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RESEARCH ARTICLE

Female Mice Exposed to Pyriproxyfen Since Prepuberty Showed Reproductive Impairment During Sexual Maturity and Increased Fetal Death in Their Offspring

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ABSTRACT

Pyriproxyfen (PPF) is an insecticide used in agriculture, which is approved for use in drinking water tanks for human consumption. However, some studies indicate that it may act as an endocrine disruptor and affect nontarget organisms. This study aimed to evaluate the effects of PPF on reproduction and general health status in female mice exposed from pre-puberty to adulthood. In the first experiment, females were treated by gavage from postnatal day (PND) 23 to (PND) 75 and were distributed into three experimental groups: control (vehicle), PPF 0.1 mg/kg, and PPF 1 mg/kg. Female mice were assessed for the age of puberty onset, body mass, water and food consumption, and the estrous cycle. On PDN 75, a subgroup was euthanized, when vital and reproductive organs were collected and weighed. The thyroid, ovary, and uterus were evaluated for histomorphometry. The other subgroup was assessed in relation to reproductive performance and fetal parameters. In a second experiment, the uterotrophic assay was performed with juvenile females (PND 18) using doses of 0.01, 0.1, or 1 mg/kg of PPF. PPF treatment reduced thyroid mass and increased liver mass. Furthermore, there was an increase in ovarian interstitial tissue and, in the uterus, a decrease in the thickness of the endometrial stroma with reduced content of collagen fibers. There was also a reduction of 30% in pregnancy rate in the treated groups and an increase in the frequency of fetal death. This study suggests that, based on this experimental model, the insecticide may pose a reproductive risk for females chronically exposed to the substance from the pre-pubertal period until adulthood. These results raise concerns about prolonged exposure of women to the same compound.

1 | Introduction

Pyriproxyfen (4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether) is a larvicide used in agriculture and horticulture to combat the California red scale insects, silverleaf whitefly, cutworms, aphids, and bollworm [1]. Because it is previously considered of low toxicity for mammals, the World Health Organization (WHO) approved the use of pyriproxyfen (PPF) in drinking water,

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in which the maximum tolerable concentration in water tanks should be less than 0.01 mg/L. Then, this pesticide is mainly used to control disease-vector mosquitoes such as *Aedes aegypti* [2]. PPF acts on the endocrine system of the insect and mimics the juvenile hormone, an insect hormone produced by the *corpora allata*, which inhibits the genes that initiate the development of adult characteristics; thus, it acts by controlling the immature stages of the insect, and preventing metamorphosis from happening [3]. In addition, it causes morphological and functional changes in adults, such as inhibition of reproduction [3].

According to the WHO studies, PPF has a LD_{50} of 5000 mg/kg of body mass in mice and rats [2]. Regarding the NOAEL (No Observed Adverse Effect Level), two studies carried out on dogs, lasting 1 year, showed that the overall NOAEL was 10 mg/kg of body mass per day [2]. Therefore, an uncertainty factor of 100 was applied to this NOAEL value, resulting in a maximum permitted daily dose of 0.1 mg/kg[2].

Since 2014, the Brazilian Ministry of Health, following WHO recommendations, adopted the use of PPF at a dose of 0.01 mg/L in drinking water reservoirs to combat *Aedes* mosquitoes. According to the epidemiological bulletin, in 2021, about 30 to 45 tons of PPF were distributed to the Brazilian states, to combat the breeding sites of these disease-vector mosquitoes [4, 5].

Despite their relevance in agriculture and in controlling disease-vector mosquitoes, pesticides have been shown to provoke harmful effects on nontarget organisms, such as aquatic organisms, birds, domestic animals, and humans [6, 7]. Insecticides can affect the female genital system in different manners, causing alterations in sex hormone levels, declining female fertility, generating menstrual disorders, causing ovarian damage, and increasing the rates of congenital anomalies by affecting embryonic and fetal development [7, 8]. In light of this, researches performed on model organisms, such as the mouse and zebrafish, contribute to understanding the damages caused by these pesticides and the possible adverse effects on humans and animals [9, 10].

In different animal models, PPF has been shown to cause reproductive toxicity. This larvicide disrupts the homeostasis of sex hormones and induces histopathological changes in the female and male gonads of zebrafish [9]. Moreover, it can induce oxidative stress and impair follicle maturation in zebrafish, by increasing the rate of early stages and decreasing the later stages of follicle maturation [11].

Furthermore, a metabolite of PPF, named 4′-OH-PPF, may affect the thyroid activity in *Xenopus laevis*, by acting as a thyroid hormone T3 antagonist; consequently, exposure to this metabolite affects the development of cerebral compartments in this animal model [12]. In rodents, during pregnancy, this insecticide reduced maternal weight gain and increased the duration of gestation [10]. In this same study, there were augmented fetal mortality and histopathological alterations in the liver, kidney, heart, and brain of the fetuses [10].

Considering that PPF may present reproductive toxicity and act as a potential endocrine disruptor, and that humans are exposed to this pesticide through their feeding during various life stages, this study aimed to highlight the potential risks of PPF exposure for female reproductive health by using the mouse as the experimental model, and administering environmentally relevant doses of this pesticide since pre-puberty.

2 | Materials and Methods

2.1 | Animals

The procedures and handling of the mice were carried out following with the guidelines provided by the national council for animal control and experimentation (CONCEA) and by the ethics committee on animal use of the federal university of santa catarina (protocol number 1963200721). The animals were maintained under controlled temperature (23 ± 1°C) and photoperiod (12 h light/dark cycle) at the Biotherium of Embryology (Room 110C/First floor/Biological Sciences Center/UFSC). Filtered water and standard rodent chow were offered ad libitum during the experiments.

2.2 | First Experiment

2.2.1 | Experimental Design

Female *Swiss* mice were randomly distributed in three experimental groups ($n = 20$ *per* group): control group, that is, animals that received vehicle (975 μL of 0.9% saline solution +25 μL of dimethyl sulfoxide); and PPF-treated groups at the doses of 0.1 or 1 mg/kg, whose animals received PPF diluted in the vehicle at the respective doses (Figure 1). PPF was purchased from Sigma-Aldrich as a white powder with at least 98.0% of purity. The treatments were daily performed by gavage from postnatal day (PND) 23 to PND 75. This period represents an exposure from pre-puberty (PND 23), 2 days after weaning of the mice, until sexual maturity on PND75. Considering that estrous cycle regularity starts approximately on PND 60, its assessment could be performed for at least 3 cycles, from PND 60 until PND75. On alternated days, the animal body mass was evaluated to monitor the mass gain and growth of the females. In addition, water intake and food consumption were monitored throughout the experiment.

The PPF doses used in this study were obtained using the dose conversion methodology between different species, based on the body surface area of the species, humans and mice [13]. Thus, the doses administered to the mice are equivalent to the maximum acceptable dose of PPF allowed by WHO for human daily intake (0.1 mg/kg of human body mass) and a dose 10 times lower than the maximum level allowed by WHO [14, 15].

2.2.2 | Puberty Onset

From PND 23 the females were daily evaluated to identify the complete vaginal opening and first estrus, which are considered physical signs of puberty onset in females [16]. When vaginal opening occurred, the mice were weighed and evaluated about the day of first estrus through the content of cells in the vaginal smears, which were obtained by introducing 10 μL of saline solution in the vagina of the mice by using a micropipette followed by the aspiration of this fluid. This fluid was added to a microscope slide

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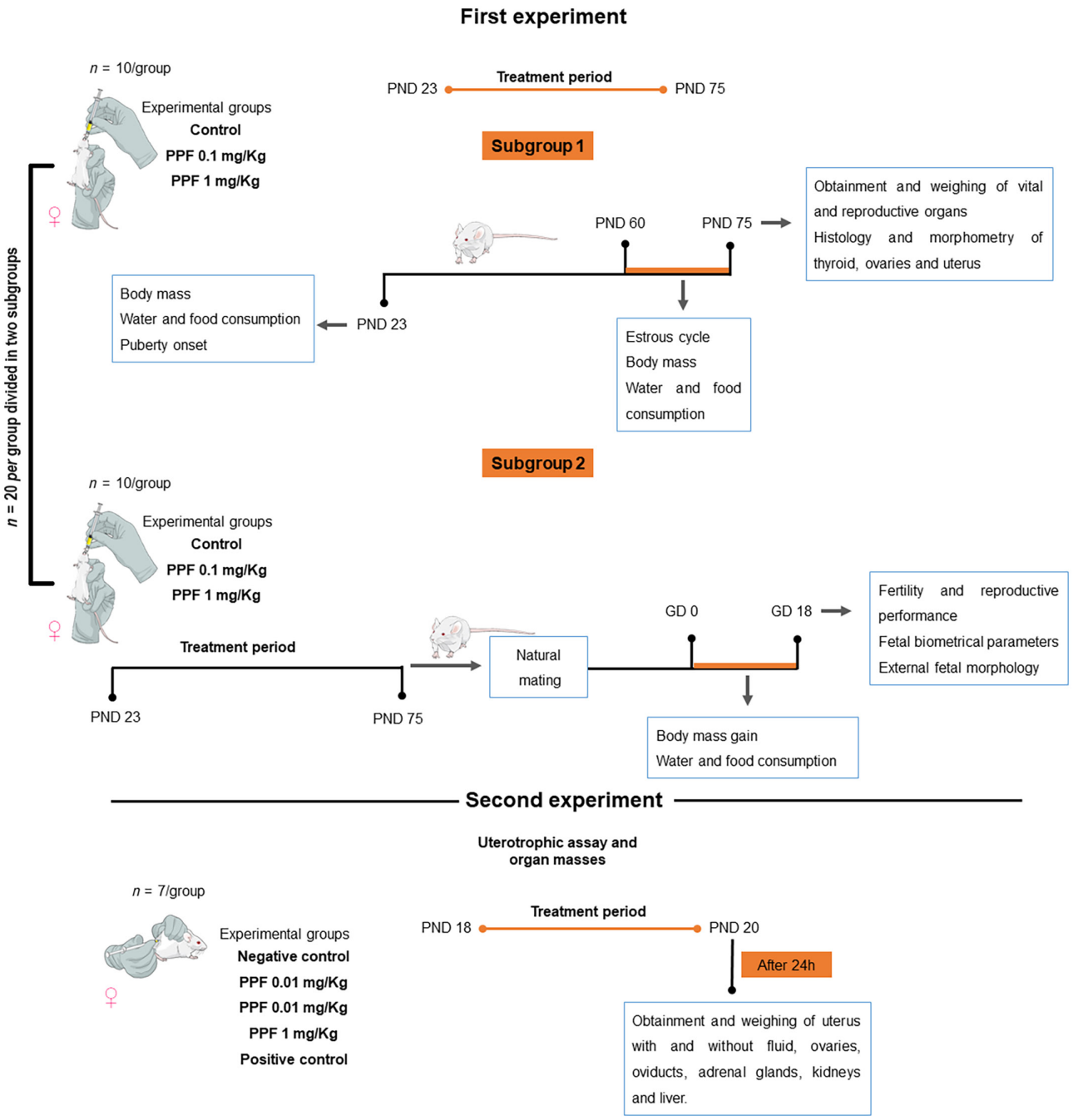

FIGURE 1 | Experimental design of the two experiments.

and was covered with a cover glass. Posteriorly, the distribution of the cells present in the vaginal smear was assessed under phase-contrast microscopy. The presence of cornified cells allowed the identification of this phase of the estrous cycle [17]. On the day of the first estrus, the females were also weighed.

2.2.3 | Estrous Cyclicity

From PND 60 to PND 75, the cellular composition of the vaginal smears was daily examined to determine the phase of the estrous cycle and its regularity. The procedures for obtainment of vaginal smear and its evaluation were performed as previously described for assessing the first estrus. Proestrus was characterized by the predominance of nucleated epithelial cells; estrus was determined by the abundance of cornified epithelial cells; metestrus was identified by a similar distribution of nucleated and cornified epithelial cells, and leukocytes; and diestrus was characterized by the predominance of leukocytes [17]. The data obtained were used to estimate the frequency (in days) of proestrus, estrus, metestrus, and diestrus, the duration of the estrous cycle, and the number of cycles during the period evaluated [18].

2.2.4 | Euthanasia and Organ Masses

After PND 75, one subgroup ($n = 10$ *per* group) was euthanized, in the estrus phase, by isoflurane inhalation followed

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by cervical dislocation and, subsequently, reproductive organs such as ovaries, oviducts, and uterus (both uterine horns) were collected and weighed. Bilateral ovaries and oviducts were weighed together. To assess signs of systemic toxicity, vital organs such as kidneys, adrenals glands, liver, thyroid, brain, and pituitary gland were removed and weighed. The left kidney and adrenal gland were standardized for weighting in all animals.

2.2.5 | Histological Procedures and Histomorphometric Analyses

The thyroids, left ovaries, and uterine horns were collected and fixed in modified Davidson's fixative solution, embedded in paraffin, and sectioned in three nonserial transverse sections ($n = 6$/group) with a thickness of 4 μm, with a spacing of 35 μm. For uterine sectioning, it was used the medial region of the organ. Sections were stained with hematoxylin and eosin (HE) to assess ovarian, uterine, and thyroid general morphology under microscopy. Moreover, thyroids were stained with Periodic Acid–Schiff (PAS) and hematoxylin to highlight colloid content inside the follicles. The uteri were also stained with Picrosirius Red and hematoxylin to identify the collagen fibers content and with toluidine blue for mast cell count. The ovaries were also stained with toluidine blue and used for mast cell quantification.

Ovarian follicles were counted and classified concerning the different maturation stages, taking into consideration the structure and number of granulosa cell layers of the different follicular types. The classification of ovarian follicles was performed as described by Borgeest et al. [19] and Talsness et al. [20]. Corpora lutea were identified by the presence of large and acidophilic lutein cells, as well as by their vascularization [21].

Regarding the uterine morphological evaluation, the structure of the different layers of the organ was analyzed, including perimetrium, myometrium, and endometrium (endometrial stroma, uterine glands, and luminal uterine epithelium) [18]. The endometrial projection towards the lumen was qualitatively assessed by comparing the appearance of endometrium concerning the presence and the apparent length (short or long) of these endometrial projections toward the lumen.

For morphometric analysis, the ovary, uterus, and thyroid slides were photodocumented under an Olympus BX 41 optical microscope with an attached 3.3 megapixels digital camera, and measurements were performed on a personal computer using ImageJ software. The left ovary area was measured to obtain the number of ovarian follicles and corpora lutea per ovary area (mm^2).

Furthermore, the quantity of ovarian interstitial tissue was assessed through degrees, considering the presence and abundance of this tissue in the cortical and medullary regions of the organ, as follows: 0—indicates absence of interstitial tissue, 1—represents a small amount of this tissue, 2—indicates moderate quantity of ovarian interstitial tissue, and 3—represents a large quantity of this tissue. After classification for the presence and distribution of interstitial tissue in each ovarian section (three sections per animal), the average grade per animal was calculated, following the same approach as performed by Leite et al. to estimate the degree of seminiferous epithelium development [22].

In addition, in the uterus, morphometric assessments were performed to measure the thickness of the perimetrium, myometrium, endometrial stroma, luminal uterine epithelium, and total area of the lumen. Moreover, a stereological evaluation of uteri stained with Picrosirius Red and hematoxylin, and thyroid stained with PAS and hematoxylin was performed to determine the frequency of the different compartments of these organs. In the uteri, the following compartments were considered, focusing on myometrium and endometrium stroma: circular myometrium layer, longitudinal myometrium layer, connective tissue between the myometrium layers, collagen fiber in the myometrium, endometrium stromal cells, uterine glands, and collagen fibers in the endometrium stroma.

Mast cell quantification was performed in three nonserial sections of the ovaries and uterus of each animal. In the ovaries, mast cells were quantified in the cortex and medulla of the organ, and, in the uterus, these cells were counted in the myometrium and endometrium. Then, the mean value of mast cells obtained in the three sections was calculated for each region of the organ in each animal assessed. For the thyroid, stereological assessment considered the follicular epithelium, colloid content, parafollicular cells, and the remaining interstitial components of the organ.

2.2.6 | Reproductive Performance and Fetal Evaluations

From PND 75, the other subgroup ($n = 10$/group) was used for the assessment of reproductive performance and fetal parameters (Figure 1). Thus, when proestrus was identified, the females were placed for mating with untreated males and on the following morning after mating, the presence of a vaginal plug or the presence of sperm in the vaginal smear was considered to confirm pregnancy, and this day was considered as gestational day (GD) 0. The body mass gain was daily monitored and maternal mass gain was calculated by the difference between body masses observed on GD 0 and GD 18. Water intake and food consumption were also monitored during gestational days.

On GD 18, females were weighed and euthanized by isoflurane inhalation followed by cervical dislocation. Then, the gravid ovaries and uterus were removed and weighed. In addition, gravidic corpora lutea, implantation sites, resorptions, and the number of live and dead fetuses were counted. Fetal and placental masses were also measured in an analytical balance. From these results, the following indexes were obtained: pregnancy rate = number of pregnant females/number of inseminated females × 100; fertility potential (implantation efficiency) = implantation sites/corpora lutea × 100; pre-implantation loss rate: number of corpora lutea − number of implantations/number of corpora lutea × 100; post-implantation loss rate = number of implantations − number of live fetuses/number of implantations × 100; sex ratio = number of male fetuses/number of female fetuses × 100 [18].

After being removed on GD 18, the fetuses were fixed in 70% alcohol. Then, they were externally examined, with the use of a Zeiss stereomicroscope, for the position and shape of the eyes

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Parameter	Differences among the groups
First experiment	
Treatment from PND 23 to PND 75	
Vaginal opening	=
Body mass at the day of vaginal opening	=
First estrus	=
Body mass at the day of first estrus	=
Estrous cycle	=
Absolute organ masses	Thyroid – ↓ 1 mg/Kg
Relative organ masses	Liver – ↑ 0.1 and 1 mg/Kg
Body mass	=
Water intake	=
Food consumption	=
Ovarian mast cells	=
Total ovarian area	=
Primordial and primary follicles	=
Preantral follicles	=
Antral follicles	=
Atretic follicles	=
Corpora lutea	=
Ovarian histopathology	Interstitial tissue in the medullary and cortical regions – ↑ 1 mg/Kg
Perimetrium	=
Myometrium	=
Endometrial stroma	↓ 0.1 and 1 mg/Kg
Luminal epithelium	=
Luminal area	=
Myometrium collagen fibers	=
Endometrium collagen fibers	↓ 1 mg/Kg
Uterine histopathology	Endometrial projections – ↓ 1 mg/Kg
Thyroid stereology	Glandular epithelium and colloid masses – ↓ 1 mg/Kg
Fertility and reproductive performance	
Body mass gain	=
Water intake	=
Food consumption	=
Pregnancy rate	↓ 0.1 and 1 mg/Kg
Fetal death	↑ 0.1 mg/Kg
Other fertility rates	=
Fetal biometric parameters	=
External fetal morphology	=
Second experiment: Uterotrophic assay	
Body mass	=
Uterus mass with fluid	↑ Positive control
Uterus mass without fluid	↑ Positive control
Other organ masses	=

FIGURE 2 | Description of the main results obtained in the experiments. No differences among the groups are indicated by the symbol of equal (=).

and mouth, height of ear implantation, cranial contour, presence and appearance of forelimbs and hind limbs, anal perforation, aspect of the tail and neural tube defects such as spina bifida [23].

Furthermore, fetal biometric parameters such as the relative anogenital distance, craniocaudal distance, and cephalic circumference were obtained. The relative anogenital distance was calculated as follows: anogenital distance/cube root of body

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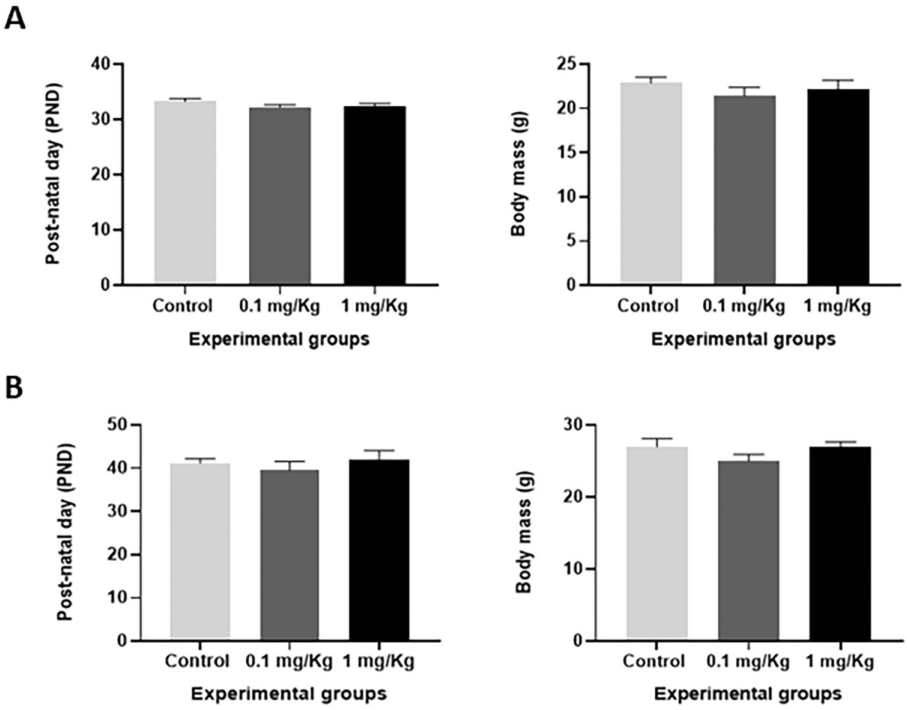

FIGURE 3 | Age and body mass during puberty onset. (A) Age and body mass on the day of vaginal opening. (B) Age and body mass on the day of the first estrus values are expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

TABLE 1 | Estrous ciclicity in the animals from the experimental groups.

	Experimental groups ($n = 20$/group)		
	Control	**0.1 mg/kg**	**1 mg/kg**
Estrous cycle length (days)	4.44 ± 0.18	4.65 ± 0.25	4.94 ± 0.30
Number of estrous cycles	2.73 ± 0.00	3.20 ± 0.18	3.14 ± 0.17

Note: Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

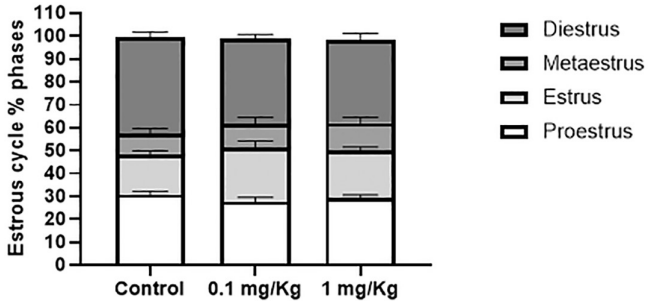

FIGURE 4 | Frequency of the estrous cycle phases of mice from the experimental groups. Values are expressed as median and interquartile range. Kruskal-Wallis followed by Dunn's post hoc test ($p > 0.05$).

mass ($mm/g^{1/3}$) [24]. Cephalic circumference was calculated after obtaining the biparietal and fronto-occipital distances. For the obtainment of these measurements, a digital pachymeter was used.

2.3 | Second Experiment

2.3.1 | Uterotrophic Assay, Body Mass, and Organ Masses

In parallel to the other experiment, the uterotrophic assay was performed as previously reported [25]. Female mice on PND 18 were randomly distributed into five experimental groups ($n = 7$/group): negative control, whose animals received vehicle (corn oil); PPF at the doses of 0.01, 0.1, or 1 mg/kg diluted in vehicle; and positive control, whose animals received 17β-estradiol at the dose of 0.5 mg/kg diluted in vehicle (Figure 1). The treatments were performed subcutaneously for 3 days. Twenty-four hours after the last administration, the mice were euthanized by isoflurane inhalation followed by cervical dislocation. Then, the uterus with and without fluid were collected and weighted to assess the estrogenic activity of PFF. Additionally, ovaries, oviducts, adrenal glands, kidneys, and liver were collected and weighed to evaluate the possible toxic effects of PPF in these organs.

2.4 | Statistical Analysis

The data were tested for normality and homoscedasticity by the Shapiro–Wilk and Levene tests, respectively. Two-way ANOVA accompanied by Tukey's post hoc test was used to evaluate data with monitoring throughout the experiment, such as body mass gain, and water and food intake. The other data were evaluated by One-way ANOVA followed by Tukey's post hoc test if the data presented normality and

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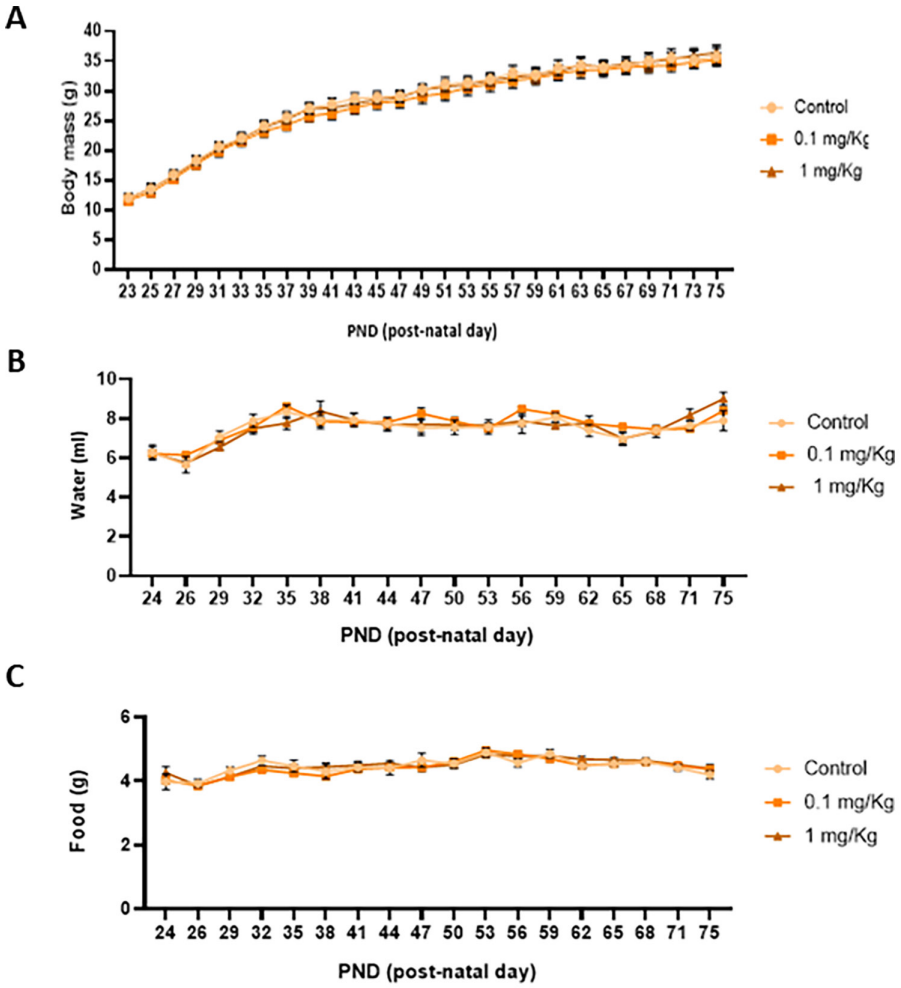

FIGURE 5 | Body mass gain and water and food intake in females from the experimental groups from pre-puberty until sexual maturity. (A) Body mass gain during treatment. (B) Water ingestion along the exposure period. (C) Food intake during treatment. Values expressed as mean ± SEM. Two-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

homoscedasticity. However, when these characteristics of the data were not confirmed, the Kruskal–Wallis test followed by Dunn's post hoc test was used. The Fisher exact test was used to assess the frequency of external congenital anomalies. Data were expressed as mean ± standard error of the mean, median and interquartile range, or in percentage. Statistical assessments were performed in the statistical program GraphPad Prism version 8 and the differences were considered significant when $p \leq 0.05$.

3 | Results

A synthetic description of the main results obtained in both experiments, after female mice were exposed to the different treatments, is shown in Figure 2.

3.1 | First Experiment: Female Reproductive Parameters

The age of puberty onset was not affected by PPF exposure. Females showed similar age and body mass on the day of vaginal opening and first estrus (Figure 3). About the regularity of the estrous cycle, the length and number of cycles showed no differences among the groups (Table 1). In addition, the frequency of each phase of the estrous cycle showed a similar distribution among the groups (Figure 4).

Considering the evaluations performed from pre-puberty until sexual maturity, such as body mass gain and water and food ingestion, there were no differences among the experimental groups (Figure 5). Regarding the vital organ masses, PPF caused a decrease in absolute thyroid mass at the higher dose, when compared to the control group, and an increase in relative liver mass at both doses ($p < 0.05$). However, the absolute and relative masses of the other organs showed similar among the experimental groups (Tables 2 and 3).

Ovarian morphometric parameters such as total ovarian area, quantification of follicles at different stages of maturation and atresia, and the number of corpora lutea were similar among the experimental groups (Figure 6). However, ovarian histopathological evaluation exhibited an increase of interstitial tissue in the gonadal cortex and medulla of the mice treated with PPF 1 mg/kg, when compared to the other groups (Figure 7).

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TABLE 2 | Vital organ masses of females from the experimental groups at the first estrus after PND 75.

	Experimental groups ($n = 10$/group)		
	Control	**0.1 mg/kg**	**1 mg/kg**
Final body mass (g)	35.88 ± 1.43	35.20 ± 0.70	35.60 ± 1.18
Brain (mg)	450.10 ± 10.82	446.10 ± 12.21	478.90 ± 10.86
Brain (mg/g BM)	1.26 ± 0.03	1.27 ± 0.03	1.36 ± 0.04
Pituitary (mg)	3.57 ± 0.48	3.70 ± 0.54	3.00 ± 0.00
Pituitary (mg/g BM)	9.99 ± 1.10	10.50 ± 1.54	8.13 ± 0.39
Thyroid (mg)	3.60 ± 0.37[a]	2.89 ± 0.26[ab]	2.50 ± 0.22[b]
Thyroid (mg/g BM)	10.10 ± 1.00	9.26 ± 1.16	7.06 ± 0.60
Heart (mg)	128.50 ± 4.41	127.40 ± 3.61	130.60 ± 5.12
Heart (mg/g BM)	360.30 ± 11.33	354.70 ± 8.93	366.60 ± 5.98
Lung (mg)	196.60 ± 6.00	194.00 ± 4.55	190.60 ± 6.99
Lung (mg/g BM)	550.80 ± 12.68	551.70 ± 11.00	536.30 ± 14.05
Liver (g)	1.40 ± 0.08	1.48 ± 0.03	1.50 ± 0.08
Liver (g/g BM)	3.98 ± 0.10[a]	4.20 ± 0.08[b]	4.21 ± 0.10[b]
Adrenal gland (mg)	7.20 ± 0.57	7.60 ± 0.34	7.90 ± 0.90
Adrenal gland (mg/g BM)	20.09 ± 1.48	21.61 ± 0.92	22.26 ± 2.31
Kidney (mg)	187.70 ± 6.46	194.90 ± 3.76	200.10 ± 7.98
Kidney (mg/g BM)	524.30 ± 5.26	550.00 ± 15.84	562.40 ± 14.69

Note: Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Different letters indicate statistically significant differences among the groups ($p > 0.05$). The superscript values a, b, and ab indicates the presence or absence of differences between the groups.
Abbreviation: BM = body mass.

TABLE 3 | Reproductive organ masses of mice from the experimental groups in the first estrus after PND 75.

	Experimental groups ($n = 10$/group)		
	Control	**0.1 mg/kg**	**1 mg/kg**
Ovaries (mg)	12.56 ± 0.93	15.90 ± 1.31	14.60 ± 0.97
Ovaries (mg/g BM)	38.79 ± 4.42	45.52 ± 4.11	40.83 ± 2.02
Oviducts (mg)	9.78 ± 0.36	11.90 ± 0.95	10.00 ± 1.01
Oviducts (mg/g BM)	27.04 ± 1.31	33.93 ± 2.80	27.91 ± 2.54
Uterus (mg)	167.90 ± 7.68	160.20 ± 5.00	171.90 ± 4.33
Uterus (mg/g BM)	469.40 ± 20.75	454.60 ± 11.30	488.20 ± 21.86

Note: Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).
Abbreviation: BM = body mass.

In the uterine histopathology, there was a reduction in the endometrial projections towards the lumen and a diminution in the proportion of collagen fibers in the group exposed to PPF 1 mg/kg, in comparison to the other two groups (Figures 8 and 9). Additionally, morphometric assessment demonstrated a reduction in endometrial stroma in both experimental groups in comparison to the control group ($p < 0.05$) (Figure 8). However, the other uterine morphometric parameters were comparable among the groups (Figure 8).

The quantification of mast cells performed in the cortex and medulla of the ovary and the uterine myometrium and endometrium stroma were similar among the experimental groups (Table 4). Furthermore, Figure 10 shows representative photomicrographs of ovaries and uteri stained with toluidine blue and mast cells identified in these organs, whose cell population was greater in the uterus than in the ovary, in both experimental groups.

Concerning thyroid histology, both experimental groups showed similar follicular epithelium structures with colloid inside them, as well as similar interstitial tissue with parafollicular cells (Figure 11). However, thyroid stereology showed a reduced mass of glandular epithelium and colloid in the group exposed to the higher dose of PPF, in comparison to the other groups ($p < 0.05$) (Table 5).

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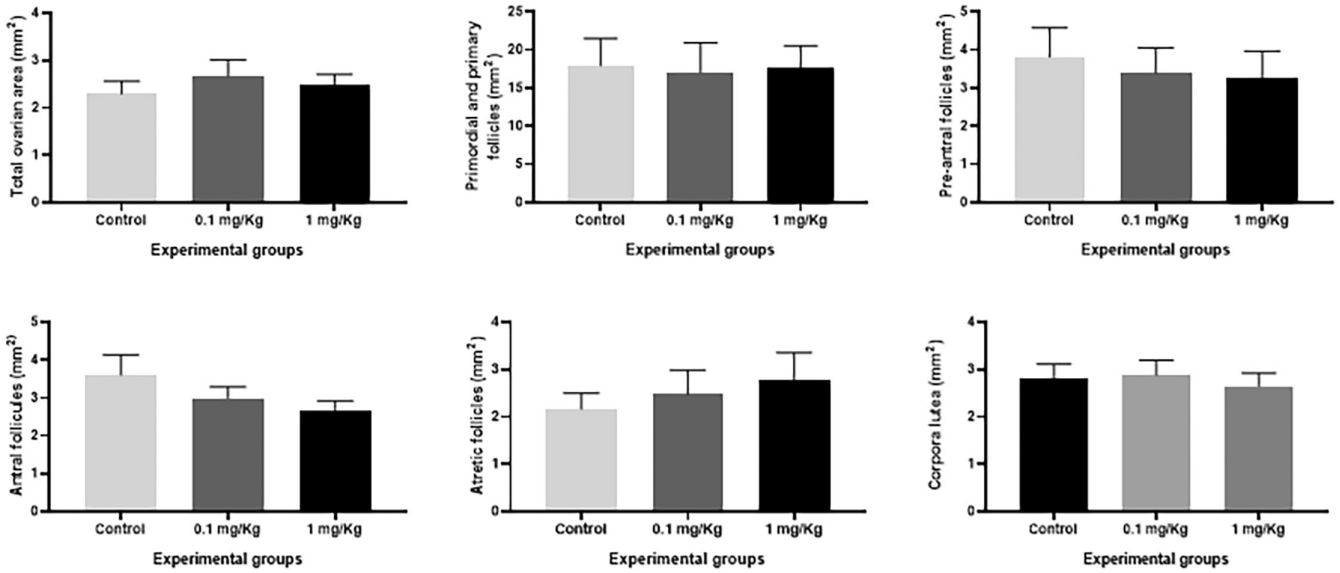

FIGURE 6 | Quantification of follicles and corpora lutea in the ovary of the animals of the experimental groups in the first estrus phase after PND 75. Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

3.2 | First Experiment: Reproductive Performance and Fetal Evaluations

Body mass gain, water intake, and food ingestion in pregnant mice from the experimental groups were similar during pregnancy (Figure 12). Regarding reproductive performance, females from both treated groups showed a reduction of 30% in the gestational rate, when compared to the control group (Table 6). Additionally, it was observed as an augmented frequency of fetal death in the offspring of females previously exposed to 0.1 mg/kg of PPF ($p < 0.05$) (Table 6). On the other hand, no differences were found for the other indexes assessed in this study (Table 6).

The fetal biometric parameters evaluated, as well as the frequency of congenital anomalies and early developmental milestones, were similar among the groups, showing that PPF did not affect fetal development (Tables 7 and 8).

3.3 | Second Experiment: Uterotrophic Assay, Body Mass, and Organ Masses

Females used in the second experiment had similar initial and final body masses among the experimental groups (Figure 13). As expected, the mass of the uterus with and without fluid of the animals that received 17β-estradiol increased compared to the other groups ($p < 0.05$), showing the efficacy of the test, although uteri masses among the other groups were similar (Figure 13). The mass of the other collected organs showed no differences among the groups (Table 9).

4 | Discussion

It is known that endocrine disruptors act by mimicking or antagonizing the action of endogenous hormones, such as estrogen [26]. When evaluating the in vitro estrogenic activity of 32 pesticides, it was found that PPF exhibited estrogenicity [27]. However, Ji et al., observed in vitro that only PPF metabolites had intense estrogenic activity [28]. The uterotrophic assay is a test validated by the Organization for Economic Cooperation and Development, which consists of evaluating the ability of a compound to induce an agonist or antagonist activity to endogenous estrogens [29, 30]. In this study, the mass of the uterus of the PPF-exposed females did not change after the exposure, indicating that PPF showed no estrogenicity in the doses and conditions tested.

In female rodents, the vaginal opening and the first estrus are considered external markers of puberty onset [31]. Some endocrine disruptors already described may affect this critical phase of postnatal development, resulting in early or late puberty [32]. Therefore, in this study, PPF did not affect puberty onset, since the females from the experimental groups showed similar ages of vaginal opening and first estrus. Considering that puberty onset is dependent on increased levels of estradiol and that PPF showed no estrogenicity in the tested doses, it was expected that the age of puberty onset would be similar among the groups. Thus, the absence of estrogenic action of the insecticide may explain the similar ages of puberty onset.

Determining the regularity of the estrous cycle is considered a useful marker for assessing the integrity of the hypothalamic–pituitary–gonadal axis and ovarian activity [33]. When there are changes in the estrous cycle, such as a prolonged diestrus, reduced proestrus, and shorter estrus, it may be due to an imbalance in the estrogen/progesterone ratio, as suggested by Baligar and Kaliwal (2002) [34]. In this study, PPF-treated females showed no changes in estrous cyclicity, which possibly indicates few impairments in the integrity of hypothalamic-pituitary-gonadal axis, although changes in hormonal levels are still possible because other reproductive effects were shown in this study. To confirm this, future studies are necessary to measure the hormonal levels in females after PPF exposure.

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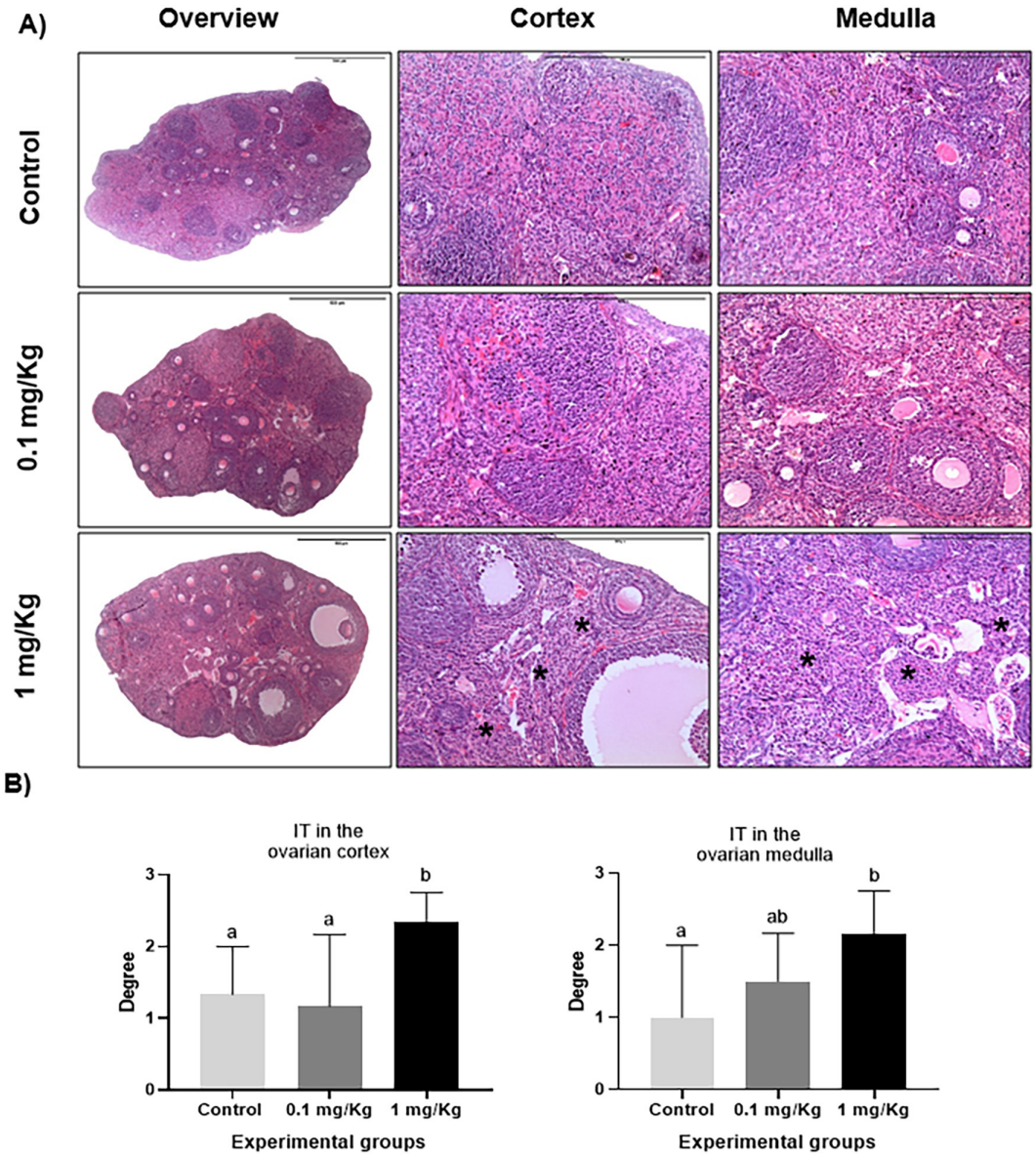

FIGURE 7 | (A) Photomicrographs of ovaries from females of the experimental groups in the first estrus after PND 75. Note the increased interstitial tissue in the cortex and medulla region of the ovary (asterisks) of mice exposed to the 1 mg/kg of PPF. Hematoxylin and eosin (HE). Scale = 500 μm in the first column and 250 μm in the second and third columns. (B) Degree of interstitial tissue (IT) in the ovarian cortex and medulla. Values are expressed as median and interquartile range. Kruskal-Wallis followed by Dunn's post hoc test. Different letters indicate statistical differences among the groups ($p < 0.05$).

Furthermore, monitoring body mass and water and food consumption during the experiment are considered crucial parameters for assessing the animal's state of health, and, in addition, the mass of vital organs can also indicate signs of systemic toxicity [35]. Herein, the treated groups showed body mass growth and consumption of water and food similar to the control group.

In the present study, a decrease in thyroid mass and the masses of glandular epithelium and colloid were observed at the dose of 1 mg/kg. Thyroid hormones play an important role in maintaining different biological processes, such as metabolism, reproduction, and development [36]. In the ovaries, adequate levels of thyroid hormones modulate the action of FSH and LH, as they are involved in the survival of follicles and granulosa cells [37, 38]. In the uterus, through intracellular receptors, these hormones regulate the responsiveness of the organ to estrogen [39].

Thyroid dysfunction is associated with reproductive disorders because thyroid hypofunction may reduce the number of ovarian growing follicles, increase follicular atresia in the ovaries, decrease the proliferative rate of uterine epithelial and stromal cells, reduce endometrial thickness, and it is also related to augmented oxidative stress in the ovary [39]. Furthermore, a previous study showed that *Xenopus laevis* exposed to PPF metabolite (4′-OH-PPF) exhibited decreased thyroid hormone signaling with an antagonistic activity to T3 [12]. Furthermore, another study performed with zebrafish embryos exposed to

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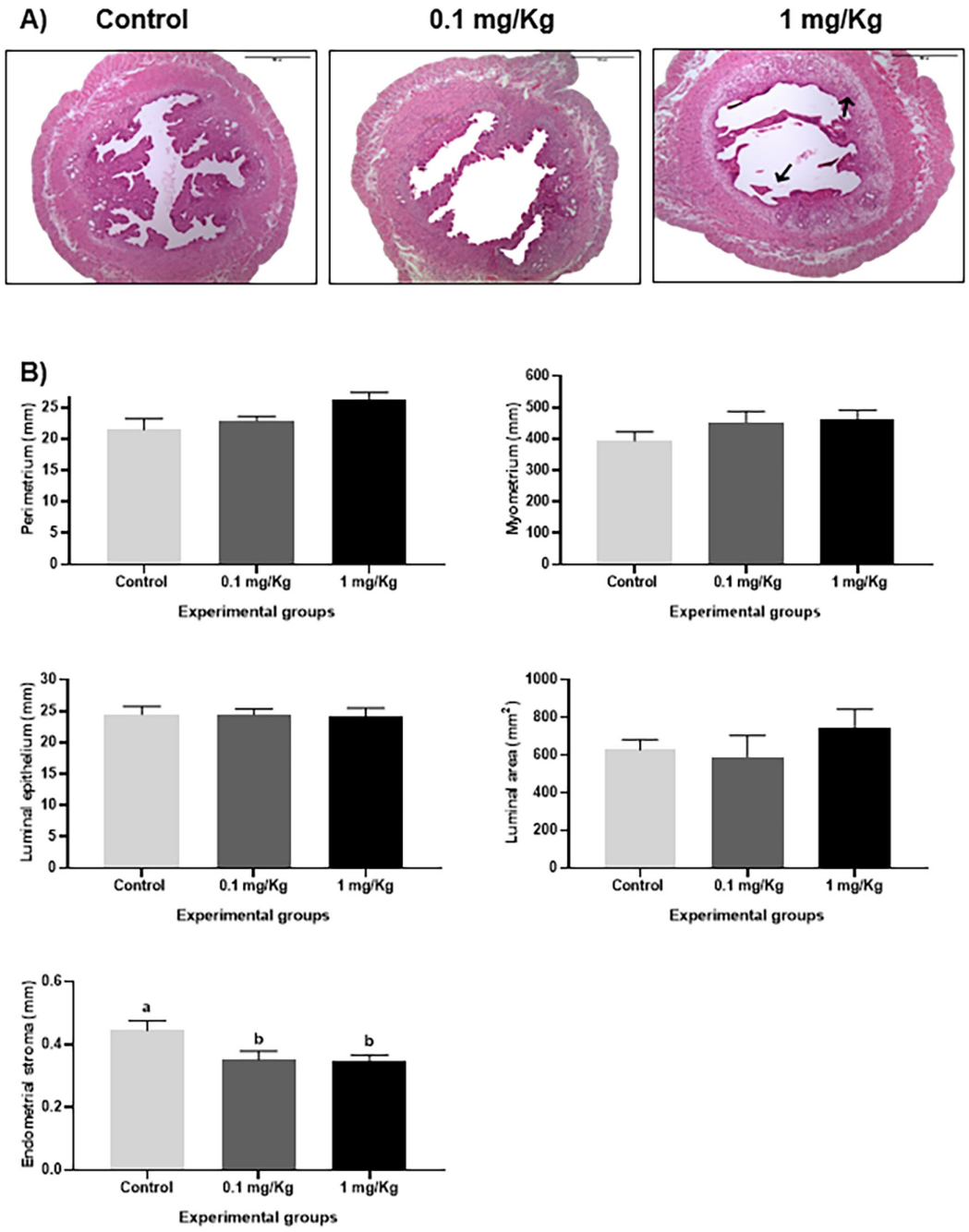

FIGURE 8 | (A) Uterine photomicrographs of animals from the experimental groups in the first estrus after PND 75. Note the decrease of endometrial projections towards the lumen (arrows) in females exposed to the higher dose of PPF. Hematoxylin and eosin (HE). Scale = 500 μm. (B) Uterine morphometry of the animals of the experimental groups in the first estrus phase after PND 75. Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test. Different letters indicate statistical differences among the groups ($p < 0.05$).

PPF showed a decrease in thyroid hormone-related gene expression and indicated that the toxic effects provoked by PPF are mediated by its thyroid hormone-disrupting action [40].

Then, we suppose that the thyroid may be a target for this toxicant like its metabolite was shown in the previous study performed with frogs. Herein, PPF seems to act as an antagonist of thyroid hormones and, then, it may provoke some of the reproductive adverse effects observed in the female reproductive system, such as reduced endometrial stroma and diminished gestational rate possibly through impairing oocyte quality. Additional studies are necessary to elucidate the pathways used by PPF to provoke toxicity in the thyroid.

Additionally, relative liver mass was increased at both doses of exposure. It is known that the liver is involved in the response to most xenobiotics due to its ability to biotransform chemical compounds [41]. After analyzing PPF metabolites in rat livers in vitro, it was shown that their toxicity was greater than that of PPF [42]. In addition, WHO studies have shown an increase

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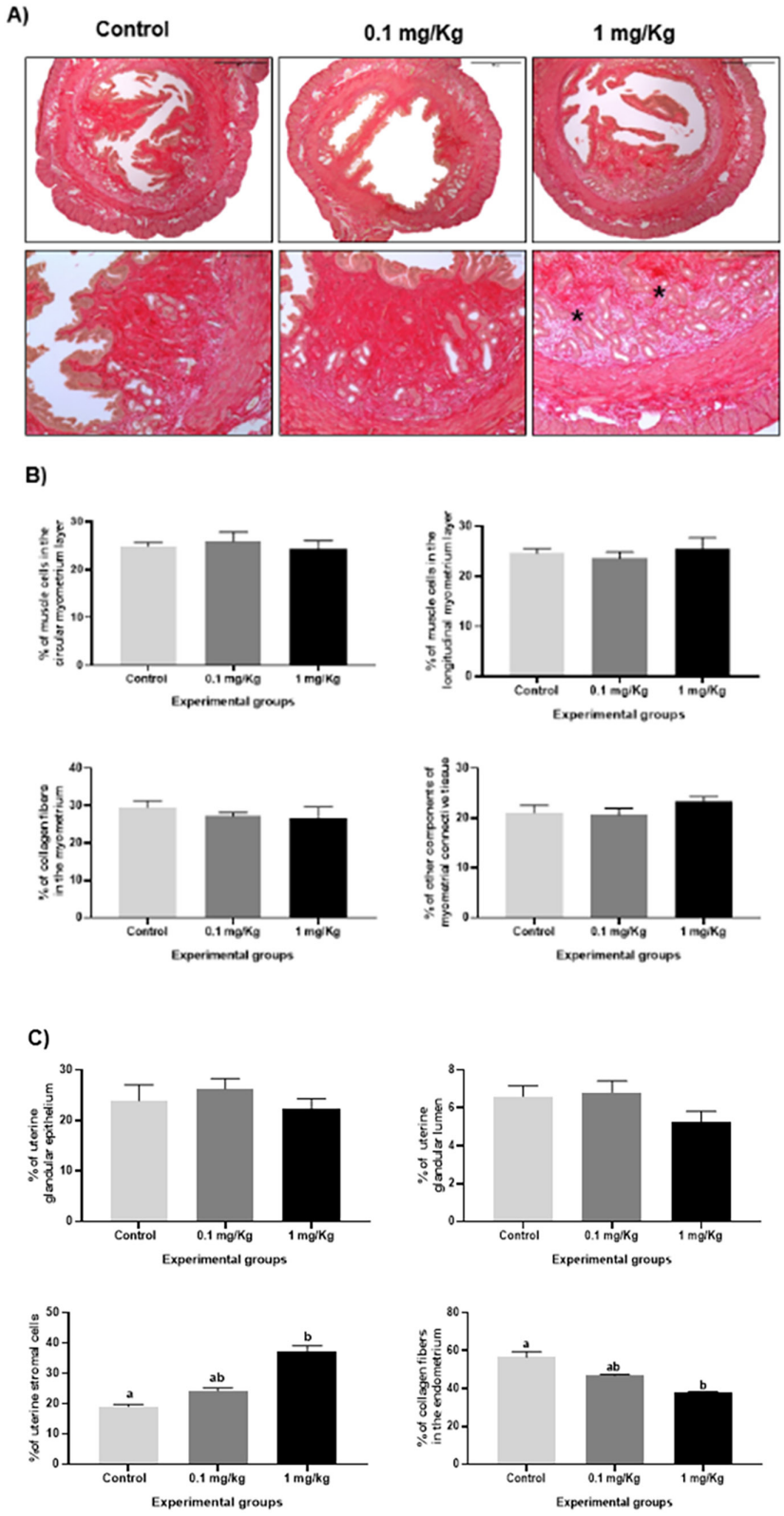

FIGURE 9 | (A) Uterine photomicrographs of animals from the experimental groups in the first estrus after PND 75. Note the decrease in the content of collagen fibers in the endometrium stroma (asterisks) of females exposed to 1 mg/kg of PPF. Picrosirius red and hematoxylin (PSR-H). Scale = 500 μm in the first line and 200 μm in the second line. (B and C) Proportion of different cells and tissues and collagen fibers in the myometrium and endometrial stroma of animals of the experimental groups in the first estrus after PND 75. Values are expressed as median and interquartile range. Kruskal-Wallis followed by Dunn's post hoc test. Different letters indicate statistical differences among the groups ($p < 0.05$).

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TABLE 4 | Quantification of mast cells in the ovary and uterus of females from the experimental groups in the first estrus after PND 75.

	Experimental groups ($n = 6$)		
	Control	**0.1 mg/kg**	**1 mg/kg**
Ovarian cortex	1.00 ± 0.35	0.94 ± 0.34	0.27 ± 0.12
Ovarian medulla	0.05 ± 0.05	0.11 ± 0.11	0.22 ± 0.16
Myometrium	103.20 ± 9.64	110.90 ± 14.60	148.60 ± 20.20
Endometrium	5.50 ± 1.69	2.67 ± 0.56	3.78 ± 1.20

Note: Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

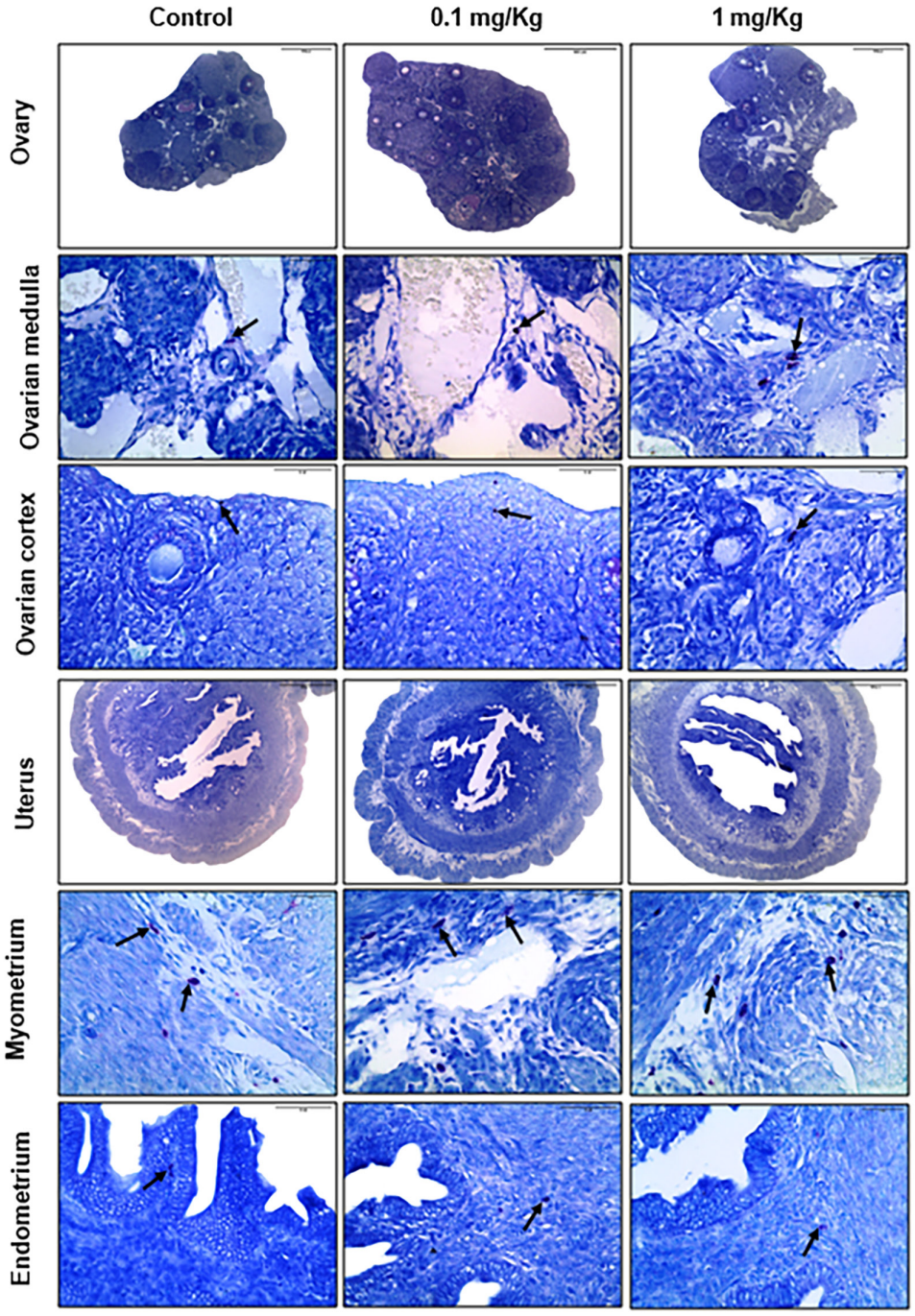

FIGURE 10 | Photomicrographs of ovaries and uteri from females of the experimental groups stained with toluidine blue. The second and third lines detail mast cells (arrows) identified in the ovarian cortex and medulla, respectively. The fifth and sixth lines show mast cells (arrows) in the myometrium and endometrial stroma. Scale = 500 μm in the first and fourth lines and 50 μm in the second, third, fifth, and sixth lines.

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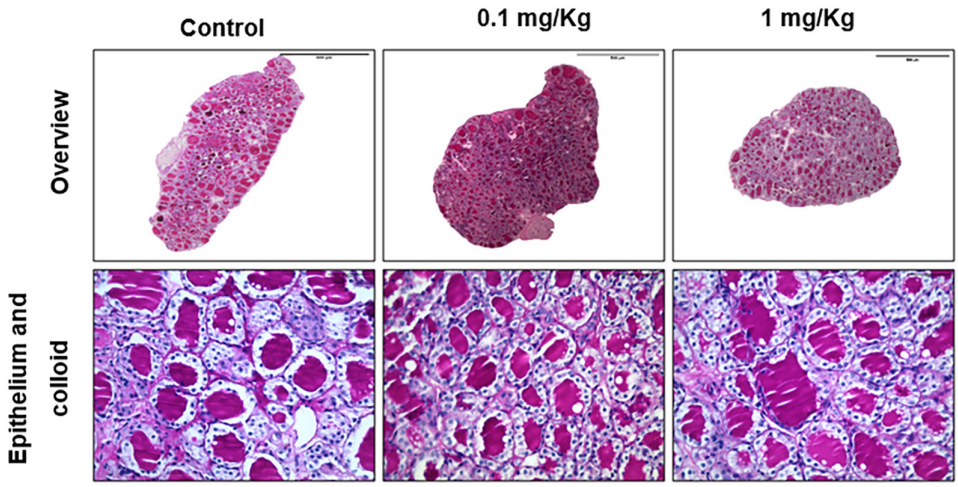

FIGURE 11 | Photomicrographs of thyroids from females of the experimental groups in the first estrus after PND 75. Periodic acid Shiff (PAS) and hematoxylin. Scale = 500 μm in the first line and 50 μm in the second line.

TABLE 5 | Thyroid stereology in the females of the experimental groups in the first estrus after PND 75.

	Experimental groups ($n = 6$/group)		
	Control	**0.1 mg/kg**	**1 mg/kg**
Glandular epithelium (%)	52.96 (47.84–54.38)	53.48 (35.41–59.78)	49.38 (35.10–62.03)
Glandular epithelium (mg)	2.00 ± 0.31^{a}	1.43 ± 0.16^{ab}	1.25 ± 0.27^{b}
Colloid (%)	42.79 (36.37–54.87)	40.29 (27.04–56.37)	42.92 (31.23–49.54)
Colloid (mg)	1.72 ± 0.20^{a}	1.18 ± 0.17^{ab}	1.02 ± 0.09^{b}
Parafollicular cells (%)	1.24 (0.46–3.57)	0.96 (0.68–1.12)	1.54 (0.46–1.73)
Parafollicular cells (mg)	0.05 ± 0.01	0.03 ± 0.00	0.05 ± 0.02
Other intersticial components (%)	5.53 (3.46–7.86)	3.01 (2.20–5.89)	5.48 (4.17–7.87)
Other intersticial components (mg)	0.22 ± 0.04	0.11 ± 0.02	0.17 ± 0.03

Note: Values expressed as mean ± SEM or median and interquartile range. One-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test. Different letters indicate statistical differences among the groups ($p < 0.05$). The superscript values a, b, and ab indicates the presence or absence of differences between the groups.

in liver mass in rats exposed to doses of 120 mg/kg/day [2]. We tested doses that did not exceed the maximum limit of PPF allowed for WHO for daily ingestion, and even using lower doses, we showed an increased relative liver mass, suggesting a possible toxic effect for this organ.

Concerning the reproductive organs, in this study, both groups exhibited a similar number of follicles and corpora lutea. However, females exposed to PPF presented an increase in ovarian interstitial tissue, in both cortex and medulla. In contrast, a study with *Danio rerio* found that PPF inhibited follicle maturation, causing an increase in the proportion of follicles in early stages containing previtellogenic oocytes [11].

In the ovary, mast cell numbers change according to the phase of the reproductive cycle and play a role in increasing vascular permeability in response to LH, interacting with estrogen and progesterone, and helping to maintain the organ's homeostasis [43]. In the uterus, mast cells also fluctuate according to the phase of the cycle and their most important role is in the implantation, by secreting substances that generate the tissue remodeling necessary during this process [44]. Females exposed to glyphosate, another pesticide, during pregnancy, showed increased mast cell numbers in the uterus, which, together with other inflammatory processes, contributed to increased post-implantation losses [45]. Despite this, no difference was observed in the number of mast cells in the ovary and uterus of PPF-exposed animals.

In the uterus, there was a decrease in the thickness of the endometrial stroma with reduced endometrial projections toward the lumen and a reduction in the content of endometrial collagen fibers. This may be attributed to the alterations shown in the thyroid in this study, which is suggestive of decreased action of thyroid hormones. It is known that the uterus expresses receptors for thyroid hormones in the luminal and glandular epithelium, as well as in the stromal cells [46]. Then, a possible reduction in endometrial stimulation by thyroid hormone could provoke diminished endometrial proliferation, reduced stromal thickness, and changes in the appearance of the endometrial tissue [39]. These uterine changes may compromise many processes related to

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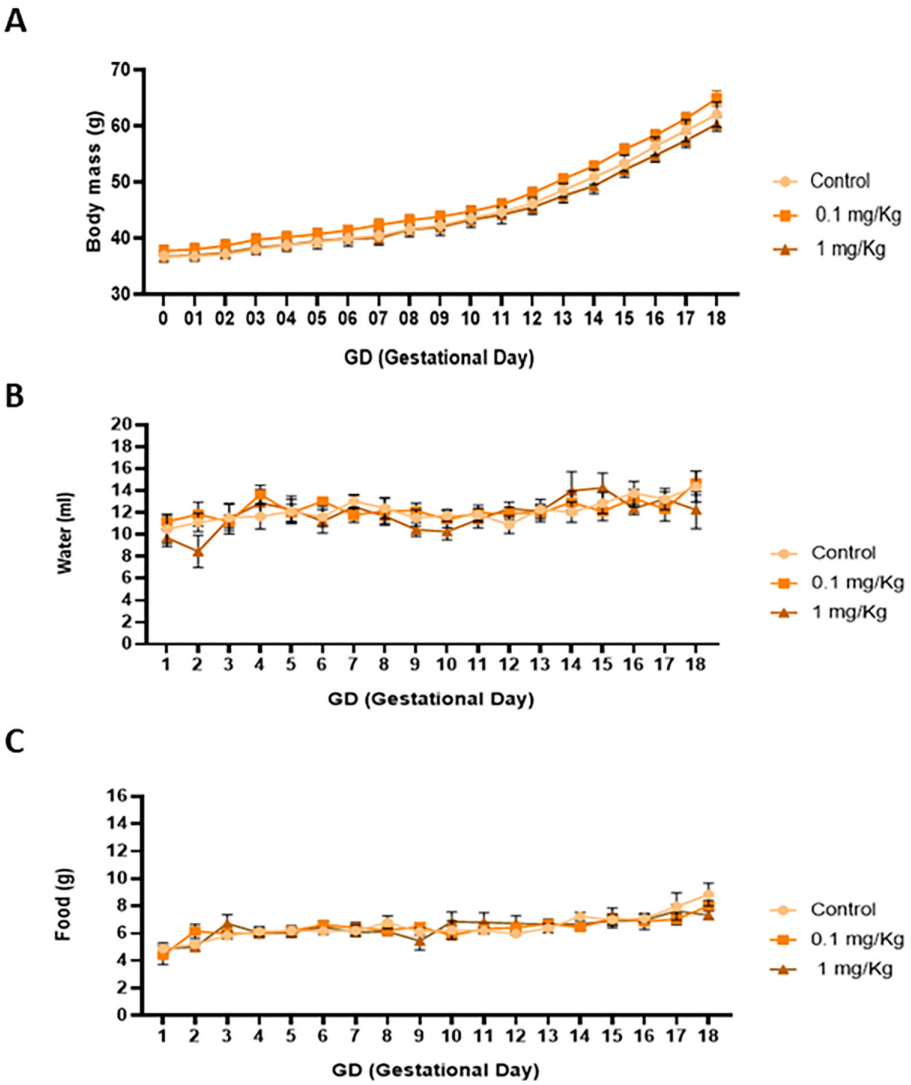

FIGURE 12 | Body mass gain and water and food intake during pregnancy (A) Body mass gain during gestation. (B) Water ingestion after mating. (C) Food intake during pregnancy. Values expressed as mean ± SEM. Two-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

embryo development, such as fertilization, differentiation, nutrition, and implantation [39], which may explain the reduced pregnancy rate and increased fetal death demonstrated in this study.

In the uterine stroma, collagen fibers are organized interspersed with cells and can have their synthesis, degradation, and organization modified by steroid hormones [47, 48]. It is known that thyroid hormones may modulate the bioavailability and metabolism of other hormones like steroid hormones and their transport proteins [49]. Whether there is a deregulation of steroid hormones with a predominance of androgens, collagen fibers undergo disarrangements in their distribution throughout the endometrium, which may impair endometrial receptivity during implantation [50].

Although a previous study showed that the administration of PPF during pregnancy caused a decrease in female mass gain and an increased duration of pregnancy [10], in this study, females demonstrated similar mass gain and water and food consumption throughout pregnancy. It is important to highlight that the doses used in this previous study were higher than the acceptable daily intake allowed by the WHO, unlike this study, in which the guidelines were followed to simulate human exposure to PPF [2, 15].

Herein, there was a reduction of 30% in the pregnancy rate of both PPF-exposed females. It is worth mentioning that females were inseminated and presented vaginal plug or sperm in vaginal smears, although the mice were not able to keep gestation. This result is associated with the endometrial impairment observed in the PPF-exposed females and also indicates a possible damage of PPF to oocytes, reducing the viability of germ cells, which, in turn, compromises fertilization and the success of pregnancy.

Recent studies have reported that PPF may induce oxidative stress in zebrafish ovaries and testis, by increasing reactive oxygen species formation and lipid peroxidation and provoking glutathione depletion [11, 51]. Furthermore, considering that PPF may increase oxidative stress, it may provoke DNA damage in

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TABLE 6 | Reproductive performance of females from the experimental groups after natural mating with untreated males.

	Experimental groups ($n = 10$/group)		
	Control	**PPF 0.1 mg/kg**	**PPF 1 mg/kg**
Gestational rate (%)	100%	70%	70%
Fertility test (%)	93.33 (86.67–100)	100 (86.67–100)	66.67 (46.15–92.86)
Pre-implantation loss (%)	6.67 (0.00–13.33)	0.00 (0.00–13.33)	33.33 (7.14–53.85)
Post-implantation loss (%)	11.11 (3.84–20.63)	11.76 (10.00–35.71)	16.67 (15.38–25.00)
Sex ratio (M:F)	1.37 (0.75–2.00)	1.40 (0.71–3.50)	1.20 (0.57–1.67)
Gravid uterus (g)	16.44 ± 1.80	18.80 ± 1.59	14.66 ± 1.50
Dead fetus (%)	0.00 (0.00–0.00)[a]	0.50 (0.00–1.25)[b]	0.00 (0.00–0.00)[a]
Male fetus mass (g)	1.41 ± 0.04	1.44 ± 0.05	1.41 ± 0.06
Female fetus mass (g)	1.31 ± 0.03	1.30 ± 0.05	1.32 ± 0.04
Placenta mass from male fetus (g)	0.11 ± 0.00	0.10 ± 0.00	0.11 ± 0.00
Placenta mass from female fetus (g)	0.11 ± 0.00	0.10 ± 0.01	0.11 ± 0.00

Note: Values expressed as mean ± SEM or median and interquartile range. One-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test. Different letters indicate statistical differences among the groups ($p < 0.05$). The superscript values a and b indicates the presence or absence of differences between the groups.

TABLE 7 | Fetal biometric parameters of male and female offspring of animals from the experimental groups.

	Experimental groups		
	Control ($n = 10$)	**0.1 mg/kg ($n = 7$)**	**1 mg/kg ($n = 7$)**
Craniocaudal distance in the female fetus (mm)	23.78 ± 0.34	23.78 ± 0.32	23.40 ± 0.33
Craniocaudal distance in the male fetus (mm)	24.60 ± 0.36	24.30 ± 0.28	24.42 ± 0.36
Head circumference in the female fetus (mm)	47.83 ± 0,59	48.48 ± 1.43	46.90 ± 0.93
Head circumference in the male fetus (mm)	48.73 ± 0.66	50.28 ± 0.90	49.73 ± 0.70
AGDi in the female fetus (mm)	0.72 ± 0.04	0.69 ± 0.07	0.70 ± 0.05
AGDi in the male fetus (mm)	1.12 ± 0.07	1.08 ± 0.08	1.08 ± 0.04

Note: Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

TABLE 8 | Frequency of congenital anomalies and anticipation of developmental milestones in the offspring of animals from the experimental groups.

	Experimental groups		
	Control	**0.1 mg/kg**	**1 mg/kg**
Total congenital anomalies	5.62	8.84	5.26
Curly tail	2.25	0.00	1.75
Elongated snout	1.12	1.23	0.00
Exencephaly	1.12	0.00	0.00
Macroglossia	1.12	0.00	0.00
Congenital anomalies of limbs	0.00	3.70	1.75
Gastroschisis	0.00	2.47	1.75
Exophthalmos	0.00	1.23	0.00
Open eyelid	16.85	15.07	14.04

Note: Values expressed in percentage (%). Fisher's exact test ($p > 0.05$).

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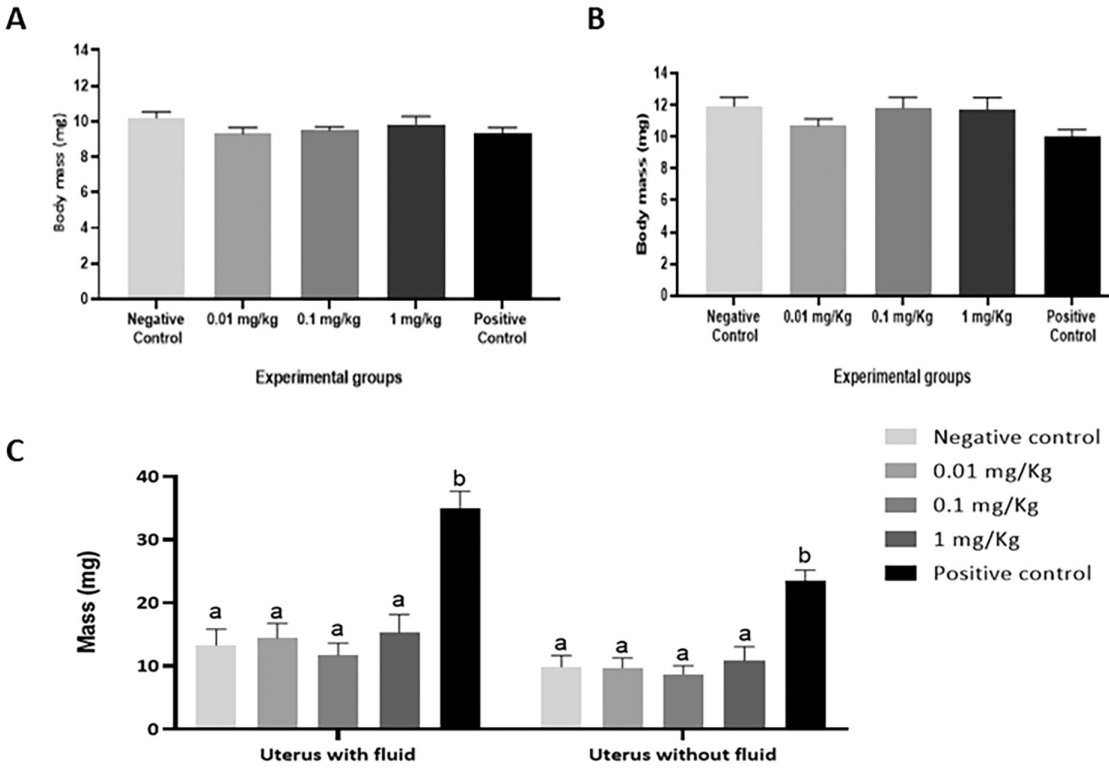

FIGURE 13 | Female body mass and mass of uterus with and without fluid on uterotrophic assay. (A) Initial body mass of females during uterotrophic assay. (B) Female final body at the end of uterotrophic assay. (C) Uterine mass with and without fluid at the end of uterotrophic assay. Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

TABLE 9 | Reproductive and vital organ masses of females used in the uterotrophic assay.

	Experimental groups ($n = 7$/group)				
	Negative control	**0.01 mg/kg**	**0.1 mg/kg**	**1 mg/kg**	**Positive control**
Ovaries (mg)	3.00 ± 0.22	2.43 ± 0.20	2.57 ± 0.30	2.57 ± 0.20	2.71 ± 0.29
Oviducts (mg)	3.00 ± 0.00	2.43 ± 0.20	3.00 ± 0.00	2.86 ± 0.26	3.00 ± 0.00
Adrenal gland (mg)	2.43 ± 0.20	2.00 ± 0.00	2.00 ± 0.00	2.14 ± 0.26	2.57 ± 0.20
Kidneys (mg)	102.80 ± 3.83	82.29 ± 4.23	85.71 ± 3.89	96.29 ± 5.40	94.14 ± 6.56
Liver (mg)	556.00 ± 33.70	432.30 ± 24.44	470.40 ± 30.56	511.50 ± 28.96	527.00 ± 44.54

Note: Values expressed as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test ($p > 0.05$).

oocytes, which could be transmitted to the zygote whether fertilization was successful. Then, embryo or fetal development may be compromised during critical phases of the prenatal period, which, at last, may increase fetal death in the offspring. Shahid and Saher (2020) when administered PPF to pregnant mice also reported augmented fetal death and changes in the mass and histology of liver, kidney, brain, and heart in the offspring of these females [10]. Despite this, in this study, when females were exposed to PPF until pre-conception, there were no changes in the frequency of congenital anomalies or fetal biometric parameters.

In this work, several reproductive adverse effects were found when females were exposed to the higher dose of PPF, which include reduction in thyroid mass and the mass of colloid and thyroid epithelium, increased relative mass of the liver, augmentation in the ovarian interstitial tissue, reduced endometrial stroma thickness with diminished content of collagen fibers and endometrial projections, as well as reduction in the pregnancy rate. In the lower dose of PPF, we only showed part of the effects that were shown in the higher dose, such as the augmented relative mass of the liver, diminished endometrial stroma thickness, and reduction in the pregnancy rate. Additionally, this dose presented an increased fetal death. Considering the main outcomes demonstrated in this study, PPF exposure seems to manifest crescent dose-dependent effects, suggesting that higher doses of this compound may provoke more toxic effects for female reproduction.

Then, PPF may impair endometrial stroma, which indicates a possible antagonist action for thyroid hormones; thus, it demonstrates an endocrine-disruptive action of this pesticide. As a limitation of

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this study, we were unable to perform the sex steroids and thyroid hormonal measurements; however, the results are consistent and indicate this action. When oocytes are exposed to reduced thyroid hormone stimulation and increased oxidative stress, as we suppose these are part of the adverse outcome pathways of PPF, then, the events such as fertilization and successful pregnancy may be harmed. Whether embryo development progresses, fetal viability may be impaired and fetal death can be augmented.

5 | Conclusion

In summary, female mice exposed to PPF from pre-puberty to sexual maturity exhibited systemic toxic effects, as demonstrated by reduced thyroid mass, diminished mass of the thyroid glandular epithelium and colloid, and increased liver mass. In addition, PPF decreased endometrial stroma thickness and the content of collagen fibers. Together, the results indicate a possible antagonist action of this pesticide for thyroid hormones. Moreover, PPF reduced the pregnancy rate in both groups and increased fetus death in the group exposed to the lower dose, suggesting diminished viability of oocytes and an impairment to fetal viability mediated by maternal factors. Then, based on this experimental model and considering that this study was performed with environmentally acceptable doses of PPF, the insecticide may pose a reproductive risk for females chronically exposed to the substance from the pre-pubertal period. These results raise concerns about prolonged exposure of women to the same compound.

Author Contributions

Alice Santos da Silva: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing of the original draft. **Tainara Fernandes de Mello:** methodology, investigation, and formal analysis. **Henrique Frederico Enz Fagá:** investigation and formal analysis. **Jennyfer Karen Knorst:** investigation and formal analysis. **Fátima Regina Mena Barreto Silva:** conceptualization, resources, and writing of the original draft. **Gabriel Adan Araújo Leite:** conceptualization, resources, methodology, project administration, supervision, visualization, and writing of the original draft. The manuscript was revised and approved by all authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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