Spermiogenesis is the process by which haploid round spermatid completes an extraordinary series of events to become streamlined motile spermatozoa.
- The different steps of spermiogenesis are distinguished by the morphological appearance of the developing acrosome and the change in the shape of the nucleus.
- Sperm is divided into 3 main regions—head, neck and middle piece and tail.
- Alkalinisation of sperm cytoplasm is the elementary change that occurs during capacitation where increase in pH levels takes place, mainly in the flagellum.
- Mammals are diploid organisms whose cells possess two matched sets of chromosomes, one inherited from the mother and one from the father. Thus, mammals have two copies of every gene.
- Fertilization process in animals can occur either internally or externally.
- In Sea urchin, the activation of egg and egg metabolism involves the following two mechanisms: Early Responses and Late Responses.
- Cryopreservation is an applied aspect of cryobiology which permits low temperature for the maintenance of diversity of the cells.

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- Process of cryopreservation involves following steps:
  i. Slow freezing
  ii. Vitrification
  iii. Sub-zero non-freezing storage
  iv. Preservation in dry state.
- Equilibrium vitrification requires the formulation of multimolar cryoprotectant mixtures and their injection into the cell suspension.
- Non-equilibrium vitrification which is further divided into carrier-based system includes the former plastic straws, quartz micro capillaries and cryoloops for obtaining a minimum drop volume and carrier-free systems involves the use of an extremely high freezing rate along with lower concentrations of the CPA mixture.
- Egg transport refers to the movement of the oocyte from the moment of expulsion from the ovarian follicle to entry into the distal segment of the fallopian tube before fertilization takes place.
- Sex determination is the result of the translation of genetic sex into gonadal sex. The H-Y antigen is known to play a pivotal role in gonadal differentiation.
- In the dioecious, there are two types of chromosomal sex determination have been found, viz; Heterogametic males (e.g., Hemipteran and Orthopteran insects which has XX/XO type) and Heterogametic females (e.g., *Drosophila* sp. and (*Coccinia indica*).

## 3.10 KEY TERMS

- **Spermatogenesis:** It refers to the developmental process from germ cell that ends at formation of haploid spermatid, which begins at the puberty and occurs in the seminiferous tubules of the testis.
- **Gamete:** It is a haploid cell that fuses with another haploid cell during fertilization in organisms that reproduce sexually.
- **Embryo:** It is the early stage of development of a multicellular organism.
- **Sperm:** It is the male reproductive cell and is derived from the Greek word 'sperma' (meaning seed).
- **Spermatozoon:** It is a uniflagellar sperm cell that is motile is referred to as a spermatozoon.
- **Semen**: It is an organic fluid created to contain spermatozoa.
- **Fertilisation:** It is also known as generative fertilisation, insemination, pollination, fecundation, syngamy and impregnation. It is the process that involves the fusion of gametes to initiate the development of a new individual organism or offspring.
- **Acrosome:** It is an organelle that develops over the anterior half of the head in the spermatozoa (sperm cells) of many animals including humans.

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- **Vitrification**: It refers to the transformation of a substance into a glass, that is to say, a non-crystalline amorphous solid.
- **Cryopreservation:** It is an applied aspect of cryobiology which permits low temperature for the maintenance of diversity of the cells.

## 3.11 SELF-ASSESSMENT QUESTIONS AND EXERCISES

**Short-Answer Questions**

1. Distinguish between internal and external fertilisation.
2. Write a short note on proliferative phase.
3. What is the composition of semen?
4. What is the disadvantage of using slow freezing technique during cryopreservation?
5. How is an ovum transported in mammals?

**Long-Answer Questions**

1. Describe the mechanism of fertilization in different animals.
2. Diagrammatically explain the process of formation of haploid spermatids with suitable diagrams.
3. Discuss and illustrate the process of cryopreservation.
4. Analyse the difference between gonads in mammals and invertebrates.

## 3.12 FURTHER READING

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# UNIT 4 GAMETE BIOLOGY - II

NOTES

**Structure**



## 4.0 INTRODUCTION

The science of embryology is concerned with the study of animal development. The term 'development' is used in embryology to describe the events that occur during the transition of a fertilised egg into a new adult being. Ontogenetic development is the name given to this form of personal growth. Phylogenetic development, on the other hand, is the steady historical evolution of a species over a lengthy period of time. Embryology may be described as the study of an organism's

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ontogenetic development. In this unit, we will discuss the multiple ovulation and embryo transfer technology, along with assisted reproduction technologies. We will also focus in the transgenic animals and knock-outs and care and breeding of experimental animals, along with the concept of immunocontraception.

## 4.1 OBJECTIVES

After going through this unit, you will be able to:

- Describe multiple ovulation and embryo transfer technology
- Explain the biology of sex determination and sex differentiation
- Discuss the various assisted reproduction technologies
- Describe the concept of immunocontraception

## 4.2 MULTIPLE OVULATION AND EMBRYO TRANSFER TECHNOLOGY

As the ruminant animal like (cow) produces one egg at time, it can carry only one pregnancy at a time. Hence, it is possible to increase the production of female animals by increasing the number of mature eggs from a given female, fertilize them and transfer (implant) the embryos (fertilized eggs) into a foster mother (recipient). The foster mother serves as an incubator, and does not make any genetic contribution to the offspring. Embryo transfer is a costly technique, and is selectively used for the production of animals of high genetic or economic valve. In the normal reproductive cycle of a non--pregnant female, a single ovarian follicle (out of the 20 ovarian follicle that develop during the cycle) matures and ruptures and releases out one fertile egg at a time. The time of ovulation differs in different animal species i.e., 21 days for cow as well as horse to 16 days for sheep and goat.

The circulating gonadotrophic hormone is closely related to two processes i.e., with ovulation as well as with release of egg. By increasing the concentration of gonadotrophic hormone, a large number of ovarian follicles can be induced to ripen as well as to produce more eggs. This phenomenon is referred to as superovulation or multiple ovulations. Superovulation may yield approximately 8-10 eggs at a time. A few animal breeders were highly successful in super ovulating animals to produce as many as 60 eggs at a single time. This mostly depends upon the breed, nutrition as well as the health status of the animal, besides the environmental factors. By administering prostaglandin as well as follicle stimulating hormone (FSH), estrous can be induced. A process discussed in detail below i.e., artificial insemination is carried out in the super ovulated females. Al is chosen as compared to natural mating as the purpose of superovulation is to genetically improve the progeny. As the eggs are fertilized, they undergo development to form embryos.

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Occasionally, the process of multiple ovulations, a phenomenon also known by the name of superovulation as well as embryo transfer are considered together which is then called as MOET i.e., multiple ovulation embryo transfer technology. The embryos developed in the super ovulated animals (described above) are recovered within a time span of 6-8 days of artificial insemination. For instance: - In cattle, the recovery process is easy and can be done with the help of a catheter. A few animals having smaller reproductive tract, surgical procedures may be needed to expose the oviduct and to recover the embryos also. These embryos are examined thoroughly under the microscope and identified.

The embryos are then transferred into a synchronized recipient (i.e. the foster mother) by using a procedure, which can be compared to the phenomenon of artificial insemination. Alternatively, the embryos obtained can be frozen and stored to be used at an appropriate time and place later on. Approximately, 50-60% of pregnancy could be achieved in cattle with the help of transferred embryos. Thus, a super ovulated female may result in 5-6 pregnancies.

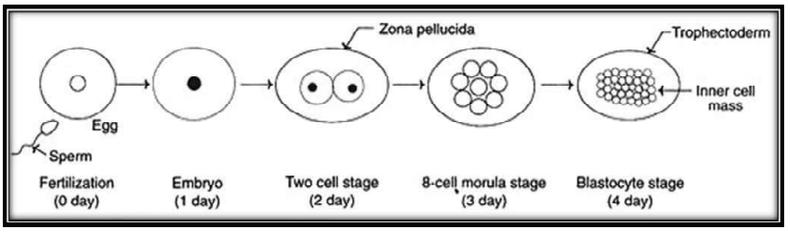


***Fig. 4.1*** *Figure depicting the early stages of embryo development*

## Discovery of Embryo Transfer Technology

The ability to produce transgenic animals is dependent on several components. One of the first things which is required to generate transgenic animals is the ability to transfer embryos. The first successful transfer of embryos was achieved by Walter Heape in Angora rabbits in the year 1891. Another important component is the ability to manipulate the embryo. For the first time, in vitro manipulation of embryos in mice was reported in the year 1940s by using a culture system. What is also vital is the ability to manipulate eggs. The ability to manipulate eggs was made possible only due to huge efforts put in by Ralph Brinster, attached to the University of Pennsylvania, who in the year, 1963 developed a reliable system to culture eggs, and that of Teh Ping Lin, who is based at the California School of Medicine, who in 1966 outlined a technique to micro-inject fertilised mouse eggs which enabled the accurate insertion of foreign DNA.

## Embryo Transfer Technology in Dairy Animals

The first embryo transfer technology (ETT) project in the country was initiated by NDDB (National Dairy Development Board) in 1987 by the establishment of a central ET laboratory at Sabarmati Ashram Gaushala (SAG), Bidaj. The project was funded by the Department of Biotechnology (DBT), Ministry of Science & Technology, GoI for 5 years i.e. from April 1987 to March 1992. Under this project, NDDB established one Main ET Lab at SAG Bidaj and four Regional

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ET Labs at CFSP&TI, Hessarghatta (Karnataka), ABC, Salon (UP), Shri Nashik Panchavati Panjrapole, Nashik (Maharashtra) and Buffalo Breeding Centre, Nekarikallu (AP). NDDB also assisted in establishment of 14 State ET centres across the country.

***Table 4.1*** *Table depicts the history of embryo transfer technology*

| Events | Species | Scientist | Year |
|---|---|---|---|
| First successful ET | Rabbit | Walter Heape | 1890 |
| First successful ET | Rat | JS Nicholas | 1933 |
| First successful SOV | Cattle | Casida | 1940 |
| First successful ET | Sheep and Goat | BL Warmick & RO Berry | 1949 |
| First successful ET | Pig | AV Kvansnickii | 1951 |
| First ET reported in cattle | | Umbaugh | 1949 |
| First successful ET | Cattle | EL willett | 1951 |
| Baby girl born through ET | | Steptoe & Edwards | 1979 |
| Calf -Frozen thawed Embryo | Cattle | Wilmut & LEA Rowson | 1973 |
| Calf born by ET | Buffalo | Drost | 1983 |
| Calf born by IVF | Buffalo | Madan et al | 1990 |
| Calves through surgical and non-surgical ET in Asia/India<br>Calf born by frozen thawed embryo | Buffalo | Misra et al<br>Misra *et al.,* | 1988<br>1991 |

'Embryo transfer' (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals. Usually, one can get one calf from a superior quality female dairy animal in a year. However, by using 'MOET'/'ET' technology, dairy workers can get 10-20 calves in a year from a cow/buffalo. A genetically superior cow/buffalo is administered artificial hormones with FSH-like activity to induce super-ovulation. Under the influence of the FSH-like hormone, the genetically superior female produces numerous eggs instead of single egg produced in general. The super-ovulated female is artificially inseminated multiple time (2-3 time) at 12-hour interval during estrus and on 7th day post insemination the uterus of the artificially inseminated female is flushed with a medium to recover the developing embryos. Embryos are collected along with flushing medium in a specific filter. Thereafter, the quality of the developing embryos is assessed under the microscope. Superior quality embryos are either preserved or frozen for the purpose of transfer in future or they are transferred into recipient females (surrogate mother) roughly seven days post the heat date. Thus, with the use of 'ET' technology numerous genetically superior calves can be produced in a year.

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SAG has done ground-breaking work in this field and so far, has produced 14,388 viable embryos and 755 calves, which is maximum by any organisation in the country. Of these, 1026 embryos are of indigenous cattle breeds, from which 122 calves have been born. Besides these, around 3000 embryos of buffalo breeds have also been produced. Under the project, the first buffalo calf of India from frozen thawed embryo was born in the year 1991.

### 4.2.1 Procedure of MOET Technology in Dairy Animals

MOET is carried out in the following steps.

**Programming**

Multiple Ovulation Embryo Transfer of simply known as MOET involves the use of several hormones like Follicle Stimulating Hormone to stimulate the release of multiple eggs from the ovaries of females. This phenomenon is known as superovulation. Superovulation refers to the treatment of a donor with gonadotropin (FSH or PMSG) to produce more than a single ovum. Thus, superovulation can be defined as increased ovulatory response by exogenous hormone therapy, above a level that would be expected to occur naturally. Apart from the Follicle Stimulating Hormones, progesterone (eg., CIDRs), PMSG (eg., pregnecol), Gonadotropin Releasing Hormone (eg., Receptal), and Prostaglandin (eg., Estroplan or Estrumate injection) hormones are routinely used to synchronize the animals so that they cycle at the correct time. Synchronization refers to matching the estrous cycle of a donor and a recipient by the injection of prostaglandin (PGF2 alpha) to stimulate the onset of estrus (heat). In the first step known as programming, the donor cattle are first treated with follicle-stimulating hormone as well as prostaglandin. These hormones are injected only in the muscles. The cows need to be provided with proper nutrients before as well as after calving. They should have completed their cycle. Routine checkups are vital to ensure that they are in good health as well as clean.

**Heat Detection**

Heats need to be accurately recorded while the cows are being artificially inseminated. As the male produces millions of sperms daily, the semen can be used to produce several off-springs. This is made possible by a technique known as artificial insemination (Al) of females Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar i.e. genetically superior female. For an effective Al to produce the desired results, the following aspects must be considered.

**Semen Collection and Storage**

The ejaculated semen is collected, diluted and then examined under the microscope for checking the total number of motile sperms present in the semen sample. Usually, 0.2 ml of bull semen consist approximately 10 million motile sperms. The diluted semen can then be used fresh within few days, or cryopreserved at -196°C in liquid nitrogen for long-term storage and transport.

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Artificial insemination is done on standing animals via a technique, known a rectal palpation. Each semen ejaculate of a male bull, in theory, can be used to inseminate as many as 500 cows. Another advantage of Al is that the semen can be transported (in cryopreserved form) to different places/countries and used to develop superior animals. There are three common methods of artificial insemination (AI):

- Vaginal method
- Recto vaginal method
- The speculum method

Based on the size as well as the structure of the bovine reproductive tract, two methods namely Recto vaginal method and the speculum method are widely accepted as suitable methods for insemination in beef cattle. Below, we will discuss both the methods in detail:

- **Recto Vaginal Method:** This is one of the most widely accepted methodology in dairy industry due to its practicality as well as it is believed to be the safest for the animal. This methodology involves the manipulation of the reproductive tract, primarily the cervix, through rectal palpation.
- **The Speculum Method:** This methodology involves placing a speculum (or spectrum) into the vagina pushed up to the posterior end of the cervix.

**Synchronization of Ovulation**

The female animals are inseminated after ovulation which can be detected by their behavioural estrous. It is slightly hard to detect estrous, as it majorly occurs during the night and lasts only for few hours. The animal breeders would like to inseminate a large number of females at the same time, so that managing such animals becomes easy. For this purpose, the females are induced (synchronized) to ovulate at a set time. This is achieved by administration of progesterone and/or prostaglandins, which regulate ovulation cycle. Although total synchrony of ovulation is not possible, about 80% of the females could be made to respond by this approach.

**Sperm Sexing**

The dairy industries like to have the animals belonging to one sex only. For example, the dairy as well as beef industries require more females as compared to males. It is quite possible to produce the animals of desired sex via sperm sexing.

Sperms and ova contain half of the chromosomes of a somatic cell. Thus, it can be said that the genetic constitution of an ovum is made up of a set of autosomes with one X chromosome. On the contrary, the genetic constitution of a sperm contains one set of autosomes and one Y chromosome. Sex is determined genetically by the sex chromosomes (X and Y). X chromosome is present in all the ova, whereas half of the sperm (of a semen ejaculate) possess X and the other half Y chromosomes.

The sex of the embryo is determined by the sperm (X or Y containing) that is successful in fertilization. Thus, if the embryo contains X chromosome, it is a

female; however, it is a male if it possesses Y chromosome. In the natural breeding, the sex ratio of progeny is close to 1:1.

It is quite possible to separate the sperms containing either X or Y chromosome, and use them selectively for the desired sex of the progeny. By employing a fluorescent dye (Hoechst 33342) and an instrument namely fluorescent activated cell sorter (FACS), two populations of sperms (X or Y chromosome containing) can be separated. Using this approach as well as in vitro fertilization technique has produced pre- sexed calves. FACS separation of sperms is costly as well as time (about 24 hours)-consuming. Several sperms may die even before they are actually separated. Attempts are being made to develop better separation techniques of sperms. The benefits of artificial insemination include:

- Increase in efficiency of usage of genetically superior bull
- Increased potential for genetic selection
- Cheap and economical over a long period of time
- Protecting the interest of both dairy farmers as well as animals
- Reduction in the transmission of diseases.

The drawbacks of artificial insemination include:

- More laborious technology requires skilled labour
- Dairy farmer needs to detect the most genetically fit male to carry out the process of Artificial insemination
- It can decrease the genetic variability over a long period of time

**Embryo Collection**

The donor as well as the recipient cattle are now placed in a secured and covered place. The donor cow is injected with an epidural to relax her bowel. The rear end is washed appropriately and sterilised to prevent any bacterial, viral or fungal infection. After this, the veterinary doctor inserts its hand in the rectum of the cow and traces a catheter into one horn of the uterus. The fluid is then run into the uterine horn. The fluid is run back via the catheter and a very fine filter where the embryos are caught. Thus, the embryos are collected easily. The cow is then injected with the hormone prostaglandin after 3 days for proper cycling.

**Technique of Embryo Transfer**

This step involves watching embryos under the microscope. The filter is observed under a microscope for the embryos. The embryos detected are counted, graded and washed. The collected embryos are then either frozen or are preserved in liquid nitrogen at a temperature of -190°C for later use or are transferred into the recipients with the help of transfer guns. These two methodologies are discussed below.

**Cryopreservation**

Cell preservation refers to the entire process of extraction, processing and storage of cells so that it can be used in the future for research, scientific or medical purpose. Cell preservation techniques aim at maintaining healthy cell cultures by providing them an environment which helps the cells to retain their specific properties.

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Although, it is a difficult task to achieve as cell cultures can easily become contaminated which renders them useless. Further, preservation techniques applies very high or low temperatures (above about 600°C and below -200°C) that initiates the process of denaturation of proteins in cells leading to cell death. Hence, it is essential to apply a suitable cell preservation technique to prevent the loss of cells. Cell preservation techniques vary according to the type and age of cells. There are two primary types of cell preservation namely, cryopreservation and hypothermic preservation. All the other cell preservation methods have been derived from these two primary types of cell preservation. Hypothermic preservation works only for a couple of hours in a clinical setting with the anticipation of transplantation whereas cryopreservation is for long term storage, up to years. Cryopreservation is generally carried out at low temperatures to slow down cell's biological activity or in other words to let cells continue in the nearly animated state where there is little or no biological activity.

## Transformation

Gene delivery refers to the process of introducing foreign genetic material such as DNA or RNA directly into host cells. Genetic material either DNA or RNA must reach the genome of the host cell in order to induce gene expression· However, this is not the end of process; successful gene delivery must ensure the foreign genetic material to stay stable within the host cell. Also, the foreign material either integrates into the genome or replicate independently of it.

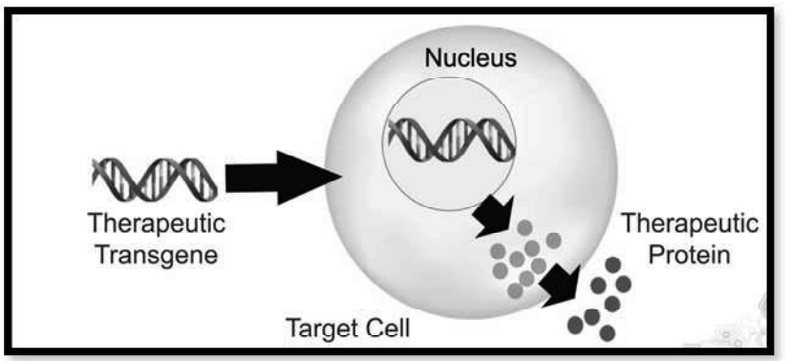


***Fig. 4.2*** *The principle of gene therapy*

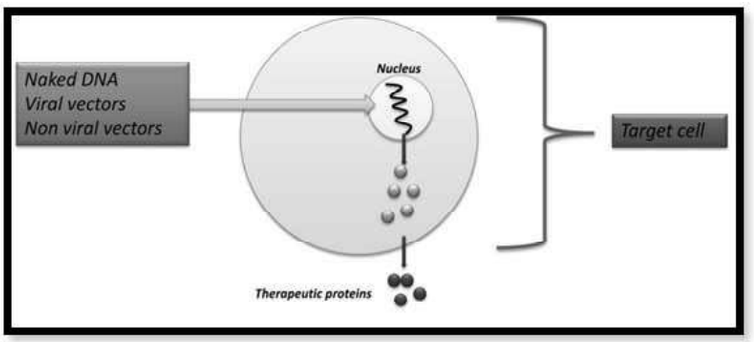


***Fig. 4.3*** *Mode of Gene Delivery*

Gene delivery is an essential step in gene therapy for introducing or silencing of a gene to encourage a therapeutic outcome in patients. It also serves in the genetic modification of crops. There are several methods of gene delivery system:

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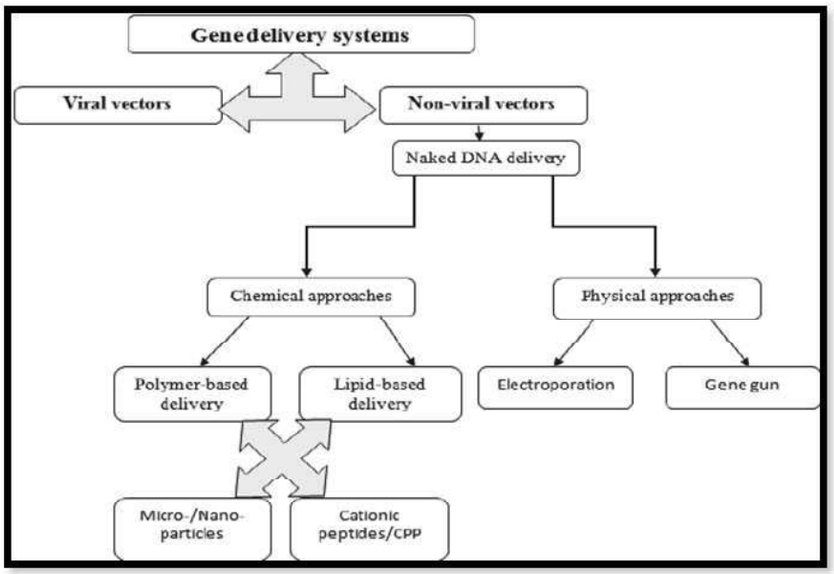


***Fig. 4.4*** *Different Gene Delivery Methods*

However, all the techniques depicted in the figure above are not suitable for transferring embryos. Embryo transfer can be done in following ways:

## Microinjection

Microinjection is a widely used technique for delivering foreign DNA into a living cell (for instance- a cell, egg, oocyte, embryos of animals) with the help a glass micropipette. During the process, one end of a glass micropipette is heated until the glass becomes somewhat liquefied. It is quickly stretched which forms a very fine tip at the heated end. The tip of the pipette attains to about 0.5 mm diameter which resembles an injection needle. The process of delivering foreign DNA is done under a powerful microscope. Firstly, cells to be microinjected are placed in a container. A holding pipette is then placed in the field of view of the microscope. The holding pipette holds a target cell at the tip when gently sucked. The tip of the micropipette is injected through the membrane of the cell. Contents of the needle are delivered into the cytoplasm and the empty needle is taken out. Microinjection is the most widely used and accepted methodology in mammals as it can be easily applied to a variety of species. However, the newly introduced foreign gene may over- or under-express depending upon the insertion of gene which is a highly random process. The manipulated fertilized ovum is then transferred into the oviduct of a foster mother that has been prepared to act as a recipient by mating with a vasectomized male.

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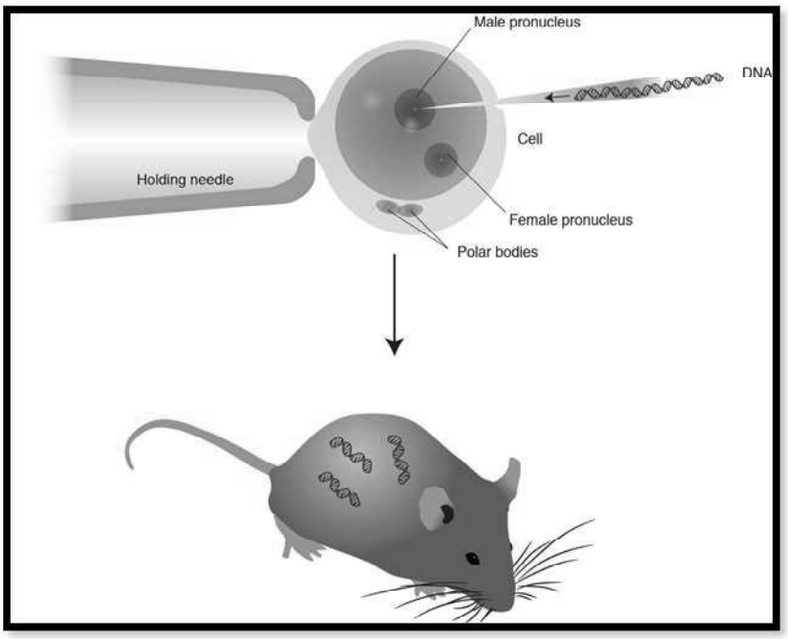


***Fig. 4.5*** *Production of Transgenic Mice by the Method of 'DNA microinjection*

## Biolistic particle delivery system

In genetic engineering, biolistic particle delivery system or simply a gene gun is a tool used to deliver exogenous DNA (transgenes), RNA, or protein inside cells. In the biolistic approach, gene of interest or other biological molecule are coated on a heavy metal like gold or tungsten and then accelerated to high velocity via source such as helium pulse and finally driven through cell wall and membranes into the target. The biolistic or gene gun approach is easy to perform and can be applied on target such as tissues, bacteria, cell cultures, plants, animals, organs as well as organelles.

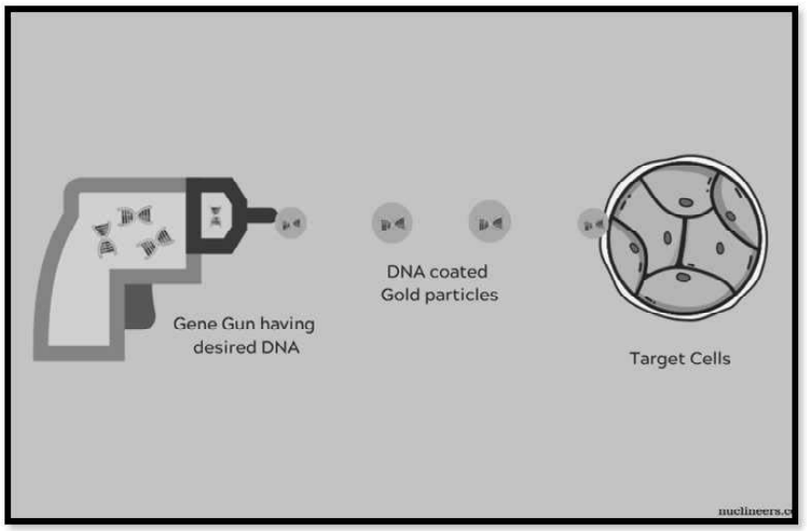


***Fig. 4.6*** *Biolistic Approach*

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The gene gun which was originally an air pistol customized to fire dense tungsten particles. It was invented by John C Sanford, Ed Wolf, and Nelson Allen at Cornell University along with Ted Klein of DuPont between 1983 and 1986. Onions, due to their large size were chosen as the first target to deliver particles coated with a marker gene which would pass on a signal if appropriate insertion of the DNA transcript takes place.

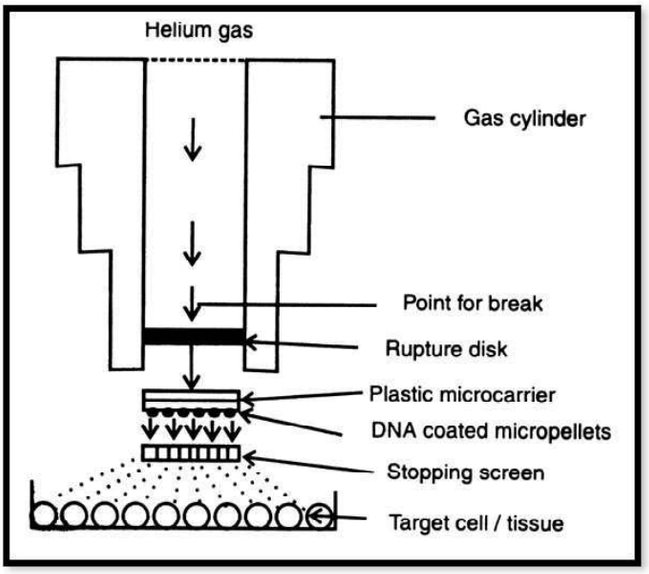


***Fig. 4.7*** *Working System of a Gene Gun*

The apparatus includes a chamber coupled to an outlet to generate vacuum. At the top, a cylinder (in which Helium gas flows) is temporarily sealed off from the rest of chamber with a plastic rupture disk. A microcarrier made of plastic is placed close to rupture disk. It consist of DNA coated tungsten particle, the microscopic pellets *(i.e.* coated microprojectiles). The target organs/organelles/ cells/tissue is placed in the apparatus. A stopping screen is positioned in between the target cells and microcarrier assembly. Helium gas is then flown in to the cylinder at a very high velocity. Whenever the pressure of cylinder goes beyond the bursting point of plastic disk, it gets ruptured. Helium shock waves drive the plastic mircrocarrier containing the DNA coated micropellets. The stopping screen allows the micropellets to pass through and deliver DNA into target organs/organelles/ cells/tissue. The transformed cells i.e. cells having the foreign DNA are then regenerated onto nutrient rich medium. The regenerated plant tissues are selected over nutrient culture media containing cither herbicide or antibiotics. The selected plants are then evaluated or examined for expression of foreign DNA. The advantages of biolistic approach include:

- Gene gun delivery method is versatile, rapid and easy to use
- In this method, transient or stable expression is possible

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- Very small amounts of nucleic acid either DNA or RNA or molecule of interest is required for efficient transformation
- Very high levels of co-transformation are possible
- Large DNA fragments may be transferred or small interfering RNAs (siRNAs) for gene silencing
- Many cell types can be transfected, including nondividing cells and plants
- Potentially toxic treatments (such as from viruses or chemical- and lipid-based systems) are avoided
- Plastid transformation has also seen great success with particle bombardment when compared to other current techniques, such as *Agrobacterium* mediated transformation

The applications of biolistic approach include:

**Plant Systems**

Biolistic technology is particularly valuable for target cells or tissues that are noncompliant to other transformation methods like agriculturally significant monocotyledonous plants. Embryos, seedlings, leaves, cultured cells, floral tissues, epidermal tissues, apical meristems etc. are among several targets that have been successfully transformed.

**Animal Systems**

Gun gene delivery system has been used effectively for the transformation of intact animal embryos, tissues, as well as animal cells in culture. Particle delivery is a suitable technique for transforming these sensitive cells as little pre- or post-bombardment manipulation is required.

**Other Biological Systems**

Biolistic skills have also been applied to targets as diverse as mitochondria, bacteria, algae, chloroplasts, fungi and pollen.

Gene gun has been further used to gain understanding of infectious disease, cancer and wound healing as well as to generate immune responses in animals. It is also used to assay gene expression and regulation both in vitro and in vivo.

The limitations of biolistic approach include:

- Biolistics approach introduces target DNA or RNA or any molecule randomly into the target cells.
- Thus, the foreign DNA, RNA or molecule of interest get transformed into whatever genomes are present in the cell i.e. nuclear, plasmid or mitochondrial

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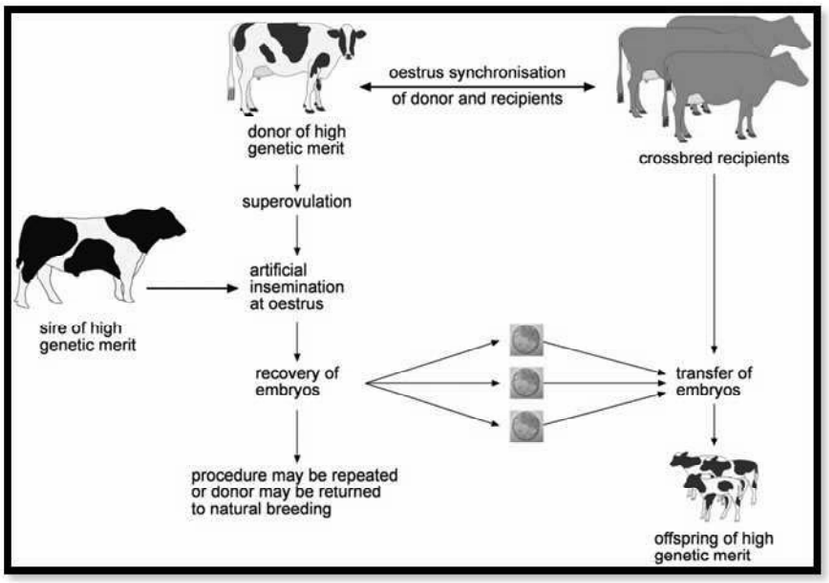


***Fig. 4.8*** *Embryo Transfer Technology*

## 4.2.2 Role of Pheromones in MOET

Pheromones are the substances that are released into the environment by an individual of a species that elicits a specific social response in members of the same species. They are the chemicals used by insects and other animals to communicate with each other. Pheromones are majorly used in cattle and pig breeding. They are sold as commercial preparations in the market based on their structural analogues. However, in some farms, nasal rings are used with substances containing pheromones. These substances are urine and mucus from cows at the height of their estrous cycle. Pheromones play an essential role in the initiation of a particular behaviour or affecting the reproductive physiology of the animal by altering the activity levels of the internal hormones. Due to this reason, pheromones are also considered as ectohormones, i.e., chemical messengers that are transmitted outside the body. They support or increase the attractiveness of opposite poles as well as promotes aggression between males, accelerate the attainment of puberty, shortens the period of anoestrus, brings about changes in the estrous cycle and also plays an essential role in inducing mating behaviour in both sexes. Additionally, bio-stimulatory effects of pheromones have been observed in domesticated as well as farm animals in terms of positive effect of the presence of breeding bulls on ovarian activity in cows and the success or failure of the conception. An increase in the ejaculate volume as well as the number of live, health and motile sperms have been observed after nasal spray application of pheromones in farm animals. Thus, from the above discussion, we conclude that pheromones not only play a significant role in natural breeding process but also in artificial breeding technologies like artificial insemination and MOET or ETT technology discussed below. Embryo transfer technology refers to a step in the process of assisted reproduction in which embryos are placed into the uterus of a female with the intent to establish a pregnancy. This technique (which is often used in connection with in vitro fertilization

(IVF)), may be used in humans or in animals, in which situations the goals may vary.

## 4.2.3 Advantages of MOET

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The advantages of MOET are as follows:

1. This technology increase in the reproductive rate of the animals.
2. No surgery is required in this technology.
3. The entire procedure of MOET or ETT can be easily carried out on a farm.
4. The embryos can be frozen and stored in the laboratory for future use.
5. The embryos can also be cryopreserved in banks for future use.
6. ETT also enables rapid genetic improvement
7. ETT Increase the number of offspring from a genetically superior female
8. MOET or Embryo transfer technology helps in controlling diseases
9. This technology helps to obtain offspring from valuable females that are infertile due to disease or injury
10. This technology also helps in the conservation of endangered species
11. This technology enables production of clones as well as genetic engineering
12. Rapid genetic change within a small population
13. Useful in utilising low producer animals by making them recipient or surrogate mother.

Embryo transfer technology is used in SCNT also, i.e., Somatic cell nuclear transfer technology. In this technique, firstly, the nucleus of a somatic (body) cell is transferred to the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed). Secondly, the somatic nucleus present inside the egg, is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg). Thirdly, zygote is stimulated to undergo division divide by an electric shock. Lastly, when the developing zygote forms a blastocyst, it is implanted via ETT (embryo transfer technology) in the womb of a surrogate mother. However, for any of the above discussed techniques the success rate in terms of live birth of animals containing the transgene is extremely low. Dolly, born on 5th July, 1996, at the Roslin Institute in Edinburgh, Scotland, was a female Finn Dorset sheep. It was the first mammal cloned from an adult somatic cell. As the Dolly's DNA came from a mammary gland cell, she was named after the country singer Dolly Parton. 'Dolly' was created using the technique of somatic cell nuclear transfer (SCNT). Dolly, born on 5th July, 1996, at the Roslin Institute in Edinburgh, Scotland, was a female Finn Dorset sheep. It was the first mammal cloned from an adult somatic cell. As the Dolly's DNA came from a mammary gland cell, she was named after the country singer Dolly Parton. 'Dolly' was created using the technique of somatic cell nuclear transfer (SCNT). Firstly, the nucleus of a somatic (body) cell is transferred to the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed). Secondly, the somatic nucleus present inside the egg, is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg). Thirdly, zygote is stimulated to undergo division

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divide by an electric shock. Lastly, when the developing zygote forms a blastocyst, it is implanted in the womb of a surrogate mother.

Dolly had three mothers, namely; first mother provided the egg, second mother provided the DNA and third mother (surrogate mother) carried the cloned embryo to term. Scottish Blackface ewes were treated with gonadotropin-releasing hormone (GnRH) to cause them to produce oocytes ready to be fertilized. Plunge a micropipette into the egg over the polar body to remove the polar body as well as the haploid pro-nucleus within the egg. Fuse each enucleated egg with a diploid cell growing in culture. Somatic cells from the mammary gland of an adult Finn Dorset ewe are grown in tissue culture. Donor cells and enucleated recipient cells are placed together in tissue culture. The cultures are then exposed to brief pulses of electricity in order to stimulate their respective plasma membranes to fuse and begin the process of mitosis i.e. cell division. Zygotes are then developed into tissue culture media until they grow into a blastocyst. Numerous blastocysts were transferred into the uterus of Scottish Blackface ewes for implantation. One ewe gave birth to Dolly after 148 days. 277 diploid somatic cells were fused with 277 enucleated unfertilized eggs. 29 viable reconstructed eggs survived and were implanted in surrogate Blackface ewes. One gave birth to Dolly. Dolly lived her entire life at the Roslin Institute in Midlothian. 'Dolly' was bred with a 'Welsh Mountain ram'. She produced six lambs. Bonnie (born in April 1998) was her first lamb. Subsequently, she produced twin lambs named Sally and Rosie. In the year 2000, she gave birth to triplets- Lucy, Darcy and Cotton. On 14 February 2003, Dolly was euthanised as she had developed a progressive lung cancer called ovine pulmonary adenocarcinoma as well as severe arthritis. Dolly lived for 6.5 years.

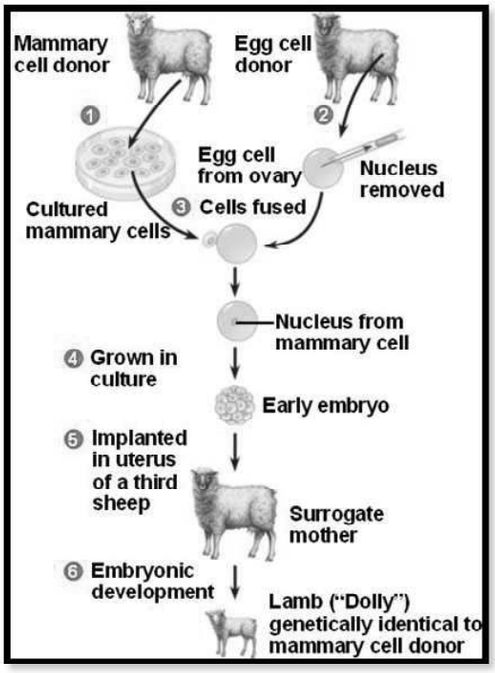


***Fig. 4.9*** *Steps involved in the cloning of Dolly-Sheep*

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Embryo transfer technology helps in the production of transgenic animals which can then be used for research and development purpose. Transgenic farm animals are also being explored as a means to produce huge quantities of complex human proteins for the treatment of rare human disease. Such therapeutic proteins are presently produced in mammalian cell-based reactors; however, this production process is expensive. In order to produce biologically significant compounds in large quantities bioreactors are used. A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH level, oxygen levels and stirring rate to yield the maximum cell growth and productivity. Bioreactors are generally used for making pharmaceutical products such as antibiotics and insulin in large quantities. Bioreactors are basically systems or devices that supports a biologically active environment. They are commonly cylindrical, ranging in size from liters to cubic meters, and are often made of stainless steel. Inside the Bioreactor, chemical or biochemical process is carried out under homogenous environment by constantly stirring the contents. Bioreactor ensures the controlled environment by maintaining the same temperature, pH, as well as oxygen levels. The reaction carried out inside a bioreactor can be either aerobic or anaerobic A cheaper option would be to develop a means to produce recombinant proteins in the milk, blood or eggs of transgenic animals. Development in this field is slow till date. Only two biomedical products have so far received regulatory approval. The first is human antithrombin III, a therapeutic protein produced in the milk of transgenic goats, which is used to prevent clots in patients with hereditary antithrombin deficiency receiving surgery or undergoing childbirth. A relatively small herd of goats (about 80) can supply enough human antithrombin III for all of Europe. The second product is a recombinant human C12 esterase inhibitor produced in the milk of transgenic rabbits. This is used to treat hereditary angiodema, a rare genetic disorder which causes blood vessels in the blood to expand and cause skin swellings. Embryo transfer technology (ETT) can also be used in the production of monoclonal antibodies.

The limitations of embryo transfer technology include:

- There is a limited supply of embryo from super ovulated donors.
- Freezing as well as thawing of embryos requires a lot of care to keep them functionally intact.
- Embryo transfer requires highly skilled technician
- The cost of the technology is very high

## 4.2.4 In-Vitro Oocyte Maturation (IVM)

In vitro oocyte maturation (also known as IVM, or IVM) refers to a procedure where immature eggs mature in vitro, i.e. in the laboratory. To perform the procedure of IVM, the most common is that the patient does not undergo a hormonal treatment of ovarian stimulation (if she receives it is at low doses) and this is one of its main advantages. However, IVF laboratories do not perform IVM routinely and hence it can be regarded as an experimental technique. Thus, we can say that In vitro maturation is an assisted reproductive technology by which immature eggs

are matured in the laboratory until they reach the stage of maturity, also known as metaphase II.



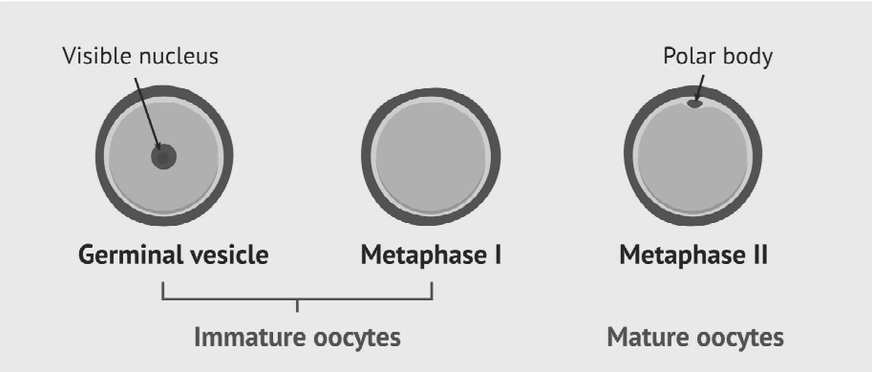


***Fig. 4.10*** *Stages of Oocyte Maturation*

The IVM process comprises of incubating the immature oocytes for a time span of 24 to 48 hours in a culture medium with controlled conditions. The major difference compared to classic IVF cycles is that in IVM the patient does not receive (or receives at very low doses) the previous hormonal treatment. Therefore, IVM does not require ovarian stimulation, unlike regular IVF cycles. This is one of the main advantages of IVM, as it minimizes the risk of ovarian hyper-stimulation syndrome that can occur as a consequence of ovarian stimulation.

IVM is a technique that avoids ovarian stimulation and hence reduces the risk of ovarian hyper-stimulation. Consequently, some of the situations in which IVM would be indicated are the following types:

- IVM is seen as suitable treatment for women suffering from Polycystic Ovarian Syndrome (PCOS) or polycystic ovaries as these women do have a high follicle count (the structures where the eggs are located in the ovary) and a higher risk of ovarian hyper-stimulation with conventional IVF.
- Patients at risk of ovarian hyper-stimulation or who wish to avoid ovarian stimulation to reduce the discomfort and costs associated with medication.
- **Fertility Preservation:** IVM is seen as an alternative for patients who cannot undergo ovarian stimulation due to a hormone-dependent tumour or because they should not delay the beginning of cancer treatment. If ovarian stimulation can be performed, IVM of immature oocytes can increase the number of oocytes available for carrying out the process of preservation. Additionally, IVM can also be performed in conjunction with ovarian tissue preservation.
- **Low Responder Patients:** In these patients, it is generally have been seen to cancel a classic IVF cycle (with ovarian stimulation) due to low response, however after obtaining immature oocytes and their IVM can be an alternative to cancellation.

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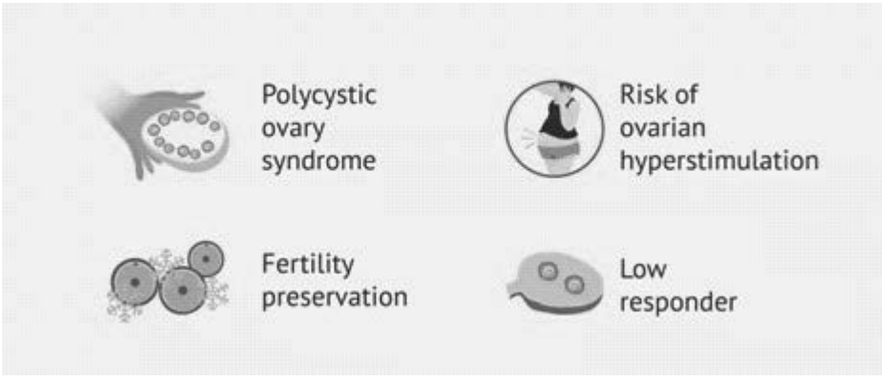


***Fig. 4.11*** *Phases of In-Vitro oocyte Maturation*

**Advantages of IVM**

The following are the advantages of IVM technique:

- It does not require ovarian stimulation, hence reducing the risk of ovarian hyperstimulation syndrome.
- It reduces the discomfort, costs as well as time which is required in a fertility treatment.
- It offers an alternative for preserving fertility when ovarian stimulation is to be avoided.

**Disadvantages of IVM**

Currently, IVM is very restricted to a very few special cases. The disadvantages of using IVM technique are as follows:

- IVM is still regarded as an experimental technique
- IVM has not been optimised as it is supposed to deal with some issues like ovarian hyper-stimulation
- There are reliability issues associated with this techniques when used in women having PCOS as compared to IVF i.e., In-vitro fertilization
- It presents worse results in assisted reproductive technologies and a higher rate of miscarriage

## 4.2.5 Superovulation

Super ovulation is a critical requirement for successful assisted reproduction technology in horses. Management techniques to optimize fertility in the horse have been limited by mares ovulating only one follicle per cycle refer, figure 4.12. Induction of multiple ovulations in the mare would increase the number of oocytes available for fertilization. Consequently, increasing the ovulation rate of sub fertile mares or of normal mares bred to subfertile stallions may increase the probability of establishing a pregnancy. Superovulation also may enhance embryo collection rates from donor mares, increasing the efficiency of embryo transfer programs. Finally, stimulation of multiple large follicles may increase the collection efficiency of oocytes which can be used for In-Vitro Fertilization (IVF), Gamete Intra Fallopian Transfer (GIFT) and Intra Cytoplasmic Sperm Injection (ICSI). Super

ovulation also known as Controlled Ovarian Hyperstimulation, is the process of inducing a woman to release more than one egg in a month. It is different from ovulation induction where the goal is to release one egg per month.

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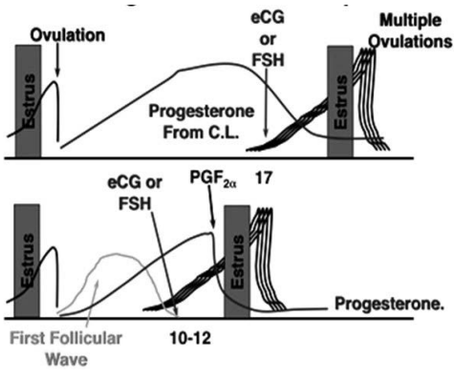


***Fig. 4.12*** *Stimulation of Follicular Development for Super-Ovulation*

## Needs of Superovulation

Woman with open fallopian tubes and whose partner has adequate sperm count are candidates for super ovulation. If a woman already ovulates and is not conceiving, doctors can increase her chances of getting pregnant by causing her to release more eggs by super ovulation. Similarly when a woman has been ovulating with an oral medication such as Clomiphene and is still not conceiving by stimulating her ovaries to release more egg.

## Procedure of Superovulation

Woman who naturally ovulates may release extra eggs when they take oral medications such as clomiphene. This is a mild super ovulation and is generally less expensive and is low risk bearing. Initially an ultrasound is performed around the time of ovulation to determine how many follicles are growing if the result of ultrasound reveals that the woman is producing just one follicle the dose or medication might be changed in the next cycle. Sometime Gonadotopins are also used for super ovulation. Gonadotropins are those hormones that cause eggs to grow. Gonadotropins are injected inside the woman (or in form of liquid) e.g., Bravelle, Follistin, Gonal-F, etc. One main disadvantage of administration of gonadotropins is the growth of multiple eggs at one time. To prevent this situation, close and regular monitoring of ovaries is required. Once the desired number of eggs gets mature, the woman is given Human Choroinic Gonadotropin injection to cause ovulation.

## Risks of Superovulation

Following are the risks of superovulation:

- **Multiple Births:** The most common form of human multiple birth is twins (two babies), but cases of triplets (three), quadruplets (four), quintuplets (five), sextuplets (six), septuplets (seven), and octuplets (eight) have all been recorded with all siblings being born alive. Multiple births can occur

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due to many reasons. It has occurred naturally too but of late the fertility treatments seem to have caused a few more multiple births. Multiple births of more than 6 babies are heard from time to time and many times it is because of one of the fertility treatments. The fertility treatments lead to a spurt of egg production in a woman during her ovulation cycle. As it is too risky to fertilize and place only one embryo in the uterus, it is the usual practice in fertility treatment to place 5, 6 or more embryos. Now, if all or more than one embryo develops, it becomes multiple births.

- **Adnexal Torsion (Ovarian Twisting):** Rare complication that occurs in one percent of cycles. As the ovaries gets enlarge, they may twist, cutting off their blood supply and ceasing severe abdominal pain, nausea, vomiting and sometimes low grade fevers. It can be treated by surgical untwisting of ovaries.
- **Ectopic Pregnancy:** An ectopic pregnancy occurs when a fertilized egg implants itself outside the uterus. Egg may implant in the fallopian tube or less commonly in the ovary, cervix or pelvic cavity. This condition occurs in 1-2% of all pregnancies. Most of the infertility treatments results in the ectopic pregnancies as many woman with infertility dysfunction and treatment leads to release of multiple eggs thereby increasing the possibility that not all fertilized eggs move through the tubes into the uterus. Ectopic pregnancies require emergency medical treatment and the pregnancy must be ended soon as the life of both mother and fetus is on stake.

## 4.2.6 In-Vitro Fertilization

In-Vitro Fertilization (IVF) is a type of Assistive Reproductive Technology (ART) which involves retrieving eggs from a woman's ovaries and fertilizing them with sperm in a laboratory dish. This fertilized egg is known as an embryo which can then be frozen for storage or transferred to a woman's uterus (Figure 4.13).

**Significance of IVF**

IVF is a blessing for the people with following problems

- Women with ovulation disorders, premature or ovarian failure, uterine fibroids
- Blocked or damaged male or female ducts
- Women who have had their fallopian tubes removed
- Male factor infertility including decreased sperm count or sperm motility
- Incompatibility between the sperm and the milieu of the egg or the reproductive tract
- Unexplained infertility

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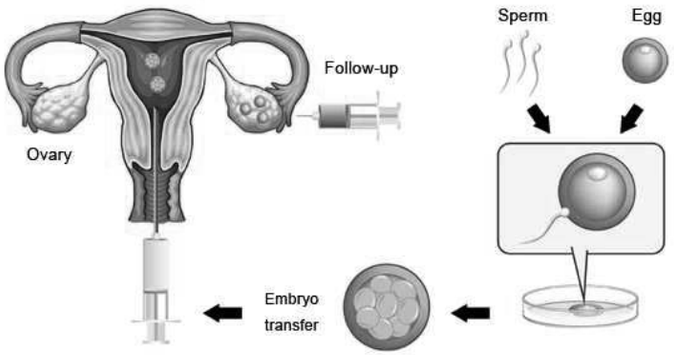


***Fig. 4.13*** *Diagram Showing IVF Technique*

## Procedure of performing In-Vitro Fertilization

**Ovarian Hyper-Stimulation:** The process of IVF requires multiple eggs because some eggs will not develop or fertilize after retrieval. Proper examination of ovaries is done through trans-vaginal ultrasound and a hormone level is checked through blood testing. For preliminary maturation of follicles, ovaries are hyper-stimulated to produce mature oocytes by injecting gonadotropins approximately for 10 days. Ovarian hyper-stimulation also includes suppression of spontaneous ovulation, (Refer Figure 4.14) for which two main methods are available:

- **GnRH against Protocol:** It is usually longer. In a standard long GnRH agonist protocol the day when hyperstimulation treatment is started and the expected day of later oocyte retrieval can be chosen to conform to personal choice.
- **GnRH antagonist Protocol:** In this case, it must be adapted to the spontaneous onset of the previous menstruation. GnRH antagonist protocol has a lower risk of ovarian hyper-stimulation syndrome (OHSS), which is a life-threatening complication. It is shorter than previous protocol.

**Final Maturation Induction:** When the ovarian follicles have reached a certain degree of development, induction of final oocyte maturation is performed, generally by an injection of human chorionic gonadotropin (HCG). Commonly, this is known as the 'trigger shot.' HCG acts as an analogue of luteinising hormone and ovulation would occur between 38 and 40 hours after a single HCG injection, but the egg retrieval is performed at a time usually between 34 and 36 hours after HCG injection; that is, just before the rupture of follicles.

**Egg Retrieval:** The eggs are retrieved from the patient using a trans-vaginal technique called trans-vaginal oocyte retrieval, involving an ultrasound-guided needle piercing the vaginal wall to reach the ovaries. Through this needle follicles can be aspirated and the follicular fluid is sent to an embryologist to identify ova. It is common to remove between ten and thirty eggs. The retrieval procedure usually takes between 20 and 40 minutes, depending on the number of mature follicles and is usually done under conscious sedation or general anaesthesia.

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**Egg and Sperm Preparation:** In the laboratory, the identified eggs are stripped of surrounding cells and prepared for fertilisation. An oocyte selection may be performed prior to fertilisation to select eggs with optimal chances of successful pregnancy. Semen samples are collected 2 hours before the oocytes are retrieved. Semen is prepared for fertilisation by removing inactive cells and seminal fluid in a process called sperm washing and thus, the sperms are capacitated.

**Co-Incubation:** The sperm and the egg are incubated (12-18 h) together at a ratio of about 75,000:1 in a culture media for the actual fertilisation to take place. In certain situations, such as low sperm count or motility, a single sperm may be injected directly into the egg using Intra Cytoplasmic Sperm Injection (ICSI). The fertilised egg is passed to a special growth medium and left for about 48 hours until the egg consists of six to eight cells.

**Embryo Culture and Selection:** The main durations of embryo culture are until cleavage stage (day two to four after co-incubation) or the blastocyst stage (day five or six after co-incubation). To optimise pregnancy rates, the embryo quality is determined by morphological scoring system.

**Embryo Transfer:** Embryos are graded by the embryologist based on the number of cells, evenness of growth and degree of fragmentation. The number to be transferred depends on the number available, the age of the woman and other health and diagnostic factors. Different countries follow different regulations for embryo transfer. In countries, such as Canada, UK, Australia and New Zealand, a maximum of two embryos are transferred except in unusual circumstances. According to HFEA regulations, a woman over 40 may have up to three embryos transferred in UK, whereas in the USA, younger women may have many embryos transferred based on individual fertility diagnosis. The embryos judged to be the 'best' are transferred to the patient's uterus through a thin, plastic catheter, which is inserted through her vagina and cervix. Several embryos may be passed into the uterus to improve chances of implantation and pregnancy.

**Adjunctive Medication:** In order to attain good success rate in IVF certain supporting procedures are followed like Luteal Support (LS). LS is the administration of medication, generally progesterone, progestin or GnRH agonists, to increase the success rate of implantation and early embryogenesis; thereby complementing and/or supporting the function of the corpus luteum.

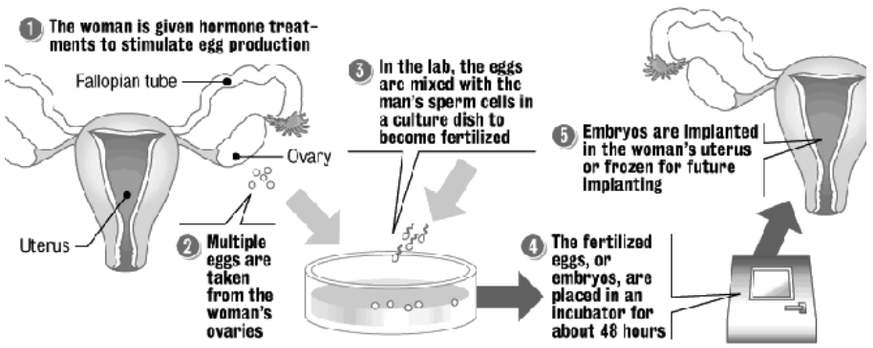


***Fig. 4.14*** *Procedure of IVF*

## Risks Associated with IVF

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The risks associated with IVF are:

- **Multiple Births:** The major complication of IVF is the risk of multiple births. This is directly related to the practice of transferring multiple embryos at embryo transfer. Multiple births are related to increased risk of pregnancy loss, obstetrical complications, prematurity and neonatal morbidity with the potential for long term damage. Strict limits on the number of embryos that may be transferred have been enacted in some countries (for example, Britain, Belgium) to reduce the risk of high-order multiples (triplets or more), but are not universally followed or accepted. Another risk is spread of infectious disease (such as Hepatitis B, HIV/AIDS).
- **Other Risks to the Egg Provider/Retriever:** A risk of ovarian stimulation is the development of ovarian hyper-stimulation syndrome, particularly if HCG is used for inducing final oocyte maturation. This results in swollen, painful ovaries. It occurs in 30% of patients. Mild cases can be treated with over the counter medications and cases can be resolved in the absence of pregnancy. In moderate cases, ovaries swell; fluid gets accumulated in the abdominal cavities and may have symptoms of heartburn, gas, nausea or loss of appetite. In severe cases patients have sudden excess abdominal pain, nausea, vomiting and will result in hospitalisation. During egg retrieval, there is a small chance of bleeding, infection and damage to surrounding structures like bowel and bladder (trans-vaginal ultrasound aspiration) as well as difficulty in breathing, chest infection, allergic reactions to medication, or nerve damage (laparoscopic techniques). Ectopic pregnancy may also occur if a fertilised egg develops outside the uterus, usually in the fallopian tubes and requires immediate destruction of the foetus.
- **Birth Defects:** Infants resulting from IVF (with or without ICSI) have a relative risk of birth defects of 1.32% compared to naturally conceived infants. The birth defects include septal heart defects, cleft lip with or without cleft palate, oesophageal atresia, and anorectal atresia. In 2017 studies revealed that there is prevalence of Cerberal Palsy in offsprings produced through in vitro fertilization technique. Cereberal palsy is a congenital neurological disorder which affects the movement and the muscle coordination of the child, in many cases vision, hearing and sensation are also get affected. In total of 211,660 live births were included in study and prevalence of cereberal palsy was increased in children born after ART (7.2 /1000 live births compared with natural conceive births 2.5/1000.

### Side Effects of IVF

Side effects of IVF may include: Discharge of fluid may be clear or blood-tinged after the procedure from vagina, mild cramping, Constipation, Breast tenderness, Nausea or vomiting, Decreased urinary frequency, Shortness of breath, Faintness, and severe stomach pains and bloating.

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**Check Your Progress**

1. Does the foster mother in case of embryo transfer make any genetic contribution to the offspring?
2. State one of the first things required to generate transgenic animals.
3. What is used to induce super-ovulation?
4. Mention three common methods of artificial insemination.
5. What are the two types of cell preservation techniques?
6. Name the chemicals used by insects and other animals to communicate with each other.
7. What is one of the major difference between classic IVF cycles and IVM?
8. When does an ectopic pregnancy occur?

## 4.3 TRANSGENIC ANIMALS: PRODUCTION

Many generations of selective mating are required to improve livestock and other animals genetically for traits such as milk yield, wool characteristics, egg-laying frequency, etc., at each generation, animals with superior characters are used as breeding stock. This combination of mating and selection, through succession, is costly and time-consuming. With the development of r-DNA technology, methods are available that allows foreign or synthetic genes to be introduced into and expressed in higher plants and animals. The DNA that is introduced is called transgene, the animal is transgenic and the process is called Trans-genesis.

When the DNA from foreign origin is introduced within the genome of another organism, the process is referred to as trans-genesis. Animals whose genome is permanently engineered with foreign DNA using either the conventional breeding methods or the recombinant DNA tools are referred to as Transgenic Animals. These animals are very crucial to study different biological aspects, such as organogenesis, gene expression, and function, aging, the onset of diseases, etc. In the year 1981, Richard D. Palmiter from University of Washington reported the development of the First Transgenic Animal. He synthesized a chimera of the promoter region of metallothionein-I and the growth hormone gene and microinjected into the pro-nuclei of fertilized Mouse eggs leading to the development of Seven Chimeric Mice that carried the fusion gene product.

In scientific research, Mice are generally used to study the *in-vivo* gene functions as well as a mammalian model. Therefore, most of these transgenic experiments have been conducted using Mice models. Transgenic technology has been perfected in the laboratory mouse. Hundreds of genes were introduced into the Mouse since 1980, contributing to an understanding of gene regulation, tumor development, immune specificity, etc. DNA can be introduced into Mice by microinjection or engineering embryonic stem cells or through retroviral vectors. Transgenic Mice can be developed mainly using three approaches. The first method involves the use of retroviral vectors to manipulate the gene structure and function.

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This technique can only transfer a small fragment (<8 kb) of DNA. Due to many technical issues, this method is no longer in practice for routine Transgenic Mice production. The second method involves the transfer of foreign gene into the male pro-nucleus post fertilization using the microinjection. Micro-needle is used as an instrument to inject the gene of interest (70-100 kb) within the male pro-nucleus and the gene of interest is randomly integrated into the host DNA. However, the gene of interest mainly integrates after one or two successive cell divisions leading to the formation of a Mosaic Mouse containing both non-transfected and transfected cells. The microinjected embryos are transferred into the oviducts of foster pseudo-pregnant mice, leading to the subsequent development of Transgenic Mice. These Mice are then confirmed using sensitive techniques like Southern blot, polymerase chain reaction. The *third* method describes the targeted manipulation of Mouse Embryonic Stem (ES) cells by introducing loss or gain of function mutation by altering the nucleotide(s). The embryonic stem cells are derived from Inner Cell Mass (ICM) of Mouse blastocysts. By nature, these cells are pluripotent and can form all cell lineages of the embryo once injected into the recipient blastocyst. In order to demarcate the donor and recipient Mice, different coat colour Mice are used. After the genomic manipulation, the resultant offspring known as chimeras display the patchy coat colours. The gene of interest is incorporated into the ES cells by the process of homologous recombination using a targeting vector that contains a modified version of the endogenous gene. This technique is most commonly used to produce the gene knockouts. The targeted vectors are used to precisely remove one or more exons from a gene, resulting in the production of truncated and non-functional protein (Refer Figure 4.15).

The use of ES cell-based method leads to the modification of genes at their normal location in the chromosome. In contrast to this, the pronuclear injection method leads to random transgene insertion within the genome leading to either misexpressed spatially and temporally or overexpressed than the normal gene.

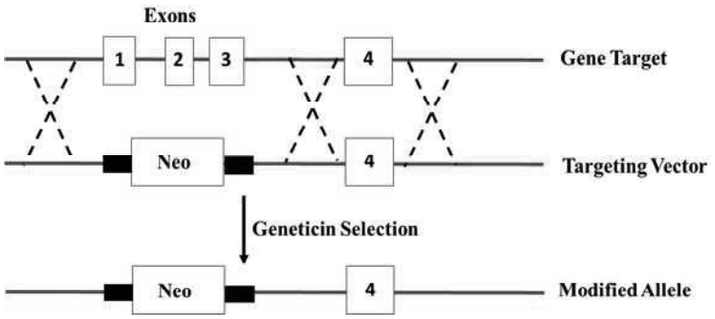


***Fig. 4.15*** *The Homologous Recombination Method used for Gene Inactivation or Knockout Mutants*

## 4.3.1 Process of Development of Gene Knockout Mice

To develop the knockout Mice, the following steps can be followed (Refer Figure 4.16):

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- Extraction of ES cells from the ICM of the blastocyst and their culturing.
- Mutation of ES cells using homologous recombination method.
- Injection of the recombinant ES cells into the recipient blastocyst.
- Surgical implantation of the recombinant blastocyst into the pseudo-pregnant Mouse.
- Mating of chimeric heterozygous offspring to obtain the homozygous knockout Mice.

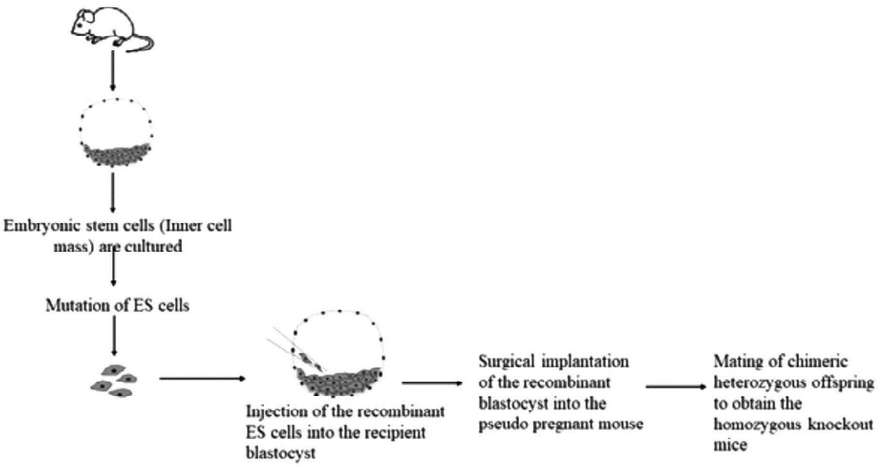


***Fig. 4.16*** *Experimental Approach to Generate Knockout Mice*

## 4.3.2 Applications of Transgenic Mice

Transgenic Mice are produced routinely in laboratories throughout the world, they provide valuable tools for the study of gene expression in mammals. They stimulate both the onset and progress of human disease and provide a system for testing therapeutic agents. Mouse models for human diseases, such as, Alzheimer's disease, Arthritis, Hypertension, and Coronary Diseases have been developed.

Alzheimer's disease is a degenerative brain disorder characterized by loss of memory accompanied by personality changes and language disturbances. Neurons accumulate fibrillary tangles of amyloid bodies in the blood vessels of the brain and senile plaques develop at the ends of axons. The principal component of the senile plaques is a β-protein cleaved from an Amyloid Precursor Protein (APP). It is not known what causes the accumulation of β-protein. Some families with a high incidence of Alzheimer's disease have a mutation in the APP Gene, a finding that implicates the APP Gene in Alzheimer's Gene.

The vector constructed consists of a promoter region from a brain-specific virus ligated to a portion of the human APP Gene that encodes last 100 amino acids at the C terminus of APP (for β-protein). Transgenic Mice were established with this construct and the expression of the transgene was limited to neurons. The Mice showed the expression of β-protein derived from the transgene.

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Transgenic mice have also been used as models for expression systems that are designed to secrete transgene product into milk, for example large quantities of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein are needed to understand and treat Cystic Fibrosis. The CFTR gene was cloned into the middle of a defective goat β-casein gene. β-casein is a major milk protein and the gene is actively expressed in mammary glands. Transgenic Mice carrying the CFTR gene produced milk with CFTR as predicted. The protein is glycosylated and extracted for scientific studies.

### Transgenes in Growth Promotion

Increased growth was observed when Rat, Bovine or Human Growth Hormone (GH) transgenes were expressed in Mice (super mouse: double the size of normal mice), this prompted animal breeders to introduce extra copies of homologous or heterologous GH genes to obtain enhanced growth rates. Transgenic Salmons (Fish) increased in weight 0 to 37 times the weight of normal siblings. Transgenic Pigs were found to be leaner than controls because GH favors protein synthesis instead of fat synthesis (low cholesterol levels). Unfortunately, Transgenic Animals exhibited several side effects like sterility, susceptibility to Arthritis, Gastric Ulcers, etc. Clearly, the native levels of GH represent the end product of several generations of natural selection for optimum fitness and any changes induced in hormone levels by transgenes are likely to decrease fitness by upsetting the normal metabolic balance.

### Transgenic Sheep, Goats, and Pigs

Transgenic research with Sheep and Goats devoted to developing mammary glands as bioreactors for the production of pharmaceutical proteins. Although the quantity of milk produced by a Sheep or Goat is less than that of a Cow, lactation in Sheep or Goat produces hundreds of litres of milk. Transgenic Sheep were produced that secrete a variety of human proteins like blood clotting factor IX, elastase inhibitor, antitrypsin (deficiency causes emphysema), interleukin, long-acting Tissue Plasminogen Activator (TPA), etc., the coding sequences of these proteins were inserted into b-lactoglobulin (a milk protein) gene of Sheep and chimeric construct was microinjected into fertilized egg. The injected eggs were implanted into foster mothers to produce transgenic lambs. Though the concentrations of these proteins are small, the Sheep exhibited no side effects. Scientists believe that the concentrations can be increased by manipulating the regulating the regulatory sequences of Chimeric Genes.

With a construct that consisted of the regulatory region from human β-globin joined to two Human Gene clusters: one α-globin gene and one human β-globin gene, healthy Transgenic Pigs were produced that expressed human haemoglobin (Hb) in their blood cells. The Hb produced as a result of Transgenesis from Pigs had the same properties as human Hb. Although preliminary, these results point to the possibility of replacing whole blood used for transfusion with haemoglobin produced by Transgenesis.

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## Transgenic Cattle

If the mammary glands are to be used as a bioreactor, dairy cattle with annual milk production of 10,000 L are good candidates for transgenesis. Transgenesis in cattle involves: collecting oocytes from slaughterhouse killed Cows, *in-vitro* fertilization, and centrifugation of fertilized egg, microinjection of DNA and non-surgical implantation of one embryo into one foster mother.

One of the goals of transgenesis of dairy cattle is to change the constituents of milk. The amount of cheese produced from milk is directly proportional to casein content, increasing casein production with an over-expressed casein transgene is a reasonable likelihood. For a different end-use, expression of a lactose transgene in mammary glands could result in milk that is free of lactose. Such milk would be welcomed by many people who are lactose intolerant and experience severe indigestion after consuming milk. Efforts are on to introduce a gene that confers resistance to bacterial and parasitic diseases and production of antibodies to provide biological protection. The term 'pharming' is coined to convey the idea that milk from transgenic farm animals is a source of human protein drugs.

## Transgenic Birds

Microinjection of DNA into eggs is not possible in birds to identify male pronucleus because of polyspermy. Further, the avian ovum is enveloped by albumin and membranes rapidly after fertilization. However, blastoderm cells can be removed from donor chickens, transfected with DNA and reintroduced into subgerminal space of the embryos of freshly laid eggs. The progeny consists of a mixture of cells. Transgenic lines can then be established by rounds of mating's. The proportion of donor cells can be enhanced by irradiating the embryos prior to the introduction of transfected cells. The radiation destroys some blastomeres increasing the ratio of transfected cells.

Avian Leukosis Virus (ALV) is a major viral pathogen of chickens causing $100 million loss to poultry. Transgenic chickens were produced that carry a defective ALV genome. These chickens produce viral RNA and envelop protein but no progeny viruses. The synthesis of large amounts of retroviral envelope protein somewhat blocks the reproductive cycle of intact pathogenic viruses. Most importantly they are resistant to infection by ALV. The ALV resistance has been transmitted to several generations of progeny indicates the trait is stable.

Avian researchers have also suggested that the egg with its high protein content could be used as a source of pharmaceutical proteins. By analogy with mammary glands, the expression of transgenes in the oviduct of a hen that normally secretes large amounts of albumin could lead to the accumulation of transgene-derived proteins that become encased in the eggshell.

## Transgenic Fish

In fish, the pronuclei are not rapidly seen under a microscope after fertilization. So, linearized transgenic DNA is microinjected into the cytoplasm of either fertilized egg or 4-celled embryos. As the development is external there is no need for implantation. Human growth hormone gene transfected to fish allowed growth that gave twice the size of normal control fish. Similarly, the Anti-Freeze Protein

(AFP) gene was transferred to several fishes. It was shown that the level of AFP gene expression is still low to provide protection against freeze.

## 4.3.3 Knock-Outs

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A Gene Knock-Out (KO) is a genetic technique in which one of an organism's genes is made inoperative ('knocked out' of the organism), (Refer Figure 4.17). However, KO can also refer to the gene that is knocked out or the organism that carries the gene knockout. Knockout organisms or simply knockouts are used to study gene function, usually by investigating the effect of gene loss. Researchers draw inferences from the difference between the knockout organism and normal individuals.

The KO technique is essentially the opposite of a gene knock-in. Knocking out two genes simultaneously in an organism is known as a Double Knock-Out (DKO). Similarly the Terms Triple Knock-Out (TKO) and (QKO) are used to describe three or four knocked out genes, respectively. However, one needs to distinguish between heterozygous and homozygous KOs. In the former, only one of two gene copies (alleles) is knocked out, in the latter both are knocked out.

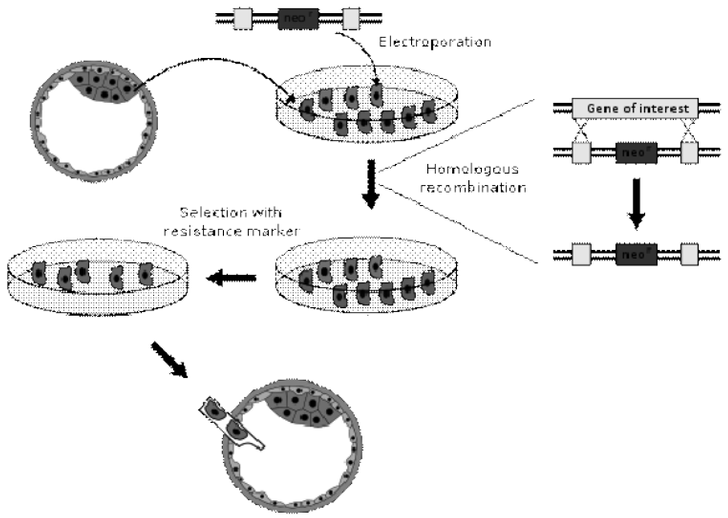


***Fig. 4.17*** *Diagram Showing Breeding Scheme for Producing Knock-Out Mouse*

### Gene Knock-out Techniques

Knockouts are accomplished through a variety of techniques. Originally, naturally occurring mutations were identified and then gene loss or inactivation had to be established by DNA sequencing or other methods, (Refer Figure 4.18).

- **Homologous Recombination:** Traditionally, homologous recombination was the main method for causing a gene knockout. This method involves creating a DNA construct containing the desired mutation. For knockout purposes, this typically involves a drug resistance marker in place of the desired knockout gene. The construct will also contain a minimum of 2kb of homology to the target sequence. The construct can be delivered to stem cells either through microinjection or electroporation. This method then relies

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on the cell's own repair mechanisms to recombine the DNA construct into the existing DNA. This results in the sequence of the gene being altered, and most cases the gene will be translated into a nonfunctional protein, if it is translated at all. However, this is an inefficient process, as homologous recombination accounts for only $10^{-2}$ to $10^{-3}$ of DNA integrations. Often, the drug selection marker on the construct is used to select for cells in which the recombination event has occurred.

- **Site-Specific Nucleases:** There are currently three methods in use that involve precisely targeting a DNA sequence in order to introduce a double-stranded break. Once this occurs, the cell's repair mechanisms will attempt to repair this double stranded break, often through Non-Homologous (NHEJ), which involves directly ligating the two cut ends together. This may be done imperfectly, therefore sometimes causing insertions or deletions of base pairs, which cause frameshift mutations. These mutations can render the gene in which they occur nonfunctional, thus creating a knockout of that gene. This process is more efficient than homologous recombination, and therefore can be more easily used to create biallelic knockouts, for example, Zink-finger nuclease, TALENs (Transcription Activator-Like Effector Nucleases), CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats).

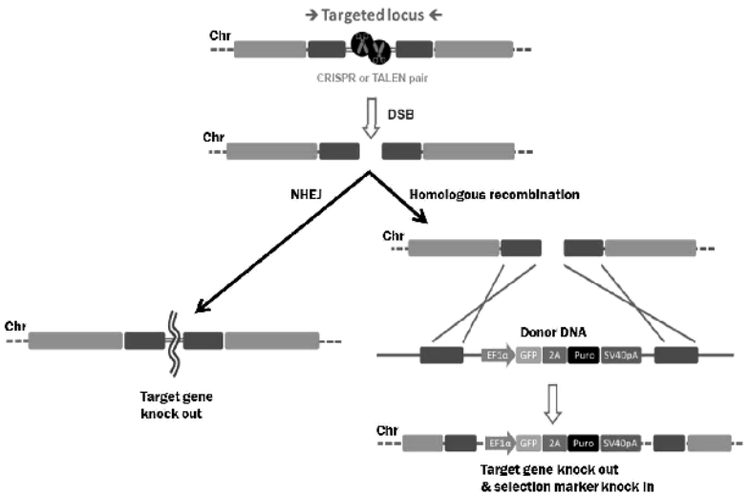


***Fig. 4.18*** *Diagram Showing Knock-Out by CRISPR vs Knock-Down by SiRNA*

## Conditional Knock-Out

A conditional knock-out allows gene deletion in a tissue in a time specific manner. This is required in place of a gene knockout if the null mutation would lead to embryonic death. This is done by introducing short sequences called loxP sites around the gene. These sequences will be introduced into the germ-line via the same mechanism as a Knock-Out. This germ-line can then be crossed to another germline containing Cre-recombinase which is a viral enzyme that can recognize these sequences, recombines them and deletes the gene flanked by these sites.

### Uses of Knock-Out

Knock-Outs are primarily used to understand the role of a specific gene or DNA region by comparing the knockout organism to a wild type with a similar genetic background. Knock-Out organisms are also used as screening tools in the development of drugs, to target specific biological processes or deficiencies by using a specific knockout, or to understand the mechanism of action of a drug by using a library of Knock-Out organisms spanning the entire genome, such as in *Saccharomyces cerevisiae*.

## 4.3.4 Embryonic Stems Cells

Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body. As, we can see in the figure below, they can differentiate to produce different types of cells:

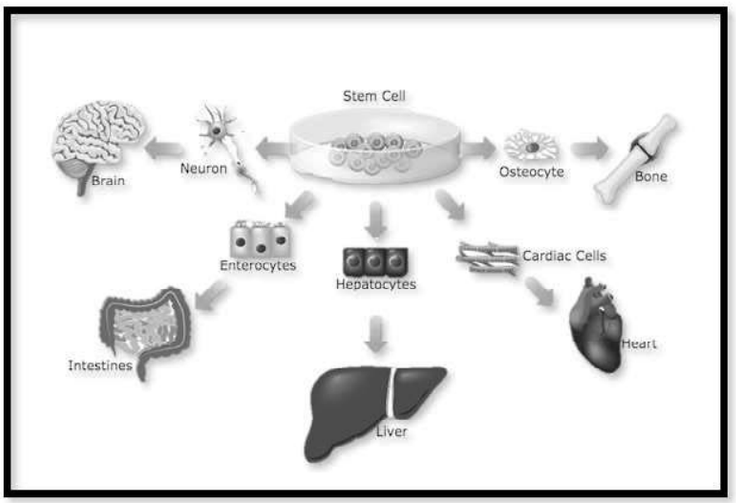


***Fig. 4.19*** *Differentiation of Stem Cells to form Different Types of Cells*

Stem cells have unique properties:

- They are unspecialized cells
- They can undergo self-replication via asymmetric cell division.
- They can undergo differentiation to produce specific cells in accordance with the need of the body as well as signal received by the stem cells

### Types of stem cells

Stem cells can be classified. The Figure 4.20 below summarizes different types of stem cells on the basis of potency as well as origin:

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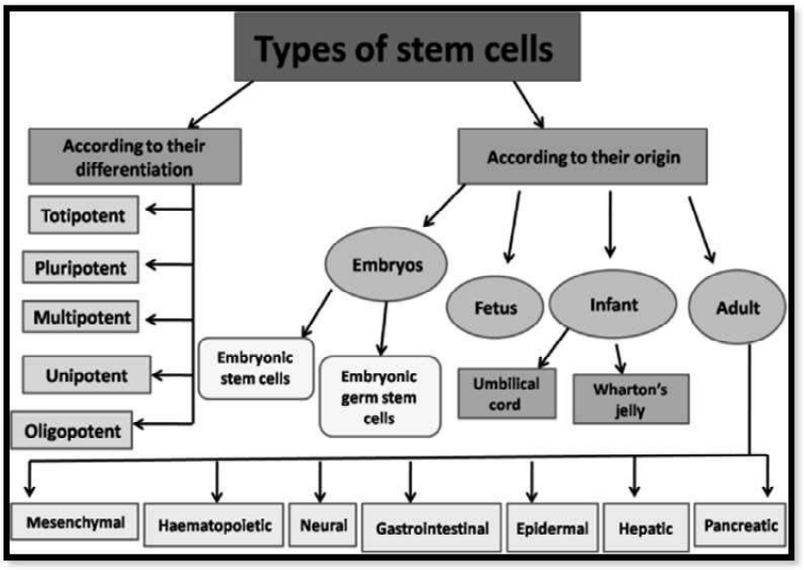


***Fig. 4.20*** *Classification of Stem Cells*

Let us discuss both the ways one by one:

1) **On the Basis of Potency**: Based on the potency, i.e. their ability to differentiate and form different types of cells, stem cells are of following types:

   a. **Totipotent**: These stem cells can differentiate into all possible cell types. Oocytes and sperm are the best differentiated cells in our body and they are capable of forming any tissue in the body

   b. **Pluripotent**: These cells can turn into almost any cell. Cells derived from the early embryo i.e. blastocyst are referred to as pluripotent.

   c. **Multipotent**: These cells are capable of differentiating into a closely related family of cells, i.e., these cells that can only give rise to cells of the tissue from which they are isolated. For example, Adult hematopoietic stem cells can differentiate to become red and white blood cells or platelets.

   d. **Oligopotent**: These cells can differentiate into a few different cell types. For example- Adult lymphoid or myeloid stem cells.

   e. **Unipotent**: These cells are capable of producing only one kind of cell, i.e., their own cell type. Their self-renewal property helps in repair of the damaged adult tissue. For examples, adult muscle stem cells.

2) **On the basis of their Origin:** Based on their origin, stem cells are of following types:

   a) **Embryonic Stem Cells (ESCs)**

   - They are derived from early stages of embryo development i.e. from mouse or human blastocyst
   - They are pluripotent, self-renewing cells

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- They can be stored in culture for a long duration of time as undifferentiated cell lines
- These undifferentiated cells can be stimulated to differentiate into any cell line
- ESCs can differentiate into endoderm, mesoderm, and ectoderm embryonic germ layers or any type of somatic cells.
- They are of wide significance in tissue regeneration therapy
- Embryonic stem cells are also derived in-vitro as shown in the figure 4.21 below:

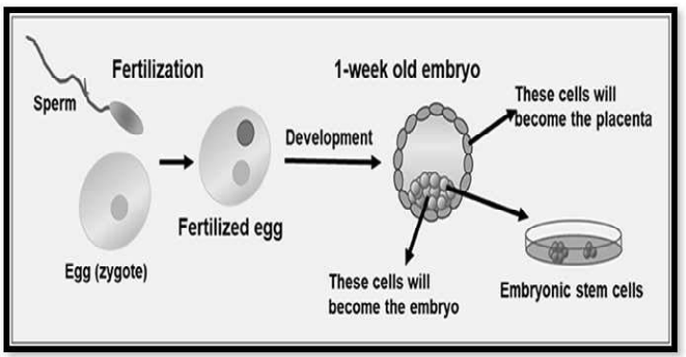


***Fig. 4.21*** *In-vitro Isolation of Embryonic Stem Cells*

**b) Embryonic germ stem cells**

- They are derived from Primordial Germline Cells (PGCs) in the early development.
- The PGC-derived cells are pluripotent.

**c) Foetal stem cells**

- These cells are present in the organs of the foetuses.
- They are capable of differentiating into two different types of stem cells i.e. pluripotent stem cells and hematopoietic stem cells.

**d) Umbilical cord stem cells**

- They are derived from the Umbilical cord
- Stem cells derived from Umbilical cord blood varies when compared to stem cells derived from bone marrow and adult peripheral blood
- Umbilical Cord cells are Multipotent
- They can differentiate into neurons and liver cells
- Below, the figure 4.22 depicts the methodology of collecting blood form umbilical cord:

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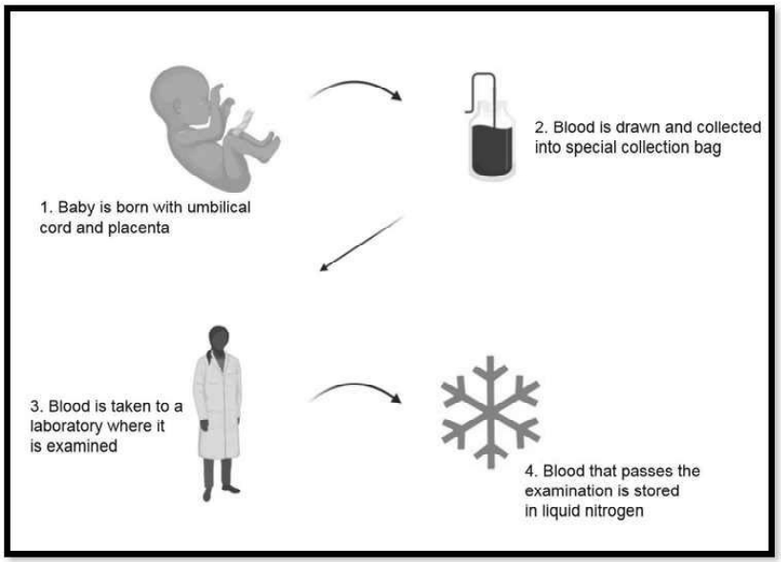


***Fig. 4.22*** *Collection of Blood from Umbilical Cord*

**e) Wharton's jelly**

- Wharton's jelly, refers to the umbilical cord matrix
- It serves as a source of mesenchymal stem cells.
- Wharton's jelly cells can be propagated for long duration
- They can be induced to differentiate *in vitro* into neurons

**f) Adult Stem Cell**

- Adult stem cells are the stem cells which are derived from mature tissue
- They can be isolated from the tissues of a fully developed child (whole embryo) or adult
- They are multipotent in nature
- They play a crucial role in the physiological processes like tissue repair, regeneration etc.

Below we have mentioned, different types of adult stem cells:

- **Mesenchymal Stem Cells**: Initially, Mesenchymal Stem Cells (MSCs) were described as adherent cells with a fibroblast-like appearance which can differentiate into several other cell types such as osteocytes, chondrocytes, adipocytes, tenocytes and myocytes. MSCs can be derived from the connective tissue or stroma that surrounds the body's organs and other tissues. MSCs exhibits property of long-storage without major loss of their potency.
- **Hematopoietic Stem Cells:** Due to their self-renewal properties, Hematopoietic stem cells (HSCs) are capable to differentiate into different cells of all hematopoietic lineages. They can be used to treat hematologic disorders. The following figure 4.23depicts the mode of division of HSCs:

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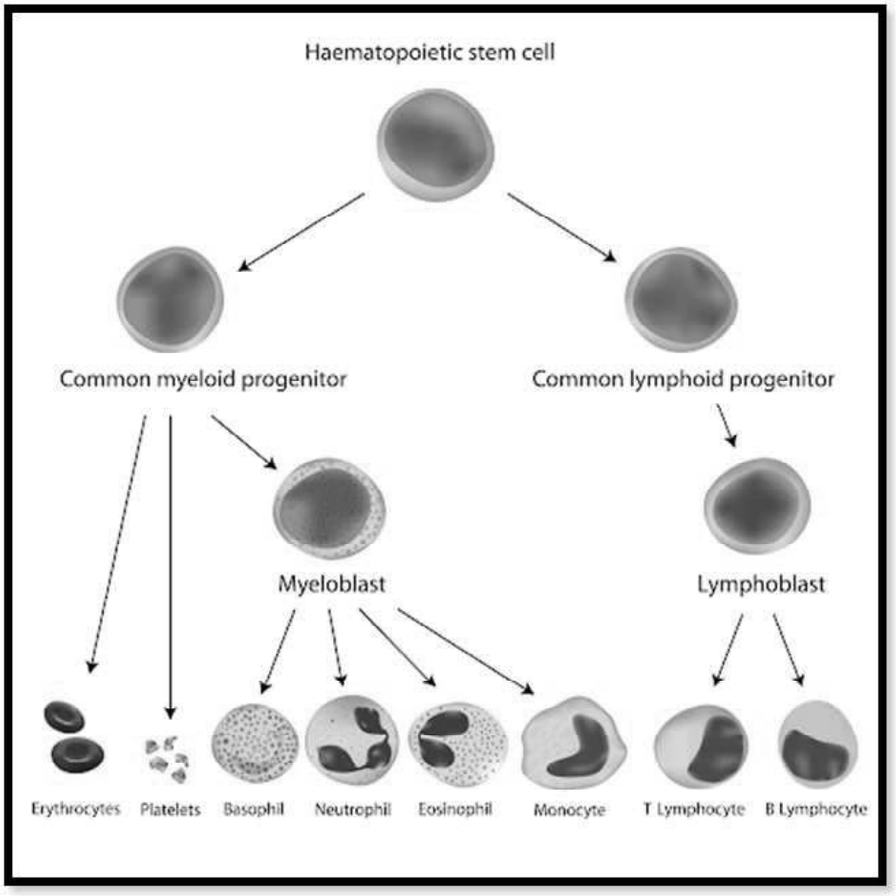


***Fig. 4.23*** *Lineage of Blood Cells*

**Neural Stem Cells:** They are present in specialized molecular microenvironments in the adult mammalian brain. Due to their multipotency and self-renewal properties, they can be potentially used in cellular therapy of the brain

**Gastrointestinal Stem Cells:** Gastrointestinal Stem Cells (GSCs) are located in the intestinal crypts and gastric glands. However, the position and mechanism of these stem cells is widely debated.

**Epidermal Stem Cells:** Epidermis undergoes a lot of wear and tear and hence it is a rapidly rejuvenating tissue. Epidermal stem cells are established in the basal layer and due to their self-renewal properties participate significantly in maintaining homeostasis and cellular regeneration of normal skin; wound healing as well as neoplasm formation

**Hepatic Stem Cells**: As we all know, hepatic (liver) tissue possesses a strong regenerative capacity and can utilizing diverse means of regeneration depending upon the kind and degree of the wound or injury.

Mature liver cells or hepatic cells can propagate to replace the damaged tissue and hence let the revival of the parenchymal function

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As we have learned in the previous section that scientists have found stem cells in tissues, including:

- Bone marrow
- Brain
- Skeletal muscles
- Blood and blood vessels
- Liver
- Skin

These adult stem cells can divide or self-renew indefinitely. Thus, it can be concluded that an adult person's body contains stem cells throughout the life and the body can utilize these stem cells in accordance with the need and signal received. Adult stem cells remain in a non-specific state, till the body requires them for a definite purpose such as regeneration or maintenance of body tissues or repair. However, adult stem cells are difficult to be located and isolated. They can remain in a non-dividing and non-specific state for years until the body signals them to repair, regenerate or develop some new tissue.

### Donating or Harvesting Stem Cells

Stem cell can be derived from following sources:

1) **Bone Marrow**: These cells are usually taken from the hip or pelvic bone. Technicians then isolate the stem cells from the bone marrow for donation or storage.
2) **Peripheral Stem Cells**: The donor receives numerous injections that stimulate their bone marrow to liberate stem cells into the blood. Subsequently, blood is taken out from the body, stem cells are separated from other blood cells via a machine and finally the filtered blood (blood without stem cells) is returned to the body.
3) **Umbilical Cord Blood**: This is a harmless and painless technique in which stem cells can be easily harvested from umbilical cord after delivery. Some people donate the cord blood cells whereas others store it for future purpose. The advantages of storing stem cells for future needs include:
   - Easily available when required in urgency like serious illness
   - Very low chances of transplanted tissue being rejected when stem cells comes from the recipient's own body

### Cell Culture Technique Used for Stem Cells

Cell culture is one of the major molecular techniques used in life sciences. It involves the removal of cells, tissues or organs from a plant or an animal and its culturing in an artificial environment. Cell requires optimal conditions for their survival and proliferation like:

- A substrate for cell attachment
- An appropriate growth medium/nutrient media that provides correct pH as well as osmolality

- An incubator that maintains controlled temperature, pressure, humidity, etc.

The most critical step of *in vitro* cell culture is the selection of an appropriate growth medium. By definition, a culture medium/growth medium refers to a liquid or gel produced to maintain the growth and propagation of cells, microorganisms and small plants. All Cell culture media/growth media has a general composition comprising of an appropriate source of energy (carbohydrates) and other compounds which helps in regulate the cell cycle. Generally, a typical culture medium is composed of a glucose (as energy source), amino acids, essential vitamins, inorganic salts, serum (which serves as a source of growth factors), hormones, and attachment factors (aids in the attachment of cells to surface). As mentioned above, growth medium also provides the optimum pH and osmolality for the survival and propagation of cells.

Cell culture is a multistep process which involves isolation of cells from desirable animal or plant tissues/organs either by mechanical or enzymatic application or they may be derived directly from established cell lines or cell strains followed by their proliferation under optimum conditions in a suitable cell culture media. The classic hanging drop culture is a small drop of liquid, like plasma or some other media permitting tissue growth, suspended from an inverted watch glass. Thereafter, hanging drop is suspended by gravity and surface tension, rather than spreading across a plate. This permits tissues or other cell types to be thoroughly observed without being squashed against a dish. Initially, this methodology was developed to study about bacteria in a confined system. However, 'Ross Granville Harrison' used this technique to show the growth of nerve cells.

Other major applications of this technique comprise stem cells cultures as well as *in vitro* cultures of whole embryos. This technique allows the stem cells to be cultured easily without being pressed against a plate. This is particularly advantageous when the 3D structure of a tissue is preferred. In a similar way, entire embryos can be cultured conveniently by following this technique. For embryology studies, it is essential to maintain the original structure of the embryo, hence hanging drop methodology permits the embryo to develop without getting pressed against a dish. The capability to resolve 3D structures was a significant progress that made this technique a widely used one for research purpose.

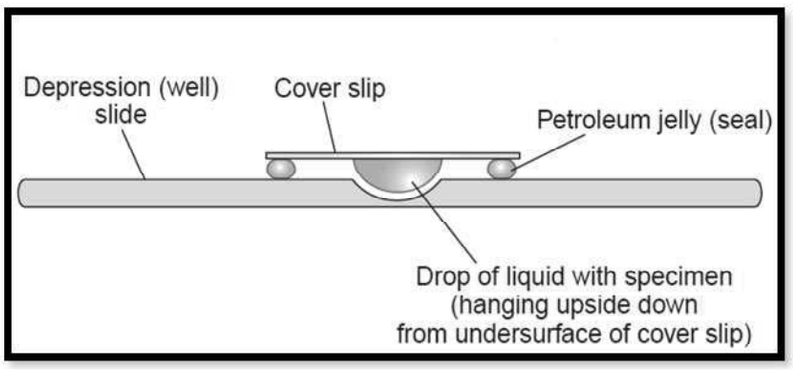


***Fig. 4.24*** *Principle of Hanging Drops Technique*

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Cell culture provides outstanding model systems for carrying out the research on normal physiology and biochemistry of cells involving concepts like metabolic studies, aging, mutagenesis, carcinogenesis and also the effects of drugs and other toxic compounds on the cells. Besides this, it also helps in drug screening as well as development and production of active biological compounds like vaccines and therapeutic proteins in large quantity. The applications of stem cell culture system are as follows:

**I. Stem Cell Culture as Model System**

- Cell culture are used regularly as model system to study the concepts of basic cell biology as well as biochemistry
- To study the interaction between cell and pathogen like bacteria, viruses etc.
- To carry out the drug screening
- To carry out the research on metabolic processes like ageing
- Cell culture system can be used to carry out the cancer research i.e. it can help the researchers to reveal the mechanism behind the conversion of normal functioning cells to cancerous
- Further, it can also help to screen the drugs that can be effectively used to destroy cancer cells

**II. Stem Cell Culture to Study Virology**

- Animal cell culture system can be used to replicate the viruses as an alternative to animals for the mass production of vaccine.
- Cell culture system can also be used to identify and isolate viruses as well as to study growth and development cycle of viruses.
- Cell culture system can also be used to study the mode of infection used by different viruses.

**III. Stem Cell Culture System in Toxicity Testing**

- Animal cell culture system can be used to study the effects of newly discovered drugs, cosmetics as well as chemicals on survival, growth and development of different cell types.
- Cell culture system can also be used to determine the maximum permissible safe dosage of newly discovered drugs.

**IV. Stem Cell Culture System in Vaccine Production**

- Cell culture system can be used in the production of viruses and these viruses can be used to produce vaccines for diseases.

**V. Stem Cell Culture System in Production of Genetically Engineered Protein**

- Cell culture system can be used to manufacture genetically engineered biological compounds such as insulin, hormones, monoclonal antibodies etc.

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### VI. Stem Cell Culture System as Substitute for Vital Tissue or Organs

- Cell culture system can be used as a substitute for vital tissues as well as organs. For instance, skin produced by cell culture system can be used to treat patients having severe burns and ulcers.
- Advance researches are going in the direction to produce organs like kidney, liver, pancreas etc.
- Advance researches are going on in the field of organ culture techniques on both embryonic as well as adult stem cell culture. These cells are pluripotent i.e. they have the unique ability to differentiate into several different types of cells as well as organs.

### VII. Stem Cell Culture System in Genetic Counselling

- Foetal cell cultures extracted from pregnant women are generally used to study or examine the abnormalities of chromosomes.
- Such culture can help in determining the chromosomal abnormalities as well as in the detection of foetal disorders.

### VIII. Stem Cell Culture System in Genetic Engineering

- Cell culture system can be used to develop transgenic cells i.e. cells having new genetic material like DNA or RNA.
- Such transgenic cells can be used to study the expression of new genes as well as its effect on cell.
- Such cell lines can be developed commercially to extract the desirable protein product.
- Further, such genetically altered cells can be used in gene therapy techniques.
- Such genetically altered cells having the desirable functional gene can be introduced into the patient lacking or missing that functional gene.

### Application of stem cells

Stem cells under optimum conditions and on receiving proper signal can regenerate damaged tissue which potentially could save several lives or repair wounds or can heal tissue damage in people after some serious illness or injury. Embryonic stem cells are pluripotent in nature, i.e., they are more adaptable in research studies when compared to adult stem cells. One of the major advantages of embryo research include discovering new ways of treating diseases, injuries as well as organ failure. These cells can be manipulated in labs in a variety of ways to develop into any other type of cell present in the body. Researchers involved in embryo research enables scientists to understand how to prevent injected stem cells from growing abnormally and causing tumours. Further, stem cell therapies could possibly increase dopamine in the brains of those suffering from Parkinson's disease. These cells can possibly restore the functioning of patients suffering from disorders like heart diseases, stroke, cancer, tumour, spinal cord injuries, diabetes, and osteoarthritis. Alzheimer's disease or any other degenerative disease like

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amyotrophic lateral sclerosis (ALS). Below, we have listed some potential use of stem cells.

### 1) Embryonic Stem Cells can be used in the Production of Transgenic Animals

This method involves insertion of the desirable foreign DNA via homologous recombination into an in vitro culture of embryonic stem (ES) cells. These cells are then allowed to develop in tissue culture media and incorporated into an embryo at the blastocyst stage of development. This results in a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method. This methodology is of utmost importance for the study of the genetic control of developmental processes. This methodology has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

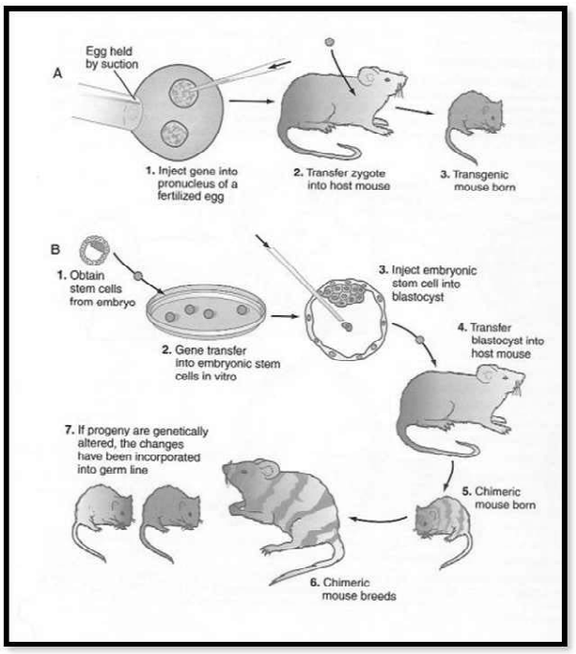


***Fig. 4.25*** *'DNA Microinjection' Method of Creation of Transgenic Mice*

'Embryonic Stem Cell' Mediated Gene Transfer Method of Producing Transgenic Mice

Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding.

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The steps involved in creating a transgenic organism are as follows:

**a. Identification of the Desirable Gene**

The first step is the identification of the desirable gene or gene of interest that codes for a specific target protein. A gene that codes for a desirable trait or protein must first be identified. Several molecular techniques like Gene Chips (Microarray) and DNA sequencing can be used to identify the desirable gene.

**b. Isolation of Desirable Gene**

The second step involves the isolation of the desirable gene from the target species. It can be achieved either via mechanically breaking the cells or with the aid of chemical agents like detergents. The entire DNA can be then separated from the other cell components via technique known as cell centrifugation. Now, to separate the target gene from the total DNA content following steps needs to be followed:

- Separation of DNA fragments according to size via Gel Electrophoresis
- Identification of the gene of interest using a DNA probe
- Cut out of the gel and amplified (copied) using PCR
- Alternatively, gene of interest could be inserted into a bacterial plasmid using the enzyme DNA Ligase
- Bacteria would automatically copy the gene while undergoing cell division- a technique popularly called as Gene Cloning
- However, if enough information is available regarding the gene of interest then it might be possible to create specific DNA primers and copy the gene using PCR without isolating it on a gel

**c. Transformation of the Desirable Gene**

- Finally, a vector (varies according to cell type) is used to transfer the gene of interest into the organism being modified.
- The final DNA sequence that is prepared consisting of target gene and associated regulatory sequences (promoter and termination) sequences is referred to as Gene Construct.
- However, the success rate at which transgene is expressed is very low.
- For the target gene to be expressed, it must make its way into the nucleus.
- For it to be passed on during cell division (mitosis and meiosis) it must integrate itself into the target cells genome via recombination /crossing over.
- For verifying, whether, the target gene has been inserted into the genome or not- researchers incorporate a second gene known as reporter gene into the gene construct. This second gene codes for an easily selectable / observable characteristic like antibiotic resistance or glow in the dark protein.

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- This enables the researchers to easily verify whether the integrated gene is expressing or not.
- Transgenic animals produced from embryonic stem cells have several potential use:
- Transgenic animals are widely used as disease model: Historically, mice have been used to model human disease because of their physiological, anatomical and genomic similarities to humans. Transgenic animals such as mice are quite popular with researchers to study critical diseases such as Alzheimer's, cancer, AIDS. Transgenic animals enable scientists to be aware of the role of genes in specific diseases.
- Transgenic animals as food: The FDA suggested that cloned animals and their products are edible.
- Transgenic animals in Drug and Industrial production: Transgenic animals are routinely used for production of therapeutic proteins used in the treatment of diseases such as emphysema or cystic fibrosis. Industries are investing a lot of money in research and development to derive therapeutic proteins like monoclonal antibodies from the milk of transgenic animals such as rabbits, goats and cows to administer drugs in treatment protocols for disorders such as cancer, rheumatoid arthritis and other autoimmune disorders.
- Transgenic animals in Disease control: Researchers developed the mice by altering the genes of the mouse pox virus in Australia.
- Transgenic animals in Xenotransplantation: Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of precious human organs like hearts and kidneys. Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients.
- Researchers are also discovering the use of cell transplantation therapy for patients with critical disorders such as Parkinson's or spinal cord injury. Genetic manipulation of stem cells which involves the growth of tissues on scaffolding can be utilized as a provisional skin alternate for healing wounds or burns. Tissue engineering is rapidly becoming an appropriate substitute in methodology that involves replacement of human structure such as cerebrospinal shunts, heart valves, cartilage and other organs.
- Transgenic combinations might help researchers to develop vaccines against deadly diseases. For instance, the DNA of human tumour fragments is inserted into tobacco plants in order to develop a vaccine against non-Hodgkin's lymphoma.
- Likewise, researchers have produced a flu vaccine using human DNA and tobacco plants.
- Transgenic animals in Blood replacement- Transgenic swine are used to produce human haemoglobin. Transgenic animals are used in toxicity testing.

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- **Transgenic Animals in Agriculture:** Transgenic animals are used for milk production. Generally, transgenic mice help in improving the composition of milk. Similarly, transgenic pigs are used to augment milk production by changing the composition of lactose. Further, transgenic sheep are also used for production of wool. Researchers all over the world are putting in serious efforts to generate disease-resistant animals like influenza-resistant pigs.

### 2) Tissue Regeneration

The most common example for use of stem cell is tissue regeneration. Until now, if anyone needs a new kidney for replacing the damaged one, he or she had to wait for a donor before undergoing transplantation. However, if stem cells are available, one can instruct stem cells to differentiate in a certain way to produce a specific type of tissue or organ. For ex- surgeon uses stem cells from just beneath the skin's surface to make new skin tissue. These newly transplanted cells can easily repair or regenerate skin injury or other severe burn.

### 3) Cardiovascular Disease Treatment

In a study conducted in 2013, a team of researchers reported in *PNAS Early Edition* (a scientific journal) that they had created blood vessels in laboratory mice, using human stem cells. It was reported that a network of newly formed blood vessel were established after 2 weeks of transplanting the stem cells. Researchers believed that this technique could be developed to treat people having chronic vascular and cardiovascular diseases.

### 4) Brain Disease Treatment

Researchers are trying to differentiate embryonic stem cells into specific brain cells and tissues for treating specific brain disorders such as Parkinson's and Alzheimer's.

### 5) Cell Deficiency Therapy

Researchers are trying to develop healthy heart cells in a laboratory to treat people having serious cardiovascular disorders. These newly transplanted cells could easily repair heart damage. Similarly, people with type I diabetes could receive pancreatic cells to replace the insulin-producing cells that their own immune systems have lost or destroyed.

### 6) Blood Disease Treatments

Researchers use adult hematopoietic stem cells to treat serious disorders like sickle cell anemia, leukemia and other immunodeficiency problems. Hematopoietic stem cells can undergo self-replication and differentiation to form all type of blood cells such as RBCs which carry oxygen and carbon dioxide in blood and WBCs that fight diseases.

### 7) Research and Scientific Discovery

Stem cells are widely used for research fields related to regeneration, repair mechanism, replication, differentiation, cell signaling, cell division, abnormal cell

division leading to cancer as well as testing drugs on organ derived from stem cells rather than using them on human volunteers.

**8) Stem Cell Therapies**

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Researchers have suggested that stem cell therapies can be used to treat many life-threatening diseases like leukemia. Till date, scientists rely only on gene therapy. The methodology of gene therapy initially developed in 1972 refers to the procedure where a foreign DNA containing a functional gene is introduced into a patient to treat a genetic disorder. In the process, DNA is carefully chosen to rectify the effect of a mutated gene that is causing genetic disease like cystic fibrosis and muscular dystrophy. On the basis of cell type treated, gene therapy is of two types:

- **Somatic Gene Therapy:** It refers to the transfer of a section of functional DNA to any somatic cell of the body that doesn't produce any gamete i.e. either sperm or eggs. Thus, the effects of somatic gene therapy will not be passed onto the patient's offsprings.
- **Germline Gene Therapy:** It refers to the transfer of a section of functional DNA to gametes i.e. either eggs or sperm. Thus, the effects of gene therapy will be passed onto the patient's offspring as well as subsequent generations.

Research suggests that stem cell therapy can help the body to heal on its own. Majority of the cells present in the human body are specific in nature i.e., they are associated with a particular organ to perform a specific function. In case the cells die or malfunction, the body is capable of replenishing lost cells. Illness, organ failure as well as death can occur if the number of diseased and dying cells surpasses production of new cells.

Normal cells can replicate several times. Researchers are refining methods that can begin the production of healthy cell. For instance, normal pancreatic cells transplanted into a patient suffering from diabetes could restore the ability to produce insulin as the cells multiply.

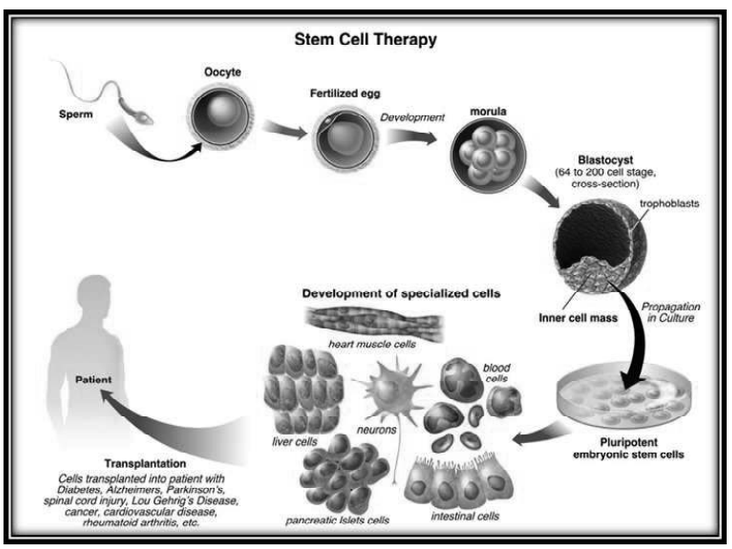


***Fig. 4.26*** *Procedure of Stem Cell Therapy*

**9) Tissue grafts**

- Stems cells can also be used successfully for tissue grafts. Stem cells can be used to repair and replace damaged cells.

**10) Stem cell research**

- Research on stem cells can possibly help the doctors in future to treat several birth defects.
- Research on stem cells helps the doctors to get better idea about the progression of deadly diseases.
- It can bring huge changes in the field of regenerative medicine.
- Stem cells can be possibly used for culturing new organs in labs. It can benefit a lot of people waiting for organ transplant worldwide.
- Stem cell culture can be used for testing the effectiveness of new drugs. For instance: - They can be used for test the drug related to blood disorders.

**Risks associated with stem cell therapy**

- The U.S. Food and Drug Administration warns people to not to be part of any stem cell clinical studies or treatments if it is not approved by FDA.
- Further, it warns people not be excited with claims that stem cell therapies offer a phenomenal cure.
- A lot of adverse reactions are likely to occur by following stem cell therapies that are comparatively untested.
- For instance, in the year 2016, the FDA was informed that a patient went blind after receiving an injection of stem cells for curing an eye problem
- Further, the chances are high that the stem cells injected in to a patient's body might migrate from the site of injection and start behaving or multiplying into an unexpected cell type
- Further, results obtained by carrying in-vitro studies are very different to real population. Hence, stem cells might not follow the same maturation pathway as expected in experimental trials.
- Stem cell therapies can lead to development of tumours.
- Further, the patient's immune system might recognize the stem cells as foreign substance and starts attacking them. Even if the stem cells are derived from the patient's own body, as seen in the case of an autologous transplant, there could be several complications
- The process of manipulating, removing as well as returning stem cells can introduce bacterial contamination and cause illness or abnormalities.

**Ethics of Embryo Research**

The use of human embryos for stem cell research has several ethical concerns like:

- One of the major issues is does life begin at the moment of conception.
- Another concern is that should a blastocyst be considered as human or not

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- Can embryonic stem cell research justified if it may save lives of dying patient

However, a section of intellectuals believe that embryos have rights as they have the potential to develop into a complete human being. However, researchers in favour of embryonic stem cell research suggest that huge number of embryos from fertility clinics are either of poor quality or fails to implant in the uterus and in both the scenarios fails to develop in to a foetus. Hence, they can be used for research purpose for the betterment of society in general. Further, it has been argued that donated embryos were scheduled for destruction even before the donation was made. Induced pluripotent cells can act as research alternatives to Embryonic Cells.

As mentioned above, human Embryonic Stem Cells (hES) are vital to for carrying out stem cell research. Human embryonic stem cells are pluripotent in nature as compared to other cells present in the body. However, using embryonic stem cells for research purposes have a lot of concerns and other ethical issues. Thus, scientist is also working upon the production of iPS, i.e., induced pluripotent cells from the adult stem cells. Further, advances are made in the field of how to use a patient's own stem cells to treat diseases. Substitutes to hES cells may reduce use of human embryonic stem cells in future. Apart from hES, another option is to use *perinatal* stem cells. These cells were first discovered in umbilical cord blood as well as in amniotic fluid which is drawn during an amniocentesis method. However, further research is required in this field to determine how perinatal stem cells could be used in experimental studies and treatment.

**Check Your Progress**

9. How can transgenic mice be used in the context of human diseases?
10. How are stem cells differentiated on the basis of their origin?
11. What are multipotent stem cells?
12. State the most critical step of in vitro cell culture.
13. Define germline gene therapy.

## 4.4 ASSISTED REPRODUCTION TECHNOLOGIES

### 4.4.1 GIFT and ICSI

Assisted Reproduction Technology (ART) issued to treat infertility. It includes fertility treatments that handle both a woman's egg and a man's sperm. In the following section we will discuss its types.

GIFT is a procedure initially developed by Dr. Ricardo Asch in the 1980s. In this technique the physician extracts the ova and sperm with the same procedures as in IVF, but the ova are not fertilized in vitro (outside of the body). Instead, the

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collected ova and sperm are not mixed until the physician inserts each into the fallopian tubes where they come into contact and allow fertilization, (Refer Figure 4.27). The likelihood of successful fertilization leading to a pregnancy is lower with GIFT than with IVF or ZIFT, but some patients prefer this method because it costs less and is considered a more 'natural' method since fertilization occurs inside the body as it would normally. Thus in GIFT, the sperms and eggs are placed in fallopian tube to allow fertilization in natural site. So that woman must have at least one normal open fallopian tube.

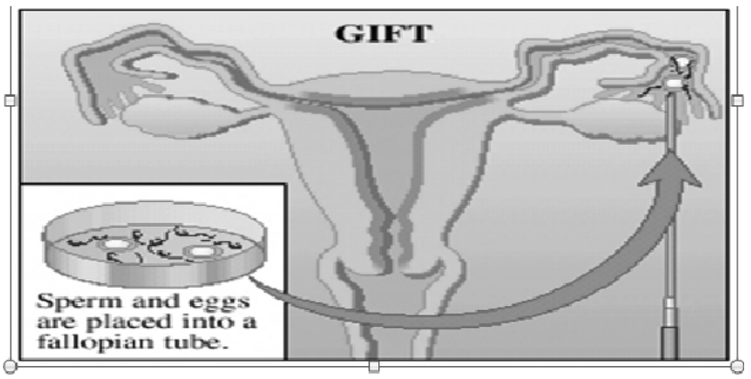


***Fig. 4.27*** *Showing Gamete Intra Fallopian Transfer (GIFT)*

Gamete Intra Fallopian Transfer (GIFT) has emerged as one of the major Assisted Reproductive Technologies (ART). It began in 1979 with a case report in which clomiphene citrate was given to a woman on cycle days 5–9 and artificial insemination performed on cycle day 12. A laparotomy was performed the following morning to reanastomose the ligated fallopian tubes. Six follicles were aspirated and the follicular fluid divided equally and transferred into each reopened tube. The first successful transfer of both sperm and oocytes was reported in 1983 in six patients with a history of Pelvic Inflammatory Disease (PID). As in the past, it still requires less laboratory equipment and less complexity than IVF and therefore remains an important procedure for a specific category of patients.

GIFT was developed out of a desire to place gametes directly into their natural physiologic environment in order to enhance the potential for fertilization. It is not a procedure that can be used for all patients because at least one patent fallopian tube is required and severe oligospermia is a relative condition. In general, success rates for IVF and GIFT are comparable. Because GIFT requires general anesthesia and a laparoscopy in most instances, most centers prefer to focus the majority of their cases on IVF to reduce operative risk, time and recovery, and to verify fertilization.

As a result of these considerations, GIFT is now used only in niche situations, and it is likely that, over time, GIFT will become an even smaller percentage of ART. Nevertheless, GIFT will continue to be an important option for those individuals who either for personal or religious reasons are opposed to IVF and for those centers that cannot afford or do not have the laboratory equipment, space and technical expertise needed to perform IVF. Various techniques constitute

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assisted reproduction, one of which is Gamete Intra Fallopian Transfer (GIFT). The first example of GIFT involved primates during the 1970s, however, the technology was unsuccessful until 1984 when an effective GIFT method was invented by Dr R. Asch at the University of Texas Health Sciences Center and the procedure resulted in the first human pregnancy. The GIFT technique was created in hopes of generating an artificial insemination process that mimicked the physiological sequences of normal conception. The technique was further advanced at the Center for Reproductive Health at the University of California, Irvine, when Asch and his associate Jose Balmaceda employed a newly developed catheter into the GIFT procedure that eliminated the need for general anesthesia in the later stages of the procedure.

On average, the GIFT cycle takes four to six weeks before fertilization occurs. Women undergoing GIFT begin the procedure with hormonal treatments similar to patients undergoing In-Vitro Fertilization (IVF). The hormonal treatments are administered in order to promote the development of oocytes, which are the precursor to eggs stored in the ovaries. For the highest chance of success with GIFT, fully mature eggs are essential. The male's sperm is also manipulated in order to promote sperm capacitation so it is primed to fertilize the egg. Once the sperm is capacitated, 100,000 to 500,000 motile sperm are utilized in the GIFT procedure. For the highest chance of success with GIFT, an estimated 1.5 million sperm should be motile with at least thirty percent having normal morphology.

The GIFT process begins by obtaining the father's semen two hours before the mother undergoes a laparoscopic procedure to harvest her eggs. A small incision is made near the woman's navel and her eggs are harvested with the use of a fiber-optic viewing device known as a laparoscope. Once the sperm and eggs are collected from both parents, they are immediately placed in the woman's fallopian tubes through a catheter. The catheter contents are separated by air to prevent fertilization prior to the transfer. Depending on the patient's age and the maturity of the oocytes, two to five oocytes are transferred into the fallopian tubes along with the sperm. The transfer of multiple oocyctes carries the possibility of multiple pregnancies, which occurs in an estimated thirty percent of assisted reproductive pregnancies. After the sperm and oocytes are delivered to the woman's fallopian tubes and consequently mixed, the hope is that the resulting embryo or embryos will divide normally, move down to the uterus to implant, and result in a healthy live birth.

The GIFT procedure is considered to be very similar to the process of normal conception since fertilization occurs within the woman's body. Because the GIFT procedure closely resembles natural or unassisted reproduction, it is one of the few reproductive technologies approved by the Vatican; no decisions are made as to which embryos are implanted or discarded, the embryo itself is not manipulated, and fertilization occurs naturally in vivo rather than artificially in vitro. However, one point of contention with Catholic doctrine results from obtaining sperm through masturbation. Gift is an available treatment for infertility caused certain ovary disorders, endometriosis and cervical problems, but it does not treat women with untreated fallopian tube blockages. GIFT requires at least one healthy

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fallopian tube, whereas treatments such as in vitro fertilization do not. Results of GIFT vary depending on the age of the patients and the quality of the sperm. Women have decreased fertility odds and increased miscarriage risks with increasing age and most successful cases are with women having thirty-five years of age or younger. The GIFT technique is generally more expensive and more invasive than IVF because the former requires surgical procedures. According to the 2004 report from the Center for Disease Control and Prevention on Assisted Reproductive Technology, GIFT is the least selected technique with only one percent of 94,242 couples undergoing the procedure. Of the one percent of couples undergoing a GIFT procedure, twenty-three percent result in a live birth.

Although GIFT is seldom chosen among the different assisted reproduction techniques, it remains an option for treating infertility. GIFT is one artificial insemination technique that is accepted by the Vatican, making this technology an appropriate choice for patients abiding by certain religious doctrines. Drawbacks of GIFT are that there is no diagnostic test to determine whether fertilization has occurred and there is an increased chance of having an ectopic pregnancy. Although GIFT is generally more invasive than traditional IVF; it constitutes one of many choices in pursuing assisted reproductive technology.

### Zygote Intra Fallopian Transfer (ZIFT)

ZIFT is a combination of IVF and GIFT. The sperm and ova are extracted with the same procedures as IVF and GIFT, and the ova are fertilized outside of the body as with IVF. ICSI may also be used in conjunction with ZIFT, (Refer Figure 4.28). During a ZIFT procedure, the developing embryo is placed in the fallopian tubes at the zygote stage (in contrast to IVF where the developing embryo is placed in the uterus later in its development, at the blastocyst stage). Since the developing embryo is placed in the woman's body much sooner with ZIFT, it is also considered more 'natural' than IVF.

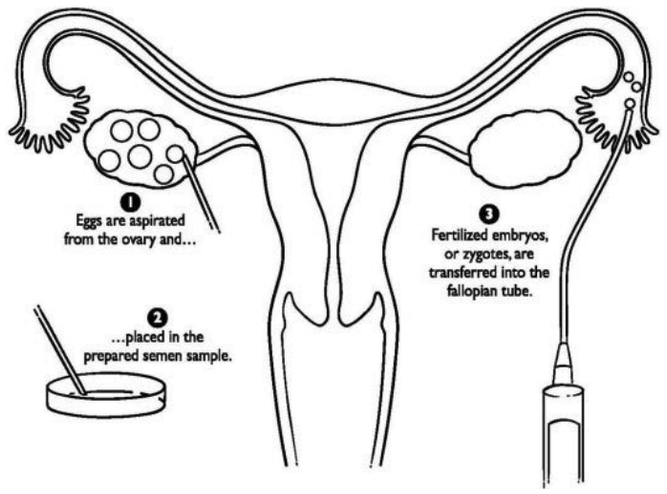


***Fig. 4.28*** *Showing Zygote Intra Fallopian Transfer (ZIFT)*

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Zygote Intra Fallopian Transfer (ZIFT) is an Assisted Reproductive Technology (ART) first used in 1986 to help those who are infertile to conceive a child. ZIFT is a hybrid technique derived from a combination of In-Vitro Fertilization (IVF) and Gamete Intra Fallopian Transfer (GIFT) procedures. Despite a relatively high success rate close to that of IVF, it is not as common as its parent procedures due to its costs and more invasive techniques. Some patients prefer ZIFT, however, considering it more natural because the fertilized oocyte, the zygote, is placed in the woman's body for implantation much sooner than with IVF. To be a suitable candidate for ZIFT, a woman must have at least one healthy fallopian tube where the physician can implant the zygote. The entire ZIFT process takes approximately four weeks, including the period when the patient must first undergo hormone treatment called superovulation. With superovulation, the physician administers fertility medications such as Clomid to stimulate the ovaries to produce several mature eggs, or ova. Clomid will increase the amount of follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) in the female that are required for oocyte maturation. If Clomid is not enough to stimulate oocyte maturation, the physician can also inject the patient with additional FSH and LH intravenously to supplement the oral Clomid medication for a more aggressive hormone therapy.

Once the hormone treatment helps produce several mature ova, the physician extracts the ova through a non-invasive procedure called transvaginal oocyte retreival, the same technique used with IVF and GIFT. For transvaginal oocyte retreival, a thin needle guided by sonogram is inserted through the vaginal wall and enters the ovaries to extract several mature ova. Then shortly before implantation the physician obtains sperm from the male either by masturbation, by using a collection condom or with surgical methods if there is an obstruction preventing the normal ejaculation of sperm.

Once the sperm and ooctyes are prepared, the physician allows the sperm to fertilize the oocyte in a petridish either naturally or manually with a procedure called Intra Cytoplasmic Sperm Injection (ICSI). If there is a male fertility problem such as low sperm count, a high concentration of misshapen sperm, or low sperm motility; ICSI is a good procedure to be used in conjunction with ZIFT. With ICSI, the sperm is injected directly into the egg in the petri dish to increase the chances of fertilization. Studies are conflicted on whether ICSI will increase the chances of birth defects in newborns, but any increased chance is too low to determine accurately.

After fertilization, in vitro the physician monitors the fertilized oocyte (s) for approximately twenty-four hours until cell division begins. With ZIFT, the physician then implants the zygote into the fallopian tube. This differs from IVF, where the physician waits until the fertilized egg has divided into eight cells before implanting it into the uterus. The location where the physician implants the developing embryo depends on the stage of the embryo's development and thus models the path that the developing embryo would follow after natural conception.

During ZIFT, the physician places one to four zygotes in the fallopian tubes through a surgical technique called laparoscopy, an invasive procedure utilizing a small abdominal incision unlike IVF, where the physician places the 8-cell embryo

in the uterus by entering through the cervix. The zygote then travels down the fallopian tube and may implant on the uterine wall. In a healthy young woman, there is approximately a 32–36% chance that the fertilized egg will implant in the uterine wall and result in pregnancy. The number of zygotes the physician places in the fallopian tube will depend on the patient's age as well as her preference. The greater the woman's age, the more difficult it becomes for pregnancy to occur, thus physicians may insert more zygotes to increase the chance of a successful implantation and resulting live birth.

As with GIFT, there is a greater chance of an ectopic pregnancy (the fertilized egg implants anywhere other than inside the uterus) when using ZIFT. Although the probability of pregnancy with ZIFT is close to that of IVF, it makes up only 1% or less of all ART currently used in the United States. The invasive surgery of laparoscopy and the relatively higher costs have made ZIFT less appealing than IVF, which due to its popularity has attracted more research and resulted in higher success rates for both fertilizations and live births. ZIFT, like GIFT, followed the development of IVF as another technique to help achieve pregnancy. Currently, every year in the United States approximately 250–280 babies are born as a result of ZIFT compared to 40,000 babies born from all assisted reproductive technologies.

### Advantages of ZIFT

Following are the advantages of ZIFT:

- Confirmation of fertilization and selection of only normally fertilized zygotes for transfer.
- Embryo cleavage and development occur in the natural and physiological environment of the fallopian tube
- Better synchronization between embryonic and endometrial development
- Avoidance of suboptimal in vitro culture systems
- Prevention of zonal hardening, especially in couples with advanced female partner age
- Prevention of microtrauma to the endometrium by uterine transfer catheters
- Prevention of embryo expulsion following UET induced by Sub endometrial myometrial contractions
- Prevention of the detrimental effects of cervical microorganisms associated with UET
- Important diagnostic information provided by laparoscopy

### Disadvantages of ZIFT

Following are the disadvantages of ZIFT:

- Risks and complications inherent with laparoscopy
- Increased cost compared with uterine embryo transfer
- Longer hospital stay compared with uterine embryo transfer

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- Lack of the ability to select the morphologically best-cleaving embryos compared with uterine embryo or blastocyst transfer.

ZIFT normally requires general anesthesia and endotracheal intubation. Intrafallopian transfer with local anesthesia and continuous sedation has also been described. ZIFT is performed 18–48 hours after oocyte aspiration using a three-puncture video laparoscopic Technique. During the ZIFT procedure, pronuclear embryos are normally selected for transfer based only on the visualization of two pronuclei 18–24 hours after egg retrieval and insemination.

### Intra Cytoplasmic Sperm Injection (ICSI)

One kind of assisted reproductive technology is ICSI that is helping numerous individuals to get rid off from male infertility. Spermatozoa sometimes fail to fertilize even when they are artificially placed in close proximity to eggs during conventional In-Vitro Fertilization (IVF). Fertilization failure in IVF is particularly common where there are grossly abnormal semen parameters or when the number of spermatozoa is insufficient. The ability of ICSI to achieve higher fertilization and pregnancy rates regardless of sperm characteristics makes it the most powerful micro manipulation procedures to treat male factor infertility. In fact, the therapeutic possibilities of ICSI go from cases in which, after sperm selection, the spermatozoa show poor progressive motility, to its application to azoospermicmen where spermatozoa are micro-surgically retrieved from the epididymis and the testis.

Injection of single mature immobilized normal spermatozoa into the cytoplasm of a mature metaphase II oocyte is known as Intra Cystoplasmic Sperm Injection (ICSI). Since the introduction of ICSI, it has revolutionized the treatment of male factor infertility and excellent pregnancy and implantation rates are achieved in couples for whom there were no treatment option except donation or adoption. ICSI was first used successfully in patients whose oocytes failed to become fertilized after insemination with motile spermatozoa, (Refer Figure 4.29). Then, it became evident that ICSI might equally be well applied in couples with too few spermatozoa for conventional IVF. Finally researchers tried for azoospermic men by injecting sperm, which obtained from epididymis (Obstructive Azoospermia, OA) and testes (Non Obstructive Azoospermia, NOA). It was also successful in terms of normal fertilization, embryo development and implantation rates as well as birth of healthy offspring 10-12. Before 1992, the majority of severe male factor infertility was virtually untreatable. Due to establishment of ICSI as a routine it is now possible to treat the whole spectrum of male infertility from such optimal ejaculate samples or ejaculatory failure to obstructive and non-obstructive azoospermia.

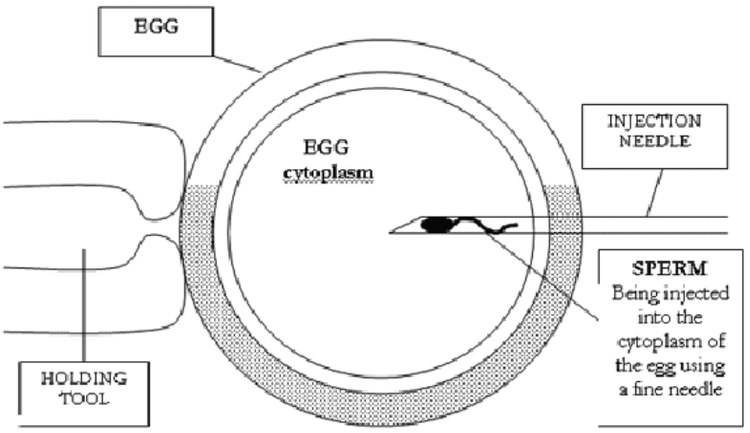


***Fig. 4.29*** *Showing Intra Cytoplasmic Sperm Injection (ICSI)*

## Significance of ICSI

It is used in case of:

- Couples who have suffered from recurrent failure of fertilization due to disorder at functional level of gametes, barrier at the level of acrosome reaction, zona binding or interaction, zona penetration or fusion with oolema. In ICSI all these steps are bypassed and only requirement is the decondensation of spermatozoa inside the oocyte. Severe oligospermia where sperm count is less than 5million/ml., severe oligospermia and testicular failure. Oligopsermia due to hypogonadotropic hypogonadism, environmental factors, drugs or due to any disease can be corrected by behavioural changes and specific treatment. Otherwise repeated low sperm count with high FSH and without any specific reason (idiopathic) or Y chromosomal micro deletion are the candidates for ICSI. Severe asthenospermia including patients with ultra-structural abnormalities such as Kartagener's syndrome.
- Teratospermia where >70% sperms are morphologically abnormal.
- Obstructive azoospermia due to congenital absence of Vas deference, vasectomy or post inflammatory obstruction of the vas deference. Sperm can be retrieved by Per Epididymal Sperm Aspiration (PESA), Testicular Sperm Aspiration (TESA) or Testicular Sperm Extraction (TESE).
- Non-Obstructive Azoospermia. Sperm can be retrieved by TESA, TESE or open biopsy of the testis.
- Ejaculatory dysfunction such as retrograde ejaculation.
- Paraplegeic male if electro ejaculation is not satisfactory, then TESE and ICSI can be done.
- Immunological factors-Anti-sperm antibody in both male and female partner.
- Frozen semen sample in patients having chemotherapy and radiotherapy. Testicular biopsy specimen may also be cryopreserved as backup where quality of ejaculation is inadequate for freezing.

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Thus, ICSI is one of the most successful technique to overcome infertility as it involves injection of mechanically immobilized spermatozoa achieves fertilization at a higher rate than the injection of motile spermatozoa directly inside the woman.

## 4.4.2 Cloning of Animals by Nuclear Transfer

Nuclear cloning, also referred to as nuclear transfer or nuclear transplantation, denotes the introduction of a nucleus from an adult donor cell into an enucleated oocyte to generate a cloned embryo. When transferred to the uterus of a female recipient, this embryo has the potential to grow into an infant that is a clone of the adult donor cell, a process termed 'reproductive cloning'. Nuclear transplantation is a method in which the nucleus of a donor cell is relocated to a target cell that has had its nucleus removed (enucleated). Nuclear transplantation has allowed experimental embryologists to manipulate the development of an organism and to study the potential of the nucleus to direct development. Nuclear transplantation, as it was first called, was later referred to as somatic nuclear transfer or cloning. Yves Delage first wrote about nuclear transplantation in 1895, speculating that if one were to replace an egg nucleus with another egg's nucleus, full development would occur. Later in 1938, H. Spemann suggested an experiment whereby, using technologies not yet available to him, one could remove the nucleus of an egg and replace it with a different nucleus extracted from a developed cell. Thomas King and Robert Briggs were the first to perform experimental nuclear transplantation. The technique was soon after used by J. Gurdon and eventually led to the first clone of a mammal, 'Dolly' the sheep, by I. Wilmut in 1996.

Nearly fifteen years after Spemann wrote about the possibility of nuclear transplantation, Briggs and King, using northern leopard frogs (*Rana pipens*), performed the first nuclear transplantation experiment. They transplanted the nucleus from an early stage embryo to an unfertilized egg that had been enucleated. The egg cell was pricked with a clean glass needle in order to induce a fertilization-like response. The faux activation of fertilization allowed for extraction of the nuclear material inside while also activating the host egg cell. Meanwhile, the nucleus of a donor cell was extracted and then inserted into the newly enucleated and activated egg cell. That process induced development of the host egg according to the instructions of the newly inserted nucleus, resulting in the formation of an organism with the same genetic material as the donor cell, or a clone. Briggs and King continued to examine the potential of differentiated cells throughout the 1950s. They found that if the donor nucleus was extracted later in development, the potential of directing full development in the activated egg cell was greatly reduced. After the Briggs and King experiments it was generally accepted that the nuclear material in developing cells slowly loses its potential for full development.

That view was challenged in 1958 when Gurdon's experiments with African claws frogs (*Xenopus levis*) produced fully developed frogs from the transferred nucleus of cells much later in development. Gurdon allowed the cloned frogs to develop to sexual maturity and was then able to mate two sexually mature clones, suggesting that the donor nuclei were able to fully redirect development. Gurdon's

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experiments were widely accepted by the scientific community but questions remained for several decades. Scientists were concerned about whether the nucleus of the host egg cell was truly enucleated. The question of whether remnants of the host egg cell or the inserted nucleus directed development remained unanswered from 1958 to 2002, despite many attempts by Gurdon to prove it was the inserted nucleus. In 2002, however, K. Hochlelinger and R. Jaenisch published an experiment using nuclear transplantation of mature white blood cells to generate mouse clones. Hochedlinger and Jaenisch were able to show that the inserted nucleus induced development in the host egg cell.

Although experimental embryologists continued to use nuclear transplantation to create clones of several species, Ian Wilmut's cloning experiment in 1996 was a controversial and widely publicized cloning experiment. Dolly was cloned using the nucleus of a mammary gland cell from an adult sheep and transplanting it into an enucleated egg cell from another sheep. The activated egg cell was then transferred into a third surrogate sheep that carried Dolly to term. Dolly died at the age of six due to lung disease and severe arthritis, and although her death was not attributed to the fact that she was a clone, many believe that the relationship between telomeres and ageing was the reason for her demise. Nuclear transplantation may have begun as a subtle idea in the late 19th and early 20th centuries, but it evolved into a feasible and widely used process by experimental embryologists in the late 1990s. The cloning of Dolly the sheep worried many about the possibility of human cloning and the moral boundaries of modern advances in science. In the context of the embryonic stem cell discourse of the late 1990s and early twenty-first century, somatic nuclear transfer has been contrived into moral arguments about rights of the human embryo. Furthermore, nuclear transplantation has spurred ethical discussion on the value of a human life during all stages of development. Many scientists have abandoned the methods involved in nuclear transplantation and have adopted methods set forth by S. Yamanaka in his experiments involving induced pluripotent stem cells.

### 4.4.3 Embryo Cloning

A clone depicts a population of cells or organisms derived from a single ancestor cell. They are genetically identical to each other as well as to their common ancestor. Cloning usually refers to the means of the production of identical copies of an individual or we can say that it refers to the production of exact genetic replica copies of an individual. Thus, it is highly advantageous in increasing the number of embryos from a specific desirable embryo having some superior quality genes. However, clones cannot be considered as an offspring but just the copy of a given individual. Much work has been done lately on cloning in plants as well as microorganisms. However, the techniques used in plants cannot be applied for animals. Moreover, numerous animals from a single genetically superior embryo can be produced by the process of cloning. Still there is no mode of finding out which embryos are capable of cloning. It is useless to clone an embryo if it is not superior. Two approaches are in use for embryo cloning.

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## (1) Nuclear Transplantation or Transfer

Nuclear transplantation (also known as nuclear transfer) involves the removal of a single blastomere from a cleavage stage embryo with the help of a fine micropipette of made up of glass, and then placing it under the outer membrane of an unfertilized mature enucleated oocyte (whose haploid nucleus has been removed by using micropipette or destroyed by UV light). Robert Briggs and Tom King at Cancer Research Institute, Philadelphia (USA), for the first time in the year 1955, carried out the nuclear transplantation experiment on embryonic cells of frog. They transferred nucleus of undifferentiated blastula (a stage soon after fertilization of egg) into an enucleated egg cell. Surprisingly, they observed the normal development of the embryo. However, when they carried out serial transplantation of differentiated nucleus from late gastrula (a stage which comes after blastula and is formed by the morphogenetic movement of cells) into a nucleus-free unfertilized egg, abnormal embryos were formed. This shows that cell nucleus is differentiated with embryo development. In 1960s, J.B. Gurdon at Oxford University, U.K. transferred differentiated intestinal nucleus of a frog into nucleus-free unfertilized egg of different amphibian species (Xenopus laevis). The embryo developed into tadpole and matured into frog (Gurdon, 1962). This new enucleated cell developed into normal embryo. Any damage to the donor nucleus during transplantation leads to abnormal development.

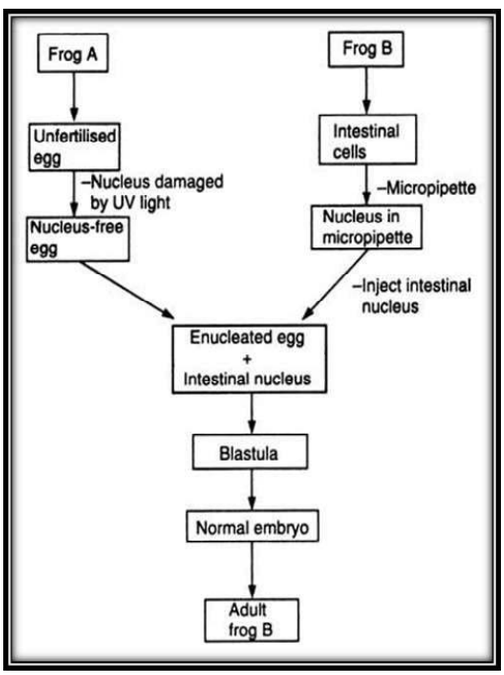


***Fig. 4.30*** *Nuclear Transplant in Frog Details of the Experiment are Mentioned Above*

Further, Dolly the sheep was created by SCNT (Somatic cell nuclear technology). The details of technique are discussed below:

- Firstly, the nucleus of a somatic (body) cell is transferred to the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed)

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- Secondly, the somatic nucleus present inside the egg, is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg)
- Thirdly, zygote is stimulated to undergo division divide by an electric shock
- Lastly, when the developing zygote forms a blastocyst, it is implanted via ETT (embryo transfer technology) in the womb of a surrogate mother

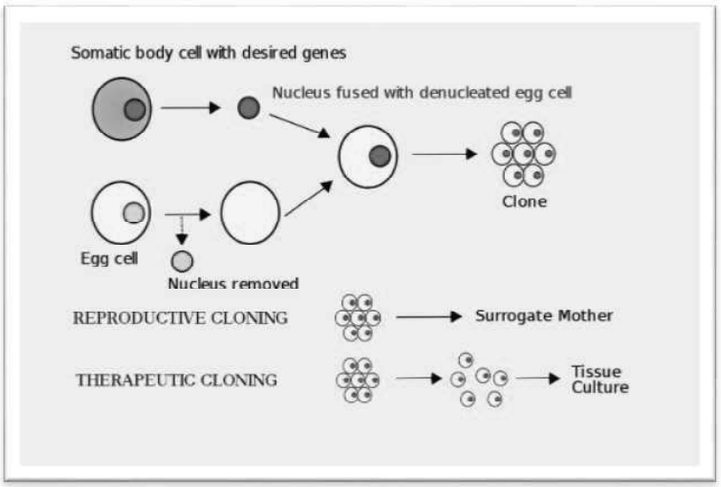


***Fig. 4.31*** *Cloning Process*

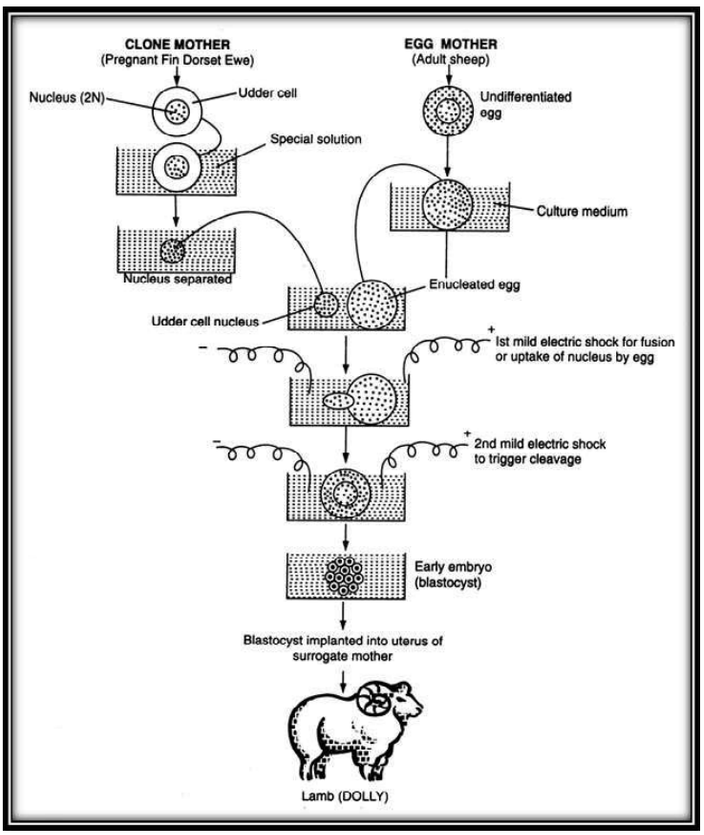


***Fig. 4.32*** *Cloning of 'Dolly' Sheep.*

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Dolly, born on 5th July, 1996, at the Roslin Institute in Edinburgh, Scotland, was a female Finn Dorset sheep. It was the first mammal cloned from an adult somatic cell. As the Dolly's DNA came from a mammary gland cell, she was named after the country singer Dolly Parton. 'Dolly' was created using the technique of somatic cell nuclear transfer (SCNT).

The steps involved in the production of Dolly are:

1) Dolly had three mothers namely:
   - First mother provided the egg
   - Second mother provided the DNA
   - Third mother (surrogate mother) carried the cloned embryo to term.
2) Scottish Blackface ewes were treated with gonadotropin-releasing hormone (GnRH) to cause them to produce oocytes ready to be fertilized.
3) Plunge a micropipette into the egg over the polar body to remove the polar body as well as the haploid pronucleus within the egg.
4) Fuse each enucleated egg with a diploid cell growing in culture.
5) Somatic cells from the mammary gland of an adult Finn Dorset ewe were grown in tissue culture.
6) Donor cells and enucleated recipient cells were placed together in tissue culture.
7) The cultures are then exposed to brief pulses of electricity in order to stimulate their respective plasma membranes to fuse and begin the process of mitosis i.e. cell division.
8) Zygotes were then developed into tissue culture media until they grows into a blastcyst
9) Numerous blastocysts were transferred into the uterus of Scottish Blackface ewes for implantation.
10) One ewe gave birth to Dolly after 148 days

    The probability of Dolly's birth was:
    - 277 diploid somatic cells were fused with 277 enucleated unfertilized eggs
    - 29 viable reconstructed eggs survived and were implanted in surrogate Blackface ewes.
    - 1 gave birth to Dolly.

    Major events of Dolly's life:
    - Dolly lived her entire life at the Roslin Institute in Midlothian.
    - 'Dolly' was bred with a 'Welsh Mountain ram'
    - She produced six lambs
    - Bonnie (born in April 1998) was her first lamb.
    - Subsequently, she produced twin lambs named Sally and Rosie

- In the year 2000, she gave birth to triplets- Lucy, Darcy and Cotton.
- On 14 February 2003, Dolly was euthanised as she had developed a progressive lung cancer called ovine pulmonary adenocarcinoma as well as severe arthritis.
- Dolly lived for 6.5 years

As seen from the above data, that even though, behind this great success the rate of success is very slow, yet it has given some hope to embryo-biotechnologists to bring about refinement. Out of 277 nuclei transferred singly to enucleated egg, only 29 eggs grew into embryos. Out of these, only 13 embryo could be successfully transplanted into surrogate mothers. Of these only one ewe was successful in giving birth to an offspring, Dolly (Wilmut et al, 1996)

The significant conclusions that can be derived from the cloning experiment are as follows:

- Firstly, the genes of differentiated cells have inherent property of totipotency.
- The interaction between the regulatory system of the genome present inside the nucleus as well as the cytoplasmic factors of egg may make a cell totipotent in nature.
- Perhaps it is the cytoplasm of enucleated egg which makes the transplanted nucleus totipotent like that of normal fertilized egg nucleus.
- The egg cytoplasm contains maternally derived information which plays a huge role in the process of cleavage- a phenomenon which usually occurs after fertilization.
- Therefore, it is the egg cytoplasm but not the nucleus which regulates the process of cleavage.
- As the udder cell nucleus is having only limited potential for mitosis; it is the egg cytoplasm that interacted intracellularly with udder cell nucleus and stimulated to undergo repeated mitosis.
- Cloning of the adult sheep is possible even without involving sperms from male partner.
- The cloned animal which is produced via the process of nuclear transplantation technique will be capable of restoring fertility as in 1998 Dolly gave birth to a little lamb named Bonny.

**2) Embryonic Stem (ES) cells are used for the production of clones such as that of mice**

Cloning of mice could not be done as in sheep via the process of nuclear transplantation. The reason being accelerated development programme of the embryo. However, it is obvious that even before first embryonic division the cell has started its process of differentiation. Consequently, for the purpose of cloning of mice an alternative approach has been made, i.e., the use of embryonic stem cells. In this approach, a blastocyst of mouse is place in culture condition. The

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Inner Cell Mass (ICM) that form future foetus will continue to divide and remain in undifferentiated totipotent state as embryonic stem cells only. There is a peptide growth factor referred to as the leukaemia inhibitory factor (LIF) which helps in the establishment as well as maintenance of ES cell lines. As explained in the previous section, the ES cell lines will be very useful in the area of production of transgenic animals. However, the ES cells are used in two different ways: a small number of ES cells can be injected into blastocoel space of a blastocyst. The ES cells get mixed with inner mass of cells of blastocyst to produce a chimera mouse which is a mixture of two cell genotype having the patches of different coloured fur. Crossing of male and female chimera will allow selection of homozygous mice derived from ES cells.

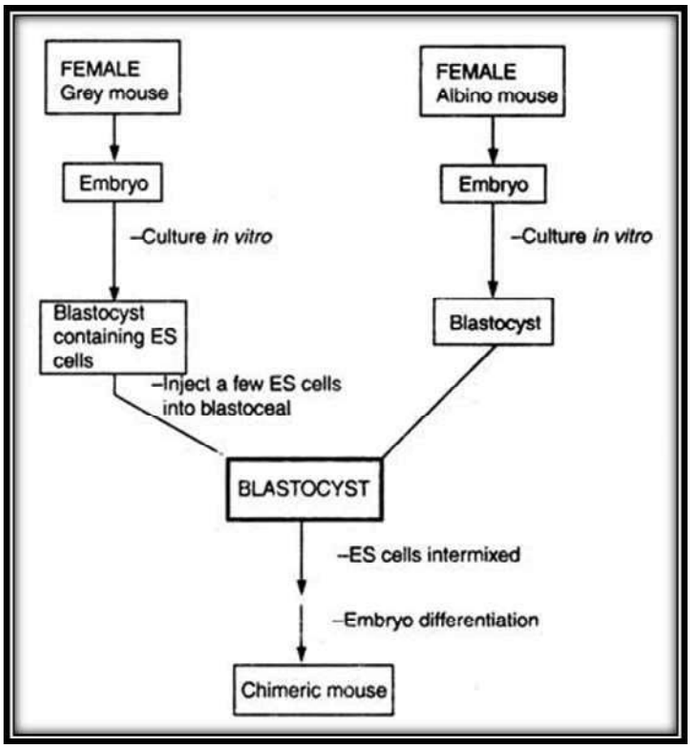


***Fig. 4.33*** *Production of Chimeric Mouse by Embryonic Stem Cell Transplantation*

## Applications of Cloning

The applications of cloning are:

1. Cloning has bio-medical applications, like in the production of pharmaceuticals in the blood or milk of transgenic cattle.
2. Cloning may also be useful in the production of research models.
3. Uses in agriculture comprises of several applications of the cloning technology which include:
    a) Making genetic copies of elite seed stock as well as prize winning show cattle.

b) Other purposes ranges from "insurance" to making copies of cattle that have little sentimental value, similar to cloning of pets.

c) Increased selection opportunities available with cloning may provide for improvement in genetic gain.

d) The primary goal of the cloning has usually been projected as a system for producing quantity as well as uniformity of the perfect dairy cow. However, this is possible only if heritability were 100%, thus, clone mates would have complete uniformity. Changes in the environment may have huge influence on the productivity as well as the longevity of the resulting clones. Changes in consumer preferences as well as economic input costs may all change the definition of the perfect cow. The cost of producing such superior quality animals via the process of cloning must be economically achievable to meet the proposed applications.

**Limitations of the Cloning Technology**

The limitations involve:

1 Present inadequacy limit the cloning opportunities to only highly valued animals. hence, improvements are must to move the technology toward the commercial application.

2 Cloning technology has additional difficulties to overcome. Social as well as other regulatory acceptance of cloning is chief to its utilization in the production of agricultural products. Regulatory acceptance will need to address the animal, its products, as well as its offspring.

3 Loss of pregnancies due to developmental abnormalities is another major concern of cloning technology. These developmental abnormalities appear to be due the presence of faulty epigenetic reprogramming as well as gene expression in the genes of different cells.

## 4.4.4 Embryo Sexing

Embryo biopsy involves the removal of a few cells from the embryo (mostly from the trophoblastic cells of the trophectoderm) for analysis to determine its sex a technique known as embryo sexing. In recent years, the technique of embryo sexing has become popular for the detection of genetic disorders. By using embryo biopsy, it is possible to stop the transfer of embryos with genetic abnormalities and undesirable traits. Embryo sexing is essential before the implantation of embryo. The principle for embryo sexing is very common. It is based on the principle of presence or absence of Y chromosome. The presence of Y chromosome denotes that the embryo is a male on the contrary absence of Y chromosome makes the embryo female. In recent years, the presence DNA sequences specific to Y chromosome are being detected for embryo sexing. Another development is the use of polymerase chain reaction to amplify the DNA sequence (of Y chromosome) even from a single cell and determination the sex. Handy side et al. (1989) isolated single blastomere from early embryo from a womb, amplified DNA sequences of Y chromosome and carried out embryo sexing before implantation into uterus.

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## Steps in Embryo Sexing

The steps of embryo sexing are as follows:

**1) Collection of Embryonic Cells**

- Collection of embryos produced either in vitro or in vivo.
- Selection of grade one or grade two embryos.
- The embryo thus obtained are washed thoroughly with PBS & placed in a drop containing 200 mM sucrose under micromanipulator.
- Zona pellucida is cut open with the help of fine micro blade.
- Few blastomere are sucked with the help of fine aspiration pipette.
- These blastomeres are then washed in KCl & transferred to Eppendorf tube.

**3) Isolation of Embryonic DNA**

This step involves the isolation of DNA from the embryonic cells. The steps of DNA isolation are as follows:

- Biopsy in 0.5 ml Eppendorf tube
- Add Proteinase-K
- Add 9μ of lysis buffer.
- Overlaid with 25μ of mineral oil
- The entire solution is incubated at a temperature of 37°C for 10- 60 minute
- Inactivation of proteinase-K at 98°C for 10 min.
- The solution is cooled at a temperature of 4°C

**4) Amplification of DNA**

Next step of the process is to amplify the isolated DNA. Amplification of the DNA is carried out by using PCR machine. The process involves amplifying the target region i.e., 'DNA barcode' region via Polymerase chain reaction 'PCR'. The methodology of 'PCR' is as follows:

The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis.

This method is used in molecular biology to make millions of copies of (amplify) short sections of DNA or a gene.

Five core 'ingredients' are required to set up a PCR:- the DNA template to be copied, primers that initiate the PCR reaction, DNA bases (A, C, G and T), Taq polymerase enzyme to insert in the new DNA bases, DNA buffer to make sure the optimum conditions for the PCR reaction.

PCR involves a repeated process of heating and cooling referred to as 'thermal cycling' which is carried out by PCR machine.

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There are three main stages of a PCR reaction:

- **Denaturing:** This is first step of the process in which double-stranded template DNA is heated to 94-95p C which ensures the breaking of hydrogen bonds between the two strands and thus separating it into two single strands. This step usually takes between 15-30 seconds.
- **Annealing:** During this stage, temperature is lowered to 50-65p C to enable the DNA primers (short sequences of DNA 20 to 30 base in length) to attach to the template DNA. Cooling down help the primers to attach to a specific location on the single-stranded template DNA by way of hydrogen bonding. This step usually takes between 10 to 30 seconds.
- **Extending:** During this final step, the heat is increased to a temperature of 72p C to facilitate the new DNA to be made by a special Taq DNA polymerase enzyme which keeps on adding new DNA bases.

All the above mentioned three steps are repeated 20-40 times, thus doubling the number of DNA copies every time. It usually takes a few hours to complete the entire PCR reaction. The entire three step PCR reaction is summarized below:

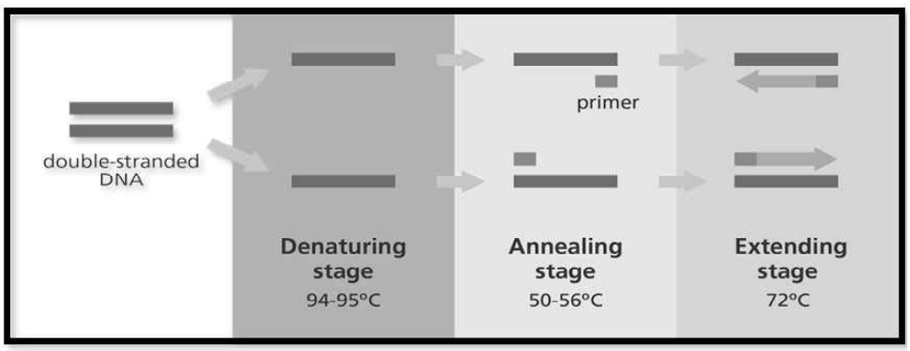


***Fig. 4.34*** *PCR reaction*

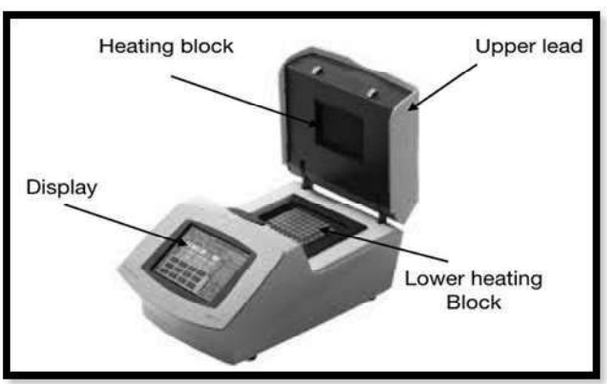


***Fig. 4.35*** *Parts of a PCR machine*

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The details of the PCR reaction for embryonic DNA are as follows:

- 15µ of PCR reaction mixture (PCR reaction buffer, primers, 1.5µ of Taq DNA polymerase & 125µ of Ethidium bromide) is added to the tube
- Subjected to PCR cycling
- Three minute denaturation at 94°C followed by 10 cycles of denaturation at 92°C. This is followed by annealing at 50°C (80 seconds). Annealing is followed by Extension at 72°C for 20 seconds. Further, 40 cycles at 60°C of annealing temperature. Final extension achieved by 5 min. incubation at 72°C

### 4) Identification of Sex

After the PCR reaction has been completed, another methodology referred to as electrophoresis is used to check the size as well as quantity of amplified product.

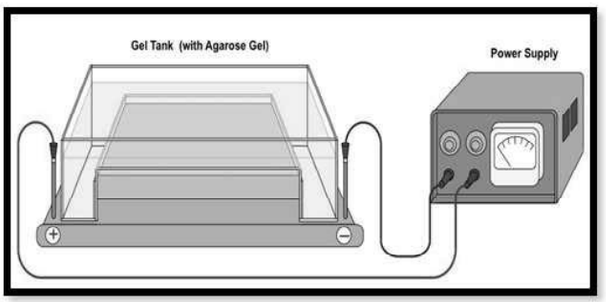


***Fig. 4.37*** *Agarose gel electrophoresis apparatus*

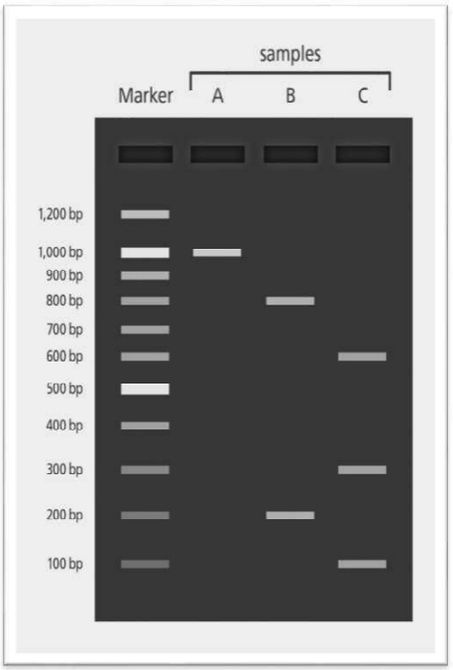


***Fig. 4.37*** *Agarose Gel having DNA Markers of different Sizes and DNA Samples Marked as A, B and C*

After electrophoresis, sex identification of embryo can be done in two ways:

- **Electrophoretic Method:** In PCR, second pair of primer added to increase accuracy. After electrophoresis Y-specific bands are observed Autosomal primer commonly used is C1C2
- **Direct observation under UV Light:** Tubes having male DNA show bright pink fluorescence.

## 4.4.5 Screening for Genetic Disorders

Genetic screening determines whether a couple is at increased risk of having a baby with a hereditary genetic disorder. Hereditary genetic disorders are basically the disorders of chromosomes or genes that are passed down from one generation to other generation. Genetic screening involves the assessment of couple's family history and, if required, assessment of blood or tissue samples (like that of cells from the inside of the cheek). Genetic testing involves examining the DNA, the chemical database that carries instructions for body's functions. Genetic testing can reveal changes (mutations) in the genes that may cause illness or disease. Any couple who are going to have a baby, can go for genetic screening, however, screening is particularly recommended when:

- Either one or both the partners are aware that they do have some genetic issue or abnormality
- When the couple are aware of their family tree and suspects a genetic disorder which keep on passing from generation to generation.
- When the partners belong to a high-risk ethnic group.

However, a few genetic disorders are not hereditary and hence cannot be identified by screening the parents. To determine whether a couple going for genetic screening has an increased risk of having a baby with a genetic disorder, doctors or a medical practitioner ask the couple about the following:

- Genetic disorders that runs among the family members
- The cause of death in any family members
- The health status of all living first-degree relatives (i.e., parents, siblings, and children) as well as that of second-degree relatives (aunts, uncles, and grandparents)
- History of miscarriages, stillborn babies, or babies who have died soon after birth in the couple or in family members
- Presence of babies with birth defects in the family
- Aware of any Intermarriages among relatives (which further increases the risk of having the same abnormal gene especially the disorders which are caused by recessive genes
- Ethnic background (specific groups are at higher risk of certain disorders)

Information regarding three generations is generally required. If the family history is complex, information related to more distant relatives may be required. Sometimes to be assure, doctors also review the medical records of relatives who

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may have had a genetic disorder. Further carrier screening is also required to pinpoint a genetic disorder. Carriers are people who have an abnormal gene for a disorder however, they do not show any visible symptoms or evidence of the disorder. Usually, it has been observed that in carriers, the abnormal gene is recessive in nature—that is, two copies of the gene are required to develop the disorder. Such carriers individuals comprises of one normal gene and one abnormal gene for the disorder. However, it should be noted that only women can carry an X-linked (sex-linked) recessive gene as they do have two X chromosomes. Thus, on the other X chromosome, the corresponding gene may be normal and protect women from developing the disorder. As men contains only one X chromosome, all men who have an abnormal X-linked recessive gene have the disorder that the abnormal gene causes. The process of carrier screening involves testing people who does not exhibit any symptoms yet are at a higher risk of carrying a recessive gene for a specific genetic disorder. Risk is usually higher either one or both the partners have a family history of certain disorders or have characteristics (like as ethnic background or racial or geographic group) that increase the risk of having certain disorders. However, carrier screening is usually done only if the following criteria are also met:

- The disorder caused has to be very debilitating or lethal.
- Only, if the reliable test is available for carrying out the screening.
- The foetus can be treated, or reproductive options (such as abortion or elective sterilization) are available and acceptable to the parents.

Genetic disorders like sickle cell anaemia, the thalassemia, Tay-Sachs disease, and cystic fibrosis falls under this category. The number of hereditary disorders that can be screened is progressively increasing, and some laboratories offer carrier screening for dozens of disorders (called expanded carrier screening). People should meet with a genetics professional before deciding whether to pursue expanded carrier screening.

Carrier screening generally comprises of analyzing the DNA from a blood sample. However, occasionally, cheek swab i.e., a sample of cells obtained from the inside of the cheek can also be analysed. The patient has been asked by the doctor to swirl a specific fluid in their mouth, then spit it into a specimen container, or also by rubbing a cotton swab inside their cheek. Generally, it is advantageous to do carrier screening before a woman becomes pregnant. However, if it is done after the female gets pregnant and if the screening indicates that both partners carry a recessive gene for the same type of disorder, then they are suggested to have prenatal diagnostic testing. In this case, the foetus may be tested for the disorder even before birth. If the foetus has the disorder, treatment of the foetus may be possible, or termination of the pregnancy may be considered.

### Types of genetic testing

Genetic testing plays an essential role in assessing the risk of developing certain disorders as well as screening and sometimes medical treatment. Different types of genetic testing can be done for different reasons:

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- **Diagnostic Testing:** If the patient shows some kinds of symptoms of a disease that may be caused by genetic changes, generally known as mutated genes, genetic testing can disclose if the patient is having that specific disorder or not. For instance, genetic testing can be used to confirm a diagnosis of cystic fibrosis or Huntington's disease.
- **Pre-symptomatic and Predictive Testing:** If the patient is aware of his family tree and is having a family history of a specific genetic condition then genetic testing is usually recommended even before the appearance of any symptoms. This helps in assessing whether the concerned individual is at the risk of developing a particular genetic disorder or not. For instance, Pre-symptomatic and predictive testing type of test may be useful for identifying the risk of certain types of colorectal cancer.
- **Carrier Testing:** This is usually carried out only if the concerned person has a history of specific genetic disorder like that of sickle cell anemia or cystic fibrosis — or if the concerned person is in an ethnic group that has a high risk of a specific genetic disorder. An expanded carrier screening test can detect genes which are associated with a broad range of genetic diseases as well as mutations and can recognize if both the partners are carriers for the same genetic conditions.
- **Pharmacogenetics:** Pharmacogenetics can be done if the concerned individual is having a specific health condition or disease. This type of genetic testing helps in determining the kind of medication which is necessary and the amount of dosage that will be most effective and beneficial for the concerned individual.
- **Prenatal Testing:** Prenatal testing is done to detect genetic disorder of the foetus if any. For instance: Down syndrome and trisomy 18 syndromes are two genetic disorders that are usually screened for as part of prenatal genetic testing. Conventionally, this is done looking at markers in blood or by invasive testing like amniocentesis. Newer testing called cell-free DNA testing looks at a baby's DNA via a blood test done on the mother.
- **Newborn Screening:** Newborn screening is one of the most common types of genetic testing. In the United States, all states need that newborns be tested for specific genetic as well as metabolic abnormalities that can cause specific conditions. This type of genetic testing is essential as the results depicts the disorder like congenital hypothyroidism, sickle cell disease or phenylketonuria (PKU), care and treatment can begin right away.
- **Pre-implantation Testing:** This kind of testing is done when someone tries to achieve pregnancy via in-vitro fertilization. This kind of testing is also known as pre-implantation genetic diagnosis. The embryos are screened for genetic abnormalities. Embryos without abnormalities are implanted in the uterus in hopes of achieving pregnancy

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## Risks Associated with Genetic Testing

There is little or almost negligible physical risk is involved in genetic testing. Blood sample and cheek swab tests have almost no risk. However, prenatal testing like amniocentesis or chorionic villus sampling has a small risk associated with the loss of pregnancy loss/ miscarriage. Genetic testing can also have emotional, social as well as financial risks. The concerned person is recommended to discuss all risks as well as benefits of genetic testing with the doctor, a medical geneticist or a genetic counsellor before he/she plans to have a genetic test done.

## Samples Used for Genetic Screening

Depending on the type of test, a sample of blood, skin, amniotic fluid or other tissue will be collected and sent to a lab for genetic analysis.

- **Blood Sample:** A medical practitioner collects the blood sample by inserting a needle into a vein of the concerned person. For newborn screening tests, a blood sample is collected by pricking the baby's heel.
- **Cheek Swab:** For some kind of genetic screening, a swab sample from the inside of the concerned person's cheek is collected.
- **Amniocentesis:** In the process of amniocentesis doctor inserts a thin, hollow needle via the abdominal wall into the uterus of the concerned person to collect a small amount of amniotic fluid for carrying out genetic testing.
- **Chorionic Villus Sampling:** For this prenatal genetic test, the doctor collects a tissue sample from the placenta. Depending on the situation of the concerned individual, the sample may be taken with a tube (catheter) via the cervix or through the abdominal wall and uterus using a thin needle.

## Preparation for Genetic Screening

In case, an individual is planning to go for a genetic screening, he/she must collect maximum information about his/her family's medical history. After this, an individual can discuss his family's medical history with a genetic counsellor or medical practitioner to better understand his/her risk of developing that particular disorder. If the genetic disorder runs frequently in the family, then the individual might like to take the test along with his/her family members or would like to discuss and assess the situation with his/her family. Having these kind of discussion and conversation can give an idea to the individual about his family's reaction as well as its effect on them. Not all health insurance policies pay for genetic testing. So, before you have a genetic test, check with your insurance provider to see what will be covered.

## Techniques of genetic testing

Genetic testing involves the laboratory analysis of human genetic material including chromosomes, deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) to detect genetic material and/or identify genetic changes. Chromosomes are composed of DNA. Specific DNA segments called genes serve as templates to make (transcribe) RNA. Genetic changes are referred to as 'variations' or 'variants' (sometimes called 'mutations').and they can have many different effects on the body. While most genetic variations do not affect a person's health, they are sometimes related

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to disease. Before, studying further, let us discuss the common properties of DNA and RNA which help them to be ideal for genetic screening. Nucleic acids are of two types - deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), both of which primarily serve as reservoir and transmitters of genetic information. Johann Friedrich Miescher, a Swiss researcher, discovered DNA in 1869. Avery, Macleod and MacCary first demonstrated in 1944 that DNA contained genetic information. Friedrich Miescher (1869) first isolated nucleic acids from pus cells. Initially named nuclein, Hertwig (1884) believed them to be the carrier of hereditary traits. Owing to their acidic nature the term nucleinic acids was used for them which was later replaced by nucleic acids (Altmann, 1899). Chemical analysis of highly purified DNA has shown that it is made of four kinds of monomeric building blocks, each of which contains three types of molecules:

## (i) Phosphoric acid

- The phosphoric acid ($H_3PO_4$) is biologically called phosphate and it was discovered by Levene in 1910.
- Phosphoric acid consists of three reactive hydroxyl groups (—OH), out of which two are involved in forming sugar phosphate backbone of both DNA as well as RNA.
- A phosphate group binds to the 52 carbon of one and 32 carbon of the other adjacent pentose sugar molecule to make the phosphate di-ester. The phosphate makes a nucleotide (see latter) negatively charged.

Therefore, a DNA becomes a polyanionic structure:

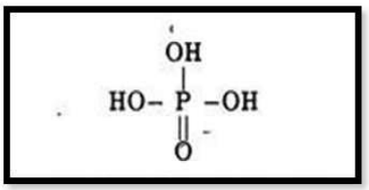


***Fig. 4.38*** *Structure of Phosphoric Acid*

### (ii) Pentose Sugar:

DNA contains β D 22 -deoxyribose sugar.

It is a five-carbon sugar; hence it is a pentose sugar. Since one oxygen atom at the 22 carbon is missing it get its name 22 -deoxy. The 22 - deoxy-containing backbone is more resistant to hydrolysis as compared to the riboform.

D-ribose does not mean dextrorotary ribose. It is actually a form of stereoisomer. Here the prefix D is used to refer to the configuration of sugar due to presence of asymmetric carbon atom. Any D-sugar is the mirror image of L-sugar with respect to the orientation and the position of monovalent atom or group linked with asymmetric carbon atom, i.e., monovalent atom or group in one form of sugar is exactly opposite in position (left- right) in other form of sugar. In D-ribose there are three asymmetric carbon atoms.

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In deoxyribose sugar, the hydroxyl group on the carbon that carries the aldehyde group can rapidly change from one position to another. The two positions are known as α and β

The ring form in which the deoxyribose sugar is always present is derived from heterocyclic furan (C4H40) structure. The carbon atoms of the deoxyribose are numbered from the end closet to the aldehyde and the numbers are given as 12, 22, 32, 42, and 52 in order to distinguish and characterise them from the corresponding position in DNA bases. It is also explained that each numbered carbon on the sugar is followed by a prime mark, therefore one speaks of 5 prime or 3 prime carbon etc.

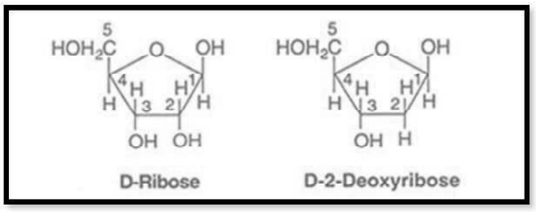


***Fig. 4.39*** *Structure of Sugars Present in Nucleic Acids*

### (iii) Organic Bases:

Many varying kinds of heterocyclic nitrogen comprising of ring compounds are found in the structure of DNA. They are known as simply bases because they can combine with H+ in acidic solution. They are also referred to as nitrogenous base because of the presence of nitrogen.

The structure of RNA is very similar to that of DNA. It also consists of sugar- phosphate bonds and similarly is a polynucleotide chain. The only difference is that it is a single stranded molecule and it contains ribose sugar. Also, a major difference between DNA and RNA is that RNA contains Uracil (U) instead of base thymine (T). Furthermore, in RNA the amount of purines is not equal to pyrimidines. Thus, the four bases in RNA are A, U, G and C. RNA shows less secondary characteristics but it does show bond formation between complimentary segments of the same chain and this leads to the formation of an antiparallel duplex structure known as Hairpin structure. To test genetic material for medical reasons, some type of sample from the body is required. This sample can be blood, urine, saliva, body tissues, bone marrow, hair, etc. The sample can be submitted in a tube, on a swab, in a container, or frozen. Once received in the laboratory, the genetic material is separated and removed from the sample. A few genetic disorders are associated to a single gene, and genetic testing has conventionally focused on testing for mutations in genes based on a person's symptoms or family history. For example: cystic fibrosis comprises of a definite set of symptoms as well as testing for mutations in one gene can generally identify the cause of those symptoms. However, there are several other genetic disorders that cannot be easily identified. These are linked to multiple genes or large sections of the genome. The current development of new gene sequencing technology and the deteriorating cost of

sequencing has led to the development of tests that can look for genetic disorders beyond a single gene. In the next section, we are going to discuss a few methodologies of genetic screening:

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### 1) Polymerase Chain Reaction

Polymerase chain reaction is one of the most common techniques which is used for amplifying the short DNA fragments or in other words generating numerous copies of short DNA sections from a very small sample of genetic material. This process is also known as "amplifying" DNA and it allows precise genes or regions of interest to be detected or measured. This technique is usually used to copy DNA so it can be sequenced or analysed with other techniques. It is often used to help look for genetic variants known to cause certain diseases, like those disease which are associated with cancer or genetic disorders.

Next step of the process is to amplify the isolated DNA. Amplification of the DNA is carried out by using PCR machine. The process involves amplifying the target region via Polymerase chain reaction 'PCR'. Five core 'ingredients' are required to set up a PCR:- the DNA template to be copied, primers that initiate the PCR reaction, DNA bases (A, C, G and T), Taq polymerase enzyme to insert in the new DNA bases, DNA buffer to make sure the optimum conditions for the PCR reaction. PCR involves a repeated process of heating and cooling referred to as 'thermal cycling' which is carried out by PCR machine. Denaturing is the first step of the process in which double-stranded template DNA is heated to 94-95p C which ensures the breaking of hydrogen bonds between the two strands and thus separating it into two single strands. This step usually takes between 15-30 seconds. During the next stage known as annealing, temperature is lowered to 50-65p C to enable the DNA primers (short sequences of DNA 20 to 30 base in length) to attach to the template DNA. Cooling down help the primers to attach to a specific location on the single-stranded template DNA by way of hydrogen bonding. This step usually takes between 10 to 30 seconds. The final step is extension where the heat is increased to a temperature of 72p C to facilitate the new DNA to be made by a special Taq DNA polymerase enzyme which keeps on adding new DNA bases. All the above mentioned three steps are repeated 20-40 times, thus doubling the number of DNA copies every time. It usually takes a few hours to complete the entire PCR reaction.

### 2) Sequencing of DNA

After the PCR reaction has been completed, another methodology referred to as electrophoresis is used to check the size as well as quantity of amplified product.

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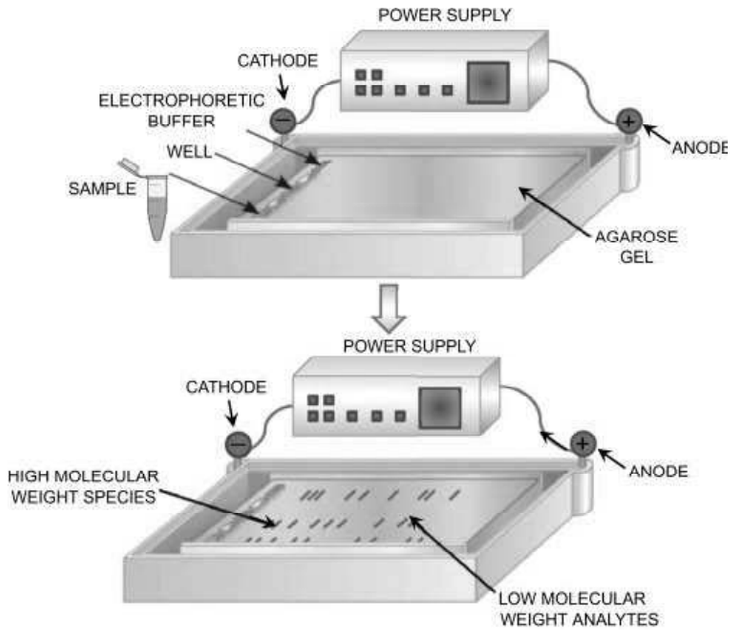


***Fig. 4.40*** *Agarose Gel Electrophoresis Apparatus*

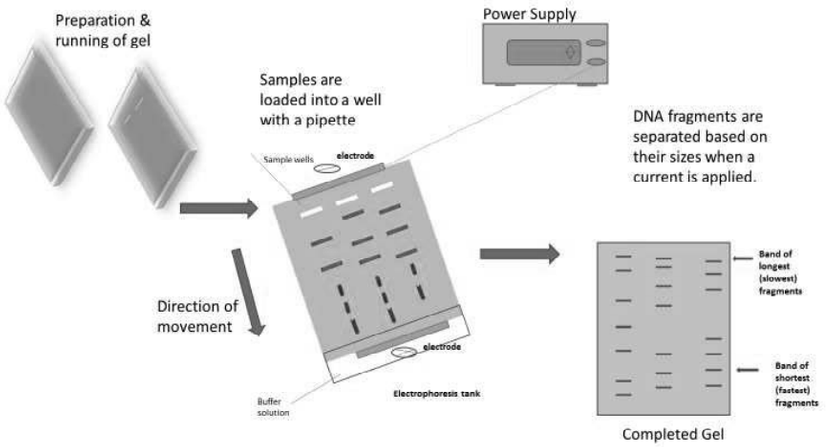


***Fig. 4.41*** *Agarose Gel having DNA Markers of different Sizes and DNA Samples Marked as A, B and C*

Next step involves the sequencing of the amplified PCR products using methods such as Sanger sequencing or amplicon pyrosequencing. Everyone has a unique genome, made up of the DNA in all of a person's genes. This complex testing can help identify genetic variants that may relate to your health. This testing is usually limited to just looking at the protein-encoding parts of DNA called the exome. DNA sequencing refers to determining the order of bases [adenine (A), thymine (T), cytosine (C) and guanine (G)] that make up DNA. Sequencing allows clinicians to determine if a gene or the region that regulates a gene (regulatory region of DNA) contains changes, or variants, linked to a disorder. The major difference between Sanger sequencing and next-generation sequencing is that Sanger sequencing processes only a single DNA fragment at a time, on the contrary,

next-generation sequencing (NGS) processes millions of fragments at the same time. Besides, Sanger sequencing is analogical while next-generation sequencing is digital, allowing the detection of the novel or rare variants with deep sequencing. Further, Sanger sequencing is best suited for low number of targets, whereas, next gen sequencing is cost effective when millions of fragments are analyzed together. Let us see both the sequencing in detail:

### a) Sanger sequencing

Sanger Sequencing (SGS) is the first-generation sequencing method which was developed by Fredric Sanger in the year 1977. Sanger sequencing involves the selective incorporation of chain terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. The amplicons thus produced are separated from each other by the process of capillary electrophoresis. Usually, Sanger sequencing acts as a quick and cost-effective technique for low number of samples say less than 100. Further, it is better for the sequencing of single genes. Furthermore, as mention above too, Sanger sequencing is an analogical method i.e., it generates a single sequence by combining signals from all DNA fragments in the sample. Sanger sequencing does not allow the isolation of individual signals. Consequently, the resultant signal is a mixed-signal, which does not allow the identification of variants, which occur below 25% frequency in a sample.

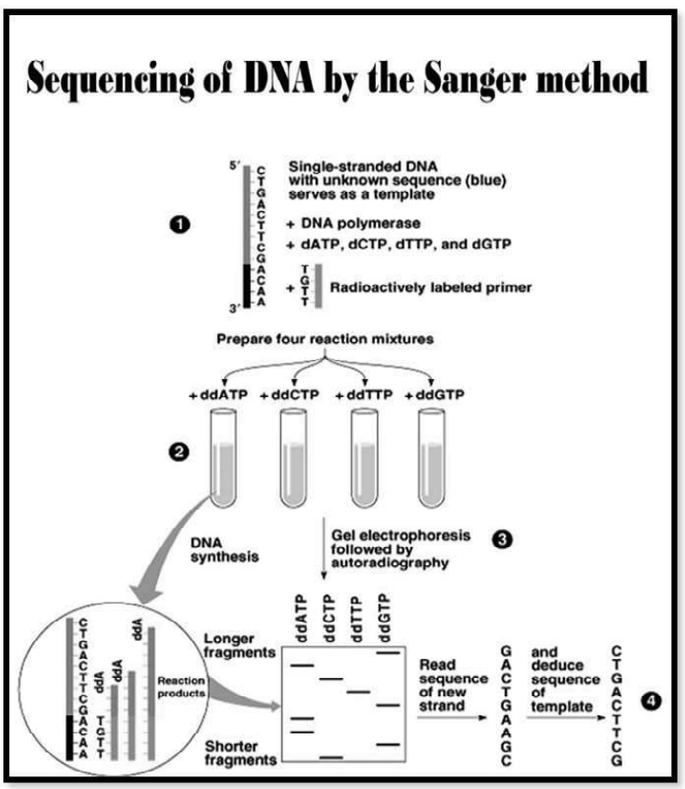


***Fig. 4.42*** *Overview of Sanger Sequencing*

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### b) Next-Generation Sequencing (NGS) (Whole Exome Sequencing and Whole Genome Sequencing)

Next-generation sequencing (NGS) is a second-generation sequencing method. Furthermore, it is a high-throughput DNA sequencing approach with the concept of massively parallel processing. Genome Analyser/HiSeq/MiSeq (Illumina, Solexa), SOLiD System (Thermo Fisher Scientific), Ion PGM/Ion Proton (Thermo Fisher Scientific), and HeliScope Sequencer (Helicos Biosciences) are the numerous platforms presently performing next-generation sequencing. Usually, they can sequence approximately 1 million to 43 billion short reads (50-400 bases each) per instrument run. Furthermore, the primary characteristic of next-generation sequencing is that it can accomplish a parallel examination of numerous targets. NGS has tremendously increased the speed as well as efficiency of detection of mutation. Mostly, in somatic cancer mutations, tumours are heterogeneous and comprises of both cancer cells as well as the normal cells. Though, the preparation of a DNA library by clonal amplification in next-generation sequencing for parallel sequencing helps to physically separate signals originating from each target DNA molecule in the library. Consequently, this allows the separation of DNA sequences of cancer cells from the DNA sequences of normal cells. Thus, all in all, the next-generation sequencing is a digital sequencing method with a higher depth of coverage variants.

When the Human Genome Project was completed in the year 2003, it took over ten years to finalize the sequence of a single person's genome using the Sanger sequencing. Today there are rapid sequencing ways to sequence millions of samples together. Such rapid technologies are known as next-generation sequencing (NGS) technologies. They are quick as they sequence millions of small DNA fragments in parallel (at the same time). NGS techniques can be used to look at an approximately 22,000 genes that code for the production of proteins. The protein-coding regions of genes are referred to as exons and all of them collectively are known as the exome. All of the genes, both the coding as well as non-coding portions of the genes, along with the areas between the genes, is called the genome. When NGS is used to evaluate the entire exome or genome, it is known as whole exome sequencing or whole genome sequencing, respectively.

NGS is broadly available now. Several commercial as well as academic laboratories presently use NGS for medical purposes. For example, whole exome sequencing or whole genome sequencing can be used to assess individuals with a personal or family history that indicates a tendency to breast cancer or ovarian cancer. One of the greatest advantages of using NGS technology is that a lot of samples can be assessed at the same time to determine whether gene variants are present that would increase risk of these cancers. Further, it should be noted, that since NGS is in-depth sequencing of the samples, hence results obtained from NGS must be analyzed carefully. NGS can recognize several genetic changes at the same time when compared to older techniques that sequence individual or select genes, however, the consequence of the changes is not always understood. Many times, genetic changes that are detected are not able to point to an identifiable disorder. That's why, when considering or undergoing genetic testing, it is essential

to seek help from a genetics expert or genetic counsellor to understand test results, implications of the results, or risk of passing genetic disorders to any children.

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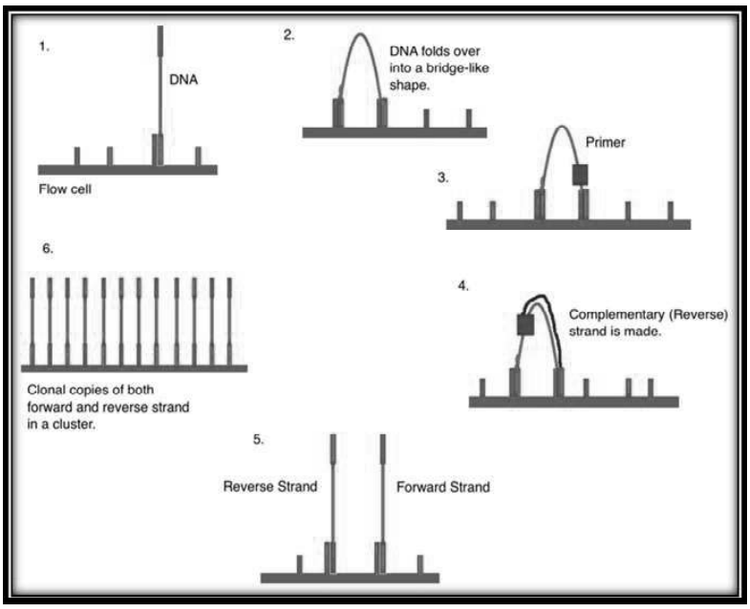


***Fig. 4.43*** *Clonal Amplification in Illumine Sequencing*

Thus, even though, the basic principle as well as function of the methods of sequencing is same, yet both these platforms differ from each other in multiple ways:

***Table 4.2*** *Sanger Sequencing Targeted NGS*

| | **Sanger Sequencing** | **Targeted NGS** |
|---|---|---|
| **Benefits** | • It is quick<br>• Cost-effective sequencing<br>• Well suited for low numbers of targets (maximum 100 targets)<br>• One of the oldest known techniques<br>• Result analysis is simple as compared to NGS. | • Higher sequencing depth enables higher sensitivity (down to 1%)<br>• Higher discovery power. Here, discovery power refers to the ability to identify novel variants.<br>• Higher mutation resolution. Here, mutation resolution refers to the size of the mutation identified. NGS can identify large chromosomal rearrangements down to single nucleotide variants.<br>• More sequencing data is produced with the same amount of input DNA.<br>• Higher sample throughput |
| **Challenges** | • Low sensitivity (i.e., limit of detection is approximately 15–20%)<br>• Low discovery power<br>• Not very cost-effective for high numbers of targets (> 100 targets)<br>• Low scalability due to increasing sample input requirements | • Not cost effective for low number of targets<br>• Time-consuming for sequencing low numbers of targets (1–20 targets)<br>• Result analysis requires computational skills. |

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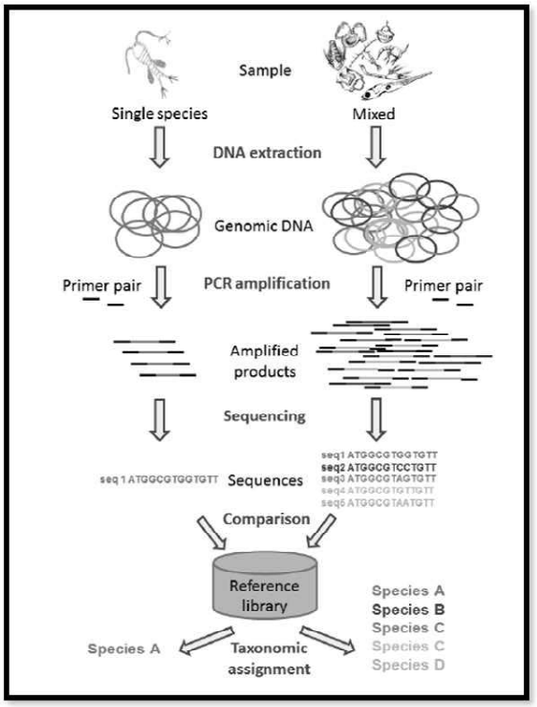


***Fig. 4.44*** *Overview of PCR and Sequencing Methodology*

The last step of the process is to compare the resulting sequences against reference databases to find the matching species. Once the whole DNA is sequenced, the result is saved into one unique file format. The inbuilt software (provided by the manufacturer) processed the data and compared it with the available data. The sequence data is compared with other available data by the software to find out variations and other mutations present in a gene.

### 3) Chromosome analysis (Karyotyping)

The cell of human beings comprises of 23 pairs of chromosomes, with 22 pairs of autosomes and one pair of sex chromosomes. The branch of science that deals with the study of these chromosomes is referred to as "cytogenetics". Trained cytogeneticists examine the number, shape, as well as staining pattern of these structures with the help special technologies. In this way, they can detect extra chromosomes, missing chromosomes, missing or extra pieces of chromosomes, or rearranged chromosomes.

Karyotyping is basically a laboratory technique that allows the doctor or medical practitioner to examine set of chromosomes. "Karyotype" refers to the actual collection of chromosomes being examined. Examining chromosomes via karyotyping allows a doctor/medical practitioner to determine whether there are any abnormalities or structural problems within the chromosomes. Chromosomes contain the genetic material i.e., DNA which is inherited from parents. Whenever, a cell divides, it needs to pass on a complete set of genetic instructions to each new cell it forms. When a cell isn't undergoing any division, the chromosomes are arranged in a spread out, unorganized way. During division, the chromosomes in

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these new cells line up in pairs. A karyotype test examines these dividing cells. The pairs of chromosomes are arranged by their size as well as appearance. This helps the doctor easily to determine if any chromosomes are missing or damaged. An unusual number of chromosomes as well as incorrectly arranged chromosomes, or malformed chromosomes can all be signs of a genetic condition. The methodology known as Karyotyping begins with placing cells on glass slides and separating whole chromosomes from the nuclei of the cells. The slides are then stained with special dyes and examined under a microscope. Pictures of these slides are taken and these pictures are cut into pieces, in such a way that the chromosome pairs can be arranged as well as matched. Each chromosome pair is assigned a special number (from 1 to 22, then X and Y) that is based on its staining pattern and size. Potential abnormalities include

- Extra chromosomes
- Missing chromosomes
- Missing portions of a chromosome
- Extra portions of a chromosome
- Portions that have broken off of one chromosome and reattached to another

Thus, the lab technician can see the chromosomes' shape, size, and number. This information is important in determining if there are any genetic abnormalities. A normal test result will show 46 chromosomes. Two of these 46 chromosomes are sex chromosomes, which determine the sex of the person being tested, and 44 of them are autosomes. The autosomes are unrelated to determining the sex of the person being tested. Females have two X chromosomes, while males have one X chromosome and one Y chromosome. Abnormalities that appear in a test sample could be the result of any number of genetic syndromes or conditions. Occasionally, an abnormality will occur in the lab sample that's not reflected in your body. The karyotype test may be repeated to confirm that there's an abnormality.

Genetic conditions vary greatly, however two examples are Down syndrome and Turner syndrome. In Down syndrome, an individual has an extra X chromosome i.e., chromosome number 21, can be easily identified by karyotyping. When three chromosomes are present together in a group, they can be easily identified and the condition is known as trisomy as seen in case of Down syndrome. Similarly, if one of the chromosomes is missing from the group, for instance in Turner's syndrome, then also it is easily recognizable and the condition is known as monosomy. Occasionally, it has been observed that a piece of a chromosome will break off and attach to another chromosome. Whenever this happens, it is known as "translocation" or "rearrangement." For instance, chronic myelogenous leukaemia (CML) is a disease caused by a translocation in which a part of chromosome 9 breaks off and attaches itself to chromosome 22 (BCRABL-1 fusion gene).

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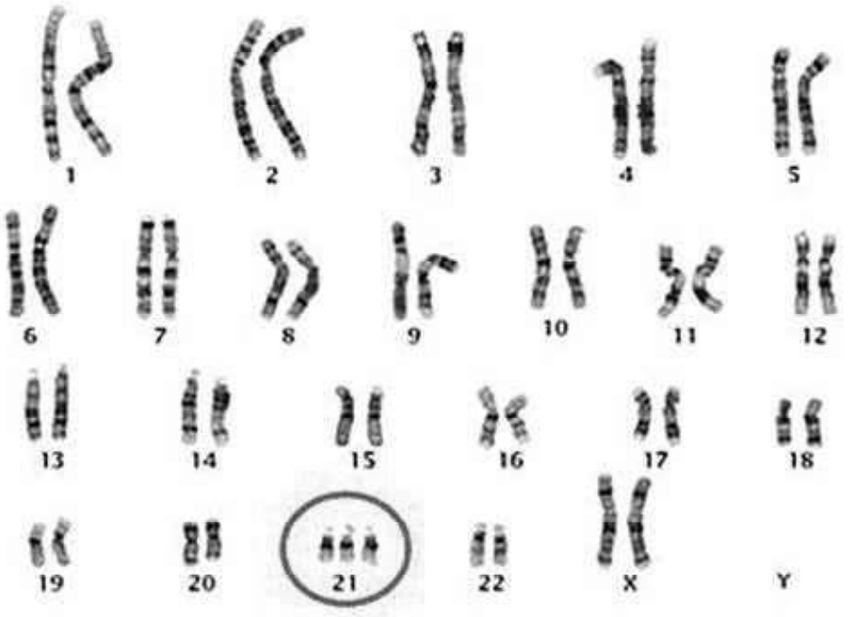


***Fig. 4.45*** *Karyotype of an Individual Suffering from Down's Syndrome*

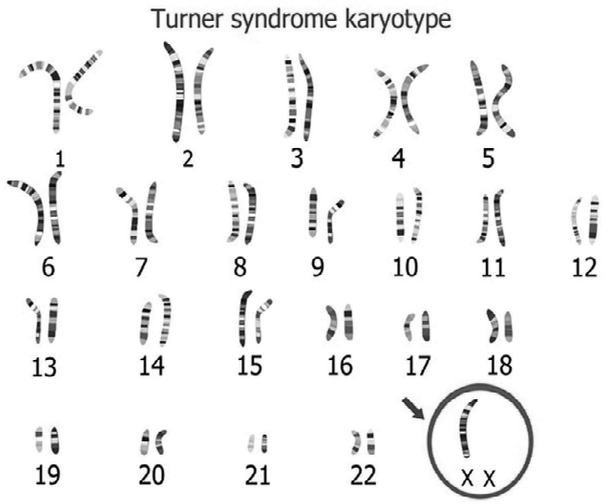


***Fig. 4.46*** *Karyotype of an Individual Suffering from Turner Syndrome*

The preparation required for karyotyping depends on the method used by the medical practitioner. Samples can be collected from:

- A bone marrow biopsy, which involves taking a sample of the spongy tissue inside certain bones
- An amniocentesis, it involves collecting the sample of amniotic fluid from the uterus

Generally, this is one of the earliest techniques to be used for studying genetic disorder. However, occasionally, a few complications can also result from these testing methods like slight bleeding or infection in case of biopsies and amniocentesis

involves a minimal risk of miscarriage, but otherwise the technique as well as its procedure is very safe.

**4) Fluorescence In-Situ Hybridization (FISH)**

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FISH is a special technique that can be used to view changes in chromosomes that result from genetic variations. In this methodology, a gene segment in a chromosome can be made to 'light up' or fluoresce when it is bound to a special probe. By using more than one probe at once, cytogeneticists can compare to see if the probes are positioned in their normal positions or if they have somehow shifted to a new position on a different chromosome, or if there are more or fewer copies of a probe as generally seen in a normal cell. The FISH technique is dependent upon hybridizing a probe with a fluorescent tag, which is complementary in sequence, to a short section of DNA on a target gene. The tag as well as the probe are applied to a sample of interest under conditions that enables the probe to attach itself to the complementary sequence in the specimen if it is present at all. After the specimen has been treated, excess of the fluorophore has been washed away and the sample can be visualized under a fluorescent microscope. By quantifying the amount of fluorescence with the help of a microscope, a skilled researcher or technician can easily determine the type of cell the probe was designed for is present, and if so, how much of it is present in a sample. Transcripts (mRNA) in microbes can also be targeted to detect if a specific gene is being expressed under the given conditions. Genetic changes in some cancers can be detected using this method. For instance, FISH is one of the methods used to determine increased copy number (amplification) of the gene ERBB2 (also known as HER2) in breast cancer.

**5) Microarrays**

This technique is used for variety of purpose. In genetic diagnostic testing, microarrays can be used to determine whether an individual's DNA contains a duplication, a deletion, or large stretches of identical DNA which can be responsible for causing some kind of disease. Similar to karyotyping, microarray testing can view all the chromosomes at once, however, it can easily detect changes that are smaller as compared to either karyotyping or FISH. The methodology of DNA microarray can be described below:

i. **Collect Samples:** This can be from a variety of organisms. Two samples i.e., cancerous human skin tissue & healthy human skin tissue

ii. **Isolate mRNA:** Extract the RNA from the samples either with the help of a column or by using methodology or by using phenol-chloroform. After isolating the RNA from the sample, an individual need to isolate the mRNA from the rRNA and tRNA. As we all know, that mRNA comprises of a 3 end poly A tail, hence, technician can use a column with beads having poly-A tail to separate the mRNA. The beads are then thoroughly washed with the buffer to separate the mRNA.

iii. **Create Labelled cDNA:** Add a labelling mix to the RNA. The labelling mix contains poly-T (oligo dT) primers, reverse transcriptase (to make cDNA), and fluorescently dyed nucleotides. Further, add cyanine 3 (cy3-

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fluoresces green) to the healthy cells and cyanine 5 (cy5- fluoresces red) to the cancerous cells. The primer and RT bind to the mRNA first, then add the fluorescently dyed nucleotides, creating a complementary strand of DNA

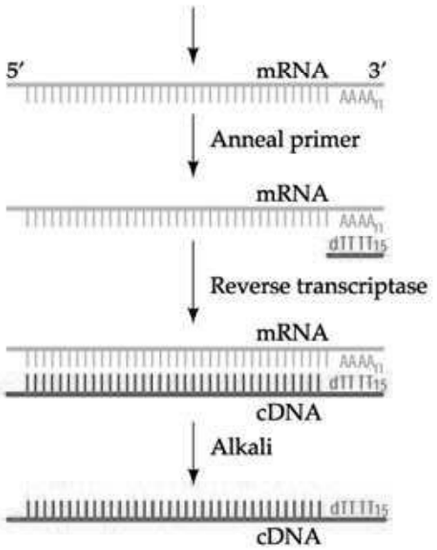


***Fig. 4.47*** *Creation of labelled cDNA*

iv. **Hybridization:** Apply the cDNA we have just created to a microarray plate. When comparing two samples, apply both samples to the same plate. The ssDNA will bind to the cDNA already present on the plate.

v. **Detect the Relative Intensities of Fluorescence Under a Microarray Scanner:** The scanner comprises of a laser, a computer, as well a camera. The laser causes the hybrid bonds to fluoresce.

The camera records the images produced when the laser scans the plate.

### 6) Data Analysis

Microarrays are capable of analysing transcriptomes as well as proteomes. Gene chips are available commercially to detect several pathogens as well as genetic disorders. It can also detect the microbes with the help of specific probes. It is employed in genotyping of genomes via SNP analysis. It can also be used to determine the gene expression by analysing cDNA produced from mRNA of specific cells at different time. To detect changes in the expression of gene levels two samples' gene expression can be compared from different samples, like from cells of different stages of mitosis. It can be used to detect genomic gains as well as losses. It can also be used to observe mutations in DNA. Chromosomal microarrays are considered a first-tier test for individuals with developmental delays, intellectual disabilities, autism spectrum disorders or multiple birth defects, and is recommended in lieu of a karyotype.

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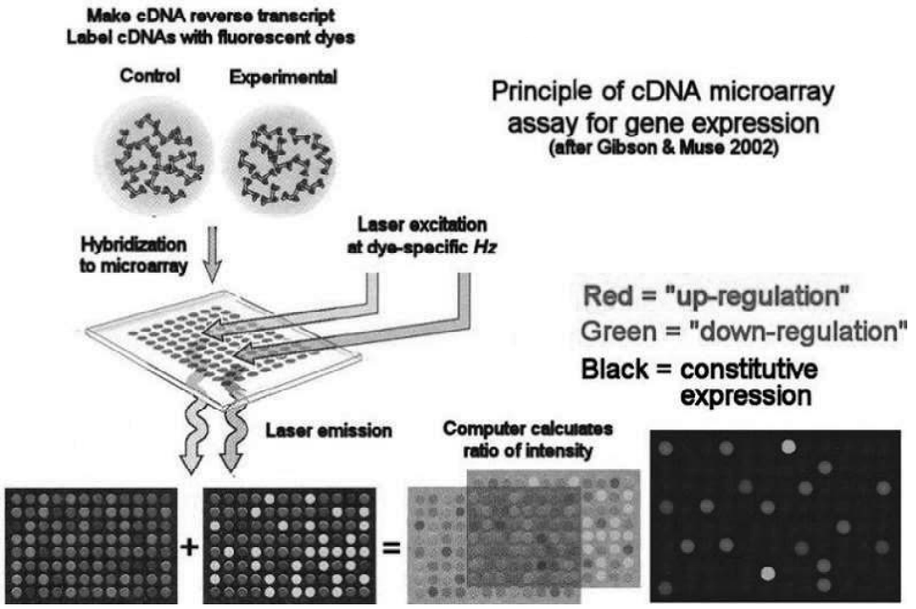


***Fig. 4.48*** *Analysis of DNA Microarray*

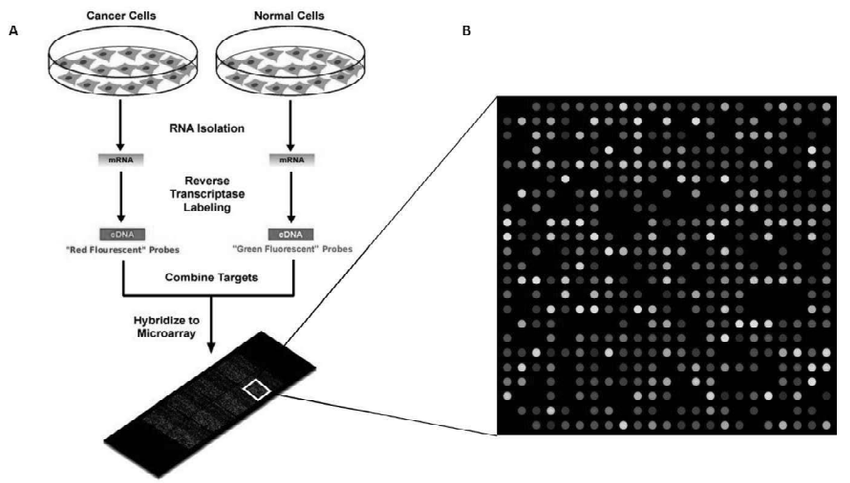


***Fig. 4.49*** *Complete Methodology of DNA Microarray in Normal and Cancerous Cells*

## 7) Gene Expression Profiling

Gene expression profiling primarily aims at looking for genes which can be turned on or off in cells. Gene expression refers to the process of making specific proteins from the information present in the genes. On the basis of their role in the body, different tissues express different sets of genes. The information obtained from the gene is then used to make a template for building RNA. RNA then undergoes certain alterations to produce the protein which is required by the cell. Gene expression tests assess the RNA in a person's tissue sample to determine which genes are actively making proteins.

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For instance; gene expression profiling tests can analyze a number of different genes within an individual cancer cells to predict his/her risk of cancer recurrence. The results obtained by this methodology help the medical practitioner who may benefit from additional (adjuvant) treatment after surgery. For instance; women with early-stage breast cancer that is sensitive to hormones, gene expression profiling tests are used to determine whether they are likely to benefit from adjuvant chemotherapy. Numerous gene expression profiling tests exist, and most of them are being researched in clinical trials. Doctors are still determining how best is to use these tests and interpret the results. One current research study states that chemotherapy might not be helpful for women with gene expression profiling test results that indicate an intermediate risk of recurrence. In the study conducted, few women received hormone therapy as well as chemotherapy after surgery and some women received hormone therapy only.

Both the groups of women had almost similar survival rates, which further indicated that the chemotherapy was unnecessary for most of the women. Chemotherapy showed some benefit for women who were 50 or younger and had gene profiling test scores in the upper end of the intermediate range.

### Results of Genetic Screening

Results of genetic testing hugely depends upon the type of test taken as well as the health care facility providing the test. It is always a good practice to discuss the results of genetic tests with a skilled doctor, medical geneticist or genetic counsellor. Even though, genetic testing can provide essential information regarding the diagnosis, treatment as well as prevention of disorder yet there are few limitations. For instance, the positive result of a healthy individual does not always be a surety that he/she will be suffering from the disorder. On the contrary, a negative result doesn't guarantee that the person might not be having the certain disorder. Talking to your doctor, a medical geneticist or a genetic counsellor about what you will do with the results is an important step in the process of genetic testing. A positive test means that the genetic change that was being tested for was detected. The steps an individual takes after being tested positive depends upon the reason of genetic testing:

- A positive test helps to diagnose a specific disease or condition and thus can help the doctor or medical practitioner to begin with appropriate treatment as well management plan.
- In case of prenatal testing, a positive test can help the parents to find out if the baby is carrying any genetic disorder as well as it can help to determine child's risk of actually developing a genetic disorder. Thus, it can surely help in family planning too.
- A positive test can also determine if the individual might suffer from the disorder in future. However, as mentioned above, a positive test doesn't necessarily mean that the individual will get that disorder. For instance, having a breast cancer gene (BRCA1 or BRCA2) means the person is at high risk of developing breast cancer at some point in her life, however, it doesn't specify with certainty that she will get the breast cancer. However, with few

conditions, like Huntington's disease, having the altered gene does specify that the disease will eventually develop.

Thus, it is always recommended to discuss the report thoroughly with the doctor or medical practitioner or genetic counsellor. In some cases, only a change in lifestyle can reduce the risk of developing a potential genetic disorder. Results may also help the individual to make choices related to treatment, family planning, careers as well as insurance coverage.

Additionally, the individual might choose to participate in research or registries related to your genetic disorder or condition. These options may help you stay updated with new developments in prevention or treatment.

A negative result indicates that a mutated gene was not detected by the genetic test, which can further be comforting, however, it does not guarantee that the individual will not develop the genetic disorder in the future. The exactness of genetic tests to detect mutated genes differs, depending on the condition being tested for and whether or not the gene mutation was previously identified in a family member.

Even, if the individual is having the mutated gene, that doesn't essentially mean that he/she will never get the disease. For instance, the majority of people who develop breast cancer don't have a breast cancer gene (BRCA1 or BRCA2). Also, genetic testing may not be able to detect all genetic defects.

In few situations, a genetic test may not provide supportive information regarding the matter of concern. Each and every individual shows some variations in the way genes appears and usually these genetic variations does not affect the health status of that individual. However, occasionally, it can be difficult to differentiate between a disease-causing gene and a harmless gene variation. These changes are referred to as the variants of indeterminate significance. In these cases, follow-up testing or periodic reviews of the gene over time may be required.

Thus, to conclude, irrespective of the results of genetic testing, an individual must discuss his/her genetic reports with the doctor or medical practitioner or genetic counsellor. This will help an individual to actually understand the results and the risk if any associated with it.

**Check Your Progress**

14. How long does the entire ZIFT process take?
15. Why ICSI is considered one of the most successful techniques to cure infertility?
16. State the two approaches used for embryo cloning.
17. Why is the technique of embryo sexing so popular?
18. Mention the genetic disorders that are usually screened for as part of prenatal genetic testing.
19. What is the difference between the structure of RNA and DNA?
20. What is the FISH technique?
21. Mention an example of gene expression profiling test and its use.

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# 4.5 BIOLOGY OF SEX DETERMINATION AND SEX DIFFERENTIATION: A COMPARATIVE ACCOUNT

In this section, we will discuss sex determination and differentiation in detail.

## 4.5.1 Sex Determination

A sex-determination system refers to a biological system which determines the development of sexual characteristics in an organism. Most of the sexually reproducing organisms have two sexes i.e., males and females. A few species are hermaphrodite i.e., having both the male and female reproductive system in a single individual.

In many species, sex determination is genetic i.e., males as well as females have different alleles or even different genes that specify their sexual morphology. In animals this is usually accompanied by chromosomal differences, via the chromosomal combinations such as XX, XY, ZW, XO, and ZO. The sexual differentiation is generally triggered by a main gene (a "sex locus"), with a multitude of other genes following in a domino effect. In few species like that of lizards and crocodile, environmental factors like temperature also plays an important role in determining the sex of developing embryo. In the following section, we shall discuss about different modes of sex determination.

Establishment of male as well as female individuals is referred to as sex determination. Sex determination can be on the basis of:

### I. Environmental or non-genetic determination of sex

a. It has been observed that marine mollusc like Crepidula becomes female if reared alone. However, if they are in the company of a female, it develops into male (Coe, 1943).

b. It has been researched that marine worm Bonellia develops into a 3 cm long female if its larva settles down in an isolated place. However, it grows into small i.e., 0.3 cm long parasitic male if it comes closer to an already established female (Baltzer, 1935). The male enters the body of the female and stays there as a parasite.

c. Ophryortocha is male in the young state and female later on.

d. In few species of Crocodiles as well as lizards' high temperature induces maleness on the contrary low tempera-ture femaleness. It has also been observed in turtles, where males are predominant below 28°C, on the contrary females above 33°C and equal number of the two sexes between 28-33°C.

### II. Non-allosomic genic determination of sex

In bacteria which is a prokaryotic organism it is the fertility factor present in a plasmid which determines the sex. Chlamydomonas possesses sex determining

genes. Maize possesses separate genes for development of tassel (male inflorescence) and cob (female inflorescence).

### III. Chromosomal determination of sex

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Sex chromosomes are referred to those chromosomes which singly or in pair determine the sex of the individual in dioecious (Dioecy is a characteristic of a species, meaning that it has distinct individual organisms that produce male or female gametes) or unisexual organism (relating to only one sex or having only one type of sexual organ). They are also known as allosomes (Gk. alios- other, soma- body) or idiochromosomes (Gk. idios- distinct, chroma- colour, and soma- body). A sex chromosome that determines male sex is also known as androsome (Gk. ander- male, soma- body), for instance, Y-chromosome in humans. All the other normal chromosomes, apart from the sex chromosomes of an individual are known as autosomes. Further, sex chromosomes can be present in homomorphic or heteromorphic condition. Individuals having homomorphic sex chromosomes produce only one type of gametes. They are also known as homogametic (for instance- human female are homogametic as they produces same type of gametes containing X chromosome as sex chromosome). On the contrary, individuals having heteromorphic sex chromosomes produce two types of gametes. They are also known as heterogametic (For instance; human male produces two different types of gametes containing either X or Y chromosome).

In the year, 1891, Henking observed that an X-body is present in 50% of the sperms of firefly. In the year, 1902, Y-body was also discovered by Stevens. In the year, 1902, McClung observed 24 chromosomes in female Grasshopper and 23 chromosomes in male Grasshopper. In the year, 1905, Wilson and Stevens together put forward the chromosome theory of sex. They referred the X- and Y-bodies as sex chromosomes, X and Y. Sex determination in higher animals is controlled by the action of one or more genes. The testes determining factor (TDF) gene is the dominant sex determining factor in human beings. Hemking a German biologist identified a particular nuclear structure throughout the spermatogenesis in some insects. He named it as 'X-body' and showed that sperm differed by its presence or absence. The X body was later found to be a chromosome that determined sex. It was identified in several insects and is known as the sex or X chromosome. In a nut shell, chromosomal theory of sex determination states that both female and male individuals differ in their chromosomes. As mentioned above, chromosomes can be differentiated as either autosomes or sex chromosomes. Sex chromosomes helps in determining the gender of an individual as they carry genes for sex. In few animals, females have one more chromosome as compared to males, therefore, they comprise of two X chromosomes on the other hand males have only one.

Hence, females are cytologically referred as XX and on the contrary, males are XO. Here, 'O' denotes the absence of X chromosome. Thus, it can be concluded, that during gamete formation, females produce only type of gamete i.e., all the gamete produced by female contains only X chromosome. On the other hand, during gamete production, male produces two different types of gametes containing either X or lacking X chromosome.

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When the female gamete and male gamete i.e., egg and sperm respectively fuses with each other two different types of zygotes can be formed:- Zygote which is formed by the fusion of egg (female gamete containing X chromosome) and sperm containing X chromosome will be having the chromosomal composition as XX. Hence, this zygote will develop into a female in future. On the other hand, the zygote which is formed by the fusion of egg (female gamete containing X chromosome) and sperm lacking X chromosome will be having the chromosomal composition as XO. Hence, this zygote will develop into a male in future. Because both of these types are equal in number, the reproductive mechanism preserves a 1:1 ratio of males to females.

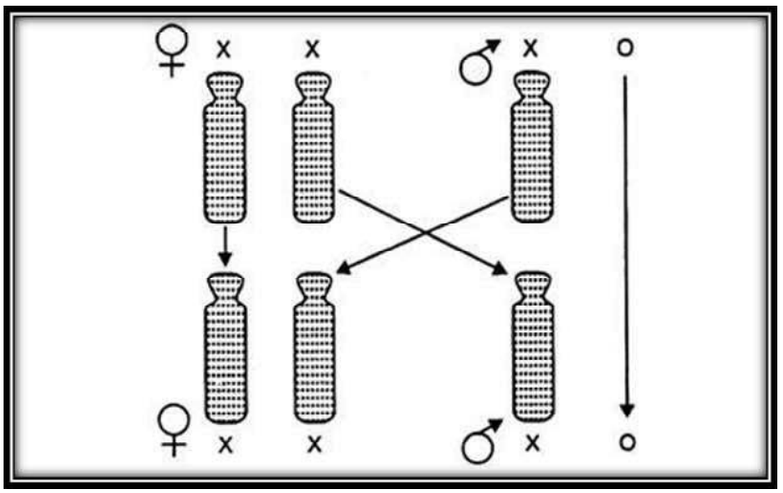


***Fig. 4.50*** *Inheritance of Sex Chromosomes in Animals having XX-XO Mechanism*

In several animals, including human beings, males as well as females have the same number of chromosomes. The total complement of human chromosomes comprises of 44 autosomes and XX as the sex chromosome in female whereas XY in the male. Eggs produced by the female in oogenesis have a complement of 22 autosomes plus an X chromosome. Sperm from the male have the same autosomal number, i.e., 22 and either an X or a Y chromosome. Eggs fertilized with sperm containing a Y chromosome result in zygotes that develop into males; those fertilized with sperm containing an X chromosome develop into females. This numerical equality is due to the presence of a chromosome in the male known as the 'Y' chromosome, which pairs with the X chromosome. During meiosis in the male, the X and Y-chromosomes separate from each other producing two types of sperm, one type with X chromosome and the other type having Y chromosome. Thus, the frequencies of the two types are approximately equal. In random fertilization, approximately half of the zygotes are with XX chromosomes on the other hand, the other half will be having the XY chromosomes leading to a sex ratio of 1:1. This mechanism is called XX – XY type of sex determination. The XY mechanism of sex determination is more common when compared to XO mechanism. This is basically a characteristic method of sex determination in higher animals and has been observed in few plants too. In human beings, the X

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chromosome is considerably longer than the Y chromosome. As mentioned above also, in animals showing the XX-XY mechanism of sex determination, females (XX) produce same type of gametes, i.e., all the eggs produced by the female will have the same chromosome composition (one X plus one set of autosomes). These females are homogametic sex as all the gametes are the same. On the contrary, male produces two different types of gametes (sperm), one gamete containing one X chromosome plus one set of autosomal chromosomes whereas the other one containing one Y chromosome plus one set of autosomes.

Chromosomal determination of sex is of the following types:

### A. Sex determination in human beings (XX—XY Type of sex determination)

As mentioned above also, in human beings, the gender of an individual is determined by the number of X chromosomes or by the presence or absence of the Y chromosome.

Sex of the offspring can be determined at the time of fertilization and it cannot be changed later on. It is also not dependent on any characteristic of the female parent as the latter is homogametic and produces only one type of eggs (22 + X), the male gametes are of two types, andosperms (22 + Y) and gynosperms (22 + X). They are produced in equal proportion.

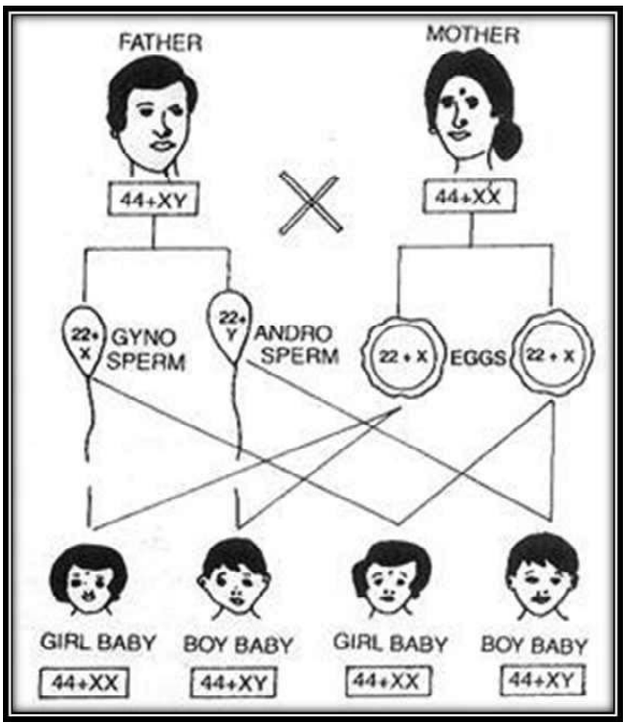


***Fig. 4.51*** *Sex Determination in Humans and Birth of either a Boy or Baby Girl*

Fertilization of the egg (22 + X) with a gynosperm (22 + X) will produce a female child (44 + XX) while fertilization with an androsperm (22 + Y) gives rise to male child (44 + XY). As the two types of sperms are produced in equal

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proportions, there are equal chances of getting a male or female child in a particular mating. As Y-chromosome determines the male sex of the individual, it is also called androsome.

In human beings as well as other placental mammals, maleness is due to a dominant effect of the Y chromosome. This dominant effect of the Y chromosome is established early during the development of the embryo when it directs the primordial gonads to differentiate into testes. After their formation, testes start secreting a hormone known as testosterone which stimulates the development of male secondary sexual characteristics.

Testis determining factor (TDF) is the product of a gene known as the SRY (Sex determining Region of Y). This SRY gene is located on the short arm of the Y chromosome of the mouse as well as that of human beings. For the first time, SRY was discovered in unusual individuals whose sex was not steady with their chromosome constitution i.e., individuals having the chromosomal composition as XX appeared to be males, on the contrary, individuals having the chromosomal composition as XY appeared to be females. Further research in these individuals, depicts that few XX males carried a small piece of the Y chromosome inserted into one of the X chromosomes. Hence, it is obvious that this small piece of Y chromosome carried the genes for maleness. On the contrary, few, XY females carried an incomplete Y chromosome. The part of the Y chromosome that was missing corresponded to the piece that was present in the XX males. These observations depicts that a specific segment of the Y chromosome was necessary for the development of the male. Additional studies showed that the SRY gene is located in this male determining segment. Like that of the human SRY gene is present in the Y chromosome of the mouse and it specifies male development. Thus, it can be concluded that TDF region of the Y chromosome brings about differentiation of embryonic gonads into testes. Later on, testes produce male specific hormone i.e., testosterone which ultimately help in the development of male reproductive tract as well as other secondary sexual characteristics of male. The hormone testosterone binds to receptors of several types of cells. Due to this, a hormone- receptor complex is formed. This complex transmits signals to the cell instructing them how to differentiate.

The combined differentiation of numerous cells leads to the development of male characteristic such as heavy beard, heavy musculature as well as a deep voice. Failure of the testosterone signalling system leads to non-appearance of the male characters and the individual develops into a female. In the absence of TDF gene, gonads differentiate into ovaries after sixth week of embryonic development. It is followed by formation of female reproductive tract. Female sex is, therefore, a default sex.

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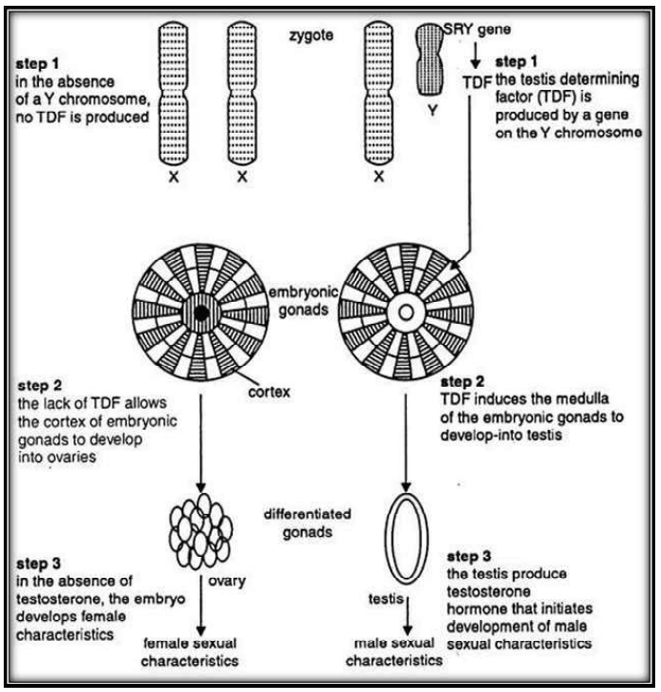


***Fig. 4.52*** *Role of TDF in Sex Determination in Human Beings*

Further, it has been observed that in human beings' irregular sex chromosome constitutions occur occasionally. This occurs due to faulty meiosis or incomplete meiosis. Thus, it is interesting to see the gender of such individuals. For instance: - an individual having more than two X chromosome but lacking Y chromosome (individual having the genetic composition as XXX or XXXX) will always develop into a female. On the other hand, an individual having more than two X chromosome along with a Y chromosome (individual having the genetic composition as XXXY or XXXXY) will develop into a male. Thus, it can be concluded that products of genes located on Y chromosome are essential for maleness, even if several X chromosomes are present and the presence of a single Y chromosome leads to maleness.

The Y chromosome induces the development of the undifferentiated gonad medulla into testis, whereas an XX chromosomal set induces the undifferentiated gonadal cortex to develop into ovaries. The gene on the Y chromosome that induces the development of testes is called as Testis Determining Factor (TDF). It has been isolated, characterized and found to encode a protein that regulates the expression of other genes.

Thus, it can be easily said that the TDF gene is the master regulator gene. It activates the expression of other numerous genes that produce male sex phenotype. In the absence of TDF gene, the genes that produce femaleness predominate and express to produce a female phenotype. The TDF exerts a very leading effect on development of the sex phenotype.

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### B. Sex determination in grasshopper (XX—X0 types of sex determination)

In roundworms as well as in few insects (like true bugs, grasshoppers, cockroaches), the females comprise of two sex chromosomes, XX, on the contrary, the males have only one sex chromosome, X. The second X chromosome is missing. Hence, the males are designated as X0. The females are homogametic as they produce only one type of eggs (A+X). Here, the alphabet 'A' stands for set of autosomal chromosomes. The males are heterogametic with half the male gametes (gynosperms) carrying X-chromo- some (A+X) while the other half (androsperms) being devoid of it (A + 0). The sex ratio produced in the progeny is 1: 1 (Fig. 4.53).

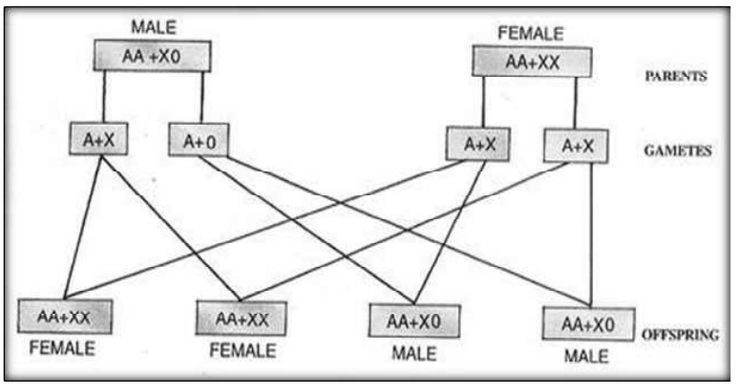


***Fig. 4.53*** *XX-XO Sex Determination*

### C. Sex Determination in Birds (ZW—ZZ Type of Sex Determination)

In birds as well as few reptiles both the sexes hold two sex chromosomes however unlike human beings where males are heterogametic and produces two different types of gametes, here females are heterogametic and produces two different types of gametes. Thus, in these species, the genetic constitution of diploid homogametic males is AA + ZZ whereas the genetic constitution of heterogametic diploid female is AA + ZW. Thus, in birds as well as few reptiles, the females are heterogametic and produce two types of eggs, (A + Z) and (A + W). Whereas, the male gametes or sperms are of one type (A + Z). 1: 1 sex ratio is produced in the offspring.

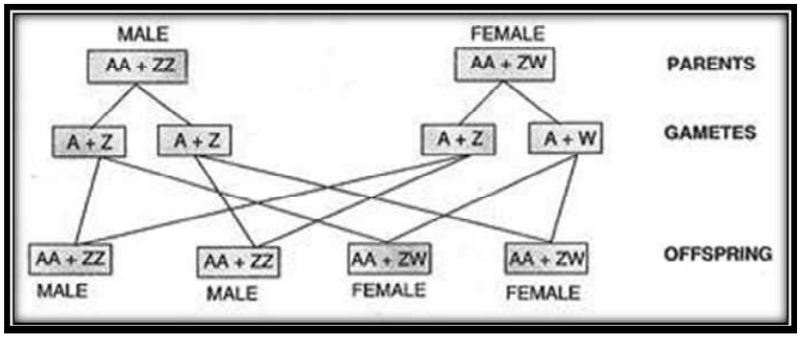


***Fig. 4.54*** *Figure Depicting Sex Determination in Birds*

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### D. Sex Determination in Moths and Butterflies (ZO—ZZ Type of Sex Determination)

ZO-ZZ type of sex determination occurs in few butterflies as well as moth species. This is exactly in contrast to the condition seen in cockroaches as well as grasshoppers. Here the females have odd sex chromosome (AA + Z) while the males have two homomorphic sex chromosomes (AA + ZZ). The females are heterogametic and hence produces two different types of gametes. They produce two types of eggs, male forming with one sex chromosome (A + Z) and female forming without the sex chromosome (A + 0). The males are homogametic, forming similar types of sperms (A + Z). The two sexes are obtained in the progeny in 1:1 ratio as both the types of eggs are produced in equal ratio.

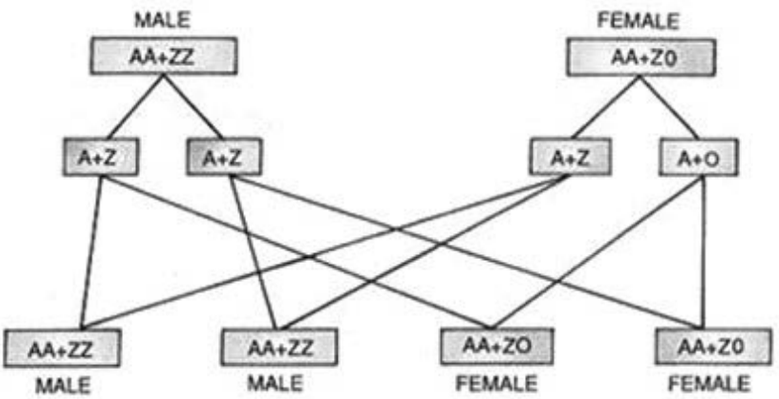


***Fig. 4.55*** *Sex Determination in Butterfly*

### E. Sex Determination in Bees, Ants or Wasps: Haplodiploidy

Haplodiploidy refers to type of sex determination where the male is haploid on the contrary female is diploid. Haplodiploidy occurs in few insects such as bees, ants and wasps. Male insects are haploid as they develop partheno-genetically from unfertilized eggs. The phenomenon is referred to as arrhenotoky or arrhenotokous parthenogenesis. Meiosis does not occur during the formation of sperms. Females grow from fertilized eggs and are hence diploid.

For instance; Haplodiploidy is seen in honey bees. The virgin queen honey bees take to nuptial flights for mating and then settles in the hive for laying eggs. Queen bee is the only female bee in the entire honey bee colony which is capable of laying approximately 2,000 to 3,000 eggs per day. However, the normal fecundity is approximately 600 eggs/day. The egg is positioned upright and falls on the side by the third day. The queen bee lays both fertilized egg as well as unfertilized egg. Queen bee can produce fertilized or unfertilized eggs on her own choice. The fertilized egg develops into female bees (worker bees) or queen bees. The unfertilized egg hatches to form male bees also known as drone bees. Males are darker, robust and hairy and larger than workers. There are about two dozen of them in a hive and chase the queen in air every time she ventures on nuptial flight.

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## F. Genic balance Theory of Sex Determination in Drosophila

The genie balance theory of sex determination in Drosophila discusses the mechanism involved in sex determination in this fly. The Y chromosome in Drosophila does not play any functional role in determination of sex. Further, sex in Drosophila is determined by the ratio of X chromosomes to autosomes which is commonly written as X/A. Normal diploid insects comprises of a pair of sex chromosomes, either XX or XY, and three pairs of autosomes. These are denoted by AA, each A representing one set of haploid autosomes.

***Table 4.3*** *The Ratio of X/A and the Possible Phenotypes in Drosophila*

| X chromosomes (X) and Sets of autosomes (A) | X : A ratio | Phenotype |
|---|---|---|
| 1X 2A | 0.5 | Male |
| 2X 2A | 1.0 | Female |
| 3X 2A | 1.5 | Metafemale |
| 4X 3A | 1.33 | Metafemale |
| 4X 4A | 1.0 | Tetraploid female |
| 3X 3A | 1.0 | Triploid female |
| 3X 4A | 0.75 | Intersex |
| 2X 3A | 0.67 | Intersex |
| 2X 4A | 0.5 | Tetraploid male |
| 1X 4A | 0.33 | Metamale |

The theory of genic balance was given by Calvin Bridges (1926). It states that instead of XY chromo-somes, sex is determined by the genic balance or ratio between X-chromosomes and autosome genomes. In his studies, Calvin Bridges observed that in diploid cells sex is determined by the number of X chromosomes and that the Y chromosome played no part in this process. We now know that Sex-lethal (Sxl) is the immediate downstream target of a chromosome counting mechanism that distinguishes one X chromosome from two. Simply stated, Sxl is the female or male switch of fly sex determination. In XX animals, Sxl is ON and its expression directs all aspects of female development. Sxl expression in females also prevents the activation of the male-specific dosage compensation system. In XY animals, Sxl remains OFF, dosage compensation is activated, and male development ensues. By virtue of sitting at the top of a regulatory cascade that includes dosage compensation, loss of Sxl function in XX animals results in female-specific lethality, and inappropriate Sxl expression in XY animals leads to male-specific lethality.

The theory is basically applicable to Drosophila melanogaster over which Bridges worked. He found that the genic ratio X /À of 1.0 produces fertile females whether the flies have XX + 2A or XXX + ÇÀ chromosome complement. A genic ratio (X /À) of 0.5 forms a male fruitfully. This occurs in XY + 2A as well as X0 + 2A. It means that expression of maleness is not controlled by Y- chromosome but is instead localized on autosomes.

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It can be depicted from the table that whenever the ratio of X chromosomes to autosomes is 1.0 or above, the sex of the fly is female. If the ratio of X/A is 1.0 then it is a normal female, however, if the ratio is more than it is known as meta-female. If the ratio of the fly is 0.5 or less than 0.5, then the fly will be a male or meta-male respectively. However, if the ratio is between 0.5 and 1.0, it is an intersex with both male and female characters. Even though, it can be seen that Y chromosome as such has no role in determining the sex of fly yet it is essential for male fertility. In Drosophila sex determination mechanism, an X-linked gene called Sex lethal (Sxl) plays an important role.

A large number of X linked genes sets the level of Sxl activity in a zygote. For instance: - If the ratio between X chromosomes and autosomes (A) is 1.0 or above, the Sxl gene will become activated and the zygote will develop into a female. However, if the ratio is 0.5 or less, the Sxl gene is inactivated and the zygote will develop into a male. A ratio between 0.5 and 1.0 leads to mixing of signals and the zygote develops into an intersex with a mixing of male and female characters.

Abnormal chromosomal behaviour in insects sometimes produces sexual mosaics or gynandromorphs. In gynandromorphs, few parts of the animal are male whereas others are female. When such abnormal chromosomal transmission involves autosomes lodging genes that control easily recognized phenotypes, individuals may also be produced that are mosaic for phenotypes unrelated to sex phenotype. A few mosaics in Drosophila are bilateral intersexes with male colour pattern; body shape as well as sex comb on one half of the body and female characteristics on the other half of the body. Both male as well as female gonads and genitalia are present in mosaics or gynandromorphs.

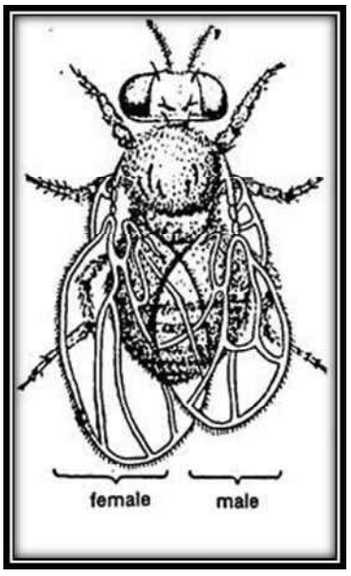


***Fig. 4.56*** *Gynandromorph in Drosophila*

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The reason behind formation of gynandromorph is faulty mitotic division at the first cleavage of the zygote. As we have already studied that in Drosphila, sex determination is based on the ratio of autosomes (A) to X chromosomes, so here, if one of the X chromosomes of an XX female zygote lags behind in the spindle, one daughter nucleus receives only one X chromosome, while the other receives two X chromosomes resulting in a mosaic body pattern.

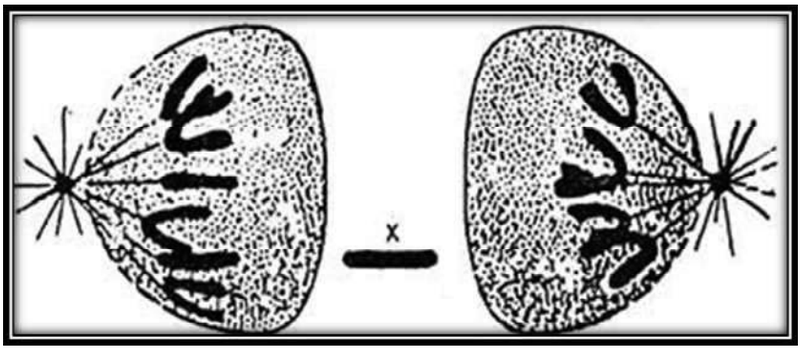


***Fig. 4.57*** *A Lagging Chromosome during the First Mitotic Division in Drosophila Embryo*

## G. Sex determination in Coenorhabditis Elegans

Coenorhabditis elegans is a nematode hermaphrodite species having two X chromosomes along with five pairs of autosomes. Sometimes, animals containing a single X chromosome as well as five pairs of autosomes are produced by meiotic non disjunction. These animals are males which are capable of producing sperms, however, they fail to produce eggs. Hermaphrodites are females in their vegetative parts (soma) but mixed in their genetic composition.

### Dosage compensation

Dosage compensation is the process by which organisms equalize the expression of genes between members of different biological sexes. Across species, different sexes are often characterized by different types and numbers of sex chromosomes. As sex chromosomes contain different numbers of genes, different species of organisms have developed different mechanisms to cope with this inequality. Different lineages have evolved different mechanisms to cope with the differences in gene copy numbers between the sexes that are observed on sex chromosomes. Dosage compensation is a process that balances expression of sex-linked and autosomal genes in the heterogametic sex. For example, one X chromosome in XX female mammals is randomly inactivated in every cell, and gene transcription levels from the X chromosome of XO or XY, male Drosophila is elevated to approximately equal the output of two sex chromosomes in XX females.

It is a mechanism by which species with sex chromosomes ensure that the homogametic sex does not have too much or the heterogametic sex too little activity of loci on the homogametic sex chromosome. The process, in organisms using a chromosomal sex determination mechanism (such as XX versus XY), that allows standard structural genes on the homogametic sex chromosome to be expressed

at the same levels in females and males, regardless of the number of homogametic sex chromosomes. In mammals, dosage compensation operates by maintaining only a single active X chromosome in each cell (see Lyon hypothesis), while in Drosophila it operates by hyper activating the single male X chromosome.

## 4.5.2 Sex Differentiation

In nature, different kind of mechanism exist for determining the sex of a species. The major aim of the sex determination is to identify the factors which are responsible for making an organism either male or female or sometimes hermaphrodite. So far, the mechanism of sex determination has been related to the presence of sex chromosomes whose composition differs in male as well as female sexes. However, in recent years, sex determination has been completely differentiated from sex differentiation. The primary basis of sex determination is on the basis of specific genes located on sex chromosomes as well as autosomes. Sex determination is recognized as a process in which signals are initiated for male or female developmental patterns.

However, during the process of sex differentiation, events occur in certain pathways which ultimately lead to the development of male as well as female phenotypes and also that of secondary sexual characters. Significant progress has been made in understanding the mechanism of sex in human beings as well as other mammals and new genes have been identified.

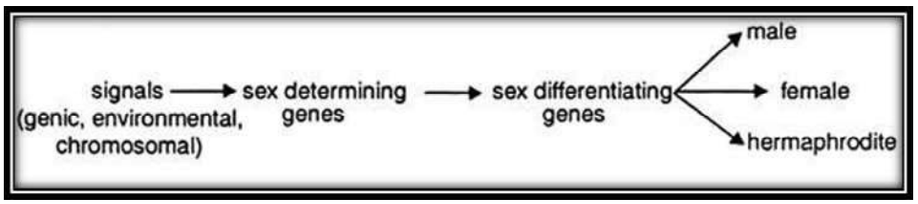


***Fig. 4.58*** *Mechanism of Sex Determination and Sex differentiation Involving Two Sets of Genes and the Signals*

The primary germ cells also known as PGCs in mammals are located on the floor of the yolk sac. From this place, these PGCs migrate to the genital ridges that ultimately develop dorsal to the hindgut region. In females, the germ cells become entirely surrounded by follicle cells.

In later stages, these germ cells increase tremendously in size to become oocytes. In males, the germ cells associate with cells of the genital ridge in a topological configuration which looks very similar to that of seminiferous tubules. In this form, the germ cells differentiate into spermatogonia.

These germ cells can differentiate into either oocytes or spermatogonia regardless of the fact that whether they carry XX (female) chromosomal component or XY (male) configuration of chromosomes. The fate of the germ cell is determined by their contact with the genital ridge.

This specifies that it is the cellular interaction which determines the pathway of differentiation. Male embryos which are obtained from genital ridge cells with the ability to direct differentiation to spermatogonia whereas female embryos form

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genital ridge cells with the ability to direct oocyte differentiation. Germ cells of either XX or XY embryos respond to these signals.

H-Y antigen plays a very significant role in determining the association of germ cells with that of genital ridge. H-Y antigen is a part of family of proteins. These antigens were determined by specific antibodies that react far more strongly with male embryonic tissue as compared to that of the female embryonic tissue. H- Y antigen is the product of a gene on the X-chromosome that is found in both males as well as females.

However, a gene on the Y-chromosome is present only in the males and it is essential to activate the H-Y gene and hence it is expressed only in the male embryo. Antibodies which are specific to H-Y antigen suggests that this particular antigen controls in males. For instance:- It has been observed that whenever the dissociated cells of new-born male rat testes are coated with the antibody, they re-associate in a form which looks very similar to that of the ovaries. The germ cells become completely surrounded by follicular cells.

However, in the absence of antibody, the testes cells associate in such a manner as to form tubules from which they come. These results depicts that the H-Y antigen which is there on the surface of the genital ridge cells of male embryo not only leads to the male tubular configuration, however, it further directs all succeeding differentiation of germ cells in the pathway to form spermatogonia.

On the other hand, the genital ridges of XX individuals direct germ cells to differentiate into oocytes whereas that of XY individuals guides germ cells to differentiate into spermatogonia regardless of their sex chromosomes complement.

The flexible nature of germ cells has also been observed in few fishes like sheep-shed fish. In this fish, all young fish begin life as small pink females. However, as they grow big, the colour of the young fish transforms to that of black and the sex changes to males. Initially, the germ cells differentiate into eggs, on the contrary, later in life these fishes differentiate into sperm. In the sea bass, females continue as females in the company of males.

The visual stimulus which is generated by the presence of the male confirms the hormonal balance guides the differentiation of the germ cells into eggs. Elimination or exclusion of the male leads to females changing their sex to become males.

The first transformed male then blocks or curbs all the others. In that individual, the germ cells now differentiate into sperm. Even though, the ultimate form of sperm as well as that of eggs varies about as much as any two differentiated cells, germ cells evidently are composed to follow either pathway. The ultimate verdict comes from the hormonal balance in the fish.

The hormonal system that controls the internal as well as physiological environment of the organism does not straightaway put an impact on the central process of sex determination. However, this hormonal system, is essential for the development of secondary sexual characters. Sex hormones are synthesized by the ovaries, testes majorly, however, some amount of sex hormones are also

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produced by the adrenal glands under the influence of hormones coming from pituitary.

In fact, steroids produced by the adrenals are chemically associated with those of gonads and that also impact the secondary sex characters. Ovaries as well as testes perform a dual function. They produce gametes as well as sex hormones. Further, sex hormones influence the development of secondary sex characters.

Sperms as well as eggs are produced within the body of an organism and need duct system to find their way out. In mammals, the genital ducts differentiate from primitive excretory ducts of the embryo. Here, the oviducts are derived from the Mullerian ducts, on the other hand, the vas deferens develop from the Wolffian duct. In early embryos, both these ducts are present, however, they are under the influence of hormones, in the later stages of development, one of these two ducts degenerates.

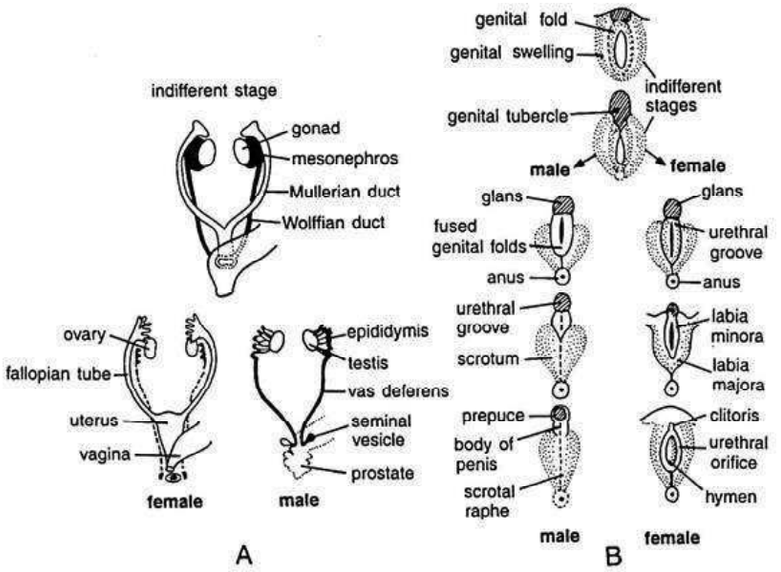


Fig. 15. Embryogenesis of the male and female phenotypes. (a) Formation of the internal organs of accessory reproduction. (b) Formation of the external genitalia.

***Fig. 4.59*** *Embryogenesis of Male as well as Female Phenotype: Figure 'A' Depicts the Formation of Internal Organs of Accessary Reproduction. Figure 'B' Represents the Formation of External Genitalia*

The primary sex hormone of the mammalian male i.e., testosterone is produced by the gonads. This testosterone circulates in the blood and transforms the Wolffian duct into the male reproductive duct i.e., vas deferens and thus stops it from deteriorating. Female embryos produce less testosterone, and in them the Wolffian duct degenerates.

The gonads of the male also produce a peptide hormone which is denoted by 'X'. This peptide hormone directs the degeneration of the Mullerian duct so that the female organs are not formed. In females, hormone X is not produced and hence, the Mullerian duct differentiates into the oviduct.

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**Check Your Progress**

22. What are autosomes?
23. Mention the animals/insects in which ZO-ZZ type of sex determination occurs.
24. What factor plays a significant role in determining the association of germ cells with that of genital ridge?

## 4.6 IMMUNOCONTRACEPTION

Immunocontraception refers to the use of an animal's immune system to prevent it from fertilizing offspring. Contraceptives of this type are not currently available for human use. Classically, immunocontraception involves the administration of a vaccine that induces an adaptive immune response that can causes an animal to become briefly infertile. Contraceptive vaccines have been used under several situations for controlling the wildlife population. However, due to the risk associated with this strategy specialists in the field do believe that major innovations are necessary before immunocontraception can become a practical form of contraception for human beings. Thus, far immunocontraception has focused majorly on mammals exclusively. There are several targets in mammalian sexual reproduction for immune inhibition. They can be organized into three categories:

- **Gamete production:** Organisms that reproduce by the phenomenon of sexual reproduction must first produce gametes, cells which have half the typical number of chromosomes of the species. Usually, immunity, that prevents gamete production also inhibits secondary sexual characteristics and thus has effects similar to castration. Castration refers to the removal of the testicles of a male animal or man.
- **Gamete function:** After the production of gametes in sexual reproduction, two gametes i.e., sperm from male and ova from female must unite during the process of fertilization to form a zygote, which again contains the typical number of chromosomes of the species. Methods that target gamete function prevent this fertilization from occurring and are true contraceptives.
- **Gamete outcome:** Shortly after the process of fertilization, a zygote develops into a multicellular embryo that in turn develops into a larger organism. In placental mammals, this phenomenon of gestation happens inside the reproductive system of the mother of the embryo. Immunity that targets gamete outcome induces abortion of an embryo while it is within its mother's reproductive system.

In the following sections, we shall discuss the different modes of immunocontraception in detail.

### 4.6.1 Gamete Specific Antigens

Gamete specific antigens comes under the category of gamete function. The process of sexual reproduction starts with production of sperm (smaller gamete produced

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by male) and egg cells (large gamete produced by female), which are produced via a process known as meiosis. These haploid cells are referred to as haploid as they contain half of the number of chromosomes as the parent. In sexual reproduction, a haploid sperm from the male parent fertilizes the haploid egg from the female parent to produce what is called a diploid zygote. Zygote is the technical term for a fertilized egg. The diploid number of chromosomes is the normal number of chromosomes found in all of the regular cells of an organism. The form of sexual reproduction which has been practiced by most of the placental mammals is anisogamous, i.e., it requires two different or dissimilar kinds of gametes, and allogamous, like each individual only produces one of the two kinds of gametes. Under this structure, fertilization requires two gametes, one from an individual of each sex, in order to occur. Immunocontraception targeting the female gamete has focused on the zona pellucida. Immunocontraception targeting the male gamete has involved many different antigens associated with sperm function.

Zona pellucida refers to a glycoprotein layer that surrounds the mammalian oocytes. Zona pellucida is present between the plasma membrane of oocytes and its protective layer known as corona radiata. Zona pellucida plays an essential role in successful fertilisation as well as the initiation of the acrosome reaction for the entry of sperm into the secondary oocyte.

There are four sperm binding proteins present in the zona pellucida namely $ZP_1$, $ZP_2$, $ZP_3$ and $ZP_4$. $ZP_1$ connects as well as forms cross-linking between $ZP_2$ and $ZP_3$. The glycoprotein $ZP_3$ is involved in sperm binding that induces the acrosome reaction. $ZP_2$ facilitates additional sperm binding. The zona pellucida layer is also referred to as the vitelline membrane or envelope in insects as well as other non-mammals. Zona pellucida plays an essential role in successful fertilisation. The major role played by zona pellucida are as follows:

- Zona pellucida is present between oocytes and the follicular cells and thus serves as a link between them at the time of oogenesis.
- Zona pellucida protects oocytes or eggs as well as embryos during the course of development.
- Zona pellucida begins the acrosome reaction for the binding as well as penetration of sperm cells.
- Zona pellucida helps in preventing the polyspermy.
- The binding to zona pellucida glycoprotein (ZP3) triggers acrosome reaction.
- Zona pellucida helps in species-specific fertilisation and thus prevents cross-breeding of different species.
- Zona pellucida regulates the interaction between free-swimming sperms as well as ovulated eggs and secondary oocytes.
- ZP1, ZP2, ZP3 and ZP4 which acts as the sperm binding protein are the primary targets for immunocontraception.
- The female mutants who lack the zona pellucida layer surrounding the egg are infertile.

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Immunity against zona pellucidae causes an animal to produce antibodies that themselves are bound by a zona pellucida. Thus, whenever, a sperm encounters an ovum in an animal immunized against zona pellucidae, the sperm cannot bind to the ovum because its zona pellucida has already been occupied by antibodies. Hence, fertilization does not occur.

In the year 1987, a pharmaceutical company known as Zonagen (later was renamed as Repros Therapeutics) began with the goal of developing zona pellucida vaccines which might act as an alternative to the surgical contraceptive method to be used for human beings. However, no vaccine was made commercially available till date.

Also, in late 1980s, researchers began working into the use of vaccines based around zona pellucidae harvested from pigs for the purpose of wildlife control. Such porcine zona pellucida (PZP) vaccines were tested in captive as well as domesticated horses in the year 1986 with fruitful results.

In placental mammals, fertilization usually occurs inside the female in the oviducts. The oviducts are located near the ovaries where the ova are produced. An ovum hence requires to travel a short distance to the oviducts for fertilization. In contrast, sperm cells must be highly mobile, as they are deposited into the female reproductive tract during copulation and thus must travel via the cervical region (in few species) and the uterus as well as the oviduct (in all the species) to reach an ovum. Sperm cells that are motile are spermatozoa.

Spermatozoa are protected from the male's immune system effectively by the blood-testis barrier. However, spermatozoa are deposited into the female in semen, which is an organic fluid ejaculated from the male reproductive tract which contains sperm cells. It also contains liquid to form a seminal plasma which helps to keep sperm viable. It primarily comprises of secretions from seminal vesicles, prostate gland, as well as bulbourethral glands. In this situation, antibodies generated by the male are deposited into the female along with spermatozoa. Due to this and the extensive travel in the female reproductive tract, spermatozoa are vulnerable to anti-sperm antibodies produced by the male in addition to waiting anti-sperm antibodies generated by the female.

In the year 1899, the discovery of the antibodies against sperm was made independently both by Serge Metchnikoff of the Pasteur Institute as well as by the Nobel prize laureate Karl Landsteiner.

### 4.6.2 Antibody Mediated Fertilization Blocks and Termination of Gestation

Majority of the research conducted into immunity has concentrated on human chorionic gonadotropin (hCG). This hormone is not required for the process of fertilization, however, this hormone is secreted by embryos shortly after the fertilization and formation of zygote. Hence, immunity against hCG does not prevent fertilization. Even though, it was found that anti-hCG antibodies prevent marmoset embryos from implanting in the endometrium of their mother's uterus.

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Human chorionic gonadotropin is another hormone that is produced naturally in women. HCG is made in the cells that make up the placenta at the time of pregnancy. hCG hormone has been observed in both urine as well as blood tests for pregnancy. Research studies indicates that levels of HCG double every third day and then reduces after the 12th week of pregnancy. The role of HCG in pregnancy is to maintain the production of progesterone that keeps the body warm. It basically helps to sustain the ovarian corpus luteum during pregnancy past the time it would normally decay as part of the regular menstrual cycle. It is the corpus luteum which secretes the hormone progesterone. It also helps in maintaining the lining of uterus during pregnancy.

Consequently, immunity against hCG during this time period would act as an aborticide, as suggested by the experiments conducted in baboons. In the scientific literature, 'birth control vaccine' rather than 'contraceptive vaccine' is used to mention hCG vaccines.

Contraception refers to an artificial method or other methods which are majorly used to prevent or avoid pregnancy as a consequence of sexual intercourse. When a sperm reaches the ova in women, the chances are that it fuses with it to form a zygote leading to the woman being pregnant. Contraception is a method that prevents this phenomenon by any of the following means:

- Restricting the egg production
- Keeping the egg distinct or far from the sperm
- By stopping the fertilized egg attaching to the lining of the womb

The choice of contraceptive method depends upon a lot of factors like:

- Effectiveness of the contraceptive method.
- Potential risks as well as side-effects associated with a specific contraceptive method
- Plans for future pregnancies.
- Personal preference.
- Medical condition
- Medicines that might interfere with the contraceptive approach

### 4.6.3 Other Contraceptive Technologies: Surgical Methods, Hormonal Methods, Physical Barriers, and IUCD

Apart from the immunocontraception, other contraceptive methods can be broadly classified into the following categories:

**1) Surgical methods**

A few surgical methods can also be used to block the gamete transfer. It comprises of blocking the vas deferens to prevent the transfer of sperms known as vasectomy. Similarly, fallopian tubes of the female can be blocked so that the egg will not reach the uterus known as tubectomy. Even though, surgical methods sound very

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effective, yet no contraception is 100% reliable. Male vasectomy also known as sterilisation is easier, as it can be done under local anaesthetic and is also more reliable. These methods are usually applied when the family is complete. However, one should discuss this with his/her partner as these methods are not reversible.

- **Female Sterilization—Tubal Ligation or 'Tying Tubes' or Tubectomy**: A woman can have her fallopian tubes tied (or closed) in such a way that both the sperm as well as eggs cannot meet for fertilization. The methodology can be performed either in a hospital or in an outpatient surgical center. The person can go home the same day. This method is effective immediately.
- **Male Sterilization–Vasectomy:** This surgical procedure is done to keep a man's sperm from going to his penis, in such a way that his ejaculate never has any sperm in it that can fertilize an egg. The methodology is usually performed- at an outpatient surgical center. The man can go home the same day. Recovery time is less than one week. After the operation, a man visits his doctor for tests to count his sperm and to make sure that the sperm count has dropped to zero. The procedure can take approximately 12 weeks. Another form of birth control must be used until the man's sperm count has dropped to zero.

  The advantages of surgical methods are:

  - Surgical methods are very effective.
  - No need to take pills, any hormones or insert any implant.

  The disadvantages of surgical methods are:

  - Irreversible methods**.** It is indeed difficult to reverse them.
  - Female sterilisation is done under a general anaesthetic. Thus, this methodology comes with slight risk associated with general anaesthesia. The tummy might feel bloated as well as sore for a few days even after the operation.
  - Men can have discomfort, bruising and swelling after the operation for a week or so.
  - Vasectomy becomes effective after a while and hence males are advised to use another form of contraception.

### 2) Hormonal Methods

In this method, tablets or drugs are taken orally. These contain small doses of hormones that prevent the release of eggs and thus fertilization cannot occur.

### a) Combined Oral Contraceptive Pill

The Combined Oral Contraceptive (COC) pill is usually when people say they are 'on the pill'. This is one of the most popular contraceptive methods used by women. However, despite taking pills, a few women become pregnant as it depends upon how well the woman uses the pill. The oral pill contains two female hormones namely oestrogen and progesterone. Different brands suit different people.

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The advantages of using combined oral contraceptive pill are:

- Oral pills are very effective.
- Side-effects are rare.
- Oral pills also help to ease painful as well as heavy periods.
- oral pills slightly reduce the chance of few cancers – like cancers of the ovary and womb (uterus).
- The effects go away quickly when you stop it.

The disadvantages of using combined oral contraceptive pill are:

- There is a small risk of serious problems (particularly related to blood clots).
- A few women show side-effects like bleeding between periods, mood swings as well as breast tenderness.
- It should be taken on time otherwise can lead to pregnancy.
- It can't be used by women with certain medical conditions like women having uncontrolled high blood pressure, specific types of migraine or a women having past history or family history of blood clots.
- There is a very slightly higher risk of breast cancer for women who take it.

**b) Progestogen-Only Pill**

The progestogen-only pill (POP) is sometimes also known as "the mini-pill". It contains only a single hormone, i.e., progestogen hormone. Approximately, 3 to 90 women in 1,000 using the POP will become pregnant.

The advantages of using progestogen-only pill are:

- Reduced risk of serious problems than the COC pill.
- Several women who can't take the COC pill due to a medical condition are safe to use the POP, like smokers over the age of 35 and women with specific types of migraine.
- Women can use it safely during breast feeding too.

The disadvantages of using progestogen-only pill are:

- Irregular periods
- A few women show major side-effects.
- A women needs to track the timings of consuming pill. With some POPs, women have to take it within three hours of the time she has taken it the day before. In others, there is a 12-hour window before it becomes a "missed pill".
- It carries a slight high risk of breast cancer

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### c) Contraceptive Patch

The contraceptive patch comprises of the same hormones as the COC pill however in patch form. It functions exactly in the same way as described for COC pill. Approximately, 3 to 90 women in 1,000 will become pregnant using it. In this methodology, a contraceptive patch is stuck on to the skin in such a way that the two hormones are continuously delivered to the body.

The advantages of using contraceptive patch are:

- Contraceptive patches are very effective
- They are easy to use
- A female need not to remember about consuming the pill everyday
- Periods gets lighter, painless as well as regular
- Even if you have sickness (vomiting) or runny stools (diarrhoea), the contraceptive patch is still effective.

The disadvantages of using contraceptive patch are:

- A few women might show skin irritation.
- Even though, it is very small and often go unnoticeable design, a few women still feel that the contraceptive patch can be seen.
- Sometimes, it falls off and hence becomes less effective
- A few women show side-effects like bleeding between periods, mood swings as well as breast tenderness.
- It can't be used by women with certain medical conditions like women having uncontrolled high blood pressure, specific types of migraine or a woman having past history or family history of blood clots.
- There is a very slightly higher risk of breast cancer for women who take it.

### d) Contraceptive Vaginal Ring

The contraceptive vaginal ring also comprises of the same hormones as the COC pill. These hormones i.e., both progesterone as well as oestrogen have effects on a female body that can prevent her from becoming pregnant. It is a flexible, see-through ring which is approximately 5 cm in diameter. This contraceptive vaginal ring sits perfectly in the vagina for around three weeks and then a female can have one week without it. After exactly one week, female needs to put in a new ring into her vagina. Contraceptive vaginal ring is about as effective as the COC pill at preventing pregnancy.

The advantages of using contraceptive vaginal ring are:

- It is very effective and easy to use methodology
- Women need not to remember to consume a pill every day.
- Even if the female is suffering from sickness (vomiting) or runny stools (diarrhoea), the contraceptive vaginal ring is still effective.
- Periods becomes light, painless as well as regular

The disadvantages of using contraceptive vaginal ring are:

- A few women (as well as their partners) find it uncomfortable during sexual activity
- It might irritate vagina and leads to soreness or discharge.
- A few women show side-effects like bleeding between periods, mood swings as well as breast tenderness.
- It can't be used by women with certain medical conditions like women having uncontrolled high blood pressure, specific types of migraine or a women having past history or family history of blood clots.
- There is a very slightly higher risk of breast cancer for women who take it.

### e) Contraceptive injections

Contraceptive injections comprise of a progestogen hormone which slowly releases into the body. They are very effective. However, approximately 3 to 60 women in every 1,000 using it will become pregnant. An injection is needed every 8-13 weeks, depending on which injection is used.

The advantages of using contraceptive injections are:

- Contraceptive injections are very effective
- Females need not to remember about consuming pills everyday
- Once the body of the female is used to the hormone, she can miss her periods or report very light periods. If the female is suffering from heavy periods, this could be really effective method.
- Females can take contraceptive injections whilst breastfeeding.

The disadvantages of using contraceptive injections are:

- In this methodology, periods become irregular or infrequent or lighter or may stop altogether.
- It takes a while for the cycle to be back to normal. This might cause delay in pregnancy, in case the women is looking to start a family. It may take up to a year for your period to come back.
- A few women report side effects like weight gain, mood swings as well as headaches.
- Cannot undo the injection, so if side-effects happen they might continue for longer than 8-13 weeks.
- The injections cause a very slight thinning of bones.
- There may be a very small increase in the risk of breast cancer as well as cancer of the neck or the womb or the cervix region.

### 3) Physical barriers

In barrier methods, the fertilization of ovum and sperm is prevented with the help of barriers. Barriers are available for both males as well as females. Condoms are

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barriers made of thin rubber that are used to cover penis in males and vagina in females.

- **Diaphragm or cervical cap:** All these physical barrier methods are positioned inside the vagina in order to cover the cervix or to block sperm. The diaphragm is shaped like a shallow cup. The cervical cap is a thimble-shaped cup. Before sexual intercourse, an individual must insert them with spermicide in order to block or kill sperm.
- **Sponge:** The contraceptive sponge comprises of a spermicide and is positioned in the vagina where it fits over the cervix. The sponge works approximately 24 hours, and must be left in the vagina for at least 6 hours after the last act of intercourse, at which time it is removed and discarded.
- **Male Condom:** One of the most popular method world-wide. It is used by the man. A male condom keeps sperm from getting into a woman's body. Latex condoms, which is one of the most common types of condoms, helps in preventing the pregnancy, spread of HIV as well as other sexually transmitted diseases. "Natural" or "lambskin" condoms also help prevent pregnancy, however, they may not provide protection against STDs, including HIV. Condoms can only be used once.
- **Female Condom:** As the name suggests, these condoms are worn by the woman. The female condom helps keeps sperm from getting into her body. It is usually packaged with a lubricant and is available at drug stores. It can be inserted up to eight hours before sexual intercourse. It also helps in preventing the transmission of sexually transmitted diseases.
- **Spermicides:** Spermicide functions by killing the sperm and thus come in many forms like foam, gel, cream, film, suppository, or a tablet. Spermicides are positioned or applied in the vagina no more than one hour before engaging in sexual activity. To reduce the risk of unwanted pregnancy, spermicide can be used in addition to a male condom, diaphragm, or cervical cap. They are easily available at drug stores.

The advantages of barrier methods include:

- There are no serious medical risks or side-effects associated with using condoms.
- Condoms help to provide protection from sexually transmitted infections also known as STDs
- Male condoms are easily available and one of the most popular contraceptive choices

The disadvantages of barrier methods are:

- They are not quite as dependable as compared to other methods.
- They need to be used properly every time a person engages in sexual activity.
- Male condoms occasionally split or come off.
- Diaphragms and caps usually need to be fitted.

- A few male reports that condoms interrupt sex or make it feel less spontaneous.

**4. Implants methods**

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Contraceptive devices like the loop or Copper-T are positioned in the uterus to prevent pregnancy. A contraceptive implant refers to a small device that can be positioned under the skin. These implants contain a progestogen hormone which slowly releases into the body. Approximately, 1 woman in 2,000 using the implant will become pregnant each year. Implant method involves a small minor operation. An injection of local anaesthetic is used to numb the skin. Each implant lasts only three years, after which it should be removed.

The advantages of implant method are:

- This method is very effective.
- Women need not to worry about consuming the pills everyday
- These implants are reversible and hence periods return quickly once they are removed.
- Periods tends to be very light or non-existent.

The advantages of implant method are:

- Irregularity of the periods. They may become lighter or stop altogether.
- A few women develop side-effects however these tend to relax after the first few months.

**a) Intrauterine contraceptive device**

An intrauterine contraceptive device (IUCD) is also referred to as a coil. A plastic or a copper device is put into the womb (uterus). This device lasts for five or more years. However, in between 6-8 women, 1 in 1,000 will become pregnant in one year of use of this method.

The advantages of using intrauterine contraceptive device are:

- This method is very effective.
- Women need not to worry about consuming the pill everday
- It can easily last for a period of 5-10 years.
- There are no hormones released in the body, hence no side effects due to hormonal changes in your body.

The disadvantages of using intrauterine contraceptive device are:

- Periods generally becomes heavier, painful
- There is a very small risk of serious problems.
- A few female report uncomfortable having the coil put in.

**b)** Intrauterine System

Intrauterine System (IUS), refers to a plastic device that contains a progestogen hormone which is put into the womb (uterus). The progestogen is released at a

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slow but uniform rate. Approximately, 1 to 2 women in 1,000 will become pregnant in one year of use of this method. The IUS is also used to treat heavy periods (menorrhagia).

The advantages of intrauterine system are:

- It is one of the most effective methods
- Females need not to worry about consuming the pills everyday
- Periods usually become light or stop altogether.

The disadvantages of intrauterine system are:

- A few side-effects might be associated with progestogen methods like seen in case of other methods like oral contraceptive pills, implant. However, they are much less likely, as little hormone gets into the bloodstream.
- It is uncomfortable having it put in and does not last as long as the other type of coil.

**5. Natural method**

Natural methods involves avoiding the chances of meeting of sperms as well as ovum. In this methodology, the sexual act is avoided from day 10th to 17th of the menstrual cycle because, during this period, ovulation is expected and therefore, the chances of fertilization are very high.

The advantages of natural method are:

- There are no side-effects or medical risks.
- Anybody can use this method safely as long as they need to.

The disadvantages of natural method are:

- It is not as reliable when compared to other methods.
- Fertility awareness needs proper instruction and takes approximately 3-6 menstrual cycles to learn properly.
- If the cycle of the females are not very regular, this method can be very unreliable.

The lactational amenorrhoea method also known as LAM is another type of natural family planning for women who are breastfeeding and not having periods. For women who recently had a baby and are breastfeeding, the Lactational Amenorrhea Method (LAM) can be used as birth control when three conditions are met: 1) amenorrhea (not having any menstrual periods after the delivery of baby), 2) fully or nearly fully breastfeeding, and 3) less than 6 months after delivering a baby. LAM is a temporary method of birth control, and another birth control method must be used when any of the three conditions are not met.

**6) IUCD**

Emergency contraception can be used if the individual had sex without using contraception, or there was some mistake associated with contraception. For instance, a split condom or missing an oral contraceptive pills. Emergency options

are a pill or an IUCD (also known as a coil). Two modes of emergency contraception are as follows:-

- **Copper IUD:** Women can have the copper T IUD inserted within five days of unprotected sex.
- **Emergency contraceptive pills:** Women can take emergency contraceptive pills up to 5 days after having unprotected sex. However, the sooner the better approach works here.

Thus, all the contraceptive methods mentioned above are effective. However, no method is 100% reliable. Further, the effectiveness of few contraceptive methodologies depends upon how the user use them. For instance: the combined oral contraceptive (COC) pill (often referred to as "the pill") is more than 99% effective if taken correctly. However, if you miss pills or are sick (vomit) then it becomes less effective. Other user-dependent methods include:

- Barrier methods (male and female condoms, diaphragms and caps).
- The progestogen-only pill (POP).
- Natural family planning.

On the other hand, a few other contraceptive methods are not so user-dependent and need to be renewed only infrequently or never. These methods tend to be more reliable and include:

- The contraceptive injection.
- Contraceptive implant.
- Intra-uterine contraceptive devices (IUCDs) - also known as 'coils'.
- Surgical methods

**Check Your Progress**

25. What does immunocontraception involve?
26. Where is the Zona pellucida located and what is its role?
27. Mention the role of hCG in pregnancy.
28. What is a contraceptive patch?
29. What is a contraceptive sponge?
30. List the conditions to be met for Lactational Amenorrhea Method (LAM) to be used as birth control.

## 4.7 CARE AND BREEDING OF EXPERIMENTAL ANIMALS

In majority of the countries, laboratory animals are protected by the Law known as 'Cruelty to Animals Act'. A researcher has to get license from the Home Department to begin experimenting on animals. The health as well as general well-being of the experimental animals depend upon a lot of factors like care, handling,

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maintenance, accommodation, quality as well as quantity of food, etc. To keep animals healthy, the care taker staff has to look after the cleanliness of the animal rooms as well as cages, timely supply of nutritious food and water for the animal, to move to a cooler or warmer place and to provide air. The general guidelines for the care and maintenance of the animals are outlined as follows:

- **Water:** Health of the animal depends not only on quality or quantity of food but also on its hydration state. Experimental animals are provided with sufficient supply of fresh clean drinking water from a bottle (250 ml capacity) which generally remains attached to the outside of the cage. The water is held in a container which is composed of 6-9 mm glass tubing through a rubber bung to an accessible position inside the cage; the outlet tubing is about 3 mm.
- **Quality and Quantity of the Food:** The fitness of the experimental animal depends upon the supply of nutritious food. A balanced diet which is rich in carbohydrate, fat, protein, vitamins, minerals as well as other trace elements has to be given regularly for ensuring good health of the experimental animal. Diet is supplied commercially in the form of cubes or pellets. Small quantities of green stuff are also to be supplied.
- **Hygiene:** Hygiene is of utmost significance to ensure the good health of the experimental animal. A clean environment helps to keep the animal disease free i.e., free from bacteria, viruses and all other pathogens. Animals, when breeding, should not have their cages changed too often. Cages should be cleaned frequently. For cleaning, the cages must be boiled in soapy water solution; alternatively, to be kept immersed in a solution of disinfectant like 3% Lysol. Lysol, however, should not be used in cleaning the cages of rabbit as the smell of this chemical distresses the animals.
- **Litter:** Here, litter refers to the dropping (faecal matter), vomit as well as to the food particle not eaten up by the animal. A layer of absorbent material (for instance: soft wood sawdust's, sugarcane piths) should be spread to a depth of approximately 1/2 to 1 inch (1.25-2.5 cm) on the bottom of the cages.
- **Cages:** Cages should be tailor made according to the routine of the animal. Each species of animal requires its own type of cage. Cage should be large enough to provide free movement as well as space for performing some exercise. An optimal cage also ensures proper ventilation.
- **Labelling:** Every case should be provided with a holder or socket for a small card of 6-9 cm for record of the experiment (date, identifying marks of animal, nature of experiment and specimen).
- **Ventilation:** Animal room where the cages are kept or breeding experiment has been set up must be properly air-conditioned or ventilated or at least ten changes of air in each hour are needed.
- **Humidity:** Each and every species requires an optimum humidity for developing properly. This requirement varies from species to species. For

instance- Humidity of animal house ranges between 45% for rabbits to 65% for mice.

- **Marking Animals:** It is essential to mark the animals for easy identification. White or light coloured animals can be marked by staining the fur with a strong dye (like carbol fuchsin, eosin etc.). Rabbits may be marked in ears with the help of a needle dipped in India ink. Rats as well as mice are punctured in ear and fowls are marked by numbered metal tags on legs clipped via the loose skin of the wing.
- **Detection of Disease in Animals:** Animals must be visited on daily basis to check for any disease as well as to ensure the timely availability of essentials. To see nose movements of the animals and to see any animal remaining quiet and still. Such animals may be separated and investigated for the cause of disease. For instance: - Diarrhoea due to *Salmonella Typhimurium* is a common natural disease of rabbits, guinea pigs and mice. Coccidiosis (Protozoan disease) and pseudo tuberculosis (caused by Yersinia pseudo tuberculosis) are also common natural disease of animals. Explosive epizootics have been observed with *S. Typhimurium* infection in which practically the whole of a colony of animals have been destroyed. Experimental animals might suffer from following common disease:
- **Foot-and-Mouth Disease (FMD):** FMD is a viral disease caused by Picornavirus or Aphthovirus. It usually affects cloven-hoofed animals such as bovid including cattle, water buffalo, antelope, bison, deer, goats, etc. Can also affect hedgehogs, llamas, elephants and alpacas. FMD is transmitted through animal-to-animal spread, close contact and even aerosol spread and fomites. Humans can also transmit this disease through clothes, skin, inanimate objects, feed supplements containing infected animal products, etc. Standing water and uncooked food can also contain the virus. Animals which are not susceptible to the disease such as wolves and dogs can also spread the disease. The symptoms include:
  - Blisters in the mouth, nose and on feet that rupture typically after 24 hours. Blisters on teats of cows has also been observed.
  - Weight loss and lack of appetite
  - Smacking of lips along with frothing of mouth.
  - Lameness
  - Drop in production of milk in cows and swelling in testicles of mature males.
  - Fever
  - Shivering

  FMD can be controlled by:
  - Vaccination is available for FMD but they don't provide cross protection against the other strains of the virus.

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- Close monitoring of the animals is required and affected animals have to be quarantined from the other animals quickly. Usually affected animals are culled to prevent further transmission of infection.
- Export restrictions are imposed on countries which have are experiencing an ongoing outbreak.
- Cleaning and disinfection of affected equipment, clothes or buildings.
- Infected carcasses should be safely disposed by burial or incineration away from other animals.
- Decontamination of clothes and equipment used by animal handlers and farmers working with animals.

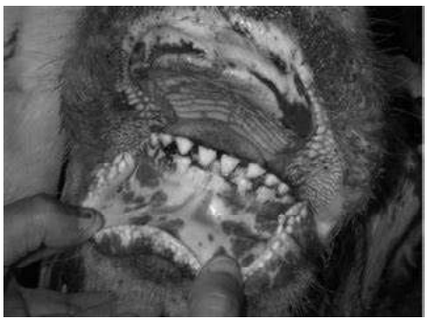

***Fig 4.60*** *Animal Suffering from Foot and Mouth Disease*

- **Bovine Spongiform Encephalopathy (BSE) or Mad cow disease:** Prions which are misfolded protein cause mad cow disease. Bovid like cattle are the main victims of the disease. It can be directly transmitted when healthy animals come in contact with tainted tissues belonging to infected animals. It can also be caused by feed given to animals containing prions. Its symptoms include:
  - Difficulty in walking and balancing
  - Behavioural changes such as aggression, nervousness, etc.
  - Weight loss
  - Decrease in milk production

  It can be controlled by:
  - Banning feeding of meat and bone meal to cattle
  - Import control and feeding regulations
  - Observing animals carefully to catch affected animals quickly
- **African Swine Fever Virus (ASFV):** It is caused by a large, double stranded DNA virus belonging to the Asfarviridae family. Warthogs, bush pigs and domestic pigs are mainly affected by it. It can be spread by soft ticks belonging to genus *Ornithodoros,* which act as a vector spreading the disease to pigs. Swine eating infected pork products also can lead to transmission. It can also be transmitted through direct or indirect contact with infected pigs, faeces or their bodily fluids. Its symptoms include:

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- High fever
- Difficulty in standing along with weakness
- Diarrhoea
- Vomiting
- Red or blue blotches around ears and snout
- Large number of pigs dying within 10 days in a pig farm

It can be controlled by:

- Quarantining the pigs when they are moved to a different place
- Keeping pigs away from wild pigs and wild boars and the areas where they are found
- Movement restriction and herd depopulation
- Culling of pigs before they can infect others

- **Equine Infectious Anaemia (EIA) or Swamp fever:** It is caused by retrovirus called Equine infectious anaemia virus and is a lentivirus. It is a disease that only affects horses. It is transmitted by bloodsucking insects such horse-fly or deer-fly. It is spread through blood, milk, body secretions of infected animal, or contaminated surgical equipment can also transmit the disease. Mares can also pass on the disease to foetus through placenta. Its symptoms include:
  - High fever
  - Anaemia due to breakdown of RBC
  - Swollen lower abdomen and legs
  - Weak pulse and irregular heart beat

  It can be controlled by:
  - There is no cure for the disease as it's a retrovirus so confirmed cases are euthanized as they are lifelong carriers of disease.
  - A vaccine is available called 'Chinese Live Attenuated EIA Vaccine' developed in China and USA is developing one.
  - Affected horses are quarantined and branded. The equipment is not shared between horses.

***Fig 4.61*** *A Horse Suffering from Equine Infectious Anaemia*

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- **Echinococcosis or Hydatid Disease:** It is caused by parasitic disease of tapeworms of Echinococcus type. Vulnerable animals include Dogs, sheep, kangaroos, pigs, dingoes, etc. which are part of the tapeworm's life cycle. It can be spread by animals scavenging on infected carcass which can consume cysts or eggs of the tapeworm and become carriers of the disease. Coprophagic flies, arthropods and carrion birds can also act as mechanical vectors for the eggs. Its symptoms include:
  - Reduced production of milk, meat and wool
  - Reduced birth rate
  - Multiple cysts in liver, kidneys, bones or testes of animals can cause organ damage.
  - Bone fractures

  It can be controlled by:
  - Preventing access of dogs to livestock carcasses.
  - Giving dogs' anthelmintic to kill adult tapeworm
  - Vaccinating sheep and other cattle to prevent development of larval stage.
  - Using anthelmintic bait for foxes to reduce number of carriers.

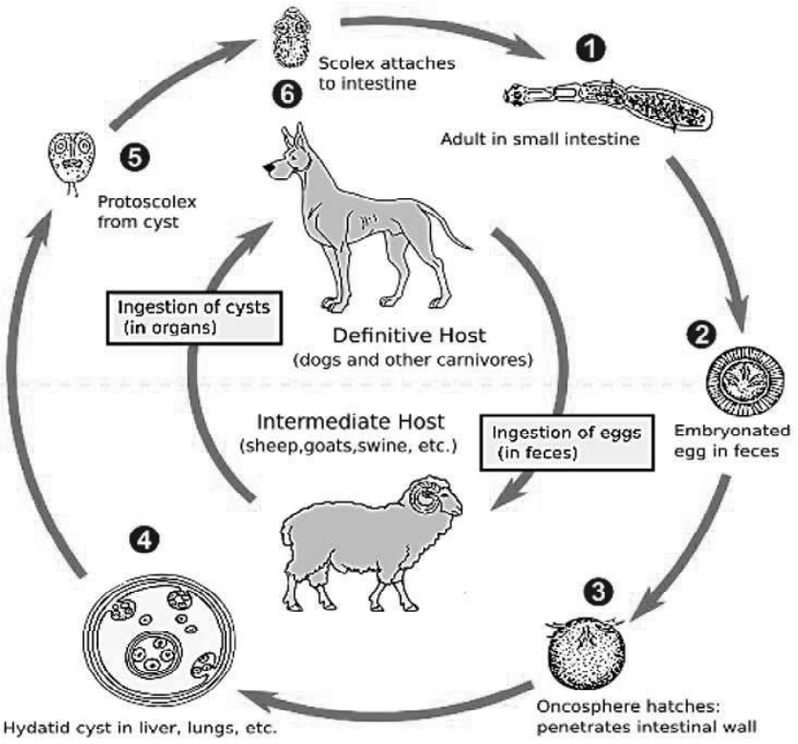


***Fig 4.62*** *Life cycle of Echinococcus*

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- **Paratuberculosis or Johne's disease:** It is caused by bacteria called Mycobacterium avium subspecies paratuberculosis. It affects ruminants but has also been found in rabbits, foxes and birds. Infected animals can spread the bacteria in their excretion which can then infect normal cattle. Infected mothers can spread it to their calves through their udder. Its symptoms include:
  - Diarrhoea and wasting
  - Weight loss
  - Decreased milk production
  - Roughening of hair coat

  It can be controlled by:
  - Pasteurization is utilized to kill the causal agent.
  - Avoid overgrazing so that cattle doesn't consume soil or manure.
  - Infected cows should be kept away from healthy cattle.

***Fig 4.63*** *Animal Suffering from Paratuberculosis or Johne's Disease*

- **Virulent Newcastle Disease (VND):** The causal agent is Newcastle disease virus (NDV) which is a variant of avian orthoavulavirus 1 that is a negative sense, ssRNA virus. It affects both domestic and wild bird species. It can spread through direct contact between healthy birds and bodily fluids of infected birds, and bird droppings and secretion from eyes, mouth and nose. Humans can also transmit it by picking up infected discharge unknowingly and bring it to healthy birds. Amazon parrots are carriers of disease but don't show symptoms. Its symptoms include:
  - Production of rough or thin shelled eggs
  - Green, watery diarrhoea
  - Swelling of tissues around eyes and neck
  - Drooping wings
  - Coughing
  - Twisting of head and neck

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It can be controlled by:

- Infected animals should be isolated immediately.
- Birds should be vaccinated before bringing them to a new flock.
- Immediate disposal of dead infected birds

***Fig 4.64*** *A Chick suffering from Virulent Newcastle Disease (VND)*

- **Rabies:** Rabies lyssavirus, a neurotropic virus causes rabies. Dogs, wolves, foxes, coyotes, lions, mongoose, skunks, bats, monkeys and cats can be affected by rabies. Rabies is transmitted through direct contact with an infected animal such as through broken skin, saliva or mucous membranes of eyes and nose. Its symptoms include:
  - Weakness
  - Fever
  - Headaches
  - Encephalitis
  - Hypersalivation
  - Hydrophobia
  - Aerophobia
  - Confusion
  - Difficulty in swallowing

  It can be controlled by:
  - Vaccination of both stray and domestic dogs
  - Keeping cattle away from wild animals or bats
  - Oral vaccine in baits to vaccinate wild animals

***Fig 4.65*** *Dog suffering from Rabies*

- **Chytridiomycosis:** Batrachochytrium dendrobatidis, a fungus is a waterborne pathogen causing the disease. This disease is a global killer of amphibians like frogs. Can also affect crayfish. The fungus releases its zoospores in the water causing its spread and enter the skin of the amphibians. Direct contact between frogs and tadpoles or infected frogs leaving spores on ground or other streams. Its symptoms include:
  - Reddening of ventral skin
  - Convulsion with hind limbs extension
  - Sloughing of epidermis and its accumulation over the body
  - Minute skin tags over the body along with ulcers
  - Lethargy
  - Loss of reflexes and posture becomes abnormal.

  It can be controlled by raising juvenile fish in concrete raceways as the parasite lives in mud.
- **White-Nose Syndrome (WNS):** The syndrome is caused by Pseudogymnoascus destructans which is a fungus. The syndrome is a fatal disease which affects bats which hibernate and are found primarily in United States and Canada. Vulnerable animals are brown bat, northern long-eared bat, gray bat, etc. A bat coming in physical contact with an infected bat is the Primary mode of infection. A bat can also get it from infected caves or rocks. Human-caused transmission is also a mode of infection by carrying the fungus inadvertently from one bat roost to another through clothes, shoes, etc. Its symptoms include:
  - Presence of white fungal growth on the membrane of wings, ears, tail and on muzzles of bats infected with the disease.
  - Scars and lesions can also be present on the wings of the bat due to the fungus.

  It can be controlled by:
  - Bats treated with probiotic bacteria, pseudomonas fluorescence are more likely to survive post-hibernation.

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- Closing caves and abandoned mines to all humans except for essential work can limit the spread of disease.
- Decontamination procedures for humans who have come in contact with bats or roosting sites.

**Chronic Wasting Disease (CWD) or Zombie deer disease:** It is caused by misfolded form of Prion Protein (PrP) called prions which are found in central and peripheral nervous systems. These prions can then cause other normally folded prion protein to abnormally folded protein. CWD affects members of the deer family such as Mule deer, red deer, elk, moose, etc. It can be directly transmitted by infected animals, their bodily fluids or tissue's contact with another animal. Infected animal's droppings can contaminate the soil and the grass growing on it and transmit the disease to the animal feeding on the grass. Maternal transmission can also occur in animals such as Rocky Mountain Elk. Sharing of food and water sources can also help spread the disease. Its symptoms include:

- Difficulty in movement.
- Weight loss
- Behavioural changes such as listlessness, lowering of head, decreased interaction with other animals, tremors and repetitive walking in a set pattern.
- Grinding of teeth and increased salivation.
- Increased drinking and urination.

It can be controlled by:

- CWD is a fatal disease and cannot be treated or prevented with vaccines as it is a neurodegenerative disease.
- As it is contagious, it is best to isolate the affected animal and then kill it. Keep the carcass away from other animals and incinerate or bury it.
- Observation of animals is crucial to catch the disease before it spreads further.

***Fig. 4.66*** *Animal Suffering from Chronic Wasting Disease*

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- **Sylvatic Plague:** The disease is caused by the plague bacterium, Yersinia pestis. It primarily affects rodents like prairie dogs and some mustelids like the black-footed ferret. Transmission primarily occurs through flea bites and contact with infected bodily tissue or fluids. Its symptoms include:
  - High body temperature
  - Weakness and chills
  - Dehydration
  - Lack of energy and appetite
  - Enlarged spleen and lymph nodes

  It can be controlled by:
  - Using pesticide in rodent dens to kill fleas is the primary method right now to control the plague in the wild.
  - Oral vaccines are being developed to control the spread of the disease.

***Fig. 4.67*** *Animal suffering from Sylvatic Plague*

- **Whirling Disease:** Myxobolus cerebralis, a parasitic protozoan, causes the disease. Juvenile salmonids are mainly affected by the disease. Stocking of infected fish with other fish can cause the spread of the parasite. It can also be spread by the alimentary tracts of fish-eating migratory birds. The symptoms include:
  - Convulsive movements along with backwards jerking movement.
  - Increased death rate in fry population
  - Increased rate of breathing.
  - Erratic darting movements and also chasing their tail.
  - Spinal curvature and skull deformation.
  - Darkening of skin from vent to tail.
  - Gill plates get shortened.

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It can be controlled by:

- Can be controlled by raising juvenile fish in concrete raceways as the parasite lives in mud.
- And taking out the infected fish from the breeding ponds.

***Fig. 4.68*** *Suffering from Whirling Disease*

- **Chytridiomycosis:** Batrachochytrium dendrobatidis, a fungus is a waterborne pathogen causing the disease. This disease is a global killer of amphibians like frogs. It can also affect crayfish. The fungus releases its zoospores in the water causing its spread and enter the skin of the amphibians. Direct contact between frogs and tadpoles or infected frogs leaving spores on ground or other streams. Its symptoms include:
  - Reddening of ventral skin
  - Convulsion with hind limbs extension
  - Sloughing of epidermis and its accumulation over the body
  - Minute skin tags over the body along with ulcers
  - Lethargy
  - Loss of reflexes and posture becomes abnormal.

  It can be controlled by raising juvenile fish in concrete raceways as the parasite lives in mud.

***Fig 4.69*** *Animal Suffering from Chytridiomycosis*

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- **Turtle fibropapillomatosis:** Chelonid alphaherpesvirus 5 (ChHV-5), a virus that causes epithelial tumours on surface of tissue. Sea turtles are mainly affected by this disease. Turtle leeches act as mechanical vectors which transmit the disease to other turtles. These are turtle ectoparasites and by feeding on an infected turtle's blood, are able to spread it to other turtles. Its symptoms include:
  - Large epithelial tumours that can affect swimming, vision, feeding, etc.
  - Wartlike masses on skin
  - Severe emaciation

  It can be controlled by:
  - Surgical removal of tumours is the treatment method mainly used along with electrochemotherapy, photodynamic therapy and CO2 laser surgery.
  - Cleaning of ocean as biotoxins can cause the disease.

***Fig 4.70*** *Animal Suffering from Turtle Fibropapillomatosis*

- **Bluetongue disease or The Dancing Disease:** Bluetongue virus (BTV) is the causative agent of this disease. Bluetongue disease affects ruminants such as sheep, cattle, yaks, goats, deer, elk, pronghorn antelope, etc. Virus is transmitted by midges such as Cullicoides imicola, Cullicoides variipennis, etc. Ticks or sheep keds can also transmit the virus. Its symptoms include:
  - Nasal discharge and stertorous respiration
  - Swelling of lips and tongue makes the tongue blue
  - Swelling of face
  - Excessive salivation
  - High body temperature
  - Foot lesions
  - Knee-walking
  - Torsion of neck

  It can be controlled by:
  - Quarantining the affected animal and providing vaccines with live attenuated virus can help stop the spread of disease.
  - Reducing the number of midges which act as vectors.

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- Preventing animals from going out when the midge activity is at its maximum.
  - o Rabbits suffering from diseases like Coccidiosis (diarrhoea), pseudo tuberculosis (caseous lymph nodes) and Taenia pisiformis (numerous cysts in omentum).
  - o Guinea pigs suffering from diseases like Protozoan diseases (coccidiosis toxoplasmosis), pseudo tuberculosis, S. typhimurium, abscess in lymph node (Streptococcus group C), haemorrhagic septicaemia (P. multocida) and viral pneumonia and paralysis.
  - o Mice suffers from disease like Mouse typhoid (caused by *S. Typhimurium, S. Enteritidis*) may be responsible for severe epizootics. Other diseases include: Ectromelia (mouse pox, caused by a pox virus related to the vaccinia virus), *Streptobacillus Moniliformis*, infection, viral infections and Taenia taeniaeformis infection (large cysts in liver).
  - o Hamsters suffers from disease like Streptococcal and staphylococcal infections, distemper (virus) and foot rot (caused by mites).
  - o Salmonellae of various types (e.g. *S. Typhimurium* causing diarrhoea), *S. Pullorum* (outbreak of typhoid), coccidiosis, avian leucosis (involving lymph node and bone marrow), parasitic infections due to lice and mite, virus diseases (infectious laryngotracheitis, fowl pest due to Newcastle virus).

- **Recording Animals' Temperature:** Regular recording or determination of animal's temperature helps to track whether the animal is suffering from any disease or not. Clinical thermometer is liberally smeared with sterile petroleum jelly as well as the blunt-ended rectal thermometer is introduced into the rectum or vagina to a depth of about 3 to 3.5 cm.
- **Prevention of Disease:** Newly arrived animals must be kept in a special quarantine room separated from other animals. They must be kept under careful watch of a skilled researcher or staff. The minimum quarantine period lasts for 10-14 days. Animals falling sick during the period should be kept in quarantine and steps must be taken to look for finding out the cause of the illness. Animal infected experimentally with bacteria or viruses or fungi or any other deadly pathogen should be kept in separate isolation rooms to prevent spread of infection to other animals. Here, it is essential to differentiate between the two terms i.e., quarantine and isolation. Quarantine refers to restriction on the movement of people, animals as well as goods which is intended to prevent the spread of disease or pests. It is often used in connection to disease and illness, preventing the movement of those who may have been exposed to a communicable disease, yet do not have a confirmed medical diagnosis. It is distinct from medical isolation, in which those confirmed to be infected with a communicable disease are isolated from the healthy population.

- **Insect pest:** Bed bugs, fleas, lice, mites, ticks, flies, mosquitoes and cockroaches may all infest the animal house. These can be controlled by 0.5% insecticidal sprays or 10% DDT.

***Table 4.4*** *Data of Experimental Animals*

| Features | Rabbit | Guinea-pigs | Mice | Rats | Hamsters | Fowl |
|---|---|---|---|---|---|---|
| Rectal temperature (No temperature below 40°C is considered as pathological) | 38.7° – 39°C | 37.6° – 38.9°C | 37.4°C | 37.5°C | 36.7° – 38°C | 41° – 60°C |
| Oestrous cycle | — | — | 4-5 days | 4-5 days | 4-5 days | — |
| Normal respiration rate | 55 | 80 | — | 210 | — | 12 |
| Pulse rate per minute | 135 | 150 | 120 | — | — | 140 |
| Gestation period | 28-31 days | 59-72 days | 19-21 days | 21-23 days | 16-17 days | — |
| Weaning age | 6-8 weeks | 14-21 days | 19-21 days | 23-28 days | 3-4 weeks | — |
| Mating age | 6-9 months | 12-20 weeks | 6-8 weeks | 70-84 days | 7-9 weeks | — |
| Litters | 4 yearly average litters – 4 | 3 yearly, average litters – 3 | 8-12 yearly, average litters 7-8 | 7-9 yearly average litters – 7 | 3-4 yearly average litters – 5 | — |
| Room temperature | 15.5°–18.5°C | 18.5°–21°C | 20°–21°C | 18.5°–21°C | 20°–22°C | — |
| Humidity | 40 –45% | 45% | 50 –60% | 45–55% | 40–50% | — |
| Weight *(adult)* | 0.9–6.7 kg | 120 g | 25–28 g | — | — | — |
| CAGES : Galvanised iron | 2' × 2' × 1 1/2' (for one) | 4' × 6' × 1' 8" (high) (for 25) | 6" × 12" × 6" (deep) (for 6)* | Same as guinea pigs | 17" × 7" × 9" (for one) | 24" tall and 20" × 20" |
| Diet *(daily)* | Pellets or daily 30 g mixture of 1 part oats plus 3 parts of bran | Pellet diet | Pellet diet | Dry pellet | Pellet diet | Pellet diet |
| Diet supplemented by | Green vegetables plus hay<br>Plenty | Cabbage 60g plus hay<br>Plenty | —<br>Plenty | —<br>Plenty | Fresh green foods. Milk added to bran/oats<br>Plenty | Green food<br>Plenty |

* Cages made of thick polypropyline (plastic) are now commercially available for mice which are commonly used

Key : foot = ' inch = "

## 4.7.1 Experimented Animals

In this section, we will discuss a few regularly used experimental animals:

### Rabbit

Animals must be handled with care. The rabbit is picked up from cage with the ears by one hand in a firm grip and another hand is placed under the hind-quarters to support the weight of the animal. The animal is then lifted softly. After removing from cage, the animal is placed in a non-slippery place as it otherwise feels insecure and becomes frightened. When the doe (female of the rabbit) is on heat, vulva and vagina become red in colour, swollen and moist. The doe is placed in the cage of buck (male).

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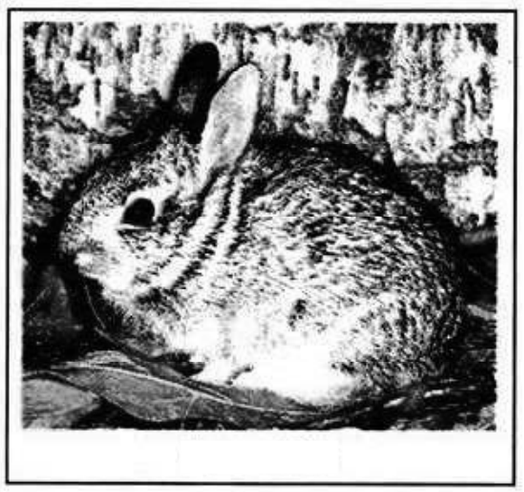

***Fig 4.71*** *Experimental Animal- Rabbit*

## Guinea Pig

One hand is placed across the back of the animal with the thumb behind the shoulders and the other fingers well forward on the opposite side. The animal is then lifted slowly supporting its entire weight with the other hand placing the palm uppermost under the hind quarters.

***Fig 4.72*** *Guinea Pig*

## Armadillo

It has been observed that whenever nine-banded armadillo is inoculated with lepra bacilli, widespread infection develops with extensive multiplication of the bacilli. Due to this, the cuts are produced which resemble lepromatous leprosy. Natural infection by a mycobacterium resembling lepra bacillus has also been observed in some wild caught armadillos in Texas and Mexico. Consequently, the wild armadillo, should be screened for mycobacterial infection and kept in quarantine for 3 months before inoculation. European hedgehog bred in captivity is suitable for propagation.

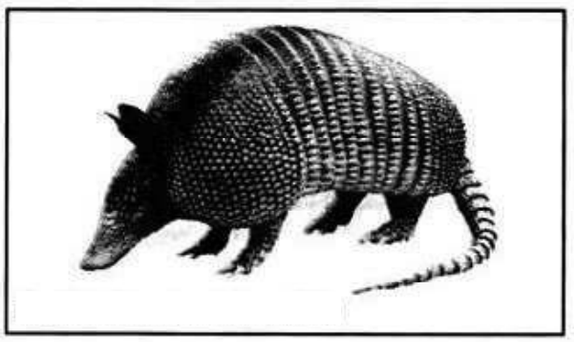

***Fig 4.73*** *Nine Banded Armadillo*

### Mice

Handling of mice is comparatively easy when compared to other large animals. An assistant can hold the tail of the mice with the left hand and slowly raises the hind limbs from floor of the cage. The mouse placed in this position cannot turn round and bite. With the help of right finger and thumb, a fold of skin close to the head is held.

### Rat

The handling of rat is very similar to that of guinea pig. It is handled in the same way as guinea pig by experienced operator.

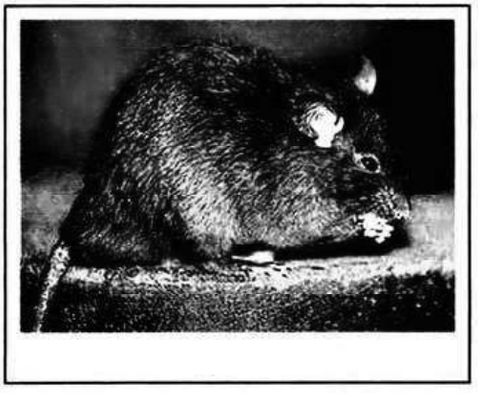

***Fig 4.74*** *Experimental Rat*

### Material Inoculated

The materials which are inoculated include:

- **Urine, CSF, Blood and Serous Fluids:** These specimens are inoculated with a medium- bore needle however in case of tenacious material, such as pus or sputum, it is injected via a wide-bore needle.
- **Culture Material:** Liquid cultures are inoculated via a medium- bore needle. Growths on solid media are first scraped off and then suspended either in broth or in saline, alternatively, the diluting fluid may be poured on the culture which is then emulsified with a wire loop.

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- **Tissues:** Small fragments of tissues like brain, spleen, liver and kidney are first homogenized. The process of homogenization includes crushing these materials with the help of a suitable diluent in a tissue grinder. When tissue is well-ground, more saline is added and allowed to stand for a short time. After the homogenized tissue has settled to the bottom of the mortar, the supernatant fluid is drawn into a syringe. When intravenous injection is to be given, care must be taken that no large particles are injected. The suspension should be centrifuged at a very low speed and the supernatant fluid is then used for the intravenous administration.

**Experimental Procedure Inoculation of Material**

The procedure involves:

- **Scarification:** In this process, the animal is first clipped and its flank is shaved to remove all the hair. Otherwise, depilating powder (mixture) may be used to remove hair from the animal. Skin is cleansed by applying alcohol and finally it is allowed to evaporate. Several parallel scratches are made by a sharp sterile scalpel, which might be sufficient to draw blood. The specimen is then rubbed into the scarified area with the side of the scalpel. This technique is majorly employed for the propagation of Vaccinia virus in rabbit.
- **Sub-Cutaneous Inoculation:** In this process, inoculation is done in the loose tissue about the flank or into the abdominal wall. Hairs are clipped. The skin is sterilised with iodine as well as alcohol and then pinched up, and finally the needle is inserted. A maximum amount of 5 ml can be introduced. However, it is recommended to use a syringe of 2 ml or 5 ml. This method is the same for rabbit as well as for guinea pig and removes superficial ulceration when tuberculosis specimen is injected. In fowls, sub-cutaneous inoculation is done in pectoral or thigh regions.
- **Intra-Cutaneous Inoculation:** Hair are removed from the flanks of the animal either by plucking or shaving or by depilating powder. For this methodology, a 1 ml all-glass tuberculin syringe, which is fitted with a short needle is used. After pinching up the skin of the animal present between the thumb and forefinger region, the point of the needle is inserted at the top of the fold in such a way that the level of the needle is towards the surface of the skin. The needle passes into the dermis only, as near as the surface. Generally, a volume of approximately 0.2 ml is injected. When a lot of tests are to be done, injections are to be given about one inch apart and not too near the middle line of the abdomen. No more than ten injections should be made in one animal. This methodology is majorly used in testing cultures of the diphtheria bacillus for toxigenicity. Generally, white guinea pigs (300-400 g) are used for carrying out this procedure.
- **Intra-Peritoneal Inoculation:** In this process, animal is held by an assistant and the material is inoculated in the peritoneum in the midline in the lower half of the abdomen. A maximum solution of 5ml can be inoculated intra-peritoneal. This methodology is usually done with guinea pigs and mice are also intra-peritoneal inoculated.

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- **Intravenous Inoculation:** In Intravenous inoculation, specimen is directly introduced into the circulation. Intravenous inoculation is done in a lot of experimental animals like rabbit, guinea pig, mouse and rat as described below:
- **Rabbit:** In rabbit, the marginal vein of the ear is easiest option for performing intravenous inoculation. The hair present over the ear is removed by the process of dry shaving with the help of a sharp razor. Vein may be distended by rubbing with a piece of cotton wool or by even by holding the ear on an electric bulb (heat dilates blood vessels). The rabbit is held in position by an assistant or placed in a special box in such a way that only the head region protrudes out. The operator faces the animal and holds the ear horizontally by left hand and injects the material into the vein by a sterile syringe. Rabbits are majorly used for diagnostic purposes. They are also used for production of immune sera which is used in diagnostic purpose.
- **Mouse and Rat:** Intravenous injection may be given into a vein at the root of the tail with the help of a fine needle. The tail vein is dilated by placing in water at a temperature of around 45°C. Hence, a maximum volume of 0.5 ml can be inoculated.
- **Fowl:** Fowl is positioned on its side and feathers over an area in the elbow are plucked. After this, a very large brachial vein can be seen on the bone which is present underneath the skin. Intravenous administration of specimen or culture is made carefully with the help of fine needle.

**Intra-cerebral inoculation:** Intra-cerebral inoculation is done in a lot of experimental animals like:

- **Rabbit:** For intracerebral inoculation, firstly, rabbit is anaesthetized with ether. Hair present over the head region is shaved. After disinfection of skin of head, a short incision is made with the help of a scalpel. Through the scalp at a point 2 mm lateral to the sagittal suture and 1.5 mm anterior to the lambdoidal suture. The skull of the animal is perforated with a trephine or mechanical drill. A 0.45 ml of material is then introduced into the occipital lobe of brain with the help of a needle. Rabbits may also be inoculated in frontal lobe of brain, at a point 2 mm lateral to the median plane joining the two external canthi of the eyes.
- **Mouse and Rat:** In mouse as well as rat, the area of skull is disinfected as mentioned above. A 0.03 ml of fluid is injected directly with the help of a one ml syringe at a site which is present midway between the outer canthus of the eye and the point of attachment of the pinna of the ear. Approximately, 1/8 to 1/6 inch point of needle is introduced via the skull.

**Intra-testicular:** Intra-testicular inoculation is done in a lot of experimental animals like:

- **Rabbit:** Testes of the rabbit lie in the abdominal region. The animal lies on its back and the testes of the animal are made to descend on scrotum by stable pressure on the belly. The testes are fixed by a lab assistant. After this, skin is disinfected and disinfection of the skin is followed by 0.2 to 0.4

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ml of inoculum which is directly introduced into the centre of a testis with the help of a hypodermic needle.

**Ophthalmic:** In rabbit, only a single eye is inoculated by dropping a drop of material via a Pasteur pipette. Before installation of the material, one eye may be scarified by anaesthesia.

**Intra-nasal inoculation:** Intra-nasal inoculation is done in a lot of experimental animals like:

- **Mouse:** Mouse should be preferably kept in an inoculating chamber which is particularly designed for the purpose. The inoculating chamber has been designed in such a way that the operator is not at a risk of inhaling the infective material. Failing this, the operator should use a mask. The animal is then anaesthetized with ether and as soon as its breathing has become deep, a volume of 0.1 ml is introduced into the anterior nares one side only with the help of an automatic pipette.
- **Ferrets and Fowls:** Under light ether anaesthesia, as soon as the regular respiration is established, 0.5 to 1.5 ml material is dropped from a Pasteur pipette into the nares. For studies of Distemper virus in ferret and New Castle virus and other virus infections in fowls.

  48 hours suckling mice are used for the isolation of herpes simplex, enteric as well as other arbo-viruses. The litters must be handled with great care as well as cleanliness to avoid biting from their mothers. Doses of 0.03 ml subcutaneously, 0.05 ml intra-peritoneal or 0.03 ml intra-cerebrally can be used depending upon the need of experiment. Route of inoculation could be intra-peritoneal or intra-cerebral or sometimes both can be performed together depending upon the requirement of experiment.

## 4.7.2 Bioethics

Ethics or moral philosophy refers to a branch of philosophy that "involves systematizing, defending, and recommending concepts of right and wrong behaviour". The field of ethics, along with aesthetics is majorly concerned with the matters of value; these fields comprise of the branch of philosophy known as axiology. Ethics seeks to resolve questions of human morality by defining concepts like good and evil, right and wrong, virtue and vice, justice and crime. As a field of intellectual inquiry, moral philosophy is related to the fields of moral psychology, descriptive ethics, and value theory. Three major areas of study within ethics recognized today are: Meta-ethics, concerning the theoretical meaning and reference of moral propositions, and how their truth values (if any) can be determined; Normative ethics, concerning the practical means of determining a moral course of action; Applied ethics, concerning what a person is obligated (or permitted) to do in a specific situation or a particular domain of action.

Bioethics refers to the study of the ethical issues arising out from the advances made in the field of biology, medicine as well as technologies. Bioethics proposes the discussion related to moral discernment in society and it is usually related to medical policy and practice, but also raises wider concerns like environment and

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well-being. Bioethics are concerned with the ethical questions that arise in the relationships among fields like life sciences, biotechnology, medicine, politics, law, theology and philosophy. It involves the study of values concerning to primary care, other branches of medicine ('the ethics of the ordinary'), ethical education in science, animal, and environmental ethics. Ethics also relates to many other sciences outside the realm of biological sciences and Bioethics is also claimed as a new ethic to answer complex questions of contemporary society.

**Ethical issues concerning experimental animals**

Experimentation on animals has been conducted for a very long duration of time. One of the major arguments for using animals for experimentation is that the data obtained can be useful for human beings and hence these animals can be used for a variety of purpose ranging from drug testing to treatment of disease. However, the utilization of non-human subjects for research purpose has often been an area of intense debate. Do we actually need to use animals for medical research or are we simply guilty of speciesism? However, those intellectuals, which are in favour of using animals in research studies argue that the data obtained by experimenting on animals cannot be used for humans as such. Thus, the potential benefits obtained by conducting animal experimentation is less when compared to harm being done to the animal. It has been suggested that the physiological system of majority of animals is simpler when compared to that of complex system of human beings. Moreover, these animals lack cognitive capabilities as well as level of our (human beings) autonomy. This would limit our obligation toward them and would also limit their rights, leading to a form of speciesism. Thus, in order to prevent undue suffering, ethical considerations in animal studies are very essential. Usually, before animals are used for research studies or before beginning of any kind of experimentation on animals the research protocol must be reviewed by animal ethics committees. The guiding principle of these committees is usually guided by the 3 R's:

- **Replacement:** Replacement of animal experimentation with alternative methods like mathematical models, computer simulation or using in vitro biological systems. These methodologies that can be used to replace or complement the utilization of animals must be taken into consideration even before beginning with any procedure or method involving use of animals.
- **Reduction:** Reduction in the number of animals used for carrying out the experiment. It involves using minimum number of animals to obtain the desired scientifically valid results. Additionally, scientific projects involving the use of animals must not be repeated or duplicated unnecessarily.
- **Refinement:** Refinement of projects and techniques used to minimize impact on animals which involves: The animals chosen for carrying out the experimentation must be of an appropriate species and quality for the scientific projects concerned taking into account their specific biological properties, including genetic constitution, behaviour, and microbiological, nutritional and general health status.

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A local animal care committee should be constituted to look after the animals that are housed for carrying out experimentation. The committee should also ensure if the animals that are required to test the hypotheses are listed or not. The animal care committee should also limit the sample sizes of the animals and methodology to be used while carrying out the experimentation. The animals should have access to veterinary care. All personnel who work with the animals should be trained enough in both the experimental methodology as well as ethical handling of the animals.

The legislation of animal experimentation should be based on the idea that it is ethically acceptable to conduct these experiments under specific conditions. This demonstrates the significance of research ethics which is what determines that how these animals should be treated while carrying out experimentation. This involves having a clear rationale for why should a hypothesis needs to be tested using animals. There must be a reasonable expectation that the experiment will generate valuable data. The study design of the experiment should be such that it seeks to minimize the number of animals which shall be used in carrying out the studies. However, it should be noted that this minimal number is sufficient to validate the data.

Further, all the researchers involved in animal handling must be trained in handling the specific species in the study. The design of the experimental study should aim at minimizing the pain of experimental animals. Experimental animals must be anesthetized before conducting any experiment. Further, repeated surgical procedures on the same animal must be avoided at all cost. All in all, the experimental animals must be treated in the most humane way. Only skilled personnel should perform surgical procedures and anesthetization of the animals in the study.

The national centre for the replacement, refinement, and reduction of animals in research (NC3Rs) has issued ARRIVE (Animal Research: Reporting of In-Vivo Experiments) guidelines which are proposed to improve the reporting of research using animals. This checklist offers information that needs to be provided in the different section of the manuscript like experimental animals, study design, experimental methodology, housing as well as husbandry, ethical statements, and many more.

However, there are intellectuals which completely oppose the use of animals for carrying experimental studies. Their major argument is that the data obtained by experimenting on animals is not sufficient or reliable or comparable enough to be used for human beings having complex physiological systems. Further, there are also those who argue that we are embarrassed of speciesism when we justify animal research since it advances human medicine. The middle ground aims at utilizing non-human subjects for highlighting the significance of animal ethics. The major arguments put forward by those in support of animal testing is the amount of data generated by conducting such studies. However, researchers should be trained to follow all the ethical guidelines before beginning with the experimental studies. It has also been observed that generally scientific journals do not publish data that has not been obtained in with appropriate ethical considerations.

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## Ethical Issues Concerning the Products of Animal Experimentation (Transgenic Animals)

Genetic engineering or genetic modification is a field of biotechnology and bioengineering which aims at uses of multiple tools and techniques to modify an organism's genetic makeup. Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. Transgenic organism are able to express the transgenes effectively due to the high similarity between the genetic sequences for proteins among different species. As we studied in the previous section, Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding. Hybrids refer to the transgenic organisms produced when germ cells (reproductive cells) from two different species combine to form an embryo which later develops into full-fledged organism. For instance- In nature, Mule is the best example observed in nature. It is produced by the copulation between a horse and a donkey. Further, chimeras are produced in labs by artificially combining genetic material from two different organisms into a single species. As studied in the above section, the field of transgenic biotechnology projects diverse possibilities in the positive way, however these possibilities are not without possible risks. Some of the issues that need to be taken into consideration are as follows:

### Social Concerns

The social concerns include:

- Provided the blending of animal and human DNA results in chimeric organisms having high degrees of intelligence never witnessed before in nonhuman animals, should these chimeric organisms be given exceptional rights as well as protection?
- What, if any of the social or legal controls or reviews should be placed on carrying out such research?
- What unintentional personal, social, and cultural consequences could result?
- One of the most important points for consideration is that who will have right to these technologies?
- How will be limited rather we can say inadequate resources like medical advances and novel treatments—be allocated to carry out such advance research.
- Various environmentalists, bioethicists and animal rights activists have argued that it is not ethically correct to generate animals that would go through pain as a consequence of genetic alteration. For instance- genetic alteration could lead to the development of a pig with no legs and hence such experimentation should be prohibited

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## Extrinsic Concerns

The extrinsic concerns include:

- What about the health risks associated with consumption of transgenics and genetically modified foods and crops?
- What if the GMOs have adverse effects on the health of human?
- The risk of creating novel diseases by combining different DNAs for which no treatment exist.
- What are the long-term effects on the natural environment when genetically modified organism (GMOs) transgenic organisms are released?
- What if this result in the development of highly resistant weeds?
- What if it leads to development of antibiotic resistance by insect pests and other pathogens?
- What if it disturbs ecosystem in irreversible ways?
- What if it leads to development of highly pathogenic viruses and bacteria which might be dangerous to the existing crops?
- Should the research and development in the field of transgenics be limited to some extent and, if so, how should the limits decided? Also, it should be taken into consideration how to impose should the limits both nationally as well as internationally?

## Intrinsic Concerns

The intrinsic concerns include:

- Are there any fundamental issues with creating new species in labs by using the methodology?
- Are species boundaries 'rigid' or 'inflexible' or they should be viewed as a scale? What, if any, the outcomes of crossing the species boundaries?
- Are chimeras as well as transgenics organisms likely to suffer than 'traditional' organisms?
- Will transgenic interference in humans generate physical or behavioural traits which may or may not be easily distinguishable from what is typically supposed to be 'human'?
- What if any, research taken in the field of genetic engineering is considered morally impermissible and banned. For instance, research and development in the military sector for destroying other nations.
- Will these interferences re-establish what it means to be 'normal'?

## Legal Implications

There are several legal implications of transgenics as cited by numerous bioethicists from time to time. A few such legal issues will be highlighted in the following section:

- Several bioethicists have expressed their concern that such molecular technologies could be used to produce a race of sub-humans that could be exploited.
- Delineation of species boundary is yet another concern.

- In future, if bioengineers succeed in creating a chimera between an animal and human, shall that creation be given all the rights as well as protection?
- Some bioethicists argue that the description of 'human being' should be more open and defensive, rather than more limiting. However, others objects argue that more open or liberal descriptions can create a monetary hindrance to patenting creations that could be of possible use. This question needs legal addressing.
- Medical fraternity has expressed concern that athletes/celebrities might start using bioengineering to get an upper hand over their competitors.
- People with enormous wealth might be willing to genetically manipulate their children in order to make them successful in their respective fields or to have better looking kids or to make them better athletes/musicians/actors/ scientists or whatever else that might give them a benefit or upper hand.
- This practice might increase the ever-widening gap between common and rich people in the society. Also, this will close the door for less fortunate (in terms of money) to achieve something great even if they are talented.

### Concept of Species Boundary

Some individuals believe that crossing species boundaries is unnatural, immoral, and in violation of God's laws, which assumes that species boundaries are rigid and delineated. However, research conducted by researchers and published in several notable books and journal articles exhibit that the notion of fixed species boundaries is a hotly debated topic. Some bioethicist's points out that species concept is a broad concept and a variety of species concepts exist in the scientific literature such as: morphological, typological, biological, evolutionary, phylogenetic and ecological. All these species concepts reflect varying theories as well purpose for which researchers conceptualize and employ different species. However, it is said then if species boundaries are just a topic of a designation and there is no as such existence of true or fixed boundaries to cross, then several philosophical oppositions to transgenics organism will become less challenging.

The queries regarding the morality of transgenic animals is highly questionable yet numerous potential health risks might be associated with the transplantation of cells or organs between species. For instance, the transplantation of cells or organs between animal and human species might cause some fatal zoonotic disease. Following the rule of precautionary principle, i.e., better safe than sorry approach, it will be in the interest of general public to ban xenotransplantation trials using non-human primates until and unless the protocols have been marked sufficiently safe and ethical issues have been publicly addressed.

**Check Your Progress**

31. Mention the causative organism of bovine spongiform encephalopathy.
32. Who are the vulnerable animals of Virulent Newcastle disease?
33. State the mode of spread of whirling disease.
34. How are guinea pigs handled?

## 4.8 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

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1. The foster mother serves as an incubator and does not make any genetic contribution to the offspring.
2. One of the first things which is required to generate transgenic animals is the ability to transfer embryos.
3. A genetically superior cow/buffalo is administered artificial hormones with FSH-like activity to induce super-ovulation. Under the influence of the FSH-like hormone, the genetically superior female produces numerous eggs instead of single egg produced in general.
4. There are three common methods of artificial insemination (AI):-
   - Vaginal method
   - Recto vaginal method
   - The speculum method.
5. Cell preservation techniques vary according to the type and age of cells. There are two primary types of cell preservation namely: - cryopreservation and hypothermic preservation.
6. Pheromones are the substances that are released into the environment by an individual of a species that elicits a specific social response in members of the same species. They are the chemicals used by insects and other animals to communicate with each other.
7. The major difference compared to classic IVF cycles is that in IVM the patient does not receive (or receives at very low doses) the previous hormonal treatment. Therefore, IVM does not require ovarian stimulation, unlike regular IVF cycles.
8. An ectopic pregnancy occurs when a fertilized egg implants itself outside the uterus.
9. Transgenic mice can be used to stimulate both the onset and progress of human disease and provide a system for testing therapeutic agents. Mouse models for human diseases, such as Alzheimer's Disease, Arthritis, Hypertension, Coronary Diseases have been developed.
10. The following is the classification of stem cells according to their origin: embryos, fetus, infant and adult.
11. Multipotent cells are capable of differentiating into a closely related family of cells i.e. these cells that can only give rise to cells of the tissue from which they are isolated.
12. The most critical step of *in vitro* cell culture is the selection of an appropriate growth medium. By definition, a culture medium/growth medium refers to a liquid or gel produced to maintain the growth and propagation of cells, microorganisms and small plants.

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13. Germline gene therapy refers to the transfer of a section of functional DNA to gametes i.e. either eggs or sperm. Thus, the effects of gene therapy will be passed onto the patient's offspring as well as subsequent generations.
14. The entire ZIFT process takes approximately four weeks, including the period when the patient must first undergo hormone treatment called superovulation.
15. ICSI is one of the most successful technique to overcome infertility as it involves injection of mechanically immobilized spermatozoa achieves fertilization at a higher rate than the injection of motile spermatozoa directly inside the woman.
16. Two approaches are in use for embryo cloning.
    - Nuclear transfer
    - Use of embryonic cells
17. In recent years, the technique of embryo sexing has become popular for the detection of genetic disorders. By using embryo biopsy, it is possible to stop the transfer of embryos with genetic abnormalities and undesirable traits.
18. Down syndrome and trisomy 18 syndromes are two genetic disorders that are usually screened for as part of prenatal genetic testing.
19. The structure of RNA is very similar to that of DNA. The only difference is that it is a single stranded molecule and it contains ribose sugar. Also, a major difference between DNA and RNA is that RNA contains Uracil (U) instead of base thymine (T). Furthermore, in RNA the amount of purines is not equal to pyrimidines.
20. FISH is a special technique that can be used to view changes in chromosomes that result from genetic variations.
21. Gene expression profiling tests can analyze a number of different genes within an individual cancer cells to predict his/her risk of cancer recurrence. The results obtained by this methodology help the medical practitioner who may benefit from additional (adjuvant) treatment after surgery.
22. All the other normal chromosomes, apart from the sex chromosomes of an individual are known as autosomes.
23. ZO-ZZ type of sex determination occurs in few butterflies as well as moth species. This is exactly in contrast to the condition seen in cockroaches as well as grasshoppers. Here the females have odd sex chromosome (AA + Z) while the males have two homomorphic sex chromo¬somes (AA + ZZ).
24. H-Y antigen plays a very significant role in determining the association of germ cells with that of genital ridge.
25. Immunocontraception involves the administration of a vaccine that induces an adaptive immune response that can causes an animal to become briefly infertile.

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26. Zona pellucida is present between the plasma membrane of oocytes and its protective layer known as corona radiata. Zona pellucida plays an essential role in successful fertilisation as well as the initiation of the acrosome reaction for the entry of sperm into the secondary oocyte.
27. The role of HCG in pregnancy is to maintain the production of progesterone that keeps the body warm. It basically helps to sustain the ovarian corpus luteum during pregnancy past the time it would normally decay as part of the regular menstrual cycle.
28. The contraceptive patch comprises of the same hormones (oestrogen and progesterone) as the COC pill however in patch form. It functions exactly in the same way as described for COC pill. In this methodology, a contraceptive patch is stuck on to the skin in such a way that the two hormones are continuously delivered to the body.
29. The contraceptive sponge comprises of a spermicide and is positioned in the vagina where it fits over the cervix. The sponge works approximately 24 hours, and must be left in the vagina for at least 6 hours after the last act of intercourse, at which time it is removed and discarded.
30. The Lactational Amenorrhea Method (LAM) can be used as birth control when three conditions are met: 1) amenorrhea (not having any menstrual periods after the delivery of baby), 2) fully or nearly fully breastfeeding, and 3) less than 6 months after delivering a baby.
31. Prions which are misfolded protein causes bovine spongiform encephalopathy or mad cow disease.
32. Virulent Newcastle disease affects both domestic and wild bird species.
33. Mode of spread of whirling disease include:
    a. Stocking of infected fish with other fish can cause the spread of the parasite.
    b. Can also be spread by the alimentary tracts of fish-eating migratory birds.
34. Handling of guinea pig is done by placing one hand across the back of the animal with the thumb behind the shoulders and the other fingers well forward on the opposite side. The animal is then lifted slowly supporting its entire weight with the other hand placing the palm uppermost under the hind quarters.

## 4.9 SUMMARY

- A gamete is a mature reproductive or sex cell with a haploid number of chromosomes (i.e., just one set of dissimilar chromosomes) and the ability to fuse with another haploid reproductive cell to generate a diploid zygote.
- As the ruminant animal like (cow) produces one egg at time, it can carry only one pregnancy at a time. Hence, it is possible to increase the production of female animals by increasing the number of mature eggs from a given female, fertilize them and transfer (implant) the embryos (fertilized eggs) into a foster mother (recipient).

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- In the normal reproductive cycle of a non--pregnant female, a single ovarian follicle (out of the 20 ovarian follicle that develop during the cycle) matures and ruptures and releases out one fertile egg at a time.
- By increasing the concentration of gonadotrophic hormone, a large number of ovarian follicles can be induced to ripen as well as to produce more eggs. This phenomenon is referred to as superovulation or multiple ovulations. Superovulation may yield approximately 8-10 eggs at a time.
- Occasionally, the process of multiple ovulations, a phenomenon also known by the name of superovulation as well as embryo transfer are considered together which is then called as MOET i.e., multiple ovulation embryo transfer technology. The embryos developed in the super ovulated animals (described above) are recovered within a time span of 6-8 days of artificial insemination.
- The first embryo transfer technology (ETT) project in the country was initiated by NDDB (National Dairy Development Board) in 1987 by the establishment of a central ET laboratory at Sabarmati Ashram Gaushala (SAG), Bidaj.
- 'Embryo transfer' (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals.
- MOET is carried out in the following steps: programming, heat detection, embryo collection, and then embryo transfer (cryopreservation or transformation).
- Gene delivery or embryo transfer can be done through microinjection, biolistic particle delivery system, etc.
- Embryo transfer technology helps in genetic improvement, conservation of endangered species, genetic engineering, somatic cell nuclear transfer, production of transgenic animals, production of monoclonal antibodies, etc.
- In vitro oocyte maturation (also known as IVM, or IVM) refers to a procedure where immature eggs mature *in vitro*, i.e. in the laboratory. To perform the procedure of IVM, the most common is that the patient does not undergo a hormonal treatment of ovarian stimulation (if she receives it is at low doses) and this is one of its main advantages.
- Super-ovulation is a critical requirement for successful assisted reproduction technology in horses. Induction of multiple ovulations in the mare would increase the number of oocytes available for fertilization.
- In-Vitro Fertilization (IVF) is a type of Assistive Reproductive Technology (ART) which involves retrieving eggs from a woman's ovaries and fertilizing them with sperm in a laboratory dish. This fertilized egg is known as an embryo which can then be frozen for storage or transferred to a woman's uterus.
- With the development of r-DNA technology, methods are available that allows foreign or synthetic genes to be introduced into and expressed in

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higher plants and animals. The DNA that is introduced is called transgene, the animal is transgenic and the process is called Transgenesis.

- Transgenic Mice are produced routinely in laboratories throughout the world, they provide valuable tools for the study of gene expression in mammals.
- A Gene Knock-Out (KO) is a genetic technique in which one of an organism's genes is made inoperative ('knocked out' of the organism).
- Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body. Stem cells can be classified by their potency or on the basis of their origin.
- Cell culture is one of the major molecular techniques used in life sciences. It involves the removal of cells, tissues or organs from a plant or an animal and its culturing in an artificial environment.
- Cell culture provides outstanding model systems for carrying out the research on normal physiology and biochemistry of cells involving concepts like metabolic studies, aging, mutagenesis, carcinogenesis and also the effects of drugs and other toxic compounds on the cells.
- Stem cells under optimum conditions and on receiving proper signal can regenerate damaged tissue which potentially could save several lives or repair wounds or can heal tissue damage in people after some serious illness or injury.
- Potential uses of stem cells include production of transgenic animals, tissue regeneration, cardiovascular disease treatment, brain disease treatment, cell deficiency therapy, blood disease treatment, research and scientific discovery, stem cell therapies, tissue, graft, etc.
- In majority of the countries, laboratory animals are protected by the Law known as 'Cruelty to Animals Act.' A researcher has to get license from the Home Department to begin experimenting on animals.
- Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. The social, extrinsic and intrinsic concerns must be addressed related to the process.
- GIFT is a procedure initially developed by Dr. Ricardo Asch in the 1980s. In this technique the physician extracts the ova and sperm with the same procedures as in IVF, but the ova are not fertilized in vitro (outside of the body).
- ZIFT is a combination of IVF and GIFT. The sperm and ova are extracted with the same procedures as IVF and GIFT, and the ova are fertilized outside of the body as with IVF. ICSI may also be used in conjunction with ZIFT.
- One kind of assisted reproductive technology is ICSI that is helping numerous individuals to get rid off from male infertility. Spermatozoa sometimes fail to fertilize even when they are artificially placed in close proximity to eggs during conventional In-Vitro Fertilization (IVF).

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- Nuclear transplantation has allowed experimental embryologists to manipulate the development of an organism and to study the potential of the nucleus to direct development. Nuclear transplantation, as it was first called, was later referred to as somatic nuclear transfer or cloning.
- Cloning usually refers to the means of the production of identical copies of an individual or we can say that it refers to the production of exact genetic replica copies of an individual.
- Two approaches are in use for embryo cloning. 1. Nuclear transfer and 2. Use of embryonic cells.
- Embryo biopsy involves the removal of a few cells from the embryo (mostly from the trophoblastic cells of the trophectoderm) for analysis to determine its sex a technique known as embryo sexing.
- Genetic screening determines whether a couple is at increased risk of having a baby with a hereditary genetic disorder. Hereditary genetic disorders are basically the disorders of chromosomes or genes that are passed down from one generation to other generation. Genetic testing plays an essential role in assessing the risk of developing certain disorders as well as screening and sometimes medical treatment.
- A few methodologies of genetic screening include polymerase chain reaction, sequencing DNA, chromosome analysis, fluorescence in situ hybridization, microarrays, gene expression profiling, etc.
- Results of genetic testing hugely depends upon the type of test taken as well as the health care facility providing the test. It is always a good practice to discuss the results of genetic tests with a skilled doctor, medical geneticist or genetic counsellor.
- In many species, sex determination is genetic i.e., males as well as females have different alleles or even different genes that specify their sexual morphology. In animals this is usually accompanied by chromosomal differences, via the chromosomal combinations such as XX, XY, ZW, XO, ZO, etc.
- Immunocontraception refers to the use of an animal's immune system to prevent it from fertilizing offspring. Contraceptives of this type are not currently available for human use.
- There are several targets in mammalian sexual reproduction for immune inhibition. They can be organized into three categories: gamete production, gamete function and gamete outcome.
- The different modes of immunocontraception include gamete specific antigen, antibody mediated fertilization blocks and termination of gestation.
- Apart from the immunocontraception, other contraceptive methods can be broadly classified into the following categories: surgical methods, hormonal methods, physical barriers, implants, natural methods, emergency contraception, etc.

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- The general guidelines for the care and maintenance of the animals are broadly based on the following elements: water, quality and quantity of food, hygiene, litter, cages, labelling, ventilation, humidity, marking animals, detection of disease, recording of animals' temperatures, prevention of disease, insect pest, etc.
- Common diseases from which experimental animals suffer include Foot-and-mouth disease (FMD), Bovine spongiform encephalopathy (BSE), Equine infectious anemia (EIA), Echinococcosis or Hydatid disease, Paratuberculosis or Johne's disease, Virulent Newcastle disease (VND), rabies, chytridiomycosis, etc.
- A few regularly used experimental animals include rabbit, guinea pig, armadillo, mice, rat, etc.
- Bioethics refers to the study of the ethical issues arising out from the advances made in the field of biology, medicine as well as technologies.
- One of the major arguments for using animals for experimentation is that the data obtained can be useful for human beings and hence these animals can be used for a variety of purpose ranging from drug testing to treatment of disease.
- Usually, before animals are used for research studies or before beginning of any kind of experimentation, the research protocol must be reviewed by animal ethics committees.

## 4.10 KEY TERMS

- **Gamete:** It is a mature reproductive or sex cell with a haploid number of chromosomes (i.e., just one set of dissimilar chromosomes) and the ability to fuse with another haploid reproductive cell to generate a diploid zygote.
- **Superovulation:** It is a phenomenon whereby increasing the concentration of gonadotrophic hormone, a large number of ovarian follicles can be induced to ripen as well as to produce more eggs.
- **Cell Preservation:** It refers to the entire process of extraction, processing and storage of cells so that they can be used in the future for research, scientific or medical purpose.
- **Embryo Transfer Technology:** It refers to a step in the process of assisted reproduction in which embryos are placed into the uterus of a female with the intent to establish a pregnancy.
- **In Vitro Oocyte Maturation:** Also known as IVM, or IVM) refers to a procedure where immature eggs mature *in vitro*, i.e. in the laboratory.
- **In-Vitro Fertilization (IVF):** It is a type of Assistive Reproductive Technology (ART) which involves retrieving eggs from a woman's ovaries and fertilizing them with sperm in a laboratory dish.
- **Transgenesis:** It refers to the process where the DNA from foreign origin is introduced within the genome of another organism.

- **Gene Knock-Out (KO):** It is a genetic technique in which one of an organism's genes is made inoperative ('knocked out' of the organism),
- **Stem Cells:** These are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.
- **Intra Cystoplasmic Sperm Injection (ICSI):** It refers to the injection of single mature immobilized normal spermatozoa into the cytoplasm of a mature metaphase II oocyte.
- **Nuclear Cloning:** Also referred to as nuclear transfer or nuclear transplantation, it denotes the introduction of a nucleus from an adult donor cell into an enucleated oocyte to generate a cloned embryo.
- **Sex-Determination System:** It refers to a biological system which determines the development of sexual characteristics in an organism.
- **Immunocontraception:** It refers to the use of an animal's immune system to prevent it from fertilizing offspring.

## 4.11 SELF ASSESSMENT QUESTIONS AND EXERCISES

### Short Answer Questions

1. Write a note on the methods of sex determination.
2. What are the pros and cons of cloning? Discuss.
3. What do you understand by transgenics?
4. Write a short note on genetic screening.
5. What are the uses of immunocontraception?

### Long Answer Questions

1. Describe IVF as an assisted method of reproduction.
2. What is gene-knockout technique? Explain in detail.
3. Discuss the concept of embryonic stem cells.
4. Examine the need of antibody mediated fertilization block.
5. Explain your understanding of bioethics.

## 4.12 FURTHER READING

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