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Pyriproxyfen induced impairment of reproductive endocrine homeostasis and gonadal histopathology in zebrafish (*Danio rerio*) by altered expression of hypothalamus-pituitary-gonadal (HPG) axis genes

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Abstract

Pyriproxyfen (PPF), a broad-spectrum of insecticide known to cause reproductive and endocrine disruption in invertebrates, while the data is scarce in aquatic vertebrates. The goal of this study is to investigate the impact of PPF on reproductive endocrine system of male and female zebrafish along hypothalamus-pituitary-gonadal (HPG) axis. In brain, PPF caused significant alteration in the transcripts of *erα*, *lhβ,* and *cyp19b* genes in male and *fshβ, lhβ,* and *cyp19b* genes in female zebrafish. The downstream genes of steroidogenic pathway like, *star, 3βhsd, 17βhsd,* and *cyp19a* expression were significantly altered in gonad of both sexes. Subsequent changes in circulatory steroid hormone levels lead to imbalance in hormone homeostasis as revealed from estradiol/testosterone (E_2/T) ratio. Further, the vitellogenin transcript level was enhanced in hepatic tissues and their blood plasma content was increased in male (16.21%) and declined in female (21.69%). PPF also induced histopathological changes in gonads such as, reduction of mature spermatozoa in male and vitellogenic oocytes in female zebrafish. The altered E_2/T ratio and gonadal histopathology were supported by the altered transcript levels of HPG axis genes. Overall, these findings provide new insights of PPF in zebrafish reproductive system and highlights for further investigations on its potential risks in aquatic environment.

Keywords: Endocrine disruption, Testosterone, Estradiol, Vitellogenin, Testes, Ovary

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1. Introduction

Pyriproxyfen (PPF), a pyridine based insect growth regulator with broad-spectrum of insecticidal activity against various pests of agriculture, horticulture and public health (Liu et al., 2019). PPF have been applied in drinking water sources at maximum concentration of 10 µg/L (WHO, 2007) and upto 100 µg/L in water bodies as vector control measures (Lajmanovich et al., 2019). Besides, the environmental occurrence of PPF was widely reported in river waters (Campo et al., 2013; Masiá et al., 2015; Ccanccapa et al., 2016) with the maximum level of 89.66 ng/L and traceable amount in fish of Jucar River, Spain (banned by EU, since 22/9/2010) (Belenguer et al., 2014). Many studies have demonstrated the adverse effect of PPF in non-target organisms at the concentrations used in mosquito control (Lawler, 2017). For instance, PPF induced toxicity has been reported in honey bees, crab, shrimp, *Daphnia* (Azevedo-Linhares et al., 2018) and zebrafish embryos (Truong et al., 2016; Maharajan et al., 2018).

Reproduction is the most important phenomenon in all life forms to maintain the ecological balance. Over the last few decades, studies have evidenced that exposure to environmental pollutants cause adverse effects in the reproductive endocrine system and are potentially linked to decline in fertility of animals and humans (Chen et al., 2016; Wang et al., 2019). PPF mimics the juvenile hormone, cause defective reproductive functions throughout all life stages of insects such as embryogenesis, metamorphosis and adult reproduction even at low concentrations (Moadeli et al., 2014; Chłopecka et al., 2018). The endocrine disruptive potential of PPF has been widely reported in various invertebrate organisms (Kakaley et al., 2017; Tanaka et al., 2018) with multiple effects on reproduction, alteration in storage lipids, changing neonates sex into male, reduction in fecundity and subsequent ecological effects in *Daphnia* (Ginjupalli et al., 2015; Chłopecka et al., 2018; Watanabe et al., 2018). The estrogenic activity of PPF was also evidenced *in vitro* system

through reporter gene assay (Kojima et al., 2005) and cell proliferation assay using the estrogen-responsive MtT/Se (rat pituitary carcinoma) cell line (Manabe et al., 2006). In addition, PPF affects the thyroid endocrine system in tadpoles of *Odontophrynus americanus* (Lajmanovich et al., 2019). Recently, Shahid et al. (2019) has revealed PPF induced defective reproductive system of male mice and damages the testicular architecture and spermatogenesis. However, the impacts of PPF on reproductive functions of aquatic vertebrates are relatively unknown. The recent FAO/WHO (2019) report has strongly emphasized the knowledge gap and lack of data to substantiate the estrogenic potential of PPF in vertebrate organisms.

The reproductive endocrine system of vertebrates are controlled by hypothalamic-pituitary-gonad (HPG) axis which plays crucial role in maintaining hormone homeostasis by regulating their synthesis, transport, and metabolism. Zebrafish (*Danio rerio*) has been widely accepted vertebrate model to assess the reproductive toxicity of various environmental contaminants (Cao et al., 2019). Hence, the aim of this study is to investigate the potential adverse effect of PPF in male and female zebrafish reproduction through transcriptional regulation along HPG axis, circulatory sex hormone and vitellogenin (Vtg) measurement and the histopathology of gonads.

2. Material and methods

2.1. Chemicals

Pyriproxyfen (4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether) was purchased from Sigma Aldrich, USA, with ≥ 98.0% purity (CAS No.95737-68-1; Cat No. 34174). Initially, PPF was dissolved in 100% DMSO as stock (20 mg/mL) and stored at 4 °C for further use. Other chemicals and reagents utilized in this study were of analytical grade.

2.2. Zebrafish maintenance and exposure

Adult zebrafish (wild type, four month old) was maintained in the laboratory condition as described by Maharajan et al. (2018). Fish were fed twice a day with commercial feed (Taiyo max). Healthy male and female fish were separated and exposed with 1, 10 and 100 µg/L of PPF for 21 days. The exposure concentrations were chosen based on the environmental occurrence and the recommended application level of PPF in water sources (WHO, 2007; Belenguer et al., 2014; Lajmanovich et al., 2019). Twelve fish of each sex were transferred to tank containing 5 L of respective exposure medium. Additionally, the male and female control groups were separately maintained without adding PPF. The exposure medium was renewed with PPF at every 24 h interval throughout the study period. The experiment was performed according to OECD 229 guidelines for fish short term reproduction assay (OECD 229, 2012). All experiments were carried out in triplicates. During exposure period, no mortalities were observed in control and treated groups.

2.3. PPF analysis in exposure medium

After PPF exposure, the nominal concentrations in the medium were measured by Gas Chromatography Mass Spectrometer (GC-MS). From each treatment group, the exposure medium was collected immediately after exposure (T_0) and prior to medium renewal (T_{24}). The extraction method of PPF was adapted from our previous work with minor modifications (Maharajan et al., 2018). In brief, 500 mL of exposure medium was collected in duplicate from each PPF treatment group at the time of exposure (T_0) and prior to renewal (T_{24}) of test medium. The samples were transferred to separating funnel and 10 g of sodium chloride was added to increase the ionic strength. Then, the samples were extracted twice with 50 mL of n-hexane and dehydrated by adding 2 g of anhydrous sodium sulfate. Further, the extract was transferred to condensation flask and it was allowed to near dryness in the rotary evaporator (Buchi Rotavapor, Switzerland). Finally, the flasks were rinsed well with n-hexane and transferred to auto sampler vial (Agilent Technologies, USA) for analysis.

Samples were analyzed by Gas Chromatography Mass Spectrometer (GC-MS/MS) (Agilent Technologies, USA; Model: GC-7890B, MS-7000C) equipped with capillary column (HP-5MS, 30 m x 0.25 mm internal diameter, 0.25 µm film thickness). The analytical separation was carried out in the following instrument conditions; Initial GC oven temperature was set at 120 ºC for 2 min and the temperature was gradually increased to 280 ºC at the rate of 10 ºC/min. Then the column oven temperature was increased to 320 ºC at the rate of 20 ºC/min and held for 2 min. Sample injection (1 µL) was performed using an autosampler (G3186) in split-less mode. Helium (99.999 % purity) was used as a carrier gas at a flow rate of 1.0 mL/min. Interface temperature was set at 280 ºC. MS was operated in electron ionization mode at 70 eV. MRM transitions, 136.1 > 96 and 186.1 > 77.1 were used as quantifier and qualifier respectively for PPF. Calibration standards were prepared from PPF stock solution by diluting with n-hexane. Nine point calibration curve was generated from 0.2 to 50 ng/mL using linear regression analyses and the linearity was qualified by linear correlation coefficient, R^2 (Fig. S1). Limit of detection (LOD) and limit of quantification (LOQ) were 0.1 and 0.3 ng/mL respectively, and the average recovery of PPF was 96.4%. The Standard Chromatogram of PPF was shown in (Fig. S2) with retention time of 14.70 min. The PPF was quantified based on peak areas of external calibration.

2.4. Sample Collection

After 21 days exposure, fish were anesthetized with 3-aminobenzoicacid ethyl ester solution (0.1 mg/L) and the target organs like, brain, liver and gonad of male and female zebrafish were carefully dissected out for the gene expression. The dissected samples were stored in -80 °C for further analysis.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from brain, liver and gonad samples using TRI Reagent (Sigma Aldrich, USA) following manufacturer's instructions. Quantity and purity of the extracted RNA was confirmed by nanodrop (Synergy H1, BioTek, USA) and the absorbance at A_{260}/A_{280} ratio of >1.95 was used for cDNA conversion. cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). qPCR was performed in Light Cycler 480 (Roche, Germany) using SYBR® Premix Ex Taq™ II (TliRNaseH Plus, TaKaRa, Japan). The primer sequences of HPG axis genes such as *erα, ar, fshβ, lhβ, cyp19b, fshr, lhr, star, 3βhsd, 17βhsd, cyp19a, vtg1,* and *β-actin* were selected from previously published literatures (Liang et al., 2015; Xu et al., 2017; Siegenthaler et al., 2017) (Table S1). Each target mRNA expression level was normalized to the level of reference gene (*β-actin*) expression. The relative quantification of mRNA transcript of the target genes were analyzed through $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. Plasma Testosterone, Estradiol and Vitellogenin measurement

Blood samples were collected for plasma sex hormone analysis and Vtg measurement by following the procedure of Ji et al. (2013a). Briefly, 6 male and 6 female fish were randomly selected from the respective treatments and blood samples (4-6 µL) were collected from caudal vein of zebrafish using heparinized glass capillary tube. Because of the small sample volume, the collected blood samples from each sex of respective treatment were pooled together and samples were centrifuged at 5000 g for 20 min for plasma separation. The obtained plasma samples were stored at -80 °C until analysis. Sex steroid hormones, testosterone (T) and estradiol (E_2) were quantified by enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemicals, USA) as per manufacturer's instruction. Plasma Vtg levels were measured by Fish Vitellogenin ELISA kit (Origin Labs, India) following manufacturer's instructions. All samples and standards were run in triplicate.

2.7. Histopathological examination of gonads

Histopathological studies were carried out in male and female gonads as described by Teng et al. (2018). Briefly, the gonad was carefully dissected out from the male and female zebrafish and fixed in 10% formalin for 48 h. Later, the tissues were dehydrated in series of graded ethanol and xylene, and finally embedded in paraffin wax. Approximately, 5 mm thin sections of the tissue was made using microtome and stained with hematoxylin and eosin. The stained slides were examined under inverted microscope (EVOS FLc, Life Technologies, USA). The histopathological changes and the stages of gonadal development were assessed by following OECD, 123 guidelines (OECD, 2010). The testes were staged into immature spermatocytes (spermatogonia and spermatocytes) and mature spermatocytes (spermatids and spermatozoa) as described by Cao et al. (2016). The relative percentage (%) of immature and mature spermatocytes in testes was obtained by measuring the surface area at 200 x magnification using ImageJ Software (National Institute of Health, Bethesda, MD). The Ovaries were staged into primary oocytes (PO), cortical alveolar oocytes (CO), early vitellogenic oocytes (EVO) and vitellogenic oocytes (VO). The numbers of each stage were analyzed at 100 x magnification and the data was presented as oocytes percentage (%) in ovary.

2.8. Statistical analysis

For statistical analysis, GraphPad Prism software (Version 5.1) was used. One way analysis of variance (ANOVA) with Tukey's post hoc test was used to calculate the statistical significance ($p < 0.05$, $p < 0.01$) between the control and PPF treated groups. All data were expressed as mean ± SEM except for chemical analyses wherein mean ± S.D. was used.

3. Results and discussion

3.1. Chemical analysis

The nominal exposure concentrations of PPF treated medium were quantified by GC-MS analysis at T_0 and T_{24}. The result shows that the measured concentrations of PPF in the medium were marginally lowered as 0.94, 9.76 and 96.71 µg/L at T_0 compared to nominal level of 1, 10 and 100 µg/L, respectively (Table 1). Whereas, the PPF concentrations at T_{24} was further declined at the rate of 31.27%, 30.93% and 35.81% than T_0 in the medium. This result was consistent with our earlier work of Maharajan et al. (2018), that PPF concentration in the embryo medium was declined after 24 h. Furthermore, Horie et al. (2017) also found the similar reduction of PPF in the measured concentration than the nominal concentration of the exposure medium. There are many reasons to be attributed with the decline in the measured level of PPF such as, adsorption, experimental error or through metabolism (Zhao et al., 2015; Shi et al., 2017). However, more toxicokinetic study on PPF metabolites or bioaccumulation aspect is necessary to substantiate the reason for the loss.

Table 1: Nominal and exposure concentration of PPF

Nominal concentration (µg/L)	Measured level of PPF in the treatment medium (µg/L) and deviation (%)[a]		
	T_0	T_{24}	Deviation (%)
Control	n.d.[b]	n.d.	n.d.
1	0.94 ± 0.05	0.65 ± 0.11	-31.27
10	9.76 ± 0.21	6.74 ± 0.49	-30.93
100	96.71 ± 2.51	62.07 ± 7.53	-35.81

Note: [a] Deviation = (Measured level (T_0- T_{24}))/Measured level (T_0) x 100%.

[b] n.d = not detected.

3.2. Effect of PPF on HPG axis gene expression

The measurement of HPG axis gene transcripts has been widely used as a functional biomarker of hormonal changes caused by endocrine disruptors (Lee et al., 2018). The estrogenic compound binds with nuclear estrogen receptor (nER) along HPG axis and impedes the reproductive functions by regulating steroid hormone synthesis (Muthulakshmi et al., 2018). The follicle stimulating hormone (FSH) and luteinizing hormone (LH) are the key gonadotropin hormones released by pituitary gland which is involved in sex hormone synthesis (Qiu et al., 2019). The endogenous estrogen biosynthesis is regulated by aromatase *cyp19b* (cytochrome P450 aromatase 19b) in brain. In this study, the expression of *erα* and *cyp19b* was significantly up-regulated by 0.54-fold and 0.97-fold, respectively in brain of male zebrafish at 100 μg/L treatment (Fig. 1A). Expression of *lhβ* gene was 0.57-fold ($p < 0.05$) lesser in 100 μg/L PPF group compared to control. Whereas, there was no obvious changes in *ar* and *fshβ* expression level was noted. In females, significant reduction ($p < 0.05$) in *cyp19b* (0.45-fold), *fshβ* (0.43-fold) and *lhβ* (0.66-fold) expressions were observed, whereas no changes in *ar* and *erα* transcripts were found in brain of fish exposed to 100 μg/L PPF (Fig. 1B). Previous findings have shown the similar trend of *ar* expression in zebrafish exposed to bisphenol S (Ji et al. 2013b) and azoxystrobin (Cao et al. 2016). Androgen receptor (AR) belongs to nuclear hormone receptor super family binds with androgen and plays major role in sex differentiation and development (Crowder et al., 2018). In the current study, the expression of *ar* reveals that PPF was not involved in the AR mediated endocrine disruption and also suggests their non-androgenic effects in zebrafish. Previously, the up-regulated *erα* expression in male and no changes in female were reported in zebrafish exposed with tris (2-butoxyethyl) phosphate (Xu et al., 2017) and 6:2 chlorinated polyfluorinated ether sulfonate (F-53B) (Shi et al., 2018), respectively. Similarly, Manabe et al. (2006) have reported *erα* mediated estrogenic activity of PPF in MtT/Se cells (rat pituitary

tumor cell line). Therefore, transcriptional elevation of *erα* in male zebrafish revealed that PPF may have potential estrogenic action and subsequent impairment of reproductive system.

The expression of *fshβ* and *lhβ* genes were regulated by HPG axis and the reduction in *fshβ* and *lhβ* transcripts in brain was consistent with previous report wherein zebrafish was exposed to Tris (2-butoxyethyl) phosphate (Xu et al., 2017). Hence, in this study, the sub-chronic exposure of PPF might probably inhibit the activity of pituitary function thereby, causing declined expression of *fshβ* and *lhβ* genes. In fish brain, E_2 driven positive auto-regulatory feedback loop controls the expression of *cyp19b* gene (Ji et al., 2013a). The increased brain aromatase *cyp19b* expression might be a response to higher circulatory E_2 which signifies the estrogenic potential of PPF in male zebrafish (Xu et al., 2017). In the same way, suppression of *cyp19b* gene transcripts was found in the brain of female zebrafish in response to tributyltin exposure (Xiao et al., 2018). Hence, the decreased level of *cyp19b* transcripts revealed that PPF might inhibit the aromatase enzyme production, which in turn disturbs the reproductive function of female fish.

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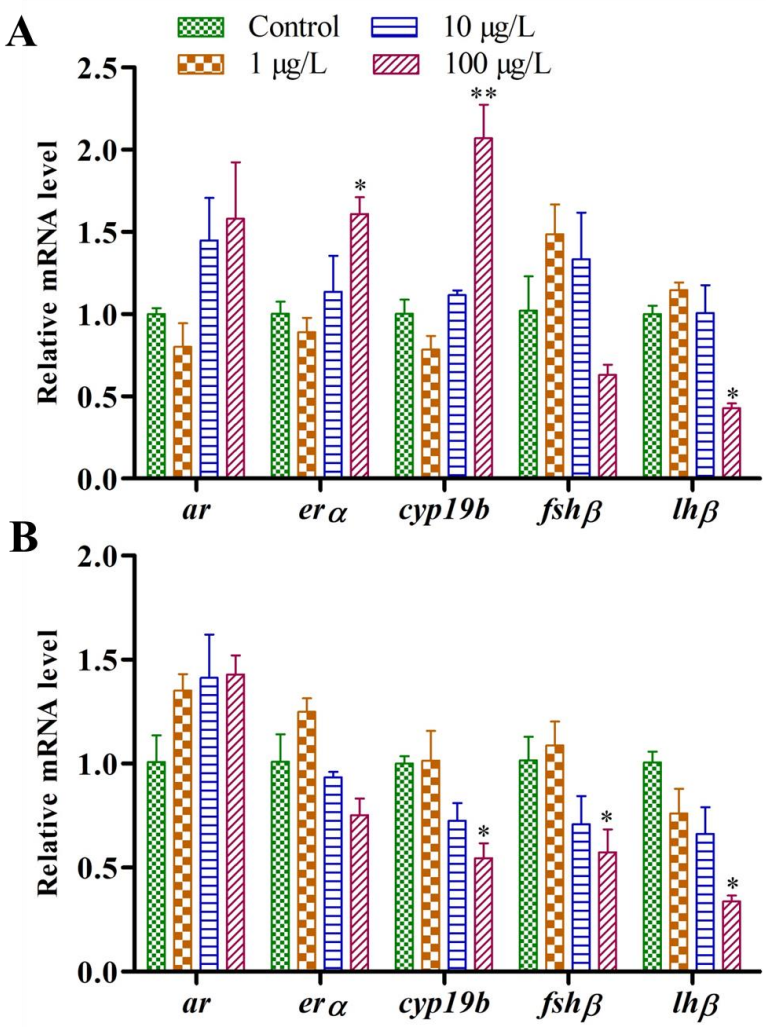

Fig. 1. Relative expression of *ar, erα, cyp19b, fshβ* and *lhβ* genes in brain of PPF exposed male (A) and female (B) zebrafish. Results are presented as the mean ± SEM of three replicates (each replicate includes four fish per gender). Asterisk, * and ** on bar represents the significance difference between control and PPF treated group at $p < 0.05$ and $p < 0.01$ levels, respectively.

In HPG axis, ER plays major role in neuroendocrine regulation of sexual development and the direct binding of estrogen or anti-estrogen controls ER in ligand dependent manner (Qiu et al., 2019; Li et al., 2019). Vtg is an important egg yolk precursor protein produced from hepatocytes and transported to the ovary via circulatory system in female zebrafish (Ahmadi et al., 2019). The *vtg1* expression is considered a biomarker of endocrine disruption

in fish in response to various environmental stressors (Qiu et al., 2019). Transcripts of *erα* gene showed no significant alteration in hepatic tissues of both male and female zebrafish in all treatments (Fig. 2A). Endocrine disruptors can modulate the natural hormones with or without binding to ER (Dai et al., 2014). Several studies have demonstrated the endocrine disruptive effect without affecting *erα* expression in hepatocytes. For instance, Shi et al. (2018) have reported the endocrine disruption with no alteration in *erα* transcript level in F-53B treated zebrafish. In this study, PPF might hinder the reproductive functions through unaltered hepatic *erα* expression. The *vtg1* mRNA expression has shown 0.58-fold ($p < 0.05$) increase in liver of male fish at 100 μg/L PPF group (Fig. 2B). In female liver, the transcriptional up-regulation of *vtg1* gene was significantly greater as 0.55-fold ($p < 0.05$) and 1.04-fold ($p < 0.01$) at 10 and 100 μg/L PPF exposure. Consistent with this result, the increased *vtg1* expression were reported in hepatic tissues of male and female zebrafish exposed to azoxystrobin (Cao et al., 2016) and acetylsalicylic acid (Baumann et al., 2020), respectively. In contrast, Tokishita et al. (2006) has revealed that PPF strongly repressed the expression of *vtg1* genes in neonate daphnids. In the current study, the induction of *vtg1* expression in liver suggests that sub-chronic exposure of PPF might interfere in Vtg synthesis that in turn leads to reproductive dysfunction in zebrafish.

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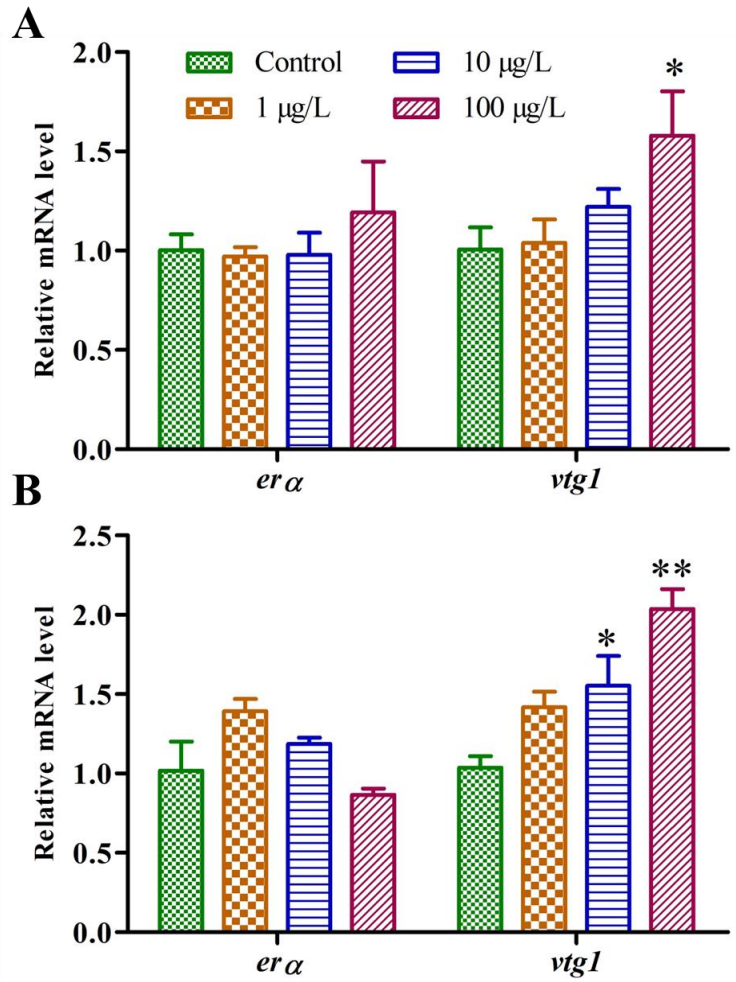

Fig. 2. Relative expression of *erα* and *vtg1* genes in liver of PPF exposed male (A) and female (B) zebrafish. Results are presented as the mean ± SEM of three replicates (each replicate includes four fish per gender). Asterisk, * and ** on bar represents the significance difference between control and PPF treated group at $p < 0.05$ and $p < 0.01$ levels, respectively.

FSH and LH acts through binding to gonadal receptors FSHR and LHR respectively to stimulate gonadal steroids and gametogenesis in fish (Ji et al., 2013a). The *de novo* biosynthesis of sex steroid hormones in gonad initiates with the steroidogenic acute regulatory (*star*) protein mediated transportation of cholesterol into the mitochondria (Teng et al., 2019). Subsequently, cholesterol is converted to testosterone by cascade process through

the involvement of multiple enzymes such as, *cyp11* (cytochrome P450 aromatase 11), *cyp17* (cytochrome P450 aromatase 17), *3βhsd* (3β-hydroxysteroid dehydrogenase) and *17βhsd* (17β-hydroxysteroid dehydrogenase). Finally, E_2 is formed from T by the action of aromatase (c*yp19a*) enzyme, which acts as key player in regulating the balance of steroid hormone level in zebrafish (Sun et al., 2019). In this study, biphasic response was observed in *fshr* mRNA level of testes with significant ($p < 0.05$) up-regulation (85.02%) at 10 μg/L group (Fig. 3A). The transcripts of *lhr* gene showed no obvious changes in male gonad when compared to control fish. In female gonad, the *fshr* and *lhr* expression was not affected by PPF exposure in all treatments (Fig. 3B). Consistently, Sun et al. (2019) and Ji et al. (2013b) have found the altered mRNA level of *fshr* and *lhr* transcripts in male and female gonad after exposed pentachlorophenol and bisphenol S, respectively. Although, *lhr* transcripts were not considerably changed in both gonads, the induction in *fshr* suggests that male fish might be more sensitive to hormonal changes after PPF exposure.

The steroidogenic enzyme producing gene transcripts like, *star, 3βhsd, 17βhsd* and *cyp19a* were significantly ($p < 0.05$) greater by 0.67, 0.41, 0.67 and 0.73 fold respectively in testes at highest treatment. In addition, *3βhsd* mRNA was raised by 0.89-fold at 10 μg/L group. In ovary, expressions of *star* (0.51-fold) and *17βhsd* (0.47-fold) were up-regulated at 100 μg/L PPF. While, *3βhsd* mRNA was increased by 1.15-fold ($p < 0.05$) at 10 μg/L group of female gonad. Interestingly, *cyp19a* expression has shown 0.58-fold decrease in ovary at 100 μg/L concentration. The enhanced level of *star* expression in both gonads reflects that PPF might interfere with the cholesterol transportation process into the mitochondria (Teng et al., 2019). In similar to our study, Cao et al. (2019) have reported azoxystrobin induced alteration in *3βhsd, 17βhsd* and *cyp19a* expression in both sexes of zebrafish. Hence, increased expression of *3βhsd* and *17βhsd* leads to the induction of *cyp19a* in male, which could subsequently affect the T to E_2 conversion (Niemuth and Klaper, 2018). However,

3βhsd enhancement might be considered as compensatory response to the inhibitory effect on other steroidogenic enzymes in female fish (Li et al., 2019). In gonad, *17βhsd* is involved in the biosynthesis of T from androstenedione and the over expression might be the related to the higher T production in female zebrafish. Further, the decreased *cyp19a* expression in ovary was consistent with azocyclotin exposed zebrafish (Ma et al., 2016). Therefore, the suppression of *cyp19a* suggests that PPF might cause the anti-estrogenic action leading to hormonal imbalance in female zebrafish.

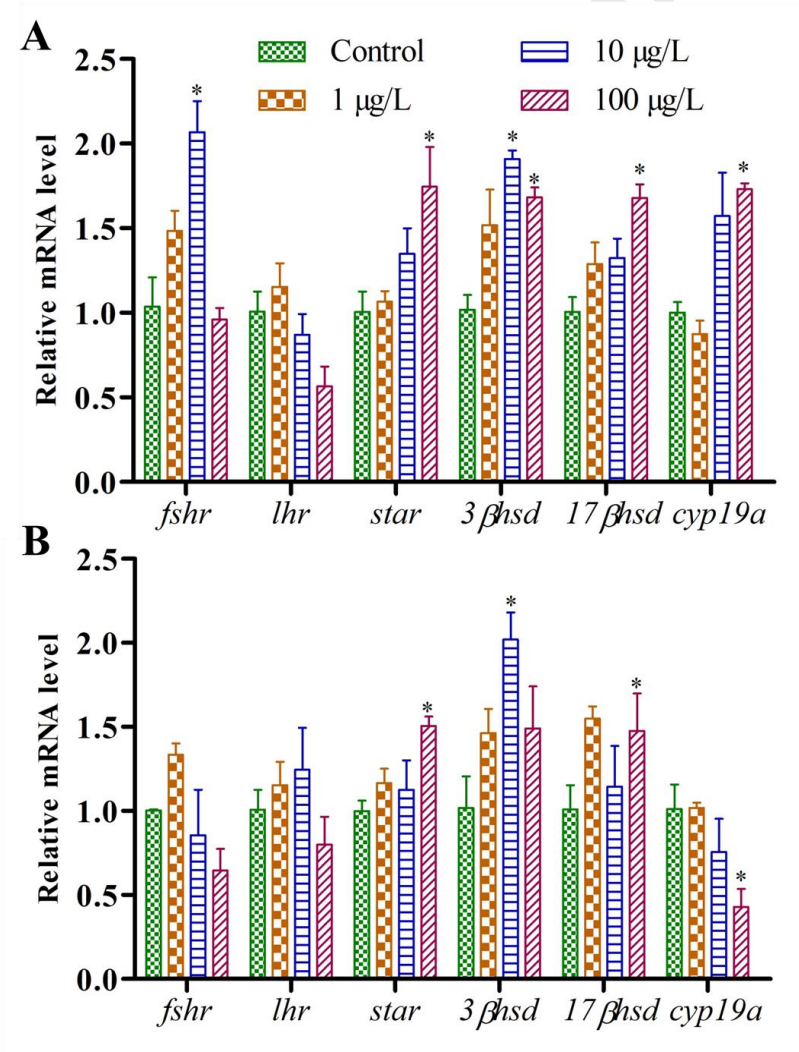

Fig. 3. Relative expression of *fshr, lhr, star, 3βhsd, 17βhsd* and *cyp19a* genes in gonad of PPF exposed male (A) and female (B) zebrafish. Results are presented as the mean ± SEM of

three replicates (each replicate includes four fish per gender). Asterisk (*) on bar represents the significance difference ($p < 0.05$) between control and PPF treated group.

3.3. Plasma sex hormone and Vtg measurement

The steroid hormones (T and E_2) regulate the reproductive processes such as gametogenesis, vitellogenesis and yolk formation in fish (Zhang et al., 2016). Sex hormone measurement has been considered as one of the most integrative and functional end point for reproductive toxicity studies (Ji et al., 2013b). The gonad synthesizes T and E_2 under the control of the HPG axis (Liu et al., 2011) and their imbalance lead to feminization or masculinization in fish (Lee et al., 2018). In this study, circulatory T level was significantly ($p < 0.05$) decreased in male zebrafish by 15.27% and 27.47% after exposed to 10 and 100 μg/L PPF, respectively (Fig. 4A). In female fish, T level was elevated by 13.34% at 100 μg/L treatment when compared to control. Further, plasma E_2 was enhanced by 22.70% ($p < 0.05$) in 100 μg/L treatment of male and suppressed by 11.26% and 19.15% ($p < 0.05$) at 10 and 100 μg/L group of female zebrafish, respectively (Fig. 4B). E_2/T ratio has revealed significant rise by 34.99% and 70.56% in male and lowered by 14.07% and 28.56% in female zebrafish at 10 and 100 μg/L treatments, respectively (Fig. 4C). These observations were in consistent with Cao et al. (2019) that decreased T and raised E_2 and E_2/T ratio in male and vice versa in female zebrafish exposed to azoxystrobin. The observed increase in the expression of *3βhsd* and *17βhsd* would primarily modulate the synthesis of androgens and affect the T concentration (Ma et al., 2012). Moreover, changes in the *cyp19* mRNA that involved in the conversion of T into E_2 would also contribute to the fluctuation in sex hormone concentrations (Cao et al., 2016). Previous reports have evidenced that reduction in *cyp19a* expression resulted to lower level of E_2 and subsequent elevation in T (Li et al., 2019). Therefore, the altered expression of *cyp19a* observed in this study might be the reason for the enhancement or inhibition of T and E_2 level of male and female zebrafish, respectively. The

sex hormone ratio serves as a sensitive biomarker in fish reproductive functions and any disequilibrium between T and E_2 balance could influence reproduction, gonad development and sex differentiation (Xu et al., 2017). Therefore, changes observed in E_2/T ratio might be attributed to the subsequent reproductive dysfunction in zebrafish by interfering with the HPG axis regulatory mechanisms (Xi et al., 2011).

Vtg synthesis is directly affected by the circulating endogenous estrogen or estrogen mimics. The measurement of Vtg level serves as best biomarker for reproductive toxicity in fish (Baumann et al., 2020). In this study, the sex specific effect of PPF was noted in plasma Vtg content that significant ($p < 0.05$) elevation by 16.21% in male and 21.69% inhibition in female zebrafish at 100 μg/L exposure compared to control (Fig. 4D). Estrogenic chemicals can trigger the *vtg* expression in liver and subsequent accumulation of Vtg in the blood of male fish (Chang et al., 2013). Similarly, elevation in *vtg* mRNA and plasma Vtg content was reported by Shi et al. (2018) in male zebrafish after exposed to F-53B which implied that PPF might have estrogenic effect. Because of increased hepatic *vtg1* transcript in female, the high plasma Vtg level was expected, while it was found be reduced in the systemic circulation. Likewise, the reduction of plasma Vtg level was reported in female zebrafish after azoxystrobin exposure (Cao et al. 2016). In contrast, Linton et al. (2009) have revealed PPF induced yolk protein synthesis in the ovaries of Christmas Island red crab, *Gecarcoidea natalis*. Vtg production is dependent on endogenous E_2 level (Shi et al., 2019), and the decreased plasma E_2 noticed in this study coincides with the lower Vtg level in female fish.

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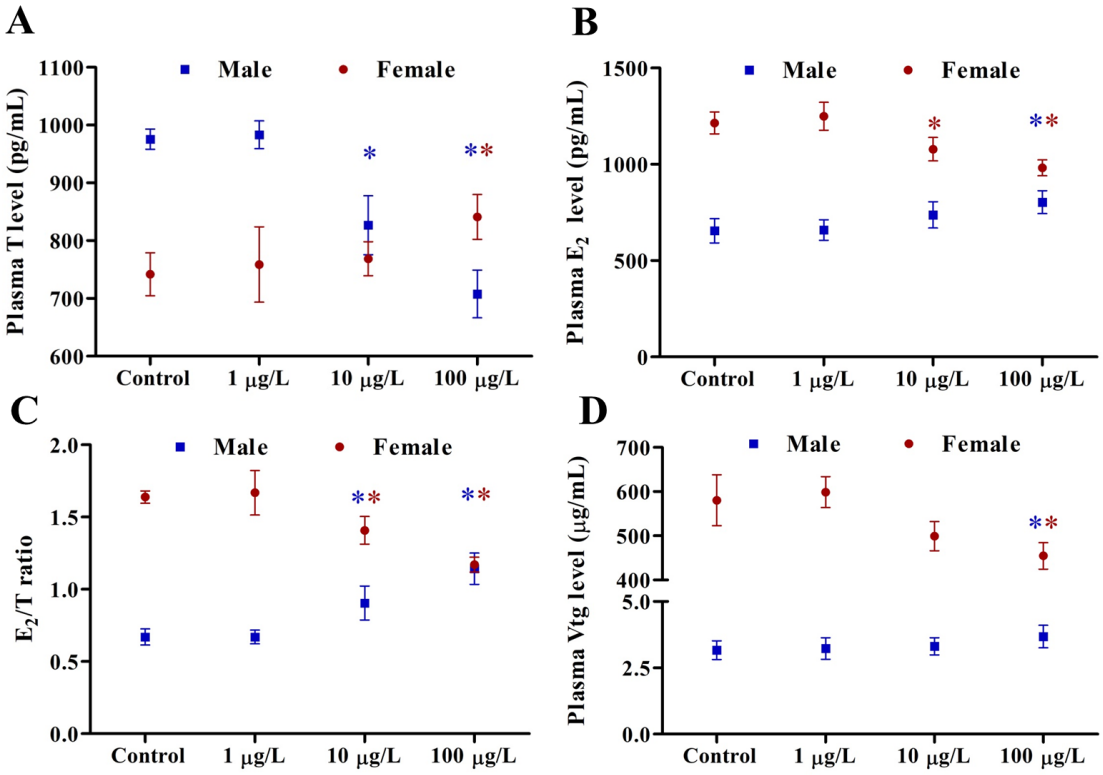

Fig. 4. Plasma level of T (A), E_2 (B), E_2/T ratio (C) and Vtg (D) in PPF exposed male and female zebrafish. Results are presented as the mean value ± SEM of three replicates (twelve fish per gender per treatment). Asterisk (*) (blue color for male & brown color female) on bar represents the significance difference ($p < 0.05$), between respective control and PPF treated group.

3.4. Histopathological effect of PPF in gonads

Assessment of gonadal histopathology is considered as a key measure of endocrine disruption potential of estrogen mimics in adult fish (Chen et al., 2017). The histopathology result of testes shows that PPF exposure at 1 µg/L was appeared to be similar to control and no obvious changes were identified. However, PPF caused the adverse effects on testes morphology, changes in spermatogonia, spermatids, and reduction in mature spermatozoa at 10 and 100 µg/L concentrations (Fig. 5). Furthermore, the relative surface area (%) measurement in testes has revealed that the mature spermatocytes (spermatids and

spermatozoa) was significantly ($p < 0.05$) reduced and the immature spermatocytes (spermatogonia and spermatocytes) were significantly increased ($p < 0.05$) in 100 µg/L treatment (Fig. 6A). Likewise, Cao et al. (2019) have reported the reduction in mature spermatocytes and increased percentage of immature spermatocytes in male zebrafish exposed to azoxystrobin. Recently, Shahid et al. (2019) have reported that PPF caused vacuolization, reduced lumen diameter, seminiferous tubules, and sperm count, nonappearance of Leydig cells, degeneration of tunica albuginea, and increased interstitial space of mice testes. FSH and LH are the fundamental hormones regulating early and late stages of spermatogenesis in male fish (Xu et al, 2017). Hence, the histopathological changes observed in testes might be correlated with the lower level of *fshβ* and *lhβ* as evidenced in gene expression study. Further, enhancement of *cyp19a* expression and lower circulatory T level also might contribute to the reduction in mature spermatozoa (Shi et al., 2018).

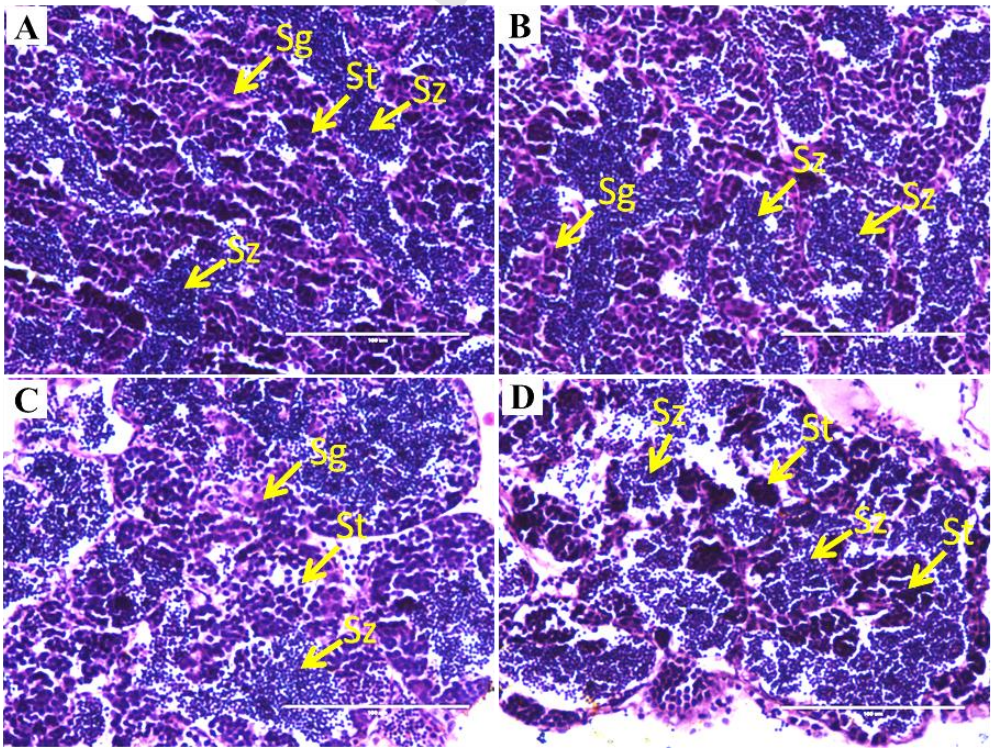

Fig. 5. Histopathological observations in the testes of control (A), 1 (B), 10 (C) and 100 µg/L (D) of PPF exposed male zebrafish. Sg - spermatogonia, St - spermatids and Sz - spermatozoa (scale bar - 100 µm).

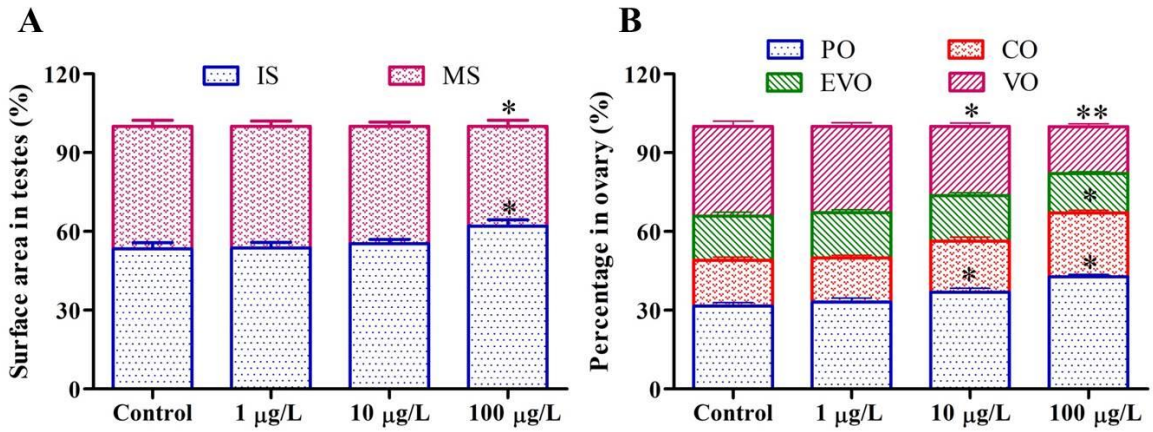

Fig. 6. Histopathological changes in each stages of testes and ovary in PPF exposed zebrafish; A. The relative surface area (%) of immature (spermatogonia and spermatocytes) and mature (spermatids and spermatozoa) spermatocytes in the testes, IS - immature spermatocytes, MS - mature spermatocytes; B. The relative number (%) of perinucleolar oocytes (PO), cortical alveolar oocytes (CO), early vitellogenic oocytes (EVO) and vitellogenic oocytes (VO) in ovary. Results are presented as the mean value ± SEM of three replicates per treatment (twelve sections and four fish per gender per treatment). Asterisk, * and ** on bar represents the significance difference between control and PPF treated group at $p < 0.05$ and $p < 0.01$ levels, respectively.

In fish, oocyte maturation depends on the coordination of the hormones produced from the HPG axis (Ahmadi et al., 2019). In this study, various histopathological alterations were observed in ovary of adult zebrafish exposed to PPF (Fig. 6B & 7). The control and 1 µg/L PPF treated zebrafish have shown normal characteristics of perinucleolar oocytes (PO), cortical alveolar oocytes (CO), early vitellogenic oocytes (EVO) and vitellogenic oocytes (VO). The relative number (%) of each stages of oocytes in ovary has revealed that

significant ($p < 0.05$) increase in PO and decrease in VO at 10 µg/L group (Fig. 6B). In addition, zebrafish exposed to 100 µg/L of PPF have shown significantly increased frequency of PO and CO ($p < 0.05$) and reduced number of VO ($p < 0.01$). Recently, Li et al. (2019) have found similar result such as, higher proportion of primary oocytes with lower level of late or mature oocyte in zebrafish after tebuconazole exposure. PPF induced histopathological changes like presence of more previtellogenic oocytes and EVO in the ovary of *Gecarcoidea natalis* were also evidenced (Linton et al., 2009). Furthermore, deterioration of follicular epithelium, vacuolated ovarioles and underdeveloped ovaries was reported in queens of Pharaoh ant after PPF treatment (Tay and Lee, 2014). In female fish, FSH and LH regulate the vitellogenesis and oocyte maturation processes, respectively (Ji et al., 2013a). Hence, the down-regulation of *fshβ* and *lhβ* expression might be correlated with the changes observed in the ovarian histology. The uptake of Vtg protein from systemic circulation determines the size of the oocytes and ratio of the follicles (Xiao et al., 2018; Ahmadi et al., 2019). Besides, the decreased E2/T ratio could cause delayed ovary development (Li et al., 2019). Therefore, in this study, the lower level of E_2 concentration and insufficient Vtg production may be attributed to the reduced number of the mature oocytes in female gonad. Hence, PPF exhibits disruptive action in the histopathology of both male and female zebrafish gonad and it may disturb the reproductive success of the subsequent generation.

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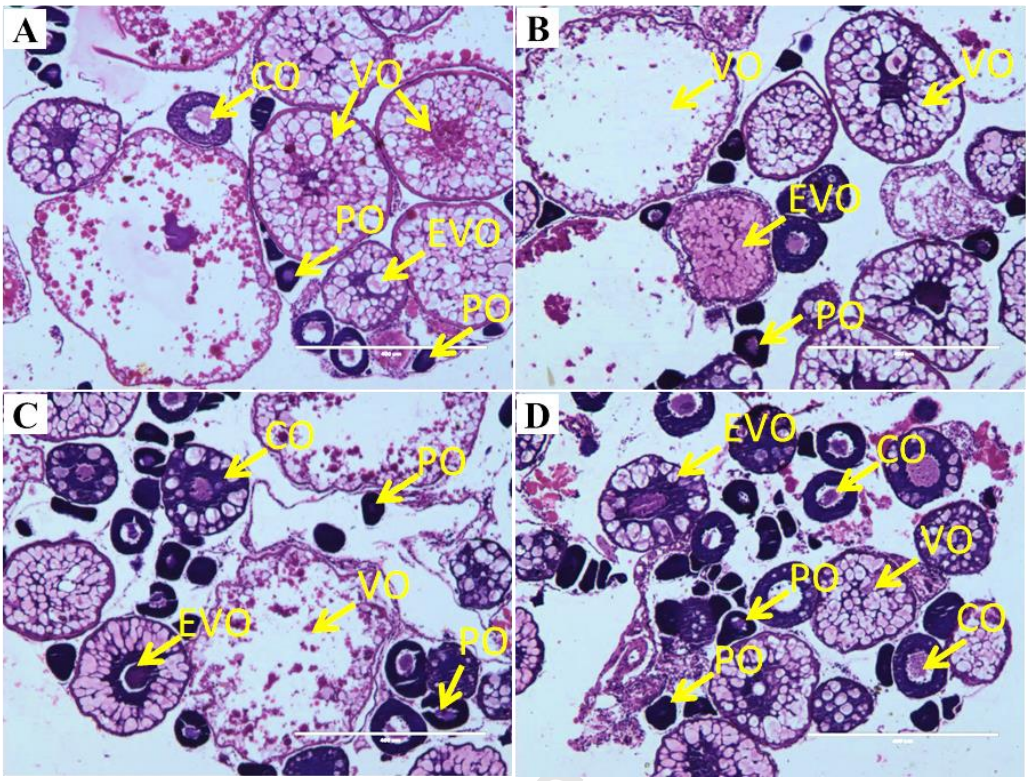

Fig. 7. Histopathological observations in the ovary of control (A), 1 (B), 10 (C) and 100 µg/L (D) of PPF exposed female zebrafish. PO - perinucleolar oocyte, CO - cortical alveolar oocyte, EVO - early vitellogenic oocyte and VO - vitellogenic oocyte (scale bar - 400 µm).

4. Conclusion

Sub-chronic exposure of PPF induced reproductive toxic effects in zebrafish through alteration of HPG axis gene transcripts, circulatory sex hormones, plasma Vtg content and histopathological changes of gonads. Moreover, both sexes of zebrafish are prone to adverse effects of PPF in reproductive endocrine system at the current recommended level (upto 100 µg/L) for the application in aquatic environment. This study provides systematic data on the impact of PPF on fish reproduction for the first time and fills the knowledge gap. Moreover, further studies on reproductive performance and trans-generational effects of PPF will provide better understanding of their health risks on aquatic wildlife and human health.

Declaration of Competing Interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found in the online version, at

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Credit Author contribution statement

Kannan Maharajan	: Conceptualization, Validation, Investigation, Methodology, Data curation; Writing - original draft
Sellamani Muthulakshmi	: Investigation, Methodology, Data curation; Writing- review & editing
Chinnannan Karthik	: Investigation, Methodology, Data curation; Writing- review & editing
Bojan Nataraj	: Methodology, Writing- review & editing
Kandan Nambirajan	: Methodology, Writing- review & editing
Devan Hemalatha	: Methodology, Writing- review & editing
Swaminathan Jiji	: Methodology, Writing- review & editing
Krishna Kadirvelu	: Resources, Supervision, Writing- review & editing
Ke-chun Liu	: Writing- review & editing
Mathan Ramesh	: Supervision, Funding acquisition, Resources, Data curation, Writing- review & editing

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Mathan Ramesh

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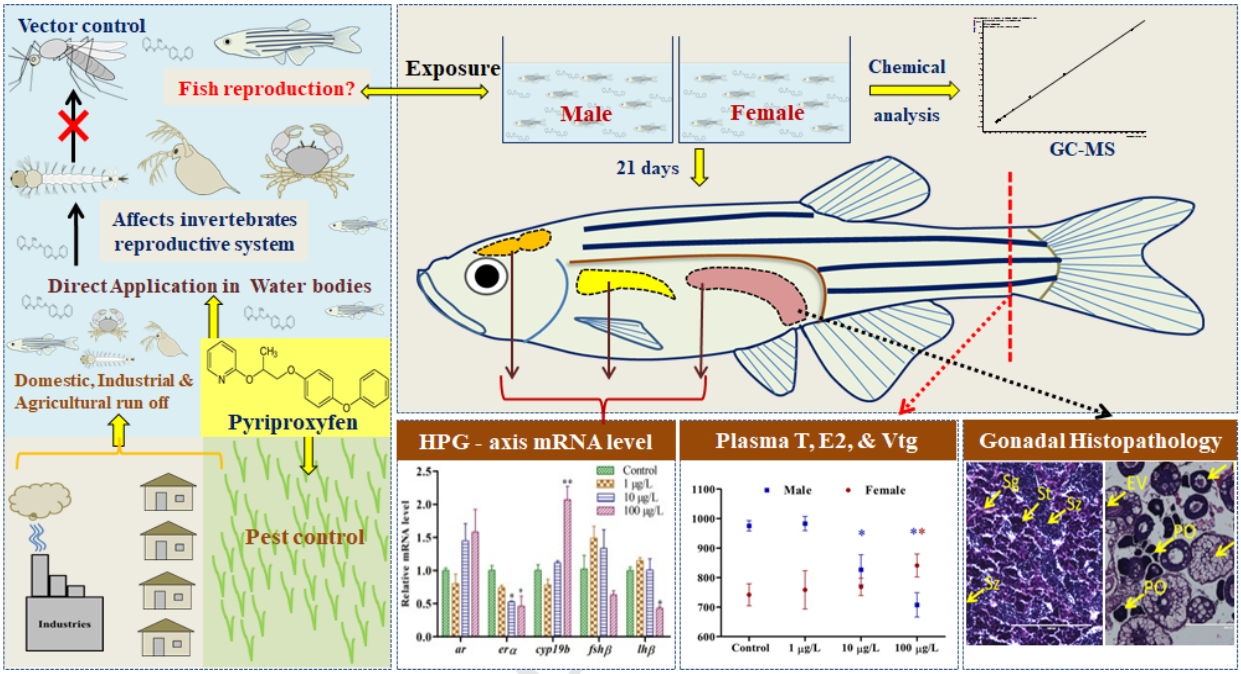
Graphical Abstract



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Highlights

- The endocrine disruptive potential of pyriproxyfen (PPF) was studied in zebrafish
- PPF altered the gonadotropin and steroidogenic transcript levels in both sexes
- Blood plasma concentration of testosterone and estradiol were significantly differed
- Vitellogenin expression was enhanced in liver and varied in blood plasma level
- PPF exposure caused histopathological damages in testes and ovary of zebrafish

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