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TRICLOSAN INFLUENCES ON REPRODUCTIVE PHYSIOLOGY OF CLIMBING PERCH IN THE SPAWNING PHASE

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ABSTRACT

The endocrine-disrupting effects of triclosan, an antimicrobial agent, and its involvement in reproductive responses mediated through different modes of activity in various fish species have been documented. The present study aims to explore whether triclosan at environmentally relevant and sublethal concentrations could influence the reproductive physiology of the fish *Anabas testudineus* in the spawning phase. Fish were exposed to triclosan at environmentally relevant (0.009 and 9 µg L^{-1}) and sublethal (176.7 µg L^{-1}) concentrations for 4, 7, 30 and 60 d during the spawning phase for evaluating the reproductive potential of the fish. Triclosan exposure caused a significant (P<0.05) reduction in the level of total protein in gonads and liver tissues while increasing blood plasma to meet the energy demand and overcome metabolic stress. Impairment in gonadal steroidogenesis was evidenced by the reduction in the activities of 3β- and 17β-hydroxysteroid dehydrogenases associated with alteration in the levels of serum gonadotropins, sex steroid hormones and vitellogenin. Triclosan exposure caused a reduction in the rate of fecundity in females and declined sperm counts, motility and viability in males. Histological lesions in gonadal tissues further confirmed the reproductive toxicity of triclosan, which in turn could contribute to the reproductive failure of the fish.

Keywords:
Anabas testudineus
Vitellogenin
Serum hormones
Fecundity
Sperm indices

How to Cite

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INTRODUCTION

Triclosan is a broad-spectrum antimicrobial, antiseptic and preservative agent widely used in healthcare, veterinary, pharmaceutical, personal care and many other consumer products, including plastic materials, toys, paints, medical devices, textiles, kitchen utensils, cosmetics and cleaning products (US-EPA, 2008). Triclosan is categorized under the emerging contaminants owing to its wide range of applications and continuous release into different environmental compartments. The occurrence of triclosan in air, soil sediments and activated sludge contributes to its persistence in the terrestrial environment with the reported range between nanogram to milligram per kilogram (Miller et al., 2008; Dhillon et al., 2015). Improper wastewater treatment and invariant discharge from the sewage effluents also contribute to the contamination in aquatic ecosystems, while the lipophilic and persistent properties of triclosan enable its negative impacts on the aquatic organisms (Oliveira et al., 2009; Nassef et al., 2010; Palenske et al., 2010; Vijitha et al., 2017; Priyatha and Chitra, 2018). In 2016, the United States Food and Drug Administration (US-FDA) reported that triclosan is not generally recognized as a safe and effective antiseptic so it was recommended to withdraw it from the global market. Some studies have documented that certain bacterial strains showing resistance to triclosan have developed adaptive tolerance to multiple antibiotics by the up-regulation of *FabI* gene expression (Copitch et al., 2010; Grandgirard et al., 2015), and thus in many developed countries the use of triclosan in the hand sanitizers has been banned since April 2019. However, the regulation of triclosan in other personal care products like liquid soaps, toothpaste and medical devices is not enforced throughout the world (Dodson et al., 2020).

The occurrence of triclosan in surface water, sewage effluents and soil sediments of Indian rivers and mangroves has been detected using the gas chromatography-mass spectrometry (GC-MS) method (Ramaswamy et al., 2011; Balakrishna et al., 2017; Sarkar et al., 2020). In the present scenario of the pandemic coronavirus disease (COVID-19), it is important to recognize that the usage and continuous release of triclosan from hand sanitizers and soaps into the environment may have increased beyond the previous detection range. Several studies have revealed that triclosan caused teratogenicity (Park et al., 2020), behavioral and developmental defects (Wirt et al., 2018; Song et al., 2020), metabolic disorders (Ho et al., 2016), neurotoxicity (Kim et al., 2018; Wang et al., 2021), histological alterations (Gyimah et al., 2020), genetic damages (Vijitha et al., 2017; Falisse et al., 2018), embryotoxicity (Jimoh and Sogbanmu, 2021), hormonal modifications (Crofton et al., 2007) and reproductive dysfunctions (Priyanka et al., 2020) in various non-target organisms. Fish is considered the most suspected non-target organism in the aquatic ecosystem as it occupies the top of the food chain, and is also the most important source of protein and other nutrients for humans. In recent years, there is an alarming decline in the fish population in both marine and freshwater habitats due to several anthropogenic activities like over-exploitation, industrialization, habitat loss, rise in temperature and release of contaminants into the water bodies. Some of the pollutants are lipophilic and disrupt the endocrine system thereby affecting the reproductive capacity of the fish, which indirectly affects survival at a population level in the natural environment. Triclosan mimics the properties of some non-steroidal estrogenic compounds like diethylstilbestrol and bisphenol A (Ishibashi et al., 2004) and has been identified as the most potential endocrine-disrupting chemical concerning its interference in the normal mechanism of hormonal actions (WHO-UNEP, 2012). The adverse effects of triclosan on the reproductive physiology of non-target organisms mediated through the hypothalamus-pituitary-gonadal (HPG) axis have been well established (Kumar et al., 2009; Stoker et al., 2010; Lan et al., 2015; Wang et al., 2018).

The endocrine-disrupting effects of triclosan have been illustrated by the induction of vitellogenin gene expression with a concomitant increase in the hepatosomatic index followed by the decline in sperm counts in the mature male mosquitofish *Gambusia affinis* (Raut and Angus, 2010). The toxic effects of triclosan assessed in swordtail fish using a multi-biomarker approach have revealed the induction in the activities of phase I and phase II metabolic enzymes, where it displayed a higher level of gene expression in the males than in the female fish (Liang et al., 2013). In adult medaka fish, the estrogenicity of triclosan has been proved by the induction of hepatic vitellogenin in the female fish that ultimately suppressed the fecundity rate (Horie et al., 2018). In the embryos of zebrafish, triclosan has been shown to interfere with the expression of the estrogen receptor-regulated brain aromatase in an ambivalent manner irrespective of direct binding and activation of estrogen receptors (Serra et al., 2018). However, a multigeneration study conducted on Japanese medaka has reported that triclosan does not act as an agonist or antagonist within estrogen, androgen, thyroid or steroidogenic pathways (Mihaich et al., 2019). A recent study has reported that exposure to triclosan at environmentally relevant concentrations activates the Nrf2-ARE signalling pathway thereby altering the oxidant-antioxidant homeostasis in mosquitofish (Bao et al., 2021).

Based on the literature reviewed, triclosan has been known to perform different modes of action in different cell types, either agonist or antagonist, depending on multiple factors including concentration and duration of exposure, mode of administration as either alone or in combination with other toxicants, age and sex of species, and different stages of reproduction (Alfhili and Lee, 2019). The choice of *Anabas testudineus* as the laboratory model in this study was based on several aspects including its high nutrient content, easy handling and acclimatization

in the laboratory, resistance to adverse environmental conditions, and also its role as a popular food fish (Manju et al., 2013). Thus the present study aimed to address the specific effects of triclosan on the reproductive potential of the fish *Anabas testudineus* during the spawning phase. Precisely, the study was designed to focus on the toxic effects of triclosan using the simple and most relevant endpoints of reproductive toxicity such as gonadosomatic index, vitellogenin, activities of steroidogenic enzymes, serum hormone concentrations, fecundity, sperm indices and gonadal histology. The current research findings could provide better insights into the mechanism of reproductive toxicity of triclosan and its role as an endocrine disruptor during the spawning phase of fish reproduction.

MATERIALS AND METHODS

Test animal

Mature male and female climbing perch *Anabas testudineus* (Bloch 1792) of the size 8 ± 1 g and 8.5 ± 0.75 cm were segregated from the stock maintained in our laboratory. As per the recommendation of APHA guidelines (1998), fish were maintained in the glass tank (40 L capacity) under laboratory conditions within the array of temperature 28 ± 2 °C, pH 7.4 to 7.6, dissolved oxygen with the range of 8.64 ± 0.6 mg L^{-1}, and salinity < 100 ppm, in well-aerated and dechlorinated water under the natural photoperiod (12: 12 h; light: dark) during the entire period of the experiment. Fish were fed daily with commercial fish pellets, and the water was renewed every alternate day. The care and use of the fish complied with the Animal Welfare Board of India and the Committee for Control and Supervision of Experiments on Animals (CPCSEA) under the Ministry of Environment, Forest and Climate Change, Government of India.

Chemicals

Triclosan (5-chloro-2-(2,4-dichloro phenoxy)-phenol; TCS of 97% purity) and dimethylsulfoxide (DMSO) used in this study were purchased from HiMedia Research Laboratories Pvt. Ltd, Mumbai, India. Hormone assay kits were procured from Bioassay Technology Laboratory, Shanghai, China, and a vitellogenin assay kit was procured from Origin Diagnostics and Research, India. All other chemicals were purchased from local commercial sources.

Experimental design and sample collection

The experiments were carried out during July and August, which was the spawning (SP) stage of the fish that showed sexual dimorphism by distinct morphological features like bulging of abdomen and modification of vent. From the stock, fishes of both sexes were randomly selected and introduced into the separate glass tanks consisting of ten fish per group. The fish were exposed to three different concentrations of triclosan namely 0.009 and 9 µg L^{-1}; environmentally relevant concentrations (Ramaswamy et al., 2011; Nag et al., 2018) and 176.7 µg L^{-1} (one-tenth of LC_{50}-96 h as sublethal concentrations) (Priyatha and Chitra, 2018) along with the negative (tap water) and vehicle (DMSO; 0.001 % v/ v) control groups for 4, 7, 30 and 60 d, in which the replicates were maintained at the semi-static condition for each concentration.

At the end of every treatment period, the weight of the fish, mucous deposition, and weights of gonads and liver were recorded immediately to evaluate the absolute and relative weights of the tissues. In addition, the reproductive indices such as fecundity of females, and sperm motility, viability and counts were also evaluated. Blood samples of both male and female fish collected from the caudal vein were kept undisturbed at room temperature for 10-20 min in a vial containing PBS heparin as an anticoagulant to obtain plasma for vitellogenin analysis. However, the serum samples were obtained by centrifuging the blood at 750-1000 g for 15 min and stored at −80 °C until hormone analyses were performed. Gonads and liver tissues were then homogenized using a glass Teflon homogenizer under crushed ice, centrifuged at 800 g for 15 min at 4 °C, and the supernatants obtained were used for the biochemical analysis, including vitellogenin. A portion of the gonadal tissues was preserved in 10% buffered formalin for histological analysis.

Relative weights of gonads (GSI) and liver (HSI) tissues

The relative weight of gonads (testes and ovary) or gonadosomatic index (GSI) and the relative weight of liver tissues (from male and female fish) or hepatosomatic index (HSI) were evaluated using the standard formula and expressed in percentage (King, 1995; Sulistyo et al., 2000).

$$\text{GSI or HSI} = (\text{Tissue weight (g)}/ \text{Fish weight (g)}) \times 100$$

Fecundity

The fecundity was estimated by the gravimetric method from the ovary of the spawning stage. The ovary was preserved in modified Gilson's fluid for 48 h to liberate the eggs completely. Then the eggs were washed thoroughly, spread on the blotting paper to air dry, and the sub-samples are counted by the given equation:

$$F = nG/ g$$

where F = fecundity, n = number of eggs in the subsample, G = total weight of the ovaries, g = weight of the subsample in the same units (Grimes and Huntsman, 1980).

Sperm indices

Male fish were gently held without stress, and the catheters were inserted into the urinogenital tract for the collection of uncontaminated milt. The collected sperm were transferred to a small Petri plate and processed for assessing sperm motility, sperm count (Caille et al., 2006) and sperm viability (Eliasson and Treichl, 1977; Wyrobek et al., 1983).

Vitellogenin and total protein

The level of vitellogenin was measured in the blood plasma, and gonadal and liver tissues using enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's instructions. Briefly, prepared antigen standards and samples were added to each well of 96-well plates pre-coated with primary antibodies. Microplates were incubated at 37 °C for 60 min after the addition of biotin conjugate and enzyme conjugate reagents. Then the plates were rinsed 5 times with wash solution and the absorbance was read at 450 nm within 15 min of chromogenic reaction. Total soluble protein was also estimated as per the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Serum hormones

The estimation of the levels of serum hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), testosterone (T) and estradiol (E2) was performed using enzyme-linked immunosorbent assay (ELISA) kits strictly according to the manufacturer's instructions.

Gonadal steroidogenic enzymes

The activities of gonadal steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were estimated (Bergmeyer, 1974). The assay mixture of 3β-HSD containing pyrophosphate buffer (100 mM), NAD (0.5 mM) and dehydroisoandrosterone (0.1 mM) was read at 340 nm by the immediate addition of the sample at 30 s interval for 5 min in a spectrophotometer against the blank. The units are expressed in µmol of NAD reduced/ min/ mg protein.

The assay mixture of 17β-HSD containing pyrophosphate buffer (100 mM), NADPH (0.5 mM) and 1,4-androstenedine-3,17-dione (0.8 mM) was read at 340 nm immediately after the addition of the sample at 30 s interval for 5 min in a spectrophotometer against the blank. The enzyme activity was expressed as µmol of NADP formed/ min/ mg protein.

Histological analysis

Gonads of male and female fish, fixed in 10% buffered formalin for 24-48 h were used for histological analysis. The tissues were dehydrated in ascending grades of alcohol and cleared in xylene until they become translucent. The tissues were embedded in molten paraffin wax for an hour for the complete impregnation to make the tissue blocks. Sections of 4 to 6 µm thickness were prepared using a rotary microtome, and then the slides obtained were double-stained with hematoxylin and eosin, and finally mounted in DPX (Roberts and Smail, 2001). Slides of gonadal tissues were examined under the microscope and photographed.

Statistical analyses

In the present study, replicates of all analyses were performed to minimize the statistical errors. Data analyses were performed using SPSS software, version 21.0. All data were expressed as mean ± standard deviation (SD) for ten animals per group. One-way ANOVA followed by Duncan's Multiple Range post-hoc was used to set the significance among the control and treatment groups. Normal distribution and homogeneity of variance were checked before conducting one-way ANOVA, and the values considered significant at $P<0.05$ were denoted as asterisks (*) in the Figures.

RESULTS

Bodyweight and mucous deposition

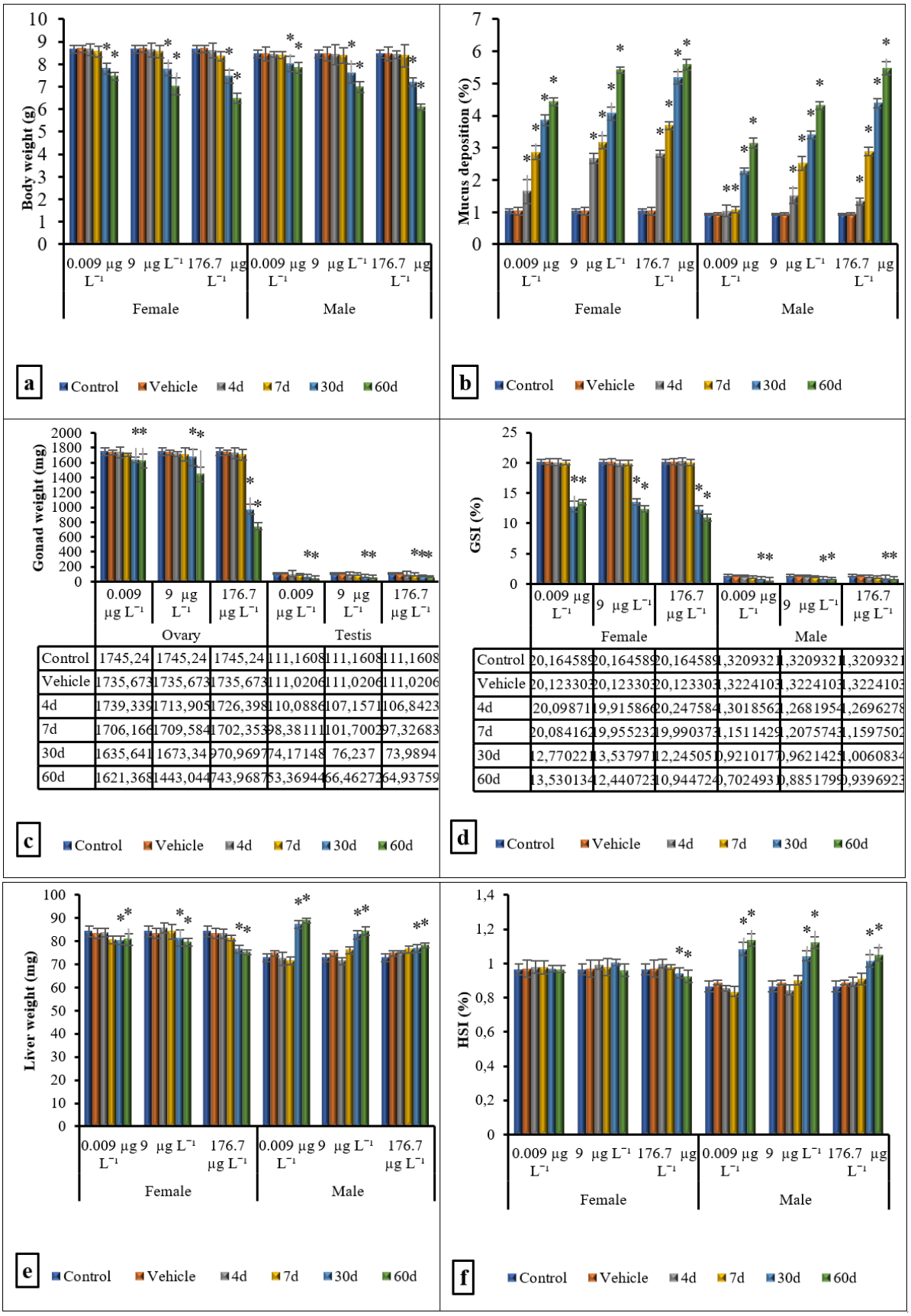
Fish exposed to triclosan at three different concentrations, namely 0.009, 9 and 176.7 µg L^{-1}, showed a significant ($P<0.05$) decrease in the weight of the animal after 30 and 60 d while no significant changes were observed after 4 and 7 d of the treatment (Fig. 1a). However, the mucous deposition increased significantly ($P<0.05$) at all concentrations after 4, 7, 30 and 60 d of triclosan exposure (Fig. 1b).

Absolute and relative weights of gonads and liver tissues

The absolute and relative weights of gonads, the ovary and testis showed significant ($P<0.05$) reduction after 30 and 60 d of triclosan treatment at all concentrations when compared with the corresponding control groups (Figs 1c and 1d). Similarly, the absolute and relative weights of liver tissue in male fish decreased significantly ($P<0.05$) in all treatment groups at the end of 30 and 60 d (Figs 1e and 1f). However, in female fish, the absolute weight decreased significantly ($P<0.05$) after 30 and 60 d in all treatment groups, whereas the relative weight of liver tissue showed a significant decrease only in the sublethal exposure group when compared to the respective control groups (Figs 1e and 1f).

Total protein

Fish exposed to triclosan significantly ($P<0.05$) increased the level of total plasma protein in a time-dependent manner in all treatment groups in comparison to the corresponding control groups (Fig. 2a). However, in the ovary, the level of total protein decreased significantly ($P<0.05$) after 30 and 60 d at 0.009 µg /L concentration, and after 7 d onwards in 9 and 176.7 µg /L concentrations (Fig. 2b). Testicular tissues showed a significant ($P<0.05$) decline in the total protein level after 30 and 60 d in all groups of triclosan exposure in comparison to that of the respective control groups (Fig. 2b). Similarly, a concentration- and time-dependent significant ($P<0.05$) reduction in the level of total protein was observed after triclosan exposure in the liver tissue of the fish in comparison to the respective control groups (Fig. 2c).



	Ovary			Testis		
Control	1745,24	1745,24	1745,24	111,1608	111,1608	111,1608
Vehicle	1735,673	1735,673	1735,673	111,0206	111,0206	111,0206
4d	1739,339	1713,905	1726,398	110,0886	107,1571	106,8423
7d	1706,166	1709,584	1702,353	98,38111	101,7002	97,32683
30d	1635,641	1673,34	970,9697	74,17148	76,237	73,9894
60d	1621,368	1443,044	743,9687	53,36944	66,46272	64,93759

	Female			Male		
Control	20,16458	20,16458	20,16458	1,320932	1,320932	1,320932
Vehicle	20,12330	20,12330	20,12330	1,322410	1,322410	1,322410
4d	20,09871	19,91586	20,24758	1,301856	1,268195	1,269627
7d	20,08416	19,95523	19,99037	1,151142	1,207574	1,159750
30d	12,77022	13,53797	12,24505	0,921017	0,962142	1,006083
60d	13,53013	12,44072	10,94472	0,702493	0,885179	0,939692

Fig 1. Effect of triclosan on the (a) Body weight; (b) Mucous deposition; (c) Weight of gonads; (d) Gonadosomatic index (GSI); (e) Weight of liver tissue and (f) Hepatosomatic index (HSI) in *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant differences against the control groups)

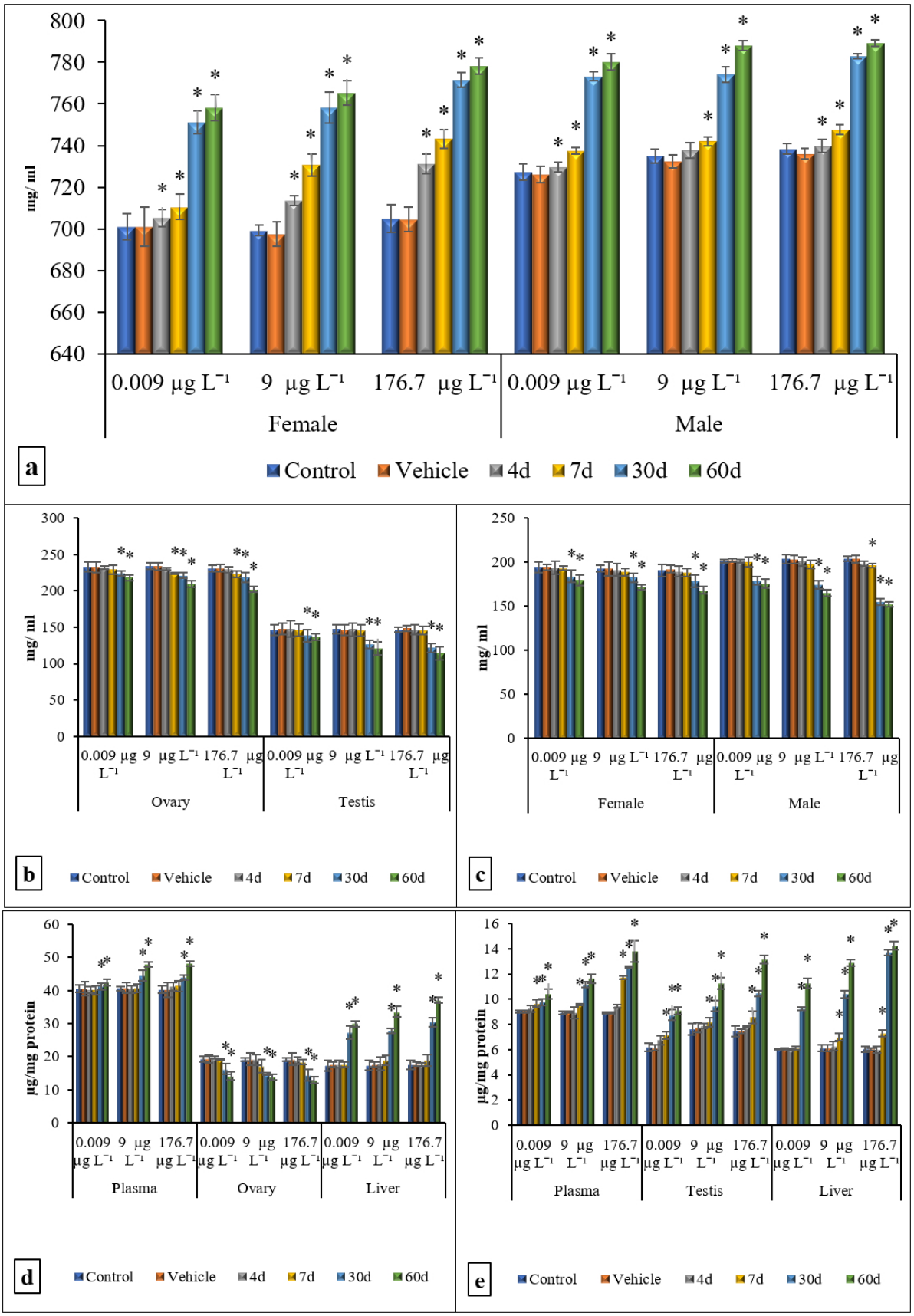


Fig 2. Effect of triclosan on the level of total protein in the (a) blood plasma; (b) gonads; (c) liver tissues; (d) level of vitellogenin in the female; (e) vitellogenin in the male *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant differences against the control groups)

Vitellogenin level

In female fish, the level of vitellogenin in the blood plasma and liver tissue increased significantly (P<0.05) after 30 and 60 d in all concentration groups in a time- and concentration-dependent manner (Fig. 2d), while the triclosan exposure in ovarian tissue showed a significant (P<0.05) decline in the level of vitellogenin after 30 and 60 d (Fig. 2d). In male fish, triclosan exposure significantly (P<0.05) increased the level of vitellogenin in blood plasma, testis and liver tissues in a time- and concentration-dependent manner when compared with the corresponding control groups (Fig. 2e).

Serum hormones

Female fish exposed to triclosan showed a significant (P<0.05) reduction in the level of estradiol at 0.009 and 9 µg/ L concentration groups after 30 and 60 d, while a significant (P<0.05) reduction was observed after 7 d onwards in the sublethal concentrations group (Fig. 3a). However, male fish exposed to triclosan caused a significant (P<0.05) rise in the level of estradiol after 60 d in the sublethal concentrations group (Fig. 3a).

The level of serum testosterone in the female fish significantly (P<0.05) reduced only after 60 d at 9 µg/ L concentration, whereas the sublethal exposure group showed a significant (P<0.05) decline after 30 and 60 d in comparison to the respective control groups (Fig. 3b). Likewise, the male fish also showed a significant (P<0.05) decrease in the level of serum testosterone in a time-dependent manner in all exposure groups (Fig. 3b).

Triclosan treatment significantly (P<0.05) decreased the levels of serum FSH and LH in both sexes in a time- and concentration-dependent manner when compared with the corresponding control groups (Figs 3c and 3d). However, the level of TSH in the serum of male and female fish significantly (P<0.05) increased, based on exposure period and concentrations (Fig. 3e).

Gonadal steroidogenic enzymes

The activities of gonadal steroidogenic enzymes, namely 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD), decreased significantly (P<0.05) in both testes and ovaries at all tested concentrations in a time-dependent manner when compared with the respective control groups (Figs 4a and 4b).

Fecundity in female fish

The rate of fecundity measured in female fish by evaluating the number of eggs showed a significant (P<0.05) reduction after 30 and 60 d at environmentally relevant concentrations, while the decline was more prominent after 7 d onwards in the sublethal concentrations group (Fig. 4c).

Sperm parameters

The sperm parameters such as sperm motility, sperm count and sperm viability decreased significantly (P<0.05), concerning the duration of triclosan exposure and concentrations in comparison with the respective control groups (Figs 4d-4f). The percentage of viable sperm was tested using trypan blue stain, which showed no stain absorption in the control groups as evidenced by the white, bright sperm, while as a result of stain absorption, dead sperm in blue color were observed in the triclosan-exposed groups at all concentrations and durations, together with some morphological abnormalities such as the absence of the tail and enlarged sperm head. Representative viable sperm from the control groups and non-viable sperm from the treatment groups were shown in Fig. 5.

Histology of ovary

The ovaries of control and vehicle-control groups in the spawning period showed mature oocytes with centrally located large oil vacuole and loosely arranged spherical eggs (Figs 6A and 6B). Triclosan exposure at 0.009 µg/ L concentration for 4, 7, 30 and 60 d showed significant histological lesions in the ovary of the fish (Figs 6C-6F). Fish exposed to triclosan for 4 d were observed with vitellogenic oocytes rather than with the expected mature oocytes, along with membrane damage and empty ovarian follicles (Fig. 6C). The intensity of oocyte membrane damage increased with the period of exposure, and at sublethal concentrations, several empty follicles were found (Figs 6D-6F). Triclosan exposed at 9 µg/ L for 4, 7, 30 and 60 d, respectively showed only vitellogenic oocytes (Figs 6G-6J) with similar lesions as observed at 0.009 µg/ L concentration but the severity of tissue damage was increased. Sublethal exposures of triclosan, i.e. 176.7 µg/ L for 4, 7, 30 and 60 d, respectively, were shown in Figs 6K-6N, in which vitellogenic oocytes with membrane damage, empty and atretic follicles, followed by the development of anucleated early-stage oocytes, were prominent (Figs 6K-6N). The representative ovarian pathologies observed in the spawning phase after different concentrations of triclosan exposure, such as the formation of anucleated perinucleolus oocyte, degenerated oocytes with membrane damage, atretic and empty follicles, were shown in Fig. 7.

Histology of testis

Histology of testis in the spawning phase of *Anabas testudineus* showed severe histological abnormalities after the triclosan exposure in comparison with the respective control groups. The histology of the control testis was normal with different stages of spermatogenesis arranged within the compact seminiferous tubules (Figs 8A and 8B). Triclosan exposed at 0.009 µg/ L concentration for 4, 7, 30 and 60 d, respectively, showed loss of spermatozoa that increased with increasing the duration of treatment.

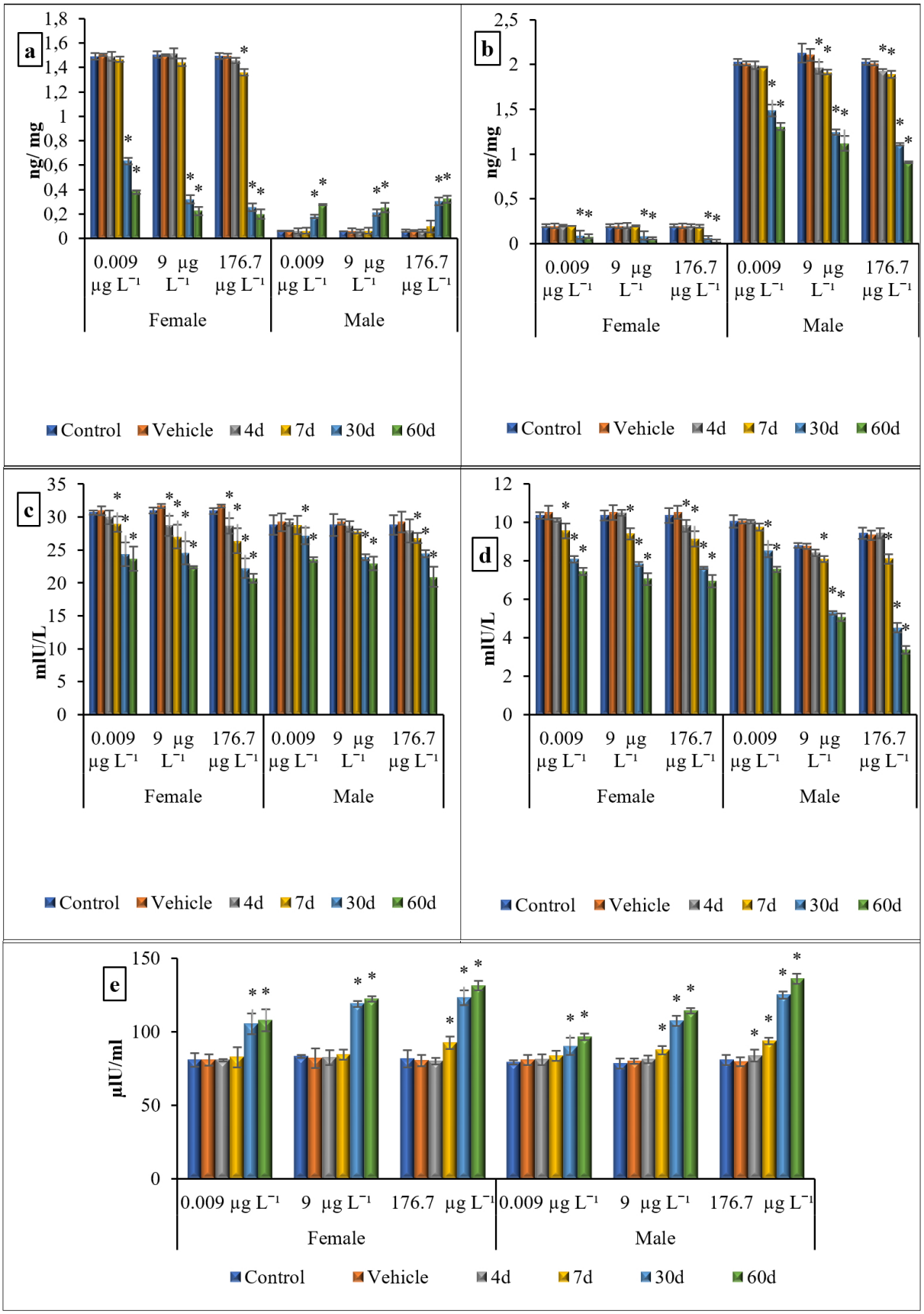


Fig 3. Effect of triclosan on the level of (A) estradiol; (B) testosterone; (C) follicle stimulating hormone; (D) luteinizing hormone; (E) thyroid stimulating hormone in *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant differences against the control groups)

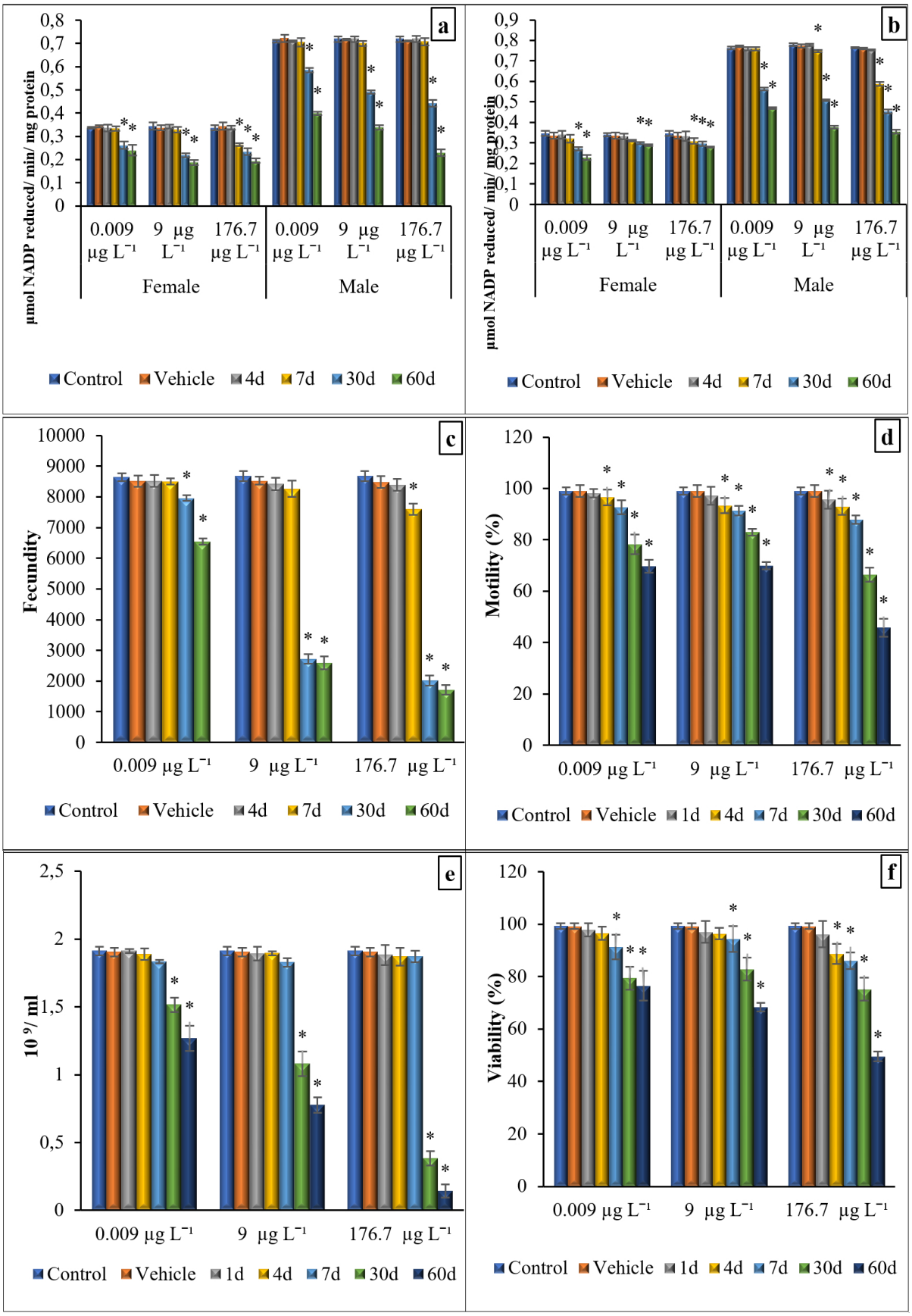


Fig 4. Effect of triclosan on the activity of (a) 3β-hydroxysteroid dehydrogenase; (b) 17β-hydroxysteroid dehydrogenase; (c) fecundity rate; (d) sperm motility; (e) sperm count; and (f) sperm viability in *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant differences against the control groups)

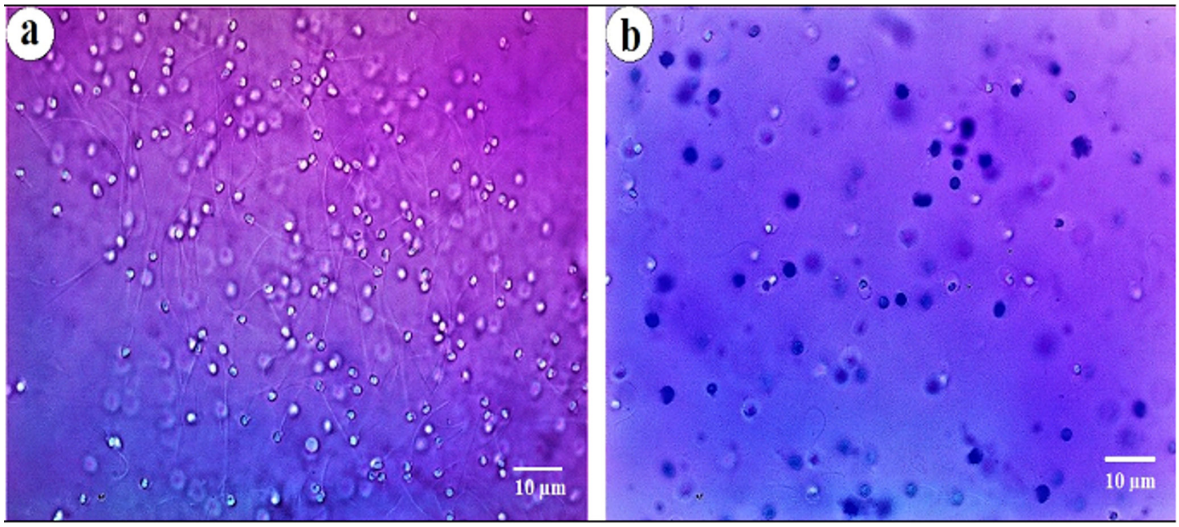


Fig 5. Effect of triclosan on the sperm viability in *Anabas testudineus* (a) Viable sperm appeared in white colour (control groups); (b) Non-viable sperm appeared in blue colour (Triclosan-exposed groups)

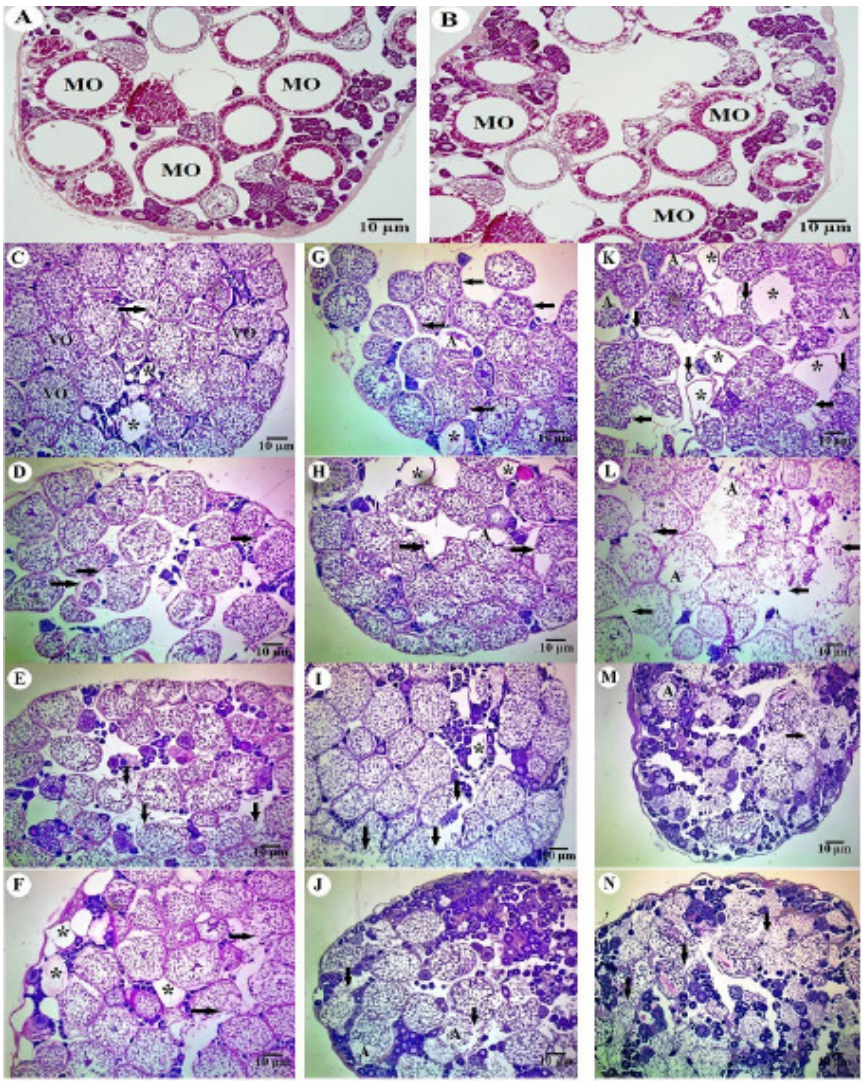


Fig 6. Photomicrographs of spawning ovary in *Anabas testudineus* exposed to triclosan. A-Control, MO-Mature oocyte; B-Vehicle, MO-Mature oocyte; C-F: Triclosan-exposed to 0.009 µg/ L for 4, 7, 30 and 60 d, respectively; G-J: Triclosan-exposed to 9 µg/ L for 4, 7, 30 and 60 d, respectively; K-N: Triclosan-exposed to 176.7 µg/ L for 4, 7, 30 and 60 d, respectively; VO-Vitellogenic oocyte, Asterisks (*)-Empty follicle (C, F, G, H, I, K), Arrow-Oocyte membrane damage (C, D, E, F, G, H, I, J, K, L, M, N), A-Follicular atresia (G, H, J, K, L, M), Arrow (↓)-Aneucleated early stage oocytes (K)

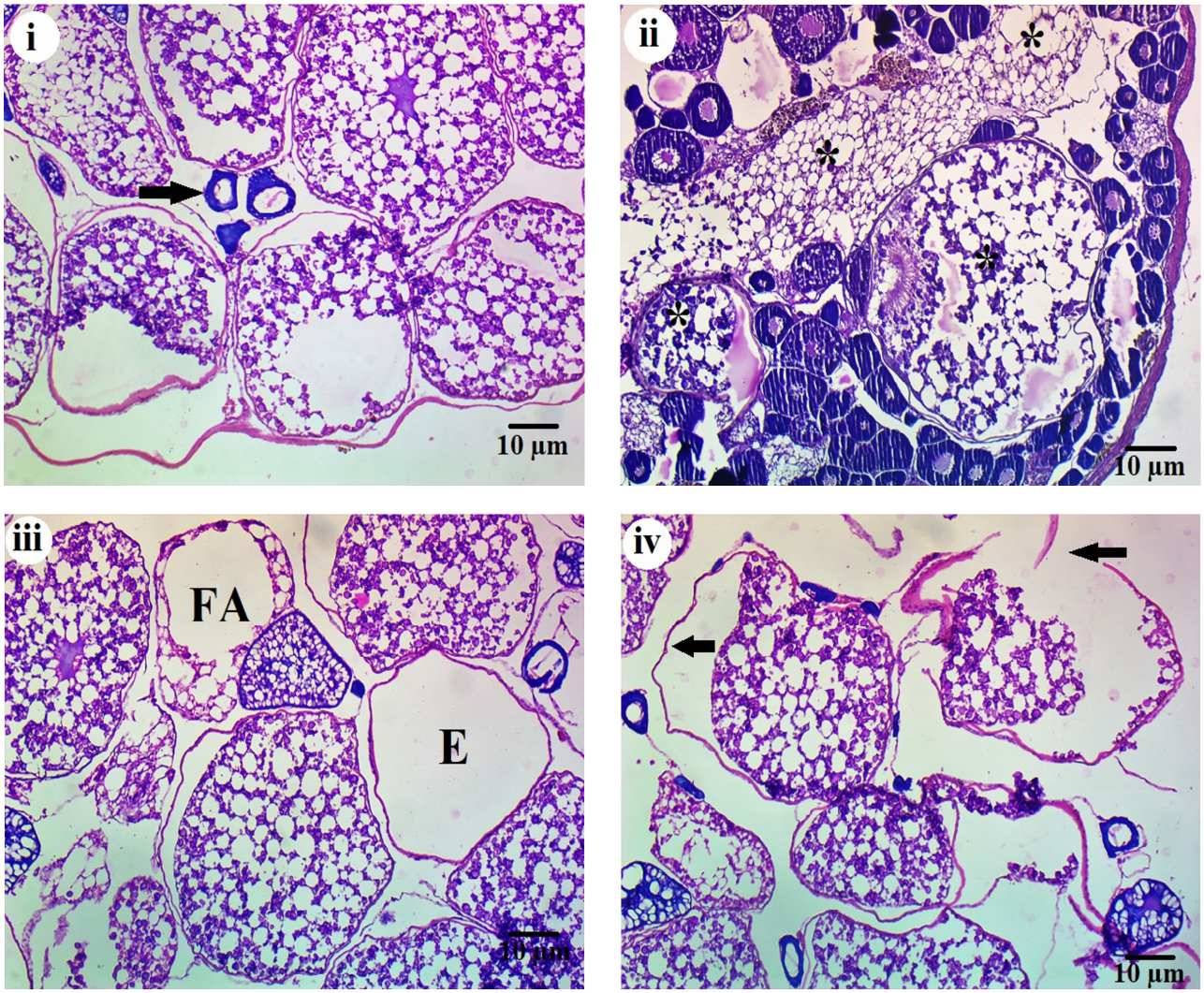


Fig 7. Photomicrographs showing representative pathologies of spawning ovary in *Anabas testudineus* exposed to triclosan. i - Anucleated perinucleolus oocyte (→); ii - Degenerated oocytes (*); iii - Follicular atresia (FA), Empty follicle (E); iv - Oocyte membrane damage (←)

Mass-like lesions named spermatocytes were found to distort the testicular architecture after triclosan exposure (Figs 8C-8E). Besides, the formation of vacuolization and thickened epithelium of seminiferous tubules were also observed in the treatment groups (Figs 8E and 8F). In the 9 µg/ L concentration group, the prominent lesions were characterized by the formation of vacuolization, thickened epithelium of seminiferous tubules, and the complete loss or reduction in the number of spermatozoa (Figs 8G-8J). The intensity of testicular damage increased at 176.7 µg/ L concentration as indicated by the loss of spermatozoa, vacuolization and thickened seminiferous tubules (Figs 8K-8N). Exposure to triclosan at 176.7 µg/ L for 60 d showed some signifying alteration in the testicular structure such as the development of ova-testis in the fish *Anabas testudineus*, as presented in Fig. 9.

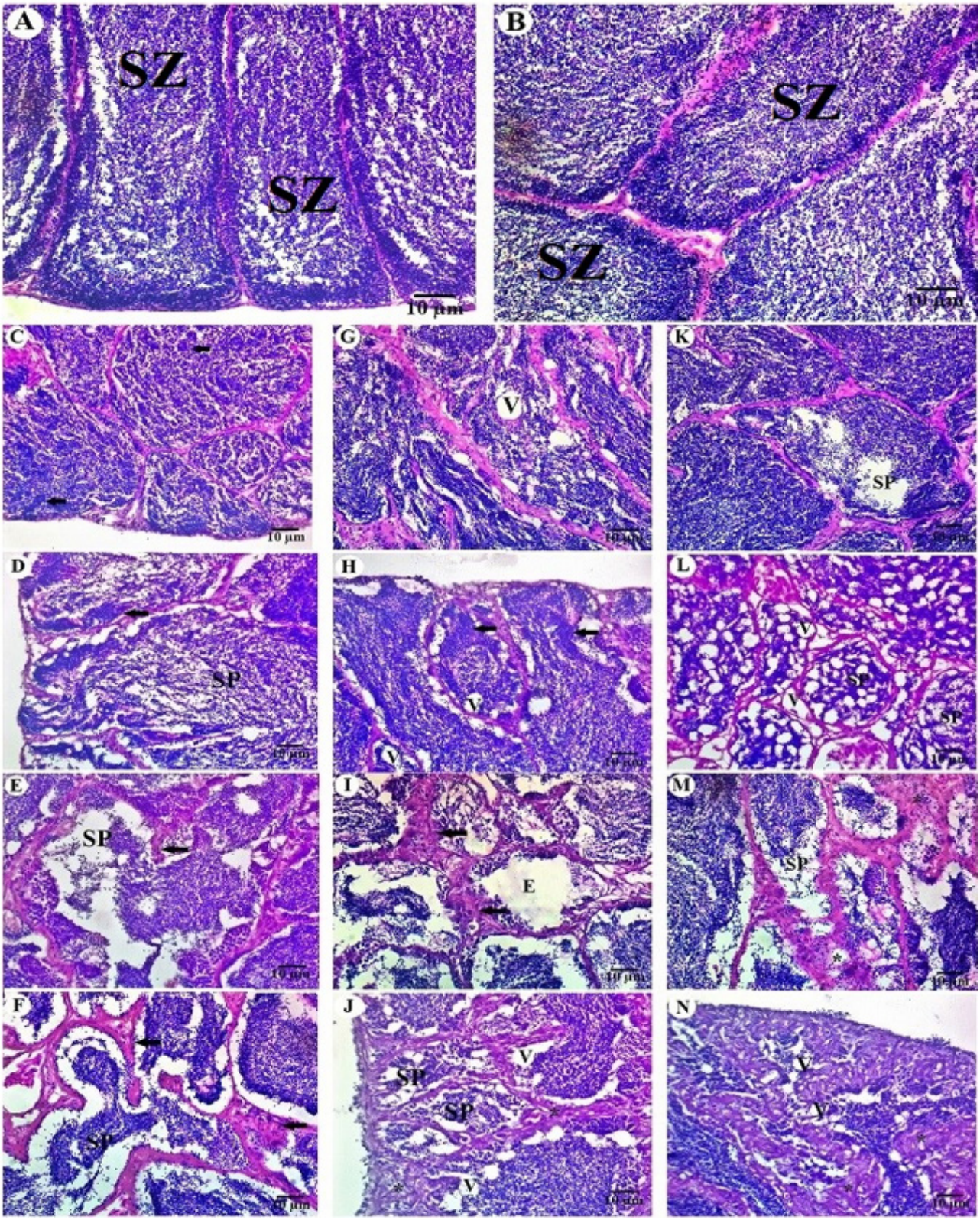


Fig 8. Photomicrographs of testis in *Anabas testudineus* exposed to triclosan. A-Control, SZ-Spermatozoa; B-Vehicle, SZ-Spermatozoa; C-F: Triclosan-exposed to 0.009 µg/ L for 4, 7, 30 and 60 d, respectively; G-J: Triclosan-exposed to 9 µg/ L for 4, 7, 30 and 60 d, respectively; Triclosan-exposed to 176.7 µg/ L for 4, 7, 30 and 60 d, respectively; SP-Loss of spermatozoa (D, E, F, J, K, L, M), Arrow (←) – Spermatocyst (C, D, H), Arrow (←) –Thickened seminiferous tubule (E, F, I), V-Vacuolization (G, H, J, L, N), E-Empty seminiferous tubule (I), Asterisks (*)-Disorganized seminiferous tubule (M, N)

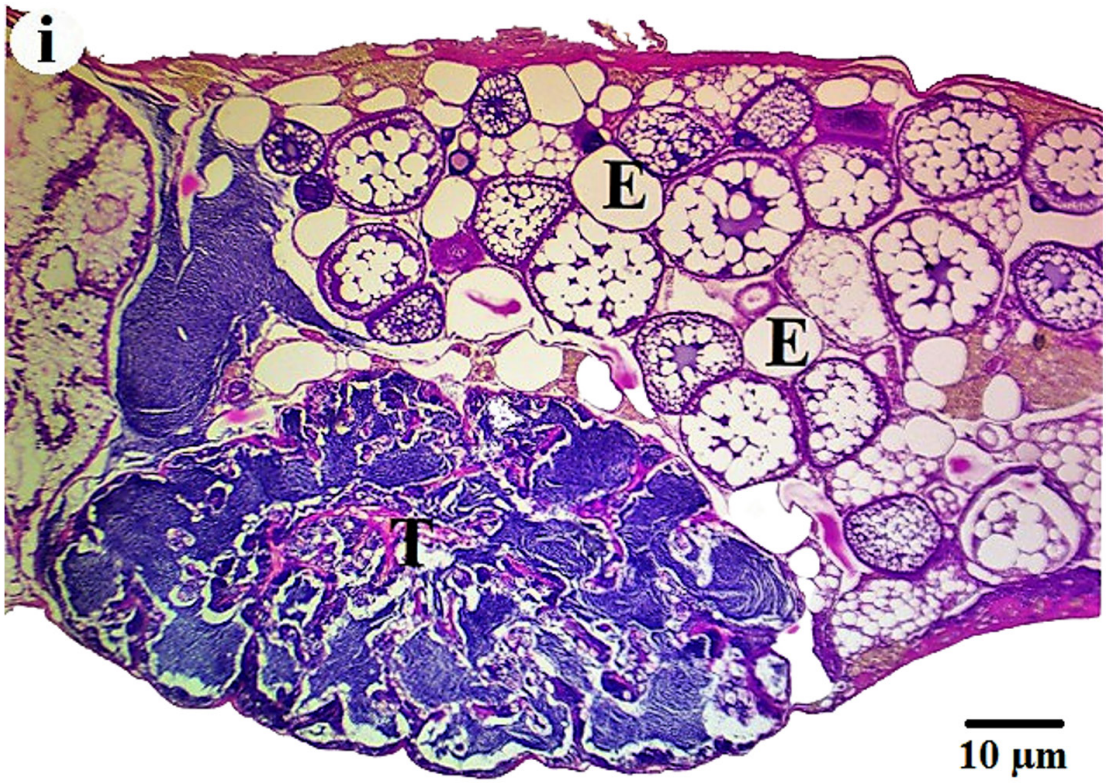


Fig 9. Photomicrographs showing development of testis-ova in *Anabas testudineus* exposed to triclosan (176.7 µg/ L for 60 d), E-Empty follicle, T-Testis

DISCUSSION

In the present findings, triclosan significantly inhibited the body weight, absolute and relative weights of gonads and liver tissues at all concentrations when exposed for a long time. The decline in the bodyweight of the fish measured at the end of every treatment period demonstrated that triclosan exposure caused anorexia, i.e. reduction in food consumption. It has been shown that there is a positive correlation between stress-activated anorexia and synthesis and secretion of cortisol through the hypothalamic-pituitary-interrenal axis (Bernier and Peter, 2001), thereby regulating gastric evacuation (Tache et al., 2001) and parasitic gut infection in fish (Kyriazakis et al., 1998). Similar growth loss in male and female fish *Oreochromis niloticus* has been reported after exposure to ethinylestradiol at environmentally relevant concentrations (Shved et al., 2008). This interpretation was further supported by another study that showed a reduction in the body weights of adult female zebrafish and cichlid fish *Pseudetroplus maculatus* after exposure to phthalate esters (Kim et al., 2015; Sajla et al., 2019). As a defensive mechanism, the surface of the fish is usually covered by a thin layer of mucous that protects from the attack of predators, pathogens and chemical stressors (Salinas et al., 2011; Rajan et al., 2011), and also plays a protective role in osmoregulation (Varsamos et al., 2005). In this study, triclosan exposure increased the mucous deposition throughout the body of the fish indicating the primary defensive mechanism to prevent the entry of the toxicant. Such a first line of defence has been exhibited by the fishes against various other chemical stressors such as phthalate plasticizers and nanomaterials (Sruthi et al., 2020; Sumi and Chitra, 2020).

Other biometric parameters such as weights of gonads, liver, gonadosomatic index (GSI) and hepato-somatic index (HSI) are important indicators used in this study to evaluate gonadal development and reproductive activity along with the involvement of the liver in vitellogenesis (Sadekarpawar and Parikh, 2013; Pandit and Gupta, 2019). A remarkable decline in the GSI of male and female fish after triclosan exposure during the spawning phase indicated failure of gonadal development and maturity in the fish. The results were in agreement with other studies on exposure to estrogenic environmental contaminants such as 17α-ethinylestradiol in adult fathead minnow, *Pimephales promelas* (Filby et al., 2007), dibutyl phthalate in *Pseudetroplus maculatus* (Sruthi et al., 2020) and sodium benzoate in *Anabas testudineus* (Vijayakumar et al., 2020). HSI is the vital indicator that provides information about the metabolic activity and the stored energy status in the fish during different phases of reproduction (Zin et al., 2011). Reduced values of HSI in the current study could be due to the mobilization of hepatic energy reserves for maintaining homeostasis to overcome the stress related to triclosan exposure. Our

results are consistent with the similar concentration- and time-dependent decrease in HSI of *Channa punctatus* exposed to malathion (Bharti and Rasool, 2021).

Plasma proteins, consisting of albumin, globulin and fibrinogen, are primarily synthesized in the liver, and a small percentage is produced by lymphocytes and plasma cells. They are involved in the control of several physiological activities and are therefore used as a diagnostic tool to detect diseases or overall animal health status (Cnaani et al., 2004). Triclosan-related stress response was evident by the rise in the level of total protein in plasma at all concentrations in a time-dependent manner to meet the energy demand. However, the level of total protein in the gonads and liver tissues declined to indicate the suppression of several biological processes. Thus the degradation of tissue protein and its release into the circulation could have been used as an alternative source of energy to overcome the stress condition, and also an attempt to detoxify the toxicant. Similar observations have been reported after exposure to the estrogenic environmental contaminants, namely phorate and chlorpyrifos, in *Channa punctatus* and *Pseudetroplus maculatus*, respectively (Singh et al., 2010; Raibeemol and Chitra, 2018).

Vitellogenin, the yolk protein, synthesized and secreted by the female liver under the influence of estrogen is widely considered the biomarker of xenoestrogens in aquatic organisms. The activation of estradiol rises the level of plasma vitellogenin during the female reproductive stage, however, the vitellogenin gene in the male liver remains silent where it gets activated only upon the stimulation of estrogen mimics (Denslow et al., 1999). Thus the gender-specific production of vitellogenin depends on the expression of estrogen receptors and differences in estrogen sensitivity (Navas and Segner, 2006). Phospholipoglycoprotein synthesized by the liver serves as a precursor of egg yolk that is transported through the bloodstream and incorporated into the growing oocytes (Arukwe and Goksoyr, 2003; Hennies et al., 2003). The increase in the level of vitellogenin in plasma and liver tissues of female fish after 30 and 60 d of triclosan exposure proved the estrogenic effects of the compound. Meanwhile, a significant reduction in the vitellogenin level of the ovary could be due to the binding of triclosan to the estrogen receptor that prevented its deposition in the oocytes. The current study showed a significant increase in the level of vitellogenin in the plasma, testis and liver of male fish after triclosan exposure, and this further confirmed the profound estrogenic effects. Similarly, some studies have reported that the administration of exogenous estrogenic compounds such as atrazine, chlordecone and dibutyl phthalate elevated the level of vitellogenin in males of *Poecilia sphenops* and *Pseudetroplus maculatus*, respectively (Vasanth et al., 2015; Asifa and Chitra, 2019; Sruthi et al., 2020). However, the disturbance of the endocrine reproductive axis by the induction of vitellogenin production mediated through the non-estrogen receptor pathway has proved the anti-androgenic action of triclosan in the male carp (Wang et al., 2018).

To prove the estrogenicity of the compound, the vitellogenin biomarker is often supplemented with serum hormone analysis. Exposure to triclosan decreased the level of estradiol by the prevention of aromatization in the female fish, while the rise in the estradiol level after 60 d of sublethal treatment proved estrogenic effects of the compound in the male fish. However, the level of serum testosterone declined in both male and female fish illustrating the negative feedback mechanism of triclosan through the hypothalamic-pituitary-gonadal (HPG) axis or by the suppression of gonadal steroidogenic enzymes (Mills and Chichester, 2005; Chang et al., 2013; Shi et al., 2015). It is well-known that pituitary gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are actively involved in gonadal differentiation, maturation and biosynthesis of sex steroid hormones, and also regulate oogenesis in females and spermatogenesis in males (Nagahama and Yamashita, 2008). Triclosan exposure caused a reduction in the levels of serum FSH and LH in both sexes of the fish, which depends on the increase in duration and concentrations thereby demonstrating the endocrine-disrupting activity of the toxicant. The action of triclosan on the HPG axis and the negative feedback inhibition on the production of sex hormones have been previously demonstrated in the female yellow river carp *Cyprinus carpio* (Wang et al., 2017). Although the serum levels of biologically active thyroid hormones, namely thyroxine (T_4) and triiodothyronine (T_3), were not detected in the present study, it was found that the level of thyroid-stimulating hormone (TSH) increased in both sexes of the fish after long-term exposure of triclosan. These results suggest that the negative feedback in the hypothalamic-pituitary-thyroid axis due to triclosan exposure could have increased TSH levels, thereby proving thyroid disrupting effects of the toxicant. In concordance, another study observed the potential disruption of the thyroid axis by triclosan during the embryonic and larval development in sheepshead minnow *Cyprinodon variegatus*, associated with retarded early development and metamorphosis (Schnitzler et al., 2016).

During the spawning phase of fish reproduction, gonadal steroidogenesis contributes a major role in the biosynthesis of circulating steroid hormones as well as helps to promote the expulsion of gametes into the surrounding water (Shanthanagouda and Khairnar, 2018). The major steroidogenic oxidoreductase enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) are predominantly expressed in gonads for the biosynthesis of sex steroids (Rasmussen et al., 2013). The present results observed a significant reduction in the activities of 3β-HSD and 17β-HSD enzymes in gonads of both sexes, illustrating the negative impact of triclosan on

gonadal steroidogenesis that ultimately resulted in the down-regulation of sex steroid hormone biosynthesis, as mentioned earlier. The findings coincided with the previous study which demonstrated the effects of estrogenic phthalate contaminants such as di-isononyl-phthalate (DINP) and di-(2-ethylhexyl)-phthalate (DEHP) in freshwater fish *Oreochromis mossambicus* (Revathy and Chitra, 2019). Similarly, another research has revealed that exposure to bisphenol A altered the gene expression associated with gonadal steroidogenesis in the ovary of rare minnow *Gobiocypris rarus* via abnormal DNA and histone methylation (Liu et al., 2020).

The reproductive performance of the fish during the spawning season may be checked using some physiological indices such as the rate of fecundity in females and sperm parameters like viability, motility and sperm count in males. In the present findings, there was a remarkable decline in the fish fecundity at all concentrations after 30 and 60 d of triclosan exposure with a concomitant reduction in the sperm motility, viability and count in the male fish, thereby leading to failure of reproductive performance causing infertility in *Anabas testudineus*. Another study has reported that the larval exposure to environmentally relevant concentrations of triclosan impaired metamorphosis, fecundity and fertility in zebrafish (Stenzel et al., 2019). An investigation conducted in the Lake Mead National recreational area has reported that triclosan, along with other environmental contaminants, altered sperm quality parameters such as motility, viability, mitochondrial membrane potential, sperm count, sperm morphology and DNA fragmentation in male common carp *Cyprinus carpio* (Jenkins et al., 2018).

Histological features of gonads after triclosan exposure exhibited severe lesions in all treatment groups. Histology of the ovary showed an increase in the number of immature oocytes with a concomitant decline in the number of vitellogenic oocytes in a time- and concentration-dependent manner. These results substantiate the reduction in vitellogenin levels observed in the ovary due to the binding of triclosan with its estrogen receptors, which could have prevented the maintenance of vitellogenic oocytes. The other morphological abnormalities observed in the current study include follicular atresia, oocyte membrane damage, formation of empty follicles and anucleated perinucleolous oocytes in almost all treatment groups. Mohapatra et al. (2020) have reported ovarian damage that includes decreased vitellogenesis, follicular atresia and disruption of the follicular wall in the freshwater fish *Anabas testudineus* exposed to sublethal concentrations of monocrotophos. Similarly, dibutyl phthalate also induced severe morphological damages in ovaries associated with apoptosis in freshwater fish *Pseudetroplus maculatus*, evident by the empty follicles, atretic oocytes, membrane blebbing, nuclear condensation, vacuolization and broken theca granulosa membrane (Sajla et al., 2019). Triclosan also caused testicular damage as indicated by remarkable pathologies like loss of spermatozoa, formation of spermatocytes, disorganized, thickened and/ or empty seminiferous tubules, and vacuolization that accounts for the reduction in sperm motility, viability and sperm count in the exposed fish. Similar observations have been reported for *Pseudetroplus maculatus* exposed to dibutyl phthalate suggesting altered reproductive performance ultimately leading to infertility in male fish (Sruthi et al., 2020). Besides, exposure to triclosan at sublethal concentrations for 60 d showed the formation of intersex, which is a condition characterized by the presence of female oocytes in testes thereby proving endocrine-disrupting effects of the toxicant. The overall results of this study indicated that the duration of exposure and concentration of the toxicant plays a critical role in the maintenance of the reproductive performance of *Anabas testudineus*.

CONCLUSION

The present findings highlight the intolerance of *Anabas testudineus* to resist the stress induced by triclosan during the spawning phase of reproduction. The results suggest that long-term exposure to triclosan even at low concentrations is sufficient to produce adverse effects on fish reproduction that affect the spawning ability and breeding fitness. The impact of triclosan on the reproductive capability of the fish in turn affects the production of offspring and maintenance of population in the natural environment. Hence, strict regulations on the widespread use and environmental discharge of triclosan must be ensured for the safety of ecological health and aquatic life.

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Ethics approval: All procedures involving animals were in compliance with the Animal Welfare Board of India and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Ministry of Environment, Forest and Climate Change, Government of India.

UTJECAJ TRIKLOSANA NA REPRODUKTIVNU FIZIOLOGIJU GRGEČA PENJAČA U FAZI MRIJESTA

SAŽETAK

U ovom radu opisano je negativno djelovanje triklosana, antimikrobnog agensa, na ometanje rada endokrinog sustava i njegova uključenost u reproduktivne odgovore posredovane brojnim načinima djelovanja kod različitih vrsta riba. Cilj ovog rada je istražiti da li bi triklosan u ekološki relevantnim i subletalnim koncentracijama mogao utjecati na reproduktivnu fiziologiju vrste *Anabas testudineus* u fazi mrijesta. Ribe su bile izložene triklosanu u ekološki relevantnim (0,009 i 9 µg L^{-1}) i subletalnim (176,7 µg L^{-1}) koncentracijama kroz 4, 7, 30 i 60 dana tijekom faze mrijesta radi procjene reproduktivnog potencijala ribe. Izloženost triklosanu uzrokovala je značajno ($P<0,05$) smanjenje razine ukupnog proteina u spolnim žlijezdama i tkivima jetre uz povećanje u krvnoj plazmi kako bi se zadovoljile potrebe za energijom i prevladao metabolički stres. Oštećenje steroidogeneze gonada dokazano je smanjenjem aktivnosti 3β- i 17β-hidroksisteroid dehidrogenaza povezanih s promjenom razine gonadotropina u serumu, spolnih steroidnih hormona i vitelogenina. Izloženost triklosanu prouzročila je smanjenje stope plodnosti u ženki i smanjila broj spermija, pokretljivost i održivost kod mužjaka. Histološke lezije u tkivima gonada dodatno su potvrdile reproduktivnu toksičnost triklosana, što bi zauzvrat moglo doprinijeti reproduktivnom neuspjehu kod ove vrste.

Ključne riječi: *Anabas testudineus*, vitellogenin, serumski hormoni, plodnost, indeksi sperme

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