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Effects of herbicide atrazine on reproductive hormonal levels, Cytochrome P450, and gonadal structure of adult male and female crayfish, *Procambarus clarkii*

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ABSTRACT

Atrazine is a herbicide commonly used on grain crops. It has been identified as a potential endocrine disruptor, especially in freshwater species. However, there is little research on its endocrine disruptor effect in crustaceans. The current study was designed to estimate the median lethal concentration of atrazine (96-h LC_{50}) and to assess the response of the freshwater crayfish *Procambarus clarkii* as a bioindicator for endocrine disruptors by measuring levels of steroid hormones, total protein, changes in Cytochrome P450 1B1, and histopathology of the ovary and testis. The results showed that the 96-h LC_{50} of atrazine for male and female *P. clarkii* was 10.62 and 12.66 mg L^{-1}, respectively. LC_{10} (1.06 and 1.27mg/l) and LC_{25} (2.66 and 3.17 mg/l) values for males and females, were chosen for sublethal studies for 28 days. The results indicated that females are more tolerant to atrazine than males. Testosterone and total protein levels were significantly lower, while estradiol and progesterone levels were significantly higher in atrazine-exposed crayfish compared to the control. Also, the levels of CYP450 1B1 increased significantly in the testis, ovary, and hemolymph. The most noticeable histopathological changes in the testis were distorted architecture, spermatogonia distribution disruption in some testicular acini, Vacuolation, and hyperplasia. In the ovary, the separation between the ovarian epithelium and oogenetic pouch and lysis in the ovarian epithelium was noted. These parameters could be used as biomarkers to assess herbicide toxicity. Furthermore, *P. clarkii* could be used as an indicator of endocrine-disrupting chemicals in waterways.

INTRODUCTION

Atrazine is one of the most extensively used herbicides. It is used to cease the appearance of grassy weeds in main crops such as wheat and corn. Atrazine has a half-life of more than 60 days in water, with concentrations ranging from 0.1 to 100 µg/L in the environment (**USEPA, 2002**). Atrazine concentrations up to 1 mg/L were found in streams and groundwater near treated fields (**Graymore *et al.*, 2001**). Despite the fact that this herbicide is rarely absorbed in sediments, the proportion linked with this substrate can be rather high (**Jablonowski *et al.*, 2011**). Because of the low persistence of atrazine, repeated applications are used to eradicate weeds in agricultural fields, resulting in huge amounts of the herbicide entering water bodies (**Nwani *et al.*, 2010**).

Atrazine is reported to be slightly to highly toxic to aquatic organisms (**Stara *et al.*, 2018**). The 96h LC_{50} of Atrazine in the decapods *Cherax destructor* is 12.10 mg/L (**Stara *et al.*, 2018**). The 96h LC_{50} for *Cyprinus carpio* and *Melanotenia fluviatilis* is 2.14 and 5.6 mg/L atrazine, respectively (**Xing *et al.*, 2015; Phyu *et al.*, 2006**). Also, the 96h LC_{50} of atrazine are 18.53 and 42.38 mg/L in *Rutilus frisii kutum* and *Channa punctatus*, respectively (**Khoshnood & Khoshnood, 2014; Nwani *et al.*, 2010**). Atrazine has possible to adversely affect aquatic organisms at environmental concentrations, comprising fishes and crayfish. It has been reported that it interferes with reproduction, growth, and development, as well as accumulating in tissues. In fish, crayfish, mammals and human, atrazine is known for its endocrine disrupting properties (**Stara *et al.*, 2018**).

The red swamp crayfish, *Procambarus clarkii*, is an endemic species of the USA and Mexico that was introduced to Egypt for aquaculture in the early 1980s but escaped and spread along the Nile River (**Ibrahim *et al.*, 1995**). *P. clarkii* is successfully cultured for human consumption in several countries across the world. It can tolerate severely contaminated water, and as a result, it may pass accumulated contaminants such as inorganic pollutants and heavy metals to humans via the food chain **(Strużyński *et al.*, 2013**). *P. clarkii* is considered as an excellent model organism for ecotoxicological investigations (**Serrano *et al.*, 2000**). *P. clarkii* has been employed as a sentinel species for biomarker research, and several studies on the impact of various contaminants have been published (**Alcorlo *et al.*, 2006; Goretti *et al.*, 2016**). However, little research on the effects of atrazine as endocrine disrupter on reproductive parameters of this common species has been done.

P. clarkii characterises the biology of most decapod crustaceans. As a result, the reproductive biology of this species has received a lot of attention. **Kulkarni *et al.*, (1991),** for example, completely characterized this crayfish's ovarian cycle, whereas **Fingerman (1995)** reviewed the roles of hormones involved in gonadal growth. **Nagaraju 2011** reported that the oocytes manufacture vitellogenin by themselves through primary vitellogenesis; nevertheless crustacean ovaries grow by absorbing vitellogenin manufactured in the digestive gland during the secondary vitellogenesis. Spermatogenesis in this crayfish was completely described by (**Moses, 1961**).

Some vertebrate-like sexual hormones, as17- β- estradiol and 17- αhydroxyprogesterone have been shown to have a role in crustacean reproduction (**Lafont & Mathieu, 2007**). The freshwater prawn *Macrobrachium rosenbergii* has been found to express numerous steroidogenesis enzymes involved in the synthesis of estrogen and progesterone (**Thongbuakaew *et al.*, 2016**). Furthermore, 17-estradiol and progesterone have been shown to stimulate ovarian development in decapod crustaceans (**Rodriguez *et al.*, 2002**). In the kuruma prawn *Metapenaeus japonicus*, 17α-hydroxyprogesterone enhanced ovarian development (**Yano, 1987**). In vivo in crayfish, 17-estradiol enhanced vitellogenesis by ovary fragments (**Coccia *et al.*, 2010**).

Following exposure to atrazine, females crayfish *P. clarkii* showed a decrease in ovarian growth and increase in estradiol levels (**Silveyra *et al.*, 2018**). Furthermore, atrazine produced a delay in ovarian rematuration and inhibition of sexual steroids in

crab *Neohelice granulata* throughout the reproductive cycle (**Alvarez *et al.*, 2015**). This herbicide impeded gonadal development in fish and other vertebrates by interfering with hypothalamic regulation of pituitary hormone release (**Tillitt *et al.*, 2010**).

The cytochrome P450 (CYP) proteins are monooxygenases that catalyze a variety of processes involved in xenobiotic detoxification and steroid synthesis. 17b-estradiol is metabolized by the enzyme expressed by this gene (**Zanette *et al.*, 2010**). Aquatic arthropods, especially crustacean have been found to contain CYP enzymatic activity (**James & Boyle, 1998**). The enzyme CYP P450 1B1 has been identified as an essential enzyme in the metabolism of estradiol (**Hayes *et al.*, 1996**). In steroidogenic organs such as the ovary and testis, CYP1B1 is expressed all of the time (**Shimada *et al.*, 1996**). The purpose of this study was to investigate the effect of atrazine as an endocrine disrupting compound on reproductive system of both male and female *P.clarkii* by measuring steroid hormone levels (Estradiol, Progesterone and testosterone), total protein levels and Cytochrome P4501B1, as well as detecting alteration in ovary and testis structures in order to determine whether this species can be used as a bioindicator of endocrine disrupting chemicals in aquatic ecosystems.

MATERIALS AND METHODS

Collection of crayfish

The red swamp crayfish *Procambarus clarkii* (approximately 200 individuals for each sex) were captured with a 0.7 cm diagonal net size from Sheba irrigation Canal in Zagazig, Sharkia Governorate, Egypt between July and September 2021.

Experimental design

The collected specimens were transported to the laboratory alive and kept in glass aquaria (40 x 40 x 40 cm). For one week, mature females and males weighing 25– 34g and measuring 9– 12cm in length were acclimated to indoor laboratory conditions. The average water quality parameters were: Temperature 29.1 ± 0.24; dissolved oxygen 6.3 ± 0.2; PH 7.4 ± 0.1 and total ammonia 0.26 ± 0.06. According to **Boyd (1984)**, all these ranges are within the acceptable limits. Suffocation was avoided by keeping a water depth of 7- 10cm and exposing the specimens to room temperature. Light tubes were used to keep the temperature at 25^{o}C and a 12:12h light– dark cycle. Aquaria's water was replaced on a daily basis with aerated tape water. Crayfish were fed carrot and minced meat until they appeared to be satiated three times a day at 9:00, 13:00 and 17:00h.

The tested herbicide

Atrazine is a white powder that dissolves in water (6-Chloro-2-ethyl-4-isopropyl-1, 3, 5-triazine-2, 4-diamine, C3H4ClN5). Its commercial name is Gesaprim (90 % atrazine as active ingredient). 1 gram of atrazine powder was mixed into 1000ml of distilled water to make the stock solution.

Determination of LC_{50}

Stock solution of atrazine was prepared by using distilled water as a solvent to obtain the following concentrations. These concentrations were obtained by serial dilution of the stock solution for atrazine 5, 10, 15 and 20mg/ l. The LC_{50} was calculated using three replicates for each concentration. Each aquarium contained ten mature individuals, either males or females. As a control, an equal number of males and females were left without treatment. Experiments were checked every 24h interval for up to 96h. The dead crayfish were counted and reported. Crayfish were considered to be dead when they failed to respond to antennal or leg stimuli. LC_{50} was calculated using **Finney (1971)** graphic method of the curve dose-effect, using the probit analysis.

LC_{10} and LC_{25} of atrazine were used and redosed every 4 days in a static renewal manner. After 28 days of exposure, living individuals that had survived the effect of the tested herbicide were sacrificed.

Haemolymph and tissue sampling

P. clarkii haemolymph was obtained by direct puncture of the heart with a syringe containing EDTA as anticoagulant for the following analysis. Following the beheading of *P. clarkii*, the ovary and testis were taken for biochemical and hormonal analysis and histopathological studies. Ovary and testis of control treated with LC_{10} and LC_{25} for 28 days were homogenized in saline solution. The homogenates were centrifuged at 5000 rpm for 5 minutes, and the supernatants were stored at -80°C. The hormones namely estradiol (E2), testesterone (T) and progesterone (PRG) were measured by ELISA method using commercial kit (Immunotech version; Beckman Coulter, Marseille, France). Total protein was determined using Biuret method.

Determination of Cytochrome P4501B1

Cytochrome P4501B1 was measured in haemolymph, ovary and testis by ELIZA technique utilizing a kit (USCN life Science Inc. Houston, Texas, and USA).

Histopathological examinations

The ovary and testis of both the control and treated *P.clarkii* were dissected out and fixed in formalin solution (10%) for 24 h. Specimens were dehydrated and embedded in paraffin wax. Sections were cut at 4- 6μm in thickness and stained with hematoxylin and eosin.

Statistical analysis

The SPSS statistic 20.0 was used to analyze all data. Before statistical analysis, all data were checked for normality and homogeneity using the Kolmogorov–Smirnov and Bartlett's tests. One-way ANOVA was done to know whether sample means were significantly different ($P< 0.05$) from each other at different concentrations of atrazine and control.

RESULTS

Toxicity test

Table (1) and Fig. (1) show that the highest mortality rate occurred at atrazine concentration of 20mg/ l, while the lowest mortality percentage occurred at atrazine concentration of 5mg/ l for both the males and females of *P. clarkii*. It was observed that, the mortality percentages increased as the concentrations of atrazine and the duration of exposure rose. It should also be noted that females are more tolerant to atrazine than males.

Table 1. Effect of different atrazine concentrations on mortality percentages of adult males and females *P.clarkii* at different exposure periods

	Mortality percentage (%)							
Time(hrs)	**24**		**48**		**72**		**96**	
&Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Conc (mg/l)								
Control	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
5	0±0.00	0±0.00	16.7±6.7	0±0.00	20±0.00	0±0.00	20±0.00	16.7±6.7
10	36.7±3.3	26.7±3.3	43.3±3.3	36.7±3.3	43.3±3.3	36.7±3.3	46.7±0.00	40±0.00
15	56.7±6.7	46.7 ±6.7	70±0.00	56.7±6.7	73.3±3.3	60±0.00	76.7±3.3	63.3±6.7
20	96.67±3.3	80±0.00	100±0.00	93.3±3.3	100±0.00	100±0.00	100±0.00	100±0.00

-Values are shown as means of samples ±SE

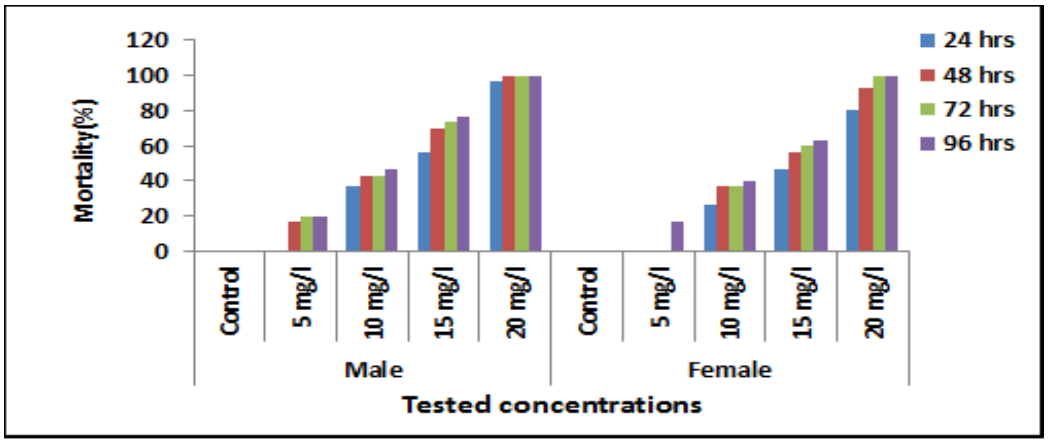

Fig.1. Effect of different atrazine concentrations on mortality percentages of adult males and females *P.clarkii* at different exposure periods

Table (2) reveals that, LC_{50} of atrazine for both males and females *P.clarkii* was 10.62 and 12.66mg/ l, respectively. On the other hand, LC_{25} was 2.66 and 3.17mg/ l, whereas LC_{10} was 1.06 and 1.27mg/ l, respectively, for both males and females *P.clarkii*.

Table 2. Lethal toxicity (LC_{50}) value and sublethal concentration of both males and females *P. clarkii* exposed to atrazine under laboratory conditions

Species	96- LC_{50} (mg/l)	1/4 LC_{50} (LC_{25}) (mg/l)	1/10 LC_{50} (LC_{10}) (mg/l)
Male	10.62	2.66	1.06
Female	12.66	3.17	1.27

Reproductive hormones and total protein levels in female *P.clarkii*

Data in Table (3) and Fig. (2) show that, there was significant increase in reproductive hormones (E2 and PRG) levels ($P< 0.05$) in the ovary and haemolymph of females exposed to LC_{10} and LC_{25} of atrazine, compared to control groups. Level of E2 and PRG in females exposed to LC_{25} atrazine was higher than females exposed to LC_{10}. The total protein content of the ovary and haemolymph decreased significantly ($P< 0.05$) in females exposed to LC_{10} and LC_{25} of atrazine, compared to control groups. Furthermore, a significantly lower content was observed in females exposed to LC_{25} atrazine than females exposed to LC_{10} and the control groups.

Table 3. Reproductive hormones and total protein levels in female *P. clarkii* exposed to sublethal atrazine concentrations for 28 days

Concentration	ovary			Haemolymph		
	Estradiol (E2) (Pg/ml)	**Progesterone (PRG) (ng/dl)**	**Total protein (TP) (g/dl)**	**Estradiol (E2) (Pg/ml)**	**Progesterone (PRG) (ng/dl)**	**Total protein (TP) (g/dl)**
Control	161.33 ± 18.8^{a}	0.9 ± 0.22^{a}	4 ± 0.00^{a}	53.22 ± 2.78^{a}	0.46 ± 0.05^{a}	4.4 ± 0.25^{a}
LC_{10}	304.83 ± 15.2^{b}	1.66 ± 0.15^{b}	3.2 ± 0.2^{ab}	60.26 ± 3.74^{b}	0.73 ± 0.08^{ab}	3.8 ± 0.3^{b}
LC_{25}	387.5 ± 12.5^{c}	2.13 ± 0.13^{b}	2.7 ± 0.3^{b}	93.06 ± 7.1^{b}	0.97 ± 0.04^{b}	2.26 ± 0.14^{b}
F-value	53.03	13.26	9.9	18.97	19.15	25.9
p- value	0.005	0.03	0.04	0.02	0.02	0.01

-Data are represented as mean ± SE (n=18 samples; each sample is a pool of 5- 6 animals). - Values with different superscripts indicate significant differences ($P< 0.05$). Means with the same letters are not significant at $P> 0.05$.

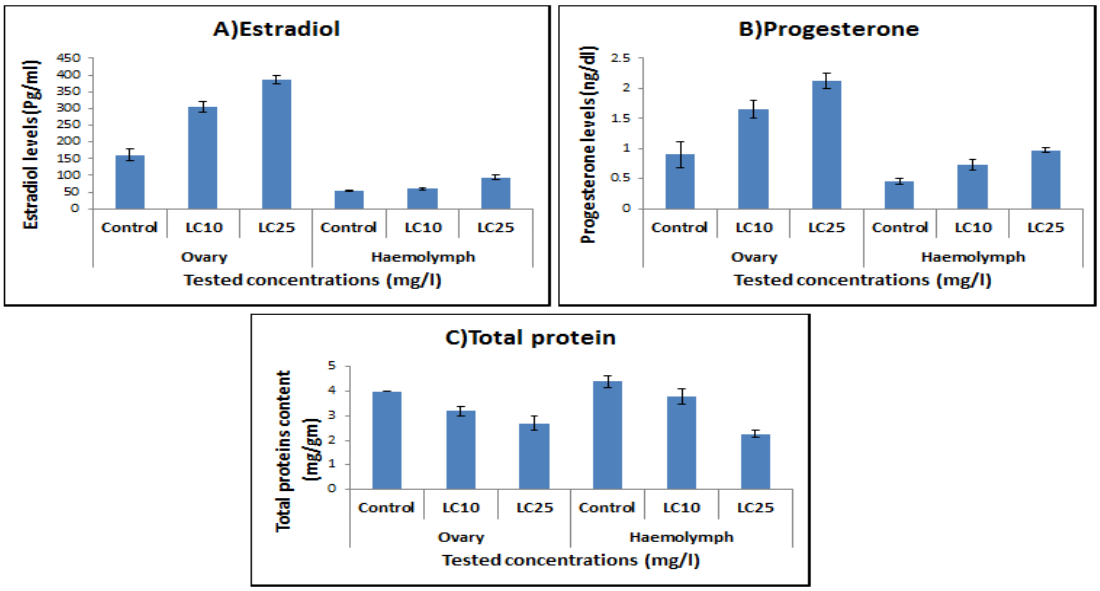

Fig.2. Reproductive hormones A) Estradiol; B) Progesterone, and C) Total protein levels in female *P. clarkii* exposed to sublethal atrazine concentrations for 28 days

Testosterone and total protein levels in male *P.clarkii*

Data in Table (4) and Fig (3) clarify that, testesterone levels in the testis and haemolymph of males exposed to LC_{25} atrazine were significantly lower ($P< 0.05$) compared to both control and LC_{10} atrazine. No significant differences ($P> 0.05$) were detected in total protein in males exposed to LC_{10} and LC_{25} atrazine, compared to control groups.

Table 4. Testesterone and total protein levels in male *P. clarkii* exposed to sublethal atrazine concentrations for 28 days

Concentration	Testis		Haemolymph	
	Testesterone (T) (ng/dl)	Total protein (TP) (mg/gm)	Testesterone (T) (ng/dl)	Total protein (TP) (mg/gm)
Control	4.2±0.2[a]	2.91±0.1[a]	2.85±0.25[a]	3.7±0.2[a]
LC10	3.43±0.13[a]	2.57±0.27[a]	2.17±0.09[a]	3.4±0.1[a]
LC25	2.9±0.1[b]	2.31±0.20[a]	1.74±0.12[b]	2.44±0.46[a]
P-value	19.2	2.32	11.3	4.31
F-value	0.02	0.25	0.04	0.13

-Data are represented as mean ± SE (n=18samples; each sample is a pool of 5- 6 animals).
- Values with different superscripts indicate significant differences ($P< 0.05$).
-Means with the same letters are not significant at $P> 0.05$.

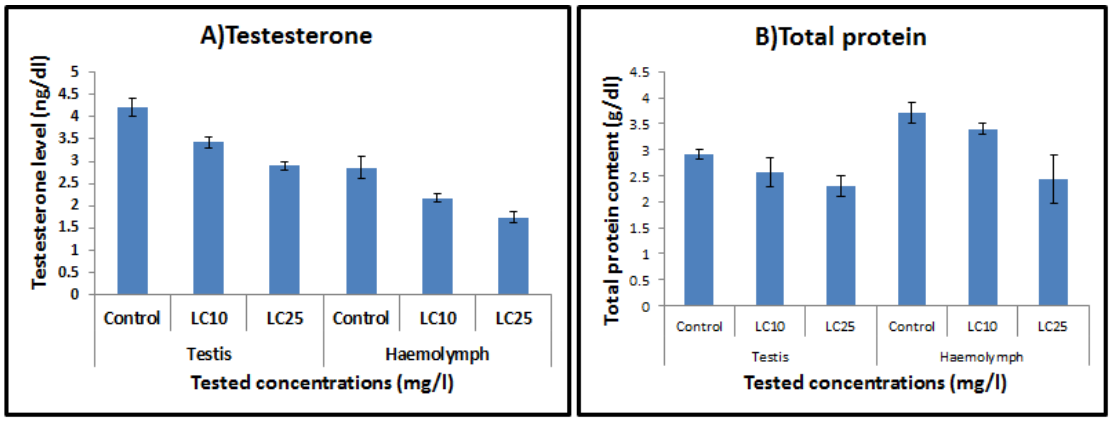

Fig.3. A) Testesterone and **B)** Total protein levels in male *P. clarkii* exposed to sublethal atrazine concentrations for 28 days

Cytochrome P450 1B1 Concentrations in ovary, testis and haemolymph of *P. clarkii*

Data in Table (5) and Fig. (4) show that, Cytochrome P4501B1 concentrations increased significantly ($P< 0.05$) in the ovary, testis and haemolymph of crayfish exposed to LC_{10} and LC_{25} of atrazine, compared to control groups. Upon exposure to LC_{10} atrazine, concentrations of Cytochrome P4501B1 increased by 27.4 %, 26.2 % and 73.6 % in the ovary, testis and haemolymph of crayfish, respectively, compared to the control. Nevertheless, exposing to LC_{25} atrazine led to an increase in the concentrations of Cytochrome P4501B1 by 38.7 %, 54.8 % and 81.9 % in the ovary, testis and haemolymph of crayfish, respectively, compared to the control groups**.**

Table 5. Cytochrome P450 1B1 Concentrations (nmol/ml) in ovary, testis and haemolymph of *P. clarkii* exposed to sublethal atrazine concentrations for 28 days

Concentration	Ovary	Testis	Haemolymph
Control	0.62 ± 0.03^{a}	0.42 ± 0.04^{a}	1.1 ± 0.07^{a}
LC_{10}	0.79 ± 0.02^{b}	0.53 ± 0.08^{b}	1.91 ± 0.06^{b}
LC_{25}	0.84 ± 0.05^{b}	0.65 ± 0.03^{b}	2 ± 0.28^{b}
F-value	12.32	18.29	13.78
p- value	0.04	0.03	0.03

-Data are represented as mean ± SE (n=18 samples; each sample is a pool of 5- 6 animals). Values with different superscripts indicate significant differences ($P< 0.05$). -Means with the same letters are not significant at $P> 0.05$

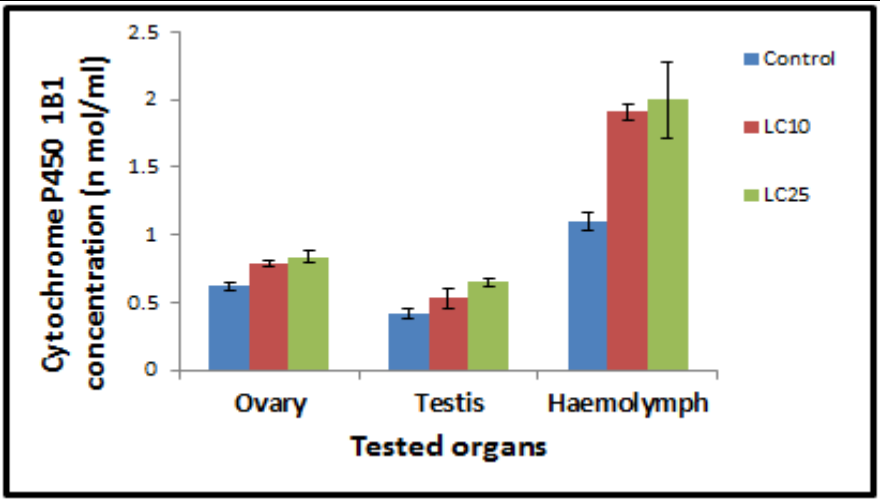

Fig.4. Cytochrome P450 1B1 concentrations in the ovary, testis and haemolymph of *P. clarkii* exposed to sublethal atrazine concentrations for 28 days

Histopathological examination of testis of freshwater crayfish

The reproductive system of the control male *P. clarkii* is located in the thoracic cavity above the digesting gland. It is composed of two white milky testes. The testis is made up of multiple testicular acini of various sizes and shapes. A basal lamina surrounds each of them on which the germinal epithelium rests. The stages of spermatogenesis that pass through the collecting tubules are spermatogonia, primary and secondary spermatocytes and spermatids. Spermatozoa are clumped together as tubular spermatophores in the vas deferens (Plate I, Fig. A, B).

The treatment of testis with LC_{10} atrazin resulted in distorted architecture, spermatogonia distribution disturbance in some testicular acini and appearance of vacuoles in primary spermatocyte (Plate I, Fig. C), as well as lysis of some testicular acini and hyperplasia (Plate I, Fig. D).

The treatment of testis with LC_{25} atrazin resulted in vacuolation and tissues necrosis in some testicular acini (Plate I, Fig. E, F).

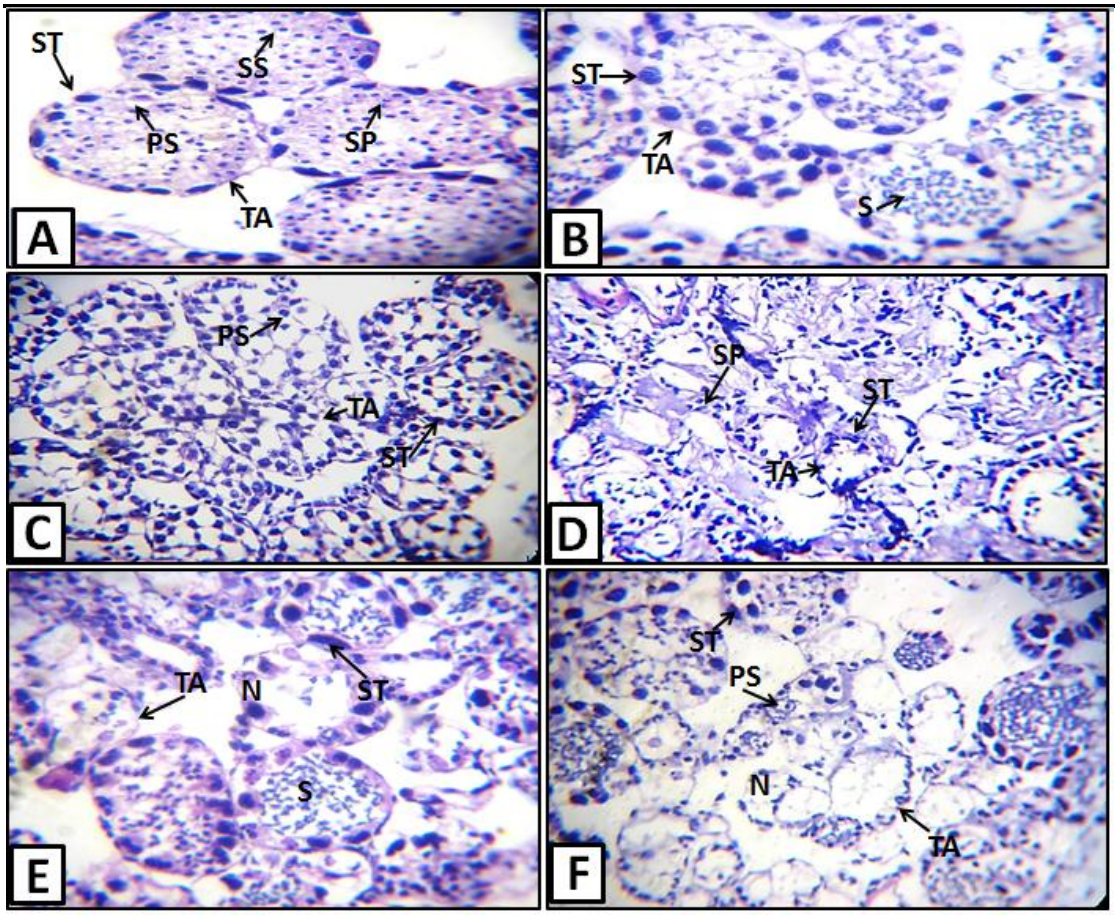

Plate I. Light micrographs of testis of male *P.clarkii*. (A and B): T.S. of untreated testis showing normal spermatogonia, testicular acini, primary spermatocytes, secondary spermatocytes, spermatides and sperm (X=400). (C and D): T.S. of the testis following treatment with LC_{10} for 28 days showing distorted architecture, spermatogonia distribution disturbance in some testicular acini, primary spermatocyte vacuoles, and hyperplasia(X=400). (E and F): T.S. of testis following treatment with LC_{25} for 28 days showing tissues necrosis in certain testicular acini (X=400).

N: necrosis; PS: primary spermatocyte; S: Sperm; SP: Spermatids; SS: Secondary spermatocyte; ST: Spermatogonia; TA: testicular acini.

Histopathological examination the freshwater crayfish ovary

A pair of anterior ovarian sacs and a single median posterior are found in the cephalothorax, dorsally to the stomach in the reproductive system of the control female *P. clarkii*. The ovary is composed of an oogenetic pouch, made up of the ovarian sac epithelium. It develops a series of oogenetic pouches of varying sizes that carry eggs or oocytes at irregular intervals. Germarium is composed of distinct germ regions of the ovarian epithelium found at the base of well-developed oogenetic pouches located throughout the ovary (Plate II, Figs. A, B). The treatment of ovary with LC_{10} atrazin resulted in the separation of the ovarian epithelium and the oogenetic pouch (Plate II, Figs. C, D) and slightly lysis in ovarian epithelium (Plate II, Fig. D). The treatment of ovary with LC_{25} atrazin resulted lysis in connective tissue (Plate II, Figs. E and F).

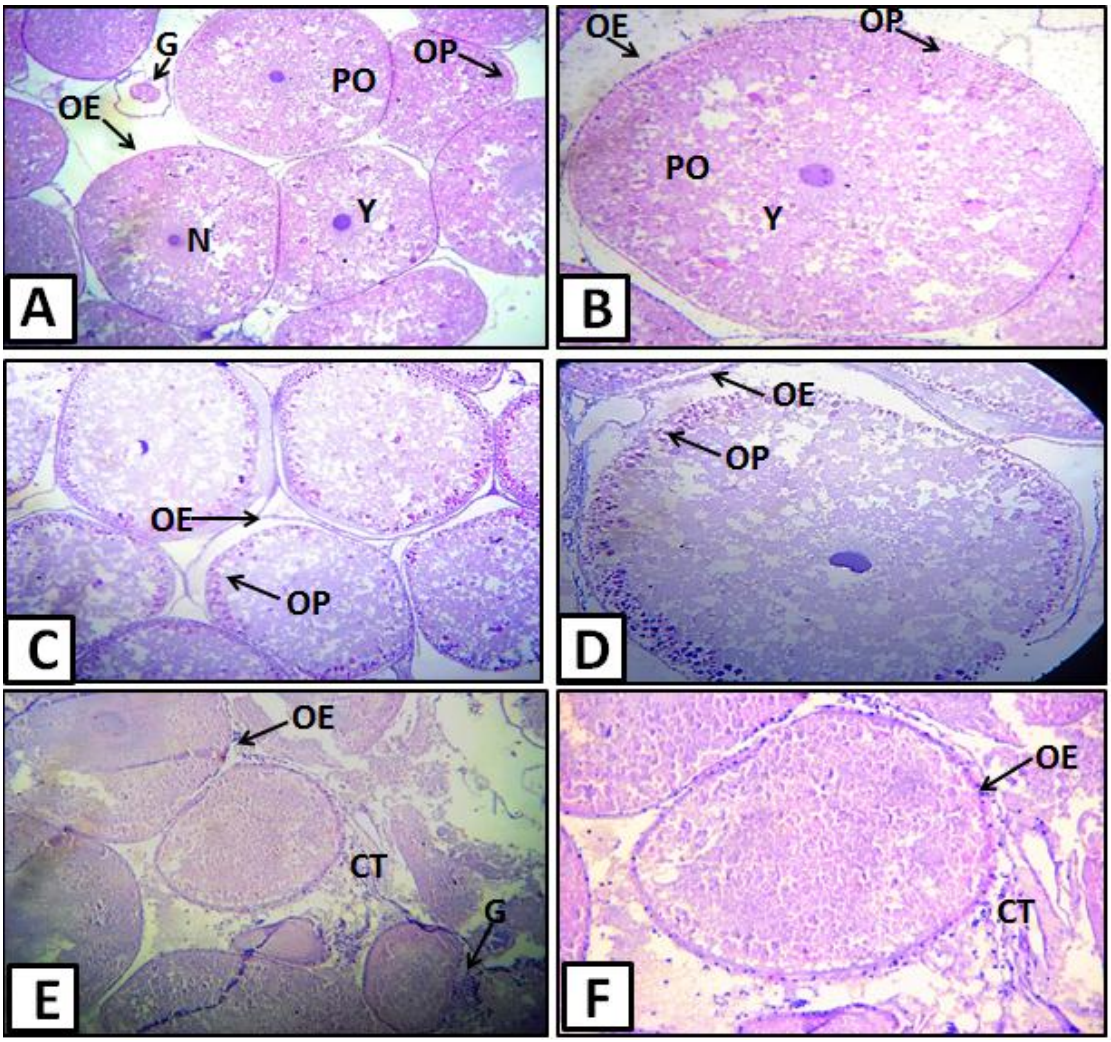

Plate II. Light micrographs of ovary of female *P.clarkii*. (A and B): T.S. of untreated ovary showing oogenetic pouch, ovarian epithelium and Previtellognic oocyte and germarium, (X=40; 200). (C and D): T.S. of ovary after treatment with LC_{10} for 28 days showing separation of the ovarian epithelium from the oogenetic pouch (X= 40; 200). (E and F): T.S. of ovary after treatment with LC_{25} for 28 days showing lysis in connective tissue (X=40; 200).

CT: Connective tissue; G: Germarium; N: Nucleus; OE: Ovarian epithelium; OP: Oogenetic pouch; PO: Previtellognic oocyte; Y: Yolk.

DISCUSSION

Invertebrates' endocrine systems regulate many processes of vertebrates, including growth, reproduction and development (**Oehlmann & Schulte-Oehlmann, 2003**). The endocrine systems of invertebrates such as development and reproduction were disrupted by several chemicals or combinations of compounds. The effects of endocrine disruptors on invertebrates may aid in predicting the potential endocrine-disrupting compounds responses in vertebrates (**DeFur, 2004**). Because of their sensitivity to toxic chemicals, ease of both manipulation and culture in addition to the short generation times,

invertebrates have been excellent models for studying endocrine systems and toxicity testing. The current study was designed to evaluate the ability of atrazine to disrupt the endocrine system of *P. clarkii* to determine the potential use of *P.clarkii* as a bioindicator for endocrine disruptor substance screening.

The results of the current study revealed a significant increase in the mortality rate of *P. clarkii* treated with lethal concentrations of atrazine. By increasing the concentrations and exposure times in both males and females, the mortality rate increased. The LC_{50} of atrazine for *P. clarkii* after 96 hours was 10.62 and 12.66mg/ l for adult male and female, respectively. In addition, it was noted that, females are more tolerant than males when treated with atrazine. The death of *P.clarkii* may be due to the immunosuppressive effects of atrazine and the direct toxic effect of atrazine on immune cells (**Galbiati *et al.*, 2021**). **Galoppo *et al.* (2020)** postulated that, the immunotoxic effects of atrazine are affected by differences in gender and time; males are more sensitive than females. These findings are consistent with the findings of **Stara *et al.* (2018)** who demonstrated that, atrazine increased the mortality rate of the crayfish *Cherax destructor*, and that LC_{50} value for atrazine was 12.1mg/ l after 96h. **Omran and Salama (2013)** reported that, the use of atrazine herbicides increased the mortality rate of *Biomphalaria alexandrina*.

The lowest concentrations chosen (LC_{10} and LC_{25}) indicate a potential environmental concentration in the herbicide's upper environmental range, i.e., water contamination around treated feilds up to 1mg/ l (**Graymore *et al.*, 2001**). Although the assayed atrazine concentrations are worst-case scenarios, determining their long-term impacts on reproductive parameters is important not only for the protection of *P. clarkii* but also for future research aiming at establishing biomarkers in other decapod crustacean species and increasing our understanding of how this herbicide affects crustacean reproduction.

Estradiol (E2) is an estrogen steroid hormone with an important role in the development and maintenance of female reproductive tissues . The present study showed that, the sublethal concentrations of atrazine increased level of estradiol in ovary and haemolymph of *P.clarkii*. Similarly, **Silveyra *et al.* (2018)** found that, estradiol level increased in haemolymph of *P. clarkii* after exposure to atrazine for one month. In this context, **Mac Loughlin *et al.* (2016)** found that the estradiol level increased in crayfish *Cherax quadricarinatus* after treatment with 2.5mg/ l atrazine. No evidence of aromatase expression in crustaceans was already found (**Swevers *et al.*, 1991**) although atrazine rises estrogen levels by inducing aromatase activity in various vertebrate species (**Hayes *et al.*, 2006**). Nonetheless, the herbicide under study may stimulate several other enzymatic pathways involved in oestrogen synthesis.

Progesterone is a steroid sex hormone that has an important role in embryogenesis. Atrazine increased haemolymph and ovary progesterone. **Foradori *et al.* (2017)** reported that, atrazine affect progesterone level by centrally activating hypothalamic-pituitary-adrenal axis levels through the corticotropin-releasing hormone receptor.

Testosterone is an androgen that is found in the testis and is responsible for spermatogenesis. Males are sterile in the absence of testosterone or functional androgen receptors because spermatogenesis rarely progresses beyond meiosis (**De Gendt *et al.*, 2004**). In the present study, atrazine decreased testis and haemolymph testosterone level. This is in line with the findings of **Silveyra *et al.* (2018)** who recorded a decrease in testosterone level in haemolymph of *Procambarus clarkii* after exposure to atrazine. **Omran and Salama (2013)** found that, the testosterone level decreased in hermaphrodite glans of *B. alexandrina* snail after treatment with sublethal concentrations of Atrazine. These results are in harmony with those of **Clair *et al.* (2012)** who stated that, atrazine decreased rat testosterone concentrations and proposed that atrazine is a potent endocrine disrupter that disrupts rat reproductive hormones. The decline in testosterone levels may be due to the inhibitory effects of atrazine on the adrenal androgens synthesis (**Zimmerman *et al.*, 2014**).

Remarkably, proteins are crucial biochemical components required for metabolic pathways and biochemical reactions. Under extreme stress, protein provides energy in metabolic processes and biochemical activities. As a result, measuring total protein levels in the hemolymph, ovary, and testis could be used as a diagnostic tool to determine an organism's physiological state (**Prasath & Arivoli, 2008**). The current study clearly demonstrated that, total protein content was significantly reduced in the hemolymph, ovary, and testis of male and female *P. clarkii* after Atrazine treatment. Total protein levels in crayfish hemolymph and ovary and testis tissues may be depleted as a result of increased proteolytic activity in these organs or energy diversification to meet the impending energy demands during toxic stress. Furthermore, protein depletion could be linked to cell death or necrosis, which would result in a breakdown in the protein production machinery (**Bradbury *et al.*, 1987**). Several studies have shown that atrazine has an inhibitory effect on total protein. **Khan *et al.* (2016)** reported a decrease of total protein in atrazine- exposed freshwater fish Grass Carp *Ctenopharyngodon idella*. **Akhtar *et al.* (2021)** reported a decrease of total protein in atrazine- exposed snow trout *Schizothorax plagiostomus*. **Opute and Oboh (2021)** noticed a decrease in total protein in *Clarias gariepinus* following chronic atrazine exposure.

Cytochrome P450 (CYP 450) is a critical biochemical marker and indication of some chemicals contamination (**Jung *et al.*, 2001**). It has a role in xenobiotic detoxification (**Uno *et al.*, 2012**). The P450 family of arthropods encodes a wide range of enzymes involved in foreign chemical metabolism as well as endocrine and ecophysiological functions (**Dermanw *et al.*, 2020**). The current study showed clearly that ovarian, testicular and haemolymph CYP 1B1 protein levels were increased after treatment with sublethal concentrations of atrazine. The increase in CYP 1B1 activities may be an adaptive mechanism to prevent harmful chemical accumulation or a reflection of the possibility of enhanced protection against atrazine toxicity. These results coincide with those of **Londono *et al.* (2004)** who found that, exposing *Chironomus tentans* to atrazine resulted in an increase in CYP 450 activity and total protein. **Dong *et al.* (2009)** stated

that, P450 content increased in male and female zebrafish *Danio rerio* after exposure to 0.01mg/ l of atrazine. **Omran and Salama (2013)** reported that, after sublethal atrazine exposure, the level of CYP4501B1-like immunoreactivity increased in the hermaphrodite gland of *Biomphalaria alexandrina*.

Histological studies are increasingly being used as environmental stress indicators since they provide a specific biological endpoint of historical exposure and indicate the toxicant's direct action in the organs (**Ramesh *et al.*, 2018**). Chronic exposure of male and female *P. clarkii* to sublethal concentration of atrazine (LC_{10} and LC_{25}) exhibited severe gonad damage. Distorted architecture, spermatogonia distribution disturbance in some testicular acini, appearance of vacuoles in primary spermatocyte, lysis of some testicular acini, hyperplasia and tissues necrosis were observed in the treated *P. clarkii* testis. Separation of ovarian epithelium and oogenetic pouch, as well as lysis in connective tissue, was observed in the ovary of treated *P. clarkii*. These results were in harmony with **Sheir *et al.* (2015)** who reported that *Trichoderma* biofungicide, caused severe alterations in ovary and testis of *Procambarus clarkii*. In this context, **Chandler *et al.* (2017)** stated that atrazine caused degradation in tubular structure of testis of juvenile *Cambarus bartonii*. After 30 days of cypermethrin exposure, **Srivastava *et al.* (2008)** found that the gonad structure of *Channa punctatus* was altered, resulting in inflammation and vacuolization of the testis, as well as necrosis of testis tissues. These histological changes could be attributed to direct toxic effects of atrazine on the gonads.

In aquatic ecosystems, crayfish are considered a keystone species. In this study atrazine was shown to have severe effects on crayfish, which may have an impact on the role of crayfish as a regulator in local aquatic ecosystems. Understanding how atrazine affects crayfish could aid efforts to preserve the health of aquatic life. In the future, it will be useful to investigate the effect of atrazine on various life stages of crayfish by using new biomarkers.

CONCLUSION

According to the findings of this study, the 96-h LC_{50} of AZ for male and female *P. clarkii* was determined to be 10.62 and 12.66 mg L^{-1}, respectively. This proved that females are more tolerant to atrazine than males. Sublethal doses of AZ caused significant changes in testosterone, estradiol, progesterone, total protein, and Cytochrome P450 1B1 levels when compared to the control. As a result, these parameters may be chosen as suitable biomarkers for assessing herbicide toxicity. Furthermore, considering the negative effect of atrazine on reproductive system of crayfish *P. clarkii*, this species could be used as a bioindicator for endocrine disruptor chemicals in aquatic ecosystems.

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