

Evaluating the transgenerational genetic and epigenetic changes in rats exposed to pyrethroids

A thesis submitted during 2025 to the University of Hyderabad in partial fulfilment of the award of a Ph.D. degree in Animal Biology

by

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2. **Priyanka, P. P.**, & Yenugu, S. (2021). Coiled-Coil Domain-Containing (CCDC) Proteins: Functional Roles in General and Male Reproductive Physiology. *Reproductive sciences (Thousand Oaks, Calif.)*, 28(10), 2725–2734. <https://doi.org/10.1007/s43032-021-00595-2>
3. Jamil Aisha, Anandha Rao Ravula, Marri Reddy Mounika, **Patra Priyadarshini Priyanka**, Kumari Sangeeta, Munipalli Suresh Babu, Suresh Yenugu. Expression of SARS-CoV2 infectivity machinery in the male reproductive tract: Possible outcomes on fertility. *Journal of Endocrinology and Reproduction*, Vol 24(2), 67-79, December 2020

4. Priyanka, P. P., & Yenugu, S. (2023). Assessment of reproductive toxicology of pesticides: studies focused on doses relevant to human exposure are the need of the hour. ISSRF Newsletter, (31), 48,49. <https://www.researchgate.net/profile/Priyanka-Patra-9/research>

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3	Society for Reproductive Biology and Comparative Endocrinology	September 14 th -16 th ,2022	CSIR-CCMB	Poster Presentation
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SL803	RESEARCH PROPOSAL AND SCIENTIFIC WRITING	4	PASS
SL804	RESEARCH AND PUBLICATION ETHICS	2	PASS


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I, **Priyanka Priyadarshini Patra**, hereby declare that this thesis entitled “**Evaluating the transgenerational genetic and epigenetic changes in rats exposed to pyrethroids**” submitted by me under the guidance and supervision of **Prof. Suresh Yenugu**, is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in Sodhganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed.

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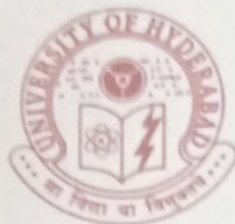
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SDG goals- Goal No. 12(Responsible Consumption and Production) and Goal No. 3(Good Health and Well-being)

The work done in this thesis as a part of the award of the doctorate degree has higher significance with the SDG goal of **good health and well-being**. The work focuses on the effect of pesticide consumption on humans, particularly focusing on the reproductive approach and how it affects not only the consumer but to further generations too. Pesticides, especially pyrethroids, are a common name for both agricultural uses and other household things. The effects of these pyrethroids have been reported previously, but the effects of doses relevant to that humans consume daily are new to its kind. For the first time, we tried to understand the effects at a generation level with a genetic and epigenetic approach. The overall study signifies the importance of rules and vigilance on consumption and production ethics. So, our major objectives [Objective 1&2] focus mainly on SDG goal no. 12, i.e., **Responsible consumption and production**, whereas our objective 3 focuses on the characterization of one of the important family of proteins, i.e., the Coiled-Coil domain-containing proteins. It majorly focuses on the role of these genes in reproductive functions. This shows the significance of the SDG goal of **good health and well-being** because understanding the genetic approach of any physiology will help us to reshape the understanding of diseases in a detailed way and can lead us to a better future. And understanding the role of uncharacterised genes will open new windows in therapeutics. All the experiments were done by keeping an eye on sustainability with minimal wastage and best of the reusability of resources wherever applicable.



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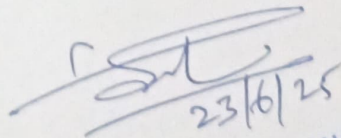
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Abbreviations

3-PBA	3-phenoxy benzoic acid
3 β -HSD	3 β -hydroxysteroid dehydrogenase
AGE	Advanced glycation end products
BAD	BCL2 associated agonist of cell death
BAK	Bcl-2 homologous antagonist/killer
BAM	Binary Alignment Map
BAX	Bcl2 Associated X
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BLAST	Basic Local Alignment Search Tool
Bt	<i>Bacillus thuringiensis</i>
BTB	Blood-testis barrier
C2D1A	Coiled-Coil and C2 Domain Containing 1A
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCDC	Coiled-coil domain-containing
cDNA	Complementary DNA
CDNB	1-Chloro-2,4-dinitrobenzene
CPGi	CPG island
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDT	Dichlorodiphenyltrichloroethane
DE	Differentially expressed
DEPC	Diethyl pyrocarbonate
DHT	Dihydrotestosterone
DMR	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphate
DSD	Disorders of sex development
ETC	Electron transport chain

FAO	Food and Agriculture Organization of the United Nations
FSH	Follicle-stimulating hormone
<i>Gapdh</i>	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
GSH	Glutathione
GST	Glutathione S-Transferase
H ₂ O ₂	Hydrogen peroxide
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferase
HPG axis	Hypothalamic-pituitary-gonadal axis
IL-6	Interleukin 6
IPM	Integrated pest management
I-TASSER	Iterative Threading ASSEmbly Refinement
JNK	c-Jun N-terminal kinases
LC	Leydig cell
LH	Luteinizing hormone
LLIN	Long-lasting insecticidal net
LPO	Lipid peroxidation
MAPK	Mitogen-activated protein kinas
MDA	Malondialdehyde
MEME	Multiple Em for Motif Elicitation
mg	Milligram
MgCl ₂	Magnesium chloride
MMP	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
MRT	Male Reproductive Tract Tissue
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
N-MRT	Non-Male Reproductive Tract tissue
NO	Nitric oxide
O ₂ ⁻	Superoxide anion

OH·	Hydroxyl radicals
PCD	Primary ciliary dyskinesia
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PKA	Protein kinase A
QC	Quality Control
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRBS	Reduced Representation Bisulfite sequencing
rRNA	Ribosomal RNA
SAM	Sequence Alignment Map
SC	Sertoli cell
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SE	Seminiferous epithelium
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SOPMA	Self-Optimized Prediction Method with Alignment
SRY	Sex-determining Region Y
SSC	Spermatogonial stem cell
StAR	Steroidogenic acute regulatory
TAE	Tris-Acetate EDTA
TBARS	Thiobarbituric acid reactive substances
TBS	Tris-buffered saline
TEP	1,1,3,3-tetraethoxypropane
TET	Ten-eleven translocation
TGFB	Transforming Growth Factor Beta
TNF- α	Tumor necrosis factor alpha
WHO	World Health Organisation
μg	Microgram
μl	Microlitre
μmole	Micromole

**Evaluating the transgenerational genetic and epigenetic
changes in rats exposed to pyrethroids**

General Introduction

Pesticides are chemical or biological substances that are used to get rid of or control pests that are detrimental to plants, humans, and the environment. Insects, weeds, fungi, rodents, bacteria, and other organisms that can damage crops, spread diseases, or disturb ecosystems are all considered as pests. Pesticides are often classified according to the pest they target, such as insecticides for insects, herbicides for weeds, fungicides for fungal infections, rodenticides for rodents, and bactericides for bacterial species (Tudi et al., 2021). Synthetic pesticides are chemically created, whereas natural pesticides are derived from biological sources such as plants, microbes, or minerals. Modern pesticides are intended to be effective at low doses and frequently target specific pests in order to reduce collateral damage to non-target creatures. However, this precision is not always ideal. Pesticides have substantially improved agricultural production and public health by eliminating disease vectors such as malaria and protecting the food supply, but they are not without downsides. Overuse and poor application can result in pesticide resistance, environmental contamination, and harm to beneficial creatures like pollinators and natural predators (Ravula & Yenugu, 2021). Pesticide residues in food and water sources can generate concerns about human health risks, such as acute poisoning and long-term impacts like endocrine disruption and cancer. The increased understanding of these consequences has fuelled interest in integrated pest management (IPM) tactics, organic farming practices, and the development of bio-based pesticides to assure long-term pest control and environmental stewardship.

Historical aspects of pesticides

Pesticides have been around for thousands of years, illustrating humanity's constant effort to safeguard food and health against pests. Natural pest control methods used by early agricultural communities included plant extracts, ashes, and sulfur compounds (Thacker, 2002). Ancient books from civilisations such as Mesopotamia, Egypt, and China describe the usage of chemicals like sulphur to repel insects and moulds. Despite their toxicity to humans and the environment; arsenic, mercury, and lead compounds were introduced as more effective pest-control agents during the Middle Ages. The introduction of chemical pesticides in the nineteenth century was a watershed moment, owing to developments in chemistry and the industrial revolution. Paris green, an arsenic-based substance, was one of the first widely used chemical insecticides to control agricultural pests such as the Colorado potato beetles. The development of chemical compounds in the twentieth century, particularly DDT (dichlorodiphenyltrichloroethane) in the 1930s, revolutionised pest management. DDT was hailed as a wonder pesticide for its efficacy against disease-carrying insects such as mosquitos,

which drastically reduced malaria and typhus in many areas. However, its broad usage resulted in serious environmental and health consequences, prompting its eventual ban in many nations following Rachel Carson's groundbreaking essay *Silent Spring* in 1962, (Carson, 1996) which highlighted the ecological risks of pesticide misuse. The post-World War II era witnessed the emergence of synthetic pesticides such as organophosphates, carbamates, and pyrethroids, which were targeted for specific insect targets and had a shorter persistence in the environment. Concerns about pesticide resistance, non-target effects, and sustainability have driven developments such as integrated pest management (IPM), genetically modified pest-resistant crops, and bio-based insecticides. This historical trend demonstrates a balance between pest management and sustainable approaches to reduce environmental and health impacts.

Classification of pesticides

Pesticides are classed using a variety of characteristic features such as target pest, chemical composition, mode of action, origin, and application method (Figure 1). Insecticides, herbicides, fungicides, rodenticides, and bactericides are used depending on the nature of pest. Chemically, they are classified as organochlorines, organophosphates, carbamates, pyrethroids, and biopesticides produced from natural sources. They can act systemically, spreading throughout the plant or pest or as contact insecticides, relying on direct exposure. Pesticides are also classified according to their origin, synthetic or natural, and application methods, such as seed treatments, foliar sprays, or soil applications.

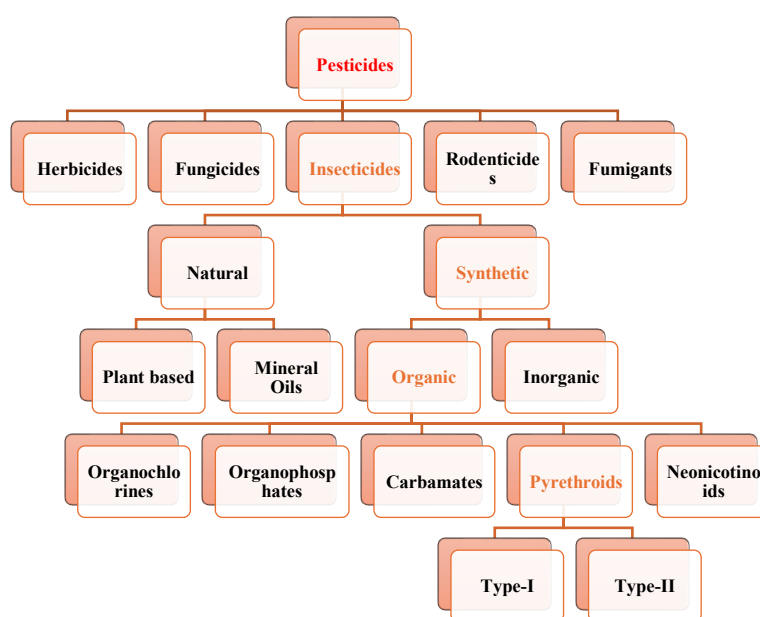


Figure 1. Classification of pesticides.

Natural pesticides

Natural pesticides, generated from natural sources such as plants, minerals, and microbes, have long been utilised as eco-friendly alternatives to synthetic pesticides. These biopesticides are generally less hazardous to non-target creatures, such as humans, beneficial insects, and wildlife, making them an important component of sustainable agriculture. Common examples include neem oil, which is extracted from neem tree seeds and functions as an insect repellent, growth inhibitor, and egg steriliser. Pyrethrins, which are generated from chrysanthemum flowers, have neurotoxic properties that make them useful against a variety of insect pests. Microbial insecticides, such as *Bacillus thuringiensis* (Bt), use microorganisms to create proteins that are harmful to specific insect larvae while being innocuous to other creatures. Mineral-based insecticides, such as sulphur and copper compounds, are commonly employed to control fungal diseases and pests. Rotenone, obtained from the roots of certain legumes, is another botanical insecticide. Essential oils such as citronella and clove oil repel or kill pests. Natural pesticides are also biodegradable and frequently degrade fast in the environment, decreasing the possibility of accumulation and long-term pollution. However, environmental conditions such as temperature, sunlight, and moisture might restrict their efficiency, and they may need to be applied on a regular basis to remain effective (Isman, 2006).

Synthetic pesticides

Synthetic pesticides are chemical compounds designed to manage or remove pests such as insects, weeds, fungi, and other dangerous organisms. These insecticides, which were first employed on a broad basis in the mid-twentieth century, transformed agriculture by considerably increasing crop yields and lowering losses due to pest infestation. The chemical structure of synthetic pesticides determines their classification, with important classes including organochlorines, organophosphates, carbamates, and pyrethroids. Organochlorines, such as DDT, were among the first extensively used synthetic pesticides, valued for their efficacy and long-lasting impact, but they were eventually banned in many countries due to environmental persistence and bioaccumulation issues (FAO, 2020; Gupta, 2004). Organophosphates and carbamates, which target pest nervous systems by blocking the enzyme acetylcholinesterase, are more biodegradable but can be very hazardous to non-target creatures, including people (Coats, 1990). Pyrethroids, modelled after natural pyrethrins, are synthetic insecticides that kill pests swiftly while being less hazardous to mammals. Synthetic herbicides, such as glyphosate and atrazine, have proven effective in weed management, particularly in

large-scale farming systems, whilst synthetic fungicides and rodenticides protect crops from fungal diseases and rodent damage, respectively.

Pesticide usage

Pesticides have played an important role in agricultural, public health, and environmental management for centuries, with significant evolution in formulation and use. Pesticides have helped farmers protect crops from pests, illnesses, and weeds, improving food security and lowering post-harvest losses (Tudi et al., 2021). Over time, herbicides such as glyphosate have permitted large-scale weed management, eased farm operations, and permitted conservation tillage, which reduces soil erosion. Insecticides such as pyrethroids and organophosphates have helped manage crop-destroying pests, while fungicides such as mancozeb have shielded crops from fungal diseases that may decimate yields. Pesticides have also played an important role in managing disease vectors in public health programs beyond agriculture (Rehwagen, 2006). Insecticides such as DDT and malathion were previously employed to combat mosquitos, resulting in a considerable reduction in the occurrence of diseases like malaria and yellow fever. Rodenticides and bactericides have been used to control the spread of rodent-borne and bacterial diseases in both urban and rural areas (Tudi et al., 2021). Pesticides have also helped to preserve goods and infrastructure, with treatments to protect timber from termite damage and stored grains from insect deterioration. Pesticide chemistry advancements have resulted in more specialized and focused applications, such as systemic pesticides, which protect plants internally, and biopesticides, which use natural pest management processes (Chandler et al., 2011).

Despite their benefits, synthetic pesticides carry substantial hazards, including the development of insect resistance, soil and water contamination, and negative impacts on non-target species such as pollinators and aquatic life (AbuQamar et al., 2024; Liang et al., 2025; Wan et al., 2025). Furthermore, long-term exposure to these chemicals has been related to human health problems such as endocrine disruption, reproductive abnormalities, and some types of cancer (Mnif et al., 2011; Soerensen et al., 2024). In response to these problems, many countries have implemented stricter measures to assure food safety during production, application, and residue levels. Advances in chemistry and biotechnology are driving the development of more selective and less damaging synthetic pesticides, with a focus on lowering environmental impact and enhancing compatibility with integrated pest management (IPM) approaches.

In the Indian context, pesticides have been a cornerstone of agricultural development, playing a crucial role in enhancing crop yields and supporting the country's food security, especially during and after the Green Revolution of the 1960s and 1970s. With the development of high-yielding variety seeds and intensive farming practices, pesticide use skyrocketed to control pests, weeds, and illnesses that would otherwise risk productivity. India is a major producer and user of pesticides, with crops such as rice, wheat, cotton, and sugarcane accounting for a big share of pesticide use. Insecticides dominate the Indian pesticide business, notably in cotton growing, where pests such as bollworms present a significant issue. Herbicides are increasingly utilised to manage weeds, particularly in labour-scarce areas, but fungicides are essential for protecting horticultural crops such as fruits and vegetables against fungal infections. Pesticides are used in public health programs to manage mosquito-borne diseases such as malaria and dengue, with insecticides such as DDT and malathion. However, India confronts specific pesticide-related difficulties, such as over-reliance on chemical pesticides, a lack of information among farmers about effective application procedures, and the prevalence of counterfeit or substandard goods on the market. These difficulties have raised environmental concerns, such as soil and water contamination, as well as health risks for farm workers and customers from pesticide residues (Aktar et al., 2009). In response, the government enacted restrictions such as the Insecticides Act of 1968, which encourages integrated pest management (IPM) approaches, organic farming, and the use of biopesticides like neem-based products. Recent efforts include endeavours to educate farmers on pesticide safety and strategies to decrease the use of very hazardous pesticides. As India seeks sustainable agricultural practices, balancing the benefits of pesticides with their potential hazards remains a significant challenge, necessitating a holistic approach that includes regulatory reforms, innovation, and farmer empowerment.

Pyrethroids

Pyrethroids are synthetic insecticides based on natural pyrethrins found in chrysanthemum flowers (Katsuda, 2011). Pyrethroids, which are known for their efficacy, broad-spectrum activity, and low toxicity to animals, are widely employed in agricultural, public health, and domestic pest management (Casida, 1980). In agriculture, they play an important role in protecting crops such as cotton, rice, and vegetables against a variety of insect pests, including aphids, beetles, caterpillars, and moths, by attacking the insects' nerve systems, causing paralysis and death (Hodoşan et al., 2023). Pyrethroids are highly effective even at low concentrations and have a quick knockdown effect, making them a popular pest management

tool. Pyrethroids such as permethrin and deltamethrin are widely employed in public health vector control programs to combat mosquitoes, ticks, and flies, thereby lowering disease transmission such as malaria, dengue, and Lyme (Kambou et al., 2024). They can also be found in treated bed nets, indoor residual sprays, and topical applications to repel and kill disease vectors. Pyrethroids are commonly used in residential pest control products such as aerosol sprays, mosquito coils, and liquid vaporisers to kill mosquitoes, cockroaches, and ants.

Routes of exposure, absorption, metabolism, and excretion of pyrethroids

Pyrethroids can enter the body through a variety of pathways, including cutaneous contact, inhalation, and ingestion, the most prevalent of which are occupational and environmental (Bradberry et al., 2005). Their absorption varies with the route of exposure; cutaneous absorption is very low due to their lipophilic nature and poor water solubility, whereas ingestion and inhalation result in more efficient absorption across the gastrointestinal and respiratory membranes, respectively (Ray & Forshaw, 2000). Pyrethroids are absorbed and transported through the bloodstream to numerous tissues, particularly those with high lipid content due to their lipophilicity (Casida et al., 1983).

Human exposure to pesticides is mostly through physical contact, ingestion, and inhalation; however, leaks at storage facilities can play a role (Costa, 2006). Pyrethroids are predominantly metabolised in the liver via two major pathways: carboxylesterases hydrolyse the ester link and cytochrome P450 enzymes oxidise it. These processes convert pyrethroids into more hydrophilic metabolites, which aids in their elimination (Anand et al., 2006). The major metabolites are chrysanthemic acid derivatives and alcohols, which are then conjugated to glucuronides or sulphates. The metabolism is stereoselective, as the cis and trans isomers of pyrethroids have different rates of metabolic transformation, which influences their toxicity profiles (Godin et al., 2010). The bulk of water-soluble metabolites is excreted in the urine, although lipophilic residues and unmetabolized chemicals may be removed in feces. Pyrethroids have a short elimination half-life in humans and animals, indicating effective metabolic detoxification; nevertheless, the parent molecules may survive in fatty tissues for a limited period (Leng et al., 1997). Despite their fast elimination, acute and chronic exposures have been linked to neurotoxicity due to their action on sodium channels (Soderlund, 2012). Understanding the kinetics of absorption, metabolism, and excretion is crucial for determining the danger of pyrethroid exposure and devising mitigation techniques in the event of accidental poisoning or overexposure (John et al., 2001).

Mechanism and toxicity of pyrethroids

One of the most predominantly studied mechanism of pyrethroids' toxicity is due to their impact on the neurological system, namely voltage-gated sodium channels in neurones. These channels are crucial for the creation and transmission of action potentials (Catterall & Catterall, 2014). Pyrethroids bind to and alter the gating characteristics of sodium channels, keeping them open and delaying inactivation. This causes continuous sodium inflow, which leads to prolonged depolarisation of the neuronal membrane. This disturbance causes repeated firing of action potentials, hyperexcitability, and, eventually, paralysis and death in insects (Bloomquist, 1996). Type I pyrethroids, such as permethrin, elicit repeated discharges and tremors (T-syndrome) by moderately extending sodium channel opening (Vijverberg & vanden Bercken, 1990). Type II pyrethroids (e.g., deltamethrin) contain an alpha-cyano group, which increases their potency and causes more pronounced effects such as membrane depolarisation, conduction block, and symptoms such as salivation and choreoathetosis (CS-syndrome) caused by stronger and longer-lasting sodium channel modification. Pyrethroids can interact with various ion channels, including chloride and calcium channels, as well as block enzymes like as ATPases, which contributes to their neurotoxic effects (Toshio, 1992). While pyrethroids are very selective for insect sodium channels, they can also damage mammals at greater dosages, especially in cases of acute exposure (Costas-Ferreira et al., 2022). In mammals, neurotoxicity manifests as tremors, ataxia, and seizures, which are predominantly caused by impacts on the central and peripheral nervous systems. Pyrethroids' selective toxicity is due to variations in sodium channel sensitivity between insects and mammals, as well as mammals' ability to rapidly metabolise pyrethroids into less toxic metabolites via hepatic enzymes. Nonetheless, resistance development in target pests, as well as potential deleterious effects on non-target species such as beneficial insects, aquatic organisms, and humans, highlight the significance of using pyrethroids with caution and additional research into their toxicity mechanisms (Weston et al., 2011).

Health hazardous of pyrethroids

Pyrethroids, which are commonly used synthetic insecticides, provide a variety of health risks across numerous biological systems. While generally regarded as less harmful to mammals than other pesticide classes, persistent or high-dose exposure might have serious consequences. Pyrethroids are strong neurotoxins that disrupt voltage-gated sodium channels, resulting in nervous system hyperexcitation. Tremors, seizures, disorientation, and paraesthesia are all

examples of acute symptoms. Chronic exposure may cause long-term neurological abnormalities, such as cognitive impairments and altered neurotransmitter activity. Pyrethroids have been found to influence immunological responses, resulting in immunosuppression or hypersensitivity (Skolarczyk et al., 2017). They can inhibit immune cell proliferation, impair cytokine synthesis, and affect macrophage and lymphocyte function, increasing susceptibility to infections and inflammatory diseases. Although most pyrethroids are not direct carcinogens, animal studies have shown that long-term exposure increases the risk of some malignancies, including liver and lung cancer. This is due to their capacity to cause oxidative stress, DNA damage, and abnormalities in cellular signalling pathways (Abdollahi et al., 2004). Through epigenetic modifications such as DNA methylation, histone acetylation, and miRNA regulation, pyrethroids can alter the expression of genes. These changes may disrupt normal cellular function and contribute to long-term health consequences, such as developmental abnormalities and a predisposition to diseases like cancer (Ravula & Yenugu, 2021).

Pyrethroids have been related to negative impacts on reproductive health in both men and women. In men, they can reduce sperm quality, lower testosterone levels, and harm testicular tissue (Meeker et al., 2009). In females, they can disturb ovarian function and menstrual cycles, potentially affecting fertility and pregnancy outcomes (Marettova et al., 2017). By generating reactive oxygen species (ROS) and impairing antioxidant defenses, pyrethroids lead to oxidative stress. This oxidative imbalance can harm lipids, proteins, and DNA, resulting in cellular malfunction, aging, and the advancement of chronic diseases. Inhaling pyrethroids can irritate the respiratory tract, resulting in coughing, wheezing, and shortness of breath (Saillenfait et al., 2015). Chronic exposure has been associated with pulmonary inflammation and impaired lung function, particularly in occupational contexts. Pyrethroids build in the liver and kidneys, causing tissue damage, enzyme malfunction, and changes in detoxification pathways. This can result in increased liver enzymes, fibrosis, and reduced renal function. Pyrethroids can behave as endocrine disruptors, interfering with hormone signalling pathways. They have been linked to changes in thyroid hormone levels, disturbances in oestrogen and androgen receptor activation, and effects on adrenal gland function. Pyrethroid exposure during pregnancy and infancy has been associated to developmental delays, behavioural abnormalities, and neurodevelopmental issues in animal models and epidemiological studies (Demicco et al., 2010). Given the wide range of toxicological effects, stringent regulation, better application procedures, and the development of alternatives are required to limit the health concerns associated with pyrethroid exposure.

The male reproductive system

The male reproductive system is a complicated network of organs and tissues that produce, store, and deliver sperm while also secreting male sex hormones, primarily testosterone (Hess & De Franca, 2009). It contains both primary and secondary reproductive organs (Figure 2). The testis is well structured and divided into two distinct regions: the interstitial space and seminiferous tubules. Specialized cell types, like Leydig cells (LCs) in the interstitial space and Sertoli cells (SCs) in the seminiferous tubules, are crucial for steroid production and sperm development. In mammals, the testis has a unique immune status, where it tolerates foreign and self-antigens by keeping antigens and antibodies away from the immune system through the blood-testis barrier. The prostate is a walnut-sized gland located below the bladder; its secretions provide nourishment for sperm. Seminal vesicles are pouch-like structures connected to the vas deferens. They create a fluid rich in fructose, which gives energy to sperm as they grow and become motile. The penis is the external male organ used for sexual intercourse and to expel sperm and urine from the body.

Spermatogenesis, or the production of sperm, and testosterone release, which regulates male secondary sexual traits, libido, and overall reproductive function, are the two roles of the testes. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are controlled by the hypothalamic-pituitary-gonadal axis, influence the processes of mitosis, meiosis, and maturation that produce sperm in tightly coiled seminiferous tubules inside the testes. The secondary reproductive organs help transport and mature sperm. Immature sperm leave the seminiferous tubules and travel to the epididymis, where they mature and gain motility. Sperm travel to the urethra during ejaculation via the vas deferens, a muscular conduit. Semen is made up of sperm and seminal fluid, which is produced by the prostate gland, bulbourethral glands, and seminal vesicles.

This fluid supplies nutrition, improves sperm motility, and neutralises the acidic environment of the female reproductive tract. The scrotum, a pouch-like sac of skin that hangs behind the penis, is one of the supporting components of the male reproductive system. The cremaster and dartos muscles, which regulate the position of the testes in response to temperature changes, and the testicular temperature, which is kept slightly lower (approximately 2-3 °C) than body temperature, allow for optimal sperm generation. (Setchell, 1998). Male fertility and hormonal balance rely on the proper functioning of this system, which is regulated by genetic, hormonal, environmental, and lifestyle variables (Skakkebaek et al., 2015).

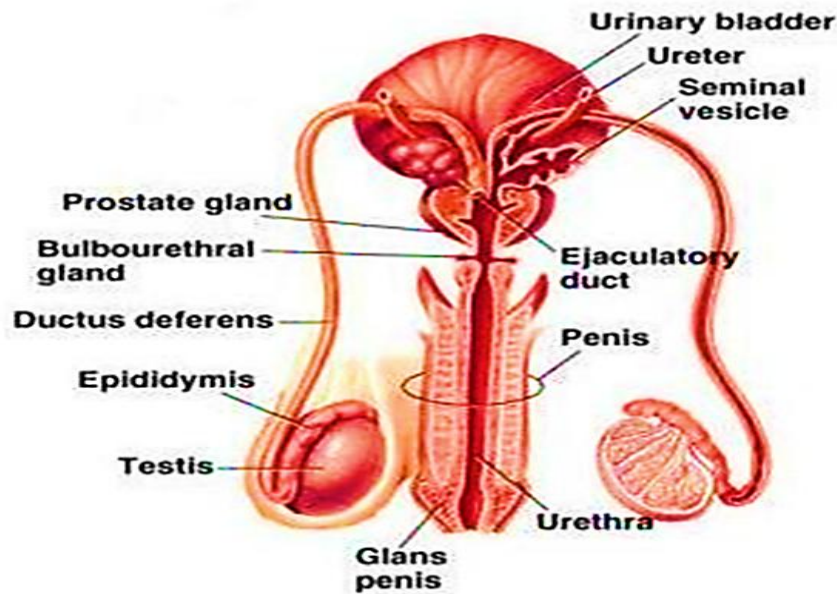


Figure 2. The Male reproductive system.

Organisation and functions of the testis

Testis is an oval-shaped structure in the scrotal sac that sits directly behind the penis. The tunica albuginea is a fibrous capsule that sheathes both testes and divides into 200-400 wedge-shaped pieces, or lobes, due to fibrous tissue segregation from the tunica albuginea (França et al., 2012). Each lobe contains three to ten coiled tubules, also known as seminiferous tubules. The testis functions as both an exocrine and endocrine gland. The Leydig cells in the testis produce testosterone, the main male sex hormone, which is used for spermatogenesis and a number of other physiological processes. (Zirkin & Papadopoulos, 2018). The testis' exocrine function is the production of spermatozoa in the seminiferous tubules.

The creation of spermatozoa in the seminiferous tubules is the exocrine function of the testis. The testes have two internal compartments: the interstitial space, which contains LCs and blood vessels, and the seminiferous tubules, which contain SCs and developing germ cells within the seminiferous epithelium (Siu & Yan Cheng, 2009). The testes are externally covered with tunica albuginea. Over 20% of the SE volume in mature rats is made up of SCs, which also have cytoplasmic extensions that aid in the growth of germ cells. One special and important ultrastructure in the testis is the blood-testis barrier (BTB) (Yan Cheng & Mruk, 2012). Actin-based tight junctions between two nearby SCs generate BTB, which promotes spermatogenesis

by establishing a suitable, specialized environment for postmeiotic germ cell growth. According to Yan Cheng and Mruk (2012), the BTB separates the SE of tubules into two compartments across the adjacent SCs: the apical or adluminal compartment, which is situated above or behind the barrier and is in charge of meiosis, spermiogenesis, and spermiation, and the basal compartment, which is situated below or outside the barrier and is in charge of the self-renewal and proliferation of spermatogonial germ cells. BTB restricts the accessibility of the immune system by acting as an immunological barrier. (Yan Cheng & Mruk, 2012). BTB works as an immunological barrier, limiting the immune system's accessibility. Steroidogenesis, or the production of testosterone, and spermatogenesis are the main roles of LCs and SCs in the seminiferous tubules, respectively. (Smith & Walker, 2014).

The transgenerational genetic and epigenetic alterations caused by pyrethroids in the male reproductive system remain unknown and, hence, need further investigation. This refers to effects passed down from one generation to the next, directly from a parent to their offspring, while it indicates effects that persist across multiple generations, even if the initial exposure only occurred in the grandparent's generation (Skinner et al., 2018). Transgenerational genetic and epigenetic changes in rats exposed to pyrethroids are an emerging topic of toxicological research, emphasising the long-term impact of these extensively used insecticides on biological systems (Ravula & Yenugu, 2022). Pyrethroids' neurotoxic action can cause oxidative stress and disrupt normal cellular processes, resulting in changes in DNA and epigenetic control (Zhang et al., 2021). According to studies, pyrethroid exposure, particularly during important developmental windows such as gestation or early postnatal periods, can cause heritable changes in gene expression and epigenetic marks that last generations (Irani et al., 2022). DNA methylation, histone modifications, and changes in non-coding RNA profiles can all influence the transcriptional activity of genes involved in neurodevelopment, metabolism, and reproduction (Jaenisch & Bird, 2003). Prenatal or early-life pyrethroid exposure in rats has been linked to altered methylation patterns in genes governing hormone synthesis and brain function, which may contribute to behavioural and cognitive deficits in offspring (Aschner et al., 2017). Changes in sperm epigenetic patterns in exposed males show that these changes can be handed down to future generations, affecting both the directly exposed individual and their offspring (Anway et al., 2005). Transgenerational impacts include decreased fertility, aberrant stress reactions, and a higher risk of metabolic and neurological problems (Manikkam et al., 2012). These findings highlight the propensity of pyrethroid exposure to modify gene-environment interactions throughout multiple generations, raising concerns about the long-

term effects on population health and genetic stability (Feil & Fraga, 2012). Determining the wider effects of pesticide exposure and creating more effective pest management strategies require an understanding of these pathways. (Ahmad et al., 2024).

References

- Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., & Rezaie, A. (2004). Pesticides and oxidative stress: a review. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, 10(6), RA141-7.
- AbuQamar, S. F., El-Saadony, M. T., Alkafaas, S. S., Elsalahaty, M. I., Elkafas, S. S., Mathew, B. T., Aljasmi, A. N., Alhammadi, H. S., Salem, H. M., Abd El-Mageed, T. A., Zaghloul, R. A., Mosa, W. F. A., Ahmed, A. E., Elrys, A. S., Saad, A. M., Alsaeed, F. A., & El-Tarabily, K. A. (2024). Ecological impacts and management strategies of pesticide pollution on aquatic life and human beings. *Marine Pollution Bulletin*, 206, 116613. <https://doi.org/10.1016/J.MARPOLBUL.2024.116613>
- Ahmad, M. F., Ahmad, F. A., Alsayegh, A. A., Zeyaulah, M., AlShahrani, A. M., Muzammil, K., Saati, A. A., Wahab, S., Elbendary, E. Y., Kambal, N., Abdelrahman, M. H., & Hussain, S. (2024). Pesticides impacts on human health and the environment with their mechanisms of action and possible countermeasures. *Heliyon*, 10(7), e29128. <https://doi.org/10.1016/J.HELİYON.2024.E29128>
- Aktar, W., Sengupta, D., & Chowdhury, A. (2009). Impact of pesticides use in agriculture: Their benefits and hazards. *Interdisciplinary Toxicology*, 2(1), 1–12. <https://doi.org/10.2478/V10102-009-0001-7>
- Aldridge, W. N. (1990). An Assessment of the Toxicological Properties of Pyrethroids and Their Neurotoxicity. *Critical Reviews in Toxicology*, 21(2), 89–104. <https://doi.org/10.3109/10408449009089874>
- Anand, S. S., Bruckner, J. V., Haines, W. T., Muralidhara, S., Fisher, J. W., & Padilla, S. (2006). Characterization of deltamethrin metabolism by rat plasma and liver microsomes. *Toxicology and Applied Pharmacology*, 212(2), 156–166. <https://doi.org/10.1016/J.TAAP.2005.07.021>
- Anway, M. D., Cupp, A. S., Uzumcu, N., & Skinner, M. K. (2005). Toxicology: Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308(5727), 1466–1469. https://doi.org/10.1126/SCIENCE.1108190/SUPPL_FILE/ANWAY.SOM.PDF
- Aschner, M., Ceccatelli, S., Daneshian, M., Fritsche, E., Hasiwa, N., Hartung, T., Hogberg, H. T., Leist, M., Li, A., Mundy, W. R., Padilla, S., Piersma, A. H., Bal-Price, A., Seiler, A., Westerink, R. H., Zimmer, B., & Lein, P. J. (2017). Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential of chemicals: Example lists and criteria for their selection and use. *ALTEX - Alternatives to Animal Experimentation*, 34(1), 49–74. <https://doi.org/10.14573/ALTEX.1604201>
- Bloomquist, J. R. (1996). Ion channels as targets for insecticides. *Annual Review of Entomology*, 41(1), 163–190. <https://doi.org/10.1146/ANNUREV.EN.41.010196.001115/CITE/REFWORKS>
- Bradberry, S. M., Cage, S. A., Proudfoot, A. T., & Allister Vale, J. (2005). Poisoning due to pyrethroids. *Toxicological Reviews*, 24(2), 93–106. <https://doi.org/10.2165/00139709-200524020-00003/METRICS>

- Carson, R. (1996). Silent Spring. In M. A. Cahn & R. O'Brien (Eds.), *Thinking About the Environment* (pp. 150–155). Routledge. <https://doi.org/10.4324/9781315698724-22>
- Casida, J. E. (1980). Pyrethrum flowers and pyrethroid insecticides. *Environmental Health Perspectives*, *VOL.34*, 189–202. <https://doi.org/10.1289/EHP.8034189>
- Casida, J. E., Gammon, D. W., Glickman, A. H., & Lawrence, L. J. (1983). Mechanisms of selective action of pyrethroid insecticides. *Annual Review of Pharmacology and Toxicology*, *Vol. 23*(Volume 23, 1983), 413–438. <https://doi.org/10.1146/ANNUREV.PA.23.040183.002213/CITE/REFWORKS>
- Catterall, W. A., & Catterall, W. A. (2014). Structure and function of voltage-gated sodium channels at atomic resolution. *Experimental Physiology*, *99*(1), 35–51. <https://doi.org/10.1113/EXPPHYSIOL.2013.071969>
- Chandler, D., Bailey, A. S., Mark Tatchell, G., Davidson, G., Greaves, J., & Grant, W. P. (2011). The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*(1573), 1987–1998. <https://doi.org/10.1098/RSTB.2010.0390;PAGEGROUP:STRING:PUBLICATION>
- Coats, J. R. (1990). Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. *Environmental Health Perspectives*, *87*, 255–262. <https://doi.org/10.1289/EHP.9087255>,
- Costa, L. G. (2006). Current issues in organophosphate toxicology. *Clinica Chimica Acta*, *366*(1–2), 1–13. <https://doi.org/10.1016/J.CCA.2005.10.008>
- Costas-Ferreira, C., Durán, R., & Faro, L. R. F. (2022). Toxic Effects of Glyphosate on the Nervous System: A Systematic Review. *International Journal of Molecular Sciences 2022*, *Vol. 23*, Page 4605, 23(9), 4605. <https://doi.org/10.3390/IJMS23094605>
- Demico, A., Cooper, K. R., Richardson, J. R., & White, L. A. (2010). Developmental Neurotoxicity of Pyrethroid Insecticides in Zebrafish Embryos. *Toxicological Sciences*, *113*(1), 177–186. <https://doi.org/10.1093/TOXSCI/KFP258>
- Elliott, M. (1976). Properties and applications of pyrethroids. *Environmental Health Perspectives*, *14*, 1–2. <https://doi.org/10.1289/EHP.76141;WGROU:STRING:PUBLICATION>
- FAO. (2020). *International Code of Conduct on the Distribution and use of Pesticides - Regular monitoring report -2008*. FAO and WHO ; <https://openknowledge.fao.org/handle/20.500.14283/cb5671en>
- Feil, R., & Fraga, M. F. (2012). Epigenetics and the environment: Emerging patterns and implications. *Nature Reviews Genetics*, *13*(2), 97–109. <https://doi.org/10.1038/NRG3142;SUBJMETA=136,176,177,199;KWRD=DEVELOPMENTAL+BI OLOGY,EPIGENETICS,EPIGENOMICS,GENE+EXPRESSION>
- França, L. R., Auharek, S. A., Hess, R. A., Dufour, J. M., & Hinton, B. T. (2012). Blood-tissue barriers: morphofunctional and immunological aspects of the blood-testis and blood-epididymal barriers. *Advances in Experimental Medicine and Biology*, *763*, 237–259.
- Gammon, D. W., Liu, Z., Chandrasekaran, A., El-Naggar, S. F., Kuryshev, Y. A., & Jackson, S. (2019). Pyrethroid neurotoxicity studies with bifenthrin indicate a mixed Type I/II mode of action. *Pest Management Science*, *75*(4), 1190–1197. <https://doi.org/10.1002/PS.5300;REQUESTEDJOURNAL:JOURNAL:15264998;WGROU:STRIN G:PUBLICATION>

- Godin, S. J., DeVito, M. J., Hughes, M. F., Ross, D. G., Scollon, E. J., Starr, J. M., Setzer, R. W., Conolly, R. B., & Tornero-Velez, R. (2010). Physiologically Based Pharmacokinetic Modeling of Deltamethrin: Development of a Rat and Human Diffusion-Limited Model. *Toxicological Sciences*, 115(2), 330–343. <https://doi.org/10.1093/TOXSCI/KFQ051>
- Gupta, P. K. (2004). Pesticide exposure - Indian scene. *Toxicology*, 198(1–3), 83–90. <https://doi.org/10.1016/j.tox.2004.01.021>
- Hess, R. A., & De Franca, L. R. (2009). Spermatogenesis and Cycle of the Seminiferous Epithelium. *Advances in Experimental Medicine and Biology*, 636, 1–15. https://doi.org/10.1007/978-0-387-09597-4_1
- Hodoşan, C., Gîrd, C. E., Ghica, M. V., Dinu-Pîrvu, C. E., Nistor, L., Bărbuică, I. S., Marin, Ştefan C., Mihalache, A., & Popa, L. (2023). Pyrethrins and Pyrethroids: A Comprehensive Review of Natural Occurring Compounds and Their Synthetic Derivatives. *Plants* 2023, Vol. 12, Page 4022, 12(23), 4022. <https://doi.org/10.3390/PLANTS12234022>
- Irani, D., Borle, S., Balasinor, N., & Singh, D. (2022). Maternal cypermethrin exposure during perinatal period dysregulates gonadal steroidogenesis, gametogenesis and sperm epigenome in F1 rat offspring. *Reproductive Toxicology*, 111, 106–119. <https://doi.org/10.1016/j.reprotox.2022.05.010>
- Isman, M. B. (2006). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annual Review of Entomology*, 51, 45–66. <https://doi.org/10.1146/ANNUREV.ENTO.51.110104.151146>,
- Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* 2003 33:3, 33(3), 245–254. <https://doi.org/10.1038/ng1089>
- John, P. J., Bakore, N., & Bhatnagar, P. (2001). Assessment of organochlorine pesticide residue levels in dairy milk and buffalo milk from Jaipur City, Rajasthan, India. *Environment International*, 26(4), 231–236. [https://doi.org/10.1016/S0160-4120\(00\)00111-2](https://doi.org/10.1016/S0160-4120(00)00111-2)
- Kambou, S. S., Valente, A., Agnew, P., de Sales Hien, D. F., Yerbanga, R. S., Moiroux, N., Dabire, K. R., Pennetier, C., Cohuet, A., & Carrasco, D. (2024). Non-contact detection of pyrethroids widely used in vector control by Anopheles mosquitoes. *PLOS ONE*, 19(7), e0298512. <https://doi.org/10.1371/JOURNAL.PONE.0298512>
- Katsuda, Y. (2011). Progress and Future of Pyrethroids. *Topics in Current Chemistry*, 314, 1–30. https://doi.org/10.1007/128_2011_252
- Laskowski, D. A. (2002). Physical and chemical properties of pyrethroids. *Reviews of Environmental Contamination and Toxicology*, 174, 49–170. https://doi.org/10.1007/978-1-4757-4260-2_3,
- Leng, G., Kühn, K. H., & Idel, H. (1997). Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: applications and limitations. *Science of The Total Environment*, 199(1–2), 173–181. [https://doi.org/10.1016/S0048-9697\(97\)05493-4](https://doi.org/10.1016/S0048-9697(97)05493-4)
- Liang, J., Xiao, F., Ojo, J., Chao, W. H., Ahmad, B., Alam, A., Abbas, S., Abdelhafez, M. M., Rahman, N., Khan, K. A., Ghramh, H. A., Ali, J., & Chen, R. (2025). Insect Resistance to Insecticides: Causes, Mechanisms, and Exploring Potential Solutions. *Archives of Insect Biochemistry and Physiology*, 118(2), e70045. <https://doi.org/10.1002/ARCH.70045;PAGE:STRING:ARTICLE/CHAPTER>
- Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, M. M., & Skinner, M. K. (2012). Transgenerational Actions of Environmental Compounds on Reproductive Disease and Identification of Epigenetic Biomarkers of Ancestral Exposures. *PLOS ONE*, 7(2), e31901. <https://doi.org/10.1371/JOURNAL.PONE.0031901>

- Marettova, E., Mareta, M., & Legáth, J. (2017). Effect of pyrethroids on female genital system. Review. *Animal Reproduction Science*, 184, 132–138. <https://doi.org/10.1016/J.ANIREPROSCI.2017.07.007>
- Meeker, J. D., Barr, D. B., & Hauser, R. (2009). Pyrethroid insecticide metabolites are associated with serum hormone levels in adult men. *Reproductive Toxicology*, 27(2), 155–160. <https://doi.org/10.1016/J.REPROTOX.2008.12.012>
- Mnif, W., Hassine, A. I. H., Bouaziz, A., Bartegi, A., Thomas, O., & Roig, B. (2011). Effect of Endocrine Disruptor Pesticides: A Review. *International Journal of Environmental Research and Public Health* 2011, Vol. 8, Pages 2265-2303, 8(6), 2265–2303. <https://doi.org/10.3390/IJERPH8062265>
- Nishizawa, Y. (1971). Development of new synthetic pyrethroids. *Bulletin of the World Health Organization*, 44(1-2-3), 325. <https://pmc.ncbi.nlm.nih.gov/articles/PMC2428056/>
- Pitzer, E. M., Williams, M. T., & Vorhees, C. V. (2021). Effects of pyrethroids on brain development and behavior: Deltamethrin. *Neurotoxicology and Teratology*, 87, 106983. <https://doi.org/10.1016/J.NTT.2021.106983>
- Ravula, A. R., & Yenugu, S. (2021). Pyrethroid based pesticides—chemical and biological aspects. *Critical Reviews in Toxicology*, 51(2), 117–140. <https://doi.org/10.1080/10408444.2021.1879007>,
- Ravula, A. R., & Yenugu, S. (2022). Transgenerational effects on the fecundity and sperm proteome in rats exposed to a mixture of pyrethroids at doses similar to human consumption. *Chemosphere*, 290. <https://doi.org/10.1016/j.chemosphere.2021.133242>
- Ray, D. E., & Forshaw, P. J. (2000). Pyrethroid insecticides: Poisoning syndromes, synergies, and therapy. *Journal of Toxicology - Clinical Toxicology*, 38(2), 95–101. <https://doi.org/10.1081/CLT-100100922;WGROU:STRING:PUBLICATION>
- Rehwagen, C. (2006). WHO recommends DDT to control malaria. *BMJ*, 333(7569), 622. <https://doi.org/10.1136/BMJ.333.7569.622-B>
- Saillénfait, A. M., Ndiaye, D., & Sabaté, J. P. (2015). Pyrethroids: Exposure and health effects – An update. *International Journal of Hygiene and Environmental Health*, 218(3), 281–292. <https://doi.org/10.1016/J.IJHEH.2015.01.002>
- Setchell, B. P. (1998). The Parkes Lecture Heat and the testis. *Reproduction*, 114(2), 179–194. <https://doi.org/10.1530/JRF.0.1140179>
- Shafer, T. J., Meyer, D. A., & Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. *Environmental Health Perspectives*, 113(2), 123–136. <https://doi.org/10.1289/EHP.7254/ASSET/6EC4539A-030B-4559-A72F-0D82DE6AE3B7/ASSETS/GRAPHIC/EHP0113-000123F4.JPG>
- Siu, M. K. Y., & Yan Cheng, C. (2009). Extracellular Matrix and Its Role in Spermatogenesis. *Advances in Experimental Medicine and Biology*, 636, 74–91. https://doi.org/10.1007/978-0-387-09597-4_5
- Skakkebaek, N. E., Rajpert-De Meyts, E., Buck Louis, G. M., Toppari, J., Andersson, A. M., Eisenberg, M. L., Jensen, T. K., Jørgensen, N., Swan, S. H., Sapra, K. J., Ziebe, S., Priskorn, L., & Juul, A. (2015). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*, 96(1), 55–97. <https://doi.org/10.1152/PHYSREV.00017.2015/ASSET/IMAGES/LARGE/Z9J0011627530021.JPEG>
- Skinner, M. K., Ben Maamar, M., Sadler-Riggelman, I., Beck, D., Nilsson, E., McBirney, M., Klukovich, R., Xie, Y., Tang, C., & Yan, W. (2018). Alterations in sperm DNA methylation, non-coding RNA and

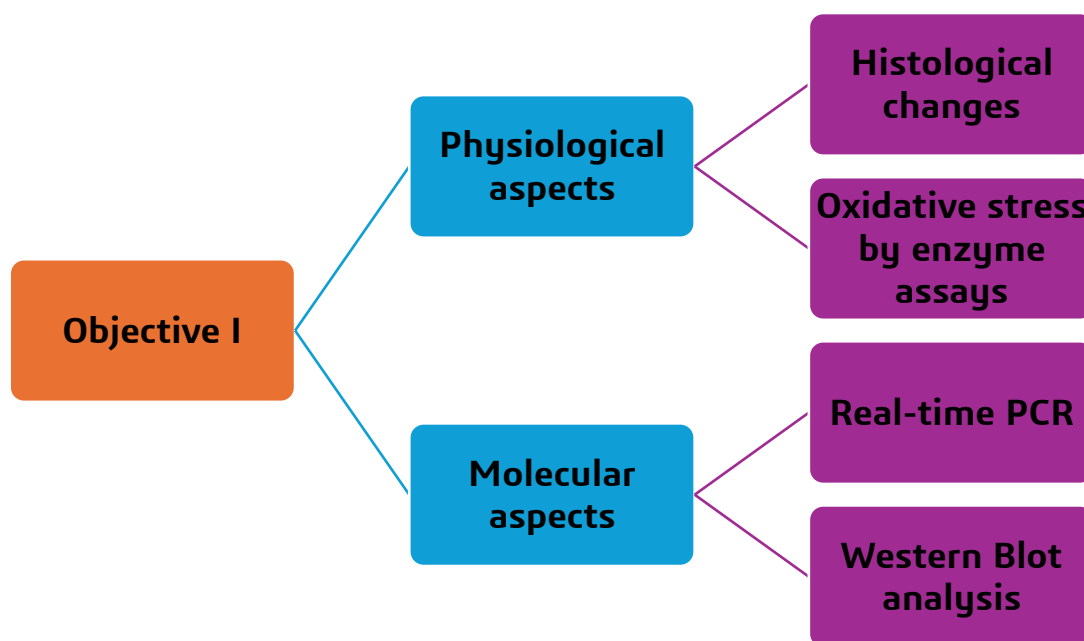
- histone retention associate with DDT-induced epigenetic transgenerational inheritance of disease. *Epigenetics and Chromatin*, 11(1), 1–24. <https://doi.org/10.1186/S13072-018-0178-0/FIGURES/10>
- Skolarczyk, J., Pekar, J., & Nieradko-Iwanicka, B. (2017). Immune disorders induced by exposure to pyrethroid insecticides. *Postepy Higieny i Medycyny Doswiadczalnej (Online)*, 71(0), 446–453. <https://doi.org/10.5604/01.3001.0010.3827>,
- Smith, L. B., & Walker, W. H. (2014). The regulation of spermatogenesis by androgens. *Seminars in Cell & Developmental Biology*, 30, 2–13. <https://doi.org/10.1016/J.SEMCDB.2014.02.012>
- Soderlund, D. M. (2012). Molecular mechanisms of pyrethroid insecticide neurotoxicity: Recent advances. *Archives of Toxicology*, 86(2), 165–181. <https://doi.org/10.1007/S00204-011-0726-X/FIGURES/4>
- Soderlund, D. M., & Bloomquist, J. R. (1989). Neurotoxic actions of pyrethroid insecticides. *ANNU. REV. ENTOMOL.*, 34(Volume 34, 1989), 77–96. <https://doi.org/10.1146/ANNUREV.EN.34.010189.000453/CITE/REFWORKS>
- Soderlund, D. M., Clark, J. M., Sheets, L. P., Mullin, L. S., Piccirillo, V. J., Sargent, D., Stevens, J. T., & Weiner, M. L. (2002). Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, 171(1), 3–59. [https://doi.org/10.1016/S0300-483X\(01\)00569-8](https://doi.org/10.1016/S0300-483X(01)00569-8)
- Soerensen, S. J. C., Lim, D. S., Montez-Rath, M. E., Chertow, G. M., Chung, B. I., Rehkopf, D. H., & Leppert, J. T. (2024). Pesticides and prostate cancer incidence and mortality: An environment-wide association study. *Cancer*. <https://doi.org/10.1002/CNCR.35572>
- Sparks, T. C., Storer, N., Porter, A., Slater, R., & Nauen, R. (2021). Insecticide resistance management and industry: the origins and evolution of the Insecticide Resistance Action Committee (IRAC) and the mode of action classification scheme. *Pest Management Science*, 77(6), 2609–2619. <https://doi.org/10.1002/PS.6254;REQUESTEDJOURNAL:JOURNAL:15264998;WGROU:STRIN G:PUBLICATION>
- Thacker, J. R. M. (2002). *An Introduction to Arthropod Pest Control*. Cambridge University Press. <http://www.cambridge.org>
- Toshio, N. (1992). Nerve membrane Na⁺ channels as targets of insecticides. *Trends in Pharmacological Sciences*, 13(C), 236–241. [https://doi.org/10.1016/0165-6147\(92\)90075-H](https://doi.org/10.1016/0165-6147(92)90075-H)
- Tracy, T. S., Chaudhry, A. S., Prasad, B., Thummel, K. E., Schuetz, E. G., Zhong, X. B., Tien, Y. C., Jeong, H., Pan, X., Shireman, L. M., Tay-Sontheimer, J., & Lin, Y. S. (2016). Interindividual Variability in Cytochrome P450–Mediated Drug Metabolism. *Drug Metabolism and Disposition*, 44(3), 343–351. <https://doi.org/10.1124/DMD.115.067900>
- Tudi, M., Ruan, H. D., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., & Phung, D. T. (2021). Agriculture Development, Pesticide Application and Its Impact on the Environment. *International Journal of Environmental Research and Public Health* 2021, Vol. 18, Page 1112, 18(3), 1112. <https://doi.org/10.3390/IJERPH18031112>
- Vijverberg, H. P. M., & vanden Bercken, J. (1990). Neurotoxicological Effects and the Mode of Action of Pyrethroid Insecticides. *Critical Reviews in Toxicology*, 21(2), 105–126. <https://doi.org/10.3109/10408449009089875>
- Wan, N. F., Fu, L., Dainese, M., Kiær, L. P., Hu, Y. Q., Xin, F., Goulson, D., Woodcock, B. A., Vanbergen, A. J., Spurgeon, D. J., Shen, S., & Scherber, C. (2025). Pesticides have negative effects on non-target organisms. *Nature Communications* 2025 16:1, 16(1), 1–16. <https://doi.org/10.1038/s41467-025-56732-x>

- Weston, D. P., Asbell, A. M., Hecht, S. A., Scholz, N. L., & Lydy, M. J. (2011). Pyrethroid insecticides in urban salmon streams of the Pacific Northwest. *Environmental Pollution*, 159(10), 3051–3056. <https://doi.org/10.1016/J.ENVPOL.2011.04.008>
- World Health Organization. (2015). World Malaria Report 2015 - World Health Organization - Google Books. *WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland*. https://books.google.com.my/books?hl=en&lr=&id=rg4LDgAAQBAJ&oi=fnd&pg=PP1&dq=http%3D%3Dwww.who.int%3Dmalaria%3Dmedia%3Dworld-malaria-report-2016%3Den%3D&ots=XTmAQRN0NF&sig=j4f1cuEWu5mLMR13c6_Msor-dl4&redir_esc=y#v=onepage&q=http%3A%3D%3Dwww.who.int%3Dmal
- Yan Cheng, C., & Mruk, D. D. (2012). The Blood-Testis Barrier and Its Implications for Male Contraception. *Pharmacological Reviews*, 64(1), 16–64. <https://doi.org/10.1124/PR.110.002790>
- Zhang, X., Zhang, T., Ren, X., Chen, X., Wang, S. Q., & Qin, C. (2021). Pyrethroids Toxicity to Male Reproductive System and Offspring as a Function of Oxidative Stress Induction: Rodent Studies. *Frontiers in Endocrinology*, 12, 656106. <https://doi.org/10.3389/FENDO.2021.656106/BIBTEX>
- Zirkin, B. R., & Papadopoulos, V. (2018). Leydig cells: formation, function, and regulation. *Biology of Reproduction*, 99(1), 101–111. <https://doi.org/10.1093/BIOLRE/IOY059>

Chapter I

Evaluating the transgenerational effect of a mixture of Pyrethroids on the **physiological (histological) and transcriptomic changes** in the male reproductive system.

Graphical presentation of Objective-I



Introduction

Pesticides, particularly pyrethroids, are widely employed in agriculture and residential settings due to their efficacy in controlling a wide range of pests and perceived safety when compared to previous pesticide classes. Pyrethroids are synthetic derivatives of natural pyrethrins that affect the neurological system of insects, causing paralysis and death. However, their widespread use has generated worries about its effects on non-target creatures, such as humans. Studies have already shown the possible effects of pyrethroids on the male reproductive system (Zhang et al., 2021). These chemicals can disrupt endocrine function, alter hormone levels, and impair sperm quality, resulting in decreased sperm count, motility, and viability. The mechanism is thought to include oxidative stress, DNA damage, and interaction with androgen signaling pathways. Furthermore, long-term pyrethroid exposure has been linked to testicular toxicity, which includes histological abnormalities such as seminiferous tubule degeneration and spermatogenesis impairment. While regulatory bodies monitor pyrethroid levels in the environment to reduce human exposure, new findings highlight the need for caution and additional research to fully understand their long-term impacts on reproductive health and further offspring (Gnambani et al., 2023).

Spermatogenesis

Spermatogenesis is the biological process by which mature spermatozoa (sperm cells) emerge from spermatogonial stem cells (SSCs) in the testes' seminiferous tubules. **(Figure 1.1)** This intricate and highly regulated process is separated into three stages: mitosis, meiosis, and spermiogenesis (Mruk & Cheng, 2004).

Mitotic Phase: SSCs divide many times to generate diploid spermatogonia. These cells develop into primary spermatocytes, providing a steady supply of cells for spermatogenesis (De Rooij & Russell, 2000).

Meiotic Phase: Primary spermatocytes enter meiosis I, giving rise to haploid secondary spermatocytes. These cells go on to conduct meiosis II, which produces haploid spermatids. This phase maintains genetic variety by homologous recombination and chromosomal reduction from diploid to haploid (Handel & Schimenti, 2010).

Spermiogenesis: Spermatids go through a series of morphological and structural changes to become mature spermatozoa, such as chromatin condensation, acrosome creation, flagellum development, and cytoplasm shedding (Griswold, 2016).

Numerous genes and signalling pathways carefully regulate the spermatogenesis process to guarantee normal sperm cell growth and activity (Linn et al., 2021). Hormonal regulation influences spermatogenesis as well, including testosterone from Leydig cells and FSH from the pituitary gland, which work together to promote germ cell development (Walker, 2011). Disruptions in the function or expression of these genes might cause infertility, emphasising their importance in male reproductive health. Spermatogenesis is controlled by a complicated network of genes and signalling pathways. These genes and hormones work together to ensure that spermatogenesis occurs efficiently and precisely. Mutations, deletions, or dysregulation of any of these variables can cause male infertility by disrupting germ cell proliferation, differentiation, or maturation (Krausz & Riera-Escamilla, 2018).

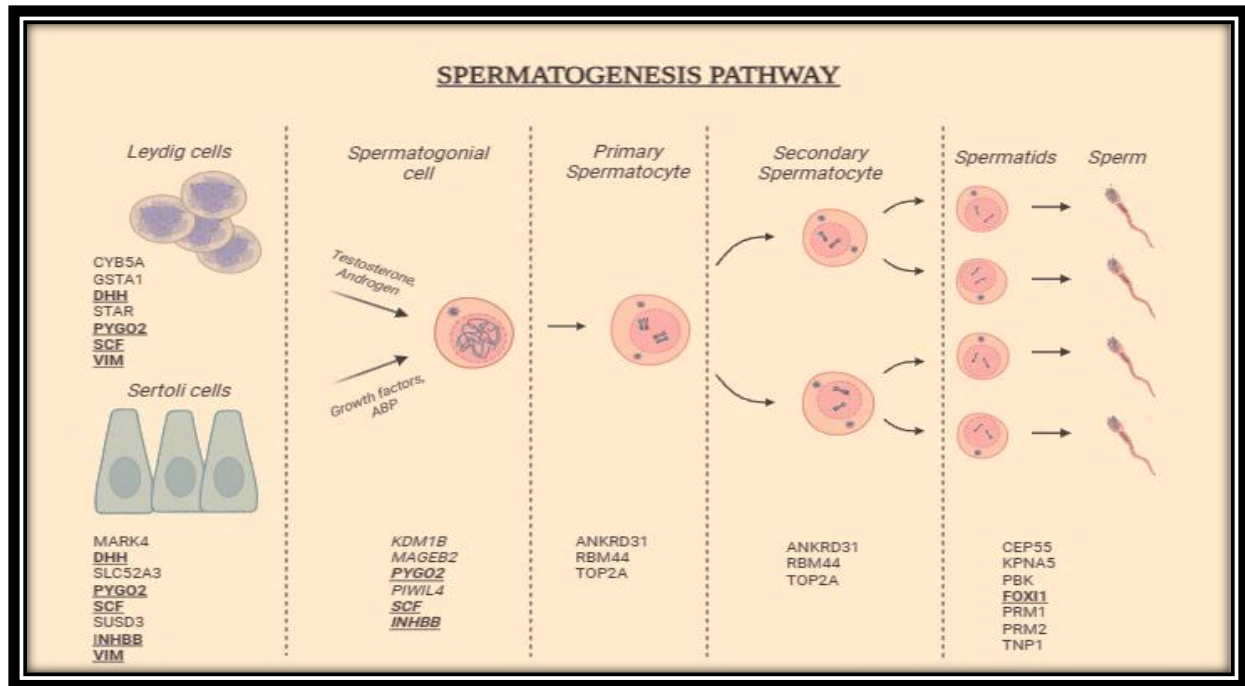


Figure 1.1: Schematic representation of genes involved in the spermatogenic Pathway.

Testis development, also known as testicular differentiation, is a complicated process that involves the formation and maturation of the male gonads (Wilhelm et al., 2007). This process begins during embryogenesis and continues postnatally, facilitated by a network of signalling pathways, transcription factors, and particular genes (Nef & Parada, 2000). **(Figure 1.2)** The testes develop by differentiating primordial germ cells (PGCs), establishing testis-specific cell types (such as Sertoli, Leydig, and germ cells), and producing the testicular architecture, which includes seminiferous tubules. The following is a comprehensive description of the stages of testis development and the genes involved (McLaren, 2003).

Phases of Testis Development

Bipotential Gonad Formation: During early embryogenesis, the gonads are bipotential and can develop into either testes or ovaries. During this stage, PGCs migrate to the gonadal ridge and integrate into the gonadal primordium (Nef & Parada, 2000).

Testis Determination: Testis development is determined by the expression level of the Y chromosome's SRY (Sex-determining Region Y) gene. The male-specific transcriptional cascade that eventually results in the differentiation of the testis's first cell type is started by the SRY gene. For example, Sertoli cells (Koopman et al., 1991).

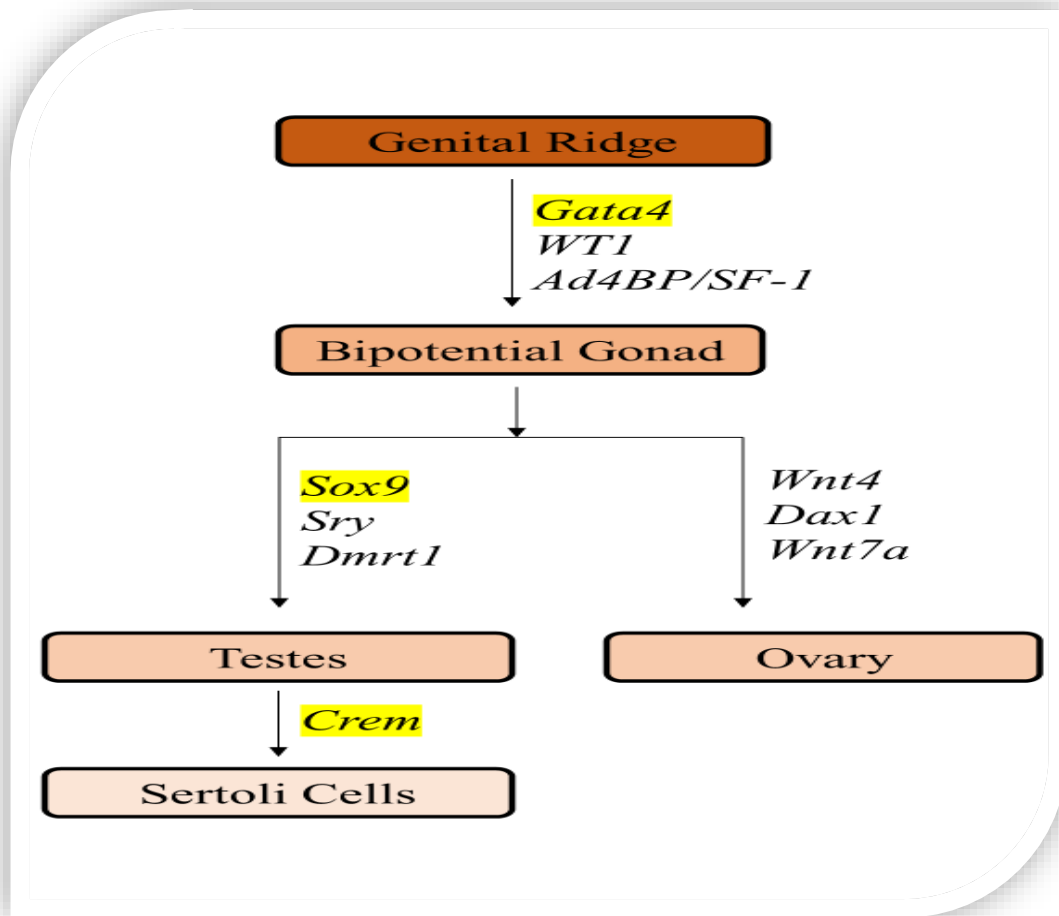


Figure 1.2: Schematic representation of genes related to the development of Testes and ovary.

The testis Differentiation & Morphogenesis: Sertoli cells form testis cords and encapsulate germ cells. Leydig cells develop in the interstitial space and initiate testosterone synthesis, which is necessary for masculinisation (Habert et al., 2001).

Postnatal Testis Development: Testicular maturation continues postnatally, with the formation of seminiferous tubules and the start of spermatogenesis at puberty (Linn et al., 2021).

Molecular Interactions and Pathways

The WNT/ β -Catenin pathway is inhibited during testis development to prevent ovarian destiny. Mutations in this system can result in sex development abnormalities (Chassot et al., 2008). Notch signalling regulates the differentiation and proliferation of Sertoli and Leydig cells (Tang et al., 2008). The TGFB (Transforming Growth Factor Beta) pathway regulates testicular architecture and germ cell development. Disruptions in these genes and processes can result in

DSDs, such as cryptorchidism, hypospadias, and infertility (Baskin & Ebbers, 2006). Understanding these pathways sheds light on male reproductive biology and suggests potential treatment targets for genetic and environmental causes of male infertility.

Steroidogenesis

Steroidogenesis is the biochemical process of the synthesis of steroid hormones from cholesterol (Miller & Auchus, 2011). (**Figure 1.3**) Leydig cells, which generate testosterone, the primary male sex hormone necessary for spermatogenesis, secondary sexual traits, and general male reproductive function, are the primary site of steroidogenesis in the testes (Ye et al., 2017). A crucial rate-limiting step in the process is the transport of cholesterol into mitochondria, which is facilitated by the steroidogenic acute regulatory (StAR) protein. Stocco (2001). The P450 side-chain cleavage enzyme CYP11A1 converts cholesterol into pregnenolone in the mitochondria. (Miller, 2013). After that, pregnenolone is sent to the smooth endoplasmic reticulum, where it is subjected to further enzyme modifications. The 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzyme transforms pregnenolone into progesterone, a precursor to androgens (Payne & Hales, 2004). CYP17A1 (17 α -hydroxylase/17,20-lyase) converts progesterone to androstenedione, an intermediary in testosterone production (Auchus, 2001). Androstenedione is transformed to testosterone via 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (Labrie, 1991). The enzyme 5 α -reductase converts testosterone to dihydrotestosterone (DHT) or oestradiol via aromatase, depending on tissue needs (Russell & Wilson, 1994). The HPG axis has considerable control over steroidogenesis. The pituitary gland secretes luteinizing hormone (LH), which interacts with its receptor on Leydig cells to activate the cAMP and protein kinase A (PKA) signalling pathways (Ascoli et al., 2002).

These pathways promote the production of steroidogenic enzymes and transport proteins such as StAR (Stocco, 2001). Disruptions in any of these processes or enzymes, whether caused by genetic mutations, environmental factors, or hormonal imbalances, can affect steroidogenesis and result in conditions such as hypogonadism, infertility, or altered secondary sexual characteristics (Miller & Auchus, 2011). Understanding the complex mechanisms of steroidogenesis is critical for detecting and treating these diseases, as well as developing reproductive medicine.

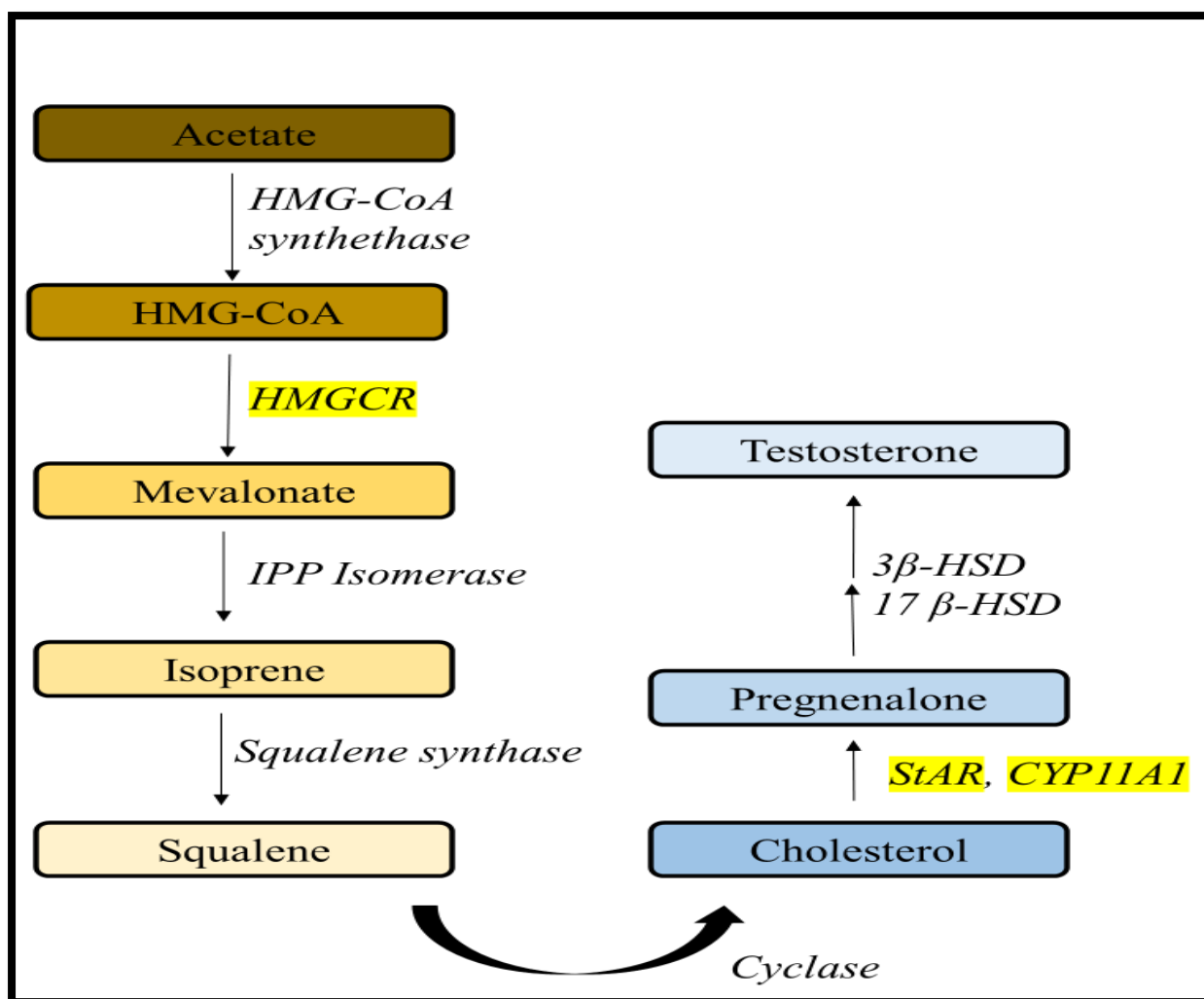


Figure 1.3: Schematic representation of Testosterone synthesis from precursors.

Pyrethroids, despite their usefulness, offer major dangers to male reproductive health by affecting numerous aspects of testicular function. The cumulative effects on spermatogenesis, steroidogenesis, sperm development, and testis development highlight the importance of using pyrethroids with caution and strict regulation, especially in populations susceptible to reproductive toxicity, such as agricultural laborers and young boys (Zhang et al., 2021). Further research into the mechanisms of pyrethroid toxicity and potential protective measures is required to reduce their influence on reproductive health.

Oxidative Stress

ROS are essential for immune defense and cellular signaling under normal physiological settings, but oxidative stress can result from either excessive ROS generation or inadequate

antioxidant defense (Dröge, 2002). Age, cancer, neurological disorders, and reproductive toxicity are all exacerbated by this syndrome's degradation of lipids, proteins, DNA, and other biological components. ROS are highly reactive chemicals that comprise hydroxyl radicals ($\text{OH}^\cdot$), superoxide anion (O_2^-), and non-radical species such hydrogen peroxide (H_2O_2). (Valko et al., 2007).

Pyrethroids are synthetic insecticides that have been shown to induce oxidative stress in both in vitro and in vivo studies. (A. K. Singh et al., 2012). (**Figure 1.4**) Cells convert pyrethroids like deltamethrin, cypermethrin, and permethrin into reactive intermediates, which then react with oxygen molecules to create reactive oxygen species (ROS). Pyrethroids also disrupt mitochondrial function by disrupting the electron transport chain (ETC), which leads to excessive ROS production and electron leakage (Gassner et al., 1997). These hazardous effects are exacerbated by pyrethroids' capacity to change the expression of antioxidant enzymes, reducing the body's ability to neutralise oxidative stress (Abdollahi et al., 2004).

Oxidative stress causes lipid peroxidation, which destroys cellular membranes, resulting in membrane integrity loss and cellular malfunction. Protein oxidation can degrade enzymatic activity and structural proteins, whereas oxidative DNA damage can cause mutations, apoptosis, and carcinogenesis (Betteridge, 2000). Pyrethroid-induced oxidative stress in reproductive systems impairs sperm integrity, motility, and viability, alters steroidogenic pathways, and causes testicular tissue damage (Joshi et al., 2007). Thus, oxidative stress is a crucial mechanism driving pyrethroid-induced toxicity, with far-reaching consequences for cellular health and physiological function (Banerjee et al., 1999).

Apoptosis

Apoptosis is a type of programmed cell death that is essential for tissue remodeling, cellular homeostasis, and development (Elmore, 2007). Apoptosis, in contrast to necrosis, is a regulated process that results in particular morphological and biochemical alterations, such as DNA fragmentation, chromatin condensation, membrane blebbing, and cell shrinkage (Kerr et al., 1972). Both extrinsic (death receptor) and intrinsic (mitochondrial) mechanisms tightly regulate the process (Green & Llambi, 2015). The intrinsic pathway involves mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome c, which in turn triggers caspase-9 and downstream executioner caspases (like caspase-3). It is mainly regulated by the balance of pro-apoptotic (like BAX, BAK) and anti-apoptotic (like BCL-2, BCL-XL) proteins. (Youle & Strasser, 2008).

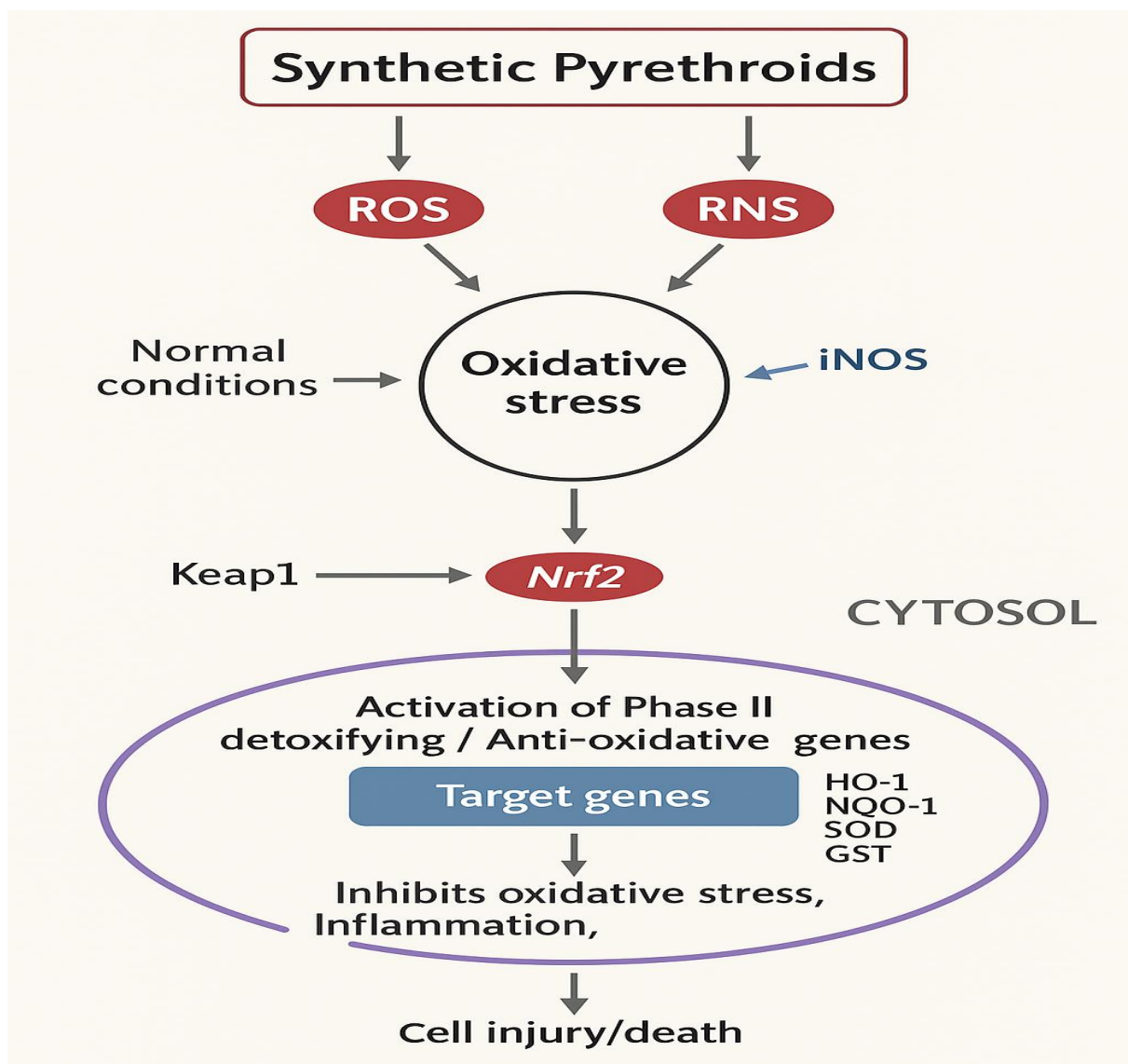


Figure 1.4: Representation of Induction of oxidative stress by Pyrethroids.

Pyrethroids, as environmental pollutants, can cause apoptosis by establishing an oxidative stress environment within cells (Banerjee et al., 1999). The cellular redox balance is upset by these chemicals because they increase the production of reactive oxygen species (ROS). Excess ROS can lead to stress reactions by harming lipids, proteins, and DNA, which are examples of biological macromolecules (Valko et al., 2006). Cytochrome C is released, and the apoptotic machinery is activated when ROS destabilize the mitochondrial membrane potential (MMP) (Ott et al., 2007). Pyrethroids can produce oxidative stress, activating signalling pathways such as p53, JNK, and NF- κ B that promote apoptosis (Kannan & Jain, 2000). Pyrethroids in

testicular tissues inhibit germ cell survival and steroidogenic activity by inducing apoptosis in Sertoli, Leydig, and spermatogenic cells. For instance, the p53 pathway is activated by pyrethroid-induced oxidative DNA damage, which directly upregulates pro-apoptotic genes and causes cell death (Joshi et al., 2007). Therefore, one of the main ways that pyrethroids cause their cytotoxic and reproductive damaging effects is through the interplay between oxidative stress and apoptosis. This emphasises the need to understand these pathways when developing preventative methods against pyrethroid-induced harm.

Testicular cancer

As environmental pollutants, pyrethroids have been linked to a higher risk of carcinogenesis, particularly testicular cancer. Because they alter hormonal signaling and the hypothalamic-pituitary-gonadal (HPG) axis, these pesticides are known endocrine disruptors and can create hormonal imbalances that promote cancer. (Gore et al., 2015). Pyrethroids have the ability to upset the balance between cell division and death in testicular tissues, which can lead to the development of tumors (Joshi et al., 2007). Disruptions in the apoptosis and oxidative stress pathways are major drivers of carcinogenesis. Apoptosis typically functions as a defense mechanism to get rid of damaged or defective cells (Elmore, 2007). However, by changing the expression of crucial apoptotic regulators like BAX (pro-apoptotic) and BCL-2 (anti-apoptotic), pyrethroid exposure may suppress apoptosis and enable cells with genetic abnormalities to survive and multiply (Naseri et al., 2015). On the other hand, prolonged exposure to pyrethroids can cause extreme oxidative stress, which overpowers antioxidant defenses and causes long-term cellular harm. The NF- κ B and MAPK signaling pathways are activated by persistent ROS production, which results in inflammation, cell survival, and proliferation all of which are linked to cancer (Taniguchi & Karin, 2018). Moreover, epigenetic modifications like DNA methylation and histone acetylation brought on by oxidative stress may activate oncogenes and silence tumor suppressor genes, speeding up the development of cancer (Baccarelli & Bollati, 2009).

One of the most prevalent diseases in young men is testicular cancer, which is marked by abnormal testicular cell proliferation, typically brought on by germ cells. Seminomas and non-seminomas are the two most common forms of testicular germ cell tumours, and the latter include embryonal carcinoma, yolk sac tumours, choriocarcinoma, and teratomas. Genetic predisposition, hormonal imbalances, environmental exposures, and lifestyle variables all have an impact on the development of testicular cancer. Disruptions in the p53 tumour suppressor

pathway, dysregulated cell cycle control, and abnormal apoptosis processes are among the key molecular pathways involved in testicular cancer (Hainaut & Hollstein, 1999). (**Table 1.1**) In the case of testicular cancer, these disturbances can cause germ cells to change malignantly. Pyrethroid exposure causes oxidative stress, endocrine disturbance, and defective apoptosis, all of which contribute to carcinogenesis (Darwish et al., 2025). This emphasises the significance of reducing exposure to environmental chemicals such as pyrethroids and conducting additional research on their long-term impacts on reproductive health and cancer risk (Gore et al., 2015).

Table 1.1: Table representing important proteins related to Testicular cancer and related functions.

Protein	Function
Lactate dehydrogenase (LDH)	Elevated LDH levels are correlated to high tumor burden in seminoma and recurrence in NSGCT
P53	It acts as a tumor suppressor, it regulates cell division by keeping cells from growing and dividing (proliferating) too fast or in an uncontrolled way.
Alpha-fetoprotein (AFP)	Testicular tumor markers (hCG, AFP, and LDH) are instrumental in the diagnosis, staging, and surveillance of seminomas and NSGCTs.
Vascular endothelial growth factor (VEGF)	A protein that promotes tumor growth and angiogenesis
Coiled-Coil Domain containing 83 (Ccde83)	CCDC83 may be a novel CT antigen that can be used as a target molecule for cancer immunotherapy.

Systemic toxicity

Pyrethroids, a popular class of synthetic insecticides, are increasingly recognised for their ability to produce systemic toxicity, notably by interfering with metabolic processes such as glucose metabolism and transport (Xiao et al., 2018). These effects are mediated by endocrine disruption, inflammation, and oxidative stress, all of which are factors in the development of metabolic diseases including diabetes and its aftereffects. (Rains & Jain, 2011).

Permethrin and deltamethrin have been shown to cause oxidative stress by increasing reactive oxygen species (ROS), decreasing insulin receptor activation, and inhibiting downstream signalling pathways such as PI3K-Akt. Pyrethroid-induced inflammation promotes insulin resistance by activating pro-inflammatory cytokines like TNF- α and IL-6. Pyrethroids can also interact with glucose transporter proteins (like GLUT4), inhibiting their translocation to the plasma membrane and impairing insulin-stimulated glucose uptake. (**Table 1.2**)

Hyperglycemia is the outcome of peripheral tissues like skeletal muscle and adipose tissue absorbing less glucose.

Pyrethroids can impair pancreatic β -cell function in addition to causing peripheral insulin resistance (Sakr & Rashad, 2023). These chemicals cause oxidative stress and mitochondrial malfunction, resulting in β -cell death, reduced insulin production, and worsened hyperglycemia. Chronic pyrethroid exposure has also been associated with the deregulation of hepatic glucose metabolism, which promotes gluconeogenesis and glycogenolysis and raises blood glucose levels. These metabolic abnormalities not only contribute to the development of diabetes but also increase its consequences. Persistent hyperglycemia and oxidative stress can harm the vascular endothelium, causing diabetic nephropathy, neuropathy, and retinopathy.

Additionally, advanced glycation end products (AGE) are produced in response to pyrethroid-induced oxidative stress, exacerbating tissue damage and inflammation in diabetics (V. P. Singh et al., 2014). Overall, pyrethroid-induced systemic toxicity indicates the chemicals' potential role in altering glucose metabolism, increasing insulin resistance and contributing to the etiology of diabetes and related comorbidities. This emphasises the necessity for strict pyrethroid control and additional research into their metabolic effects to protect public health (Wei et al., 2023).

Table 1.2: Table representing Glucose utilization-related proteins and their function

Protein	Function
Glucose transporter 4 (Glut4 ▼)	GLUT4 is the main protein that transports blood glucose into the cells of muscle and fat tissue. the skeletal expression of GLUT4 in type 2 diabetes patients is significantly reduced.
Nerve growth factor (NGF ▼)	Nerve growth factor (NGF) is a protein that plays a role in diabetic neuropathy and other metabolic processes. NGF is important for the survival of sensory and sympathetic neurons, which are often affected in diabetic neuropathy.
c-Kit receptor tyrosine kinase (C-kit ▼)	It regulates glucose metabolism and beta cell function in the pancreas, which may make it a potential target for treating diabetes
Vascular endothelial growth factor (Vegf ▲)	It is involved in the progression of diabetic retinopathy, neuropathy, and nephropathy.
Apelin (Apln ▼)	It may also help regulate glucose metabolism and improve insulin sensitivity.
Sodium glucose cotransporter 1 (SGLT1/Nagt-1 ▲)	It helps the body absorb glucose from the small intestine. In diabetes, SGLT1 inhibition can help improve glucose homeostasis.
Insulin-like growth factor (IGF-1~)	It is a hormone that helps regulate glucose levels and may play a role in the development of type 2 diabetes. IGF-1 levels that are too high or too low may be linked to impaired glucose tolerance and a higher risk of type 2 diabetes.
Heat shock protein 90 (Hsp90 ▲)	<i>It</i> plays a role in vascular changes and inflammation in diabetes. HSP90 levels increase in the blood as diabetic vascular disease worsens.
Inducible nitric oxide synthase (iNOS ▲)	It plays a role in diabetes by contributing to insulin resistance, vascular inflammation, and nerve damage.

Methodology

Pyrethroids

Cypermethrin (Cat No. 36128; CAS No. 52315-7-8), deltamethrin (Cat No. 45423; CAS No. 52918-63-5), fenvalerate (Cat No. 45495; CAS No. 51630-58-1), cyhalothrin L (Cat# 72765; CAS No. 68085-85-8) and allethrin (Cat No. 33396; CAS No. 584-79-2) were analytical grade and purchased from Sigma-Aldrich (St. Louis, USA) . For the western Blot analysis the antibodies were procured from Santa Cruz, with catalog numbers; GAPDH (Cat No. SC-25778), b-ACTIN (SC-4778), DHH (SC-271168), SCF (SC-13126), VIM (SC-6260), GATA4 (SC-25310), SOX9 (SC-166505), HMGCR (SC-271595), HIF1 α (Cat No. D2U3T), BCL2 (Cat No. D17C4), DNMT3a (SC-373905), DNMT3b (SC-376043), and HDAC1 (SC-81598)

Animal maintenance and care

Animals (male Wistar rats aged 30 days) were maintained in propylene cages that were bedded with dry rice husk at the University of Hyderabad animal house under standard conditions (room temperature at $23 \pm 3^{\circ}\text{C}$; $50 \pm 20\%$ of relative humidity; ample ventilation and 12 h photoperiod). After acclimatization for 2 weeks, rats were subjected to the treatment protocol. Two to three animals were kept in each of the propylene cages that were bedded with dry rice husks. Animals were constantly monitored for their well-being, and their body weights were noted once a week.

Dosage and treatment

Five pyrethroids, namely cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin were selected for the treatment since they are known to be present at residual levels in rice and vegetables sold in the Indian market. The amount of each of these pyrethroids present in rice and vegetables was derived from the reports of “Monitoring of pesticides at National level conducted by Government of India”. An average Indian consumes 345 and 43 grams of rice and vegetables, respectively per day (total 388 grams) (Ravula & Yenugu, 2021b). The concentration of cypermethrin, deltamethrin, fenvalerate, cyhalothrin L and allethrin in 388 grams of rice and vegetables turns out to be 598.3, 90.40, 32.32, 201.29 and 19.4 μg , respectively (totalling to 941.71 μg). Thus, an average individual with a body weight of 60 kg will be exposed to 9.97, 1.506, 0.538, 3.35 and 0.323 μg of cypermethrin, deltamethrin,

fenvalerate, cyhalothrin L, and allethrin, respectively (cumulative amount of 15.695 µg/kg body weight) per day. This dosage was used to treat the rats to reflect the human exposure. The mixture of pyrethroids was prepared in corn oil. It is widely accepted that 1 month's lifetime of rats is equal to 2.5 years of humans (Andreollo et al., 2012). In this study, rats were treated for 9 months (equivalent to exposure for 22.5 years in humans; 9 rat months × 2.5 human years), thus, a direct reflection of the long-term exposure of humans to pyrethroids through food on a daily basis starting from childhood stage to adulthood.

Animal treatments

Two groups of rats (aged 45 days), namely control and pyrethroid treated (n = 10 in each group), were maintained. To one group of rats, a mixture of pyrethroids (15.695 µg/kg body weight) dissolved in 500 µl of corn oil was administered orally for 9 months, with dosage adjustment every week based on the body weight (**Figure 1.5**). The control group received only corn oil (500 µl).

At the end of the treatment period, some F0 rats were sacrificed by euthanasia, and tissues were collected and stored at -80°C for further use; whereas some F0 generation rats were kept for mating with control females, resulting in the generation of F1 pups, and a similar trend of mating with control females was followed to generate F2 and F3 generations. Testis tissues were collected from rats of four generations (Ravula & Yenugu, 2022b). The Institutional Animal Ethics Committee of the University of Hyderabad explicitly approved this work (IAEC/UH/151/2017/01/SY/P9/Rat Wistar/M-75), and all operations involving animals were carried out in accordance with the rules for the care and use of laboratory animals to minimise suffering.

S. No.	Rat (in months)	Human (in years)
1	1	2.5
2	3	7.5
3	6	15
4	9	22.5
5	12	30
6	15	37.5

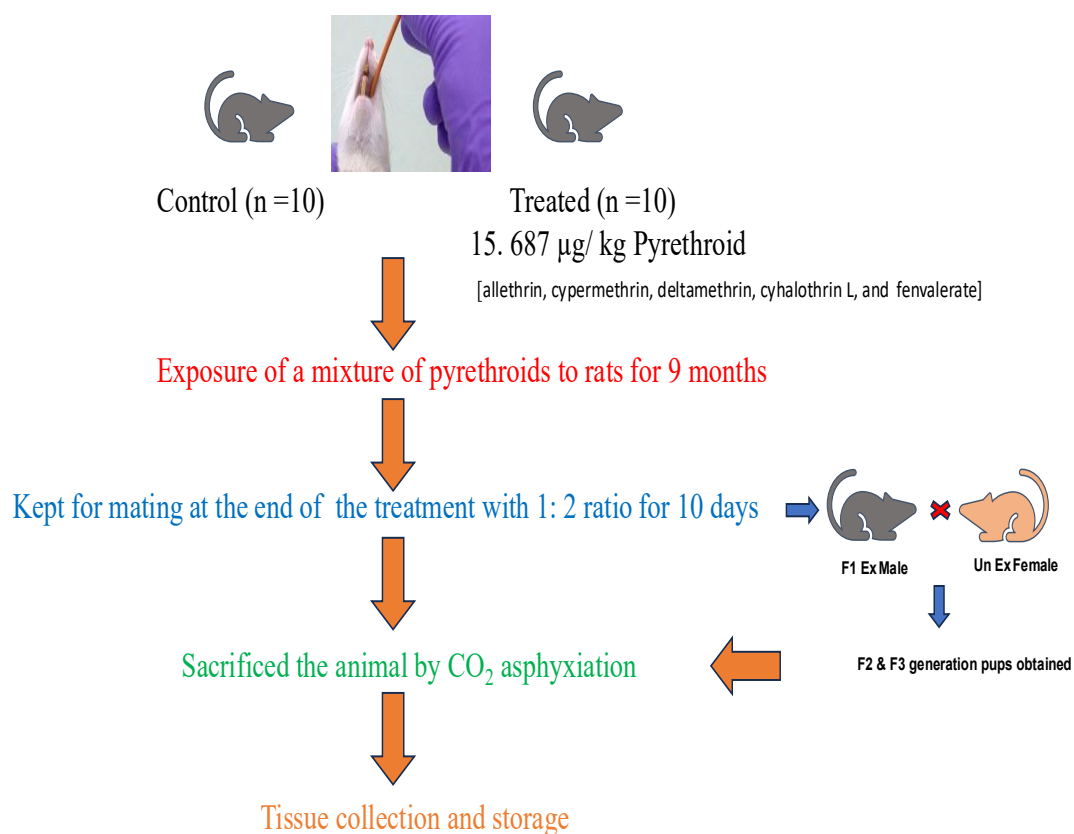


Figure 1.5: Overall animal experimentation strategy

Real-time polymerase chain reaction

Quantitative gene expression analyses were carried out by real-time polymerase chain reaction as described earlier (Ravula & Yenugu, 2021a). Testes tissues (100 mg) were homogenized in TRIzol reagent (Invitrogen), and the total RNA was isolated. Reverse transcription of the isolated RNA (2 µg) was carried out with 50 U Stratascript (Invitrogen) and 0.5 µg of oligodT (Invitrogen). Gene-specific primers, cDNA, and SYBR PCR reagent (Applied Biosystems, UK) were mixed, and real-time PCR was performed in a thermal cycler. The thermal cycling conditions, in general, were at 94° C for 10 min (initial denaturation), 40 cycles at 94° C for 15 and 60°C for 1 min. For internal control, *Gapdh* mRNA expression was determined. Relative gene expression was estimated by the ($\Delta\Delta C_t$) method. Primers used in this study are listed in Tables 1.3 to 1.7.

Table 1.3: Primer sequences to amplify genes related to spermatogenesis.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Dhh</i>	FP	TGCTACGCGGTTCTAGAGAG	159
	RP	CATTAACCTCCTCCGCCAAGC	
<i>Vim</i>	FP	AATGCTTCTCTGGCACGTCT	100
	RP	GCTCCTGGATCTCTTCATCG	
<i>Scf</i>	FP	GATGACCTCGTGGCATGTATG	190
	RP	CAGGACCTAATGTTGAAGAGAG	
<i>Inhbb</i>	FP	TAACGAAGGCAACCAGAACC	193
	RP	GGTATGCCAGCCACTACGTT	
<i>Pygo2</i>	FP	GCGAAGAAAGTCCAATACTCAGG	165
	RP	GTACAGGACTGCCAAGGAAC	
<i>Tp2</i>	FP	GGCCTCAAAGTCACACCAAT	161
	RP	GCTGGGTCTTGAAGGTGAGT	
<i>Dazap1</i>	FP	GACTGGAGTACCACTCAAGAGA	178
	RP	CATCTAGGGTGTGTGGTCTG	
<i>Hsf-2</i>	FP	TAAATGGCTCCTCCAACCTG	99
	RP	CCAACAGCTCAACCTTTCCTA	
<i>Ddx3y</i>	FP	GGATGCTGGATATGGGATTTG	129
	RP	CACGAGGCATCTGTATCTC	
<i>Ybx2</i>	FP	GTGACCAACAGCAGGGAGAT	189
	RP	GGAAGTAGGGACGGTTTCGT	
<i>Tgfb</i>	FP	GCAACAATTCCTGGCGTTAC	180
	RP	CCACGTGGAGTACATTATCTTTGC	
<i>Fshr</i>	FP	CTCATCAAGCGACACCAAGA	185
	RP	GGAAAGGATTGGCACAAGAA	
<i>Amh</i>	FP	CTAGCCACCTTCGGAGTCTG	162
	RP	AGGCTCTTGGAACCTTCAGCA	

Table 1.4: Primer sequences to amplify genes related to steroidogenesis.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Lhr</i>	FP	CTGCGCTGTCCTGGCC	103
	RP	CGACCTCATTAAGTCCCCTGAA	
<i>Sox9</i>	FP	AGGAAGCTGGCAGACCAGTA	157

	RP	TGTAATCGGGGTGGTCTTTC	
<i>Gata4</i>	FP	CTGTGCCAACTGCCAGACTA	165
	RP	AGATTCTTGGGCTTCCGTTT	
<i>Crem</i>	FP	TACTGCTTTGCCACAAGGTG	194
	RP	GATTTTCAAGCACAGCCACA	
<i>Hmgcr</i>	FP	TGCTGCTTTGGCTGTATGTC	187
	RP	CCCTTTGGGTTACTGGGTTT	
<i>Cyp11a1</i>	FP	AGCTGCCTGGGATGTGATT	155
	RP	TGTTGGCCTGGATGTTCTTG	
<i>StAR</i>	FP	CTCCTACAGACATATGCGGAAC	175
	RP	CTGGTCACTGTAGAGTGTTG	

Table 1.5: Primer sequences to amplify genes related to apoptosis and oxidative stress.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Nqo1</i>	FP	GAAGCTGCAGACCTGGTGAT	170
	RP	GTGGTGATGGAAAGCAAGGT	
<i>Ho1</i>	FP	GAAGAAGATTGCGCAGAAGG	181
	RP	GAAGGCGGTCTTAGCCTCTT	
<i>Hif1</i>	FP	TCAAGTCAGCAACGTGGAAG	198
	RP	TATCGAGGCTGTGTGCGACTG	
<i>Nrf2</i>	FP	CCTTCCTCTGCTGCCATTAG	150
	RP	GTTCAGTGAAATGCCGGAGT	
<i>Casp3</i>	FP	GGACCTGTGGACCTGAAAAA	159
	RP	GCATGCCATATCATCGTCAG	
<i>Casp9</i>	FP	CTCAGGCCAGAGGTTCTCAC	173
	RP	GGGCAGAAAGTTCACGTTGTT	
<i>Bcl2</i>	FP	CTGGCATCTTCTCCTTCCAG	183
	RP	GACGGTAGCGACGAGAGAAG	
<i>Bax</i>	FP	CTGCAGAGGATGATTGCTGA	174
	RP	GATCAGCTCGGGCACTTAG	

Table 1.6: Primer sequences to amplify genes related to testicular cancer.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Ldha</i>	FP	GCATGGCAGCCTTTTCCTTA	150
	RP	ATGTTACGTTTCGCTGGAC	
<i>Afp</i>	FP	CTGCAAACCTACCCACCATCG	195
	RP	ACAGGAAGGTTGGGGTGATT	
<i>Vegfa</i>	FP	CCAATTGAGACCCTGGTGGAC	182
	RP	CTTTGGTGAGGTTTGATCCGC	
<i>Ccdc83</i>	FP	ACTGCAGACTCGTGGATCTC	164
	RP	CAACCTGTCGTCCTCTCCT	
<i>Tp53</i>	FP	AGTGGGAATCTTCTGGGACG	178
	RP	TCTTTTGCTGGGGAGAGGAG	

Table 1.7: Primer sequences to amplify genes related to diabetes-related complications.

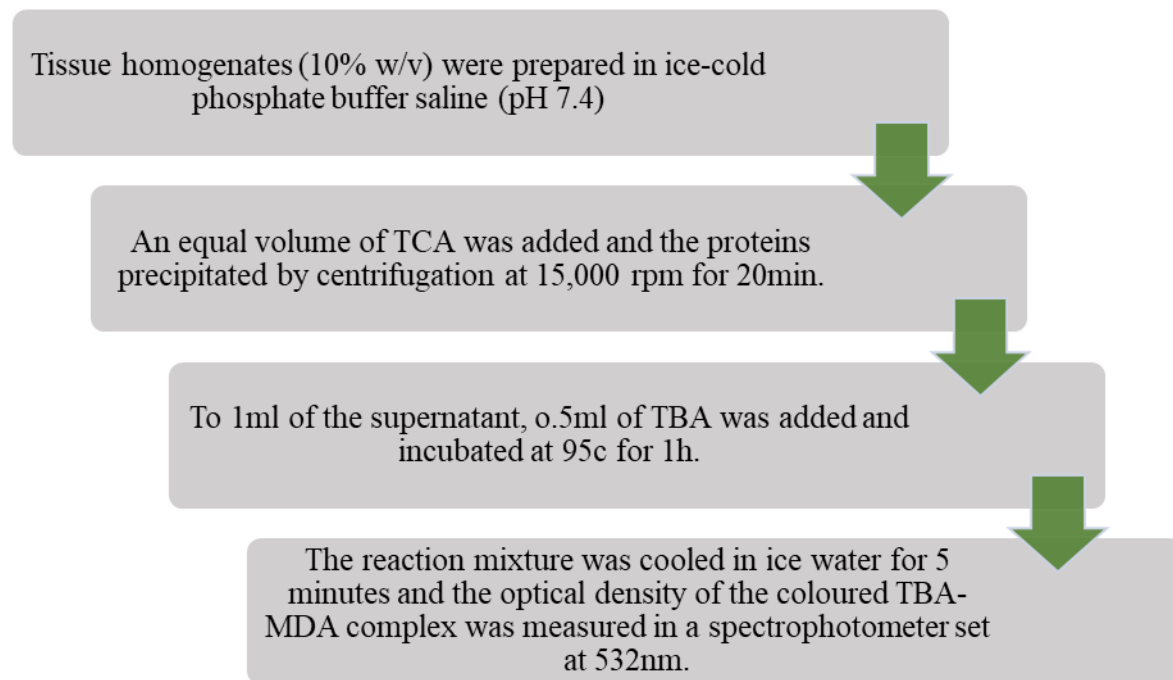
Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Slc2a3/Glut3</i>	FP	TTCGCTGTTACTGTTGCCAC	168
	RP	CACACATAACGACCAGAGCG	
<i>Nag1l</i>	FP	CACGTTGGCATGGAAGAGAG	158
	RP	AAGAAAGATGAGGCCAGGCT	
<i>Apln</i>	FP	AATCTGAGTTTCTGCGTGCA	154
	RP	CTGGTCCAGTCCTCGAAGTT	
<i>Igf1</i>	FP	TTCCGGAGCTGTGATCTGAG	162
	RP	CCTTCTCCTTGCAGCTTCC	
<i>Ngf</i>	FP	CAGTGTCAGTGTGTGGGTTG	150

	RP	CTCTCTACAGGATTCGGGGC	
<i>C-kit</i>	FP	GTCGGAACCTGAAGGTCCTGA	152
	RP	AGGAGTCACGCTTCCTTCTC	
<i>Hsp90/Cdc37</i>	FP	GCAAGGGAGCAGAAACACAAGAC	191
	RP	ATCAGGGCACACTTCTCCTCG	
<i>Inos/Nos2</i>	FP	GTTTGACCAGAGGACCCAGA	175
	RP	GTGAGCTGGTAGGTTCTCTGT	

Histology

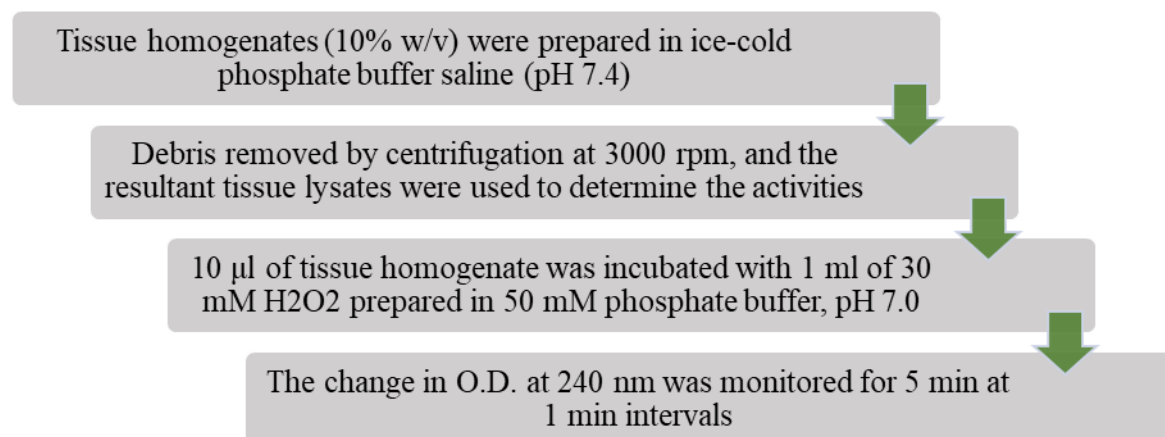
Testis tissue samples of rats treated with the pyrethroid mixture and control rats were subjected to histopathological examinations. Standard procedures were used to prepare the tissues. To get rid of the yellow tint, the samples were first soaked in Bouin's solution for 24 hours before being thoroughly cleaned with 70% ethanol. Graded ethanol solutions (80%, 90%, and 100%) were used to gradually dehydrate the tissues before they were treated in isopropanol at 60 degrees Celsius for the entire night. Following processing, the tissues were cut into five-micron sections for histological staining and fixed in paraffin wax. The sections were deparaffinized in xylene, rehydrated using graded ethanol solutions (100%, 90%, 80%, 70%, and 50%), and then rinsed in distilled water in order to prepare them for staining. Following a 10-minute staining procedure with Harris hematoxylin solution, the samples were rinsed quickly with distilled water, differentiated for 30 seconds in 1% hydrochloric acid, and then immersed for 30 to 1 minute in 0.2% ammonia water. The sections were washed with distilled water to remove excess reagents, and then counterstained for one minute with 0.2% eosin Y solution. They were dehydrated using 10%, 70%, 80%, 90%, and 100% alcohol solutions after being washed with distilled water. The pieces were mounted using a xylene-based medium after three xylene changes for cleaning. A board-certified histopathologist who was unaware of the research design or treatment plans examined and photographed the prepared slides. Using established techniques reported in prior research, at least 25 fields from each stained section were examined for histopathological alterations.

Lipid peroxidation TBARS-Assay method



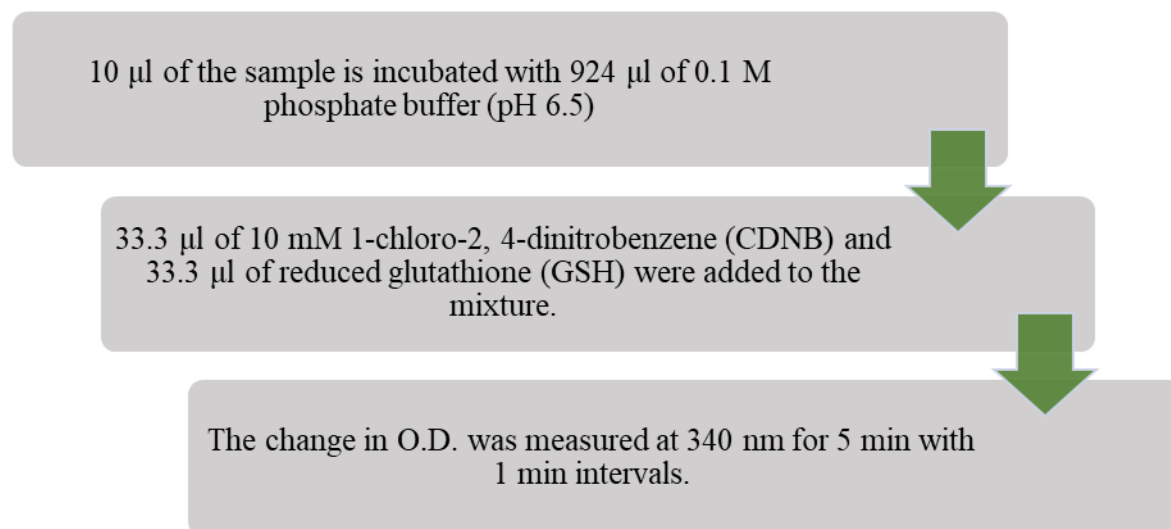
Malondialdehyde (MDA), a byproduct of lipid peroxidation, serves as a barometer for the level of lipid peroxidation in the sample. The thiobarbituric acid reactive substances (TBARS) assay was used to measure the extent of lipid peroxidation in tissues (Bernheim, 1964). To find the concentration of lipid peroxides, a standard curve was created using 1,1,3,3-tetraethoxypropane (TEP) at various concentrations. The amount of lipid peroxide was measured in nanomoles of MDA per milligram of protein.

Catalase Assay



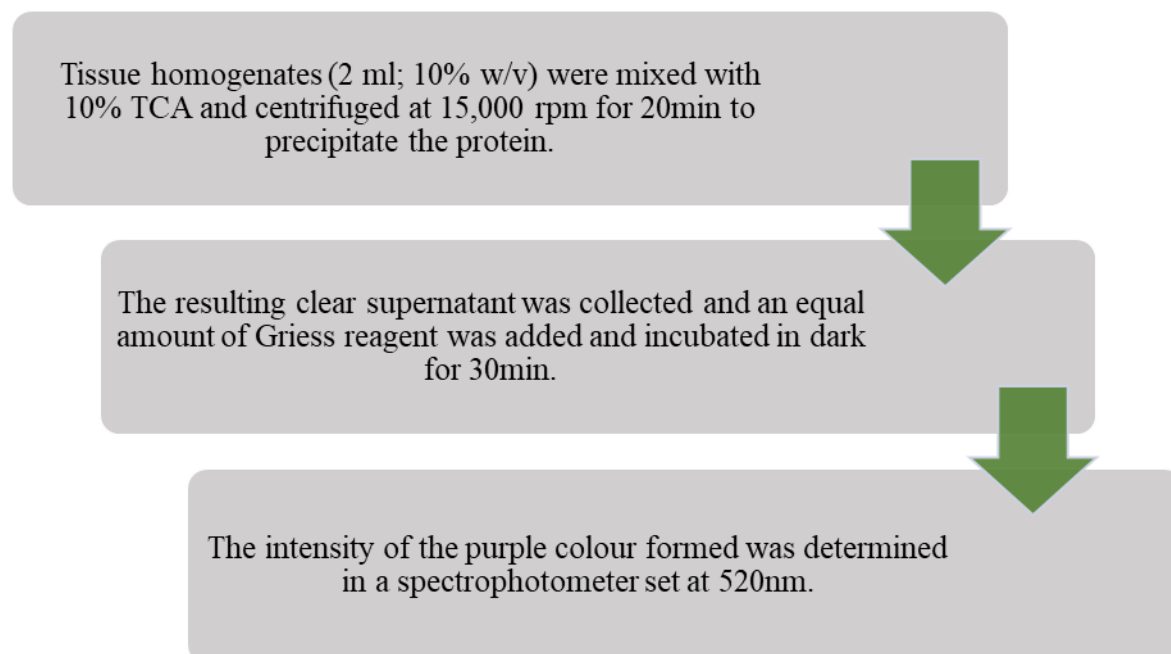
The first-order reaction is used to compute the activity, which is represented as K30 per mg protein: $K30 = (2.303/30) * \log (A1/A2)$. The maximum OD value is indicated by A1, and the lowest by A2. (Aebi, 1984).

Glutathione S-Transferase Assay



The activity was calculated using the method absorbance difference ($\Delta OD/min$) x V x 100 (dilution factor) / 9.6 x protein concentration in mg, where V is the volume of the reaction mixture and 9.6 is the molar extinction coefficient of CDNB. (Jakoby, 2006).

Nitric oxide Assay



The Griess technique was used to quantify the amounts of nitric oxide (NO). A standard curve with different sodium nitrite concentrations was used to test the nitrite levels, and the NO concentration was represented as $\mu\text{moles nitrite/mg protein}$. (Lepoivre et al., 1990).

Immunoblotting

The testes tissues of 9-month Wistar rats were homogenized in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 1% sodium dodecyl sulfate). After centrifuging the homogenates for 10 minutes at 12,857 g, Lowry's technique was used to measure the amount of protein in the supernatant. After loading the same amount of proteins in all required wells, SDS-PAGE was run. Before the transfer, the gel had to be submerged in a transfer buffer for fifteen minutes. The gel was positioned with the cathode (black) facing it, and the nitrocellulose membrane was positioned with the anode facing black. The proteins were transferred to the membrane over the course of 12–16 hours at 25V. In order to dissipate the heat produced by the high voltage and to avoid damaging the equipment and proteins, wet transfer was done at 50V for four hours while the tank was encircled by ice flakes. Ponceau stain was used to measure the effectiveness of protein transfer, and the membrane was then washed three times with TBS until the stain was gone. Following this, the membrane was blocked for three hours at room temperature using a blocking solution of 5% skimmed milk in TBST. The membrane was treated with the primary antibody in a blocking buffer for either one hour at room temperature or overnight at 4°C on a shaker without being washed after blocking. The membrane was rinsed with TBS for 15 minutes, TBS-T for 15 minutes, then TBS for an additional 15 minutes following incubation with the primary antibody. After that, it was incubated at room temperature for one to one and a half hours with the secondary antibody. The blot was then developed using developing solutions after being successively rinsed for 15 minutes with TBS, TBS-T, and TBS.

Statistical analyses

The students' t-test, available in GraphPad Prism Software version 8.0.2 (225 Franklin Street, Boston), was used to conduct statistical analyses. Mean $\pm$ SD is presented for the values obtained for each parameter. Statistical significance of $p < 0.05$ between control and treated groups is indicated by the * symbol

Results

Histopathological changes:

Histopathological analyses of the testes obtained from pyrethroid-treated rats (F0 generation) indicated 30-40% distorted seminiferous tubules with degenerated spermatogenic cells. **(Figure 1.6)** In the testes obtained from the F1 to F3 generations, around 10-20% distorted seminiferous tubules were observed. Additionally, rats of the F0 generation had edema or fluid collection in their testes, and rats up to the F3 generation also showed these kinds of changes.

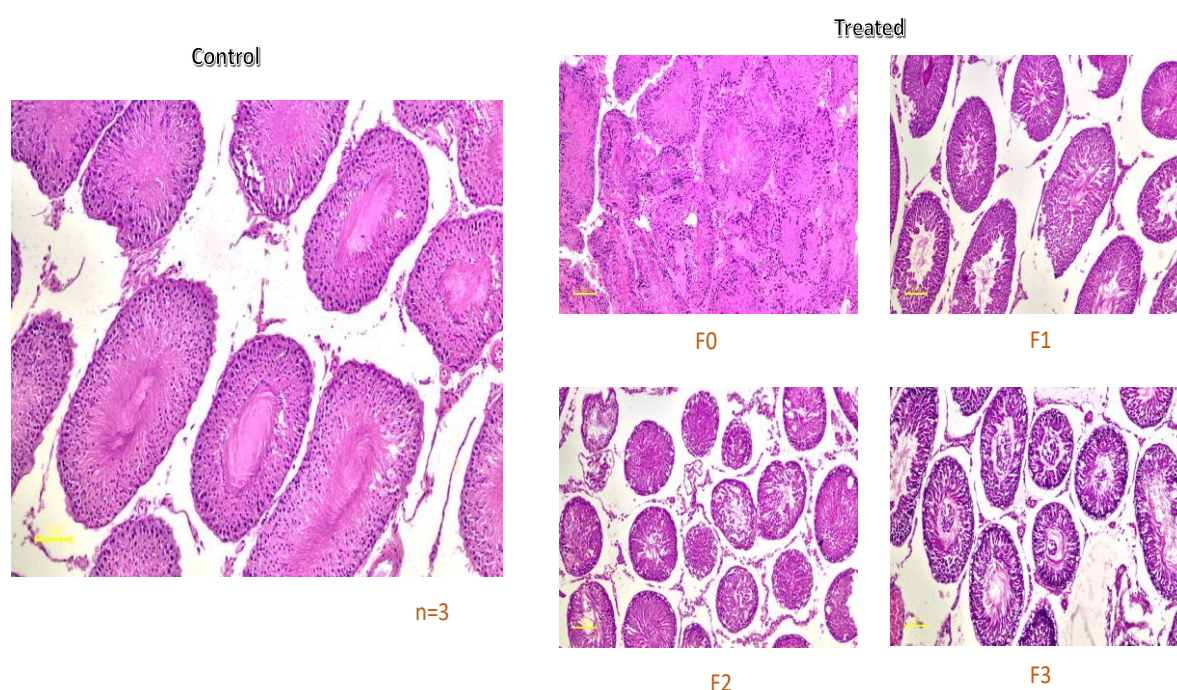


Figure 1.6: Transgenerational histopathological analyses of testis in pyrethroid-administered rats. Testis of all generations of rats were stained with hematoxylin and eosin. F0, F1, F2 & F3 represent the parental, first, second, and third generations. Scale bar: 100 μ M .

Transgenerational expression pattern of steroidogenesis associated genes.

The expression of spermatogenesis-related genes, namely, *Scf*, *Dhh*, *Vim*, *Tgf*, *Tp2*, *Inhbb*, *Dazapa*, and *Hsf2*, were significantly down-regulated in the testis of Pyrethroid-treated rats (F0 generation) and this trend was observed in the F1, F2, and F2 generations. *Amh* and *Fsh* expression was significantly upregulated transgenerationally. Genes like *Ddxy*, *Lh*, *Ybx2*, and *Pygo2* were found to be significantly downregulated in F0 generation, whereas variable changes are observed transgenerationally. **(Figure 1.7)**

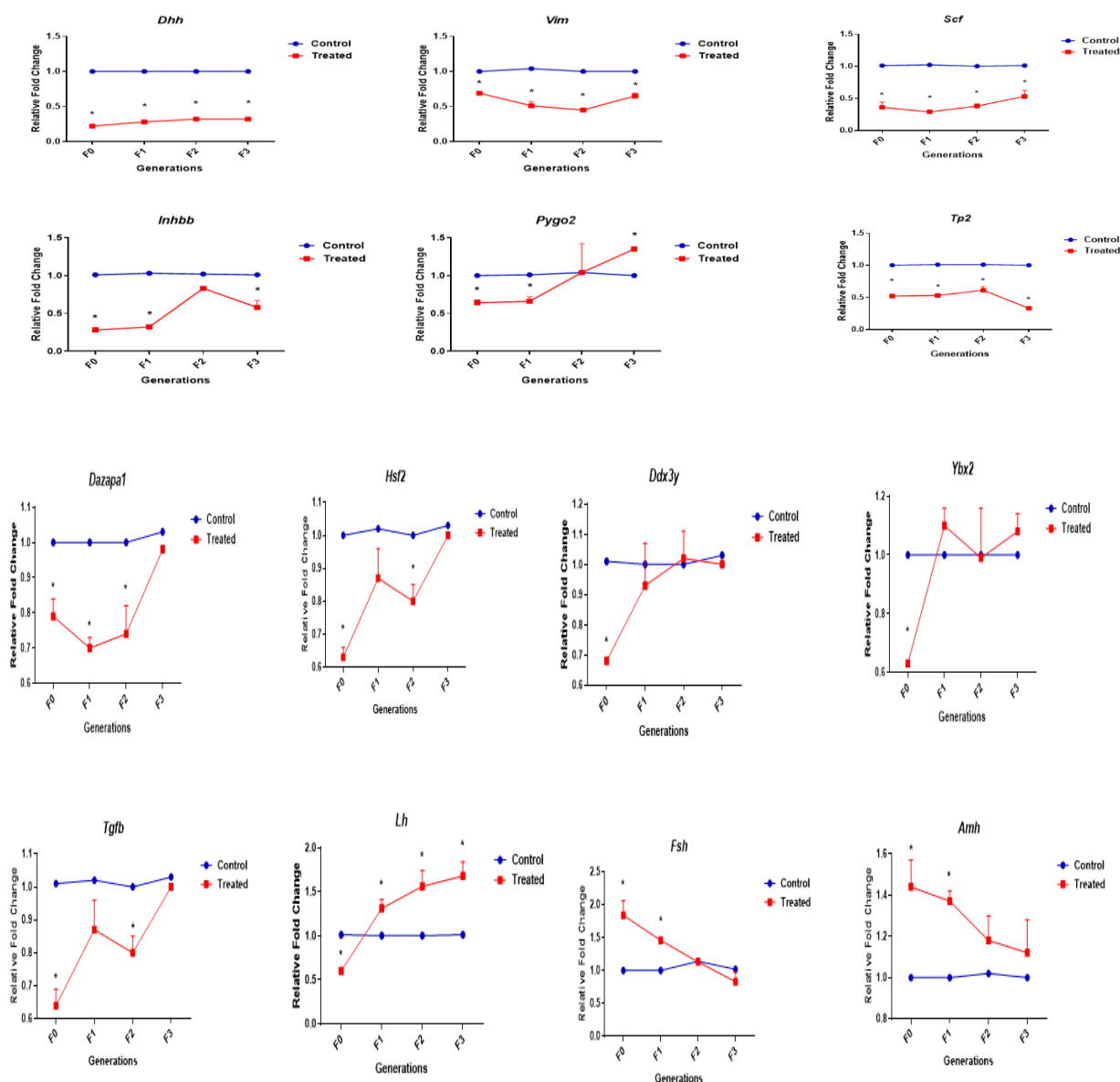


Figure 1.7: Expression pattern of spermatogenesis-related genes in the testes of pyrethroid-treated rats. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Transgenerational expression pattern of genes associated with steroidogenesis and testicular development.

We also analysed the transgenerational expression pattern of genes associated with steroidogenesis. *Sox9*, *Hmgcr*, and *Lhr* were significantly down-regulated in the testes of F0 generation rats and continued to have the same effect in the subsequent generations. *Cyp11a1*

and *Star*, which were found to be upregulated in the F0 generation, displayed the same trend of expression transgenerationally. *Gata4* and *Crem* showed variable changes across generations. (Figure 1.8)

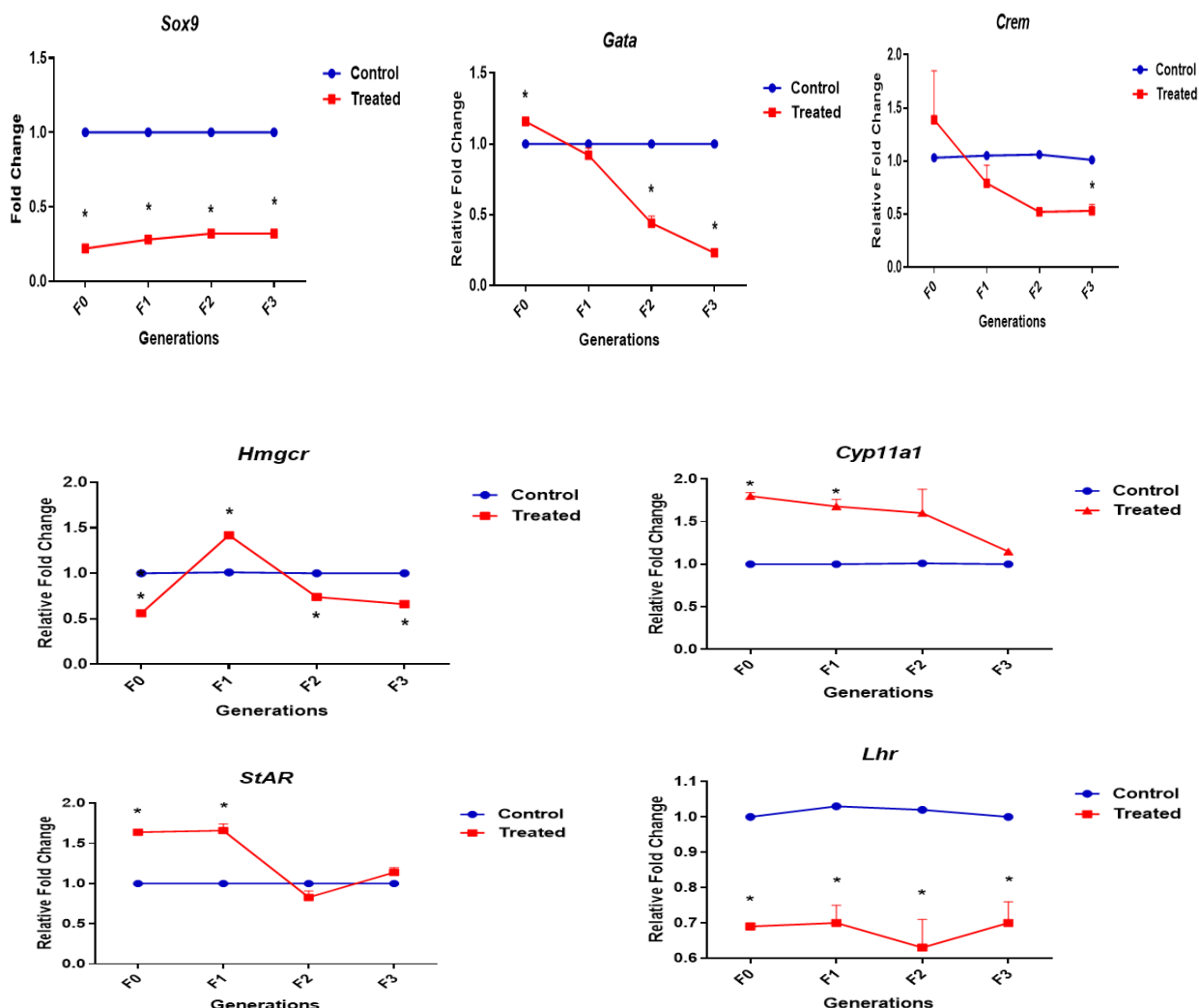


Figure 1.8: Expression pattern of steroidogenesis and testicular development-related genes in the testes of pyrethroid-treated rats. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Transgenerational antioxidant status and expression pattern of oxidative stress and germ cell apoptosis-associated genes.

Antioxidant enzyme activities were analyzed in the testes of Pyrethroid-treated rats (F0 generation) and the testes of rats obtained in subsequent generations (up to F3 generation). GST activity decreased in the tests obtained from all generations. Catalase activity was increased in

the testes of F0 and F1 generation rats, while it was decreased in the testes of F2 and F3 generations (F0[†], F1[†], F2[‡], F3[‡]). In general, lipid peroxidation (LPO) and Nitric Oxide levels were not altered significantly in any generation. (**Figure 1.9**).

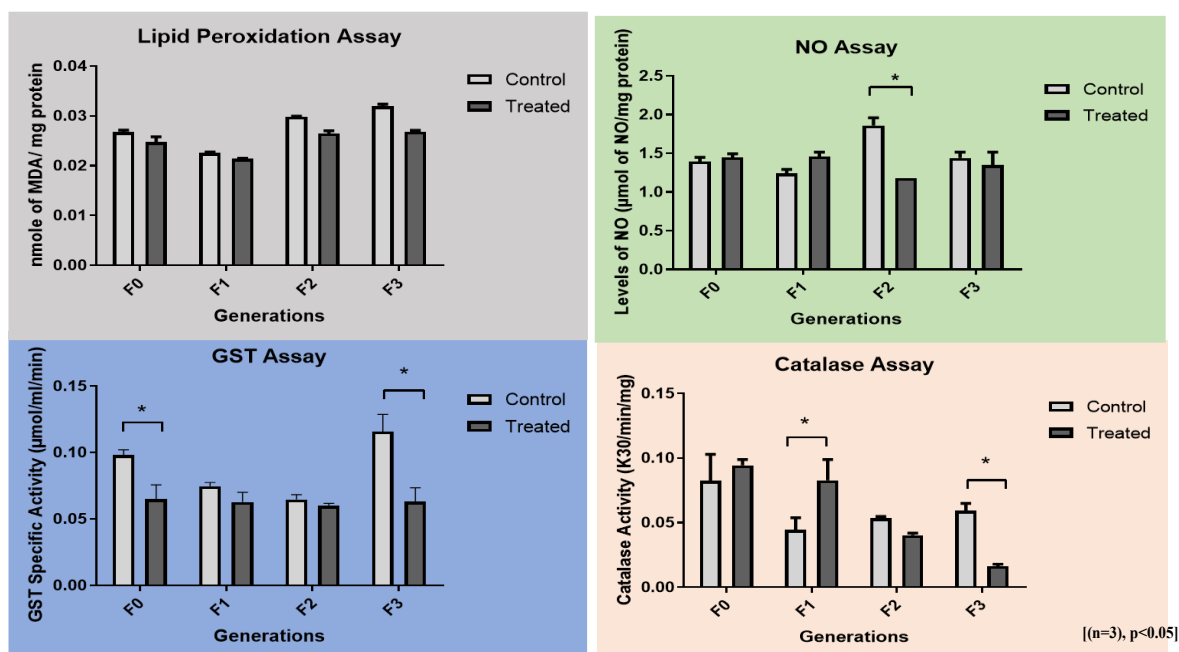


Figure 1.9: Antioxidant status in the testes of pyrethroid-treated rats and in their subsequent generations. Protein isolated by RIPA buffer from the testes of rats for all four generations was quantified and used for checking the antioxidant status via the above assays using respective compounds.

The gene expression profile of oxidative stress-related gene *Nqo1* was significantly down-regulated in the testis of pyrethroid-administered rats and continued transgenerationally (**Figure 1.10**). *Hif1a* showed significant up-regulation transgenerationally. *Nrf2* and *Ho1* displayed variable expression patterns transgenerationally (**Figure 1.10**). Since changes in antioxidant status affect apoptotic pathways, we analyzed the expression pattern of genes that govern apoptotic pathways (**Figure 1.11**). Expression of *Bcl2* was significantly up-regulated in F0 rats and continued to be up-regulated in the F1 generation. In contrast, no significant change was found in the F2 and F3 generations. *Casp3* and *Casp9* were significantly down-regulated in the testes of F0 generation rats, and the rats were obtained transgenerationally. The variable expression pattern was observed for *Bax* across generations.

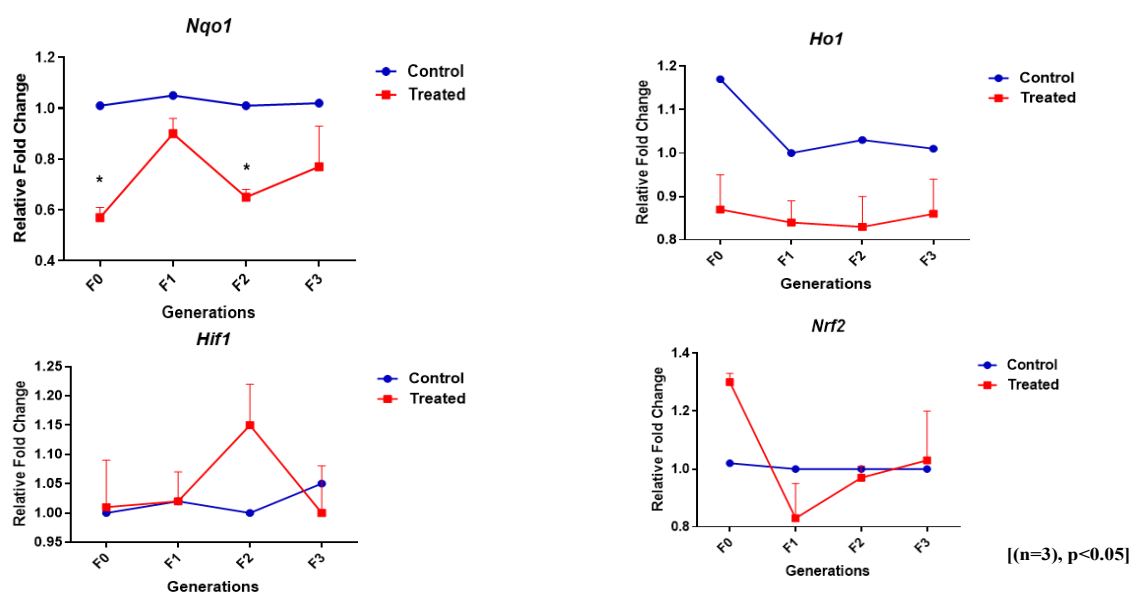


Figure 1.10: Expression pattern of oxidative stress-related genes in the testes of pyrethroid-treated rats. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

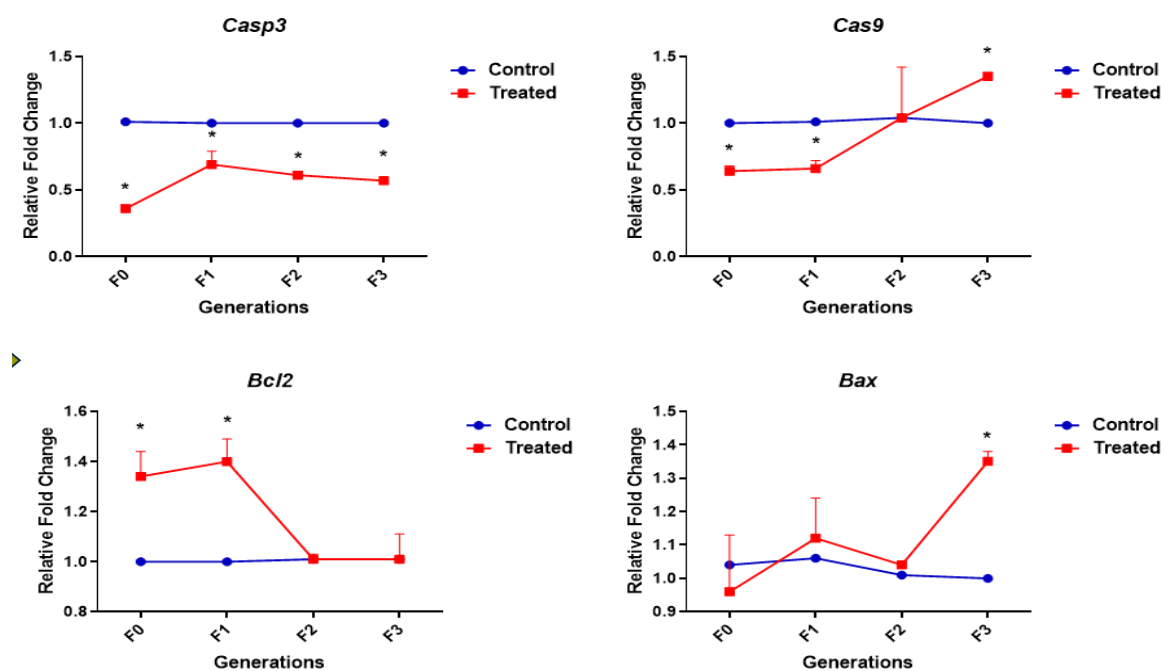


Figure 1.11: Expression pattern of apoptosis-related genes in the testes of pyrethroid-treated rats. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Transgenerational expression pattern of steroidogenesis associated genes:

Expression of testicular cancer-related genes *Afp*, *Ldh*, *Vegf*, and *Ccdc83* were upregulated in the testis of Pyrethroid-administered rats, and certain variable changes transgenerationally, whereas downregulated expression of *p53* was seen in all generations (**Figure 1.12**).

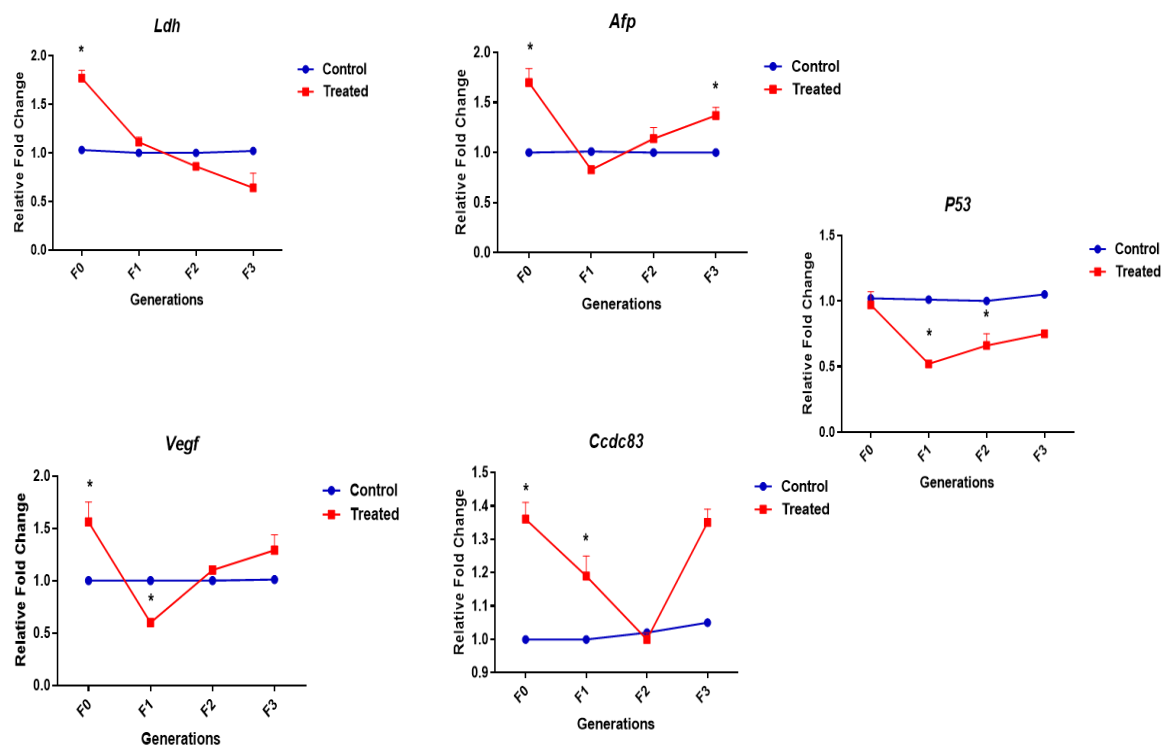


Figure 1.12: Expression pattern of testicular cancer-related genes in the testes of pyrethroid treated rats. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Diabetes-related genes like *Glut-3*, *C-kit*, *Vegf*, *HSP90*, *Apelin*, and *iNOS* were significantly upregulated in the testis of Pyrethroid-administered rats and showed certain variable changes transgenerationally, whereas *Igf1* and *Naglt-1* showed significant downregulation transgenerationally except few variations (**Figure 1.13**). The overall expression pattern for the transgenerational expression were arranged in **table 1.8**.

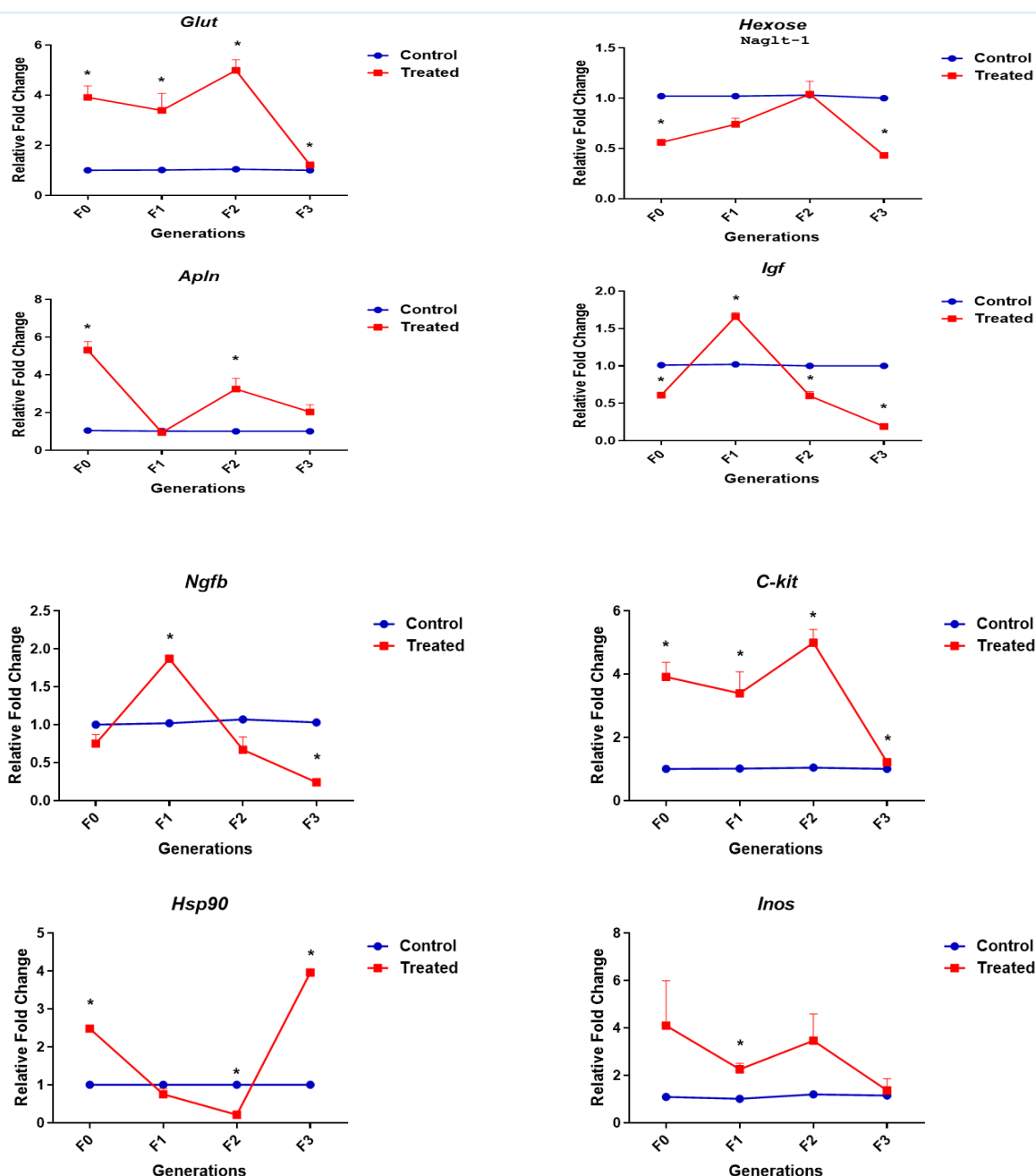


Figure 1.13: Expression pattern of diabetes-related genes in the testes of pyrethroid treated rats. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Transgenerational expression pattern of spermatogenesis, steroidogenesis, oxidative stress, and apoptosis-associated proteins.

Western blot analysis was performed only for those genes that showed a constant trend of transgenerational change. The spermatogenesis and steroidogenesis-related proteins like SCF, VIM, DHH, and SOX9 showed significant downregulation transgenerationally, whereas

certain variable transgenerational changes were seen in the case of GATA4 and HMGCR. BCL2 (F0⁺, F1⁺, F2⁻, F3⁻) and DNMT3b (F0⁺, F1⁺, F2⁺, F3⁻) also showed a similar trend as that of mRNA levels. (Figure 1.14)

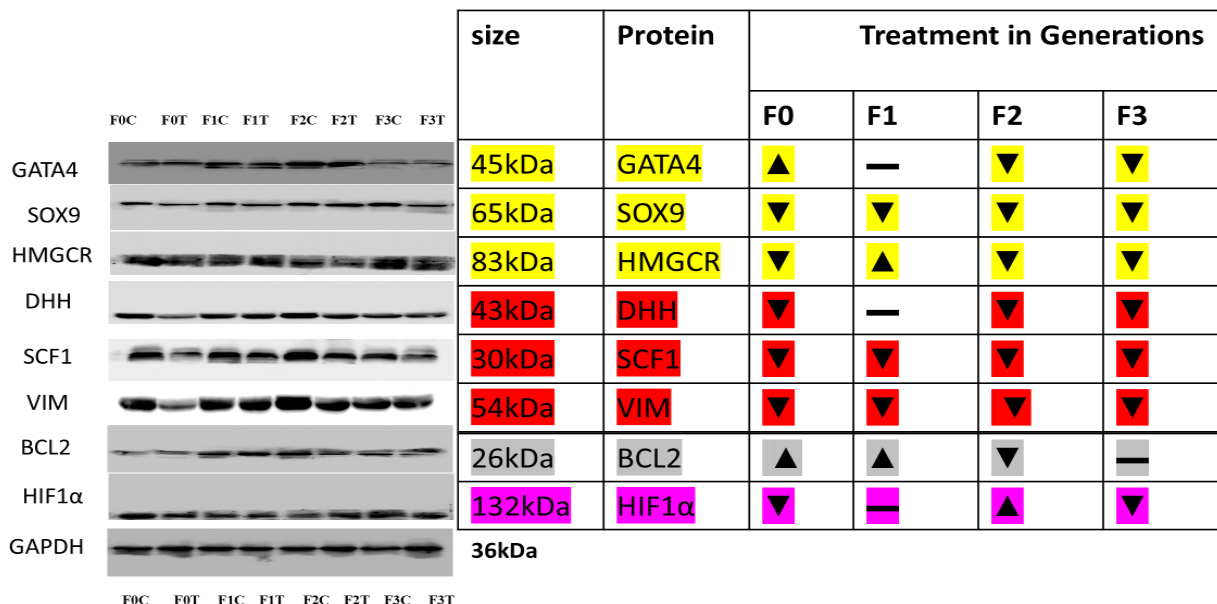


Figure 1.14: Expression pattern of steroidogenesis, spermatogenesis, apoptosis, and oxidative stress-associated proteins in the testes of pyrethroid-treated rats. ▼ -denotes significantly downregulated, ▲ - denotes significantly up-regulated, and — denotes no significant change. F0, F1, F2, and F3 represent all four generations that are studied. F0C: control of F0 generation; F0T: treated of F0 generation. . F1C: control of F1 generation; F1T: treated of F1 generation. F2C: control of F2 generation; F2T: treated of F2 generation. . F3C: control of F3 generation; F3T: treated of F3 generation.

Table 1.8: Summary of the transgenerational expression pattern of genes analyzed in this study.

Gene Name	F0	F1	F2	F3
SPERMATOGENESIS ASSOCIATED GENES				
<i>Dhh</i>	↓	↓	↓	↓
<i>Vim</i>	↓	↓	↓	↓
<i>Scf</i>	↓	↓	↓	↓
<i>Inhbb</i>	↓	↓	—	↓
<i>Pygo2</i>	↓	↓	—	↑
<i>Tp2</i>	↓	↓	↓	↓

<i>Dazapa1</i>	↓	↓	↓	—
<i>Hsf2</i>	↓	—	↓	—
<i>Ddx3y</i>	↓	—	—	—
<i>Ybx2</i>	↓	—	—	—
<i>Tgfb</i>	↓	—	↓	—
<i>Lh</i>	↓	↑	↑	↑
<i>Fshr</i>	↑	↑	—	—
<i>Amh</i>	↑	↑	—	—
STEROIDOGENESIS ASSOCIATED GENES				
<i>Sox9</i>	↓	↓	↓	↓
<i>Gata</i>	↑	—	↓	↓
<i>Crem</i>	—	—	—	↓
<i>Hmgcr</i>	↓	↑	↓	↓
<i>Cyp11a1</i>	↑	↑	—	—
<i>Star</i>	↑	↑	—	—
<i>Lhr</i>	↓	↓	↓	↓
APOPTOSIS & OXIDATIVE-STRESS ASSOCIATED GENES				
<i>Nqo1</i>	↓	—	↓	—
<i>Ho1</i>	—	—	—	—
<i>Hif1</i>	—	—	—	—
<i>Nrf2</i>	—	—	—	—
<i>Casp3</i>	↓	↓	↓	↓
<i>Casp9</i>	↓	↓	—	↑
<i>Bcl2</i>	↑	↑	—	—
<i>Bax</i>	—	—	—	↑
TESTICULAR CANCER ASSOCIATED GENES				

<i>Ldh</i>	↑	—	—	—
<i>Afp</i>	↑	—	—	↑
<i>Vegf</i>	↑	↓	—	—
<i>Ccdc83</i>	↑	↑	—	—
<i>P53</i>	—	↓	↓	—
DIABETES COMPLICATION ASSOCIATED GENES				
<i>Glut</i>	↑	↑	↑	↑
<i>Naglt1</i>	↓	—	—	↓
<i>Apln</i>	↑	—	↑	—
<i>Igf</i>	↓	↑	↓	↓
<i>Ngfb</i>	—	↑	—	↓
<i>C-kit</i>	↑	↑	↑	↑
<i>Hsp90</i>	↑	—	↑	↑
<i>Inos</i>	—	↑	—	—

(↓ Significant downregulation, ↑ - Significant upregulation, and — -denotes no Significant changes. $p \leq 0.05$ and $n=3$)

Discussion

Consumption of foods containing residual quantities of pyrethroids has become unavoidable. Even while agricultural products are cleaned in a variety of methods, ranging from straightforward tap water washing to intricate procedures, a sizable portion of the pesticides are still present. (Chauhan et al., 2014). In our previous study, we showed that oral exposure to these compounds at a dose relevant to human consumption reduced fecundity and hampered reproductive organ development and anatomical damage (Ravula & Yenugu, 2021b, 2022b). In this study, we observed similar anatomical changes like seminiferous tubule degeneration and edema in pyrethroid treated samples. Interestingly, the histological changes observed in the testes of pyrethroid treated rats were also observed in the testes obtained from F1, F2 and F3 generations. Pyrethroids like cypermethrin, permethrin, and deltamethrin interfere with

spermatogenesis by causing oxidative stress, damaging the germinal epithelium, and affecting the maturation and motility of spermatozoa (Q. Wang et al., 2020). Pyrethroids interfere with steroidogenesis by affecting the expression of enzymes such as 3 β -HSD and 17 β -HSD involved in testosterone biosynthesis, resulting in decreased testosterone levels (Zhang et al., 2021). In addition, pyrethroids are capable of disrupting testes development, especially during windows of exposure like in utero or puberty, by targeting Leydig and Sertoli cells that are critical for testicular growth and function (Delbes et al., 2022). Our analyses on the expression pattern of genes involved in spermatogenesis, steroidogenesis and testes development revealed perturbations in a large number of genes involved in these processes and that these perturbations continued transgenerationally. Any changes in the anatomy lead to physiological and stress-related changes, and our previous studies demonstrated oxidative stress due to pyrethroid toxicity in the male reproductive tract (Ali et al., 2020; Ravula & Yenugu, 2021b). Pyrethroids are known to cause oxidative stress by increasing ROS production, overwhelming antioxidant defense systems, and reducing cellular antioxidants, including glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) (El-Demerdash, 2011). In our study, the alterations in the oxidants and antioxidant enzymes (GST, LPO, NO, and CAT) were observed, which suggested that pyrethroids cause increased oxidative stress and disturbed antioxidant status. The disturbed oxidative and antioxidant status continued transgenerationally. Changes at a physiological level warrant that there might be a change at transcriptional level. Oxidative stress can cause ROS production, which may lead to changes in the expression of apoptotic factors (Martínez et al., 2018). Additionally, pyrethroid-induced oxidative stress shifts the balance in favor of apoptosis by increasing pro-apoptotic proteins (like BAX and BAD) and decreasing anti-apoptotic proteins (like BCL-2) (C. Wang & Youle, 2009). Our findings too indicated a considerable change in the expression pattern of apoptotic factors transgenerationally. Any changes in cell death machinery may lead to inflammation or tumorigenesis. Tumorigenesis due to alteration in the apoptotic pathways is reported (Cheng et al., 2024). Further, pesticides are known to cause inflammation and tumor induction (Hossain et al., 2022; Yamada et al., 2022). By generating too many reactive oxygen species (ROS), pyrethroids also contribute to oxidative stress, which can damage lipids, proteins, and DNA (Banerjee et al., 1999). Uncontrolled cell development may result from mutations in important oncogenes and tumor suppressor genes brought on by this oxidative DNA damage (Valko et al., 2006). Surprisingly, we noticed a significant upregulation of tumor promoters and a downregulation of tumor suppressors in the testes of pyrethroid-treated rats, and this trend was observed transgenerationally. It is possible that pyrethroids might have the potency to initiate

tumorogenesis in the exposed groups and transgenerationally. Pyrethroids are already known to cause systemic toxicity (Ravula & Yenugu, 2022a). New research reveals that pyrethroids can affect glucose homeostasis by changing the function of important metabolic pathways and insulin signalling (Sun et al., 2025). Environmental exposure to pyrethroids and diabetes-related changes are well-studied (Park et al., 2019). So, we screened a few genes related to glucose metabolism and diabetes-related complications. We found notable perturbations in the expression of glucose transporters, and these effects were observed transgenerationally. Long-term exposure to pyrethroids has been linked to histopathological alterations in the testes, such as tubular atrophy, decreased numbers of germ cells, and disrupted spermatogenesis, highlighting the importance of careful use and more research into these compounds' reproductive toxicity (Giannandrea, 2012).

References

- Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., & Rezaie, A. (2004). Pesticides and oxidative stress: a review. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, 10(6), RA141-7.
- Aebi, H. (1984). [13] Catalase in vitro. *Methods in Enzymology*, 105(C), 121–126. [https://doi.org/10.1016/S0076-6879\(84\)05016-3](https://doi.org/10.1016/S0076-6879(84)05016-3)
- Ali, H. F. H., El-Sayed, N. M., khodeer, D. M., Ahmed, A. A. M., Hanna, P. A., & Moustafa, Y. M. A. (2020). Nano selenium ameliorates oxidative stress and inflammatory response associated with cypermethrin-induced neurotoxicity in rats. *Ecotoxicology and Environmental Safety*, 195. <https://doi.org/10.1016/j.ecoenv.2020.110479>
- Andreollo, N. A., Santos, E. F. dos, Araújo, M. R., & Lopes, L. R. (2012). Rat's age versus human's age: what is the relationship? *Arquivos Brasileiros de Cirurgia Digestiva : ABCD = Brazilian Archives of Digestive Surgery*, 25(1), 49–51. <https://doi.org/10.1590/s0102-67202012000100011>
- Ascoli, M., Fanelli, F., & Segaloff, D. L. (2002). The Lutropin/Choriogonadotropin Receptor, A 2002 Perspective. *Endocrine Reviews*, 23(2), 141–174. <https://doi.org/10.1210/EDRV.23.2.0462>
- Auchus, R. J. (2001). THE GENETICS, PATHOPHYSIOLOGX, AND MANAGEMENT OF HUMAN DEFICIENCIES OF P450c17. *Endocrinology and Metabolism Clinics of North America*, 30(1), 101–119. [https://doi.org/10.1016/S0889-8529\(08\)70021-5](https://doi.org/10.1016/S0889-8529(08)70021-5)
- Baccarelli, A., & Bollati, V. (2009). Epigenetics and environmental chemicals. *Current Opinion in Pediatrics*, 21(2), 243–251. <https://doi.org/10.1097/MOP.0B013E32832925CC>
- Banerjee, B. D., Seth, V., Bhattacharya, A., Pasha, S. T., & Chakraborty, A. K. (1999). Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicology Letters*, 107(1–3), 33–47. [https://doi.org/10.1016/S0378-4274\(99\)00029-6](https://doi.org/10.1016/S0378-4274(99)00029-6)
- Baskin, L. S., & Ebbers, M. B. (2006). Hypospadias: anatomy, etiology, and technique. *Journal of Pediatric Surgery*, 41(3), 463–472. <https://doi.org/10.1016/J.JPESURG.2005.11.059>

- Bernheim, F. (1964). The oxidative desulfuration of thio-acids. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 90(2), 426–428. [https://doi.org/10.1016/0304-4165\(64\)90216-8](https://doi.org/10.1016/0304-4165(64)90216-8)
- Betteridge, D. J. (2000). What is oxidative stress? *Metabolism*, 49(2), 3–8. [https://doi.org/10.1016/S0026-0495\(00\)80077-3](https://doi.org/10.1016/S0026-0495(00)80077-3)
- Chassot, A. A., Ranc, F., Gregoire, E. P., Roepers-Gajadien, H. L., Taketo, M. M., Camerino, G., de Rooij, D. G., Schedl, A., & Chaboissier, M. C. (2008). Activation of β -catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Human Molecular Genetics*, 17(9), 1264–1277. <https://doi.org/10.1093/HMG/DDN016>
- Chauhan, R., Kumari, B., & Rana, M. K. (2014). Effect of fruit and vegetable processing on reduction of synthetic pyrethroid residues. *Reviews of Environmental Contamination and Toxicology*, 229, 89–110. https://doi.org/10.1007/978-3-319-03777-6_5,
- Cheng, J., Xu, J., Gu, Y., Wang, Y., Wang, J., & Sun, F. (2024). Melatonin ameliorates 10-hydroxycamptothecin-induced oxidative stress and apoptosis via autophagy-regulated p62/Keap1/Nrf2 pathway in mouse testicular cells. *Journal of Pineal Research*, 76(4). <https://doi.org/10.1111/JPI.12959>,
- Darwish, S. F., Moustafa, Y. M., Abdel Mageed, S. S., Hassan, G. S., Mangoura, S. A., Aly, S. H., Mansour, M. A., Raouf, A. A., Sallam, A. A. M., Fawzi, S. F., Atta, A. M., Elazazy, O., El-Dakrouy, W. A., El-Demerdash, A. A., Esmat, E. Z. M., Elrebehy, M. A., & Doghish, A. S. (2025). Insecticides and testicular health: mechanisms of injury and protective natural products. *Naunyn-Schmiedeberg's Archives of Pharmacology* 2025, 1–31. <https://doi.org/10.1007/S00210-025-04016-Y>
- De Rooij, D. G., & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology*, 21(6), 776–798. <https://doi.org/10.1002/J.1939-4640.2000.TB03408.X;PAGEGROUP:STRING:PUBLICATION>
- Delbes, G., Blázquez, M., Fernandino, J. I., Grigorova, P., Hales, B. F., Metcalfe, C., Navarro-Martín, L., Parent, L., Robaire, B., Rwigemera, A., Van Der Kraak, G., Wade, M., & Marlatt, V. (2022). Effects of endocrine disrupting chemicals on gonad development: Mechanistic insights from fish and mammals. *Environmental Research*, 204, 112040. <https://doi.org/10.1016/J.ENVRES.2021.112040>
- Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews*, 82(1), 47–95. <https://doi.org/10.1152/PHYSREV.00018.2001>,
- El-Demerdash, F. M. (2011). Lipid peroxidation, oxidative stress and acetylcholinesterase in rat brain exposed to organophosphate and pyrethroid insecticides. *Food and Chemical Toxicology*, 49(6), 1346–1352. <https://doi.org/10.1016/J.FCT.2011.03.018>
- Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*, 35(4), 495–516. <https://doi.org/10.1080/01926230701320337;WGROUP:STRING:PUBLICATION>
- Gassner, B., Wüthrich, A., Scholtysik, G., & Solioz, M. (1997). The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. *The Journal of Pharmacology and Experimental Therapeutics*, 281(2), 855–860.
- Giannandrea, F. (2012). Long-term pesticide exposure and the risk of testicular cancer. *Occupational Medicine*, 62(4), 309–310. <https://doi.org/10.1093/OCCMED/KQS040>
- Gnambani, E. J., Bilgo, E., Dabiré, R. K., Belem, A. M. G., & Diabaté, A. (2023). Infection of the malaria vector *Anopheles coluzzii* with the entomopathogenic bacteria *Chromobacterium anophelis* sp. nov. IRSSSOUMB001 reduces larval survival and adult reproductive potential. *Malaria Journal*, 22(1). <https://doi.org/10.1186/s12936-023-04551-0>

- Gore, A. C., Chappell, V. A., Fenton, S. E., Flaws, J. A., Nadal, A., Prins, G. S., Toppari, J., & Zoeller, R. T. (2015). EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocrine Reviews*, 36(6), E1–E150. <https://doi.org/10.1210/ER.2015-1010>
- Green, D. R., & Llambi, F. (2015). Cell Death Signaling. *Cold Spring Harbor Perspectives in Biology*, 7(12), a006080. <https://doi.org/10.1101/CSHPERSPECT.A006080>
- Griswold, M. D. (2016). Spermatogenesis: The commitment to Meiosis. *Physiological Reviews*, 96(1), 1–17. <https://doi.org/10.1152/PHYSREV.00013.2015>,
- Habert, R., Lejeune, H., & Saez, J. M. (2001). Origin, differentiation and regulation of fetal and adult Leydig cells. *Molecular and Cellular Endocrinology*, 179(1–2), 47–74. [https://doi.org/10.1016/S0303-7207\(01\)00461-0](https://doi.org/10.1016/S0303-7207(01)00461-0)
- Hainaut, P., & Hollstein, M. (1999). p53 and Human Cancer: The First Ten Thousand Mutations. *Advances in Cancer Research*, 77(C), 81–137. [https://doi.org/10.1016/S0065-230X\(08\)60785-X](https://doi.org/10.1016/S0065-230X(08)60785-X)
- Handel, M. A., & Schimenti, J. C. (2010). Genetics of mammalian meiosis: Regulation, dynamics and impact on fertility. *Nature Reviews Genetics*, 11(2), 124–136. <https://doi.org/10.1038/NRG2723>,
- Hossain, M. M., Toltin, A. C., Gamba, L. M., & Molina, M. A. (2022). Deltamethrin-Evoked ER Stress Promotes Neuroinflammation in the Adult Mouse Hippocampus. *Cells*, 11(12), 1961. <https://doi.org/10.3390/CELLS11121961/S1>
- Jakoby, W. B. (2006). The Glutathione S-Transferases: A Group of Multifunctional Detoxification Proteins. *Advances in Enzymology and Related Areas of Molecular Biology*, 46, 383–414. <https://doi.org/10.1002/9780470122914.CH6>,
- Joshi, S. C., Mathur, R., & Gulati, N. (2007). Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat. *Toxicology and Industrial Health*, 23(7), 439–444. <https://doi.org/10.1177/0748233707080908;PAGE=STRING:ARTICLE/CHAPTER>
- Kannan, K., & Jain, S. K. (2000). Oxidative stress and apoptosis. *Pathophysiology*, 7(3), 153–163. [https://doi.org/10.1016/S0928-4680\(00\)00053-5](https://doi.org/10.1016/S0928-4680(00)00053-5)
- Kerr, J. F. R., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, 26(4), 239–257. <https://doi.org/10.1038/BJC.1972.33;KWRD=BIOMEDICINE>
- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for Sry. *Nature*, 351(6322), 117–121. <https://doi.org/10.1038/351117A0;KWRD=SCIENCE>
- Krausz, C., & Riera-Escamilla, A. (2018). Genetics of male infertility. *Nature Reviews Urology*, 15(6), 369–384. <https://doi.org/10.1038/S41585-018-0003-3>,
- Labrie, F. (1991). Intracrinology. *Molecular and Cellular Endocrinology*, 78(3), C113–C118. [https://doi.org/10.1016/0303-7207\(91\)90116-A](https://doi.org/10.1016/0303-7207(91)90116-A)
- Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelander, L., & Tenu, J. P. (1990). Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. *Journal of Biological Chemistry*, 265(24), 14143–14149. [https://doi.org/10.1016/S0021-9258\(18\)77279-7](https://doi.org/10.1016/S0021-9258(18)77279-7)
- Linn, E., Ghanem, L., Bhakta, H., Greer, C., & Avella, M. (2021). Genes Regulating Spermatogenesis and Sperm Function Associated With Rare Disorders. *Frontiers in Cell and Developmental Biology*, 9, 634536. <https://doi.org/10.3389/FCELL.2021.634536/XML/NLM>

- Martínez, M. A., Ares, I., Rodríguez, J. L., Martínez, M., Roura-Martínez, D., Castellano, V., Lopez-Torres, B., Martínez-Larrañaga, M. R., & Anadón, A. (2018). Pyrethroid insecticide lambda-cyhalothrin induces hepatic cytochrome P450 enzymes, oxidative stress and apoptosis in rats. *Science of the Total Environment*, 631–632, 1371–1382. <https://doi.org/10.1016/j.scitotenv.2018.03.030>
- McLaren, A. (2003). Primordial germ cells in the mouse. *Developmental Biology*, 262(1), 1–15. [https://doi.org/10.1016/S0012-1606\(03\)00214-8](https://doi.org/10.1016/S0012-1606(03)00214-8)
- Miller, W. L. (2013). Steroid hormone synthesis in mitochondria. *Molecular and Cellular Endocrinology*, 379(1–2), 62–73. <https://doi.org/10.1016/J.MCE.2013.04.014>
- Miller, W. L., & Auchus, R. J. (2011). The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders. *Endocrine Reviews*, 32(1), 81–151. <https://doi.org/10.1210/ER.2010-0013>
- Mruk, D. D., & Cheng, C. Y. (2004). Sertoli-sertoli and sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocrine Reviews*, 25(5), 747–806. <https://doi.org/10.1210/ER.2003-0022>,
- Naseri, M. H., Mahdavi, M., Davoodi, J., Tackallou, H. S., Goudarzvand, M., & Neishabouri, S. H. (2015). Up regulation of Bax and down regulation of Bcl2 during 3-NC mediated apoptosis in human cancer cells. *Cancer Cell International*, 15(1), 1–9. <https://doi.org/10.1186/S12935-015-0204-2/FIGURES/4>
- Nef, S., & Parada, L. F. (2000). Hormones in male sexual development. *Genes & Development*, 14(24), 3075–3086. <https://doi.org/10.1101/GAD.843800>
- Ott, M., Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis*, 12(5), 913–922. <https://doi.org/10.1007/S10495-007-0756-2/METRICS>
- Park, J., Park, S. K., & Choi, Y. H. (2019). Environmental pyrethroid exposure and diabetes in U.S. adults. *Environmental Research*, 172, 399–407. <https://doi.org/10.1016/J.ENVRES.2018.12.043>
- Payne, A. H., & Hales, D. B. (2004). Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones. *Endocrine Reviews*, 25(6), 947–970. <https://doi.org/10.1210/ER.2003-0030>
- Rains, J. L., & Jain, S. K. (2011). Oxidative stress, insulin signaling, and diabetes. *Free Radical Biology and Medicine*, 50(5), 567–575. <https://doi.org/10.1016/J.FREERADBIOMED.2010.12.006>
- Ravula, A. R., & Yenugu, S. (2021a). Effect of long-term treatment with a mixture of pyrethroids on the expression of genes that govern male germ cell production in rats. *Journal of Biochemical and Molecular Toxicology*, 35(2), e22654. <https://doi.org/10.1002/jbt.22654>
- Ravula, A. R., & Yenugu, S. (2021b). Effect of oral administration of a mixture of pyrethroids at doses relevant to human exposure on the general and male reproductive physiology in the rat. *Ecotoxicology and Environmental Safety*, 208. <https://doi.org/10.1016/j.ecoenv.2020.111714>
- Ravula, A. R., & Yenugu, S. (2022a). Effect of a mixture of pyrethroids at doses similar to human exposure through food in the Indian context. *Journal of Biochemical and Molecular Toxicology*, 36(9). <https://doi.org/10.1002/JBT.23132>,
- Ravula, A. R., & Yenugu, S. (2022b). Transgenerational effects on the fecundity and sperm proteome in rats exposed to a mixture of pyrethroids at doses similar to human consumption. *Chemosphere*, 290. <https://doi.org/10.1016/j.chemosphere.2021.133242>

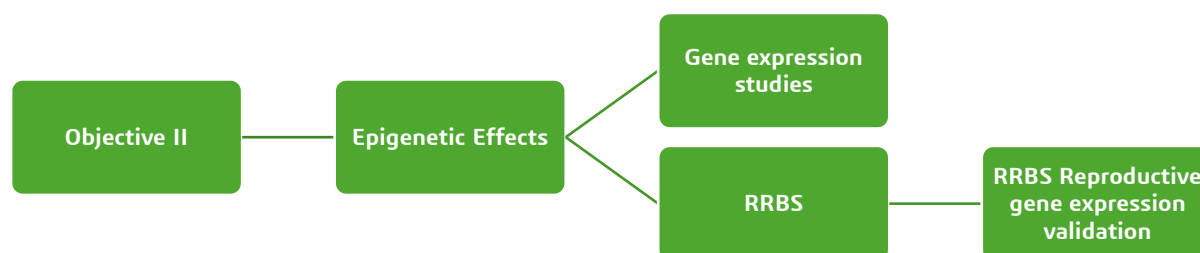
- Russell, D. W., & Wilson, J. D. (1994). Steroid 5 α -reductase: Two genes/two enzymes. *Annual Review of Biochemistry*, 63(Volume 63, 1994), 25–61. <https://doi.org/10.1146/ANNUREV.BI.63.070194.000325/CITE/REFWORKS>
- Sakr, S., & Rashad, W. A. (2023). Lambda-cyhalothrin-induced pancreatic toxicity in adult albino rats. *Scientific Reports* 2023 13:1, 13(1), 1–12. <https://doi.org/10.1038/s41598-023-38661-1>
- Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, 82(2), 291–295. <https://doi.org/10.1113/EXPPHYSIOL.1997.SP004024>
- Singh, A. K., Tiwari, M. N., Prakash, O., & Singh, M. P. (2012). A Current Review of Cypermethrin-Induced Neurotoxicity and Nigrostriatal Dopaminergic Neurodegeneration. *Current Neuropharmacology*, 10(1), 64–71. <https://doi.org/10.2174/157015912799362779>
- Singh, V. P., Bali, A., Singh, N., & Jaggi, A. S. (2014). Advanced Glycation End Products and Diabetic Complications. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, 18(1), 1–14. <https://doi.org/10.4196/KJPP.2014.18.1.1>
- Stocco, D. M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology*, 63(Volume 63, 2001), 193–213. <https://doi.org/10.1146/ANNUREV.PHYSIOL.63.1.193/CITE/REFWORKS>
- Sun, L., Cui, Z. G., Feng, Q., Muhammad, J. S., Jin, Y. J., Zhao, S., Zhou, L., & Wu, C. A. (2025). Fenvalerate exposure induces AKT/AMPK-dependent alterations in glucose metabolism in hepatoma cells. *Frontiers in Pharmacology*, 16, 1540567. <https://doi.org/10.3389/FPHAR.2025.1540567/BIBTEX>
- Tang, H., Brennan, J., Karl, J., Hamada, Y., Raetzman, L., & Capel, B. (2008). Notch signaling maintains Leydig progenitor cells in the mouse testis. *Development*, 135(22), 3745–3753. <https://doi.org/10.1242/DEV.024786>
- Taniguchi, K., & Karin, M. (2018). NF- κ B, inflammation, immunity and cancer: Coming of age. *Nature Reviews Immunology*, 18(5), 309–324. <https://doi.org/10.1038/NRI.2017.142;SUBJMETA=1909,250,256,516,580,631;KWRD=INFLAMMATION,NF-KAPPAB,TUMOUR+IMMUNOLOGY>
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, 39(1), 44–84. <https://doi.org/10.1016/J.BIOCEL.2006.07.001>
- Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, 160(1), 1–40. <https://doi.org/10.1016/J.CBI.2005.12.009>
- Walker, W. H. (2011). Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis*, 1(2), 116. <https://doi.org/10.4161/SPMG.1.2.16956>
- Wang, C., & Youle, R. J. (2009). The role of mitochondria in apoptosis. *Annual Review of Genetics*, 43(Volume 43, 2009), 95–118. <https://doi.org/10.1146/ANNUREV-GENET-102108-134850/CITE/REFWORKS>
- Wang, Q., Shen, J. Y., Zhang, R., Hong, J. W., Li, Z., Ding, Z., Wang, H. X., Zhang, J. P., Zhang, M. R., & Xu, L. C. (2020). Effects and mechanisms of pyrethroids on male reproductive system. *Toxicology*, 438, 152460. <https://doi.org/10.1016/J.TOX.2020.152460>

- Wei, Y., Liu, W., & Liu, J. (2023). Environmentally relevant exposure to cypermethrin aggravates diet-induced diabetic symptoms in mice: The interaction between environmental chemicals and diet. *Environment International*, 178, 108090. <https://doi.org/10.1016/J.ENVINT.2023.108090>
- Wilhelm, D., Palmer, S., & Koopman, P. (2007). Sex determination and gonadal development in mammals. *Physiological Reviews*, 87(1), 1–28. <https://doi.org/10.1152/PHYSREV.00009.2006>,
- Xiao, X., Sun, Q., Kim, Y., Yang, S. H., Qi, W., Kim, D., Yoon, K. S., Clark, J. M., & Park, Y. (2018). Exposure to permethrin promotes high fat diet-induced weight gain and insulin resistance in male C57BL/6J mice. *Food and Chemical Toxicology*, 111, 405–416. <https://doi.org/10.1016/J.FCT.2017.11.047>
- Yamada, T., Lake, B. G., & Cohen, S. M. (2022). Evaluation of the human hazard of the liver and lung tumors in mice treated with permethrin based on mode of action. *Critical Reviews in Toxicology*, 52(1), 1–31. <https://doi.org/10.1080/10408444.2022.2035316>;WGROU:STRING:PUBLICATION
- Ye, L., Li, X., Li, L., Chen, H., & Ge, R. S. (2017). Insights into the development of the adult Leydig cell lineage from stem Leydig cells. *Frontiers in Physiology*, 8(JUN), 258582. <https://doi.org/10.3389/FPHYS.2017.00430/XML/NLM>
- Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology* 2008 9:1, 9(1), 47–59. <https://doi.org/10.1038/nrm2308>
- Zhang, X., Zhang, T., Ren, X., Chen, X., Wang, S. Q., & Qin, C. (2021). Pyrethroids Toxicity to Male Reproductive System and Offspring as a Function of Oxidative Stress Induction: Rodent Studies. *Frontiers in Endocrinology*, 12, 656106. <https://doi.org/10.3389/FENDO.2021.656106/BIBTEX>

Chapter-II

Evaluating the transgenerational effect of a mixture of pyrethroids on the epigenetic changes in the male reproductive system.

Graphical Representation of Chapter-II



Introduction

Usage of pesticides for crop protection is increasing exponentially because of the demand for more food production. Pesticides sprayed on the crops eventually percolated into the food chain. Despite the availability of a variety of pesticides, the pyrethroid-based ones are the most favored since they display specific action on the pests and are less toxic to humans. Thus, they form the major bulk of the pesticide market globally (Isman, 2006). With the advent of pesticide-resistant pests, pesticides that contain one or more of the pyrethroids have been developed and sprayed, which has led to the presence of multiple pyrethroids in agricultural products. Studying the effects of pesticides at the cellular, anatomical, and organismal levels using different model systems has been an active area of investigation and continues to be so because of the ever-increasing usage for agricultural purposes and their consequences on human health (Freire et al., 2021; Hong et al., 2021; Rojas-Rueda et al., 2021). We presented an extensive review of the toxic effects of pyrethroids at multiple levels (Ravula & Yenugu, 2021c). Recent studies report that bifenthrin causes a reduction in pregnancy potential and defects in fetal growth and development (Curtis et al., 2021; Park et al., 2021). Malformation of male gametes, DNA damage and epimutations in sperm and reproductive toxicity were evident in animal models that were exposed to fenvalerate, cypermethrin, permethrin, deltamethrin and fenpropathrin, respectively (Abu Zeid et al., 2021; Ha et al., 2021; Hozyen et al., 2020; Huang et al., 2021; Thorson, Beck, Ben Maamar, et al., 2020). In most of the studies on pyrethroid toxicity, the effects were reported using a single compound. Reports on the toxic effects of a combination of pyrethroids, especially those that are present in the food chain, are rare, and there is an urgent need to conduct such studies.

Pyrethroids, a broad class of synthetic insecticides, have been increasingly found to possess the ability to trigger **epigenetic alterations**, which can modify gene expression and cellular processes without any modification in the underlying DNA sequence (Bordoni et al., 2019). Some of these epigenetic alterations are DNA methylation, histone acetylation, histone deacetylation, and histone methylation or demethylation, which can all affect enzyme activity and cause important physiological and pathological consequences (Song et al., 2010). (**Figure 2.1**) Exposure to pyrethroids such as permethrin causes elevation of global DNA methylation and hydroxymethylation and changes in histone modification levels, especially in the striatum and other brain areas. Such modifications are linked with abnormalities in dopaminergic signaling pathways and have been correlated with neurodegenerative diseases that are Parkinson's disease-like (Bordoni et al., 2019). In addition, research has established that such epigenetic changes are capable of being sustained through generations, thereby revealing a possibility for transgenerational inheritance of pyrethroid exposure-induced effects (Blanc et al., 2021). Also, oxidative stress caused by pyrethroids has been found to promote the acetylation of histones, which may trigger apoptosis in dopaminergic neuronal cells. This indicates that the epigenetic effect of pyrethroids is not only limited to DNA methylation but also includes histone modifications, further influencing gene expression and the overall health of cells (Song et al., 2010). Based on these results, it can be seen that exposure to pyrethroids can lead to considerable epigenetic changes, and hence, more research into their long-term impact on human health is important, as well as regulating exposure to such environmental toxins.

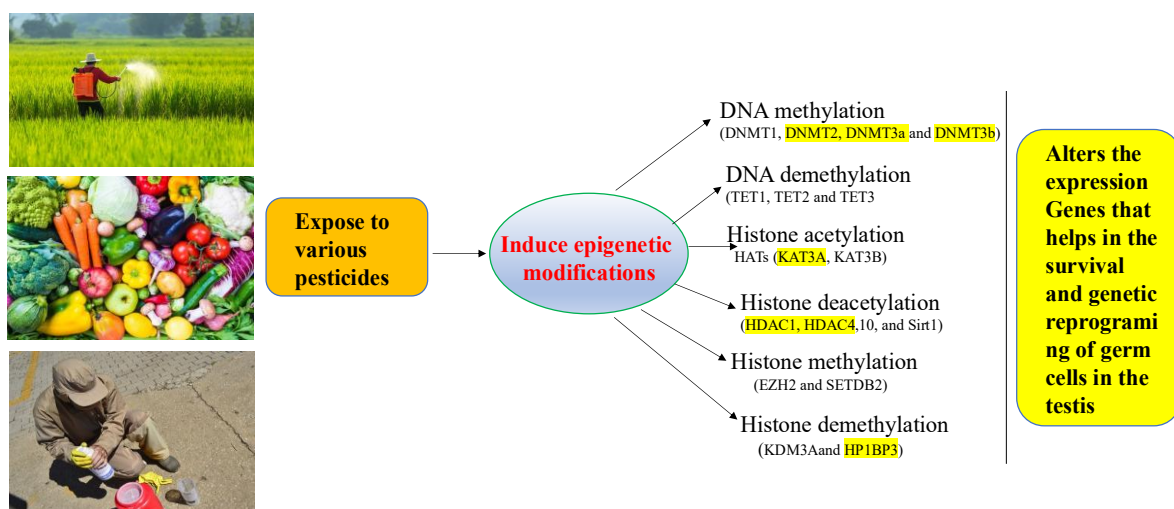


Figure 2.1: Pyrethroid exposure and the possible epigenetic effects.

DNA methylation is the enzymatic addition of a methyl group to the cytosine bases of CpG dinucleotides, which generally results in gene silence (Cedar & Bergman, 2009). The reaction is facilitated by DNA methyltransferases (DNMTs) (Li & Zhang, 2014). Pyrethroids such as permethrin and deltamethrin have been shown to impair DNMT activity, resulting in irregular DNA methylation patterns (Giambò et al., 2021). For example, hypermethylation of tumour suppressor genes can mute them, whereas hypomethylation of oncogenes can result in aberrant cell growth (Jones & Baylin, 2007). Cancer, neurodevelopmental problems, and metabolic diseases have all been linked to epigenetic changes (Feinberg & Tycko, 2004).

Histone alterations also play a vital role in chromatin dynamics and gene expression (Kouzarides, 2007). Histone acetylation, catalysed by histone acetyltransferases (HATs), indicates active transcription because it relaxes chromatin, allowing transcription factors to access DNA (Strahl & Allis, 2000). In contrast, histone deacetylation, which is catalysed by histone deacetylases (HDACs), compacts chromatin and suppresses gene expression (De Ruijter et al., 2003). Pyrethroid exposure has been associated with increased HDAC activity, which is likely to inhibit genes involved in cell cycle regulation, apoptosis, and stress response (Sule et al., 2022). This imbalance may contribute to developmental and immunological disorders.

Depending on the target site, histone methylation, the addition of methyl groups to certain histone residues, can either stimulate or inhibit gene expression. Pyrethroids have been shown to inhibit the activity of histone methyltransferase (HMT) and histone demethylase (lysine demethylase families such as KDM4 and KDM6), resulting in abnormal methylation patterns (Sharma et al., 2010). For example, modified demethylation of histones connected to developmental genes or stress response elements may result in incorrect gene activation or suppression. The interaction between histone modifications and DNA methylation amplifies the activity of pyrethroids on epigenetic control. For example, oxidative stress caused by pyrethroids has been linked to epigenetic changes that increase DNA damage and chromatin remodeling (Shaffo et al., 2018). Furthermore, a breakdown in histone code integrity may have transgenerational repercussions since some epigenetic alterations are inheritable, influencing offspring even without direct exposure (Nilsson et al., 2018). Pyrethroid-induced epigenetic alterations have significant health consequences. They cause a wide range of illnesses, including neurotoxicity, reproductive abnormalities, metabolic problems, immunological dysregulation, and cancer (G. Yu et al., 2021). These results emphasise the importance of conducting rigorous investigations to identify the molecular mechanisms behind these

epigenetic modifications and assess their long-term effect on parent and transgenerational impact on human health and biodiversity (Nilsson et al., 2018).

DNA methylation

Controlling gene expression and preserving genomic stability depend heavily on DNA methylation, a post-birth epigenetic mark in which a methyl group is appended to the cytosine residues within CpG dinucleotides. (Li & Zhang, 2014). Unusual methylation patterns, e.g., hypomethylation and hypermethylation, correlate with numerous human diseases, among which are cancers, neurological illnesses, and autoimmune conditions (Ehrlich, 2009). Hypomethylation more commonly denotes a decrease in the levels of normal methylation, usually in repetitive DNA and intergenic regions (Baylin & Jones, 2016). Loss of methylation results in chromosomal instability, transposable element activation, and ectopic expression of silent genes, such as oncogenes. In cancer, hypomethylation of the genome is a common feature, which aids tumorigenesis through the activation of oncogenes or retroviral elements and destabilization of the genome (Esteller, 2008). Hypomethylation can also occur in non-coding RNA regions, changing their control functions and still causing cellular homeostasis disruptions (Sharma et al., 2010). Hypermethylation, on the other hand, is defined as an abnormal increase in methylation levels, particularly inside CpG islands located in gene promoters (Jones & Baylin, 2007). Excessive methylation causes transcriptional repression of important genes, including tumor suppressors, DNA repair genes, and cell-cycle regulators (Baylin & Jones, 2016). For example, in cancer, hypermethylation of tumor suppressor gene promoters, such as p16, BRCA1, and MLH1, causes inactivation, allowing uncontrolled cell proliferation and survival (Esteller, 2008). Aside from cancer, hypermethylation is involved in neurodegenerative illnesses such as Alzheimer's disease, where the silencing of critical neural genes impairs cognitive function (Rao et al., 2012). The simultaneous occurrence of hypomethylation and hypermethylation in the same cellular environment demonstrates the complexities of methylation patterns since global hypomethylation can coexist with region-specific hypermethylation, causing disease via several mechanisms.

Such methylation patterns are maintained by DNA methyltransferases (DNMTs), which add methyl groups, and ten-eleven translocation (TET) enzymes, which perform active demethylation. Aberrations of such enzymes are typically caused by environmental factors such as toxin exposure, stress, or food, resulting in the creation of abnormal methylation patterns. Furthermore, methylation changes can interact with other epigenetic systems, such as

histone modifications and non-coding RNA regulation, resulting in a cascade effect on gene expression and chromatin conformation. The intricate balance of hypo- and hypermethylation is critical for understanding the pathophysiology of methylation diseases and developing appropriate treatment methods, such as DNMT inhibitors or epigenetic reprogramming tools, to restore normal methylation profiles (Sharma et al., 2010).

Reduced Representation Bisulfite sequencing (RRBS), a high-resolution method for analyzing DNA methylation across the genome with a focus on CpG-rich sections, is a bisulfite sequencing technique (Boyle et al., 2012). In order to identify and represent methylation at CpG sites, particularly in gene promoters, CpG islands, and regulatory areas that are most important for epigenetic control, RRBS uses restriction enzyme digestion and bisulfite conversion (Lister et al., 2009). MspI restriction enzymes, which target CpG-rich regions, are used to digest genomic DNA to start RRBS (Gu et al., 2011). The procedure produces segments that are mostly rich in CpG-dense sequences, which reduces the complexity of the genome for sequencing purposes while offering a concentrated representation of methylation hotspots (Bock et al., 2010). Following digestion, the pieces are ligated to adapters with barcodes specific to each sample, multiplexed, end-repaired, and adenylated. After that, the DNA is treated chemically with bisulfite, which deaminates unmethylated cytosine to uracil while leaving methylated cytosines intact (Frommer et al., 1992). Following sequencing, the conversion makes it possible to distinguish between methylation and unmethylated cytosines (Krueger & Andrews, 2011). After being amplified by PCR to yield sufficient material for sequencing, bisulfite-converted DNA is submitted to high-throughput sequencing, typically using next-generation sequencing platforms such as Illumina (Harris et al., 2010). When examining differential methylation across circumstances, organs, or disease states, RRBS is particularly helpful since it provides single-base resolution of methylation patterns (Smith & Meissner, 2013). RRBS covers a smaller but highly informative portion of the genome than WGBS, making it less expensive and computationally intensive (Cokus et al., 2008).

In the Indian context, the usage of pesticides for agricultural purposes resulted in the percolation of the active components (pyrethroids) into the food chain. This is evident from the reports of the Indian Agricultural Research Institute, New Delhi. It appears that the Indian population is consuming agricultural products containing residual amounts of pyrethroids, and this consumption is on a regular basis throughout their lifetime. Thus, it is pertinent to conduct studies that reflect human settings, that is, exposing animal models to a mixture of pyrethroids

at doses that mimic human exposure through food on a regular basis. In our previous studies, using low exposure doses that are based on residual levels present in vegetables and rice, we demonstrated pyrethroid-induced toxicity on multiple organ systems (Ravula & Yenugu, 2019, 2021b, 2021a). Further, we also reported the effect of exposure to a mixture of pyrethroids at doses equivalent to that present in the amount of rice and vegetables consumed by an average individual in the Indian context (Ravula & Yenugu, 2022).

In this study, using rats as the model system, we analyzed the toxic effects of a mixture of pyrethroids (cypermethrin, deltamethrin, fenvalerate, cyhalothrin L, and allethrin) at doses that are present in the amount of rice and vegetables consumed by an average Indian. Exposure to 9 months, which is equivalent to 22.5 years in humans, resulted in reduced fecundity and sperm count and perturbations in the reproductive hormonal profile. Further, alterations in the expression profile of genes related to spermatogenesis, steroidogenesis, germ cell epigenetic modifications, and germ cell apoptosis were evident (Ravula & Yenugu, 2021c). To further understand the genotoxic effects of the pyrethroids, we conducted an in depth analyses of the epigenetic modifications that may occur in the testes and also the effect of these modifications on the expression of genes important in male reproduction.

Methodology

DNA methylation analysis by Whole Genome Bisulfite Sequencing.

Table 2.1: Methodology for reduced representation bisulfite sequencing (RRBS).

Application	Reduced Representation Bisulfite Sequencing and Standard Analysis
Instrument	Illumina NovaSeq 6000
Library Preparation Kit	Bisulfite treatment and standard RRBS Library Prep
Library QC	Bioanalyzer and Qubit
Read Length	2 x 150 bp
Sequencing Kit	NovaSeq 6000 S4 reagent kitV1.5
Data output /sample	10 GB Raw data per sample
Standard Analysis Functional Annotation for the differentially methylated genes were done using gProfiler.	• Raw Data QC, • Clean Data quality control • MspI cutting efficiency • Alignment to the reference genome • mC-calling • Methylation level and frequency distribution • DMS detection • DMRs and DMPs detection and annotation

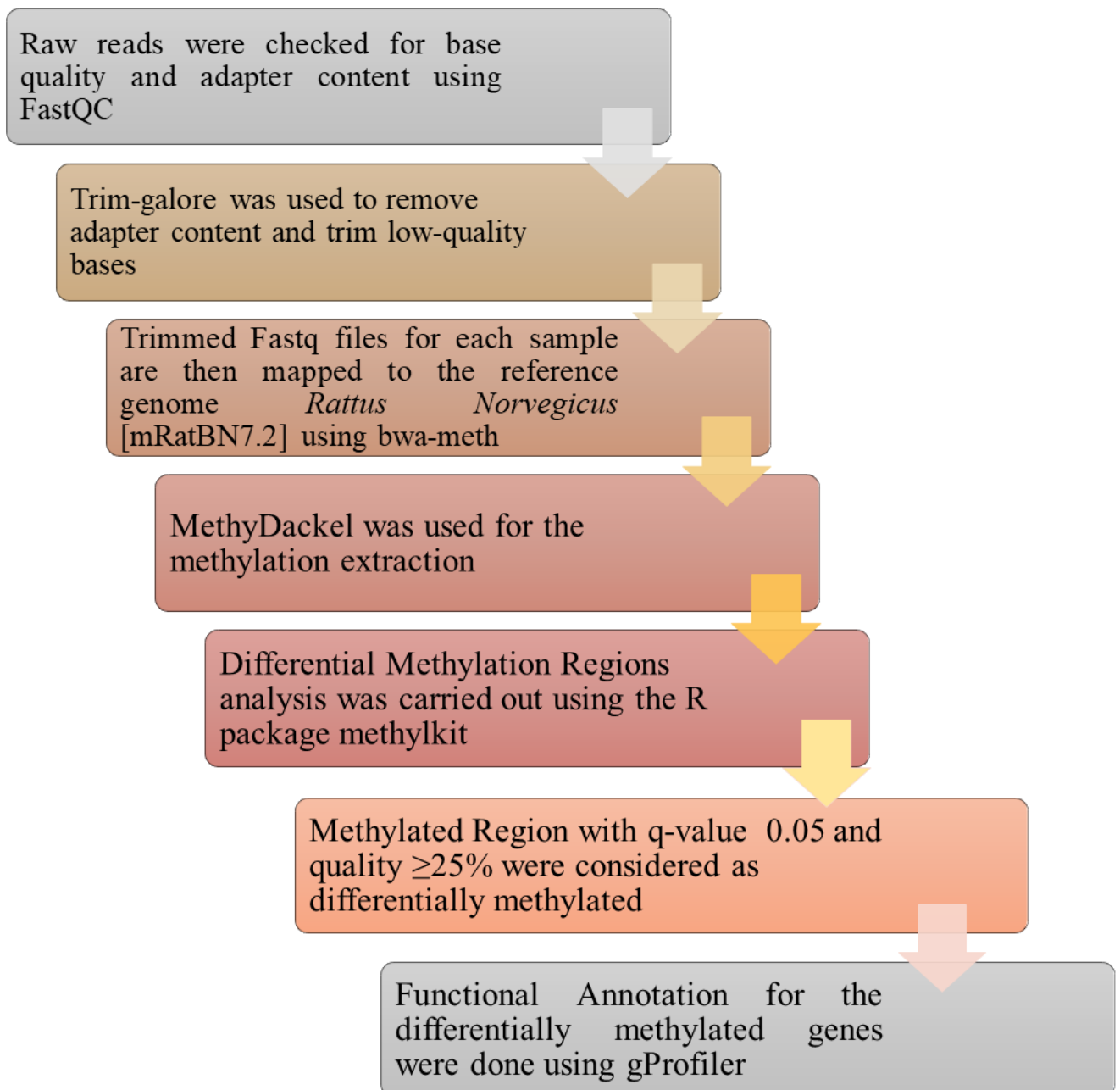


Figure 2.2: Workflow for reduced representation bisulfite sequencing (RRBS).

Data Quality Control: The overall quality of samples was checked with FastQC. Adapters were removed using the Trim Galore tool, which trims adapters by scanning bases from 5' to 3'. Trim Galore is applied tool for preparing data. The algorithm includes features for filtering by quality, adapter trimming, and quality control. The quality score is a measure of data quality. The formula $Q_{phred} = -10 \log_{10}(e)$ represents a base's quality score, also referred to as a Phred or Q score. Qphred is the base quality value, while "e" is the sequence error rate.

Mapping to the Reference Genome: For the Methylation analysis, all the clean reads were mapped to the reference genome *Rattus norvegicus* (mRatBN7.2). In this case, the reference genome was indexed using bwa-meth to create a Burrow-Wheeler Transform-based FM-Index. The alignments are reported in a SAM format (Sequence Alignment Map), and Samtools was used to sort and convert them into BAM (Binary Alignment Map).

Methylation Extraction: MethylDackel was used for the methylation extraction using the methylkit parameter from the sorted bam files. This calculates per-base CpG metrics and writes methylKit-compatible output. This requires an indexed reference genome.

Differential Methylation Analysis: Differential Methylation Analysis was performed using the R package methylKit. Differentially Methylated Genes with a q-value of 0.05 and quality $\geq 25\%$ were considered differentially expressed (DE).

Real-time polymerase chain reaction

Quantitative gene expression analyses were carried out by real-time polymerase chain reaction, as described earlier. Testes tissues (100 mg) were homogenized in TRIzol reagent (Invitrogen), and the total RNA was isolated. Reverse transcription of the isolated RNA (2 μ g) was carried out with 50 U Stratascript (Invitrogen) and 0.5 μ g of oligodT (Invitrogen). Gene-specific primers, cDNA, and SYBR PCR reagent (Applied Biosystems, UK) were mixed, and real-time PCR was performed in a thermal cycler. The thermal cycling conditions, in general were: at 94° C for 10 min (initial denaturation); 40 cycles with 94° C for 15s and 60°C for 1 min. For internal control, *Gapdh* mRNA expression was determined. Relative gene expression was estimated by the ($\Delta\Delta Ct$) method.

Statistical analyses

The students' t-test, available in GraphPad Prism Software version 8.0.2 (225 Franklin Street, Boston), was used to conduct statistical analyses. Mean $\pm$ SD is presented for the values obtained for each parameter. Statistical significance of $p < 0.05$ between control and treated groups is indicated by the * symbol.

Table 2.1: Primers used to determine the expression of genes related to epigenetic modifications.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Dnmt2</i>	FP	GCAGCAGACATTGACAGGAA	176
	RP	GTGAAGCACATGGACCTCCT	
<i>Dnmt3a</i>	FP	GGTGTGTGTCGAGAAGCTCA	181
	RP	AGCCTTGCCAGTGTCACTTT	
<i>Dnmt3b</i>	FP	ACTCGAGGAGGGAGCTTAGG	163
	RP	TTTGTCAATTCCCCAACCAT	
<i>Hplbp3</i>	FP	TTGCTGAAGGGGAAGAAGAA	173
	RP	GGTTTCCTCAGCAGACTTGC	
<i>Kat3a</i>	FP	CCGGAAGATGCATCAGATTT	166
	RP	CACTCGGTCTTCCAAATGGT	
<i>Hdac1</i>	FP	ATCGTCCTCACAAAGCCAAC	156
	RP	AGCCATCAAATACCGGACAG	
<i>Hdac4</i>	FP	ACGGTCAAGGCTTAAGCAGA	174
	RP	ACGTTGCCAGAGCTGCTATT	
<i>Mch2ta</i>	FP	AGTTTCCATCCGTGGAAGTG	159
	RP	GTGGCAGCTCCTTGTCTTTC	

Table 2.2: Primers used to determine the expression of genes related to reproductive function.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>sperm motility kinase Y-like (LOC134485089)</i>	FP	GTACATCAGAGAGTCAGGGCAC	185
	RP	GTGTTTCGGTGCTAAGCCCAA	
<i>Cfap</i>	FP	ATAGGGGAGGAGCTTCAACGAC	154
	RP	GCTGAAATCGTCTTTGTGCCCG	
<i>Tmpress</i>	FP	GAGCCCTGGACCACACAAATTT	165
	RP	TTGGGAGCACTGTAGACTGGGT	
<i>Csmd1</i>	FP	CCAATGGGACCTGGAACACTGAG	168
	RP	GGACATGGGAGCCTACAAGTGTG	
<i>Zp3r</i>	FP	ATGCGAAAGGGTATGTTGCCCA	189
	RP	GCAGGGTCCCACTTCCATCTG	
<i>Dcdc2c</i>	FP	AGGAAATCAAGCCGGTGGTACA	173
	RP	CATGGCCAGCACACTATTCC	
<i>Ace</i>	FP	ACATCCTGGCTTCCTCACGAAA	155
	RP	GGCTCCTGTGTCTGAGAAGCCA	
<i>Dis3l2</i>	FP	GTCTTGGGCAAGCTGGTGAAT	157
	RP	AGGTCTCTTCTCTTGCCACCT	
<i>Mt co-1</i>	FP	GCTAATGATTGGAGCCCCTG	164
	RP	CATGGGCTAGATTACCGGT	
<i>Adam28</i>	FP	ACTGTCACGTGGAGAATGGT	176
	RP	GGCCACCATGTCTACTCCTT	
<i>Antxr1</i>	FP	CACCACCACACACTGTTCAAGAC	201
	RP	TCTACCGTGGGCCATTTCTTCT	
<i>Selenof</i>	FP	CCTGCGATCTCCTTGACAGTT	198
	RP	GTAGACCTCTGAACAGTTTGGGT	
<i>Sntgl</i>	FP	GAAGACCTTCTCCGTGTGTGAG	200
	RP	TGCACTGTATGCGCTCCACTTC	
<i>Boll</i>	FP	CTGACCATGGAGTTCAAGCAGC	184
	RP	GGATGAAAGTGTGGGCTTCGAG	
<i>Tcp1lx2</i>	FP	GTGCTGTTTGATTTCGAGGCATG	187
	RP	TTCTTCTGTACTGGGCGGAGAG	
<i>Cdyl</i>	FP	AGTACCTGGTGGCATGGAAAGG	190
	RP	GTGGACCTGGAGATCTGCTTCC	
	FP	TGGGAAGGGGACGATTTTCAGT	174

<i>sperm motility kinase X-like (LOC134478886)</i>	RP	GGACCACTTCGACCACCTTGAT	
<i>Ros1</i>	FP	ACATTCTGAAGCAGCTGGGAGT	162
	RP	CTACACACAGCTCTACCAGGTC	
<i>Bmp6</i>	FP	GGGGCTCCAGTGCTTCAGACTA	186
	RP	GGTTGGTGGCATTTCATGTGTGC	
<i>Scaper</i>	FP	TGAACGGAAAGATGCTGAGGGATGG	201
	RP	GCAGGGGCACATCTGTCTTCAACAG	
<i>Ccdc191</i>	FP	TCCAGTACAGCCAGGAAAGG	174
	RP	GCCAGGCCTTGAAAGTCTTC	
<i>Ccdc85a</i>	FP	ACCACCACAAACATCATGCC	163
	RP	GGTCCTTTCAGTGCTTTGGG	
<i>Ccdc148</i>	FP	CAGGAACAGGAGAGGCTGAA	177
	RP	CAGCTGATGAAGAGGGTCGA	
<i>Ccdc170</i>	FP	GCAGAGAGGAGAGAACAGCA	167
	RP	CAGGGAACAAAGTGGTGGTG	

Results

The epigenetic regulation-related genes *Hdac4*, *Hp1bp3*, *Kat3a*, *Mch2ta*, *Dnmt2*, and *Dnmt3b* were significantly down-regulated in the testis of pyrethroid-administered rats, and this pattern was observed in the testes of subsequent generations with few variations, whereas *Dnmt3a* showed significant up-regulation in the parental generation. (Figures 2.3 and 2.5) Protein expression for DNMT3a (F0[~], F1⁺, F2⁺, F3⁺) and HDAC1(F0⁺, F1⁺, F2⁺, F3⁺) showed a variable change at F0 generation as compared to the non-significant shift in mRNA level. However, the changes in other generations remained the same. (Figures 2.4 and 2.6).

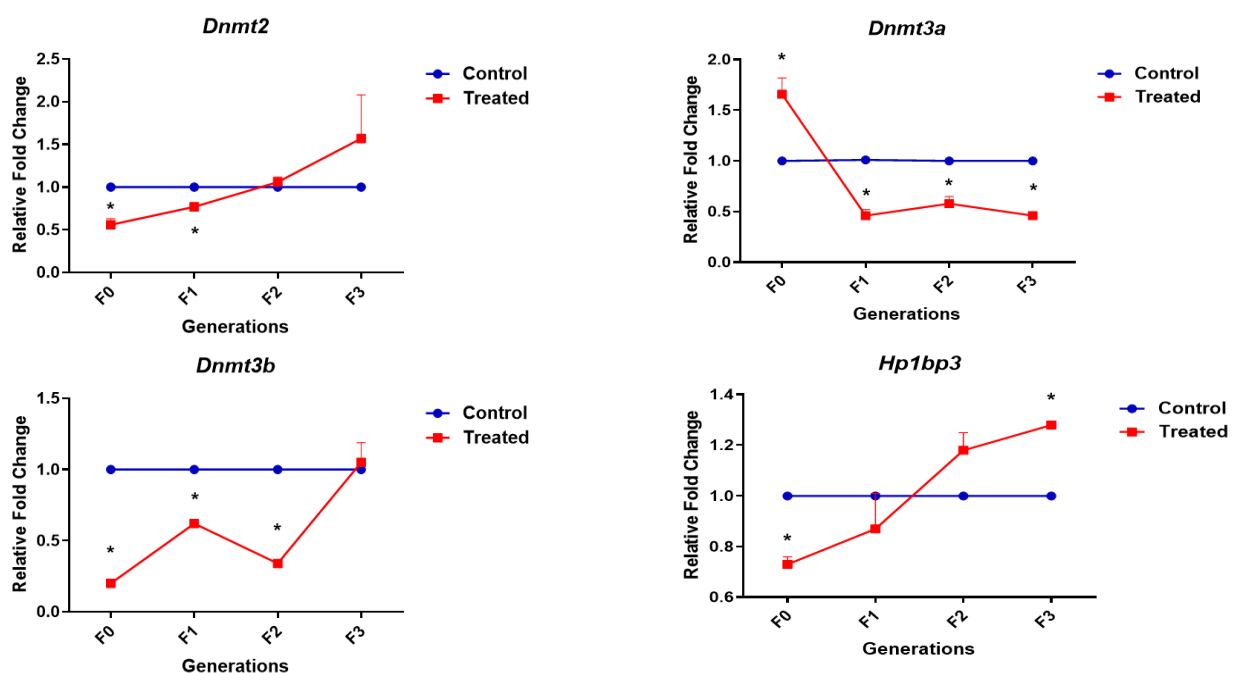


Figure 2.3: Expression pattern of DNA methylation and Histone demethylation-related genes in the testes of pyrethroid-treated rats and in the testes obtained from subsequent generations. RNA isolated from the testes of rats for all four generations was reverse-transcribed, and real-time polymerase chain reaction was performed using gene-specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

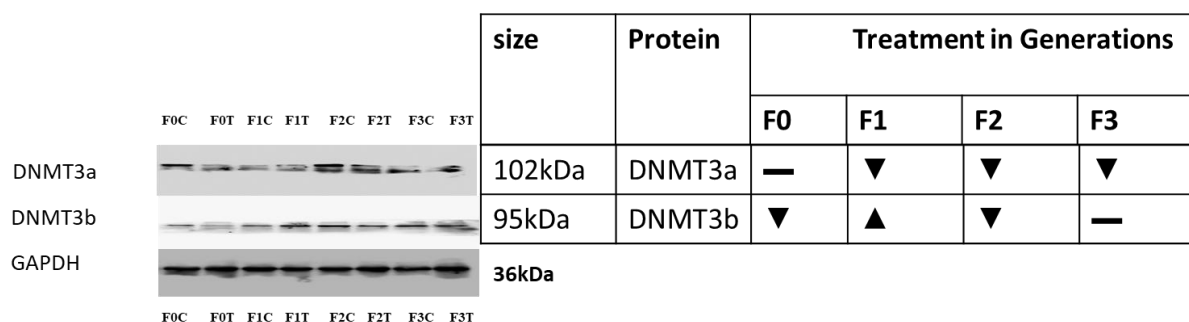


Figure 2.4: Expression pattern of DNA methylation and Histone demethylation-related proteins in the testes of pyrethroid-treated rats and in the testes obtained from subsequent generations. ▼ -denotes Significantly Downregulated, ▲ - Significantly Up-regulated, and — denotes non-significant changes ($p < 0.05$). F0, F1, F2, and F3 represent all four generations that are studied. F0C:control of F0 generation; F0T: treated of F0 generation. . F1C:control of F1 generation; F1T: treated of F1 generation. F2C:control of F2 generation; F2T: treated of F2 generation. . F3C:control of F3 generation; F3T: treated of F3 generation.

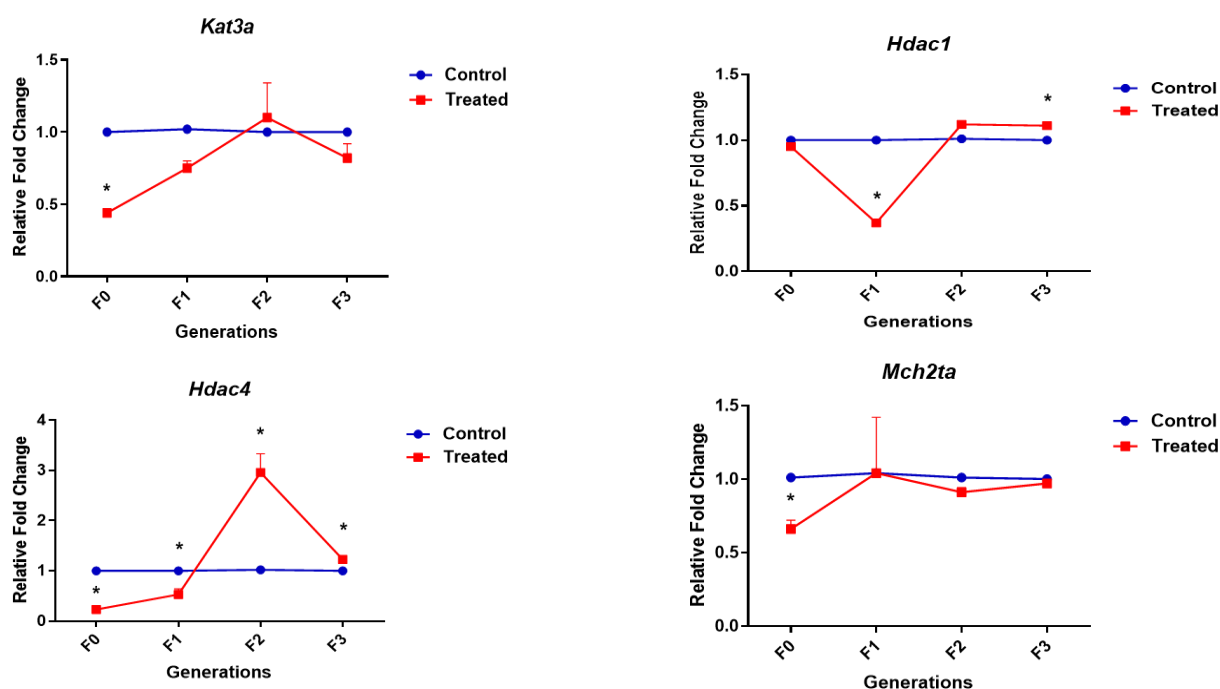


Figure 2.5: Expression pattern of histone modification related genes in the testes of pyrethroid treated rats and in the testes obtained from subsequent generations. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.



Figure 2.6: Expression pattern of histone modification-related proteins in the testes of pyrethroid-treated rats and in the testes obtained from subsequent generations. ▼ -denotes Significantly Downregulated, ▲ - Significantly Up-regulated, and — denotes non-significant changes ($p < 0.05$). . F0, F1, F2, and F3 represent all four generations that are studied. F0C:control of F0 generation; F0T: treated of F0 generation. . F1C:control of F1 generation; F1T: treated of F1 generation. F2C:control of F2 generation; F2T: treated of F2 generation. . F3C:control of F3 generation; F3T: treated of F3 generation.

Primary QC for Transgenerational Epigenome sequencing studies using genomic DNA

DNA was isolated using the DNasey Blood & Tissue kit method, & Quantification and purity of isolated DNA were checked using “Nanodrop 2000(Thermofisher Scientific, Massachusetts, USA)”. The integrity of DNA was further examined on 1% agarose (Lonza, Belgium) gel. (Figure 2.7)

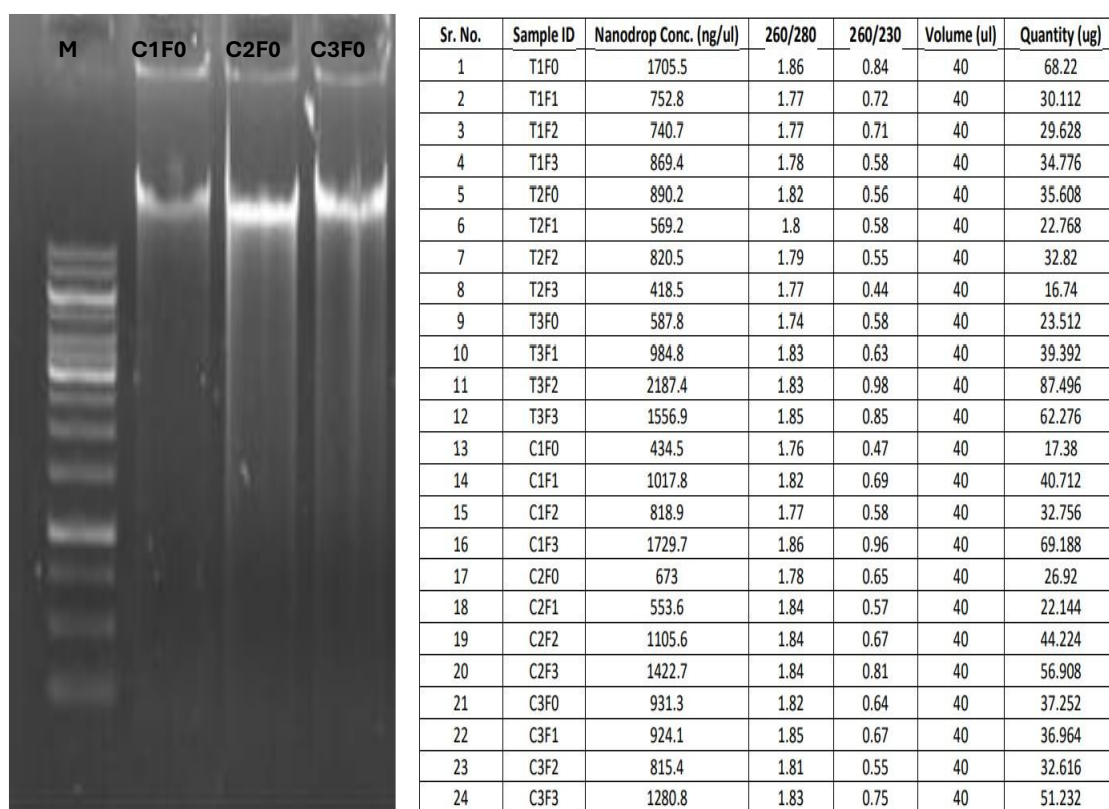


Figure 2.7: *Quality control check parameters for the DNA submitted for RRBS.*

Differential Methylation Annotation

Differential Methylation Annotation of Pyrethroid-treated testes samples across CPGi showed around 3% of the differentially methylated regions are falling on the CPGi, whereas 1% on the shore region whereas others falling on different regions (Figure 2.8).

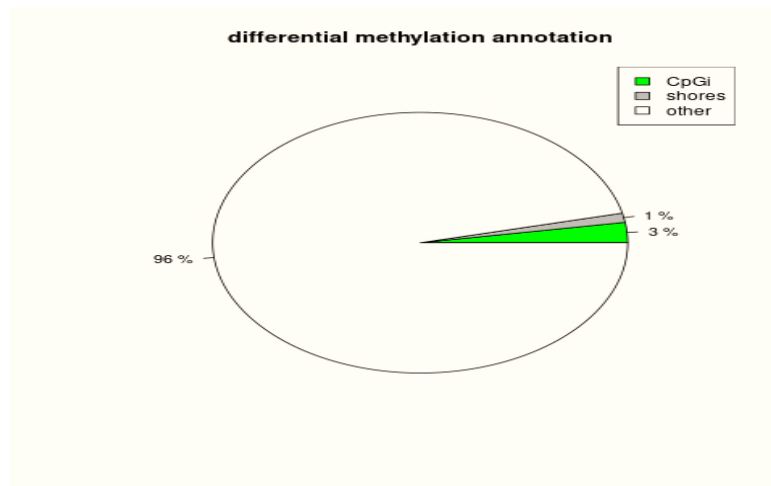


Figure 2.8: Annotation of differentially methylated regions in the CpG regions.

Differential Methylation Annotation of Pyrethroid-treated testes samples showed (**Figure 2.9**) around 14%, 12%, 13%, and 15% of the differentially methylated region falls on the intronic region for F0, F1, F2, and F3 generations respectively. Whereas around 3% in the exonic, 4% in the promoter, and around 80% in the intergenic regions in all generations.

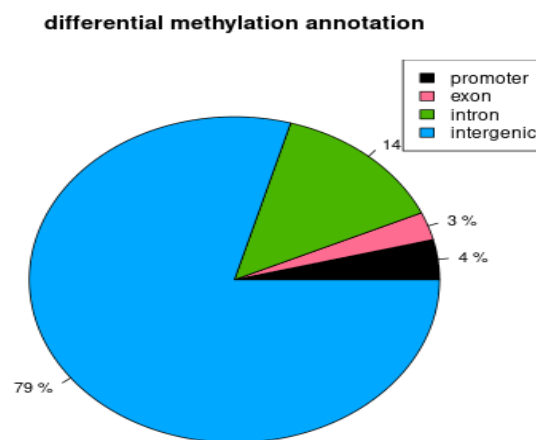


Figure 2.9: Annotation of differentially methylated regions in all the chromosomes.

The distributions of DMRs among different chromosomes showed the highest amount of DMRs in chromosome no. 9, mitochondrial chromosome, chromosome no. 9 and mt chromosome for F0, F1, F2 and F3 generations respectively. The highest amount of DMRs in the Y chromosome is found in the F0, followed by the F1-F2 and F3 generations (**Figure 2.10**).

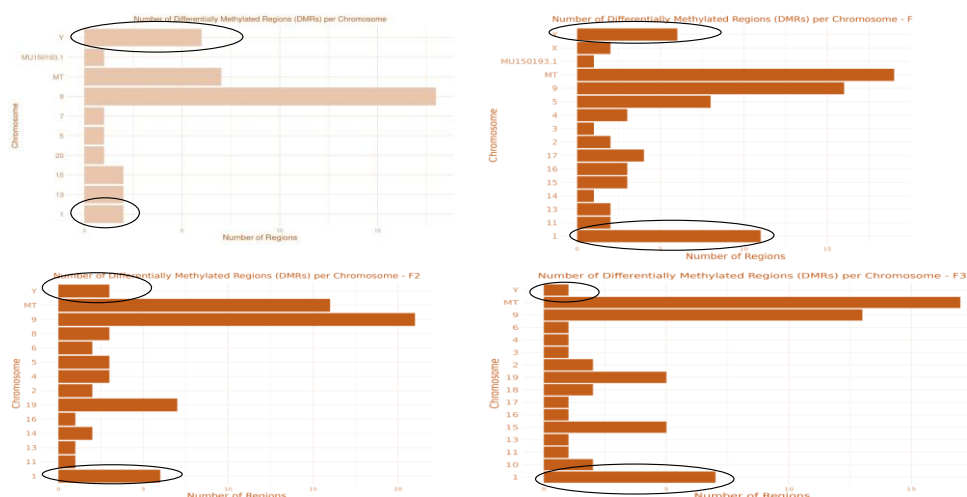


Figure 2.10: Chromosome-wise annotation of differential methylation regions.

After keeping a differential methylation cutoff score of $-5/+5$ with a p-value of 0.05; and deleting all repetitions, the differentially methylated genes were identified in the testes obtained from F0 to F3 generations (**Table 2.2**). We identified 143 genes for F0 generation that were differentially methylated, out of which 41 genes are hypomethylated and 102 genes are hypermethylated. In the testes obtained from F1 generation, we identified 149 genes; out of which 84 genes are hypomethylated and 65 genes are hypermethylated. In the testes obtained from F2 generation 142 genes were differentially methylated, out of which 71 genes are hypomethylated and 71 genes are hypermethylated. F3 generation testes displayed 116 genes that were differentially methylated. out of which 61 genes are hypomethylated and 55 genes are hypermethylated. **Tables 2.3 to 2.10** provide information on the genes that are differently methylated in the testes of rats treated with pyrethroid and in testes taken from further generations.

Table 2.2: Table showing number of differentially methylated genes and hypo-hyper methylated genes transgenerationally.

Generation	Total number of genes	Hypo	Hyper
F0	143	41	102
F1	149	84	65
F2	142	71	71
F3	116	61	55

Table 2.3: Hypo methylated genes in the testes of F0 generation rats (pyrethroid treated).

chr	pvalue	qvalue	meth.diff	gene
Y	0	0.000783	-25.2973	AABR07025152.1
MT	0	6.89E-06	-10.9584	AY172581.9
MT	0	0.000171	-6.44761	AY172581.9
MT	0.000108	0.00229	-13.6483	AY172581.9
9	0.000141	0.002725	-6.25	LOC108351885
9	0.000141	0.002725	-6.25	LOC360998
MU150193.1	0.000168	0.003093	-13.4146	LOC103694730
9	0.000183	0.003304	-27.1481	LOC103690742
MT	0.000304	0.004738	-6.62767	Mt-co1
Y	0.000331	0.004947	-22.3639	AABR07025152.1
1	0.000468	0.00663	-5.27319	Pde7b
15	0.000479	0.006731	-18.1809	Zfp385d
7	0.000519	0.007171	-6.38298	Klf21a
Y	0.000625	0.008204	-22.2368	AABR07025152.1
20	0.001091	0.0119	-6.85872	Ctnna3
Y	0.001684	0.016658	-21.4286	AABR07025152.1
MT	0.001839	0.017875	-9.21191	AY172581.24
9	0.002006	0.019316	-7.14286	LOC108351885
9	0.002006	0.019316	-7.14286	LOC360998
9	0.002093	0.019903	-7.27273	LOC108351885
9	0.002093	0.019903	-7.27273	LOC360998
13	0.002592	0.023201	-5.50734	Zp3r
9	0.002672	0.023582	-8.37662	LOC108351885
9	0.002672	0.023582	-8.37662	LOC360998
Y	0.002808	0.024343	-21.9623	AABR07025152.1
9	0.003047	0.025515	-19.8052	LOC103690742
13	0.004472	0.034019	-10.7692	Cfhr4
Y	0.00482	0.035996	-21.8703	AABR07025152.1
MT	0.00524	0.037983	-5.70497	AY172581.24
MT	0.005332	0.038368	-8.51096	AY172581.9
9	0.006066	0.041549	-17.3137	LOC103690742
5	0.006071	0.041549	-7.82353	LOC298116
9	0.006494	0.043694	-8.04749	Adgre4
9	0.006588	0.044172	-5.35714	LOC108351885
9	0.006588	0.044172	-5.35714	LOC360998
9	0.006869	0.045361	-19.88	LOC103690742
9	0.007156	0.046476	-5.48356	AABR07069611.1
9	0.007572	0.048404	-21.1329	LOC103690742
1	0.008032	0.050394	-15.9524	Hpse2
9	0.00811	0.050629	-7.64803	AABR07069611.1
15	0.008124	0.050629	-19.8946	Spetex2E

Table 2.4: Hyper methylated genes in the testes of F0 generation rats (pyrethroid treated).

chr	pvalue	qvalue	meth.diff	gene
19	0	0.000663	34.66063	LOC685989
1	0.00123	0.01308	31.19365	Tsgal0ip
9	0	0.001946	28.64847	LOC100360856
X	0	0.000266	26.05042	Cp11x2
9	0.005779	0.040332	25.69106	LOC102557419
19	0	0.000943	23.62637	LOC120098581
1	0.006091	0.041549	23.12925	LOC100910657
20	0.000121	0.002485	22.0646	Rgs1
19	0.000836	0.010027	21.85251	AABR07043389.1
1	0	0.000905	21.5033	LOC685793
X	0.021957	0.096747	19.28571	Shroom4
4	0.005055	0.036916	18.69213	Dpp6
5	0.003687	0.029143	18.27726	Prex2
15	0.045156	0.14811	17.16853	Adam28
1	0.000939	0.010802	16.76163	LOC102546375
3	0.005027	0.036916	16.11111	Gpr107
4	0.008422	0.051634	16.03284	Glicc1
8	0.012969	0.066863	15.6738	Rab6b
8	0.012969	0.066863	15.6738	Tf
16	0.01471	0.073137	14.96894	Potem
5	0.0459	0.149268	14.68732	LOC298116
Y	0.046665	0.1508	14.3197	AABR07025152.1
MT	0.006796	0.045035	12.65144	Mt-nd4
5	0.021869	0.096605	11.75339	Sntg1
1	0.032449	0.120948	11.1679	Ccdc170
1	0.009406	0.054948	10.22624	Moxd1
17	0.000769	0.009459	10.04592	Cdyl
MT	0.036425	0.130572	9.941551	Mt-nd5
8	0.043853	0.146099	9.735685	Scaper
MU15019	0.017334	0.082931	9.390693	LOC103694730
11	0.008705	0.052469	8.96145	Ncam2
7	0.018492	0.08583	8.900256	Caps2
MT	0.040106	0.138106	8.697499	Mt-co3
1	0.000972	0.010953	8.577271	Sbf2
10	0.043393	0.145357	8.253033	Cpped1
MT	0.040837	0.139725	8.199967	Mt-cyb
2	0.008209	0.050965	6.944444	Atg10
17	0.004912	0.036413	6.159955	Bmp6
MT	0.01449	0.072561	6.056071	AY172581.9
MT	0.009678	0.056185	6.055432	AY172581.24
2	0.001492	0.015175	5.955408	Adgrv1
9	0.025605	0.105834	5.660377	AABR07069611.1
9	0.021013	0.093985	5.659904	LOC103690742
9	0.009308	0.054739	5.325444	LOC108351885
9	0.009308	0.054739	5.325444	LOC360998
5	0.012393	0.065169	5.262062	Pippr1
10	0.042967	0.144423	5.004076	Cep112

Table 2.5: Hypo methylated genes in the testes of F1 generation rats.

chr	pvalue	qvalue	meth.diff	gene
MT	0	0	-57.5093	Mt-nd1
13	0	0	-49.2501	Cfap221
11	0.000209	0.018838	-29.6979	Lrch3
17	0.000382	0.023456	-29.1291	LOC108353138
17	0.000382	0.023456	-29.1291	Edaradd
17	0.000382	0.023456	-29.1291	LOC120093110
9	0.000282	0.0203	-27.037	LOC103690742
2	0.000162	0.018674	-26.5026	Rap1gds1
5	0.005695	0.09338	-24.5307	Pippr1
5	0.003627	0.072316	-21.3843	Sdf4
MT	0.000367	0.023093	-21.2997	AY172581.24
MT	0.006002	0.094802	-20.4203	LOC685793
9	0.001238	0.042803	-20.0126	AY172581.9
9	0.011963	0.128839	-19.9837	Pic12
9	0.034409	0.199208	-18.8189	LOC108351885
9	0.034409	0.199208	-18.8189	LOC360998
15	0.007233	0.100785	-18.2287	Adam28
2	0.02537	0.172093	-18.0692	Raly1
13	0.013889	0.1343	-17.4603	LOC304725
X	0.001651	0.049063	-17.4435	Hdx
1	0.050778	0.236309	-16.7113	Tsga10ip
9	0.020559	0.159721	-16.5923	LOC100360856
Y	0.019305	0.155783	-16.1497	AABR07025152.1
11	0.022527	0.165961	-14.9068	Tiam1
MU150192	0	0	-14.2108	LOC103694730
3	0.010833	0.123044	-13.8279	Syndlg1
16	0.045147	0.22268	-13.5284	RGD1564958
MT	0.025811	0.172418	-13.2956	AY172581.8
X	0.00713	0.100785	-12.7778	Tcp11x2
MT	0.018593	0.153915	-12.2457	Mt-nd5
4	0.012649	0.13284	-11.1979	Ube3c
1	0.006977	0.100075	-10.8305	LOC100910657
5	0.027011	0.17409	-10.7394	Mup4-ps3
5	0.027011	0.17409	-10.7394	LOC298116
4	0.023376	0.168617	-10.1452	Sic1a4
1	0.000153	0.018103	-9.99666	Sdf2
9	0.029199	0.179213	-9.73684	Boll
5	0.040879	0.214562	-9.22977	Sic24a2
9	0.025978	0.172925	-9.05172	AABR07069611.1
17	0.006089	0.094802	-8.98876	Dnajc1
14	0.000214	0.018838	-8.02395	Gnrhr
1	0.005685	0.09338	-7.59358	Fam168a
16	0.023844	0.169272	-6.89655	Dic1
1	0.018358	0.152242	-6.24494	Neu3
5	0.007821	0.104127	-5.95066	Zfp37-ps1
5	0.007821	0.104127	-5.95066	LOC259244
5	0.007821	0.104127	-5.95066	Mup4
4	0.021281	0.161113	-5.7971	Prickle2
MT	0.042631	0.218094	-5.61033	Mt-co2
MT	0.009344	0.113613	-5.20373	AY172581.20
MT	0.006087	0.094802	-5.08151	Pde7b

Table 2.6: Hyper methylated genes in the testes of F1 generation rats.

chr	pvalue	qvalue	meth.diff	gene
8	0	0.001826	37.26455	Myo6
MT	0.000304	0.0203	25.21368	AY172581.24
16	0.007026	0.100075	23.55991	Agpat5
14	0.023303	0.168363	21.80775	Shroom3
17	0.006933	0.100075	20	Pfkfb3
9	0.020164	0.159721	19.86264	LOC100360856
9	0.004617	0.084822	19.21039	LOC108351885
9	0.004617	0.084822	19.21039	LOC360998
15	0.004253	0.081545	18.53379	Adam28
9	0.004048	0.078973	18.53259	Inpp1
X	0.02427	0.170547	18.42483	Tenm1
9	0.044315	0.221497	17.08333	AABR07069611.1
17	0.005026	0.087735	16.11111	LOC108353138
17	0.005026	0.087735	16.11111	Edaradd
17	0.005026	0.087735	16.11111	LOC120093110
1	0.00328	0.069818	15.62352	Tsga10ip
MT	0.012821	0.133017	14.54479	AY172581.9
MT	0	0.000493	14.50821	Mt-co1
11	0.005148	0.088497	14.34708	Epha6
9	0.03086	0.185105	14.29924	AABR07068351.1
5	0.013425	0.1343	13.97531	Prex2
Y	0.044956	0.22268	13.10652	AABR07025152.1
13	0.007463	0.101461	12.41765	LOC100361907
1	0.004829	0.086319	12.41039	LOC685793
11	0.003425	0.070488	12.22989	Gap43
MT	0.011423	0.125306	11.7029	AY172581.8
7	0.013964	0.134372	11.34707	Anks1b
5	0.042253	0.216974	11.31321	Ncmmap
9	0.017444	0.147318	11.15512	LOC102557419
9	0.000949	0.038323	11.01724	Dis3l2
MT	0.000356	0.022811	10.93113	Mt-nd5
9	0.011212	0.123777	10.73218	LOC103690742
8	0.038016	0.207979	10.4811	Or7e176
2	0.002121	0.056555	10.02731	Aadacl2fm3
1	0.002938	0.066538	7.900433	Neu3
10	0.014177	0.135335	6.634315	Apoh
4	0.026476	0.17409	6.486585	Antxr1
20	0.008467	0.107177	5.553352	Ctnna3
6	0.007175	0.100785	5.319149	Rad51b

Table 2.7: Hypo methylated genes in the testes of F2 generation rats..

chr		pvalue	qvalue	meth.diff	gene
Y		0.001912	0.02456	-28.9413	AABR07025152.1
	14	0.003141	0.033395	-27.725	Acx3
	8	0	0.002827	-27.3758	Rab6b
	8	0	0.002827	-27.3758	Tf
	9	0.00278	0.030913	-24.108	Plcl2
	14	0.001216	0.019266	-22.2222	Fryl
	9	0.012797	0.076648	-21.8508	LOC100360856
MT		0	0.003591	-21.5533	Mt-co2
	9	0.021	0.102276	-20.7688	LOC103690742
	19	0	0.002776	-20.6349	LOC120098581
	1	0.036359	0.140143	-19.9744	LOC100910657
	9	0.000106	0.003591	-18.3359	LOC108351885
	9	0.000106	0.003591	-18.3359	LOC360998
	6	0	0.002776	-17.7152	Slc25a21
	19	0.024945	0.112817	-17.381	AABR07043711.1
	19	0.024945	0.112817	-17.381	AABR07043389.1
	16	0.020895	0.102124	-17.3401	Agpat5
	2	0.044487	0.157057	-17.1226	Rap1gds1
	5	0.017283	0.090604	-16.6136	Skint10
	11	0.005211	0.045123	-16.0298	Epha6
	6	0.013825	0.079092	-15.7658	Ifi27
	1	0.004609	0.042293	-15.6856	Arb1
MT		0.020661	0.101372	-15.4404	AY172581.9
	5	0.001204	0.019215	-15.4285	Mup4-ps3
	5	0.001204	0.019215	-15.4285	LOC298116
	19	0.02859	0.123092	-15.0904	LOC685989
	1	0.029273	0.124581	-13.4443	LOC685793
	13	0.010046	0.068044	-13.3837	Slc9c2
MT		0	0.002176	-12.2157	Mt-nd1
	9	0.007159	0.054814	-10.5704	AABR07069611.1
MT		0.036548	0.140712	-9.98055	AY172581.24
	4	0.0338	0.134	-9.68582	Nub1
MT		0.044954	0.157789	-9.25466	AY172581.8
	9	0.018117	0.092746	-8.41616	Adgre4
	4	0.016485	0.088055	-7.93651	Dnai7
	8	0.011588	0.073642	-6.82389	Myo9a
	2	0.015731	0.085647	-6.69775	Aadacl2fm3
	4	0.027822	0.1213	-6.11299	Ahcyl2

Table 2.8: Hyper methylated genes in the testes of F2 generation rats.

chr		pvalue	qvalue	meth.diff	gene
	1	0	0.002921	31.02273	LOC685793
	9	0.000695	0.013543	26.04118	LOC103690742
MT		0	0.000919	25.05801	Mt-co3
	19	0.003488	0.035165	24.93398	LOC120098581
	19	0.00211	0.026199	24.12465	AABR07043389.1
X		0	0.003488	20.28581	Tcp11x2
	1	0.002368	0.028083	18.71978	LOC100910657
	1	0.015573	0.085599	17.88889	LOC100912265
	12	0.000479	0.010424	17.44725	Aldh2
	4	0.002477	0.029031	17.42109	Slco1a4
MT		0.034141	0.1345	17.40741	AY172581.9
	19	0.009907	0.067841	17.33512	LOC685989
MT		0.018021	0.092614	17.28186	Mt-co1
	3	0.013455	0.07828	16.37631	Macrod2
	3	0.001171	0.018924	16.12903	Lrp1b
Y		0.033208	0.13293	16.11722	AABR07025152.1
MT		0.020664	0.101372	16.09658	AY172581.24
	15	0.0105	0.069833	15.83285	Adam28
	9	0.028501	0.123092	15.26224	AABR07069611.1
	10	0.035042	0.13659	14.26799	Abcc1
	10	0.028847	0.123333	14.03085	Luc7l3
MT		0.005215	0.045123	13.96514	Mt-co3
	2	0.024983	0.112834	13.78827	Adgrv1
MT		0.020339	0.100414	13.29821	AY172581.8
	9	0.050372	0.169	12.87879	LOC100360856
	8	0.030677	0.127673	12.18304	Zfp846
	9	0.002285	0.027565	10.76923	Col5a2
	8	0.033743	0.134316	10.74284	Scaper
	1	0.05014	0.168555	10	Neu3
	7	0.003488	0.035165	9.458509	Kif21a
	9	0.028627	0.123092	9.103841	LOC102557419
	9	0.040391	0.148917	9.098039	LOC108351885
	9	0.040391	0.148917	9.098039	LOC360998
	4	0.005374	0.045797	8.707313	Antxr1
	10	0.044814	0.157789	8.580476	Sar1b
	2	0.012727	0.076642	8.141955	Raly1
	4	0.0442	0.156699	7.872643	Magi2
	10	0.010777	0.070708	7.641629	Myo15a
	18	0.017651	0.092001	6.95902	Camk4
	13	0.002322	0.027738	6.660694	Atf6
	5	0.011482	0.07349	6.018589	Faf1
	4	0.039296	0.14712	5.769231	Ly49si2
	6	0.00398	0.0384	5.555556	Itsn2
	9	0.036335	0.140143	5.26464	Adgrb3

Table 2.9: Hypo methylated genes in the testes of F3 generation rats.

chr	pvalue	qvalue	meth.diff	gene
19	0.000992	0.043819	-30	LOC685989
1	0.000903	0.04357	-28.6946	LOC685793
9	0.00209	0.065071	-28.6885	LOC103690742
2	0	0.008856	-25.4891	Rsrc1
MT	0.000789	0.042094	-24.1514	Mt-cyb
2	0.004956	0.090239	-22.3153	Chd1l
18	0.000386	0.028274	-22.2463	Chsy3
MT	0.002234	0.065755	-21.4493	AY172581.9
10	0.001059	0.045467	-20.8333	Luc7l3
1	0.002724	0.069951	-20.7298	LOC100910657
9	0.010964	0.120038	-19.5455	LOC108351885
9	0.010964	0.120038	-19.5455	LOC360998
15	0.00527	0.092038	-16.8926	Mcpt4l1
4	0	0.007235	-16.8822	Grm8
10	0.006425	0.101052	-15.4281	Ace
18	0.006559	0.102091	-14.9798	Impa2
9	0.013302	0.13145	-14.8352	Mgat4a
Y	0.042951	0.229998	-12.8959	AABR07025152.1
MT	0.050613	0.243203	-12.7937	AY172581.24
16	0.041886	0.228439	-12.3886	Grid1
1	0.032381	0.203096	-12.0372	Arrb1
15	0.002287	0.065755	-10.9217	Adam28
3	0.00079	0.042094	-10.1118	LOC102550520
MT	0	0.002799	-9.88418	Mt-co1
9	0.011571	0.121686	-9.81969	Qis3l2
6	0.016917	0.1476	-9.49814	Dcdc2c
15	0.004492	0.086512	-8.96456	Fgf14
17	0.002901	0.071439	-8.30628	Spock1
13	0.03203	0.203096	-7.11076	Atf6
9	0.014826	0.139182	-6.32911	AABR07069611.1
MT	0.002365	0.065755	-5.7594	Mt-nd4l
1	0.039017	0.222409	-5.55876	Rrm1
9	0.003242	0.077902	-5.51652	Pard3b
MT	0.047626	0.238455	-5.50409	Mt-co3
11	0.003571	0.080883	-5.37634	Trmpss15
15	0.008342	0.108195	-5.10462	Adk

Table 2.10: Hyper methylated genes in the testes of F3 generation rats.

chr	pvalue	qvalue	meth.diff	gene
MU15018	0.001198	0.048863	31.33333	LOC108348366
9	0.000924	0.04357	29.62185	LOC108351885
9	0.000924	0.04357	29.62185	LOC360998
18	0.002245	0.065755	26.43642	Kcnn2
MT	0.014173	0.135205	23.56902	Mt-nd5
4	0.008223	0.108195	22.28261	Glicc1l
6	0.011808	0.123601	20.18042	Slc25a21
18	0.002124	0.065123	19.66667	Camk4
19	0.000352	0.026533	19.60784	LOC685989
X	0.012748	0.128942	17.15686	Hdx
7	0.038024	0.221238	16.90229	Caps2
MT	0.036599	0.216871	16.74901	AY172581.24
1	0.012086	0.125628	16.4186	LOC685793
4	0.008512	0.108705	16.41026	Ube3c
19	0.009333	0.111817	16.0418	LOC120098581
5	0.011585	0.121686	15.17783	Skint10
MT	0.043087	0.230011	12.6108	Mt-co1
MT	0.037488	0.219528	11.18421	AY172581.9
12	0.018883	0.157946	10.68023	Tmem132d
4	0.042225	0.228439	10.53114	Ccser1
15	0.029596	0.196411	9.937343	Adam28
MT	0.002484	0.066636	9.901961	Mt-co3
2	0.005079	0.091007	9.615385	Synpo2
9	0.007079	0.10388	9.52381	AABR07069611.1
MT	0.000477	0.03168	9.459459	AY172581.10
MT	0.001456	0.053999	9.290836	Mt-nd1
1	0.041468	0.228171	9.184901	Sbf2
13	0.005982	0.097902	8.407382	Zp3r
4	0.02944	0.196097	8.163265	Tmtc1
1	0.008141	0.108195	7.954545	Tnuc3
5	0.00089	0.04357	7.913669	Sntg1
16	0.004626	0.0874	7.142857	Csmd1
2	0.006094	0.097902	7.017544	Selenof
4	0.050728	0.243203	6.643978	Nub1
MT	0.002784	0.070878	5.363368	Mt-nd3
6	0.032885	0.203271	5.263158	Itsn2
2	0.043998	0.231072	5.263158	Naaladl2

After keeping a differential methylation cutoff score of -5/+5 with a p-value of 0.05; and deleting all repetitions, we observed that 11 genes were showing similar pattern of methylation in all the generations (transgenerational). Details of these genes are presented in (Table 2.11)

Table 2.11: Genes showing similar patterns of methylation transgenerationally. (Yellow, sky blue, green and white bars indicate F0, F1, F2, and F3 generations respectively).

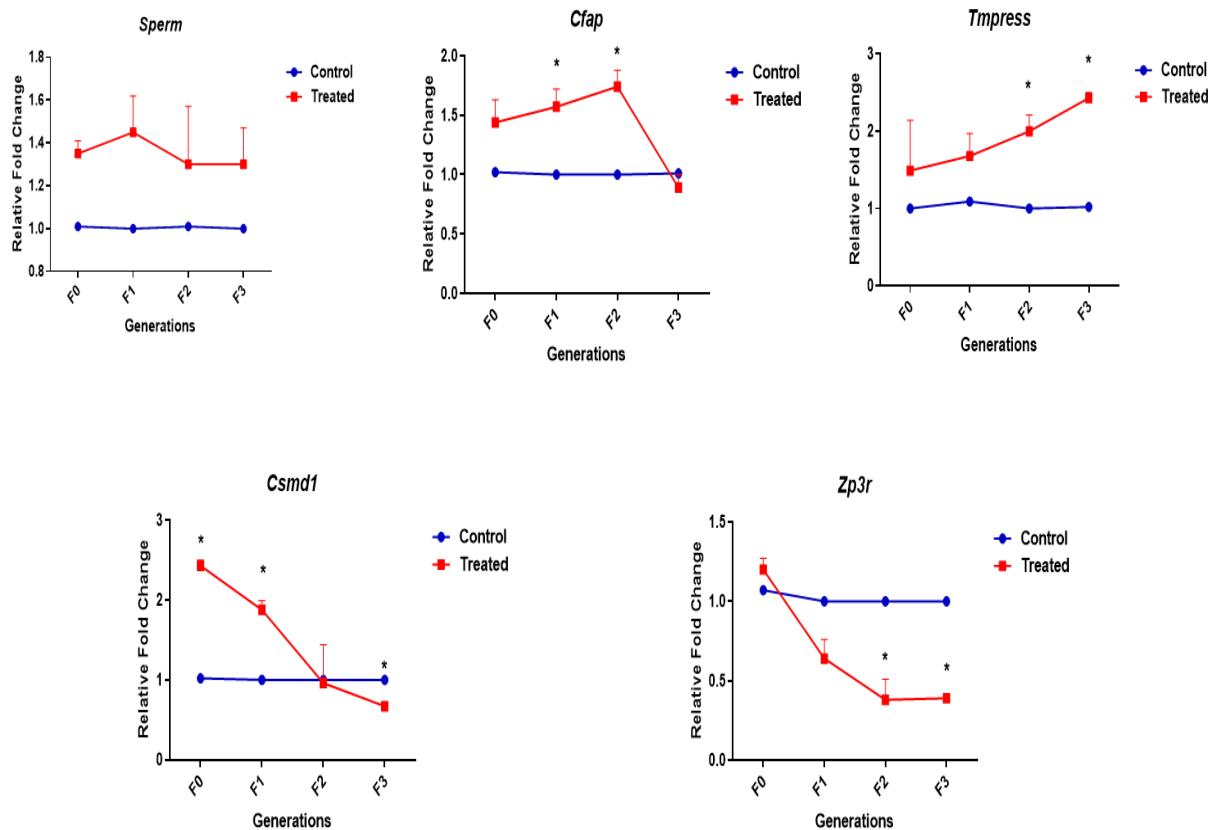
chr	pvalue	qvalue	meth.diff	gene
9	0.000183	0.003384	-27.1481	LOC103690742
9	0.000282	0.0203	-27.037	LOC103690742
9	0.021	0.102276	-20.7688	LOC103690742
9	0.00209	0.065071	-28.6885	LOC103690742
Y	0	0.000783	-25.2973	AABR07025152.1
Y	0.019305	0.155783	-16.1497	AABR07025152.1
Y	0.001912	0.02456	-28.9413	AABR07025152.1
Y	0.042951	0.229998	-12.8959	AABR07025152.1
MT	0.000108	0.00229	-13.6483	AY172581.9
MT	0.001238	0.042803	-20.0126	AY172581.9
MT	0.020681	0.101372	-15.4404	AY172581.9
MT	0.002234	0.063755	-21.4493	AY172581.9
MT	0.009678	0.056185	6.055432	AY172581.24
MT	0.000304	0.0203	25.21368	AY172581.24
MT	0.020664	0.101372	16.09658	AY172581.24
MT	0.036599	0.216871	16.74901	AY172581.24
9	0.002672	0.023582	-8.37662	LOC108351885
9	0.034409	0.199208	-18.8189	LOC108351885
9	0.000106	0.003591	-18.3359	LOC108351885
9	0.010964	0.120038	-19.5455	LOC108351885
9	0.002672	0.023582	-8.37662	LOC360998
9	0.034409	0.199208	-18.8189	LOC360998
9	0.000106	0.003591	-18.3359	LOC360998
9	0.010964	0.120038	-19.5455	LOC360998
9	0.00811	0.050629	-7.64803	AABR07069611.1
9	0.025978	0.172925	-9.05172	AABR07069611.1
9	0.007159	0.054814	-10.5704	AABR07069611.1
9	0.014826	0.139182	-6.32911	AABR07069611.1
MT	0.000304	0.004738	-6.62787	Mt-co1
MT	0	0.000493	14.50821	Mt-co1
MT	0.018021	0.092614	17.26186	Mt-co1
MT	0.043087	0.230011	12.6108	Mt-co1
1	0.006091	0.041549	23.12925	LOC100910657
1	0.006977	0.100075	-10.8305	LOC100910657
1	0.036359	0.140143	-19.9744	LOC100910657
1	0.002724	0.069951	-20.7258	LOC100910657
1	0	0.000905	21.5033	LOC685793
1	0.004829	0.086319	12.41039	LOC685793
1	0	0.002921	31.02273	LOC685793
1	0.012086	0.125628	16.4186	LOC685793
15	0.045156	0.14811	17.16853	Adam28
15	0.004253	0.081545	18.53379	Adam28
15	0.0105	0.069833	15.83285	Adam28
15	0.029596	0.196411	9.937343	Adam28

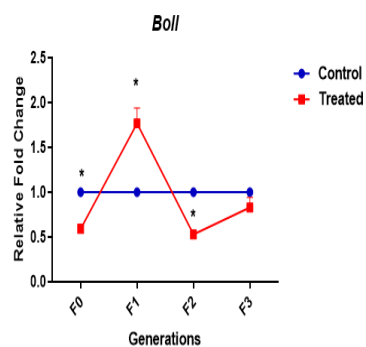
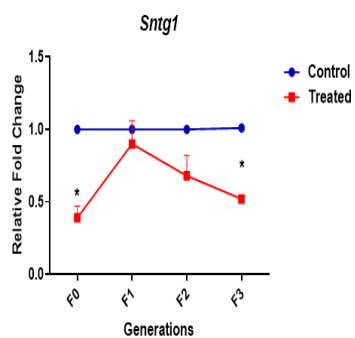
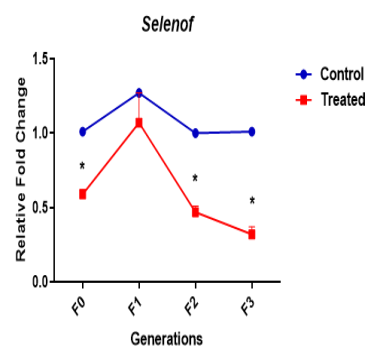
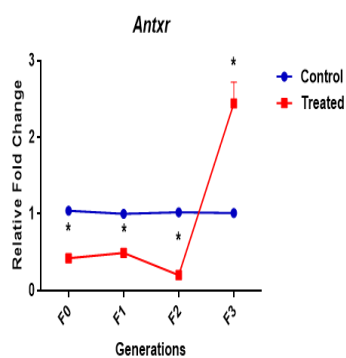
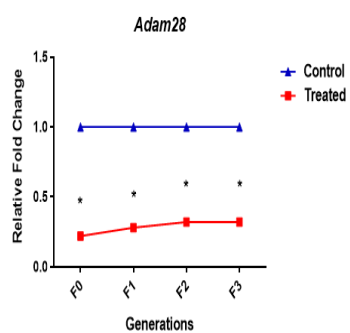
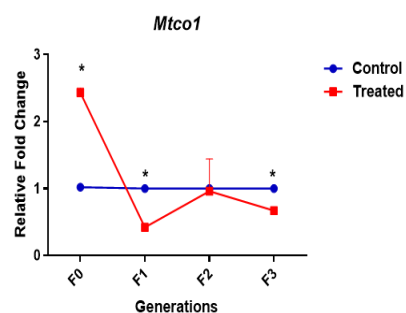
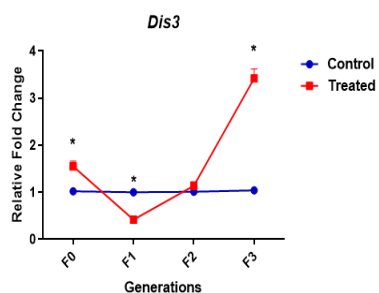
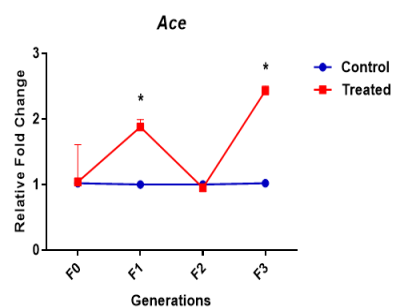
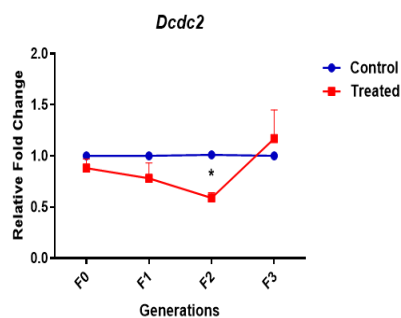
After applying a cutoff score of +5/-5 observed 20 differentially methylated genes related to reproductive function. (**Figure 2.11**). Real-time PCR was performed to cross-check the expression pattern of these 20 genes to correlate them with their methylation status. The real time analysis data showed equal variation as expected from methylation pattern transgenerationally. Genes like *TCPX11*, *Sntg1*, *Selenof*, *Dcdc2*, *Antxr*, and *Adam28* are significantly down-regulated. Whereas genes like *Cdyl*, *Cfap*, *Bmp6*, *Dis3*, and *Ace* showed significant up-regulation. Genes like *Zp3r*, *Sperm motility factor*, *Ros1*, *Scaper*, *Boll*, *Csmd1*, *Impress* and *Mtco1* showed variable changes in all generations. (**Figure 2.12**). Genes like *Ccdc170*, *Ccdc191* and *Ccdc85a*, *Ccdc148* are found to be hypo and hyper methylated respectively. The expression of *Ccdc54* and *Ccdc96* are found to be significantly downregulated (**Figure 2.13**). **Table 2.12** summarises the expression profile of differentially methylated genes in the testes of rats treated with pyrethroid and in testes from later generations.

Figure 2.11: Differentially methylated genes related to reproductive function.

chr	pvalue	gene	
13	0.002592	Zp3r	F0
X	0	Tcp11x2	
1	0.006091	LOC100910657	
20	0.000121	Ros1	
5	0.021869	Sntg1	
17	0.000769	Cdyl	
8	0.043853	Scaper	
17	0.004912	Bmp6	F1
13	0	Cfap221	
17	0.000382	LOC108353138	
17	0.000382	LOC120093110	
X	0.00713	Tcp11x2	
1	0.006977	LOC100910657	
9	0.029199	Boll	
17	0.005026	LOC108353138	F2
17	0.005026	LOC120093110	
9	0.000949	Dis3l2	
4	0.026476	Antr1	
1	0.036359	LOC100910657	
X	0	Tcp11x2	
1	0.002368	LOC100910657	F3
8	0.033743	Scaper	
4	0.005374	Antr1	
1	0.002724	LOC100910657	
10	0.006425	Ace	
9	0.011571	Dis3l2	
6	0.016917	Dcdc2c	
11	0.003571	Tmprss15	
13	0.005982	Zp3r	
5	0.00089	Sntg1	
16	0.004626	Csmd1	

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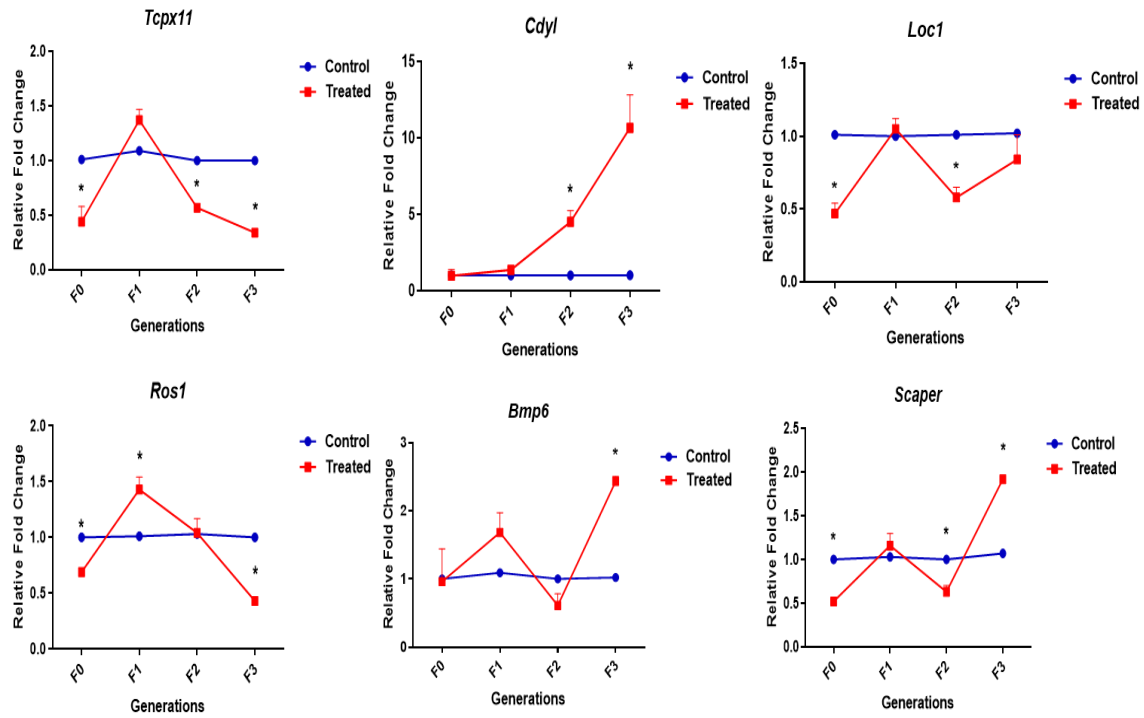


Figure 2.12: Expression pattern of reproductive function-related genes. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

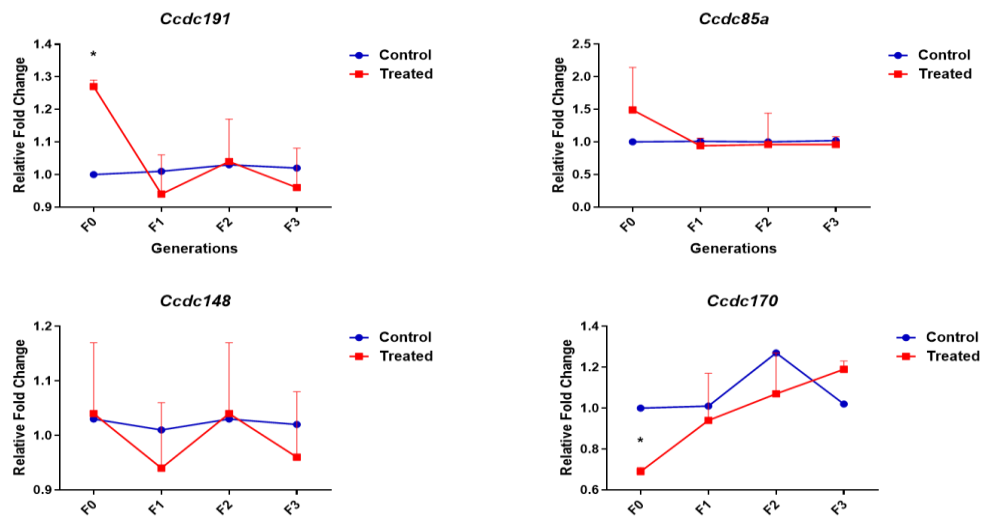


Figure 2.13: Expression pattern Ccdc genes. RNA isolated from the testes of rats for all four generations and was reverse-transcribed and real-time polymerase chain reaction was performed using gene specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Table 2.12: Expression pattern of genes analyzed in this part of the study.

Gene Name	F0	F1	F2	F3
<i>Dnmt2</i>	↓	↓	—	—
<i>Dnmt3a</i>	↑	↓	↓	↓
<i>Dnmt3b</i>	↓	↓	↓	—
<i>Hp1bp3</i>	↑	↓	—	—
<i>Kat3a</i>	↓	—	—	—
<i>Hdac1</i>	—	↓	—	↑
<i>Hdac4</i>	↓	↑	↑	↑
<i>Mch2ta</i>	↓	—	—	—
<i>Sperm</i>	—	—	—	—
<i>Cfap</i>	—	↑	↑	—
<i>Tmpress</i>	—	—	↑	↑
<i>Csmd1</i>	↑	↑	—	↓
<i>Zp3r</i>	—	—	↓	↓
<i>Dcdc2</i>	—	—	↓	—
<i>Ace</i>	↑	—	↑	—
<i>Dis3</i>	↑	↑	—	↑
<i>Mtcol</i>	↑	↑	—	↑
<i>Adam28</i>	↓	↓	↓	↓
<i>Antxr</i>	↓	↓	↓	↑
<i>Selenof</i>	↓	—	↓	↓
<i>Sntg1</i>	↓	—	—	↓
<i>Boll</i>	↓	↑	↓	—
<i>Tcpx11</i>	↓	—	↓	↓
<i>Cdyl</i>	—	—	↑	↑
<i>Loc1</i>	↓	—	↓	—

<i>Rosl</i>	↓	↑	—	↓
<i>Bmp6</i>	—	—	—	↑
<i>Scaper</i>	↓	—	↓	↑
<i>Ccdc191</i>	↑	—	—	—
<i>Ccdc85a</i>	—	—	—	—
<i>Ccdc148</i>	—	—	—	—
<i>Ccdc170</i>	↓	—	—	—

(↓ - Significant downregulation, ↑ - Significant upregulation, and — -denotes no Significant changes. $p \leq 0.05$ and $n=3$)

Discussion

Pyrethroids cause epigenetic modifications and disturb the methylation and acetylation patterns by affecting the enzymes associated with these processes (Hodoşan et al., 2023). The ability of pyrethroids to cause epigenetic changes that are passed down to subsequent generations even in the absence of direct chemical exposure is known as transgenerational pyrethroid effects (Skinner, 2015). These occur when pyrethroids alter the germ cells' (eggs or sperm) epigenetic markers, such as DNA methylation, histone modification, or non-coding RNA activity, leading to heritable changes in gene expression. For example, Thorson *et al.* found that pregnant rodents exposed to pesticides, including pyrethroids, during pregnancy changed their DNA methylation throughout the germline development (Thorson, Beck, Maamar, et al., 2020). These changes manifested as increased susceptibility to illnesses such as obesity, reproductive disorders, and neurological impairment and were perpetuated by the children and even subsequent generations (Manikkam et al., 2012). When we analysed the effect of pyrethroids on the epigenetic changes (DNA methylation) we found significant changes in the parental group and in subsequent generations, indicating a transgenerational effect. A scientific study by (Lei et al., 2022) examined the effects of deltamethrin, a widely used pyrethroid, on zebrafish reproductive processes. The researchers observed that deltamethrin exposure altered the germline's methylation levels, resulting in developmental issues and decreased fertility in subsequent generations (Lei et al., 2022). Furthermore, research on humans and wildlife suggests that species exposed to pyrethroids in their surroundings may experience population-level losses as a result of transgenerational impacts (Sanchez-Bayo & Goka, 2014). For

instance, aquatic animals such as fish and frogs exhibit long-term epigenetic alterations that are passed down through generations, and exposure to pyrethroids in agricultural runoff has been linked to decreased reproductive success in these species (Nicoletta & de Assis, 2022). Long-term pyrethroid exposure in farm labourers has been linked to biomarkers of altered DNA methylation in spermatozoa, which may have implications for human progeny (Zhang et al., 2012).

RRBS is a standard technique to study epigenetics in relation to environmental effects, developmental biology, and cancer etiology because of its capacity to target functionally important regions (Gu et al., 2011). Additionally, RRBS is a potent high-throughput method for single-base resolution DNA methylation analysis in CpG-rich genomic areas (Lister & Ecker, 2009). The epigenetic consequences of pyrethroid exposure have been clarified by recent research employing RRBS, especially with regard to changed DNA methylation patterns (Bertogliat et al., 2020). It has been demonstrated that pyrethroids, including permethrin, deltamethrin, and cypermethrin, alter DNA methylation in genes linked to neurodevelopment, endocrine regulation, and reproductive function. (Ijomone et al., 2020). RRBS studies have revealed that exposure to pyrethroids can result in hypomethylation or hypermethylation of promoter regions in genes critical for steroidogenesis, spermatogenesis, and testicular function, such as *StAR*, *CYP11A1*, and *AR* (X. Yu et al., 2024). Furthermore, in prenatal or early-life exposure models, RRBS has demonstrated that pyrethroids can induce persistent epigenetic modifications in developmental genes, suggesting long-term consequences on growth and fertility (Blanc et al., 2021). These findings underscore the significance of RRBS in uncovering the molecular underpinnings of pyrethroid toxicity and provide a basis for understanding how environmental chemicals can modulate the epigenome, with potential implications for transgenerational effects (Nilsson et al., 2022).

Hence, we analyzed the transgenerational epigenetic changes in the testes due to pyrethroid exposure. We observed changes in the methylation pattern of a large number of genes associated with male reproductive function, and these changes were associated with altered expression of these genes. When examined using bisulfide sequencing, it was discovered that samples treated with pyrethroids had impacted members of the *Ccdc* gene family. Thus, it can be assumed that the perturbations in gene expression due to pyrethroid exposure in the testis are modulated at the epigenome level, which further percolates to affecting spermatogenesis, sperm function, and fecundity. The influence of pyrethroids on the epigenome status was reported previously (Bordoni et al., 2019). However, our observations are novel because of the

fact that such effects in the male reproductive system were not reported earlier. However, there are certain drawbacks to RRBS, such as incomplete genome coverage, bias towards CpG-dense regions, and potential sequence loss during library formation (Chatterjee et al., 2017). Despite the aforementioned challenges, RRBS remains a valuable instrument for determining methylation dynamics and their effects on health and disease (Meissner et al., 2008). These findings highlight the broader effects of pyrethroid use, demonstrating that their effects extend beyond acute toxicity to include transgenerational and long-term risks (Skinner et al., 2015). They also stress how important it is to incorporate transgenerational and epigenetic research into pesticide regulation frameworks in order to assess their safety (Skinner, 2011).

References

- Abu Zeid, E. H., El Sharkawy, N. I., Moustafa, G. G., Anwer, A. M., & Al Nady, A. G. (2021). The palliative effect of camel milk on hepatic CYP1A1 gene expression and DNA damage induced by fenpropathrin oral intoxication in male rats. *Ecotoxicology and Environmental Safety*, 207, 111296. <https://doi.org/10.1016/J.ECOENV.2020.111296>
- Baylin, S. B., & Jones, P. A. (2016). Epigenetic Determinants of Cancer. *Cold Spring Harbor Perspectives in Biology*, 8(9), a019505. <https://doi.org/10.1101/CSHPERSPECT.A019505>
- Bertogliat, M. J., Morris-Blanco, K. C., & Vemuganti, R. (2020). Epigenetic mechanisms of neurodegenerative diseases and acute brain injury. *Neurochemistry International*, 133, 104642. <https://doi.org/10.1016/J.NEUINT.2019.104642>
- Blanc, M., Antczak, P., Cousin, X., Grunau, C., Scherbak, N., Rüegg, J., & Keiter, S. H. (2021). The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (*Danio rerio*). *Science of The Total Environment*, 779, 146404. <https://doi.org/10.1016/J.SCITOTENV.2021.146404>
- Bock, C., Tomazou, E. M., Brinkman, A. B., Müller, F., Simmer, F., Gu, H., Jäger, N., Gnirke, A., Stunnenberg, H. G., & Meissner, A. (2010). Quantitative comparison of genome-wide DNA methylation mapping technologies. *Nature Biotechnology* 28:10, 28(10), 1106–1114. <https://doi.org/10.1038/nbt.1681>

- Bordoni, L., Nasuti, C., Fedeli, D., Galeazzi, R., Laudadio, E., Massaccesi, L., López-Rodas, G., & Gabbianelli, R. (2019). Early impairment of epigenetic pattern in neurodegeneration: Additional mechanisms behind pyrethroid toxicity. *Experimental Gerontology*, 124, 110629. <https://doi.org/10.1016/J.EXGER.2019.06.002>
- Boyle, P., Clement, K., Gu, H., Smith, Z. D., Ziller, M., Fostel, J. L., Holmes, L., Meldrim, J., Kelley, F., Gnirke, A., & Meissner, A. (2012). Gel-free multiplexed reduced representation bisulfite sequencing for large-scale DNA methylation profiling. *Genome Biology*, 13(10), 1–10. <https://doi.org/10.1186/GB-2012-13-10-R92/TABLES/3>
- Cedar, H., & Bergman, Y. (2009). Linking DNA methylation and histone modification: Patterns and paradigms. *Nature Reviews Genetics*, 10(5), 295–304. <https://doi.org/10.1038/NRG2540;KWRD=BIOMEDICINE>
- Chatterjee, A., Rodger, E. J., Stockwell, P. A., Le Mée, G., & Morison, I. M. (2017). Generating multiple base-resolution DNA methylomes using reduced representation bisulfite sequencing. *Methods in Molecular Biology*, 1537, 279–298. https://doi.org/10.1007/978-1-4939-6685-1_16/FIGURES/2
- Cokus, S. J., Feng, S., Zhang, X., Chen, Z., Merriman, B., Haudenschild, C. D., Pradhan, S., Nelson, S. F., Pellegrini, M., & Jacobsen, S. E. (2008). Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature*, 452(7184), 215–219. <https://doi.org/10.1038/NATURE06745;KWRD=SCIENCE>
- Curtis, G. H., Nogueiro, S., Schneider, S., Bernhofer, M., McDermott, M., Nixon, E., Perez, K. N., Reeve, R. E., Easterling, M. R., & Crespi, E. J. (2021). Trans-ovo permethrin exposure affects growth, brain morphology and cardiac development in quail. *Environmental Toxicology*, 36(7), 1447–1456. <https://doi.org/10.1002/TOX.23141;PAGE:STRING:ARTICLE/CHAPTER>
- De Ruijter, A. J. M., Van Gennip, A. H., Caron, H. N., Kemp, S., & Van Kuilenburg, A. B. P. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochemical Journal*, 370(3), 737–749. <https://doi.org/10.1042/BJ20021321>
- Ehrlich, M. (2009). DNA hypomethylation in cancer cells. *Epigenomics*, 1(2), 239–259. <https://doi.org/10.2217/EPI.09.33;SUBPAGE:STRING:ACCESS>
- Esteller, M. (2008). Epigenetics in Cancer. *New England Journal of Medicine*, 358(11), 1148–1159. <https://doi.org/10.1056/NEJMRA072067;CTYPE:STRING:JOURNAL>
- Feinberg, A. P., & Tycko, B. (2004). The history of cancer epigenetics. *Nature Reviews Cancer*, 4(2), 143–153. <https://doi.org/10.1038/NRC1279;KWRD=BIOMEDICINE>
- Freire, C., Suárez, B., Vela-Soria, F., Castiello, F., Reina-Pérez, I., Andersen, H. R., Olea, N., & Fernández, M. F. (2021). Urinary metabolites of non-persistent pesticides and serum hormones in Spanish adolescent males. *Environmental Research*, 197, 111016. <https://doi.org/10.1016/J.ENVRES.2021.111016>
- Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L., & Paul, C. L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences*, 89(5), 1827–1831. <https://doi.org/10.1073/PNAS.89.5.1827>
- Giambò, F., Leone, G. M., Gattuso, G., Rizzo, R., Cosentino, A., Cinà, D., Teodoro, M., Costa, C., Tsatsakis, A., Fenga, C., & Falzone, L. (2021). Genetic and epigenetic alterations induced by pesticide exposure: Integrated analysis of gene expression, microrna expression and dna methylation datasets. *International Journal of Environmental Research and Public Health*, 18(16), 8697. <https://doi.org/10.3390/IJERPH18168697/S1>

- Gu, H., Smith, Z. D., Bock, C., Boyle, P., Gnirke, A., & Meissner, A. (2011). Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. *Nature Protocols*, 6(4), 468–481. <https://doi.org/10.1038/NPROT.2010.190>;SUBJMETA=1647,2210,2213,2254,2261,514,631,666;KW RD=LIBRARY+CONSTRUCTION,METHYLATION+ANALYSIS,NEXT-GENERATION+SEQUENCING
- Ha, M., Huang, X., Li, L., Lu, D., & Liu, C. (2021). PKC α mediated by the PI3K/Akt-FOXO1 cascade facilitates cypermethrin-induced hyperthyroidism. *Science of The Total Environment*, 757, 143727. <https://doi.org/10.1016/J.SCITOTENV.2020.143727>
- Harris, R. A., Wang, T., Coarfa, C., Nagarajan, R. P., Hong, C., Downey, S. L., Johnson, B. E., Fouse, S. D., Delaney, A., Zhao, Y., Olshen, A., Ballinger, T., Zhou, X., Forsberg, K. J., Gu, J., Echipare, L., O'Geen, H., Lister, R., Pelizzola, M., ... Costello, J. F. (2010). Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. *Nature Biotechnology*, 28(10), 1097–1105. <https://doi.org/10.1038/NBT.1682>;SUBJMETA=1647,1948,2210,2213,2254,514,61,631;KWRD=DNA+SEQUENCING,METHYLATION+ANALYSIS,NEXT-GENERATION+SEQUENCING
- Hodoşan, C., Gîrd, C. E., Ghica, M. V., Dinu-Pîrvu, C. E., Nistor, L., Bărbuică, I. S., Marin, Ştefan C., Mihailescu, A., & Popa, L. (2023). Pyrethrins and Pyrethroids: A Comprehensive Review of Natural Occurring Compounds and Their Synthetic Derivatives. *Plants* 2023, Vol. 12, Page 4022, 12(23), 4022. <https://doi.org/10.3390/PLANTS12234022>
- Hong, D., Min, J. Y., & Min, K. bok. (2021). Association between pyrethroids and prostate endpoints; stratified according to renal function. *Environment International*, 153, 106489. <https://doi.org/10.1016/J.ENVINT.2021.106489>
- Hozyen, H. F., Khalil, H. M. A., Ghandour, R. A., Al-Mokaddem, A. K., Amer, M. S., & Azouz, R. A. (2020). Nano selenium protects against deltamethrin-induced reproductive toxicity in male rats. *Toxicology and Applied Pharmacology*, 408, 115274. <https://doi.org/10.1016/J.TAAP.2020.115274>
- Huang, S., Lu, Y., Li, S., Zhou, T., Wang, J., Xia, J., Zhang, X., & Zhou, Z. (2021). Key proteins of proteome underlying sperm malformation of rats exposed to low fenvalerate doses are highly related to P53. *Environmental Toxicology*, 36(6), 1181–1194. <https://doi.org/10.1002/TOX.23117>
- Ijomone, O. M., Ijomone, O. K., Iroegbu, J. D., Ifenatuoha, C. W., Olung, N. F., & Aschner, M. (2020). Epigenetic influence of environmentally neurotoxic metals. *NeuroToxicology*, 81, 51–65. <https://doi.org/10.1016/J.NEURO.2020.08.005>
- Isman, M. B. (2006). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annual Review of Entomology*, 51, 45–66. <https://doi.org/10.1146/ANNUREV.ENTO.51.110104.151146>,
- Jones, P. A., & Baylin, S. B. (2007). The Epigenomics of Cancer. *Cell*, 128(4), 683–692. <https://doi.org/10.1016/J.CELL.2007.01.029>
- Kouzarides, T. (2007). Chromatin Modifications and Their Function. *Cell*, 128(4), 693–705. <https://doi.org/10.1016/J.CELL.2007.02.005>
- Krueger, F., & Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*, 27(11), 1571–1572. <https://doi.org/10.1093/BIOINFORMATICS/BTR167>

- Lei, L., Zhu, B., Qiao, K., Zhou, Y., Chen, X., Men, J., Yang, L., Wang, Q., Han, J., & Zhou, B. (2022). New evidence for neurobehavioral toxicity of deltamethrin at environmentally relevant levels in zebrafish. *Science of The Total Environment*, 822, 153623. <https://doi.org/10.1016/J.SCITOTENV.2022.153623>
- Li, E., & Zhang, Y. (2014). DNA Methylation in Mammals. *Cold Spring Harbor Perspectives in Biology*, 6(5), a019133. <https://doi.org/10.1101/CSHPERSPECT.A019133>
- Lister, R., & Ecker, J. R. (2009). Finding the fifth base: Genome-wide sequencing of cytosine methylation. *Genome Research*, 19(6), 959–966. <https://doi.org/10.1101/GR.083451.108>
- Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q. M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R., Ruotti, V., Millar, A. H., Thomson, J. A., Ren, B., & Ecker, J. R. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, 462(7271), 315–322. <https://doi.org/10.1038/NATURE08514;KWRD=SCIENCE>
- Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, M. M., & Skinner, M. K. (2012). Transgenerational Actions of Environmental Compounds on Reproductive Disease and Identification of Epigenetic Biomarkers of Ancestral Exposures. *PLOS ONE*, 7(2), e31901. <https://doi.org/10.1371/JOURNAL.PONE.0031901>
- Meissner, A., Mikkelsen, T. S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B. E., Nusbaum, C., Jaffe, D. B., Gnirke, A., Jaenisch, R., & Lander, E. S. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature*, 454(7205), 766–770. <https://doi.org/10.1038/NATURE07107;KWRD=SCIENCE>
- Nicolella, H. D., & de Assis, S. (2022). Epigenetic Inheritance: Intergenerational Effects of Pesticides and Other Endocrine Disruptors on Cancer Development. *International Journal of Molecular Sciences* 2022, Vol. 23, Page 4671, 23(9), 4671. <https://doi.org/10.3390/IJMS23094671>
- Nilsson, E. E., Ben Maamar, M., & Skinner, M. K. (2022). Role of epigenetic transgenerational inheritance in generational toxicology. *Environmental Epigenetics*, 8(1), 1–9. <https://doi.org/10.1093/EEP/DVAC001>
- Nilsson, E. E., Sadler-Riggelman, I., & Skinner, M. K. (2018). Environmentally induced epigenetic transgenerational inheritance of disease. *Environmental Epigenetics*, 4(2), 1–13. <https://doi.org/10.1093/EEP/DVY016>
- Park, W., Park, S., Lim, W., & Song, G. (2021). Bifenthrin reduces pregnancy potential via induction of oxidative stress in porcine trophectoderm and uterine luminal epithelial cells. *Science of The Total Environment*, 784, 147143. <https://doi.org/10.1016/J.SCITOTENV.2021.147143>
- Rao, J. S., Keleshian, V. L., Klein, S., & Rapoport, S. I. (2012). Epigenetic modifications in frontal cortex from Alzheimer’s disease and bipolar disorder patients. *Translational Psychiatry* 2012 2:7, 2(7), e132–e132. <https://doi.org/10.1038/tp.2012.55>
- Ravula, A. R., & Yenugu, S. (2019). Long term oral administration of a mixture of pyrethroids affects reproductive function in rats. *Reproductive Toxicology*, 89, 1–12. <https://doi.org/10.1016/J.REPROTOX.2019.06.007>
- Ravula, A. R., & Yenugu, S. (2021a). Effect of long-term treatment with a mixture of pyrethroids on the expression of genes that govern male germ cell production in rats. *Journal of Biochemical and Molecular Toxicology*, 35(2), e22654. <https://doi.org/10.1002/jbt.22654>

- Ravula, A. R., & Yenugu, S. (2021b). Effect of oral administration of a mixture of pyrethroids at doses relevant to human exposure on the general and male reproductive physiology in the rat. *Ecotoxicology and Environmental Safety*, 208. <https://doi.org/10.1016/j.ecoenv.2020.111714>
- Ravula, A. R., & Yenugu, S. (2021c). Pyrethroid based pesticides—chemical and biological aspects. *Critical Reviews in Toxicology*, 51(2), 117–140. <https://doi.org/10.1080/10408444.2021.1879007>,
- Ravula, A. R., & Yenugu, S. (2022). Transgenerational effects on the fecundity and sperm proteome in rats exposed to a mixture of pyrethroids at doses similar to human consumption. *Chemosphere*, 290. <https://doi.org/10.1016/j.chemosphere.2021.133242>
- Rojas-Rueda, D., Morales-Zamora, E., Alsufyani, W. A., Herbst, C. H., AlBalawi, S. M., Alsukait, R., & Alomran, M. (2021). Environmental Risk Factors and Health: An Umbrella Review of Meta-Analyses. *International Journal of Environmental Research and Public Health* 2021, Vol. 18, Page 704, 18(2), 704. <https://doi.org/10.3390/IJERPH18020704>
- Sanchez-Bayo, F., & Goka, K. (2014). Pesticide Residues and Bees – A Risk Assessment. *PLOS ONE*, 9(4), e94482. <https://doi.org/10.1371/JOURNAL.PONE.0094482>
- Shaffo, F. C., Grodzki, A. C., Fryer, A. D., & Lein, P. J. (2018). Mechanisms of organophosphorus pesticide toxicity in the context of airway hyperreactivity and asthma. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 315(4), L485–L501. <https://doi.org/10.1152/AJPLUNG.00211.2018/ASSET/IMAGES/LARGE/ZH50081874790002.JPEG>
- Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis*, 31(1), 27–36. <https://doi.org/10.1093/CARCIN/BGP220>
- Skinner, M. K. (2011). Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics*, 6(7), 838–842. <https://doi.org/10.4161/EPI.6.7.16537;PAGE:STRING:ARTICLE/CHAPTER>
- Skinner, M. K. (2015). Environmental Epigenetics and a Unified Theory of the Molecular Aspects of Evolution: A Neo-Lamarckian Concept that Facilitates Neo-Darwinian Evolution. *Genome Biology and Evolution*, 7(5), 1296–1302. <https://doi.org/10.1093/GBE/EVV073>
- Skinner, M. K., Guerrero-Bosagna, C., & Haque, M. M. (2015). Environmentally induced epigenetic transgenerational inheritance of sperm epimutations promote genetic mutations. *Epigenetics*, 10(8), 762–771. <https://doi.org/10.1080/15592294.2015.1062207>
- Smith, Z. D., & Meissner, A. (2013). DNA methylation: roles in mammalian development. *Nature Reviews Genetics* 2013 14:3, 14(3), 204–220. <https://doi.org/10.1038/nrg3354>
- Song, C., Kanthasamy, A., Anantharam, V., Sun, F., & Kanthasamy, A. G. (2010). Environmental Neurotoxic Pesticide Increases Histone Acetylation to Promote Apoptosis in Dopaminergic Neuronal Cells: Relevance to Epigenetic Mechanisms of Neurodegeneration. *Molecular Pharmacology*, 77(4), 621–632. <https://doi.org/10.1124/MOL.109.062174>
- Strahl, B. D., & Allis, C. D. (2000). The language of covalent histone modifications. *Nature*, 403(6765), 41–45. <https://doi.org/10.1038/47412;KWRD=SCIENCE>
- Sule, R. O., Condon, L., & Gomes, A. V. (2022). A Common Feature of Pesticides: Oxidative Stress—The Role of Oxidative Stress in Pesticide-Induced Toxicity. *Oxidative Medicine and Cellular Longevity*, 2022(1), 5563759. <https://doi.org/10.1155/2022/5563759>
- Thorson, J. L. M., Beck, D., Ben Maamar, M., Nilsson, E. E., & Skinner, M. K. (2020). Epigenome-wide association study for pesticide (Permethrin and DEET) induced DNA methylation epimutation

biomarkers for specific transgenerational disease. *Environmental Health: A Global Access Science Source*, 19(1), 1–17. <https://doi.org/10.1186/S12940-020-00666-Y/FIGURES/8>

Thorson, J. L. M., Beck, D., Maamar, M. Ben, Nilsson, E. E., McBirney, M., & Skinner, M. K. (2020). Epigenome-wide association study for atrazine induced transgenerational DNA methylation and histone retention sperm epigenetic biomarkers for disease. *PLOS ONE*, 15(12), e0239380. <https://doi.org/10.1371/JOURNAL.PONE.0239380>

Yu, G., Su, Q., Chen, Y., Wu, L., Wu, S., & Li, H. (2021). Epigenetics in neurodegenerative disorders induced by pesticides. *Genes and Environment* 2021 43:1, 43(1), 1–12. <https://doi.org/10.1186/S41021-021-00224-Z>

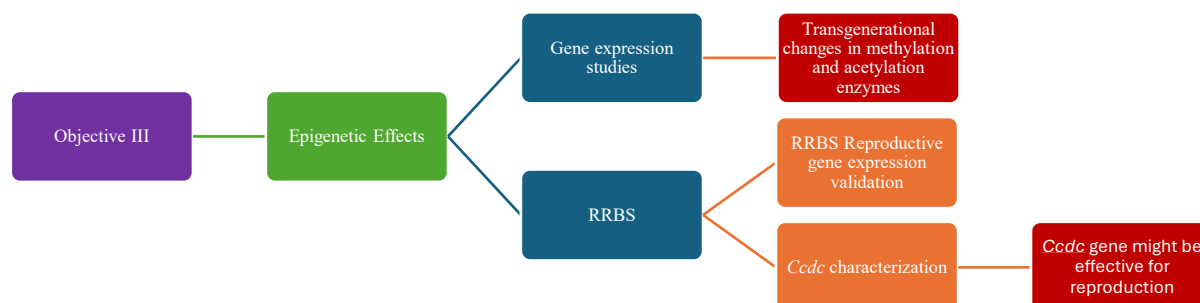
Yu, X., Xu, J., Song, B., Zhu, R., Liu, J., Liu, Y. F., & Ma, Y. J. (2024). The role of epigenetics in women's reproductive health: the impact of environmental factors. *Frontiers in Endocrinology*, 15, 1399757. <https://doi.org/10.3389/FENDO.2024.1399757/XML/NLM>

Zhang, X., Wallace, A. D., Du, P., Kibbe, W. A., Jafari, N., Xie, H., Lin, S., Baccarelli, A., Soares, M. B., & Hou, L. (2012). DNA methylation alterations in response to pesticide exposure in vitro. *Environmental and Molecular Mutagenesis*, 53(7), 542–549. <https://doi.org/10.1002/EM.21718;WGROU:STRING:PUBLICATION>

Chapter-III

Characterizing *Coiled coil domain containing*
(*Ccdc*) genes in the male reproductive tract.

Graphical Presentation



Introduction

Reproduction is one of the fundamental biological processes for the production of offspring. The evolution of distinct male and female reproductive systems facilitated sexual reproduction. The male reproductive system is a combination of organs and glands that include the testes, epididymis, vas deferens, seminal vesicle, prostate gland, and a pair of Cowper's and bulbourethral glands. The epithelial surfaces of the male reproductive tract secrete an array of proteins into the lumen. These proteins belong to a variety of families such as defensins, cathelicidins, lipocalins, and RNases (Hall et al., 2007; Zhou et al., 2018). While a large number of proteins secreted by the male reproductive tract were characterized in the last two decades, this domain of research continues to be an active area of investigation. A diverse nature of biological processes is carried out by these proteins, beyond their role in reproductive function. The coiled-coil domain-containing (CCDC) proteins have been investigated in the last decade for their multifunctional roles in general and male reproductive physiology as well. However, there is no dedicated review that describes the functional role of CCDC proteins. This review presents a comprehensive compilation of the reported functions of CCDC proteins. Since a great number of CCDC proteins are not yet characterized for their functional role in general and reproductive physiology, the information provided in this review helps to design strategies to explore the uncharacterized CCDC proteins. Further, analyzing the role of these proteins in the male reproductive tract helps to design strategies for identifying novel targets for contraception, in vitro fertilization, and pathological conditions.

General Features of CCDC Proteins

CCDC proteins essentially contain the coiled-coil domain, a highly conserved superhelical protein motif that is characterized by the presence of one or more alpha-helical peptides is wrapped around each other in a superhelical fashion. It is estimated that about 10% of an organism's proteome is comprised of the coiled-coil-forming sequences (Rose et al., 2005). In general, the coiled coils contain two to six helices that may be arranged in parallel or antiparallel orientation. They may be homo- or hetero-oligomers formed from helices of the same chain or from different chains. The supercoiling in these domains is subjected to varying degrees due to the insertion of extra amino acids, thus resulting in local deformations (Lupas et al., 2017). CCDC proteins are expressed in a wide range of tissues, and their cellular localization is diverse, thereby exhibiting functional roles in most of the physiological processes (Truebestein & Leonard, 2016). At the cellular level, they are implicated in chromosome segregation, centriole architecture, DNA recognition and cleavage, protein scaffolding, mediating vesicle tethering, and separating functional domains in proteins (by acting as molecular spacers), etc (Truebestein & Leonard, 2016). Further, these proteins are implicated in the migration, proliferation, and metastasis of different cancers (Chen et al., 2009; Farfsing et al., 2009; Gong et al., 2015; Kim et al., 2007; Park et al., 2012; Tanouchi et al., 2016; Yin et al., 2016). In the human male reproductive system, the majority of them are found to be expressed in the testis, epididymis, seminal vesicle, and prostate. In the testis, CCDC proteins are localized in the seminiferous duct cells, Leydig cells, and in the maturing spermatocytes. In the epididymis, seminal vesicle, and prostate, they are abundant in the glandular cells. CCDC proteins exhibit diverse functions in general and in male reproductive physiology as well. The functional role of CCDC proteins in different physiological processes is presented in **Figure 3.1**. It is very much evident that these proteins are implicated in normal and pathological processes of different organs and cellular systems.

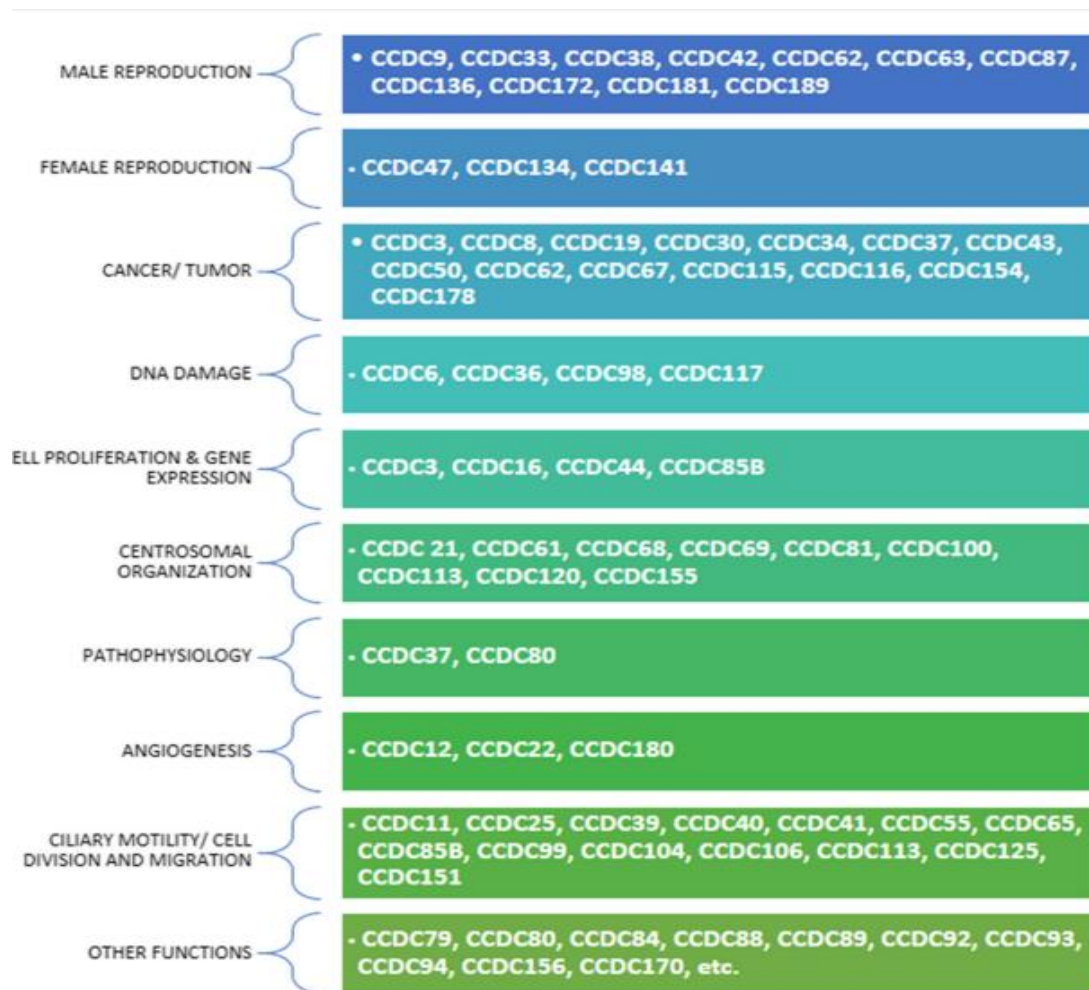


Figure 3.1: Involvement of CCDC proteins in various physiological processes

Role of CCDC Proteins in Male Reproduction

Abundant expression of *Ccdc33* is evident during mouse spermatogenesis, while a definite role for the mouse *Ccdc33b* variant is implicated in controlling spermatogenesis (Kaczmarek et al., 2010). *CCDC33* in humans is described as a cancer/testis gene, whose expression is restricted to germ cells (Kaczmarek et al., 2010). Similarly, *CCDC83* is a novel testis antigen for the detection of colorectal cancer (Song et al., 2012). *Ccdc38* mRNA expression is testis-specific, and the encoded protein is mostly confined to the nuclei of spermatogonia and spermatocytes. Increased expression of *CCDC38* was observed postnatally (2–8 weeks) in the mice testes, indicating that it is developmentally regulated and might contribute to spermatogenesis (Lin et al., 2016). *Ccdc136*, also a testis-specific gene, is expressed in an age-dependent manner and is known to influence spermatogenesis. *Ccdc136* knockout male mice were completely infertile and displayed predominant roundheaded sperm as compared with wild-type mice. *CCDC136* is implicated in acrosome formation and fertilization (Geng et al., 2016), whereas *CCDC135*

is associated with sperm motility (Yang et al., 2011). CCDC70 and CCDC78 regulate spermatogenesis and sperm maturation (CHEN et al., 2016; Majczenko et al., 2012), while CCDC112 is a marker for differentiating spermatocytes (M. Wang et al., 2018). Enriched expression of CCDC73 is associated with fertility (Khan et al., 2018). In lower animals (roosters), CCDC108 is involved in oxidation-reduction reactions in sperm and affects fertility (Y. Wang et al., 2017). In asthenozoospermic patients characterized by damaged sperm morphology and motility, the levels of CCDC9 were found to be significantly low (Sha et al., 2019). *Ccdc42* is predominantly expressed in the testis and brain. Deletion of this gene causes sterility in male mice with malformation of the head-to-tail coupling apparatus (HTCA) and the sperm tail (Pasek et al., 2016). A homolog of CCDC42, MIA2, acts as a dynein regulator and is an essential factor for ciliary motility in *Chlamydomonas reinhardtii* (Yamamoto et al., 2013). Spermatids lacking CCDC42 are characterized by defective nuclear shaping (in spite of the presence of a manchette), HTCA dislocation from its site of implantation, and absence of flagellum from the HTCA (Pasek et al., 2016). Since CCDC42-deficient mice showed no prevention in manchette formation, it is probably involved in perfect stabilization of the manchette or is a passenger protein carried through the manchette. The accumulation of this protein in the perinuclear ring indicated a stabilizing function and its participation in the attachment of the manchette with the nuclear membrane (Hoyer-Fender & Contreras, 2019). Mating of *repro29/+* mice with *Ccdc62* *-/-* mice revealed that the infertility of *repro29/repro29* mice was due to *Ccdc62* mutation (Li et al., 2017). The intracellular distribution of CCDC62 during acrosome function proves its significant role in spermiogenesis (Li et al., 2017). *Ccdc63* knockout rendered the mice infertile with spermatozoa having shortened flagella (Young et al., 2015). The expression of *Ccdc87* mRNA is testis-specific and mainly restricted to the pachytene stage of spermatocytes and spermatids. CCDC proteins are also localized on mature sperm, suggesting a role in sperm maturation (T. Wang et al., 2018). *Ccdc87* knockout mice showed serious morphological deformations in sperm. *Ccdc87*-null male mice are completely sterile (T. Wang et al., 2018). CCDC172, a TEKT2-interacting protein is localized on the rat spermatozoa (Shimasaki et al., 2010; Yamaguchi et al., 2014). Independently it exists as a component of the mitochondria of the middle piece of sperm and is essential for normal structure and functioning of flagella. *Tekt2*-lacking spermatozoa exhibit bending of flagellum and reduced motility of sperm possibly due to dynein inner arm disruption (Tanaka et al., 2004; Yamaguchi et al., 2014). *Ccdc181* mRNA is expressed strongly in the testis and also expressed ubiquitously at very low levels. CCDC181 is a structural constituent of the axoneme of sperm flagella and motile cilia (Schwarz et al., 2017). Mutations in CCDC189 result in the absence

of C1a-32 subunits in C1a complex, thus resulting in the loss of flagellar movement in sperm. A deleterious allele of *Ccdc189* induces sterility in males by producing immotile sperms with defective mitochondria (Iso-Touru et al., 2019). A plethora of factors that span cellular, molecular, and developmental processes of male gamete formation affect fertility (Gheldof et al., 2019).

Pyrethroids' impact on CCDC proteins is worsened by systemic toxicity, which interferes with endocrine function and metabolic functions (Yoshinaga et al., 2014). Disrupted hormonal balance, particularly testosterone decreases, further suppresses CCDC protein expression and action in reproductive organs (Saillenfait et al., 2015). These findings demand further investigation into the molecular mechanisms behind pyrethroid-induced modifications in CCDC proteins in order to understand their function in reproductive toxicity and to develop preventive strategies against such environmental exposures (Mnif et al., 2011).

Single nucleotide polymorphisms (SNPs) in coiled-coil domain-containing (CCDC) proteins are among the genetic variations commonly linked to infertility, a complex disorder (Coutton et al., 2015). The majority of the roles that CCDC proteins perform in reproductive biology, including spermatogenesis, sperm motility, flagella development, and centrosomal functions, are essential (Ge et al., 2024). Infertility can result from problems in the reproductive process caused by SNPs in the genes encoding CCDC proteins, which can alter their structure and function. For example, primary ciliary dyskinesia (PCD) is associated with mutations in CCDC39 and CCDC40, which are essential for the construction and maintenance of the axonemal dynein arms of sperm flagella (Knowles et al., 2016). One of the main reasons of male infertility, asthenozoospermia, is brought on by the disease's impairment of sperm motility. The regulation of spermatogonial stem cell development has also been linked to SNPs in CCDC62 (Wu et al., 2024). Aberrant spermatogenesis can arise from mutations in this gene that disrupt its interaction with vital signalling pathways, such as Wnt/ β -catenin (Cheng et al., 2018). Another example is the protein CCDC63, which is crucial for the development of sperm tails (Sironen et al., 2019). Mutations in this gene have been linked to morphological abnormalities in sperm flagella, such as short or absent tails, which further impair fertility (Graziani et al., 2024). Defective meiotic development in germ cells has also been connected to SNPs in CCDC88C, the gene encoding the cell polarity and signalling protein DAPLE, which results in fewer and lower-quality sperm (Glazenburg et al., 2024).

Furthermore, faulty acrosome development a crucial element for fertilization has been connected to SNPs in *CCDC146*, the gene involved in the last stages of sperm maturation

(Aitken & Baker, 2020). Infertility can be made worse by mutations in this gene that result in abnormal sperm-egg contact (Sawada & Saito, 2022). Infertility is one of the more common syndromic presentations brought on by SNPs in CCDC genes (Coutton et al., 2015). For instance, due of faulty axoneme formation, mutations in CCDC103, which is associated with PCD, affect sperm motility in addition to respiratory cilia (Zariwala et al., 2025). These SNPs' effects are exacerbated when they are combined with environmental stressors, such as exposure to endocrine disruptors or pesticides like pyrethroids, which alter the expression or function of the CCDC protein (Hilz & Gore, 2023). In order to identify at-risk individuals and create targeted treatment interventions, these findings emphasise the critical role that genetic variants in CCDC proteins play in male infertility (Kuroda et al., 2020). They also emphasise the necessity of molecular analysis and genetic screening (Pizzol et al., 2014).

Methodology

In silico analyses

The NCBI website's BLAST program was used to search the rat genome for the uncharacterized *Ccdc* genes. Using a variety of *in silico* methods, the primary, secondary, and tertiary structures of the rat CCDC genes were analyzed.

Secondary Structure Prediction

To predict the secondary structure of proteins, the Self-Optimized Prediction Method with Alignment (SOPMA) tool was employed. SOPMA predicted the secondary structure of a protein based on its primary sequence.

Structure Prediction

I-tasser, a fully automated protein structure prediction server was utilized for structural prediction of the CCDC proteins.

Model Validation

The quality of the protein structures was validated using PROCHECK, which determined the stereochemical quality by analyzing atomic contacts, cysteine bridges, torsion angles, planarity, residues, secondary structure, bond angles, and Ramachandran plots.

Table 3.1: *In silico* tools used for characterizing *Ccdc* genes and proteins.

Analysis	Tool used	Website
Sequence retrieval	UCSC Genome Browser assembly	https://genome.ucsc.edu/
Similarity search	NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Multiple sequence alignment	T-COFFEE	http://tcoffee.crg.cat/
Pairwise alignment	CLUSTALW	http://www.genome.jp/tools/clustalw/
General properties	EXPASY server	http://expasy.org/proteomics

Post translational modifications	Sequence manipulation suite	http://www.bioinformatics.org/sms2/
Genomic neighbourhood	Ensembl	http://asia.ensembl.org/index.html
Secondary structure prediction	SOPMA	https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html
Molecular modelling	GPCR-I-TASSER	https://zhanglab.ccmb.med.umich.edu/GPCR-I-TASSER/
Structure validation	PROCHECK	https://www.ebi.ac.uk/thornton-rv/software/PROCHECK/

Methodology used for characterization of *Ccdc* genes in vivo

1. Isolation of total RNA from the testes.
2. Checked the purity of isolated RNA and the concentration determined.
3. cDNA was prepared by reverse transcription.
4. PCR for *Gapdh* was performed to confirm the quality of cDNA.
5. PCR for the above-mentioned *Ccdc* genes using different sets of primers.
6. Amplicons were electrophoresed and purified by commercial kits.
7. Purified amplicons were sequenced.
8. The sequence obtained was deposited in GenBank.
9. Tissue distribution analysis was made for all *Ccdc* genes.
10. Cloning of *Ccdc54* and *Ccdc96* were done using pQE80mod Vector.
11. Induction and Purification procedures were further followed for the immunization study.
12. An SNP study is performed to check for a functional role of *Ccdc54* in male fertility.

Total RNA isolation from tissue

TRIzol® reagent (Gibco) was used to extract total RNA from tissues. Liquid nitrogen was used to crush and powder the tissues. Each tissue received one millilitre of TRIzol (100 mg) and vortexed for one minute before being incubated for five minutes at room temperature. The homogenate was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was pipetted up and down multiple times to ensure it was thoroughly mixed. By adding 200 µl of chloroform

and centrifuging for 10 minutes at 4 °C and 13,000 rpm, phase separation was achieved. After the aqueous top layer was carefully scraped off and transferred to a fresh tube, the sample was incubated for ten minutes at room temperature. For precipitation, an equivalent volume of isopropanol was then added. The samples were centrifuged for 10 minutes at 4 °C at 13,000 rpm, and the supernatant was disposed away. Before the ethanol was pipetted out entirely, the pellet was formed in 70% ethanol (produced in DEPC water), dissolved in it, and centrifuged at 10,000 g for five minutes. After being dissolved in 70% ethanol (produced in DEPC water) and centrifuged at 10,000 g for five minutes, the pellet was completely free of ethanol by pipetting and air drying. At last, the pellet was dissolved in 20 µl of DEPC-treated water.

RNA Integrity test

A 0.7% agarose gel made in TAE or TBE buffer was used for the RNA integrity test. The gel was electrophoresed at 70V after 2 µg of RNA were loaded onto it after being combined with RNA loading dye. After that, the banding pattern was examined under the right imaging circumstances. A consistent smear with a size of 0.5–11 kb was thought to be a sign of intact RNA. RNA integrity was verified by the existence of well-defined bands at 4.5 kb (28S rRNA) and 1.9 kb (18S rRNA), with an intensity ratio of at least 2:1 between 28S and 18S rRNA. RNA degradation was indicated by a downward shift in the RNA smear or a decreased ratio of 1:1. For these tests, denaturing formaldehyde gel was frequently used. A band close to the well indicated genomic DNA contamination, but high smearing beginning from the well indicated degraded RNA. In certain instances, DNA loading dye was also employed as a substitute.

Quantification of RNA for cDNA Preparation

The RNA was resuspended at a concentration of 1 µg/µl to allow equal input during the cDNA synthesis process. A Nanodrop spectrophotometer was used to quantify the RNA. While lower ratios indicated the presence of impurities such proteins, phenol, or other compounds absorbing at 280 nm, a 260/280 ratio of roughly 2.0 was thought to be indicative of pure RNA. It was anticipated that the 260/230 ratio for pure nucleic acids would fall between 2.0 and 2.2, with lower values suggesting possible contamination at 230 nm. Each RNA sample's name, preparation date, and quantification information were recorded in an Excel file.

Reverse transcription and polymerase chain reaction

The cDNA synthesis was carried out using 2 µg of RNA, combined with 1 µl of oligo dT (100 µM) and water to make up a total volume of 10 µl. At 65°C, this mixture was incubated for five minutes. Following incubation, the reaction mixture was mixed with a cocktail that contained 1 µl of reverse transcriptase (20 U/µl), 0.25 µl of RNase inhibitor (40 U/µl), 4 µl of 5x RT buffer, 2.5 mM MgCl₂, 1 µl of 10 mM dNTPs, and 1.25 µl of water to reach a final volume of 20 µl. Using a thermal cycler set to 65°C for 5 minutes, then chill on ice, 37°C for 60 minutes, 70°C for 10 minutes, and finally hold at 4°C, the reverse transcription reaction was carried out. After being properly labelled, the cDNA products were kept at either -20°C or -80°C. The reverse transcriptase manufacturer's recommendations were followed while modifying the software. Depending on the requirements of the experiment, the cDNA was either utilised directly or diluted in a 1:2 to 1:5 ratio. A 25 µl reaction mixture including 2.5 µl of 10X Taq buffer, 1.5 µl of 10 mM dNTPs, 1 µl of Taq polymerase (10 U/µl), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of cDNA, and 17 µl of water was made for the PCR reaction that followed. 32 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds comprised the PCR protocol. The initial denaturation lasted two minutes at 94°C. The reaction was maintained at 10°C after a final extension step that lasted five minutes at 72°C. Adjustments to the primer concentration were made to optimize product formation and reduce primer dimers. The annealing temperature was modified based on the GC content of the primers, and the extension time was adjusted according to the expected product size. For semi-quantitative PCR, around 25 cycles were used to avoid saturation, ensuring bands of varying intensities for better comparative analysis. Primers used for amplifying the predicted *Ccdc* genes are listed in

table 3.2. The primers used for qualitative and quantitative detection of *Ccdc* genes under different conditions are listed in **table 3.3**.

Recombinant protein production

With the help of gene-specific primers, the cDNA of rat *Ccdc54* and *Ccdc96* without the signal peptide was amplified and cloned into a pQE80m vector with an N-terminal His-tag (MRGSHHHHHHGS). After introducing the cloned plasmid into *E. coli BL21*, the bacteria were cultivated until they reached the mid-log stage. To express the recombinant protein, bacteria were treated with 1 mM IPTG for three hours. A commercial Ni-NTA agarose affinity protein purification kit (Qiagen, Valencia, CA, USA) was used to purify the recombinant protein after the bacterial cells were lysed. Using an anti-His tag antibody (Santa Cruz

Biotechnology, Dallas, USA; sc-57598 RRID: 26 AB_831408), western blotting confirmed the purified recombinant protein.

Immunoblotting

RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 1% sodium dodecyl sulphate) was used to homogenise the testes tissues of 9-month Wistar rats. After centrifuging the homogenates for 10 minutes at 12,857 g, Lowry's technique was used to measure the amount of protein in the supernatant. After loading the same amount of proteins in all required wells, SDS-PAGE was run. Before the transfer, the gel had to be submerged in a transfer buffer for fifteen minutes. The gel was positioned with the cathode (black) facing it, and the nitrocellulose membrane was positioned with the anode facing black. The proteins were transferred to the membrane over the course of 12–16 hours at 25V. In order to dissipate the heat produced by the high voltage and to avoid damaging the equipment and proteins, wet transfer was done at 50V for four hours while the tank was encircled by ice flakes. After measuring the efficiency of protein transfer using Ponceau stain, the membrane was rinsed three times with TBS until the stain was removed. The membrane was then blocked using a blocking solution consisting of 5% skim milk in TBST for three hours at room temperature. Either an hour at room temperature or an overnight period at 4°C on a shaker without washing was used to treat the membrane with the primary antibody in a blocking solution. The membrane was rinsed with TBS for 15 minutes, TBS-T for 15 minutes, then TBS for an additional 15 minutes following incubation with the primary antibody. The secondary antibody was then added, and it was incubated for one to one and a half hours at room temperature. The blot was then developed using developing solutions after being successively rinsed for 15 minutes with TBS, TBS-T, and TBS.

Genomic DNA isolation for the SNP study

Fertile and infertile men had their blood (2–10 ml) drawn into tubes, which were then centrifuged for 10 minutes at 3,000 rpm. After collecting the plasma, an equal volume of 2X lysis mix was added, and the mixture was incubated for 10 minutes to lyse the red blood cells. After that, the samples were centrifuged for 10 minutes at 3,000 rpm. 200 µl of proteinase K (10 mg/ml) and 450 µl of WBC lysing solution were added to the white cell pellet that had formed at the tube's bottom, vortexed, and incubated overnight at 37 °C. The sample and equal

quantities of phenol (saturated with 0.1 M Tris HCl, pH 8.0) were mixed, vortexed, and then centrifuged for five minutes at 3,000 rpm.

Another equal volume of phenol was added after the top aqueous layer was transferred to a clean tube. Following centrifugation, vortexing, and aqueous layer removal, the product was moved to a fresh tube containing an equivalent volume of chloroform and centrifuged for five minutes at 3,000 rpm. For DNA precipitation, the supernatant was taken out and combined with 1 µl of 4 M ammonium acetate and 2X volume of 100% ethanol. After mixing, it was centrifuged for 20 minutes at 4°C and 5000 rpm. The pellets were washed with 500 µl of 70% ethanol after the ethanol was decanted. To allow the DNA to dissolve, the pellet was dissolved in distilled water and then placed at 4°C for four to five hours. The quantity and purity of the DNA were estimated using NanoDrop (Thermo Scientific NanoDrop2000 UV visible spectrophotometer). This section of the study was approved by the University of Hyderabad's Institutional Ethics Committee (UH/IEC/2021/07).

Single nucleotide polymorphism analyses

- Genomic DNA was isolated.
- Primers were designed in such a way that they span all the exons of *Ccdc54* (*Ccdc54* consist of a single exon) (**Table 3.4**).
- Standardized the PCR conditions with different temperatures and genomic DNA dilutions.
- Purified samples were sequenced and multi-aligned using INRA MultAlin software and compared to controls.

Statistical analyses

The students' t-test, available in GraphPad Prism Software [version 8.0.2] (225 Franklin Street, Boston), was used to conduct statistical analyses. Mean ± SD is presented for the values obtained for each parameter. Statistical significance of $p < 0.05$ between control and treated groups is indicated by the * symbol.

Table 3.2: Primers used for amplifying the predicted full length *Ccdc* genes.

Name of the gene	Accession number	Seq 5'-3'	length	GC content %	Tm °C	Amp size
CCDC54 set2 FP	MT038367	ATGACAGACCTGGAAAACCAG	21	48	52.4	627bp
CCDC54 set2 RP	MT038367	TACTTGAAACAGGCTACATTT	21	33	46.5	627bp
CCDC67 Set2 FP	NM_001373921.1	GAAGTTAAAGTCGGCCGTGAGT	22	50	54	727bp
CCDC67 Set2 RP	NM_001373921.1	TTACCCATCTGTGCTCCAATCA	22	45	53	727bp
CCDC96 set2 FP	MT981183	CGATTGTTGAGGAGGCGGCTG	21	62	58.3	707bp
CCDC96 set2 RP	MT981183	ACGCACGGCGCTCATCTCCTT	21	62	58.3	707bp
CCDC157 set2 FP	MT981182	GCTGCTACTTCAGAGTTGTGCCA	23	52	57.1	798bp
CCDC157 set2 RP	MT981182	TGCCTGTCTATGCTCGAACTGGG C	23	61	60.6	798bp

Table 3.3: Primers used for detecting the expression of *Ccdc* genes.

Gene	FP/RP	Primer Sequence (5'→3')	Amplicon size (bp)
<i>Ccdc54</i>	FP	GGACTGCAAACCTCCACAAG	191
	RP	GAGTTCCGTTTGTCCCGTTT	
<i>Ccdc67</i>	FP	GCCTTTGCCATTCAGACCA	170
	RP	TGTGTGTGTTGAGCAGCTTC	
<i>Ccdc96</i>	FP	GGCGTCCACTGAAGAGTTTG	165
	RP	TTGCGTAGGGTCTCGTGAAT	
<i>Ccdc157</i>	FP	ATTCTCAGGGTTGACAGGCA	172
	RP	TATGGGATTCTGGGTGCAGG	

Table 3.4: Primers used to amplify *CCDC54* coding sequence to determine SNPs.

Name of the gene	Seq 5'-3'	length	GC content %	Tm °C	Amp size
<i>Ccdc54</i> FP	ATGTACACACTTCACACCAAAAGG	24	42	54	978bp
<i>Ccdc54</i> RP	CTATAGTCGAGTTACTTCTTCTTT	24	33	50.6	978bp

Results

Our preliminary results suggested an altered expression pattern of these genes due to pyrethroid toxicity (**Figure 3.2**). The expression of these *Ccdc* genes in pyrethroid-treated samples showed significant downregulation in F0 generation (*Ccdc54*, *Ccdc67*), whereas *Ccdc96* and *Ccdc157* did not show many significant changes.

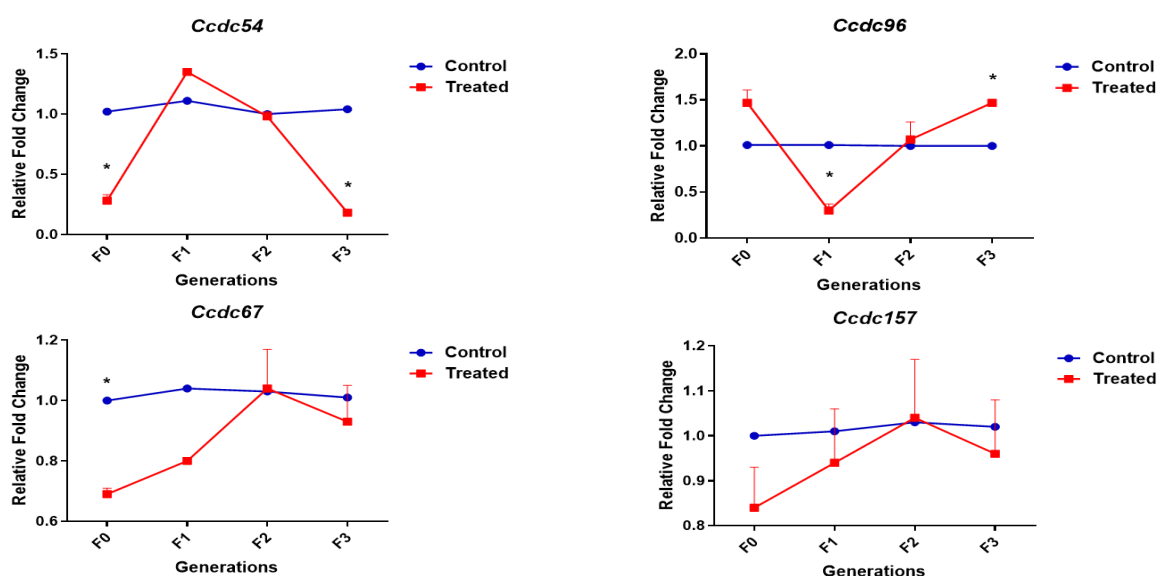


Figure 3.2: Transgenerational expression pattern of *Ccdc* genes in the testes of pyrethroid-treated rats and in the testes obtained from subsequent generations. RNA isolated from the testes of rats for all four generations was reverse-transcribed, and real-time polymerase chain reaction was performed using gene-specific primers. Values presented are mean $\pm$ S.D. *denotes $p < 0.05$.

Since we observed perturbations in the expression of *Ccdc* genes due to pyrethroid toxicity, the predicted *Ccdc* genes, namely, *Ccdc54*, *Ccdc64*, *Ccdc96*, and *Ccdc157* were considered for further characterization. As a first step, the full-length sequence of the four *Ccdcs* were amplified from the testes of 90-day old rats. The uncharacterized *Ccdc54*, *Ccdc96*, and *Ccdc157* were sequenced and submitted to the GenBank and were assigned the accession numbers MT038367, MT981183, and MT981182, respectively (**Table 3.5**).

Table 3.5: Accession numbers for *Ccdc54*, *Ccdc96*, and *Ccdc157* genes provided by GenBank.

Genes Submitted	Accession numbers
<i>Ccdc54</i>	MT038367
<i>Ccdc96</i>	MT981183
<i>Ccdc157</i>	MT981182

The confirmed sequences were translated by Expasy tool and the general characteristic features of all the three rat *Ccdc* genes and their protein products were compiled based on the information available in the rat genome at NCBI and are given in **table 3.6**.

Table 3.6: General predicted and deduced features of rat *Ccdc* genes and their encoded proteins.

Attribute	<i>Ccdc54</i>	<i>Ccdc67</i>	<i>Ccdc96</i>	<i>Ccdc157</i>
Chromosome	11q21	8q12	14q21	14q21
Genome size	3098	61914	2441	17069
Gene ID	498073	315438	289716	681091
Total number of exons	2	18	2	12
mRNA accession number	MT038367.1	NM_001014087.1	MT981183.1	MT981182.1
Length of mRNA (bp)	1221	2615	2285	3189
Coding sequence (CDS)	178-1170	131-1936	121-1893	13-2568
Protein accession number	QTQ94996.1	NP_001014109.2	UDP68720.1	UDP68719.1
Number of amino acids	330	601	590	851
Molecular Weight (Da)	37908.20	70105.22	67562.19	95257.07
Localization	–	cytoplasm	Axoneme and ciliary basal body	–
Isoelectric point (pI)	6.67	6.23	4.82	9.32
GRAVYindex	-0.508	-0.985	-1.177	-0.701
Aliphatic Index	80.67	75.02	68.63	78.18
Instability Index	50.46	56.12	57.42	77.81
Extinction coefficient	1.101/1.091	0.519/0.512	0.424/0.421	0.696/0.687
Signal peptide (amino acid range)	No	No	No	No

Secondary structure prediction

Secondary structure prediction by SOPMA tool showed that all three proteins predominantly contain alpha helix structure (**Table 3.7**). The predicted three-dimensional structures produced by I-tasser tool are shown in **figure 3.4**. Structures with highest c-score were selected (and subjected to Ramachandran plot analysis using PROCHECK, which revealed that a maximum number of amino acids were in the allowed regions (**Figure 3.5**) falling majorly above 90% category (**Table 3.8**).

Table 3.7: Secondary structure prediction for CCDC proteins.

Protein name	Alpha helix (%)	Extended strand (%)	Beta turn (%)	Random coil (%)
CCDC54	51.21	7.88	1.82	39.09
CCDC67	81.70	0.17	1.50	16.64
CCDC96	67.12	2.88	2.03	27.97
CCDC157	57.46	3.53	2.23	36.78

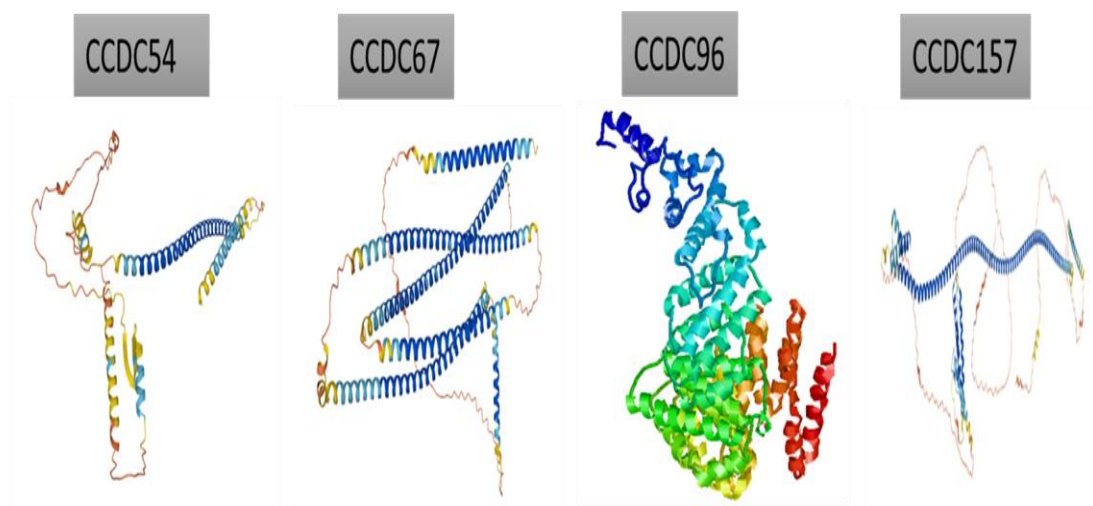


Figure 3.4: Predicted models by i-tasser on the basis of protein sequence.

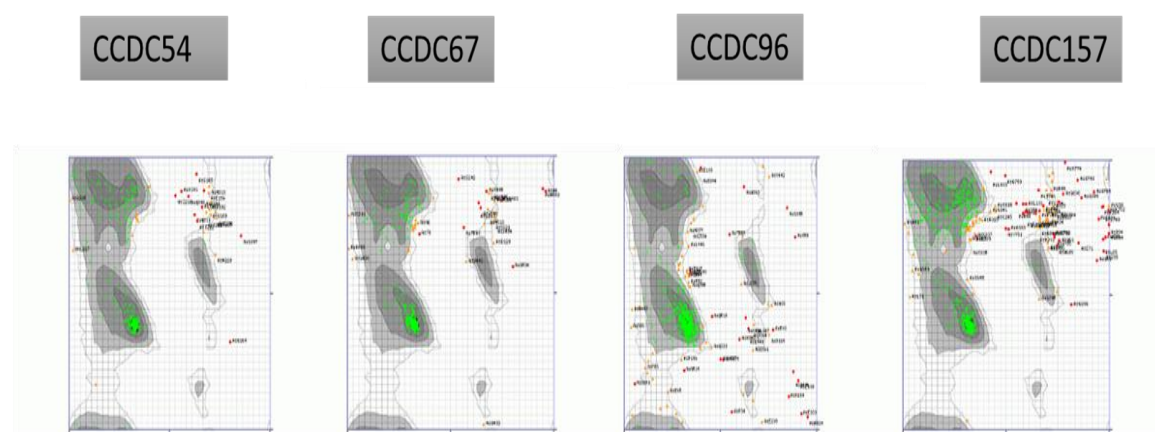


Figure 3.5: Ramachandran plots showing the allowed regions of the CCDC proteins.

Table 3.8: Ramachandran plot analyses showing the percentage of amino acids in allowed regions.

Protein name	Highly Preferred region (%)	Preferred region (%)	Questionable region (%)
CCDC54	91.15	5.79	3.04
CCDC67	94.32	4.00	1.66
CCDC96	87.92	8.50	3.57
CCDC157	88.81	6.83	4.35

Phylogenetic analysis of CCDC proteins

A phylogenetic tree, organizes various species based on evolutionary relationships. Each node represents a taxonomic group, and branches signify shared ancestry. The tree is color-coded to differentiate taxonomic categories. The scale bar indicates evolutionary distance, and the tree emphasizes the diversity and interrelatedness. The idea that the *Ccdc* gene family may have amplified early in vertebrate evolution is backed up by the existence of several *Ccdcs* in the genomes of all mammalian species as well as a number of other vertebrate species (**Figures 3.6 to 3.9**). According to phylogenetic study, the CCDC proteins are widely distributed in many different taxa, particularly in vertebrates. It appears that rodents, placentals, and primates have orthologs for the rat CCDC proteins.

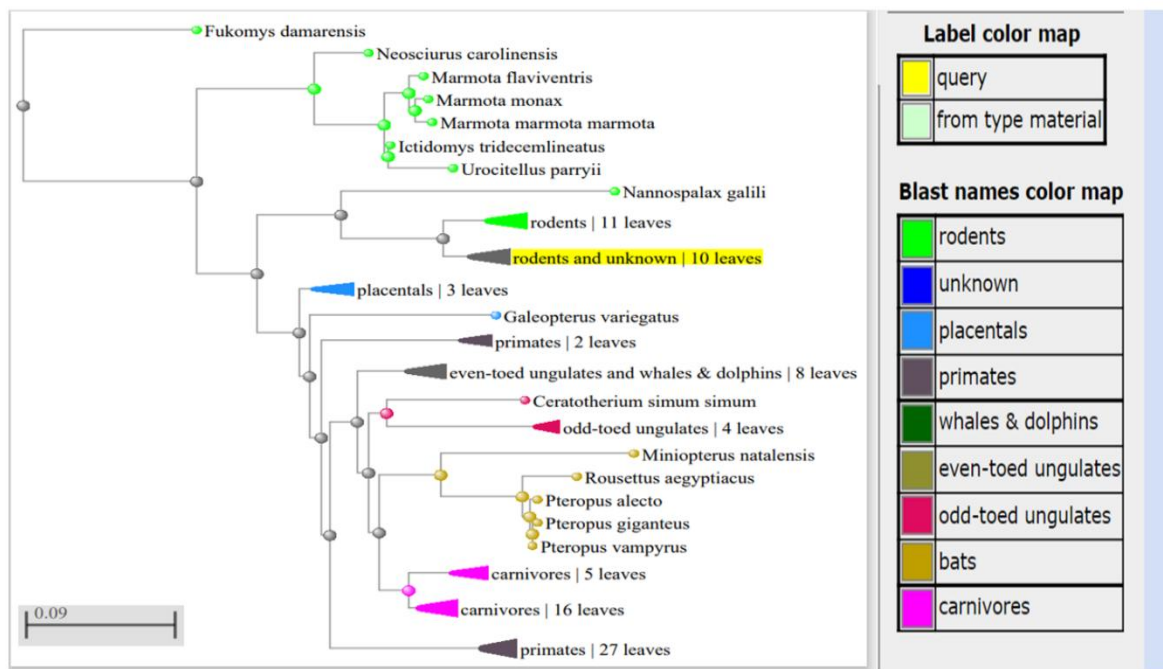


Figure 3.6: Phylogenetic analysis for the rat *Ccdc54*. Color map illustrates its distribution and conservation throughout the animal kingdom.

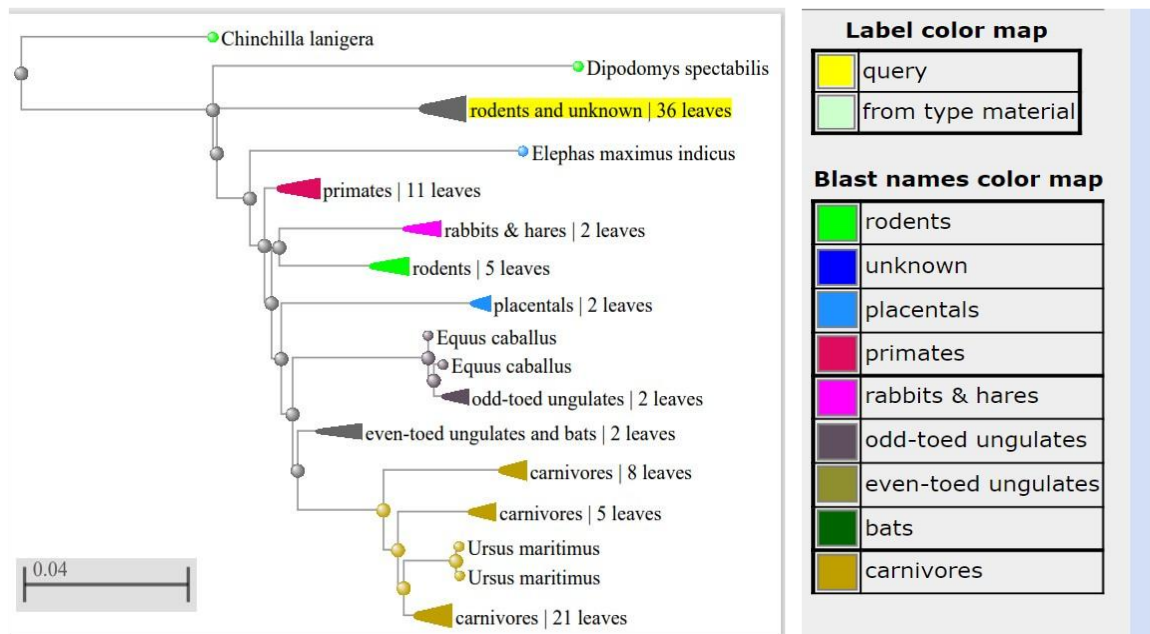


Figure 3.7: Phylogenetic analysis for the rat *Ccdc67*. Color map illustrates its distribution and conservation throughout the animal kingdom.

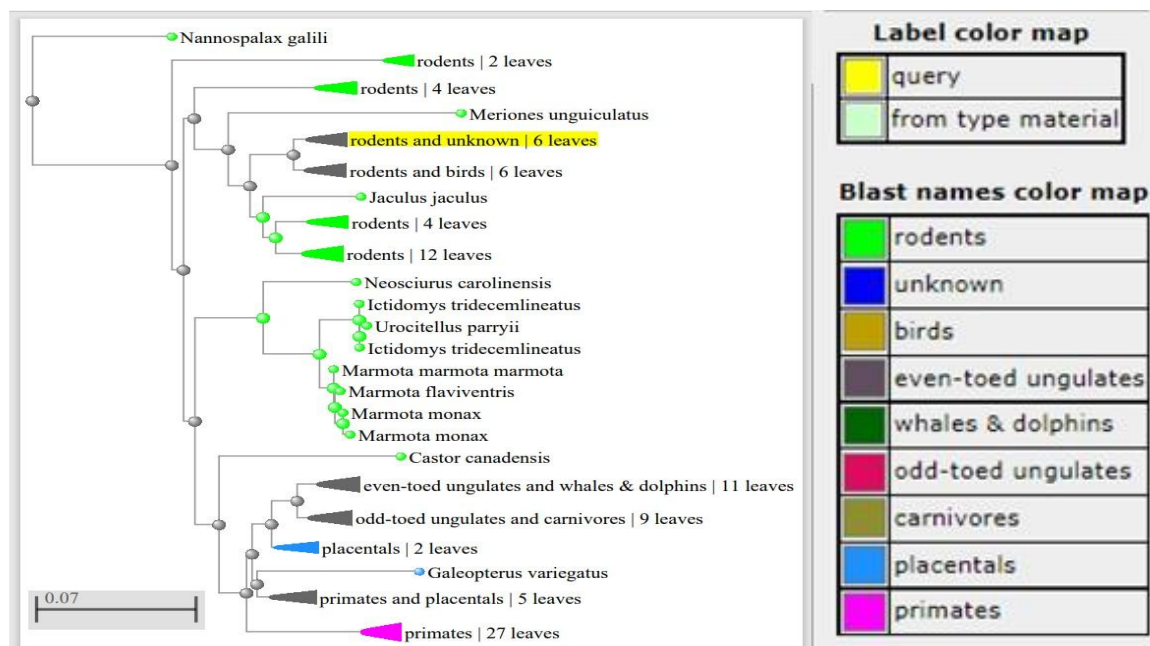


Figure 3.8: Phylogenetic analysis for the rat *Cdc96*. Color map illustrates its distribution and conservation throughout the animal kingdom.

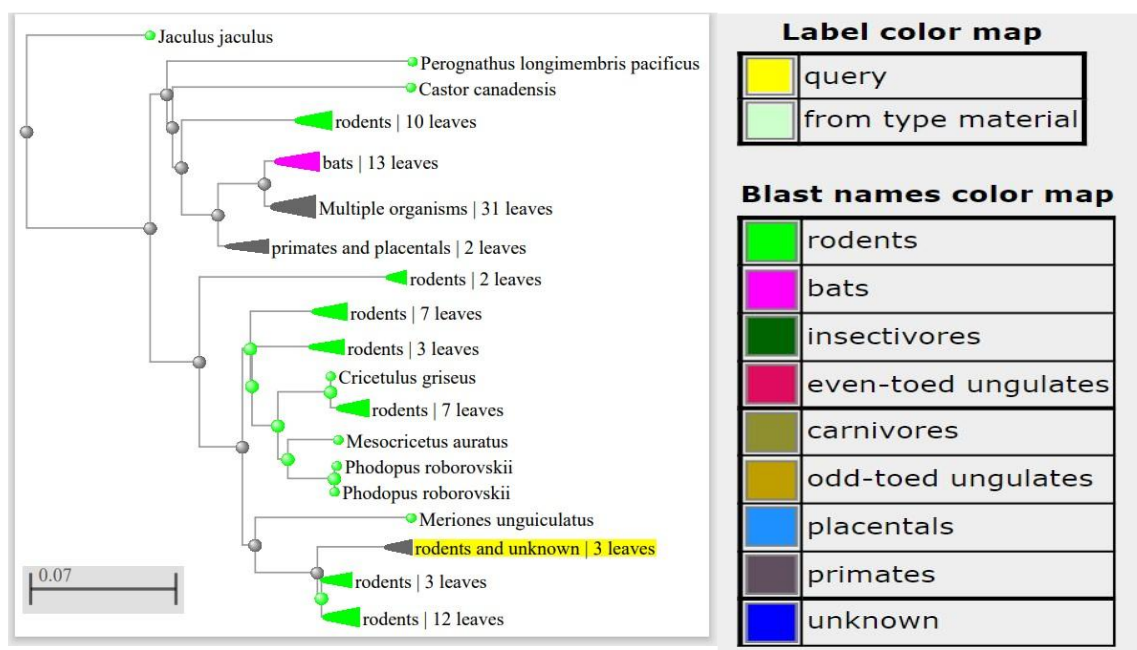


Figure 3.9: Phylogenetic analysis for the rat *Cdc157*. Color map illustrates its distribution and conservation throughout the animal kingdom.

Pairwise identity and similarity analysis

Information on the potential functional activities of a group of proteins can be obtained by analysing their sequence similarity. The rat CCDC proteins do not have such an analysis. The T-coffee tool was used to align the rat CCDCs' sequences in order to assess how similar they

were. (**Figure. 3.10**). Among all CCDC proteins screened, the highest similarity percentage of 94.2 is observed among animals, i.e. among rat CCDC67 and mouse CCDC67. The least among animals were observed among rat and human CCDC157 protein with a good score of 63.9%, and the similarity and identity index among CCDC proteins showed that though they seem to be evolutionarily conserved, they are not identical in nature.

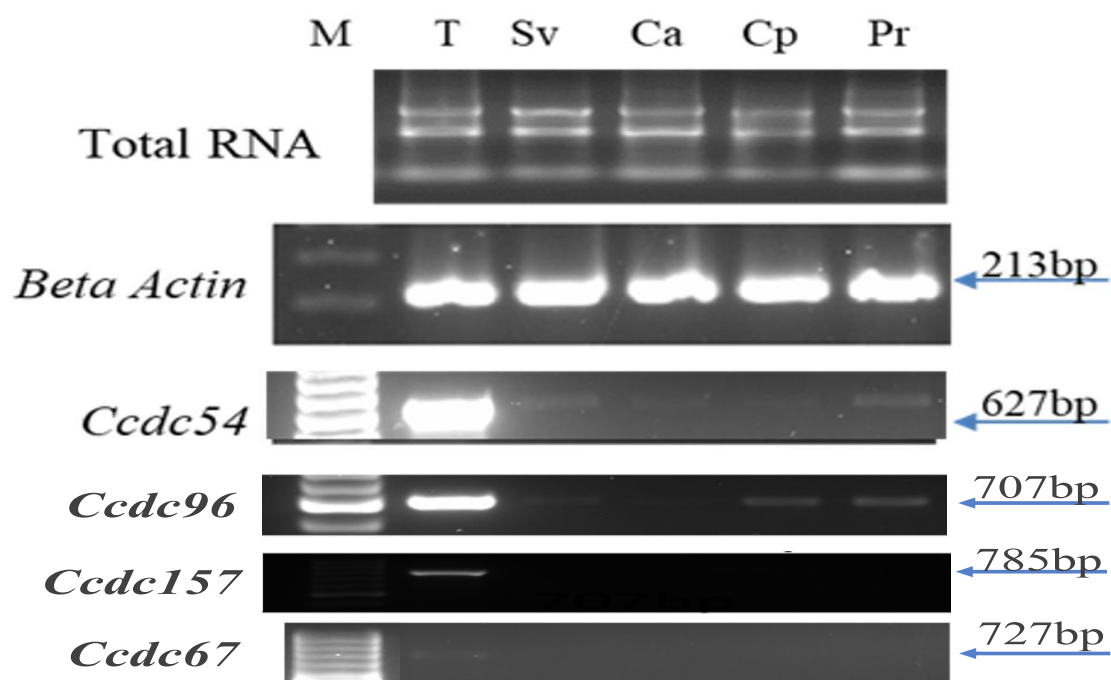
	Rat				Mouse				Human			
	CCDC54	CCDC67	CCDC96	CCDC157	CCDC54	CCDC67	CCDC96	CCDC157	CCDC54	CCDC67	CCDC96	CCDC157
CCDC54	100	13.2	14	11.4	86.7	13.5	14.5	14.7	71.8	13.9	16.9	11.3
CCDC67	6.4	100	27	21.7	7.3	94.2	23.3	21.8	8.9	86.3	18.6	25.5
CCDC96	7.6	15.7	100	17.4	7.9	16.2	85.3	20.9	7.6	15	68.6	20.6
CCDC157	5.9	12.3	10.1	100	4.9	10.1	9.7	80.4	5.5	9.7	9.9	63.9

 Similarity score among the rat proteins;
  Identity score;
  Similarity Score

Figure 3.10 Pairwise identity and similarity analysis of rat, mouse, and human CCDC proteins.

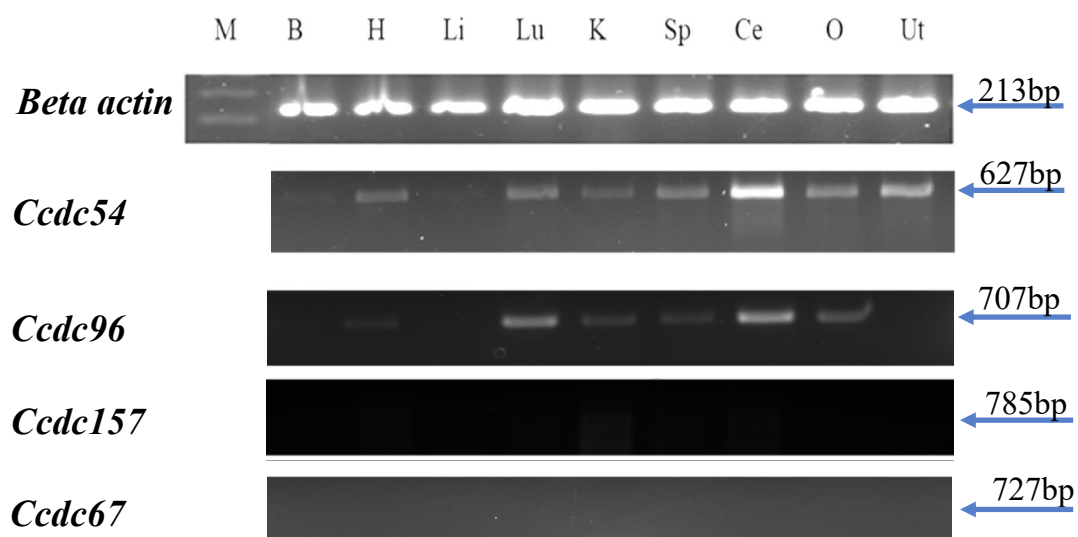
***Ccdc* gene expression**

The expression patterns of the *Ccdc* genes were examined in various organs of both adult and developing rats in order to ascertain their expression patterns (**Figures 3.11 to 3.13**). While *Ccdc54* was present in every tissue of the male reproductive tract, its expression was highest in the testes of adult rats, whereas *Ccdc96* was dominant in the testes. All other non-male reproductive tract tissues, including the brain, heart, liver, lungs, kidney, spleen, cervix, ovary, and uterus, also expressed *Ccdc54*, with the ovary expressing it the most and the liver the least. In the epididymis, *Ccdc54* expression was not controlled by development. Nearly all tissues of the male reproductive tract (MRT) expressed *Ccdc96*, although the testes are the most conspicuous. All of the non-male reproductive tract tissues, the brain, heart, liver, lungs, kidney, spleen, cervix, ovary, and uterus express *Ccdc96*, but the lungs and cervix express it the most, while the liver and uterus express it the least. The expression of *Ccdc96* in epididymis was not developmentally regulated. *Ccdc157* and *Ccdc67* showed testis-specific expression and were not development dependent (**Figures 3.11 to 3.13**).



M- Marker, T- Testis, Sv- Seminal Vesicles, Ca- Cauda, Cp- Caput, Pr- Prostate

Figure 3.11: Expression profiling of *Ccdc* genes in the organs of the Male Reproductive system.



M- Marker, B- Brain, H- Heart, Li- Liver, Lu- Lungs, Sp- Spleen, Ce- Cervix, O- Ovary, Ut- Uterus

Figure 3.12: Tissue distribution analysis of *Ccdc* genes in the non-male reproductive tract organs.

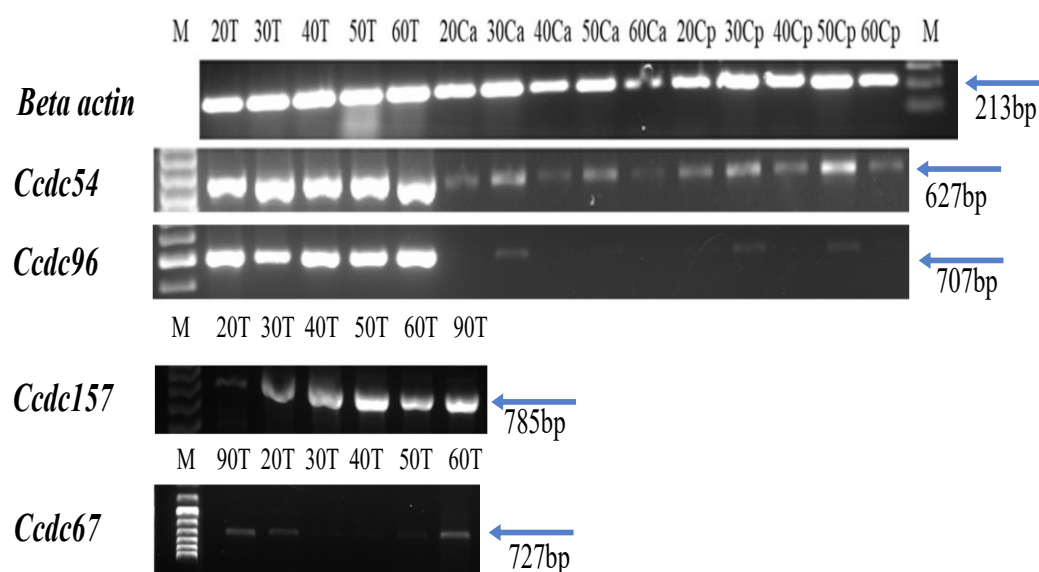


Figure 3.13: Development-dependent expression analysis of *Ccdc* genes in the epididymis and testis. The 20T, 30T, 40T, 50T and 60T represent the testis of the 20,30,40,50, and 60 days rats respectively. whereas the 20Ca, 30Ca, 40Ca, 50Ca, and 60Ca represents the Cauda of 20, 30, 40, 50 and 60 days respectively. 20Cp, 30Cp, 40Cp, 50Cp and 60Cp indicates the caput of 20, 30, 40 50 and 60 day old rats respectively.

Cloning and recombinant protein production

CCDC54 and CCDC96 were further cloned in the pQE80mod vector. Both the protein sequence was screened in 'Signal P' software and found to have no signal peptides. For both clones, *Sal-I* and *Hind-III* enzymes were selected. Clones were further confirmed with universal primers and also gene-specific primers. IPTG-induced transformation of these clones into *E. Coli BL-21*, and the recombinant protein induction occurred at 37 degrees after being induced for three hours. By using immunoblotting, the recombinant protein's induction was verified. Ni-NTA agarose was used to purify the recombinant protein.

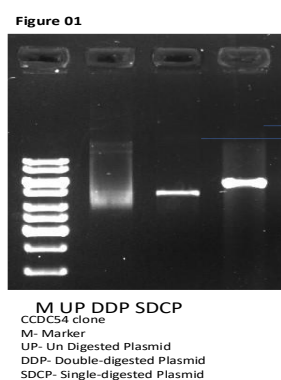


Figure 01. Gel Run of UP, DDP, and SDCP of Ccdc54 Clone.

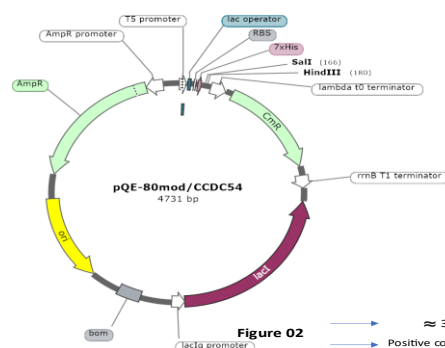


Figure 02. Vector Map of pQE80mod/CCDC54.

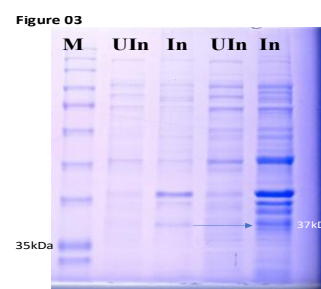


Figure 04. Western Blot analysis for Induction of CCDC54 at different time points.

Figure 3.14: Cloning and recombinant expression of CCDC54.

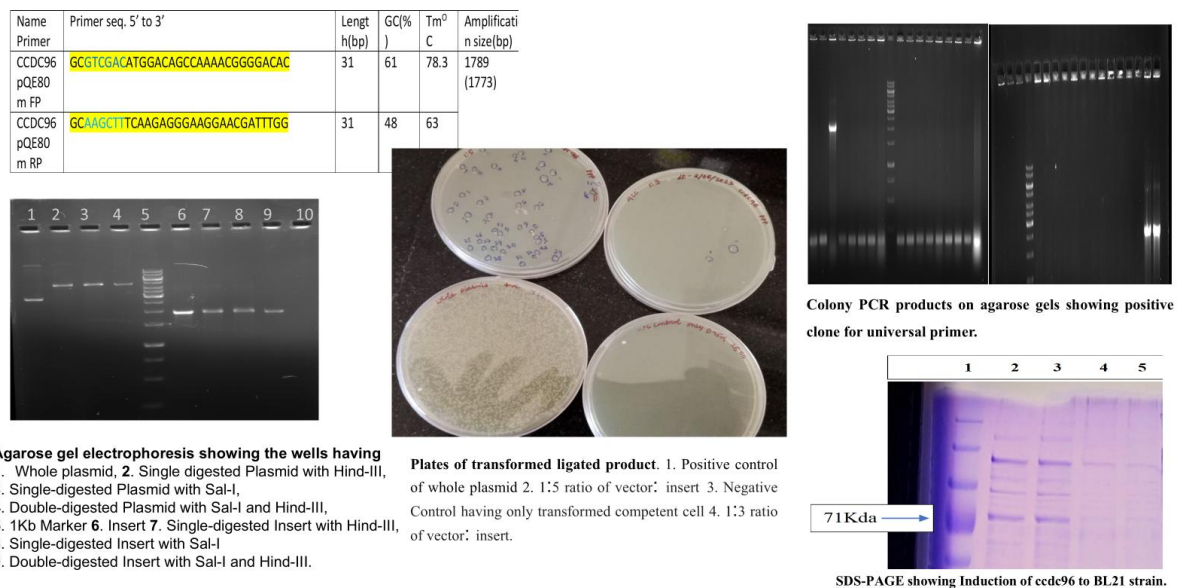


Figure 3.15: Cloning and expression of CCDC96.

Single nucleotide polymorphisms in CCDC54

The human *CCDC54* exon 1, was amplified using genomic DNA isolated from the PBMCs of fertile and infertile men and sequenced (**Figure 3.17**). The sequences were multi-aligned with the reference sequence, and SNP percentages calculated. SNP analyses of the *CCDC54* gene indicated a 71.42% G-A replacement in infertile samples compared to 14.28% in their fertile counterparts (**Figure 3.18**).

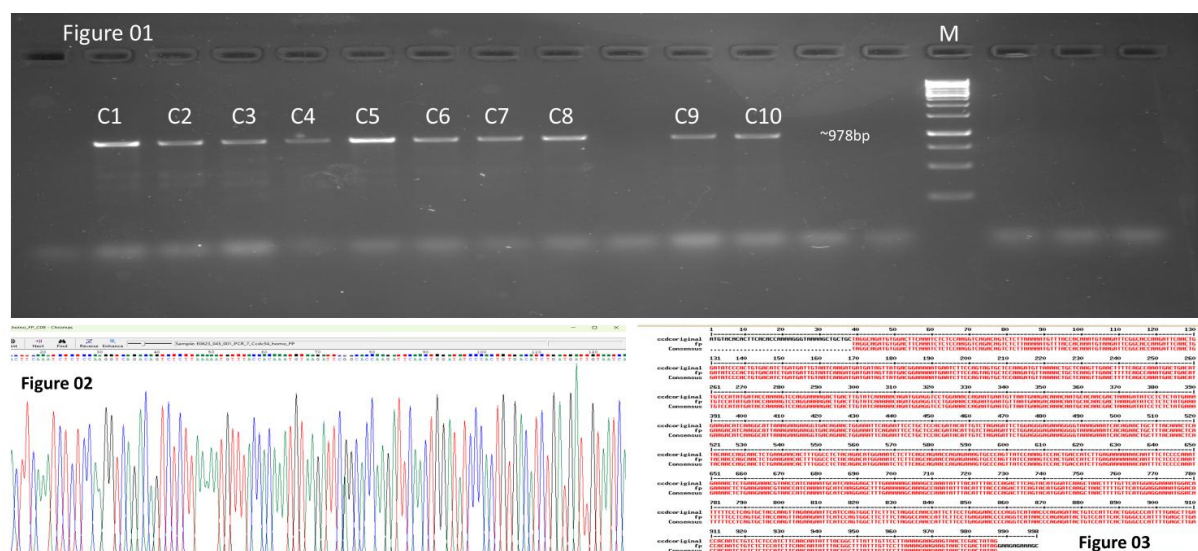


Figure 01. PCR products for control samples for *Ccdc54* gene

Figure 02. Sequencing peaks for the analysed samples

Figure 03. Multialignment of Forward Primer sequence and Reverse primer sequence with the reference genome.

Figure 3.17: Amplification and sequence analysis of exon 1 of human CCDC54. The wells in the gel represent the PCR amplified products; C1- amplified product of the 1st control sample, C2- amplified product of the 2nd

control sample, C3- amplified product of the 3rd control sample, C4- amplified product of the 4th control sample, C5- amplified product of the 5th control sample, C6- amplified product of the 6th control sample, C7- amplified product of the 7th control sample, C8- amplified product of the 8th control sample, C9- amplified product of the 9th control sample, C10- amplified product of the 10th control sample.

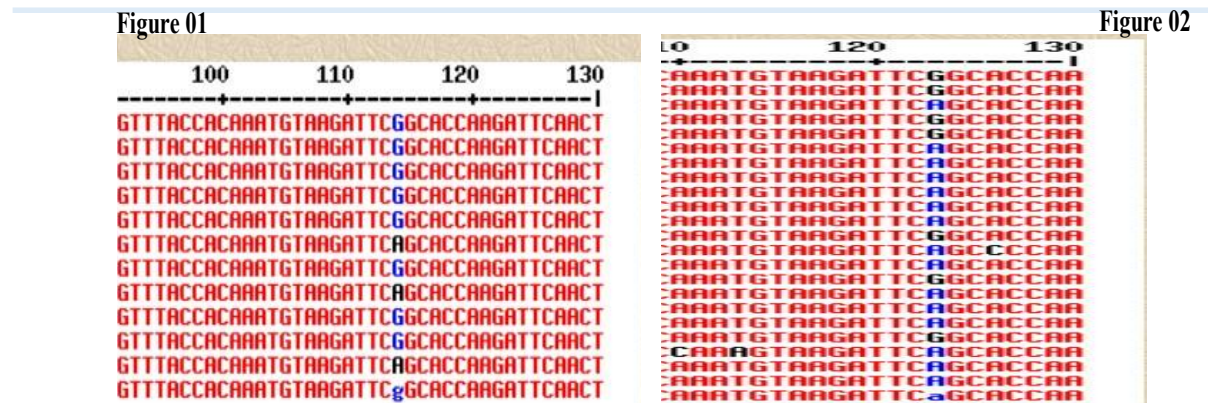


Figure 01. Multi-alignment of Sequenced nucleotide for fertile samples. Figure 02. Multi-alignment of Sequenced nucleotide for infertile samples.
Credit- INRA Multalin

Exon	Position	SNP	Genotype frequency fertile [n=10]	Genotype frequency infertile [n=21]
1	114	G/A	14.28%	71.42%

Figure 3.18: Frequency of SNPs in the exon 1 of CCDC54 in infertile men.

Discussion

The CCDC family consists of proteins that contain a specific domain, namely, the coiled-coil domain, in their structure. This study's transcriptome, proteome, and epigenome analysis showed that pyrethroid treatment altered the expression pattern of CCDC family members, which piqued our interest in learning more about this protein family. Further, members of this family are involved in development, reproduction, and cancer (Priyanka & Yenugu, 2021). It is very evident that CCDC proteins are implicated in sperm flagellum formation and movement, acrosome formation, spermatogenesis, sperm maturation, and infertility, and that these processes are influenced at the molecular level. (Shimasaki et al., 2010; Yamaguchi et al., 2014). Because of their multiple roles in male gametogenesis and development, they can be potential candidates for male contraception and also targets for addressing infertility at multiple levels. Since some CCDC proteins are identified as cancer-testis antigens, further research

studies can provide new leads on their role in the initiation and progression of male reproductive cancers as well.

Hence, we conducted an in-depth investigation on the status of these members in the testes under conditions of pyrethroid exposure, as some previous studies, including one of the studies from our lab, showed the significant differential expression of C2D1A protein transgenerationally in pyrethroid-treated rats (Ravula & Yenugu, 2022). Our methylome study also suggested that *Ccdc191* and *Ccdc170* are significantly up and downregulated in F0 generation, respectively. Whereas not many significant changes were found transgenerationally. We discovered that only a few of the CCDC proteins are uncharacterized. So, we went on checking the effect of uncharacterized *Ccdc54*, *96*, *67*, and *Ccdc157* in pyrethroid treatment transgenerationally and interestingly found *Ccdc54* and *Ccdc67* were significantly downregulated in F0 generation, and *Ccdc54* showed certain generation gap and the similar significant downregulation in the F3 generation. However, *Ccdc157* did not show significant changes in any of the generations. Since *Ccdc* genes were affected during pyrethroid toxicity, which resulted in compromised fecundity, it is possible that SNPs would have been formed.

Previous literature has already shown that any mutation in *Ccdc* genes like *Ccdc9* leads to asthenozoospermia, and mutation in *Ccdc38* leads to infertility (Mnif et al., 2011; Precone et al., 2021; Sha et al., 2019). So, to make a correlation between SNPs in *Ccdc* genes and infertility in this case, we analysed SNPs in infertile males. Interestingly, we observed 71.42% G/A mutation in infertile patients compared to 14.28% in fertile counterparts. Because CCDC comprises nearly 200 genes, many of which are known to play a role in many malignancies, reproductive problems, developmental problems, and other problems. The four uncharacterized proteins CCDC54, CCDC67, CCDC96, and CCDC157 were chosen for the study on a reproductive aspect. The sequence was first obtained from NCBI, and then it was analyzed using gene-specific primers and rat 90-day testes cDNA. The PCR-purified product was sequenced, and the sequence was submitted to NCBI. Then its structural modeling and validation, phylogenetic analysis, and other computational analysis were done for all four genes by using high-end *in silico* tools. After this, the *in vivo* studies also reported the expression of these *Ccdcs* either to be testis-specific or testis-dominant. Further protein-level studies are necessary to draw any conclusion about the direct effect of these four CCDC proteins in male reproduction.

Male infertility is one of the most common issues faced globally. Although the male reproductive system is vital for living of the organism, it is indispensable for propagation of the organism. Many CCDC proteins are already found involved in male reproductive tracts. However, there are still a lot of uncharacterized CCDC proteins, which could lead to new insights into the genetic reasons of male infertility.

References

- Aitken, R. J., & Baker, M. A. (2020). The Role of Genetics and Oxidative Stress in the Etiology of Male Infertility—A Unifying Hypothesis? *Frontiers in Endocrinology*, *11*, 581838. <https://doi.org/10.3389/FENDO.2020.581838/XML/NLM>
- CHEN, J., ZHENG, W., LI, Y., LIN, S., ZHANG, Z., WU, Y., JIANG, Z., & GUI, Y. (2016). Expression characteristics of the Ccdc70 gene in the mouse testis during spermatogenesis. *Zhonghua Nankexue*, 12–16. <http://dx.doi.org/>
- Chen, M., Ni, J., Chang, H. C., Lin, C. Y., Muyan, M., & Yeh, S. (2009). CCDC62/ERAP75 functions as a coactivator to enhance estrogen receptor beta-mediated transactivation and target gene expression in prostate cancer cells. *Carcinogenesis*, *30*(5), 841–850. <https://doi.org/10.1093/CARCIN/BGN288>,
- Cheng, J. M., Tang, J. X., Li, J., Wang, Y. Q., Wang, X. X., Zhang, Y., Chen, S. R., & Liu, Y. X. (2018). Role of WNT signaling in epididymal sperm maturation. *Journal of Assisted Reproduction and Genetics*, *35*(2), 229–236. <https://doi.org/10.1007/S10815-017-1066-4/FIGURES/4>
- Coutinho, E. M. (2002). Gossypol: a contraceptive for men. *Contraception*, *65*(4), 259–263. [https://doi.org/10.1016/S0010-7824\(02\)00294-9](https://doi.org/10.1016/S0010-7824(02)00294-9)
- Coutton, C., Escoffier, J., Martinez, G., Arnoult, C., & Ray, P. F. (2015). Teratozoospermia: spotlight on the main genetic actors in the human. *Human Reproduction Update*, *21*(4), 455–485. <https://doi.org/10.1093/HUMUPD/DMV020>
- Farfsing, A., Engel, F., Seiffert, M., Hartmann, E., Ott, G., Rosenwald, A., Stilgenbauer, S., Döhner, H., Boutros, M., Lichter, P., & Pscherer, A. (2009). Gene knockdown studies revealed CCDC50 as a candidate gene in mantle cell lymphoma and chronic lymphocytic leukemia. *Leukemia*, *23*(11), 2018–2026. <https://doi.org/10.1038/LEU.2009.144;KWRD=MEDICINE>
- Gadelha, I. C. N., Fonseca, N. B. S., Oloris, S. C. S., Melo, M. M., & Soto-Blanco, B. (2014). Gossypol Toxicity from Cottonseed Products. *The Scientific World Journal*, *2014*(1), 231635. <https://doi.org/10.1155/2014/231635>
- Ge, T., Yuan, L., Xu, L., Yang, F., Xu, W., Niu, C., Li, G., Zhou, H., & Zheng, Y. (2024). Coiled-coil domain containing 159 is required for spermatid head and tail assembly in mice. *Biology of Reproduction*, *110*(5), 877–894. <https://doi.org/10.1093/BIOLRE/IOAE012>
- Geng, Q., Ni, L., Ouyang, B., Hu, Y., Zhao, Y., & Guo, J. (2016). A Novel Testis-Specific Gene, Ccdc136, Is Required for Acrosome Formation and Fertilization in Mice. *Reproductive Sciences*, *23*(10), 1387–1396. <https://doi.org/10.1177/1933719116641762>,

- Gheldof, A., MacKay, D. J. G., Cheong, Y., & Verpoest, W. (2019). Genetic diagnosis of subfertility: The impact of meiosis and maternal effects. *Journal of Medical Genetics*, 56(5), 271–282. <https://doi.org/10.1136/JMEDGENET-2018-105513>,
- Glazenburg, M. M., Hettema, N. M., Laan, L., Remy, O., Laloux, G., Brunet, T., Chen, X., Tee, Y. H., Wen, W., Rizvi, M. S., Jolly, M. K., & Riddell, M. (2024). Perspectives on polarity - exploring biological asymmetry across scales. *Journal of Cell Science*, 137(5), 261987. <https://doi.org/10.1242/JCS.261987/344106>
- Gong, Y., Qiu, W., Ning, X., Yang, X., Liu, L., Wang, Z., Lin, J., Li, X., & Guo, Y. (2015). CCDC34 is up-regulated in bladder cancer and regulates bladder cancer cell proliferation, apoptosis and migration. *Oncotarget*, 6(28), 25856–25867. <https://doi.org/10.18632/ONCOTARGET.4624>,
- Graziani, A., Rocca, M. S., Vinanzi, C., Masi, G., Grande, G., De Toni, L., & Ferlin, A. (2024). Genetic Causes of Qualitative Sperm Defects: A Narrative Review of Clinical Evidence. *Genes* 2024, Vol. 15, Page 600, 15(5), 600. <https://doi.org/10.3390/GENES15050600>
- Hall, S. H., Yenugu, S., Radhakrishnan, Y., Avellar, M. C. W., Petrusz, P., & French, F. S. (2007). Characterization and functions of beta defensins in the epididymis. *Asian Journal of Andrology*, 9(4), 453–462. <https://doi.org/10.1111/J.1745-7262.2007.00298.X>
- Handelsman, D. J. (2022). Male Contraception. *Endotext*. <https://www.ncbi.nlm.nih.gov/books/NBK279094/>
- Hilz, E. N., & Gore, A. C. (2023). Endocrine-Disrupting Chemicals: Science and Policy. *Policy Insights from the Behavioral and Brain Sciences*, 10(2), 142–150. <https://doi.org/10.1177/23727322231196794>;REQUESTEDJOURNAL:JOURNAL:BBSA;WGROU:STRING:PUBLICATION
- Hoyer-Fender, S., & Contreras, C. T. (2019). CCDC42 localizes to manchette, HTCA and tail and interacts with ODF1 and ODF2 in the formation of the male germ cell cytoskeleton. *Frontiers in Cell and Developmental Biology*, 7(JULY). <https://doi.org/10.3389/FCELL.2019.00151>,
- Iso-Touru, T., Wurmser, C., Venhoranta, H., Hiltbold, M., Savolainen, T., Sironen, A., Fischer, K., Flisikowski, K., Fries, R., Vicente-Carrillo, A., Alvarez-Rodriguez, M., Nagy, S., Mutikainen, M., Peippo, J., Taponen, J., Sahana, G., Guldbrandsen, B., Simonen, H., Rodriguez-Martinez, H., ... Pausch, H. (2019). A splice donor variant in CCDC189 is associated with asthenospermia in Nordic Red dairy cattle. *BMC Genomics*, 20(1), 1–13. <https://doi.org/10.1186/S12864-019-5628-Y/FIGURES/3>
- Kaczmarek, K., Niedzialkowska, E., Studencka, M., Schulz, Y., & Grzmil, P. (2010). Ccdc33, a predominantly testis-expressed gene, encodes a putative peroxisomal protein. *Cytogenetic and Genome Research*, 126(3), 243–252. <https://doi.org/10.1159/000251961>,
- Khan, M., Jabeen, N., Khan, T., Hussain, H. M. J., Ali, A., Khan, R., Jiang, L., Li, T., Tao, Q., Zhang, X., Yin, H., Yu, C., Jiang, X., & Shi, Q. (2018). The evolutionarily conserved genes: Tex37, Ccdc73, Prss55 and Nxt2 are dispensable for fertility in mice. *Scientific Reports*, 8(1), 1–8. <https://doi.org/10.1038/S41598-018-23176-X>;SUBJMETA=136,1822,208,2434,631,737;KWRD=MUTATION,SPERMATOGENESIS
- Kim, H., Huang, J., & Chen, J. (2007). CCDC98 is a BRCA1-BRCT domain-binding protein involved in the DNA damage response. *Nature Structural and Molecular Biology*, 14(8), 710–715. <https://doi.org/10.1038/NSMB1277>,
- Knowles, M. R., Zariwala, M., & Leigh, M. (2016). Primary Ciliary Dyskinesia. *Clinics in Chest Medicine*, 37(3), 449–461. <https://doi.org/10.1016/J.CCM.2016.04.008>

- Kuroda, S., Usui, K., Sanjo, H., Takeshima, T., Kawahara, T., Uemura, H., & Yumura, Y. (2020). Genetic disorders and male infertility. *Reproductive Medicine and Biology*, 19(4), 314–322. <https://doi.org/10.1002/RMB2.12336;PAGE:STRING:ARTICLE/CHAPTER>
- Li, Y., Li, C., Lin, S., Yang, B., Huang, W., Wu, H., Chen, Y., Yang, L., Luo, M., Guo, H., Chen, J., Wang, T., Ma, Q., Gu, Y., Mou, L., Jiang, Z., Xia, J., & Gui, Y. (2017). A nonsense mutation in *Ccdc62* gene is responsible for spermiogenesis defects and male infertility in *repro29/repro29* mice. *Biology of Reproduction*, 96(3), 587–597. <https://doi.org/10.1095/BIOLREPROD.116.141408>,
- Lin, S. R., Li, Y. C., Luo, M. L., Guo, H., Wang, T. T., Chen, J. B., Ma, Q., Gu, Y. L., Jiang, Z. M., & Gui, Y. T. (2016). Identification and characteristics of the testes-specific gene, *Ccdc38*, in mice. *Molecular Medicine Reports*, 14(2), 1290–1296. <https://doi.org/10.3892/MMR.2016.5360>,
- Lupas, A. N., Bassler, J., & Dunin-Horkawicz, S. (2017). The structure and topology of α -helical coiled coils. *Sub-Cellular Biochemistry*, 82, 95–129. https://doi.org/10.1007/978-3-319-49674-0_4/FIGURES/7
- Majczenko, K., Davidson, A. E., Camelo-Piragua, S., Agrawal, P. B., Manfready, R. A., Li, X., Joshi, S., Xu, J., Peng, W., Beggs, A. H., Li, J. Z., Burmeister, M., & Dowling, J. J. (2012). Dominant Mutation of *CCDC78* in a Unique Congenital Myopathy with Prominent Internal Nuclei and Atypical Cores. *The American Journal of Human Genetics*, 91(2), 365–371. <https://doi.org/10.1016/J.AJHG.2012.06.012>
- Mnif, W., Hassine, A. I. H., Bouaziz, A., Bartegi, A., Thomas, O., & Roig, B. (2011). Effect of endocrine disruptor pesticides: A review. *International Journal of Environmental Research and Public Health*, 8(6), 2265–2303. <https://doi.org/10.3390/IJERPH8062265>,
- Park, S. J., Jang, H. R., Kim, M., Kim, J. H., Kwon, O. H., Park, J. L., Noh, S. M., Song, K. S., Kim, S. Y., Kim, Y. H., & Kim, Y. S. (2012). Epigenetic alteration of *CCDC67* and its tumor suppressor function in gastric cancer. *Carcinogenesis*, 33(8), 1494–1501. <https://doi.org/10.1093/CARCIN/BGS178>,
- Pasek, R. C., Malarkey, E., Berbari, N. F., Sharma, N., Kesterson, R. A., Tres, L. L., Kierszenbaum, A. L., & Yoder, B. K. (2016). Coiled-coil domain containing 42 (*Ccdc42*) is necessary for proper sperm development and male fertility in the mouse. *Developmental Biology*, 412(2), 208–218. <https://doi.org/10.1016/j.ydbio.2016.01.042>
- Pizzol, D., Ferlin, A., Garolla, A., Lenzi, A., Bertoldo, A., & Foresta, C. (2014). Genetic and molecular diagnostics of male infertility in the clinical practice. *Frontiers in Bioscience - Landmark*, 19(2), 291–303. <https://doi.org/10.2741/4208/PDF>
- Precone, V., Cannarella, R., Paolacci, S., Busetto, G. M., Beccari, T., Stuppia, L., Tonini, G., Zulian, A., Marceddu, G., Calogero, A. E., & Bertelli, M. (2021). Male Infertility Diagnosis: Improvement of Genetic Analysis Performance by the Introduction of Pre-Diagnostic Genes in a Next-Generation Sequencing Custom-Made Panel. *Frontiers in Endocrinology*, 11, 605237. <https://doi.org/10.3389/FENDO.2020.605237/BIBTEX>
- Priyanka, P. P., & Yenugu, S. (2021). Coiled-Coil Domain-Containing (CCDC) Proteins: Functional Roles in General and Male Reproductive Physiology. *Reproductive Sciences 2021 28:10*, 28(10), 2725–2734. <https://doi.org/10.1007/S43032-021-00595-2>
- Qian, S. Z., & Wang, Z. G. (1984). Gossypol: A potential antifertility agent for males. *Annual Review of Pharmacology and Toxicology*, VOL. 24(Volume 24, 1984), 329–360. <https://doi.org/10.1146/ANNUREV.PA.24.040184.001553/CITE/REFWORKS>
- Ravula, A. R., & Yenugu, S. (2022). Transgenerational effects on the fecundity and sperm proteome in rats exposed to a mixture of pyrethroids at doses similar to human consumption. *Chemosphere*, 290. <https://doi.org/10.1016/j.chemosphere.2021.133242>

- Rose, A., Schraegle, S. J., Stahlberg, E. A., & Meier, I. (2005). Coiled-coil protein composition of 22 proteomes - Differences and common themes in subcellular infrastructure and traffic control. *BMC Evolutionary Biology*, 5(1), 1–21. <https://doi.org/10.1186/1471-2148-5-66/TABLES/11>
- Saillenfait, A. M., Ndiaye, D., & Sabaté, J. P. (2015). Pyrethroids: Exposure and health effects – An update. *International Journal of Hygiene and Environmental Health*, 218(3), 281–292. <https://doi.org/10.1016/J.IJHEH.2015.01.002>
- Sawada, H., & Saito, T. (2022). Mechanisms of Sperm–Egg Interactions: What Ascidian Fertilization Research Has Taught Us. *Cells* 2022, Vol. 11, Page 2096, 11(13), 2096. <https://doi.org/10.3390/CELLS11132096>
- Schwarz, T., Prieler, B., Schmid, J. A., Grzmil, P., & Neesen, J. (2017). Ccdc181 is a microtubule-binding protein that interacts with Hook1 in haploid male germ cells and localizes to the sperm tail and motile cilia. *European Journal of Cell Biology*, 96(3), 276–288. <https://doi.org/10.1016/J.EJCB.2017.02.003>
- Sha, Y., Xu, Y., Wei, X., Liu, W., Mei, L., Lin, S., Ji, Z., Wang, X., Su, Z., Qiu, P., Chen, J., & Wang, X. (2019). CCDC9 is identified as a novel candidate gene of severe asthenozoospermia. *Systems Biology in Reproductive Medicine*, 65(6), 465–473. <https://doi.org/10.1080/19396368.2019.1655812;PAGE:STRING:ARTICLE/CHAPTER>
- Sharma, K., Ravi, K., Wani, A. W., Kumar, S., & Qureshi, S. (2025). Introduction to Herbal Pharmacopeia. *Herbal Pharmacopeia: Nanotechnology and Advancing Drug Discovery*, 1–18. <https://doi.org/10.1201/9781003544418-1/INTRODUCTION-HERBAL-PHARMACOPEIA-KUSHAGRA-SHARMA-KONDLE-RAVI-AB-WAHEED-WANI-SANJEEV-KUMAR-SHUJA-QURESHI>
- Shimasaki, S., Yamamoto, E., Murayama, E., Kurio, H., Kaneko, T., Shibata, Y., Inai, T., & Iida, H. (2010). Subcellular localization of Tektin2 in rat sperm flagellum. *Zoological Science*, 27(9), 755–761. <https://doi.org/10.2108/ZSJ.27.755>,
- Sironen, A., Shoemark, A., Patel, M., Loebinger, M. R., & Mitchison, H. M. (2019). Sperm defects in primary ciliary dyskinesia and related causes of male infertility. *Cellular and Molecular Life Sciences* 2019 77:11, 77(11), 2029–2048. <https://doi.org/10.1007/S00018-019-03389-7>
- Song, M. H., Ha, J. M., Shin, D. H., Lee, C. H., Old, L., & Lee, S. Y. (2012). KP-CoT-23 (CCDC83) is a novel immunogenic cancer/testis antigen in colon cancer. *International Journal of Oncology*, 41(5), 1820–1826. <https://doi.org/10.3892/IJO.2012.1601>,
- Tanaka, H., Iguchi, N., Toyama, Y., Kitamura, K., Takahashi, T., Kaseda, K., Maekawa, M., & Nishimune, Y. (2004). Mice Deficient in the Axonemal Protein Tektin-t Exhibit Male Infertility and Immotile-Cilium Syndrome Due to Impaired Inner Arm Dynein Function. *Molecular and Cellular Biology*, 24(18), 7958–7964. <https://doi.org/10.1128/MCB.24.18.7958-7964.2004>,
- Tanouchi, A., Taniuchi, K., Furihata, M., Naganuma, S., Dabanaka, K., Kimura, M., Watanabe, R., Kohsaki, T., Shimizu, T., Saito, M., Hanazaki, K., & Saibara, T. (2016). CCDC88A, a prognostic factor for human pancreatic cancers, promotes the motility and invasiveness of pancreatic cancer cells. *Journal of Experimental and Clinical Cancer Research*, 35(1). <https://doi.org/10.1186/S13046-016-0466-0>,
- Tian, X., Ruan, J., Huang, J., Fang, X., Mao, Y., Wang, L., Chen, X., & Yang, C. (2016). Gossypol: phytoalexin of cotton. *Science China Life Sciences*, 59(2), 122–129. <https://doi.org/10.1007/S11427-016-5003-Z/METRICS>
- Truebestein, L., & Leonard, T. A. (2016). Coiled-coils: The long and short of it. *BioEssays*, 38(9), 903–916. <https://doi.org/10.1002/BIES.201600062>

- Waites, G. M. H., Wang, C., & Griffin, P. D. (1998). Gossypol: reasons for its failure to be accepted as a safe, reversible male antifertility drug. *International Journal of Andrology*, 21(1), 8–12. <https://doi.org/10.1046/J.1365-2605.1998.00092.X>
- Wang, M., Liu, X., Chang, G., Chen, Y., An, G., Yan, L., Gao, S., Xu, Y., Cui, Y., Dong, J., Chen, Y., Fan, X., Hu, Y., Song, K., Zhu, X., Gao, Y., Yao, Z., Bian, S., Hou, Y., ... Qiao, J. (2018). Single-Cell RNA Sequencing Analysis Reveals Sequential Cell Fate Transition during Human Spermatogenesis. *Cell Stem Cell*, 23(4), 599–614.e4. <https://doi.org/10.1016/J.STEM.2018.08.007>,
- Wang, T., Yin, Q., Ma, X., Tong, M. H., & Zhou, Y. (2018). Ccdc87 is critical for sperm function and male fertility. *Biology of Reproduction*, 99(4), 817–827. <https://doi.org/10.1093/BIOLRE/IOY106>,
- Wang, Y., Li, J., Feng, C., Zhao, Y., Hu, X., & Li, N. (2017). Transcriptome analysis of comb and testis from Rose-comb Silky chicken (R1/R1) and Beijing Fatty wild type chicken (r/r). *Poultry Science*, 96(6), 1866–1873. <https://doi.org/10.3382/ps/pew447>
- Wu, B., Long, C., Yang, Y., Zhang, Z., Ma, S., Ma, Y., Wei, H., Li, J., Jiang, H., Li, W., & Liu, C. (2024). CCDC113 stabilizes sperm axoneme and head-tail coupling apparatus to ensure male fertility. *ELife*, 13. <https://doi.org/10.7554/ELIFE.98016>
- Yamaguchi, A., Kaneko, T., Inai, T., & Iida, H. (2014). Molecular Cloning and Subcellular Localization of Tektin2-Binding Protein 1 (Ccdc 172) in Rat Spermatozoa. *Journal of Histochemistry and Cytochemistry*, 62(4), 286–297. <https://doi.org/10.1369/0022155413520607>,
- Yamamoto, R., Song, K., Yanagisawa, H. aki, Fox, L., Yagi, T., Wirschell, M., Hirono, M., Kamiya, R., Nicastro, D., & Sale, W. S. (2013). The MIA complex is a conserved and novel dynein regulator essential for normal ciliary motility. *Journal of Cell Biology*, 201(2), 263–278. <https://doi.org/10.1083/JCB.201211048>,
- Yang, Y., Cochran, D. A., Gargano, M. D., King, I., Samhat, N. K., Burger, B. P., Sabourin, K. R., Hou, Y., Awata, J., Parry, D. A. D., Marshall, W. F., Witman, G. B., & Lu, X. (2011). Regulation of flagellar motility by the conserved flagellar protein CG34110/Ccdc135/FAP50. *Molecular Biology of the Cell*, 22(7), 976–987. <https://doi.org/10.1091/MBE.E10-04-0331>,
- Yin, D. T., Xu, J., Lei, M., Li, H., Wang, Y., Liu, Z., Zhou, Y., & Xing, M. (2016). Characterization of the novel tumor-suppressor gene CCDC67 in papillary thyroid carcinoma. *Oncotarget*, 7(5), 5830–5841. <https://doi.org/10.18632/ONCOTARGET.6709>,
- Yoshinaga, J., Imai, K., Shiraishi, H., Nozawa, S., Yoshiike, M., Mieno, M. N., Andersson, A. M., & Iwamoto, T. (2014). Pyrethroid insecticide exposure and reproductive hormone levels in healthy Japanese male subjects. *Andrology*, 2(3), 416–420. <https://doi.org/10.1111/J.2047-2927.2014.00202.X>;SUBPAGE:STRING:FULL
- Young, S. A. M., Miyata, H., Satouh, Y., Kato, H., Nozawa, K., Isotani, A., Aitken, R. J., Baker, M. A., & Ikawa, M. (2015). CRISPR/Cas9-Mediated Rapid Generation of Multiple Mouse Lines Identified Ccdc63 as Essential for Spermiogenesis. *International Journal of Molecular Sciences* 2015, Vol. 16, Pages 24732–24750, 16(10), 24732–24750. <https://doi.org/10.3390/IJMS161024732>
- Zariwala, M. A., Desportes, K. A., & Davis, S. D. (2025). Primary Ciliary Dyskinesia. *GeneReviews®*. <https://www.ncbi.nlm.nih.gov/books/NBK1122/>
- Zhou, W., De Iuliis, G. N., Dun, M. D., & Nixon, B. (2018). Characteristics of the epididymal luminal environment responsible for sperm maturation and storage. *Frontiers in Endocrinology*, 9(FEB), 341053. <https://doi.org/10.3389/FENDO.2018.00059/XML/NLM>

Overall Conclusion from the study

The testes of rats treated with pyrethroids and the testes of litter derived from three generations further showed deteriorated spermatogenic cells in distorted seminiferous tubules. Perturbations in oxidant status leading to oxidative stress in pyrethroid-treated rats could be a reason for decreased fertility. In the testes of rats exposed to pyrethroids, transgenerational transcriptome alterations in the genes regulating diabetes and testicular cancer suggest molecular harm. In the testes of rats exposed to pyrethroids, transgenerational transcriptome alterations in the machinery involved in steroidogenesis, spermatogenesis, epigenetic regulation, apoptosis, and oxidative stress suggest changes at the molecular level. The transgenerational epigenomic studies suggested many important genes related to biological processes, like reproductive function, were affected. A common family named the *Ccdc* gene family is found to be affected in all generations. Fertility's transgenerational impacts are a major worry that will impact future generations. This transgenerational study's findings offer firsthand knowledge of potential outcomes in human settings.

Take home message

Transgenerational effects on fertility are a critical worry for future generations. The findings of this transgenerational study provide firsthand information on what can happen in human settings. In-depth studies of human samples are necessary. The findings of this study clearly urge that agricultural methods should be revisited by minimising pesticide use and considering eco-friendly alternatives such as **organic farming**. There is also a need to change policies that would result in better food processing technology and lower pesticide content.

A mixture of pyrethroids induces reduced fecundity and increased testicular genotoxicity in rats

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Abstract

To achieve crop protection and higher agricultural yield, pesticides are used; and among them pyrethroid based ones are the most preferred choice because of their specificity on the pests. Uncontrolled use of pesticides resulted in contamination of food products. Presence of residual amounts of pyrethroids was reported in agricultural products sold in Indian market, indicating that humans are constantly exposed to these chemicals through food on a routine basis. Studies that determine the toxic effects of pyrethroids at doses equivalent to human exposure are rare. We orally administered a mixture of pyrethroids (detected in the rice and vegetables of Indian market) to male rats for 15 months to mimic the long-term exposure in humans. We observed reduced fecundity, sperm count and 13 β -hydroxysteroid dehydrogenase enzyme activity. The serum concentrations of hormones involved in male reproductive function were altered. Further, testicular genotoxicity as reflected by perturbations in the expression pattern of genes involved in the molecular processes of gametogenesis was evident. Such toxic effects may also be occurring in humans who consume agricultural products that contain residual amounts of pyrethroids on a regular basis throughout their lifetime.

KEYWORDS

fecundity, hormonal profile, pesticides, pyrethroids, sperm count

1 | INTRODUCTION

Usage of pesticides for crop protection is increasing exponentially because of the demand for more food production. Pesticides sprayed on the crops eventually percolated into the food chain. Despite the availability of a variety of pesticides, the pyrethroid based ones are the most favoured, since they display specific action on the pests and are less toxic to the humans. Thus, they form the major bulk of the pesticide market globally (Isman, 2006). With the advent of pesticide resistant pests, pesticides that contain one or more of the pyrethroids have been developed and sprayed, which led to the presence of multiple pyrethroids in the agricultural products. Studying the effects of pesticides at the cellular, anatomical and organismal level using different model systems has been an active area of investigation; and continues to be so because of the ever increasing usage for agricultural purposes and their consequences on human health (Freire et al., 2021; Hong et al., 2021; Rojas-Rueda

et al., 2021). We presented an extensive review on the toxic effects of pyrethroids at multiple levels (Ravula & Yenugu, 2021c). Recent studies report that bifenthrin causes reduction in pregnancy potential and defects in fetal growth and development (Curtis et al., 2021; Park et al., 2021). Malformation of male gametes, DNA damage and epimutations in sperm and reproductive toxicity were evident in animal models that were exposed to fenvalerate, cypermethrin, permethrin, deltamethrin and fenpropathrin, respectively (Abu Zeid et al., 2021; Ha et al., 2021; Hozyen et al., 2020; Huang et al., 2021; Thorson et al., 2020). In most of the studies on pyrethroid toxicity, the effects were reported using a single compound. Reports on the toxic effects of a combination of pyrethroids, especially that are present in the food chain are rare and there is an urgent need to conduct such studies.

In the Indian context, usage of pesticides for agricultural purposes resulted in percolation of the active components (pyrethroids) into the food chain. This is evident from the reports of Indian Agricultural

37. Pocate-Cheriet K, Santulli P, Kateb F, Bourdon M, Maignien C, Batteux F, Chouzenoux S, Patrat C, Wolf JP, Bertho G, Chapron C. The follicular fluid metabolome differs according to the endometriosis phenotype. *Reprod Biomed Online*. 2020 Dec;41(6):1023-37.
38. Braga DPAF, Montani DA, Setti AS, Turco EGL, Oliveira-Silva D, Borges E Jr. Metabolomic profile as a noninvasive adjunct tool for the diagnosis of Grades III and IV endometriosis-related infertility. *Mol Reprod Dev*. 2019 Aug;86(8):1044-52.
39. Vouk K, Hevir N, Ribić-Pucelj M, Haarpaintner G, Scherb H, Osredkar J, Möller G, Prehn C, Rižner TL, Adamski J. Discovery of phosphatidylcholines and sphingomyelins as biomarkers for ovarian endometriosis. *Hum Reprod*. 2012 Oct;27(10):2955-65.
40. Letsiou S, Peterse DP, Fassbender A, Hendriks MM, van den Broek NJ, Berger R, O DF, Vanhie A, Vodolazkaia A, Van Langendonck A, Donnez J, Harms AC, Vreeken RJ, Groothuis PG, Dolmans MM, Brenkman AB, D'Hooghe TM. Endometriosis is associated with aberrant metabolite profiles in plasma. *Fertil Steril*. 2017 Mar;107(3):699-706.e6.
41. Vouk K, Ribić-Pucelj M, Adamski J, Rižner TL. Altered levels of acylcarnitines, phosphatidylcholines, and sphingomyelins in peritoneal fluid from ovarian endometriosis patients. *J Steroid Biochem Mol Biol*. 2016 May;159:60-9.
42. Sun Z, Song J, Zhang X, Wang A, Guo Y, Yang Y, Wang X, Xu K, Deng J. Novel SWATH-MS technology for follicular fluid metabolomics in patients with endometriosis. *Pharmazie*. 2018 Jun 1;73(6):318-23.
43. Othman ER, Markeb AA, Khashbah MY, Abdelaal II, ElMelegly TT, Fetih AN, Van der Houwen LE, Lambalk CB, Mijatovic V. Markers of Local and Systemic Estrogen Metabolism in Endometriosis. *Reprod Sci*. 2021 Apr;28(4):1001-11.
44. Li J, Guan L, Zhang H, Gao Y, Sun J, Gong X, Li D, Chen P, Liang X, Huang M, Bi H. Endometrium metabolomic profiling reveals potential biomarkers for diagnosis of endometriosis at minimal-mild stages. *Reprod Biol Endocrinol*. 2018 Apr 30;16(1):42.
45. Li J, Gao Y, Guan L, Zhang H, Sun J, Gong X, Li D, Chen P, Ma Z, Liang X, Huang M, Bi H. Discovery of Phosphatidic Acid, Phosphatidylcholine, and Phosphatidylserine as Biomarkers for Early Diagnosis of Endometriosis. *Front Physiol*. 2018 Jan 23;9:14.
46. Zou W, Wen X, Xie C, Nie L, Zhou Q, Chen X, Fang C, Wang Y, Zhang L. LC-Q-TOF-MS based plasma metabolomic profile of subclinical pelvic inflammatory disease: A pilot study. *Clin Chim Acta*. 2018 Aug;483:164-9.
47. Heinonen HR, Mehine M, Mäkinen N, Pasanen A, Pitkänen E, Karhu A, Sarvilinna NS, Sjöberg J, Heikinheimo O, Bützow R, Aaltonen LA, Kaasinen E. Global metabolomic profiling of uterine leiomyomas. *Br J Cancer*. 2017 Dec 5;117(12):1855-64.
48. Tonoyan NM, Chagovets VV, Starodubtseva NL, Tokareva AO, Chingir K, Kozachenko IF, Adamyan LV, Frankevich VE. Alterations in lipid profile upon uterine fibroids and its recurrence. *Sci Rep*. 2021 Jun 1;11(1):11447.
49. Tonoyan NM, Tokareva AO, Chagovets VV, Starodubtseva NL, Kozachenko IF, Adamyan LV, Frankevich VE. Possibilities for predicting recurrent uterine myoma by plasma lipidomic analysis. *Obstet Gynecol*. 2019;11:136-51.
50. Subramani E, Dutta M, Jothiramajayam M, Joshi M, Srivastava S, Mukherjee A, Chakravarty B, Chaudhury K. Identification of serum metabolic markers for diagnosis of women with dormant genital tuberculosis. *Metabolomics*. 2016 Jun;12(6):1-9.
51. Subramani E, Jothiramajayam M, Dutta M, Chakravorty D, Joshi M, Srivastava S, Mukherjee A, Datta Ray C, Chakravarty BN, Chaudhury K. NMR-based metabolomics for understanding the influence of dormant female genital tuberculosis on metabolism of the human endometrium. *Hum Reprod*. 2016 Apr;31(4):854-65.

ASSESSMENT OF REPRODUCTIVE TOXICOLOGY OF PESTICIDES: STUDIES FOCUSED ON DOSES RELEVANT TO HUMAN EXPOSURE ARE THE NEED OF THE HOUR



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Introduction

Reproductive toxicity refers to defining the adverse effects of an environmentally detrimental agent on fertility; and when the toxic effects studied during pre-natal development are known as developmental toxicity. As the global population is ever increasing, the demand for food is also on the rise. Human welfare is defined on the availability of adequate amount of food and the quality of environment. In order to meet the food demands, changes in the agricultural practices have evolved in the last few decades. These practices were focused on using pesticides mainly to protect the crops from vector-borne diseases and thereby enhancing crop yield. Among the pesticides used, pyrethroid-based pesticides emerged as the best option, since they are known to be less toxic to humans and act specifically on the pests. While pesticides increased living quality by way of enhancing food production, they also posed serious health hazards. Humans come into contact with pesticides on a daily basis in different modes. Rampant use of pesticides resulted in their permeation into the food chain and drinking water. Further, at the domestic front, pesticides are used to prevent vector borne diseases, especially those diseases spread by small insects. Almost all known pesticides have health risks that are dependent on the dosages used and exposure durations. Synthetic pyrethroids occur in a variety of chemical forms and can cause health hazards that range from simple nausea to a variety of cancers. On the other hand, exposure during pre-natal and early post-natal developmental stages can happen during pregnancy, through breast milk, and through contaminated food. Since the most of the organ systems that contribute to immunity, reproduction, etc are not well defined in the pre-natal and early post-natal developmental stages when compared to adults, the effects of pesticides are more profound in these stages. Strong evidence suggests that exposure to chemicals during pregnancy or in the first few months after birth might harm the growing body as well as increase the risk of later acquiring fatal conditions like diabetes, metabolic syndrome, Alzheimer's disease and Parkinson's disease. We have previously summarized the historical, epidemiological, chemical and biological (physiological, biochemical, and molecular) features of pyrethroid-based insecticides [1]. In the recent years, assessment of toxicity studies has become a global challenge [2].

Expression of SARS-CoV2 Infectivity Machinery in the Male Reproductive Tract: Possible Outcomes on Fertility

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Abstract

Emergence of the COVID-19 pandemic continues to rage and rattles the entire world causing multifaceted hardships. Though initially thought to be a disease that primarily affects the lungs, latest evidence suggests its possible Long-term effects on multiple organ systems. SARS-Cov-2, the virus responsible for this disease infects the cells through ACE2 receptor and the serine protease TMPRSS2. In light of the fact that ACE2 expression is very high in the testis and the expression of TMPRSS2 in other reproductive organs, there has been growing interest to determine the effect of SARS-Cov-2 infection on the male reproductive system, especially on fertility. Through bioinformatics analyses, *in vitro* and cohort studies, the effects on SARS-Cov-2 infection at the molecular to physiological levels are proposed. Perturbations in hormonal levels, damage to the anatomical structure and inflammation in reproductive organs, decline in sperm count and sperm function have been reported. Thus, the significance of COVID-19 on global reproductive health has gained importance. In this article, we summarize the reported facts related to SARS-Cov-2 infectivity on male reproductive system. Such a comprehensive summation herein will help the researchers to have an up to date knowledge in this area of research and to coronavirus newer studies to address the effects of the COVID-19 pandemic on male reproduction, especially fertility.

Keywords: COVID-19, Fertility, Male Reproductive System, SARS-Cov-2

1. Introduction

The emergence of COVID-19 pandemic affected almost all the countries in a number of ways. As on 24th April, 2020, the pandemic caused 3,101,540 deaths and 146,312,705 infections worldwide to cause severe hardships in terms of health and economic crisis (<https://www.worldometers.info/coronavirus/>). COVID-19 is caused by the novel corona virus, SARS-Corona virus 2 (SARS-Cov-2), which primarily affects the respiratory system to cause severe lung disease¹. The infections primarily originated from the Wuhan city of Hubei province, China and culminated to infect millions of people across the world. In the last one and half years after the first outbreak of this disease in

December 2019, vast number of research studies reported the etiology and molecular mechanisms of this infection in the primarily affected organ i.e. the lung². Further, the susceptibility of other organs and the risks of post infection were also reported^{3,4}. Over a period of time it is emerging that this disease is not only fatal, but can cause High-risk mortality and morbidity in patients who have recovered from the infection. Considerable interest has also been generated to identify the effect of SARS-Cov-2 infections on the male reproductive tract. The gender bias for higher incidence of COVID-19 in men evinced interest to study the relation between the etiology of this disease and male reproductive physiology⁵. The purpose of this review is to consolidate the known aspects on the

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Coiled-Coil Domain-Containing (CCDC) Proteins: Functional Roles in General and Male Reproductive Physiology

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Abstract

The coiled-coil domain-containing (CCDC) proteins have been implicated in a variety of physiological and pathological processes. Their functional roles vary from their interaction with molecular components of signaling pathways to determining the physiological functions at the cellular and organ level. Thus, they govern important functions like gametogenesis, embryonic development, hematopoiesis, angiogenesis, and ciliary development. Further, they are implicated in the pathogenesis of a large number of cancers. Polymorphisms in *CCDC* genes are associated with the risk of lifetime diseases. Because of their role in many biological processes, they have been extensively studied. This review concisely presents the functional role of CCDC proteins that have been studied in the last decade. Studies on CCDC proteins continue to be an active area of investigation because of their indispensable functions. However, there is ample opportunity to further understand the involvement of CCDC proteins in many more functions. It is anticipated that basing on the available literature, the functional role of CCDC proteins will be explored much further.

Keywords Coiled-coil domain-containing · Male fertility · Spermatogenesis · Ciliary motion · Tumorigenesis

Introduction

Reproduction is one of the fundamental biological processes for the production of offspring. Evolution of distinct male and female reproductive systems facilitated sexual reproduction. The male reproductive system is a combination of organs and glands that include the testes, epididymis, vas deferens, seminal vesicle, prostate gland, and a pair each of Cowper's and bulbourethral glands. The epithelial surfaces of the male reproductive tract secrete an array of proteins into the lumen. These proteins belong to a variety of families such as defensins, cathelicidins, lipocalins, and RNases [1, 2]. While a large number of proteins secreted by the male reproductive tract were characterized in the last two decades, this domain of research continues to be an active area of investigation. A diverse nature of biological processes is carried out by these proteins, beyond their role in reproductive function. The coiled-coil domain-containing (CCDC) proteins have been investigated in the last

decade for their multifunctional roles in the general and male reproductive physiology as well. However, there is no dedicated review that describes the functional role of CCDC proteins. This review presents a comprehensive compilation of the reported functions of CCDC proteins. Since a great number of CCDC proteins are not yet characterized for their functional role in general and reproductive physiology, the information provided in this review helps to design strategies to explore the uncharacterized CCDC proteins. Further, analyzing the role of these proteins in male reproductive tract helps to design strategies for identifying novel targets for contraception, in vitro fertilization, and pathological conditions.

Review Preparation Strategy

This review is a compilation of studies that were conducted in the last decade to understand the biological importance of CCDC proteins and to evaluate the potential possibility of further research studies. The strategy adopted to prepare the review is provided as Supplementary Information 1. As of 8th April 2021, a total of 6274 results were identified in PubMed. After thorough screening by applying the criteria, only original research reports; no data on functions; reviews; meeting

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Aging of Human Organs: Mechanisms, Consequences, and Therapeutic Approaches

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Abstract: All living things, including humans, are subject to the unavoidable and intricate biological process of aging. The steady decline in organ function, which can result in a variety of age-related disorders and a reduction in overall quality of life, is one of the most important effects of aging. This study investigates the causes and effects of organ aging, as well as prospective therapeutic strategies to lessen the effect of aging on organ function.

Keywords: Aging, Human, Therapy, Human organ aging.

INTRODUCTION

A rise in susceptibility to various diseases and a time-dependent decline in physiological function are two characteristics of aging, which is a universal and natural process. The aging of human organs stands out among the many effects of aging as a crucial factor that profoundly affects a person's health and well-being. Developing solutions to encourage healthy aging and enhance the quality of life in an aging population requires an understanding of the mechanisms and effects of organ aging.

Mechanisms of Organ aging:

The immunological and endocrine systems are intertwined and collaborate to keep the body's equilibrium. The immune system is significantly influenced by hormones made by the endocrine system, including insulin, growth hormone, prolactin, and thyroid hormone. For instance, after LPS stimulation in macrophages, insulin stimulates the production of inflammatory cytokines like IL-6. On the other hand, immune cells like T and B cells as well as senescent cells have the ability to create hormones that control immune function and communicate with the endocrine system. Senescent cells, for instance, release growth factors and cytokines through the senescence-associated secretory phenotype (SASP), which interact with the local and systemic environment. Paracrine signaling is used by the immune and endocrine systems to coordinate cellular responses to any stress in distal bodily regions.

Cellular Senescence:

As organs age, cellular senescence is a key mechanism. Age-related organs develop an accumulation of senescent cells, which produce pro-inflammatory chemicals and cause tissue malfunction and chronic inflammation. The

regeneration and repair of tissues may be hampered by this process.

Telomere Shortening:

Telomeres, which act as chromosome ends' protective caps, get shorter with each cell division. Telomere shortening, a sign of age, reduces cells' ability to replicate. Organ degeneration results from cells entering a senescent state or going through apoptosis when telomeres are potentially short.

Oxidative Stress:

Oxidative stress, which harms cellular components and hastens organ aging, is caused by an increase in the generation of reactive oxygen species (ROS) and a decrease in antioxidant defenses. Over time, the cumulative consequences of oxidative stress led to organ malfunction and functional deterioration.

Accumulation of DNA Damage:

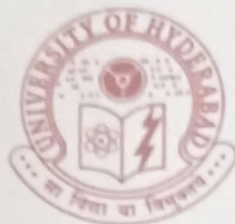
Aging cells develop DNA damage from a variety of sources, such as radiation and toxic environmental exposure. Age-related disorders like cancer are linked to mutations and genomic instability, which are brought on by ineffective DNA repair systems in aged tissues.

Lifestyle variables:

Age-related diseases are more likely to develop as a result of factors like physical inactivity, poor nutrition, smoking, excessive alcohol use, and exposure to environmental contaminants.

Psychosocial Elements:

Through a variety of causes, such as heightened inflammation and hormonal changes, chronic stress, social isolation, and psychological variables can hasten aging.



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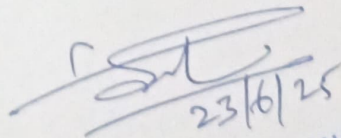
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by Priyanka Patra

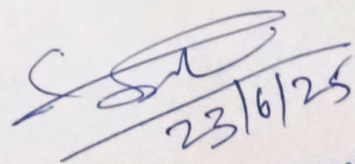
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