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

**Safaa Ezzat Nassar** ( safaanassar40@yahoo.com )
Zagazig University
**Aya Ali Mohamed**
Zagazig University
**Radwa Mohamed Said**
Zagazig University

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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 are little researches on its endocrine disruptor effect in crustaceans. The current study was designed to estimate the median lethal concentration of atrazine (96-h LC50) 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 LC50 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 male and female, 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 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 and 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 and 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 and 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, and 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

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 25–34 g and measuring 9–12 cm 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 of these ranges are within acceptable limits. Suffocation was avoided by keeping a water depth of 7 - 10 cm and exposing the specimens to room temperature air. 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 17:00 h.

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 1000 ml 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 20 mg/l. The $LC_{50}$ was calculated using three replicates per 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 24 h intervals for up to 96 hr. 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 and 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 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 hr. Specimens were dehydrated, and embedded in paraffin wax. Sections were cut at 4-6 µm in thickness, and stained with Hematoxylin and Eosin.

**Statistical analysis**

The SPSS statistic 20.0 was used to analyze all of the 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 showed that the highest mortality rate occur at atrazine concentrations of 20 mg/l, while the lowest mortality percentages occur at atrazine concentrations of 5 mg/l for both males and females *P. clarkii*. The mortality percentages rise as the concentrations of Atrazine and the duration of exposure rise. It should also be noted that females are more tolerant of atrazine than males.

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

| Time(hrs) &Sex Con(mg/l) | Mortality percentage (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24 | | 48 | | 72 | | 96 | |
| | Male | Female | Male | Female | Male | Female | Male | Female |
| 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 | | | | | | | | |

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

| 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 showed 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 significant ($P < 0.05$) 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.

| Concentrations | 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.

**Figure 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 showed that testesterone levels in the testis and haemolymph of males exposed to $LC_{25}$ atrazine were significantly lower ($P < 0.05$) compared to either control or $LC_{10}$ atrazine. There were no significant differences ($p > 0.05$) 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.

| Concentrations | Testis | | Haemolymph | |
|---|---|---|---|---|
| | Testesterone (T) (ng/dl) | Total protein (TP) (mg/gm) | Testesterone (T) (ng/dl) | Total protein (TP) (mg/gm) |
| Control | $4.2 \pm 0.2^{a}$ | $2.91 \pm 0.1^{a}$ | $2.85 \pm 0.25^{a}$ | $3.7 \pm 0.2^{a}$ |
| $LC_{10}$ | $3.43 \pm 0.13^{a}$ | $2.57 \pm 0.27^{a}$ | $2.17 \pm 0.09^{a}$ | $3.4 \pm 0.1^{a}$ |
| $LC_{25}$ | $2.9 \pm 0.1^{b}$ | $2.31 \pm 0.20^{a}$ | $1.74 \pm 0.12^{b}$ | $2.44 \pm 0.46^{a}$ |
| F-value | 19.2 | 2.32 | 11.3 | 4.31 |
| p- 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 $P > 0.05$.

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

Data in Table 5 and Fig. 4 showed 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, exposure to $LC_{25}$ atrazine, concentrations of Cytochrome P4501B1 increased 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.

| Concentrations | Ovary | Testis | Haemolymph |
|---|---|---|---|
| Control | $0.62 \pm 0.03^{a}$ | $0.42 \pm 0.04^{a}$ | $1.1 \pm 0.07^{a}$ |
| $LC_{10}$ | $0.79 \pm 0.02^{b}$ | $0.53 \pm 0.08^{b}$ | $1.91 \pm 0.06^{b}$ |
| $LC_{25}$ | $0.84 \pm 0.05^{b}$ | $0.65 \pm 0.03^{b}$ | $2 \pm 0.28^{b}$ |
| 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.

# 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 and 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 and 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 of ovary of freshwater crayfish

A pair of anterior ovarian sacs and a single median posterior is 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, Fig. A and B). The treatment of ovary with $LC_{10}$ atrazin resulted in the separation of the ovarian epithelium and the oogenetic pouch (Plate II, Fig. C and 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, Fig. 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 of the same processes as those of vertebrates, including growth, reproduction and development (Oehlmann and Schulte-Oehlmann 2003). The endocrine systems of invertebrates, including development and reproduction, have been discovered to be 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 manipulation, ease of culture, and 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*. In order 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 LC50 of atrazine for *P. clarkii* after 96 hours was determined to be 10.62 and 12.66 mg/l for adult male and female, respectively. It could be also 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) indicated that immunotoxic effects of atrazine affected by differences in gender and time, males 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 LC50 value for atrazine was 12.1 mg/l after 96 hr. Omran and Salama (2013) demonstrated 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 1 mg/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 aimed 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 and has an important role in the development and maintenance of female reproductive tissues. The present study showed that 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) who found that the Estradiol level increased in crayfish *Cherax quadricarinatus* after treatment with 2.5 mg/l atrazine. Although atrazine has been shown to raise estrogen levels by inducing aromatase activity in various vertebrate species (Hayes et al. 2006), no evidence of aromatase expression in crustaceans was already found (Swevers et al. 1991). 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). The present study showed that atrazine decreased testis and haemolymph testosterone level. This is in line with the findings of Silveyra et al. (2018) who reported that 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. Also, these results were in harmony with 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 decrement in testosterone levels may be due to the inhibitory effects of atrazine on the adrenal androgens synthesis (Zimmerman et al. 2014).

Proteins are crucial biochemical components that are 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 and 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) and has 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 are in harmony with Londono et al. (2004) who found that exposing of *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 0.01 mg/l 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 because 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 improved 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.

# Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable


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### Data availability statement

Data included in the article.

### Conflict of Interest

The authors declare that they have no conflict of interest.

### Additional information

Not applicable

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# Plate

Plate I-II are available in the Supplementary Files section

# Figures

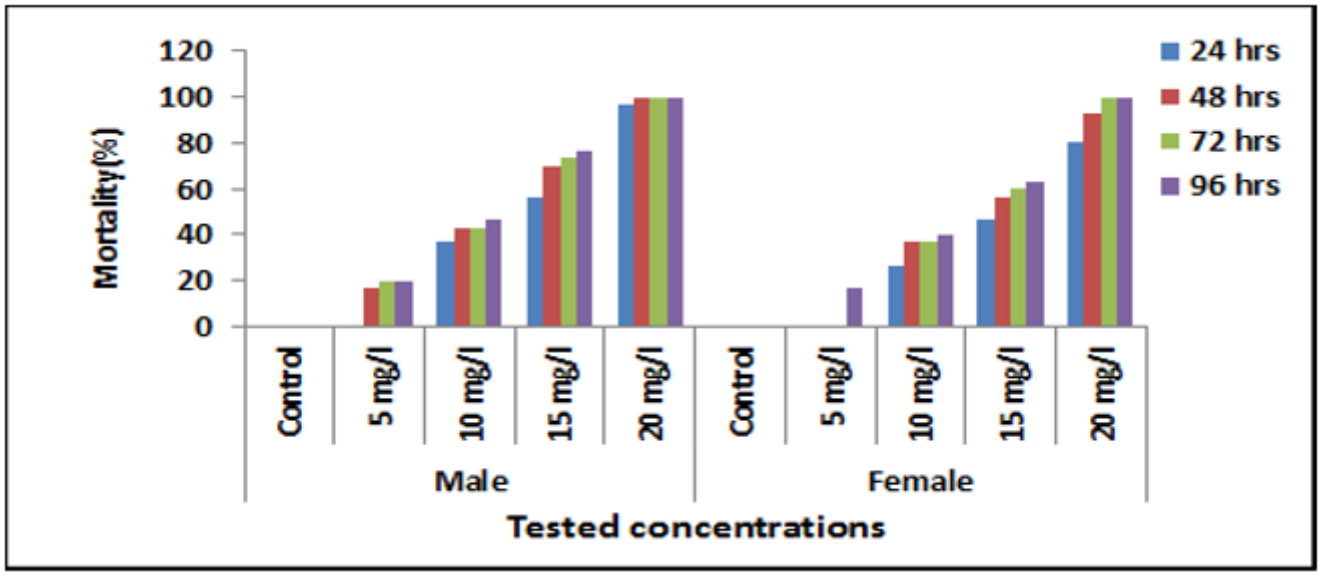


Figure 1

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

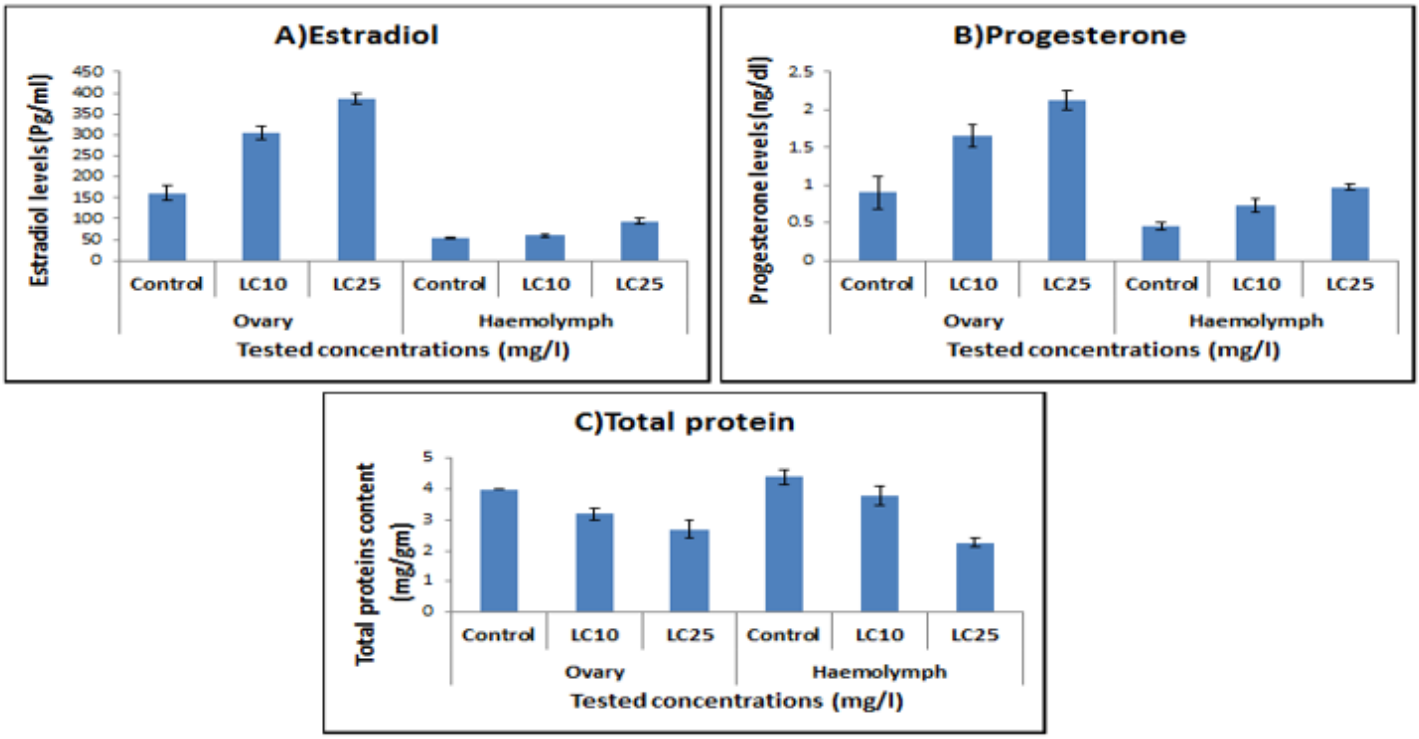


Figure 2

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

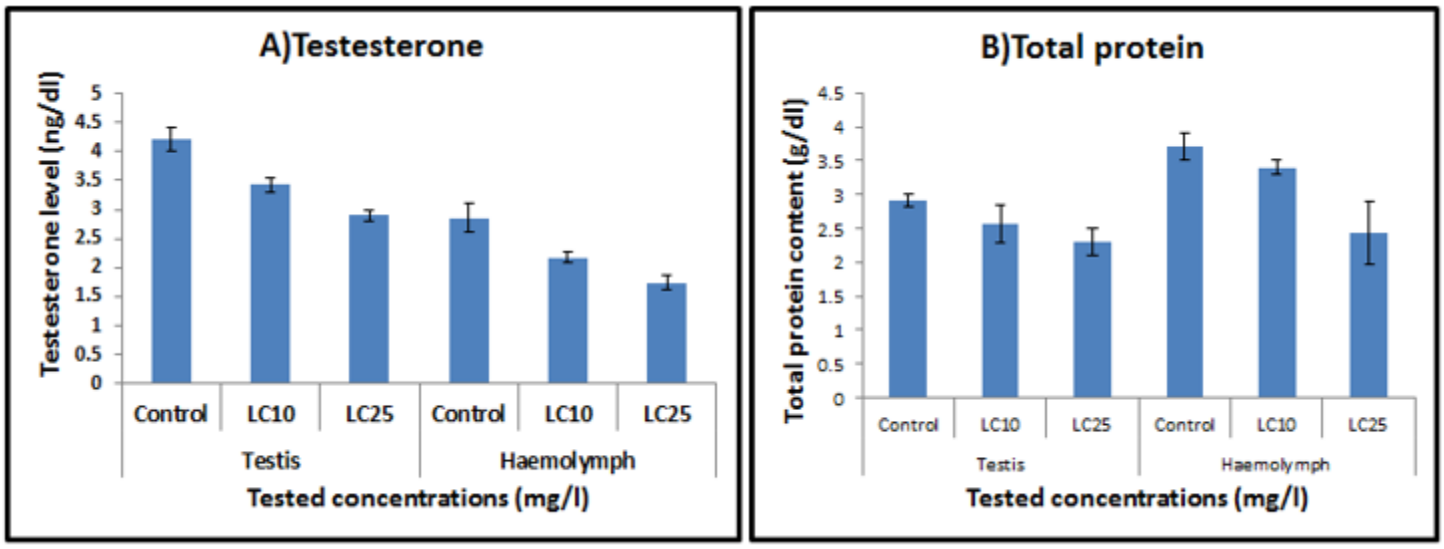


Figure 3

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

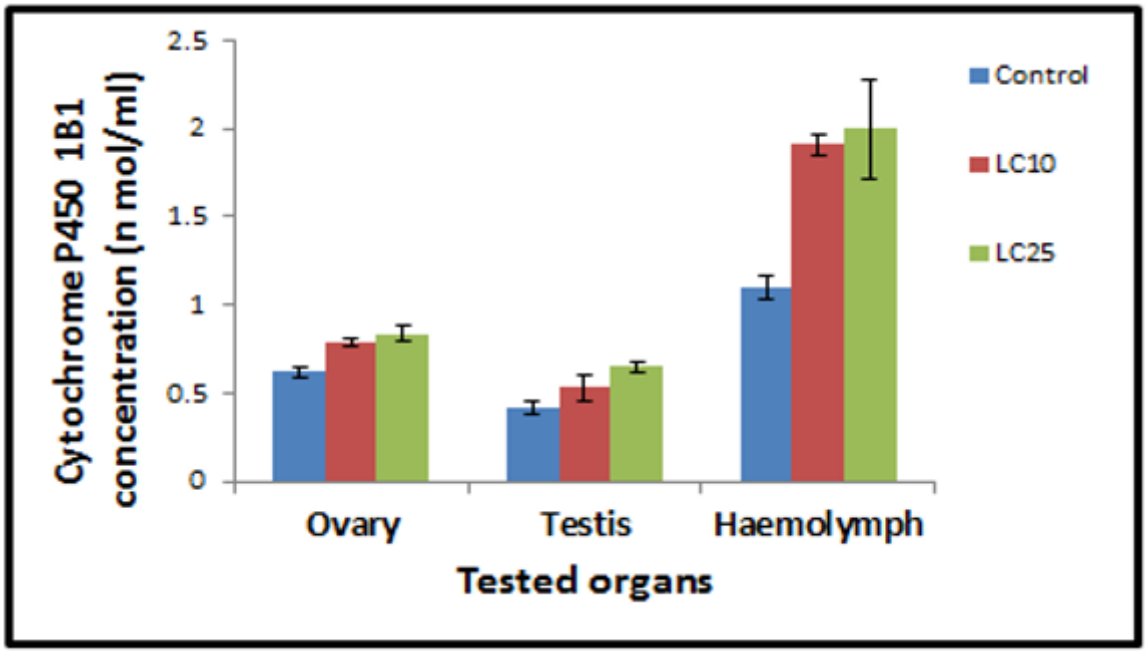


Figure 4

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

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Platel.png
- PlateII.png