# Evaluation of oral subchronic administration of Pyriproxyfen on steroidogenic activity of ovaries in adult Sprague Dawley rats.


**By**

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**DEPARTMENT OF**
**ZOOLOGY**
**FACULTY OF BIOLOGICAL SCIENCES**
**QUAID-I-AZAM UNIVERSITY ISLAMABAD,**
**PAKISTAN**
**2023**

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A dissertation submitted in the partial fulfillment of the requirements for the Degree of Master of Philosophy


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***"In the Name of ALLAH, the most Beneficent, the most Merciful"***


***Dedicated to My Parents Who have given me the opportunity to Study from the best institutions andSupported throughout my life.***

# DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is my original work. I have not previously presented this work elsewhere for any other degree.

***JALWAH FATIMA***

# CERTIFICATE

This dissertation Titled "Evaluation of oral subchronic administration of Pyriproxyfen on steroidogenic activity of ovaries in adult Sprague Dawley rats" submitted by **Jalwah Fatima** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy in Reproductive Physiology.

**Supervisor:**

Prof. Dr. Sarwat Jahan

**External Examiner:**

**Chairman:**

Prof. Dr. Amina Zuberi

Dated

**Dated:**

## *ACKNOWLEDGMENTS*

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***Jalwah Fatima***

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **ANOVA** | One way analysis of variance |
| **BMI** | Body Mass Index |
| **CDER** | Center for Drug Evaluation and Research |
| **DDT** | dichlorodiphenyltrichloroethane |
| **EDCs** | Endocrine Disrupting Chemicals |
| **EPA** | Environmental Protection Agency |
| **FAO** | Food and Agriculture Organization |
| **FDA** | Food and Drug Administration |
| **HPA** | hypothalamus-pituitary gland-adrenal |
| **HPA** | hypothalamic-pituitary-adrenal (HPA) |
| **HPG** | hypothalamus-pituitary gland-gonads |
| **HPG** | hypothalamic-pituitary-gonad |
| **HPI** | Hypothalamic-pituitary-interrenal |
| **HPT** | hypothalamus-pituitary gland-thyroid |
| **JMPR** | Joint Food and Agriculture Organization/WHO Meeting on Pesticide Residues |
| **NRs** | nuclear receptors |
| **PBBs** | polybrominated biphenyls |
| **PBS** | Phosphate buffer saline |
| **PCBs** | Polychlorinated biphenyls |
| **PPF** | Pyriproxyfen |
| **PPPs** | Plant Protection Products |
| **SEM** | Means ± standard errors of means |
| **UNEP** | United Nations Environment Programme |

| | |
|---|---|
| **Vtg** | vitellogenin |
| **WHO** | World Health Organization |

## Abstract

Pesticide exposure such as PPF in a variety of animals is known to possess an endocrine disruptive potential with effects on multiple aspects of reproduction. PPF is a pyridine ring-containing heterocyclic molecule derived from natural sources and categorized to the group of biopesticides. The aim of present study is to evaluate the toxic effects of Pyriproxyfen on reproductive system of adult rats by determining ovarian function. For this purpose, twenty adult female Sprague Dawley rats (n=5/group) weighted (160±15g) were taken. Control received distilled water, while Group I, II, and III received 62mg/kg, 124mg/kg and 186mg/kg of Pyriproxyfen for 28 days. On day 29, animals were weighed, decapitated, and blood was collected, centrifuged and plasma was stored at -20˚C until analysed. Reproductive organs, including, ovaries and uterus were dissected and weighted. The length was measured using measuring tape and the subsequent formula was used to determine the BMI. Determination of blood glucose and estrous cyclicity was carried out with 2 weeks gap. The blood plasma levels for cortisol, progesterone and estradiol were also determined using multiple ELISA. The results showed a decrease in ovarian weight and BMI as compared to control while a remarkable increase in weights of reproductive organs and body organs was noticed. The significant increase ($P<0.001$) in blood glucose levels of G2 and G3 from day 1 to day 14 while a significant decrease ($P<0.001$) from day 14 to day 28 was observed. Alterations in estrous cycle was noticed with prolonged diestrus phase and shortened proestrus phase. The hormonal analysis depicted a reduction in cortisol, estradiol and progesterone concentration in PPF treated groups as compared to control. The study concluded that PPF decreases body weight, ovarian weight and BMI, increases the weight of other

reproductive and body organs such as heart, liver, pancreas, kidneys and brain, alterations in blood glucose levels and irregularity in the phases of estrous cycle, decreases concentrations of blood plasma cortisol, estradiol and progesterone. It can be suggested from the present findings that PPF exerts toxic effects on reproductive health of females and act as an endocrine disruptor, however, further similar studies can be designed to elucidated the underlying mechanisms responsible for inducing reproductive toxicity in animals, by monitoring actions of PPF on cellular interactions in ovaries and other target organs and their molecular pathways.

# INTRODUCTION

## Pesticide

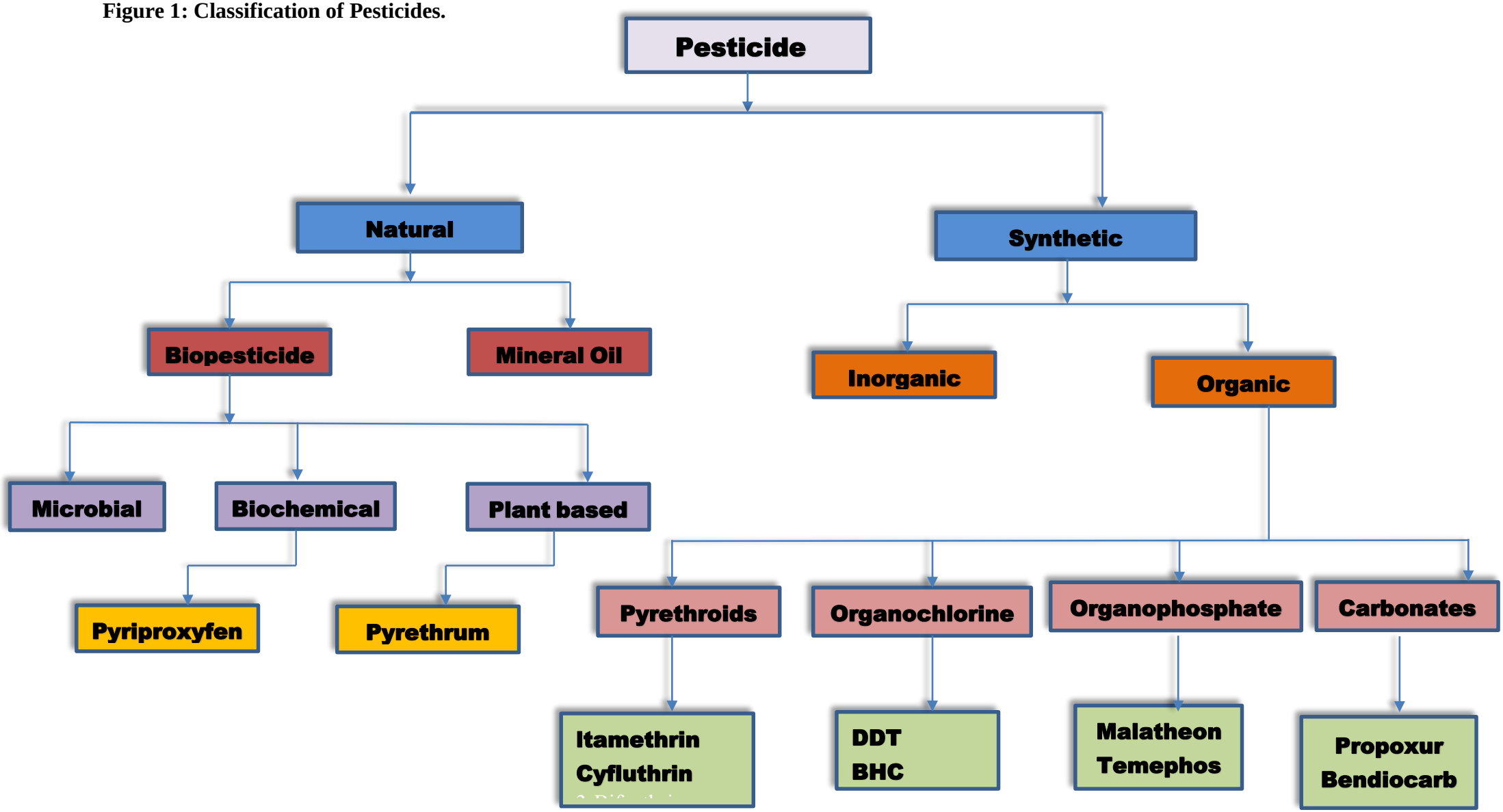
Pesticide is a substance that is administered to public spaces such as gardens, farms, and other outdoor spaces to eradicate undesired species. A broad term used to describe a variety of classes of insecticides, fungicides, herbicides, garden chemicals, household disinfectants, and rodenticides that are used to both eliminate and guard against pests is "pesticide." (Eldridge, 2008; Nemr *et al*., 2012; Eddlestone, 2020; Jia *et al*., 2020). Increased field sizes, a decline in the variety of crops grown, and a decrease in the amount of semi-natural habitats are all characteristics of modern agriculture's expanded use of land. Furthermore, they are exposed to significant inputs of agrochemicals, primarily Plant Protection Products (PPPs) used to protect agricultural productivity from pests (Hahn *et al.,* 2015). Agricultural producers use over 3 million tons of pesticides annually, which is equivalent worth $40 billion, globally (Popp *et al.,* 2012). The WHO estimates that about a million people worldwide suffer from acute poisoning brought on by pesticide exposure. The annual death rate due to pesticide exposure ranges from 0.4% to 1.9% (Qiu *et al.,* 2017; Eddleston, 2020, Jia *et al.,* 2020). Typically, pesticides are extremely sensitive to the reproductive and nervous systems of the animals. Because these processes resemble those of the physiological system in a healthy human, it is possible that these chemicals can likewise have an impact on a human body (Gore *et al.,* 2014).

## Types of pesticides

Pesticides can be categorised in a variety of ways, including chemical classes, functional groups, modes of action, and toxicity (Gunnell *et al*., 2007; Garcia *et al.,* 2012). Carbon (organic), copper sulphate, ferrous sulphate, copper, lime, sulfur and other compounds are active ingredients of most pesticides . Compared to inorganic

pesticides, organic pesticides' compounds are typically more complex and less water-soluble (Legrand *et al.,* 2016). Additionally, pesticides be classified into two categories, the first one is called biopesticides so due to imitative property from natural bases i.e microorganisms, fungi and plants. These are further divided into major three sub classes that are Microbial, biochemical pesticides and plant incorporated protectants. The second category of pesticides are chemical pesticides. Depending on their sources, the chemical pesticides are further classified four subgroups: orgochlorine, organophosphate, carbamate and pyrethroid pesticides and synthetic pesticide (Gerolt, 1969). By the kind of insect that they are used to control, pesticides are sometimes categorized as miticides, insecticides, and herbicides and fungicides. In order to manage pests, additional pesticides have been created that work on the endocrine or hormonal systems, or that affect the neurological system. (Mnif *et al.,* 2011). The schematic representation of classification of pesticides is shown in the given figure 2.

**Figure 1: Classification of Pesticides.**



**Routes of exposure of pesticide to human**

Pesticide exposure can occur due to occupational exposure, farming, and domestic use, as well as indirectly through diet. Additionally, due to pesticides being used on golf courses, in the vicinity of busy roads etc, the general public may be exposed to them. The main ways that people are exposed to pesticides are through the air, soil, food chain, water, and animals (Anderson and Meade, 2014). Pesticides can enter the body through four routes: the skin, the mouth, the eyes, and the respiratory system. Depending on the route of exposure, pesticide toxicity can change the risk of pesticide contamination that may often rises with dosage and target chemical's toxicity, as would be predicted in general. (Sharon et al., 2012).

**Pesticides and their effects**

In addition to being useful, pesticides can cause a risk to the health of both humans and animals due to their toxicity. They cause pollution and are bad for all living things. In addition to being rapidly hazardous to target and non-target species, broad-spectrum pesticides can manage a large variety of pests (World Health Organization, 2012).

**Pesticide Toxicity to Plants**

The herbicides come into touch with both target plant species and non-target plant species. These toxins destroy plant diversity (Isenring, 2010). Pesticides can harm a plant's vegetative growth during several phases, including the seedling stage, the later stages of seed production, the reproductive organs of the plant and frequently in F1 generation the somatic components of the plant (Boutin *et al.,* 2014).

**Pesticide Toxicity to Animals**

Many nonhuman biota, including bees, birds, amphibians, fish, and small mammals, die as a result of pesticide residues being dispersed throughout our

environment, (Carvalho, 2017). Animals exposed to atrazine experience reproductive damage and delayed sexual maturity (Nicolopoulou-Stamati *et al.,* 2016). Fish, migratory animals, and birds have all been found to have harmful effect due to exposure to pesticides. This contamination can reduce the number of these organisms. According to reports, pesticides are the main cause of wild mammal death and harm roughly 52.5% of birds (Pesiakov *et al.,* 2017).

**Pesticide Effects on Humans**

The United Nations Environment Programmed (UNEP) predicated that at least 3 million agriculture workers in poor developed countries are intoxicated by pesticides each year, and at least 300,000 workers in the United States are at the very least affected (Miller and Spoolman, 2017). According to the WHO, about 1000,000 human beings are affected by acute poisoning by contact with pesticide. All living things are poisonous to pesticides at specific concentrations. They interfere with regular bodily responses needed for metabolism when they enter the human body, inhibiting enzymatic activity (Saeed, 2017). Numerous harmful health impacts linked to pesticides have been identified. The same effects of pesticides are seen in the skin, gastrointestinal tract, respiratory system, central nervous system, reproductive system, renal system, and other organs and systems. Some of its side effects include endocrine-related issues, carcinogenicity, teratogenicity, mutagenicity, and other harmful outcomes (Nicolopoulou-Stamati *et al.,* 2016).

**Pyriproxyfen**

Insect growth regulator (IGR): Pyriproxyfen has broad-spectrum efficacy against mosquitoes, cockroaches, whitefly and other public health hazard insects. In the 1990s, Sumitomo Chemical Co., Ltd. created and synthesized it for the first time. Pyriproxyfen mimics the juvenile hormone action to act on the insect endocrine

system, preventing moulting and ultimately inhibiting reproduction. It is widely used throughout the world, especially in developing nations. Pyripoxyfen interferes with adult emergence and embryogenesis as a result. The juvenile hormone is essential for insects' metamorphosis, sex differentiation, courting, movement, behavior, and nervous system function (Baumann *et al.,* 2017). Consequently, pyriproxyfen has been recognized by the U.S as a Compact risk insecticide and a substitute to organophosphates. It is also the only pesticide recognized by the WHO for use in the treatment of drinkable water to prevent mosquito breeding. Due to its unique mechanism of action, it is more selective than traditional insecticides and consequently less dangerous to organisms other than the target species. Due to Pyriproxyfen high environmental stability and negative impacts on non-target species due to its presence in the food chain have been reported. Moreover, pyriproxyfen can vitiate into many metabolites in different environmental matrices (soil, water, plants, insects and mammals) documented by the Joint Food and Agriculture Organization/WHO Meeting on Pesticide Residues (JMPR). The risk that pyriproxyfen and its metabolites represent to the environment and dietary intake must therefore be understood.

**Synonyms of Pyriproxyfen**

Some of the most common Synonyms of PPF are:

(+--)-pyriproxyfen, 2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy) pyridine, 4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether,Juvinal pyridine, 2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy)-pyriproxyfen, (R)-isomer pyriproxyfen, (S)-isomer.

**Structure of Pyriproxyfen**

Pyriproxyfen (PPF) is an analogue of juvenile hormone and belongs to the subgroup of biopesticides that is organic, heterocyclic, and has an unfused pyridine ring (Manabe *et al*., 2006; Wei *et al*.,2021). The structure of PPF has been shown as under:

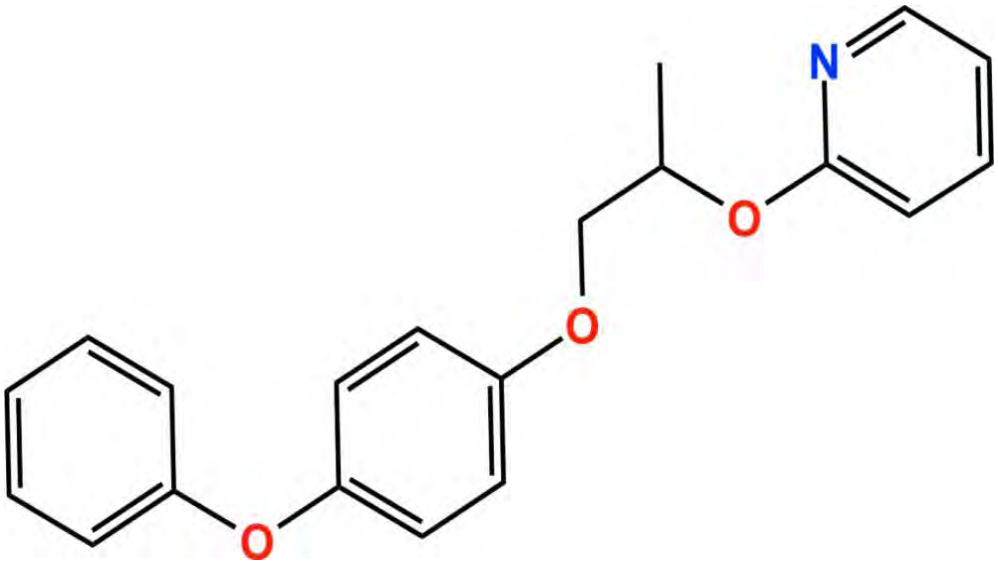



**Figure 2: Structure of Pyriproxyfen**

**Chemical and physical properties of Pyriproxyfen:**

Pyriproxyfen has high lipid solubility (with kow of 5.37 at 25℃) and long half-life in aerobic water about 23.1days due to this PPF having greater ability of bioaccumulation and classified as persistent pollutant (Sullivan, 2000; Sullivan and Goh, 2008). They also claimed that PPF (0.5% GR) had a substantially longer lasting impact at low dosage concentrations. Under an aerobic lake water sediment system, PPF has a half-life of between 16 and 21 days, making it a relatively stable aromatic chemical (WHO, 2008). The recommended usage of PPF in drinking water sources is at a maximum final concentration of 0.01 mg/L, which is the suggested guideline value for acceptable daily intake of PPF (Trounge *et al*., 2016; WHO, 2007).

**Actions of pyriproxyfen**

Juvenile hormone and its analogues are known to bind to the vertebrate retinoic acid receptor, and retinoic acid is known to imitate some of the effects of

juvenile hormone when injected into insects (Němec *et al.,* 1993; Palli *et al.,*1993). Therefore, it is probable that pyriproxyfen, a potent mimic of a juvenile hormone, will bind to the retinoic acid receptor. If this occurs PPF may either operate as an activator of the receptor or as a blocker, preventing the normal retinoic acid from binding to the receptor at the appropriate time. So if activation of receptor occurs,gene expression will turn on normally during development or is expected to cause developmental abnormalities if either inhibition of receptor occurs (Dhadialla *et al.,* 1998).

**Toxicology of pyriproxyfen**

Pyriproxyfen shown possible dangers to non-target animals, as such that as developmental toxicity to *Hippodamia convergens,* behavioural problems in bees, endocrine disruption in land crab and *Odontophrynus americanus*, and dysontogenesis in zebrafish (Linton *et al.,* 2009; Fourrier *et al.,* 2015, Truong *et al.,* 2016; Lajmanovich *et al.,* 2019; Iftikhar *et al.,* 2020). Pyriproxyfen residues are shown to be a food contaminant and act as potential dietary risk factor. A recent controversy regarding a potential link between microcephaly and PPF in humans has attracted consideration in the public and scientific environment due to the fact that it has been identified as an endocrine disruptor, exhibiting nutritive risk, effect on teratogenic, impact of aquatic toxins and the potential to enhance viral production in large quantities (Parens, 2017).

Pyripoxyfen influences the immune system of mammals. High dosages of pyriproxyfen markedly boosted the specific total IgG immunological response. IgG2a titers, TNF-alpha, and gamma interferon responses were all improved by pyriproxyfen.(Sharmin *et al.,* 2013) There is not much data on pyriproxyfen's toxicity to vertebrates. At exceptionally exposure to high concentrations of pyriproxyfen,

*Xiphophorus maculatus's* erratic swimming and embryo abnormalities have been documented (Caixeta *et al.,* 2016, Truong *et al.,* 2016).

Despite being widely used, pyriproxyfen has been reported to have impacts on the central nervous system. The main metabolite of pyriproxyfen (4'OH-pyriproxyfen), changes the expression of gene in mouse neurosphere cells suggesting potential influence on neurodevelopment (Spirhanzlova *et al.,* 2018). Additionally, arhinencephaly and lower brain weight were seen in rat pups that were continuously exposed to pyriproxyfen. (Evans *et al.,* 2016).

Recent research suggests that Cortisol is a stress biomarker and PPF exposure is known to disturb the endocrine system by inhibiting acetylcholinesterase enzyme concentration in fish and freshwater larvicides altering the release of cortisol (Arajo *et al.,* 2018; Ghelichpour *et al.,* 2018; Maharajan *et al.,* 2018). Cortisol that is primarily produced by the hypothalamic-pituitary-interrenal (HPI) axis in fish and the hypothalamic-pituitary-adrenal (HPA) axis in mammals. (Pijanowski *et al.,* 2015; Adam *et al.,* 2017). PPF also has an impact on the endocrine system of thyriod in *Odontophrynus americanus* tadpoles (Lajmanovich *et al*., 2019).

**Reproductive toxicity**

Reproduction is the most imperative function of all living organisms in maintaining balance of ecosystem. Environmental contaminant exposure has been linked to detrimental changes in the reproductive endocrine system, which may lead to a decline in both animal and human fertility, according to studies conducted over the past few decades (Chen *et al*., 2016; Wang *et al*., 2019). PPF impairs insect reproduction at all phases of development, including metamorphosis, embryogenesis and adult reproduction, at low concentrations (Moadeli *et al.,* 2014; Chłopecka *et al.,* 2018). There have been numerous reports of the endocrine disruptive potential of PPF

in a variety of invertebrate organisms with effects on multiple aspects of reproduction, including changes to storing lipids, the gender switch in neonates, a decrease in fecundity, and consequent ecological effects in Daphnia (Ginjupalli *et al.,* 2015; kakaley *et al*., 2017; Chłopecka *et al.,* 2018; Tanaka *et al*., 2018; Watanabe *et al.,* 2018). Recently, it was discovered that PPF causes faulty spermatogenesis and testicular architecture in male mice, as well as other reproductive system defects (Shahid *et al.,* 2019). The hypothalamic-pituitary-gonad (HPG) axis controls the reproductive endocrine system in vertebrates and is essential for regulating hormone synthesis, transport, and metabolism. The widely used vertebrate species zebrafish (Danio rerio) is used to evaluate the reproductive toxicity of numerous environmental pollutants (Cao *et al.,* 2019). The PPF may have potential hazard effects on reproduction of male and female zebrafish by changing gonads histopathology, alters the concentrations of estrogen and testosterone concentrations by impairment in HPG axis regulation genes (Maharajan *et al*., 2021).

**Female reproductive toxicity of Pyriproxyfen**

Surprisingly, despite being an endocrine disruptor, there aren't many studies examining how pyriproxyfen affects the reproductive system (Ji *et al*., 2020). Researchers recently discovered that during in vitro experiments on cell cultures pyriproxyfen and several of its products have shown potent estrogen-disrupting effects. In contrast, administration of this insecticide in the drinking water to male rats resulted in a reduction in serum concentrations of Follicle stimulating hormone, LH and testicular mass, while in female rats, pyriproxyfen exposure throughout pregnancy and lactation increased the likelihood of stillbirths and decreased the number of uterus implants (WHO, 2007; Mehrnoush *et al*., 2013). More recently, it was discovered that pyriproxyfen reduces fertility and increases the ratio of male

progeny in Daphnia magna, potentially lowering the number of neonates that mothers of this species give birth to (Chłopecka *et al*., 2018; Tanaka *et al.,* 2018; Watanabe *et al.,* 2018). Using the estrogen-responsive MtT/Se cell line of rats and reporter gene assays, the estrogen action of PPF was also demonstrated in vitro (Manabe *et al.,* 2006). The information gap and lack of evidence to support the estrogenic potential of PPF in vertebrate organisms have been clearly addressed in the most recent report of FAO/ WHO (2019).

In this regard, the toxic effects of PPF on the reproductive system of adult female rats has not been studied. Therefore, the current study was designed to evaluate the reprotoxic effects of Pyriproxyfen on adult female Sprague Dawley rats by monitoring estrus cyclicity, hormonal production by ovaries and BMI in rats. Based on the above studies, it is hypothesized that Pyriproxyfen might decrease the steroidogenic activity of ovaries in adult Sprague Dawley rats.

## Aims and Objectives

The experiment aims to evaluate a biochemical approach of toxic effects of Pyriproxyfen on reproductive system of adult female Sprague Dawley rats. The objectives of this study are:

1. To evaluate the ovarian steroidogenic efficiency in rats caused by the Pyriproxyfen.
2. To find out the toxic effects of PPF on blood cortisol levels.
3. To determine the harmful effects of PPF on estrous cycle.

## Materials and Methods

The current study was conducted in the Laboratory of Reproductive Physiology, Department of Zoology, Quaid-i-Azam University, Islamabad, Pakistan. As permitted by the ethical committee of the department, handling of animals and all procedures were assessed. While performing all the processes in the study, the recommendations for the appropriate maintenance and usage of research lab animals were considered.

### Animals

Twenty adult female Sprague Dawley rats (Rattus norvegicus) with an average weight of 160±15g were taken from the Zoology Department of Quaid-i-Azam University. Rats were kept randomly into four groups (n=5) and housed in separate stainless steel cages. During the experimentation female rats were placed in proper aerated room with temperature of 20-26˚C for 28 days. Food chaw and tap water was fed to the animals throughout experiment.

### Pyriproxyfen

PPF used in the experiment purchased from ANQA AGRO Multan. Having PPF (10.8 % EC) has an active ingredient. The daily oral dose of 62mg/kg (G1) ,124mg/kg (G2), 186mg/kg (G3) body weight of Pyriproxyfen was selected for sub chronic exposure (Shahid and Saher, 2019).

### Preparation of Pyriproxyfen stock solution

3ml PPF was mixed in 27ml of distal water to make the final doses. The same process was used to make fresh dose for 28 days.

### Experimental design

For present study, the animals (n=5) were grouped into four. All the doses were given orally between 10-11am, for about 28 consecutive days, as shown in figure (4).

**Control group**

This group was treated with distilled water.

**Group I**

Animals received 62mg/kg of Pyriproxyfen dissolved in distilled water.

**Group II**

Rats were provided with 124mg/kg of Pyriproxyfen dissolved in distilled water.

**Group III**

Animals were administered with 186mg/kg of Pyriproxyfen dissolved in distilled water.

**Blood and Tissue collection**

The rats were decapitated on 29$^{th}$ day of experiment. Following decapitation, heparinized syringes were used to collect trunk blood directly and kept in heparinized tubes. For 15 minutes, centrifiguation of blood samples was done at 3000 rpm. Plasma was separated and kept at -20˚C till analyzed. Immediately after blood collection, reproductive organs, including, ovaries and uterus, were separated, and weighed after removal of accessory fatty tissues.

**Determination of body weight**

By using top loading Sartorius Digital Balance, rats were weighted on day 1$^{st}$, 14$^{th}$ and 28$^{th}$ of the experiment.

**Determination of organs weight**

The weight of body organs such as heart, liver, pancrease, kidneys and brain was determined on 29$^{th}$ day of experiment using top loading Sartorius Digital Balance.

**Determination of BMI**

For calculating rat's BMI (g/cm$^2$), their body weight and body length were measured (Novelli et al., 2007). The length was measured using measuring tape, and the subsequent formula was used to determine the BMI. The normal BMI for adult female rat ranges between 0.4504−0.5044 g/cm$^2$ (Engelbregt et al., 2001).

Body mass index= Body weight (g)/Body length( cm$^2$).

**Determination of glucose level in blood**

Glucose in blood drawn from rats tail on day 1st, 14th and 28$^{th}$ and was noted using glucometer.

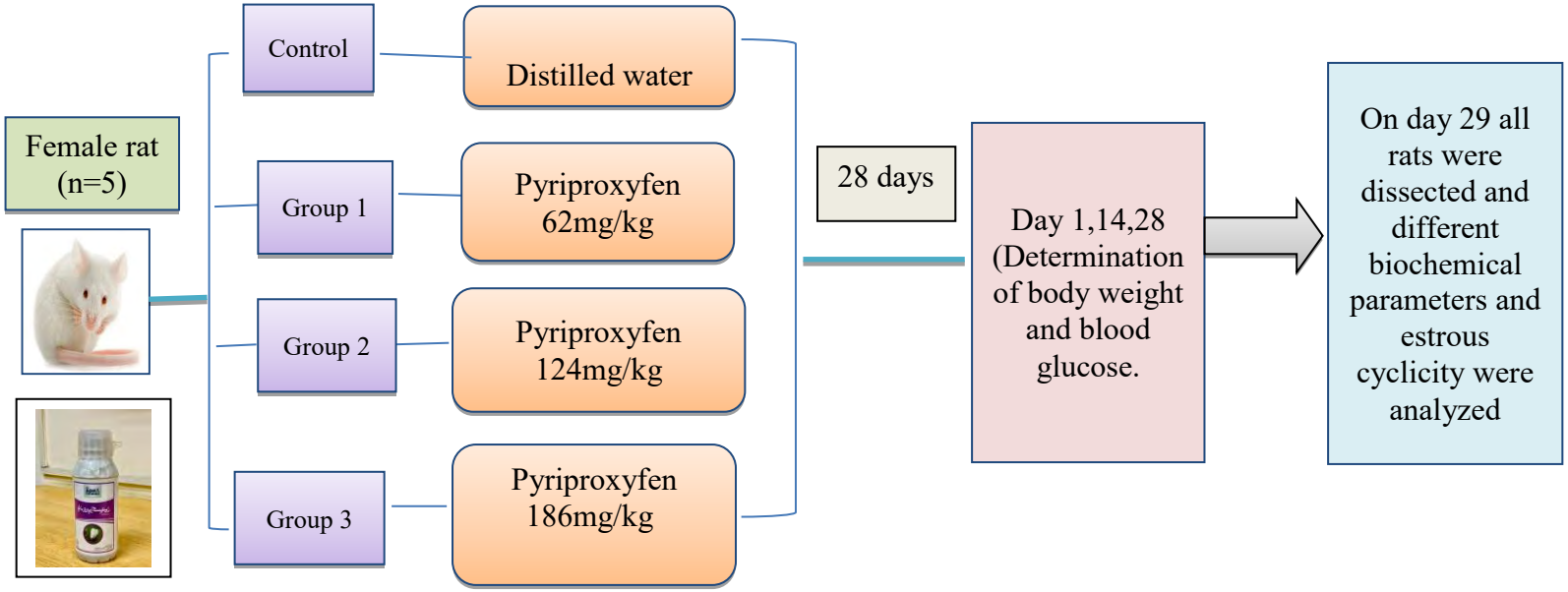



**Figure 4. Schematic representation of oral sub chronic administration of different doses of Pyriproxyfen in adult female rats.**

**Estrous cyclicity**

At the onset of puberty, the estrous cyclicity of all female rats was evaluated through different types of cells present in vaginal smear, collected two weeks gap over a period of 28 days. For examination of alterations that appear during the reproductive cycle, the short length of estrous cycle in rats, renders them an ideal animal model. As an indicator of ovarian activity, vaginal smear histology has consistently been used (Long and Evans, 1922).

**Vaginal smears**

Vaginal smear of the females was observed on every $14^{th}$ day for 28 days period.

**Procedure**

For estrous cyclicity a 100 µL micropipette was used in which Saline of 10-20 µL was filled in pipette and the tip of pipette was placed on the vaginal opening of female rat. Vagina was flushed with saline for three to five times and at the sixth time it was collected with the pipette. Used 10µL of normal saline for observation of vaginal cytology and the vaginal fluid was placed on glass slide (Caligioni, 2009).

**Staining**

1. The vaginal smear was spreaded on the slide to make a smear.
2. When the smear gets dried up, it was stained with few drops of haematoxylin stain.
3. Shaked the slide for few seconds, then extra stain was removed from the slide by tilting the slide.

4. Further put few drops of eosin stain on the smear, and again shaked it well to spread the stain on whole smear.
5. Tilted the slide again to remove extra stain. Then slide was washed gently with water four to five times to remove excessive stain.
6. All the procedures were done carefully to avoid washing of the vaginal cells.
7. This process is repeated for each slide.

**Hormonal analysis**

**Quantitative Determination of Progesterone and Estradiol Concentration:**

Progesterone and estradiol concentration in plasma were determined following the instructions provided by the manufacturers on the Pro ELISA kit obtained from (Bio check Inc, USA).

**Principle of Progestrone:**

Pro kit is based on competitive method, coat micro well plates with goat anti-rabbit to form solid face anti-body. Add pro antibody, Pro calibrator and HRP=Pro to form secondary antibody – antibody – HRP-Pro complex. The binding amount of HRP-Pro is reversely proportional to Pro content in serum. Remove the unbound Pro-HRP. Add substrate and detect absorbent value. Calculate Pro content of serum through computer or plotting fitting concertation—absorbent value curve.

**Procedure of Progesterone:**

1. Marked the microtitration strips to be used. All the Calibrators and controls should set duplicates.
2. *50µl* of calibrators / controls / samples dispensed into respective wells.
3. Dispensed *50µl* of HRP Conjugate to each well.
4. *50µl* of antibody was dispensed to each well.

5. Covered the strips with a plate sealer.Microtiter plate was swirled on flat bench in order to gently mixed it. The plate was Incubated at 37℃ for 1 Hour.
6. Washed each well for three times for 10 seconds each time.
7. Into each well *50µl* of chromogen A was dispensed.
8. *50µl* of chromogen B was dispensed to each well.
9. Covered the strips with a fresh plate sealer. Mixed it gently by swirling the microtiter plate on flat bench. Incubated the plate 37℃ for 15 Minutes.
10. *50µl* of stop solution was dispensed to each well and mixed completely.
11. Read the absorbance of the plate within 10 Minutes.

**Principle of Estradiol:**

E2 kit is based on competitive method, coat micro well plates with goat anti-rabbit to form solid face anti-body. Add E2 antibody, E2 calibrator and HRP=E2 to form secondary antibody – antibody – HRP-E2 complex. The binding amount of HRP-E2 is reversely proportional to E2 content in serum. Remove the unbound Pro-HRP. Add substate and detect absorbent value. Calculate E2 content of serum through computer or plotting fitting concertation—absorbent value curve.

**Procedure of Estradiol:**

1. Marked the microtitration strips to be used. All the Calibrators and controls should set duplicate.
2. In each well, dispensed *50 µl* of calibrators / controls / samples.
3. Distributed *50 µl* of HRP Conjugate to respectively well.
4. Plate sealer was used to covered the strips and mixed the strip gently on bench by swirling the microtiter plate. Incubated the plate at 37℃ for 1 Hour.
5. Each well was washed for three times and10 seconds each time.

6. *50 µl* of chromogen A was dispensed into each well.
7. *50 µl* of chromogen B was dispensed into each well.
8. Covered the strips with a fresh plate sealer. Mix it gently by swirling the microtiter plate on flat bench.15 minutes incubated the plate at 37°C.
9. *50 µl* of stop solution was poured to each well, mixed completely.
10. Read the absorbance of the plate within 10 Minutes.

**Quantitative Determination of Cortisol Concentration**

Using enzyme immunoassay (EIA) kits (Bio check Inc, USA) cortisol concentrations were evaluated quantitatively. The assay works on the following principle:

**Principle of Test:**

The basic principle of Cortisol ELISA lies in the competitive method. To the wells coated with streptavidin, the samples, a working solution of the HRP conjugate of cortisol, and added a solution of anti-cortisol-biotin. For binding sites, cortisol in the patient's serum competes with the cortisol enzyme (HRP) conjugate. Unbound cortisol was removed and cortisol enzyme conjugate by washing buffer. The concentration of cortisol is indirectly proportional to the intensity of the color upon tallying of substrate. A standard curve associating color strength to the concentration of cortisol was drawn.

**Procedure**

- Added 25µl of cortisol standard, control, and animal plasma. After this added 50µl of biotin reagent and 100µl of cortisol enzyme conjugate to each well, one by

one then mixed for about 10 seconds and incubation for about 60 minutes at room temperature were done.

- 300µl of wash buffer was used for washing. Then 100µl of TMB substrate and incubated for 15 minutes at room temperature.
- Finally added 50µl stop solution to all wells, mixed completely.
- Read the absorbance at 450 nm within 20 minutes.

**Statistical analysis**

SPSS 21 Software was used to compare the values of control and experimental groups, applying oneway analysis of variance (ANOVA). Dunnett's multiple comparison test was done post-ANOVA. Any number of $P<0.05$ was taken as statistically significant. Means ± standard errors of means (SEM) were determined for all value.

# Results

## Body weight

Mean ± SEM body weights of female rats exposed to different concentrations of Pyriproxyfen are presented in Table 1. Decrease in body weight of rats of all groups was seen towards end of our experiment (day 28) as compared to initial weight (day 1), however the change was not significant. Moreover, no significant difference in final body weight of pyriproxyfen treated groups was observed as compared to control.

**Table 1. Mean ± SEM body weight (g) of rats treated with different doses of Pyriproxyfen.**

| Treatment | Day 1 | Day14 | Day 28 |
|---|---|---|---|
| **Control** | 155.28±10.87 | 153.31±11.47 | 151.10±11.4 |
| **G1 (62mg/kg)** | 147.27±6.87 | 158.58±8.26 | 144.53±8.73 |
| **G2 (124mg/kg)** | 165.27±12.57 | 170.70±16.5 | 164.07±15.9 |
| **G3 (186mg/kg)** | 169.25±8.77 | 162.10±8.43 | 153.02±9.74 |

## Organs and reproductive Organs weights

## Ovaries weight

Table 2 shows the mean ± SEM weights of organs and reproductive organs weights in control and treated groups. A dose dependent significant reduction ($P<0.01$) in the weight of ovaries in all treated groups as compared to control.

## Uterus Weight

An increase was observed in weight of uterus in G1 and G3 as compared to control, however the change was not significant. There was significant ($P<0.05$) elevation was observed in the weight of uterus in G2 with reference to control (Table no. 2).

**Kidneys weight**

A significant increase ($p<0.05$) in kidneys weight was seen in all the pyriproxyfen treated groups as compared to control. No significant change was observed within treated groups (Table no. 2).

**Liver weight**

There was observed a little increase in liver weight in G1 as compared to control, however, the change was not significant. A significant increase in liver weight was indicated when G2 ($p<0.05$) and G3 ($p<0.001$) were compared to control. Non-significant difference among G1 and G2, while, a remarkable increase ($p<0.001$) was evident among G1 and G3. A significant elevation ($p<0.01$) was detected in liver weight of G2 as compared to G3 (Table no. 2).

**Heart weight**

No significant change was observed in weight of heart among G1 and G3 as compared to control, however, there was observed a significant increase ($P<0.05$) in G2 rats when compared to control. Non significant change was observed in weight of heart within treated groups (Table no. 2).

**Table 2. Effect of Different doses of Pyriproxyfen on Mean ± SEM weights of ovaries, uterus, kidneys, liver and heart among experimental groups of adult female rats.**

| Groups (n=5) | Ovaries(g) | Uterus(g) | Kidneys weight(g) | Liver weight(g) | Heart weight(g) |
|---|---|---|---|---|---|
| **Control** | 0.12±0.007 | 0.312±0.07 | 0.49±0.03 | 5.79±0.29 | 0.64±0.05 |
| **G1 (62mg/kg)** | 0.086±0.009[a**] | 0.484±0.09 | 0.67±0.07 [a*] | 6.26±0.26 | 0.78±0.06 |
| **G2 (124mg/kg)** | 0.076±0.005[a**] | 0.574±0.08 [a*] | 0.69±0.06 [a*] | 7.14±0.42 [a* c**] | 0.75±0.07 [a*] |
| **G3 (186mg/kg)** | 0.072±0.006[a**] | 0.434±0.09 | 0.68±0.04 [a*] | 8.81±0.50 [a*** b***] | 0.71±0.06 |

Values are expressed as mean ± SEM *p< 0.05 **p< 0.01, ***p< 0.001

a = Value vs control b = Value vs G1= Value vs G2

**Body Mass Index**

When compared to control non significant decrease was observed in BMI of G1. A significant reduction was seen in G2 ($p<0.01$) and G3 ($p<0.05$) as compared to control. No significant change was observed when comparison was made within groups (table 3).

**Table 3. Effect of Different doses of Pyriproxyfen on Mean ± SEM BMI (g/cm$^2$) of control and treated adult female rats.**

| Groups (n=5) | BMI(g/cm$^2$) |
|---|---|
| **Control** | 0.147±0.009 |
| **G1 (62mg/kg)** | 0.130±0.003 |
| **G2 (124mg/kg)** | 0.124±0.006 $^{a**}$ |
| **G3 (186mg/kg)** | 0.128±0.005 $^{a*}$ |

*p< 0.05, **p< 0.01, ***p< 0.001, values are stated as mean ± SEM showing significant Variance respectively.
a value used in comparison of control, while b value used in the comparison of G1 and c value used in comparison of G2.

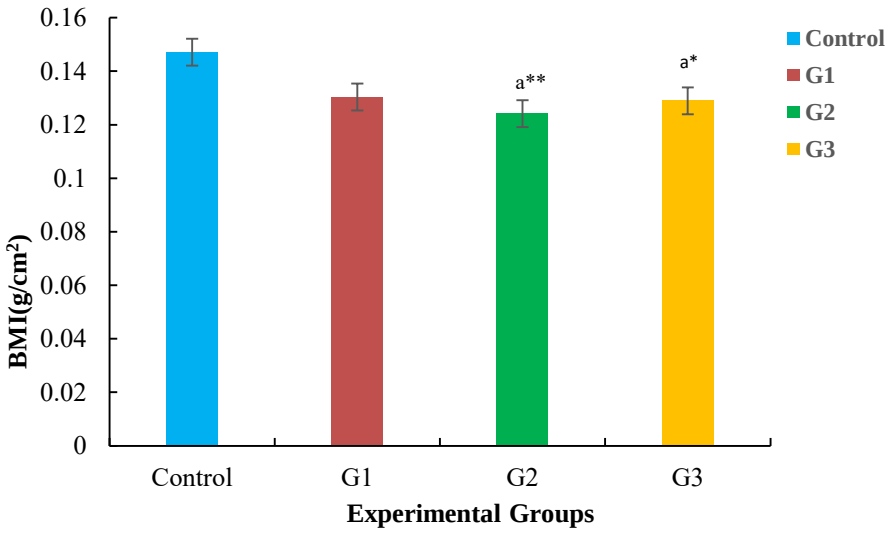



**Figure 4: Mean±SEM BMI(g/cm$^2$) among adult female Sprague Dawley rats following treatment with Pyriproxyfen.**

**Blood Glucose**

The level of glucose in the blood of rats exposed to different concentrations of pyriproxyfen is presented in Table 4, A significant increase (P<0.001) in blood glucose levels of G2 and G3 from day 1 to day 14 was observed while high significant decrease (p<0.001) was seen in blood glucose level of all treated groups between day 14 and 28.

**Table 4. Mean ± SEM blood glucose level (mg/dl) of rats at different days of estrous cycle treated with different doses of Pyriproxyfen for 28 days.**

| Treatment | Day 1 | Day14 | Day 28 |
|---|---|---|---|
| **Control** | 107.4±2.01 | 115.2±1.56 | 104±2.07 |
| **G1 (62mg/kg)** | 114.6±4.10 | 108.2±2.43 [a***] | 73.6±3.32 [a***] |
| **G2 (124mg/kg)** | 96±6.48 [a***] | 107.6±7.16 [a***] | 93.4±4.11 [a***] |
| **G3 (186mg/kg)** | 104±4.11 [a***] | 110±3.30 [a***] | 85.4±2.52 [a***] |

Values are stated as Mean ± SEM. *, **, *** showing significant variance at P<0.05, P<0.01 and P<0.001 vs control.

**Estrous cyclicity**

Estrous cycle regularity was determined through vaginal smears, obtained every 7$^{th}$ day morning during a period of 28 days, for Pyriproxyfen treated groups as well as for control females. Comparison of control and PPF treated animals' vaginal smears was made. Animals treated with low dose (62mg/kg) PPF remained at metestrus stage for maximum days, displaying abnormality in the reproductive cycle in comparison to control group. The length of proestrus shortened and the length of metestrus phase increased in animals treated with dose of PPF (124mg/kg) compared with control. Prolonged diestrus phase was observed in animals' vaginal smears treated with doses (186/kg) presenting irregularity of the sexual cycle when matched

with control group presented in Table 7. Estrous cycle of control group showed all stages, with different types of cells as shown in Figure and exhibit normal estrous cycle of 4-5 days duration.

**Table 5: Different phases of estrous cycle of rats treated with Pyriproxyfen taken on different days of experiment.**

| **Gr*oup*s** | **Day 1** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
|---|---|---|---|---|---|
| **Control** | **+** | **+++** | **++++** | **++** | **+++** |
| **G1** | ++ | +++ | +++ | +++ | +++ |
| **G2** | +++ | + | +++ | +++ | + |
| **G3** | ++++ | ++ | ++++ | +++ | ++++ |

+ = Proestrus, ++ = Estrus, +++ = Metestrus, ++++ = Diestrus

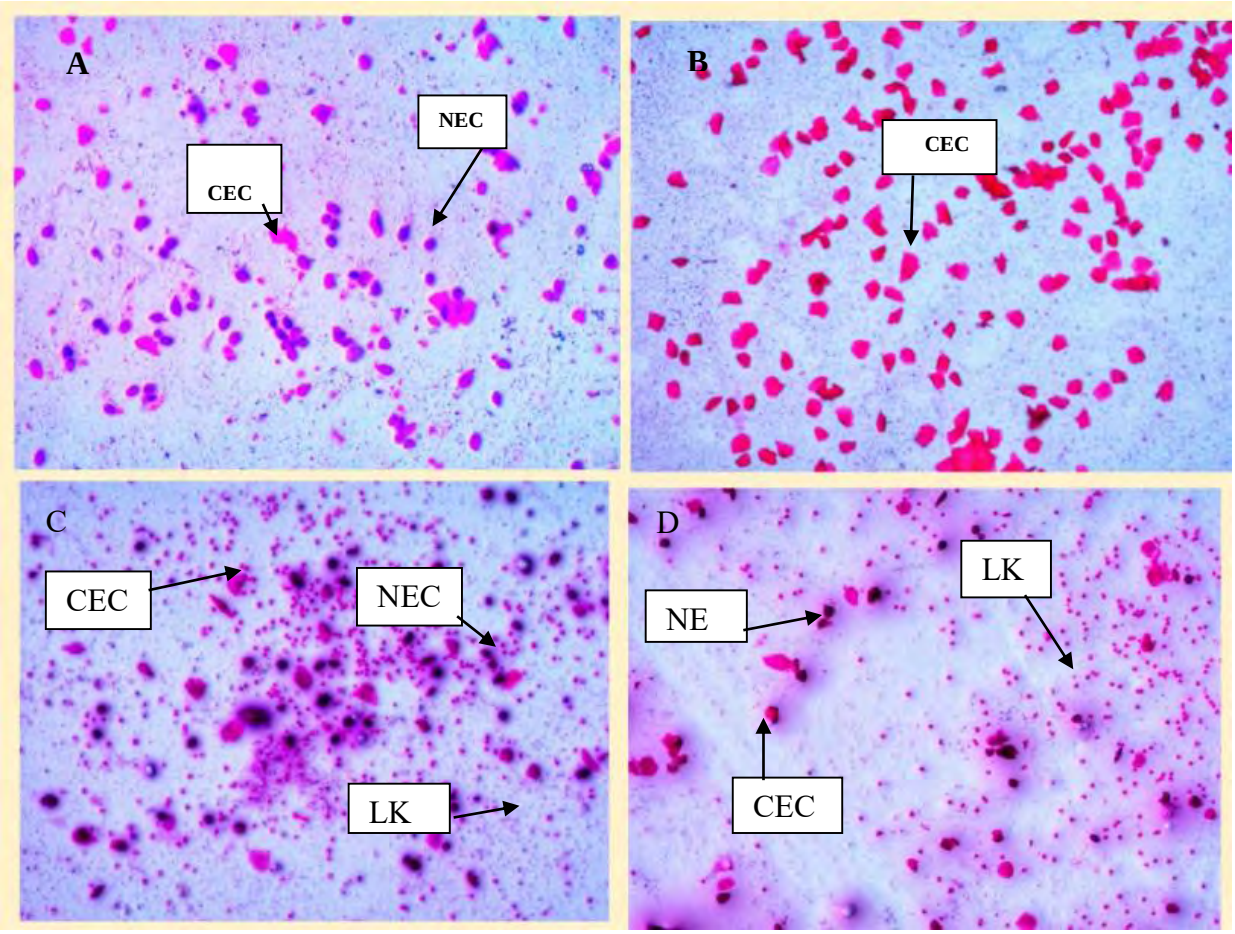



**Figure 5: Photomicrograph of vaginal smears at different stages of estrous cycle. (A) Proestrus, with numerous round nucleated epithelial cells (NEC). (B) Estrus, clumps of cornified epithelial cell (CEC). (C) Metestrus, with round nucleated epithelial cells, cornified epithelial cells and a high number of leukocytes. (D) Diestrus, with numerous leukocytes (Lkc). (10X magnification).**

**Hormonal Analysis**

The adjustment in plasma Estradiol, progesterone and cortisol (ng/ml) in adult female rats following 28 days of treatment has been given in Table 5. The decrease in concentration of plasma Estradiol and progesterone concentrations were seen as compared to control, while increase was observed in G3 as compared to G1and G2, however, the change was not significant.

A highly significant reduction ($p<0.001$) in plasma cortisol level between control and all the treated groups. Non significant change was observed in plasma cortisol level within all the treated groups (figure 5).

**Table 6: Mean ± SEM plasma concentrations of progesterone (ng/ml), estradiol (ng/ml) and cortisol (ng/ml) concentration in control and treated groups after 28 days of treatment.**

| Groups (n=5) | Progesterone (ng/ml) | Estradiol (ng/ml) | Cortisol (ng/ml) |
|---|---|---|---|
| **Control** | 77.30±4.87 | 50.71±2.23 | 84.8±8.40 |
| **G1 (62mg/kg)** | 63.14±3.74 $^{a*}$ | 29.66±3.04 $^{a**}$ | 39.8±2.70 $^{a***}$ |
| **G2 (124mg/kg)** | 62.98±4.18 $^{a*}$ | 27.90±3.39 $^{a**}$ | 33.6±2.44 $^{a***}$ |
| **G3 (186mg/kg)** | 74.96±2.21 | 32.42±3.71 $^{a*}$ | 31.6±3.47 $^{a***}$ |

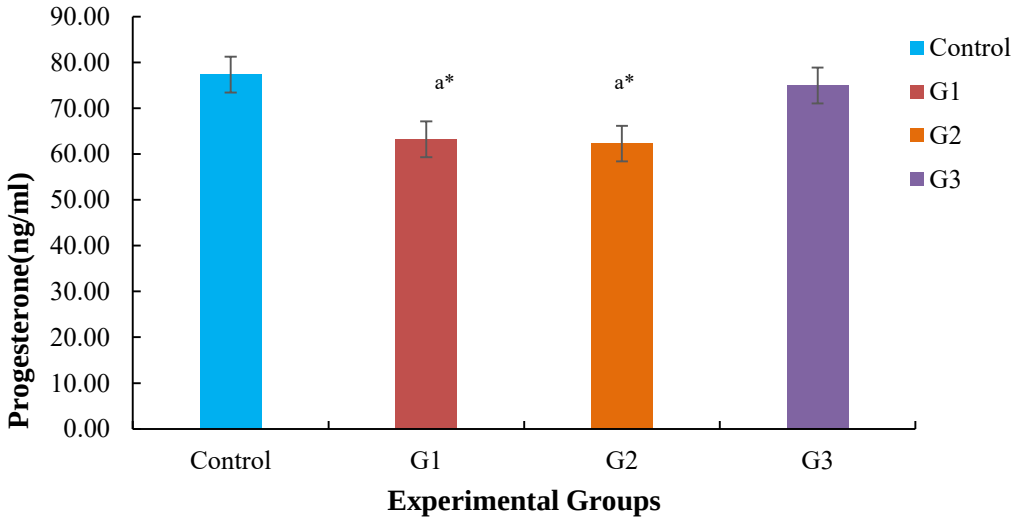



**Figure 6: Mean±SEM plasma progesterone concentrations(ng/ml) among adult female Sprague Dawley rats following treatment with Pyriproxyfen.**

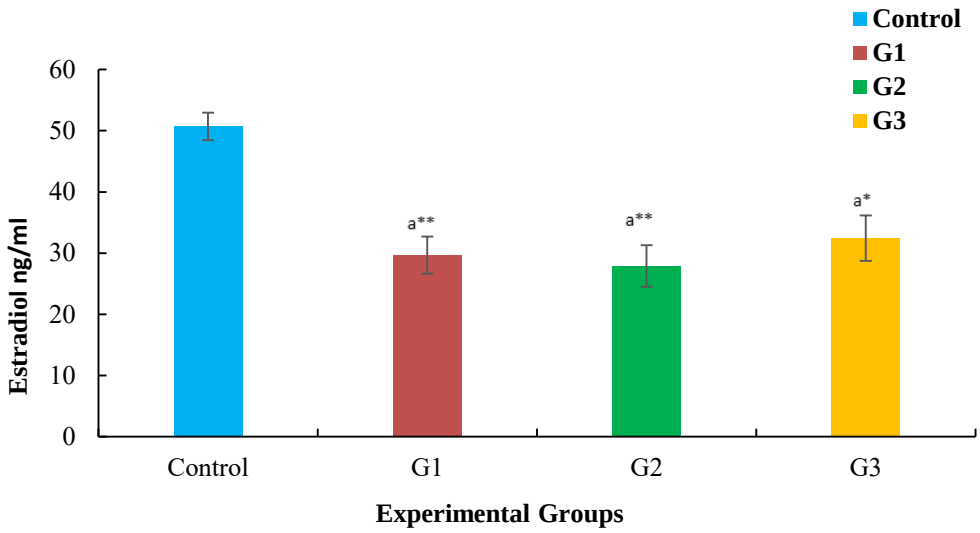



**Figure 7: Mean±SEM Estradiol concentrations(ng/ml) among adult female Sprague Dawley rats following treatment with Pyriproxyfen.**

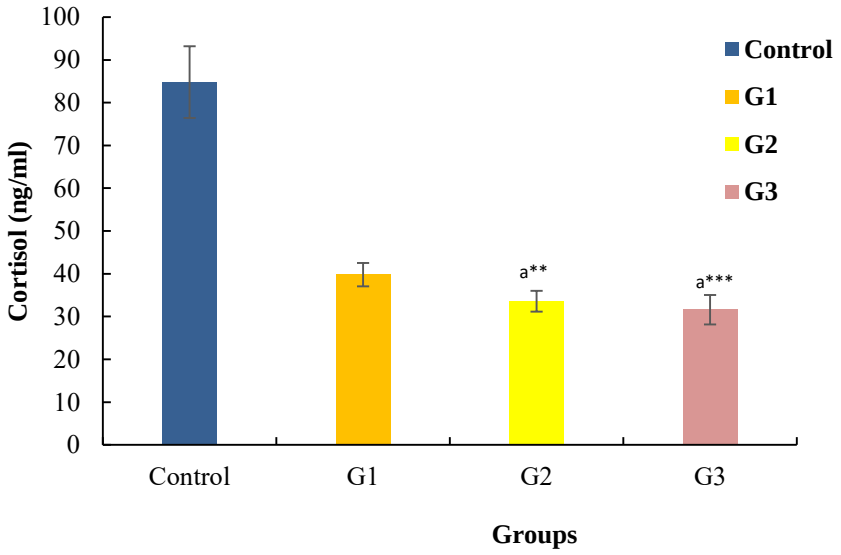



**Figure 8: Mean±SEM Cortisol concentrations (ng/ml) among adult female Sprague Dawley rats following treatment with Pyriproxyfen.**

## DISCUSSION

Agricultural products have increased due to the use of pesticides, which are used in agriculture to protect plants from pests. Despite the fact that their use is crucial for crop yield, their excessive use also pollutes our environment. These substances have an impact on the cardiovascular system, neurological system, reproductive system, skin, liver, spleen, kidneys and lungs. (Hashimi *et al*., 2020). The pesticide Pyriproxyfen is an analog of juvenile hormone, has endocrine disrupting potential seen through its actions in causing insufficient hormone production, irregular estrous cycles, which can lead to subfertility or infertility. PPF has greater stability in environment and tenacity through food chain causes harmful effects on non-target species (Mehrnoush *et al*., 2013). Therefore, the present study aimed to documented the reproductive toxicity potential of PPF in adult female Sprague Dawely rats through monitoring estrous cycle and ovarian steroidogenic efficiency. In order to determine reprotoxic effect of pesticide, rats were orally administered with different doses of PPF for 28 days to see its profound effects on female organs and reproductive organs under different concentrations of the dose. The three doses were calculated according to the body weight of rats and were 62mg/kg, 124mg/kg and 186mg/kg of PPF.

The current study revealed the effect on the body weight of female rat treated with different doses of PPF. The decline in the finale body weight gain was observed in the rats of all the groups when compared to control. Our results are in according with previous studies conducted by Shahid *et al*., (2018), exposure to different doses of PPF (1200, 600, 320, 200, 100, 40, 20, 0 mg/kg) in male mice for 28 consecutive days caused a significant decline in body weight. Recent studies have also shown that

PPF exposure to pregnant female mice also results in lowered body weight (Shahid and saher, 2020).

PPF is known to act as endocrine disruptor as it is shown to be deposited in the ovaries (Linton *et al*., 2009). Previous studies by Fowler *et al* 2021 showed that PPF exposure reduces reproductive fitness in mosquitos by affecting ovarian function. No studies regarding PPF toxicity on ovarian weight have been published in rats. In the current study a dose-dependent significant decrease was observed in the weight of ovaries in all the experimental groups as compared to control. The decrease in ovarian weight might be due to the accumulation of PPF in the ovarian tissue, as PPF accumulation could be due to the absence of specific catalytic enzymes (Homola and Chang, 1997; abdu *et al*., 2001). Similarly, an increase was observed in the weight of uterus in G1 and G3 as compared to control. This increase might be due to pyriproxyfen deposition in the uterine tissue. However, the underline mechanisms are unknown. In our study, the kidney, liver and heart weights were increased in the all dose dependent groups with reference to control. Recently, literature also reported that female pregnant mice exposed to different doses of PPF resulting in the increase of organs weight (Shahid and saher, 2020). The change in PPF treated kidneys weight might be due to edema, increased Bowman's space, glomerular degeneration, and degeneration of the lumen of renal tubules (Naseem *et al*., 2022). The increase in liver weight might be due to production of pyriproxyfen metabolites and slow metabolic rate of these metabolites in rat liver. This may be due to its enrichment ability and higher fat solubility. Future research should, however, focus on the molecular mechanisms of PPF and metabolite toxicity to better understand the PPF-induced cytotoxic pathway (Liu *et al*., 2020). The elevation in the weight of heart reported in our study might be because PPF is known to induce cardiotoxicity by removing

attachment between heart and common cardial vein to migrate dorsally and therefore heart muscles are stretched mechanically (Antkiewicz *et al*., 2005). No report regarding PPF toxicity on heart weight have been published in rats.

A significant decrease was also seen in BMI of G2 (124mg/kg) and G3 (186mg/kg) groups as compared to control. Previous studies on human have showed similar results where insecticide exposure is known to be a possible cause of reduced BMI, however, no data regarding correlation of PPF and BMI has been reported using rat model.

Our current results revealed effective increase in glucose level of female rats after exposure to PPF doses from day 1 to day 14 but effective decrease was noticed in glucose level of treated groups from day 14 to 28 when compared with control. These findings resembled with the previous work of Naseem *et al*. (2022), where pyriproxyfen at a dose of 300,600 and 900µl were administered to *labeo rohita* fish, glucose level was observed to be increased. Our results are also in accordance with the earlier studies by Etebari *et al*. (2007), which showed reduction in the glucose level in silkworm larva when they fed on melberruy leaves sprayed by PPF after 24 hours. The change in glucose level might be due to starvation stress because pyriproxyfen has antifeedant characteristics to prevent animals from feeding (Etebari *et al*., 2007).

Female rats are polyestrous. The reproductive cycle in rat is about 4-5 days and the length of each phase of cycle is as follows: proestrus 12-14 hours, estrus 25-27 hours, metestrus 6-8 hours, and diestrus 55-57 hours (Long and Evans, 1922; Antunes *et al*., 2016; Sanabria *et al*., 2019). Sexual cyclicity is regulated through activation of HPG axis by a cascade of neuroendocrine events. When analyzing the hypothalamus-pituitary-ovary reproductive axis, the estrous cycle can be a useful

tool.PPF treated rats among G1 and G3 groups in the present analysis revealed irregularity in the estrous cycle with prolonged diestrus phase, while G2 showed increased in Metestrus phase and shortened proestrous phase when compared with control group. Our results are in agreement with outcomes of Baligar and Basappa, (2002), who reported that pesticide carbofuran administered orally to virgin female mice for 30 days, the number of estrous cycle was decrease and there is elongation in diestrus phase in among control and treated groups. The irregularity in estrous cycle might be due to effect of Pyriproxyfen on impairment of HPG axis which in turn effect gonadal female hormones. Thus there will be decrease in number of healthy follicles and increase in atretic follicles number.

For studies of reproductive toxicity, measurement of sex hormone has been deliberated as one of the most functional and integrative point (Ji *et al*.,2013). Pyriproxyfen might been witnessed to cause reproductive and endocrine impairment that interfere with HPG axis, Maharajan *et al*. 2021, have documented in their studies that male and female zebrafish exposed separately to different doses of PPF for 21 days, the estradiol level was suppressed in female zebrafish. In contrast to previous work of Manabe *et al*. (2006), mixture of two pesticide prothiofos/pyriproxyfen increased estradiol level by using MtT/Se cells proliferation assay. In the present findings, all the treated groups showed reduction in plasma estradiol level as compared to control. The decrease in estradiol level might be due to PPF may inhibit aromatase enzyme production so the expression of CYP19a gene might be decreased that may affect the female reproduction (Cao *et al*., 2016; Li *et al*., 2019). Similarly, a reduction was observed in the concentration of plasma progesterone level as compared to control. Our results are supported by Neito *et al*. (2021), who documented decrease in progesterone serum level in virgin adult rats exposed to

different doses of another insecticide chlorpyrifos. The decrease in progesterone concentration might be due to PPF effect on steroidogenic pathway by inhibiting expression of Cytochrome P450 proteins and Steroidogenic Acute Regulatory Protein genes (Fei *et al*., 2010).

In the current study, a prominent decrease in cortisol concentrations were detected in the all treated groups when a comparison was made with the control group. Other studies that are in support of our present results include efforts of Gusso *et al*. (2020), where exposure of different doses of PPF (0.125, 0.675, and 1.75 mg/l) in zebrafish showed a reduction in cortisol level of treated group as compared to control. however, our results showed decrease in cortisol level.

**Conclusion:**

It is concluded from the present study that exposure to PPF for 28 consecutive days resulted in decrease in body weight, ovarian weight and BMI, while increase in o reproductive and body organs. We also found alteration in blood glucose concentrationsand irregularity in the phases of estrous cycle by looking at vaginal smears with some days gap (after each 14 days). Furthermore, PPF exposure resulted in reduction of blood plasma cortisol, estradiol and progesterone concentrations in rats. It can be suggested from the present findings that PPF exerts toxic effects on reproductive health of females and act as an endocrine disruptor, however, further similar studies can be designed to elucidated the underlying mechanisms responsible for inducing reproductive toxicity in animals, by monitoring actions of PPF on cellular interactions in ovaries and their molecular pathways.

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