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Acute and chronic effects of Bisphenol A on hormonal disruption and histological alterations in the freshwater clam, *Caelatura nilotica* (Cailliaud, 1827)

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ABSTRACT
Endocrine disruptors have been widely reported in vertebrates, but their impacts on the ultrastructure of the freshwater invertebrates were not reported. So, this study was designed to investigate the hazardous effects of 0.25 and 2 mg/l concentrations of Bisphenol A (BPA) on the freshwater clam, *Caelatura nilotica* for 1 and 4 weeks. Biometrics, sex hormones, histology and ultrastucture of gills and gonads as fundamental tools were investigated. Some biometric parameters increased with exposure and time (Chohen`s d test). condition index, testosterone and 17β-estradiol decreased significantly with concentration and time (1 and 4 weeks, $P \leq 0.05$, ANOVA). Histological examination showed pathological signs which were more severe at 4 weeks than 1 week manifested as necrosis, inflammation, fibrous tissue, and hyperplasia formation, fat droplets accumulations in gills, gonads and intestine. Scanning electron microscope examination showed erosion of cilia, irregularity and foci of the gills. In addition, transmission electron microscope examination showed decrease in protoplasmic enzymes, blebbing (apoptosis) of flagella and degeneration of mitochondria of sperms. In addition, increased yolk granules, fat droplets, gelatinous protection layer and decreased number of microvilli of the oocytes were noticed. So, BPA induced not only hormonal disruption in sex hormones and organs but also caused biometric and gill alterations in the clams which may interfere with bivalves` biology and aquatic environmental conservation. This study also spotted the ultrastructural effects of BPA for the first time in the freshwater clams.

INTRODUCTION

Freshwater bivalves play important ecological roles, especially in shallow waters; rivers, lakes, and estuaries. Bivalves are widely used to assess environmental pollution because they respond with measurable physiological and morphological change. Additionally, they cannot escape pollution and therefore serve as sessile bioindicators. So, they can tolerate and accumulate high levels of heavy metals, pesticides and hormonal disrupting chemicals and then show signs of pathology (Tran *et al.*, 2003; Fabbri *et al.*, 2014;

Chmist *et al*., 2019; Brahma and Gupta, 2020). Two species of the freshwater clam, genus *Caelatura* were identified in the River Nile, Egypt, *nilotica* and *teretiuscula*. *Caelatura nilotica* was the most widely distributed species and suitable for laboratory experiments (Ibrahim *et al*., 1999; Sheir, 2005). The freshwater bivalve, *Caelatura nilotica* is an ancient genus found in the River Nile and its tributaries (Pallary, 1924; Ibrahim *et al*., 1999). Its distribution and abundance in Africa, Egypt and Menoufia Province were discussed by Mothersill *et al*., (1980), Pallary (1924), Ibrahim *et al*. (1999), Abd El-Wakeil *et al*. (2013), Sheir (2005) and Sheir *et al*. (2018).

Bisphenol A (BPA) is widely used in the manufacturing of polycarbonate plastics and epoxy resins. It enters the aquatic environment (receiving water and sediment) mainly through wastewater effluents (Staples *et al*., 1998). BPA can be leached from plastic products and food/drink packaging and canned food (Kang and Kondo, 2003). The main factors affecting the leaching of BPA from can surfaces were acidity, heating times and temperature used in the manufacturing process. Increase in the use of products based on BPA caused increase in the environmental contamination by BPA (Kang *et al*., 2003; Goodson *et al*., 2004). A Japanese study reported the levels of BPA leached from polyvinyl chloride (PVC) products and synthetic leather reached 139 µg/g (Yamamoto and Yasuhara, 1999). Moreover, BPA migration from PVC houses used for drainage, watering and sprinkling ranged from 4 to 1730 µg/l (Yamamoto and Yashuhara, 2000). Also, Ozhan and Kocaman (2019) reported the distribution of BPA in marine and freshwaters in Turkey, which reached 29.92 µg/l. Environmental sources of BPA can be classified as pre-consumer and post-consumer products. Pre-consumer sources include the manufacture of BPA and BPA containing products (Cousins *et al*., 2002; Klecka *et al*., 2009). However, in Egypt the only research surveyed BPA concentrations in drinking and resource water was by Radwan *et al*. (2020). They measured BPA concentrations in the River Nile which reached 85.5 µg/l in surface water and 2.230 µg/l in drinking water. They reported BPA concentrations in Menoufia governorate as 25 ng/l in the surface water and 21 ng/l in the drinking water.

Bisphenol A is a well known endocrine disruptor (Krishnan et al., 1993; Goodman *et al*., 2006) and can cause acute toxicity to aquatic invertebrates (ex. *Daphnia magna* and *Mysidopsis bahia*) at concentrations that ranged from 1.1 to 10 mg/l for freshwater and marine species (Alexander *et al*., 1988). Moriyama *et al*. (2002) identified BPA as xenobiotic endocrine disruptor, disrupting the balance of the hormones of animals. Several studies have been established to find out the effects of BPA on aquatic invertebrates at medium to high concentrations. The freshwater cnidarians, *Hydra oligactis* showed suppression of testis formation at concentration 1–4 mg/l for 35 days (Fukuhori *et al*., 2005), while exposure of the copepod, *Acartia Tonsa* to 20 µg/l for 10 days resulted in induction of eggs (Andersen *et al*., 1999). In addition, gills were considered as the main target organ for pollutants encountering the habitat of bivalves and cause accumulation and impacts such as alterations in the epithelial tissue and dilation of microvilli as discussed by Bigas *et al*. (1997).

BPA has been found to affect the aquatic molluscs such as snails and bivalves, when it is released in the environment (Jahromi *et al*., 2020). For example, BPA induced changes in invertebrates such as abnormal reproductive cells (lower number of mature sperms), organs (reduced heart rate), and imposex have been detected in freshwater snails. Super-females (ie, increased egg mass/number) and feminization of males has

been reported in females of the freshwater prosobranch, *Marisa cornuarietis* (Oehlmann *et al*., 2000; Kang *et al*., 2007, 1-100 and > 460 µg/l, respectively). Chronic exposure to low levels of BPA induced superfeminization syndrome in the freshwater ramshorn snail, *Marisa cornuarietis* (Oehlmann et al., 2006). Exposure to environmentally relevant BPA concentrations elevated the fertility in the snails, *Potamopyrgus antipodarum* and *Nucella lapillus* by increasing the cases of superfemales and reduction of sperm numbers and penis length in males (Oehlmann *et al*., 2000). In the bivalve, *Mytilus galloprovincialis* exposure of fertilized eggs to BPA delayed the formation of fully developed D-larvae (2 days old and fully shelled, Fabbri *et al*., 2014). In *Mytilus edulis*, spawning induction, oocyte and ovarian follicle damage were detected after the exposure to 50 µg/l BPA for 3 weeks (Aarab *et al*., 2006). Benjamin *et al*. (2019) reported severe histopathological signs of the soft tissues (digestive gland, gills and adductor muscles) of exposed clams, *Corbicula fluminea* to BPA (1-3 µg/l) for 21 days. Such pathologies were hypertrophy, hyperplasia, fibrosis, inflammation and necrosis. Even if the environmental concentrations were low, the effects seemed to be high on the aquatic organisms. Gust *et al*., (2014) recorded bioaccumulation of weak estrogenic compounds (Tert Octyl Phenol) in the caged mud snails, *Potamopyrgus antipodarum* in sites where it was undetectable in the water. In addition, Canesi and Fabbri (2015) concluded that the concentrations of BPA caused adverse effects mostly exceeded or in the upper ranges of the concentrations in the environment and the laboratory studies just measure a small scale of the animals life span, because EDCs were incessantly released in large amounts in the environment.

So, this work aimed to expand the data on the bio-hazardous effects of Bisphenol A for short and long terms of exposure on the freshwater clam, *Caelatura nilotica*. Biometry, sex hormones, histology, and ultrastructure of gills, gonads, and intestine will be measured to achieve the previous target.

MATERIALS AND METHODS

Experimental animal and stock aquaria

Adult freshwater clams, *Caelatura nilotica* (Cailliaud, 1827) were used in conducting the experiments in this study (Fig. 1). It was collected from Gezay village's freshwater stream, Menoufia government, Egypt during winter, 2019. *C. nilotica* were maintained in well aerated glass aquaria filled with de-chlorinated tap water and fed on field silt (mix of coarse silt and sand, 30 g/tank) for two weeks for acclimatization before the main experiment. Laboratory conditions like water temperature, 20 ± 0.9 ˚C, pH, 7.1 ± 0.2 and oxygen content, 6.8 ± 0.4 mg/l were measured after every water change. Prior to the experiment, the clams were transferred to small tanks with 4 L capacity to acclimatize on space and water volume. Environmental factors and metals analysis of the field silt (sediments) and clams was done (Sheir *et al*., 2018). BPA and pesticide concentrations were carried out at The Chromatography Laboratory, The Central Laboratories Network, The National Research Centre; Cairo, Egypt. BPA concentrations in the stream water and sediment were under detection levels. Meanwhile, pesticides (organochlorines and organophosphorus) in stream water were undetectable.

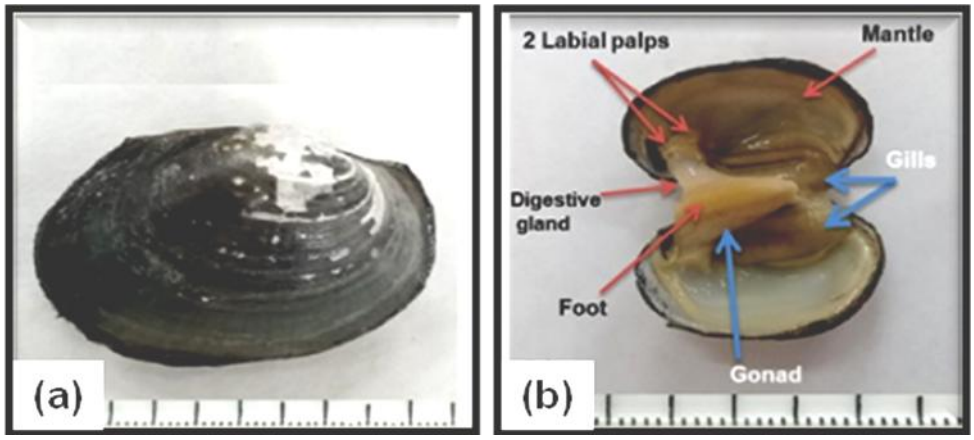

Fig. 1. The freshwater clam, *Caelatura nilotica*. **a)** Closed shell, **b)** Opened shell with organs exposed.

Experimental material

BPA (CAS No. 80-05-7, 97%) was purchased from Sigma-Aldrich Company (Taufkirchen, Germany). BPA was ultra-sonicated and dissolved in de-chlorinated tap water to avoid using toxic solvents, and was kept in the refrigerator (Mihaich *et al.*, 2009). The resulting stock solutions (1 g/l) were continuously stirred until the solutions had no un-dissolved material and freshly prepared every week.

Experimental design

In order to study the effects of BPA on clams, about 90 clams (Average length = 5.69 ± 0.072 cm, height = 3.36 ± 0.064 cm) were used. Clams were acclimatized into 4 L capacity tanks (length, 30 cm; height, 20 cm and width, 15 cm). Clams were divided into 3 groups, the control group, and two exposed groups. Each group was three replicates/treatment, 10 clams/replicate, and each tank were filled with 2 litres of water with/without BPA. The quality control (toxicity) experiment concentrations` were chosen according to Alexander *et al*. (1988) and Mihaich *et al*. (2009) as 0.25, 0.5, 1 and 2 mg/l BPA to detect lethal and sublethal concentrations on the clams for 4 weeks. The main experiment concentrations` (0.25 and 2 mg/l) were chosen according to the quality control experiment to test the acute and chronic concentrations of BPA on the freshwater clam, *C. nilotica*. The control, 1st group received no treatment and subjected to de-chlorinated tap water and a layer of the field silt (30 g/tank). The 2nd group was exposed to 0.25 mg/l BPA and silt (30 g/tank) and the 3rd group was exposed to 2 mg/l BPA and silt (30 g/tank). The experimental period lasted for four weeks. The preferred concentrations were dosed to the water tanks consciously every other day and water was changed through static method with 100% water change. Clams were collected randomly and dissected from each exposed group as well as the control at zero time, after one week (short term exposure) and 4 weeks (long term exposure) for clams` morphometry, biometry, and condition index. Sex hormones, histology of gills and gonads, SEM of gills and TEM of gametes were measured/examined after one and four weeks. Gills were selected as the first target for any pollutant (direct mode of action) and gonads represented the main target of the endocrine disruptors (direct mode of action on sex organs). The total number of dissected clams was 36 specimens.

Condition index (CI)

CI value is an index of the current nutritive status of the clams (*n* = 36, males and females collectively because there is no sexual dimorphism in bivalves) and it was measured as the relative proportions of flesh to shell weights according to Aguirre (1979) using the formula:

$$\mathbf{CI = [MW/ (TW - SW)] \times 100}$$

where: MW = wet meat (flesh) weight, TW = total wet weight, and SW = shell weight.

Hormonal assays

Testosterone and 17β-Estradiol (as an example of androgens and estrogens) concentrations in males' and females' gonads (*n* = 24), respectively were measured using Lifespan Bioscience, Inc. North America, Catalogue No. LS-F10538, Elisa Kit. (De Longcamp *et al*., 1974; Tietz, 1986). Samples in were weighted (fixed to ~ 0.03 g), frozen and thawed and then homogenized in PBS on ice. The homogenate was centrifuged at 5000 g for 5 minutes. Finally, the supernatant was collected for the assay. 50 µl of samples and standards were added to the wells in triplicates and the assay protocol was followed precisely. The plate was read at 450 nm. The data were linearized by plotting the log of the target antigen concentration on the Y-axis versus the optical density (O. D.) of the standards on the X-axis and the best fit line can be determined by regression analysis. In order to calculate the standard curve, the linear equation (Y = mx + b) was used. Y is the log of the concentration of the standard and x is the O. D. of the standard. The hormones concentrations were expressed as ng/ml.

Histological study

Clams (*n* = 24) were dissected for histological study after one and four weeks from the control and exposed tanks. Gills and gonads were immediately fixed in neutral formaldehyde (10 %) for 24 hours. Tissues were transferred to 70 % ethanol then dehydrated in ascending series of ethanol (70, 80, 90 and 100 %). Tissues were embedded in paraffin wax at 60 ˚C, and then cooled until solidification of wax according to the procedure of Romeis (1989). Serial sections were cut at 5-8 µm thick then stained with Eosin and Ehrlich`s Haematoxylin, mounted in DPX and covered with glass cover. Slides were blindly examined using Optika microscope and photographs were taken using Optika digital camera with desired magnifications. Scale bars were used for each photograph. A rating scale (0-4) was done to describe the severity of the histological impacts of BPA on each exposed group, where, 0 recorded no histological alterations and 4 the most severe histological alterations. Semi-quantitative analysis was derived by counting at least five replicates from a randomly selected area on a section from each animal.

Electron microscopy examination

Scanning electron microscopy

Gills (*n* = 24) were cleaned several times in 70 % ethanol, and then were fixed immediately in 2% glutaraldehyde buffered with phosphate (pH 7.2). Specimens were dehydrated using ascending series of alcohol and dried at critical point. Dehydrated samples were vacuumed out for 8 min, then were coated with gold. Photographs were

taken at the desired magnification using Scanning Electron Microscope (A JEOL JSM-5300 SEM (20-25) KV, Japan) at Faculty of Science, Alexandria University, El-Shatby.

Transmission electron microscopy

Males' and females' gonads ($1mm^3$, $n = 24$) were fixed in buffered glutaraldehyde (glutaraldehyde + formaldehyde) followed by post-fixation in 1% osmium tetroxide. Then, samples were dehydrated in ethanol series (50 – 100 %) and embedded in epoxy resin capsules. Capsules were put in 60 °C oven for 48 hours. Once hardened, the blocks were trimmed and sectioned using a LEICA ULTRACUT UCT microtome for semi-thin sections examinations. Ultrathin sections at 60-90 nm thick were cut and put onto copper grids. Sections were dried overnight then stained with Uranyl Acetate and Lead Citrate. Finally, grids were examined and photographed with the required magnifications using JEOL JEM-1400 Plus Transmission Electron Microscope (Japan) at Faculty of Science, Alexandria University, El-Shatby.

Statistical analysis

Morphometric, biometrics and hormones data of control and exposed groups were analyzed using Statgraphics Centurion XVI. Data were expressed as mean ± SD. Statistical analysis was carried out using one way-ANOVA, setting the probability level to $P \leq 0.05$. Variance check was performed using the multiple range tests with post-hoc test (least squares difference, LSD test). Size effects of the biometrics using Cohen`s d test was calculated where

$$\mathbf{d} = \frac{\text{Mean of group 1- Mean of group 2.}}{\text{Standard Deviation (pooled)}}$$

RESULTS

Effects of BPA on biometrics of the clams, *Caelatura nilotica*

Biometric measurements of the clams (males and females) collected from the control and exposed groups were recorded at zero time, after one and four weeks. However, no significant difference was recorded in biometrics of the control and exposed groups ($P > 0.05$), the lengths of shells increased (≥ 8.6 %) after one week but decreased after four weeks (≥ 3.8 %) of exposure with small, medium and large size effects during the time points (Cohen`s test, d = 0.2, 0.5 and 0.8). The height of the shells decreased after exposure to 0.25 mg/l BPA (11.4 %) and exposure to 2 mg/l BPA (5.5 %) after the exposure period with only small size effect after one week of exposure of 0.25 mg/l BPA exposed group. The total, shell and flesh weights increased after one and four weeks of exposure (≥ 55, 45.8 and 35.3 %, respectively). Shell and total weight recorded small size effect after one and four weeks of exposure of 0.25 mg/l BPA exposed group, respectively, while flesh weight recorded medium size effect after four weeks of exposure of 0.25 and 2 mg/l BPA exposed groups. Condition index of clams collected from exposed groups decreased over time (one and four weeks) of exposure to 0.25 and 2 mg/l BPA than the clams collected from the control group (≤ 27. 1 % reduction, $P > 0.05$, ANOVA, Table 1).

Table 1. Effect of BPA on biometric measurements of control and exposed clams to BPA for four weeks

Time (week)/ Biometrics/ Treatments	Zero	One	Four	Zero	One	Four	Zero	One	Four
	Length (cm)			Height (cm)			Total Weight (g)		
Control	5.4±0.1	5.6±0.2	5.8±0.3	3.5±0.1	3.4±0.4	3.5±0.1	16.9±1.4	25.1±2.1	28.8±3.3
0.25 mg/l	5.5±0.3	5.8±0.2	5.3±0.3	3.5±0.2	3.3±0.2	3.1±0.2	17.7±2.6	22.9±0.9	27.6±1.8
2 mg/l	5.5±0.1	5.9±0.4	5.7±0.1	3.6±0.1	3.5±0.5	3.4±0.2	16.9±2.1	27.2±1.6	32.9±7.1
Biometrics/ Treatments	Shell Weight (g)			Flesh Weight (g)			Condition index (%)		
Control	6.2±0.9	9.6±1.8	11.7±2.2	4.8±0.1	6.9±0.3	7.6±0.6	45.1	45.2	44.8
0.25 mg/l	7.2±1.2	10.1±0.9	10.5±1.4	5.1±0.8	5.3±1.1	6.9±0.8	48.6	42.8	40.2
2 mg/l	6.6±0.9	9.9±1.2	12.2±3.1	4.9±1.2	6.8±0.8	8.4±2.4	46.9	39.6	40.3

Note, $n \geq 4$ replicates, data were expressed as mean ± SD and $P > 0.05$, ANOVA.

Effects of BPA on sex hormones of the clams, *Caelatura nilotica*

Testosterone and 17β-estradiol were detected in males' and females' gonads, respectively of the control and exposed clams after one and four weeks. The testosterone and 17β-estradiol concentrations in the control were 1.17 ± 0.22, 0.72 ± 0.22 ng/ml for one week, 2.03 ± 0.09, 0.11 ± 0.01 ng/ml for four weeks. Levels of testosterone decreased under the effect of 0.25 mg/l BPA exposure (1.3-fold) but increased after exposure to 2 mg/l BPA (1.1-fold), after one week ($P > 0.05$, ANOVA) when compared to the control mussels. However, the level of the same hormone decreased significantly with exposure and time (2.5 and 1.9 folds) for 0.25 and 2 mg/l, respectively after four weeks ($P = 0.001$, ANOVA) when compared to the control mussels.

17β-estradiol levels decreased after the exposure to 0.25 mg/l BPA (1.2-fold) but increased under the effect of 2 mg/l BPA (1.9-fold) after one week significantly when compared to the control mussels. Meanwhile, 17β-estradiol levels decreased significantly under the exposure (1.8 folds) after four weeks ($P \leq 0.05$, ANOVA, Table 2) when compared to the control mussels.

Table 2. Effects of BPA on concentrations of testosterone and 17β-estradiol of control and exposed clams to BPA for four weeks

Time	Treatments (mg/l)	Testosterone	17β-estradiol
One week	**Control**	1.17±0.22	0.72±0.22
	0.25	1.03±0.09	0.62±0.22*
	2	1.24±0.08	1.34±0.23**
Four weeks	**Control**	2.03±0.09	0.11±0.01
	0.25	0.8±0.11*	0.06±0.01*
	2	1.05±0.07**	0.08±0.01**

Note, $n \geq 4$ replicates and data were expressed as mean ± SD (ng/ml). * donates significant difference between the control and exposed groups, ** donates significant difference between exposed groups when $P \leq 0.05$, ANOVA.

Effects of BPA on gills topography of the clam, *Caelatura nilotica*

In the control freshwater clam, *C. nilotica*, gill plate composed of two gill lamellae, separated dorsally by a well-developed marginal groove. Each gill lamellae composed of numerous gill filaments closed to each other. Gill filaments were distally covered by three types of cilia. Gill filaments were uniformly aligned near to each other with similar distance apart. There was a ciliary connection between each adjacent gill filaments (Fig. 2a).

In the exposed clams (0.25 mg/l BPA), some gill filaments became separated apart from each other. Others lost their uniform alignment pattern "lamellar deformation" and became irregular in shape. Frontal cilia were eroded at some places and lateral cilia were eroded between the gill filaments to form foci. Latero-frontal cilia were shortened and became more clumped. Marginal groove diminished in size after exposure to BPA. In higher magnification of the ventral end of the gill lamellae showed depressions and/or necrotic foci (Figs. 2 and 3).

The exposed clams (2 mg/l BPA) showed more damage in the gill surface than in 0.25 mg/l exposed clams. Additional pathologies were observed such as complete disappearance of latero-frontal cilia and increased cirri of the filaments` free surface. The higher magnification of the ventral end of the gill lamellae showed scattered areas which were completely devoid of cilia (Figs. 2 and 3).

Effect of BPA on histological structure of gills of the clam, *Caelatura nilotica*

In the control clams, *C. nilotica* gills consist of two gill plates, which lie in the mantle cavity. Each gill plate is made up of a pair of similar gill lamellae joined together internally by inter-lamellar tissue junctions. The lamellae are formed of numerous reflected folds of gill filaments. Each filament is lined by a layer of columnar epithelia at its free end. Chitinous rods are located on both sides of each filament where it connects to the rest of the gill lamella to support the filaments and the branchial vein. Adjacent gill filaments are joined to each other by inter-filamentar tissue junctions, which consist

of narrow bands of connective tissue. Gill filaments of the anterior end of the gill lamellae are longer than the posterior end. Gill filaments have three types of cilia; frontal, latero-frontal and lateral cilia and cirri (Fig. 4).

Exposing *C. nilotica* clams to 0.25 mg/l BPA for one week induced marked changes in gills included the erosion of frontal and latero-frontal cilia of some gill filaments and necrosis in epithelia. More damage was recorded after four weeks as some of the gill filaments became completely blocked due to the enlargement of the chitinous rods (Figs. 4 & 5, Table 3).

C. nilotica clams exposed to 2 mg/l BPA for one week presented severe effects of the gills which appeared in the blockage of some gill filaments as a result of thickened chitinious rods, necrosis in the epithelia of the filaments and erosion of frontal and latero-frontal cilia. Moreover, fibrous tissue was extensively formed at the bases of some gill filaments, the connective tissue was damaged and the posterior gill filaments became thinner than the control clams. Exposure to 2 mg/l BPA for four weeks also induced similar histopathological changes in the gills but it was more severe than one week of exposure. Severity of alterations appeared in necrosis of the epithelial layer at some parts and increased thickness of epithelia at different filaments. There was necrosis in the inter-lamellar tissue junction, irregular folding in the filaments and inter-filamentar tissue junctions as showed in Figs. (4 & 5, Table 3).

Effect of BPA on histological structure of male and female gonads of the clam, *Caelatura nilotica*

Histological structure of the gonads was examined in the control and exposed clams. The gonad (males and females) is surrounded by gonads` wall, which is composed of a layer of cuboidal epithelia, followed by a layer of concretion, then a layer of muscles internally (length, 0.29 ± 0.03 and 0.28 ± 0.02 mm for males and females, respectively).

Male gonad is composed of a number of testicular follicles, each follicle contains various developmental stages of male gametogenic cells (primary, secondary spermatogonia and mature sperms). The follicles are held together by connective tissue (Figs. 6 & 8).

In *C. nilotica* clams exposed to 0.25 mg/l BPA for one week, gonads` wall cuboidal epithelia became detached from the concretion layer, which became more thinner after one week (length, 0.21 ± 0.09 mm) then thicker (length, 0.35 ± 0.17 mm) after four weeks compared to the control ones. In addition, the muscle layer of the gonads` wall was irregularly distributed. While after four weeks, the gonads` wall increased in thickness, especially the muscular layer but the concretion layer decreased in thickness. In the exposed clams to 2 mg/l BPA for one week, necrosis arose in the cuboidal epithelium of the gonads` wall; concretion layer had some empty spaces under the cuboidal layer. The thickness of the gonads wall shrunk to reach 0.22 ± 0.08 mm after one week then expanded to reach maximum thickness after 4 weeks (length, 0.38 ± 0.21 mm, Fig. 8, Table 3).

In the males of *C. nilotica* exposed to 0.25 mg/l BPA for one and four weeks, low density of mature sperms was noted comparable to other primary stages. Loss of some sperm tails was noted as dysgenesis. Exposure to BPA induced different sizes, irregular and bizarre shapes of some the testicular follicles. Clams exposed to 2 mg/l BPA

exposure provoked similar pathological signs in addition to the appearance of completely congested follicles with fibrous tissue and necrosis of the connective tissue holding the follicles in some areas (Fig. 6, Table 3).

The female gonad composed histologically of several ovarian follicles. Each follicle contains various developmental stages of gametogenic cells including primary, secondary oogonia and mature oocytes. These follicles are held together by connective tissue (Fig. 7).

In the females of *C. nilotica* clams exposed to 0.25 mg/l BPA for one and four weeks, there was low numbers of both the ovarian follicles and the oocytes occupied them. Mature oocytes showed irregular and bizarre shapes and sizes and some clams developed many fat droplets inside oocytes. In *C. nilotica* clams exposed to 2 mg/l BPA for one and four weeks, some ovarian follicles fused together to form one large and bizarre shaped follicle. Whereas, in 2 mg/l exposed clams, mature oocytes contained more and bigger sized fat droplets than in 0.25 mg/l BPA exposed clams. Also, necrotic foci in connective tissue and fibrous bundles appeared frequently among the follicles. Irregular shapes and sizes of oocytes beside fusion of mature oocytes were detected. The early developmental stages (oogonia) increased in number more than mature oocytes in the follicles (Fig. 7, Table 3).

Effect of BPA on histological structure of intestine of the clam, *Caelatura nilotica*

In the cross section of gonads, sections of the intestine were recorded passing through the gonads` tissue. It consists of heavily ciliated columnar epithelia, which rest on a basement membrane and externally surrounded by a layer of circular muscles (Fig. 8).

In *C. nilotica* clams exposed to 0.25 mg/l BPA, the intestine showed necrosis in the epithelia with changes in shapes of nuclei. Hyperplastic changes were also detected in the intestinal epithelia with erosion of cilia. Increase in the thickness of the muscle layer and decrease in the lumen size of the intestine was observed. In exposed *C. nilotica* to 2 mg/l BPA, distorted intestinal wall, inflammatory responses underlying the muscular layer, necrotic epithelia and erosion of cilia were the most common pathological alterations (Fig. 8 and Table 3).

Effect of BPA on ultrastructure of male and female gametes of the clam, *Caelatura nilotica*

The sperm of *C. nilotica* clams is composed of three parts. Firstly, the head region starts with a conspicuous acrosomal region that is conical in shape, and the oval nucleus which is surrounded by a layer of protoplasmic enzymes. Secondly, the mid piece has four - five mitochondria and thirdly, the flagellum elaborated from both the proximal and distal centrioles. The transverse section of the flagella showed the typical structure 9+2 micro-tubules, embedded in the matrix and surrounded with a plasma membrane (Fig. 9).

In the exposed clams to 0.25 mg/l BPA for one and four weeks, sperms exhibited some changes. The protoplasmic enzymes appeared lesser than in the control sperms; and mitochondria became larger in size. In the transverse sections of flagella, several membrane blebbing were noted. Also, sperms appeared irregular in shape and size especially with nuclear blebbing and mitochondria fused with each other or had projections. In the clams exposed to 2 mg/l BPA for one and four weeks, sperms showed more severe damage than 0.25 mg/l BPA exposed clams. Some sperms showed lesser dense chromatin of the nucleus, lesser amount of protoplasmic enzymes than the control

clams. Mitochondria had membrane blebbing and projections (malformation) or complete fusion of several mitochondria and loss of cristae (Fig. 9).

In the control clams, oocytes of *C. nilotica* looked variable in size. Mature oocytes consisted of the cytoplasm, where yolk granules, fat droplets, the nucleus and its nucleolus are incorporated. Outside the cytoplasm, a vitelline envelope surrounded the mature oocytes, followed by a perivitelline space then the oocyte membrane from inside out. Numerous microvilli were protruded from the oocyte membrane and the free ends of the microvilli were embedded in a gelatinous protective layer (Fig. 10).

The exposed clams to 0.25 mg/l BPA for one and four weeks showed more yolk granules and lipid droplets in the cytoplasm of the oocytes than the controls. The oocytes membrane became highly irregular, the vitelline envelope disappeared, and number of microvilli dramatically decreased in number and size and the gelatinous coating around the oocytes increased in thickness. In addition, binucleated oocytes was noted and some nuclei with little amount or no chromatin was observed (Fig. 10). In exposed clams to 2 mg/l BPA for four weeks, the effects were almost similar but more severe than in 0.25 mg/l BPA exposed clams. These pathologies were represented in more irregularity of the oocyte membrane, much thinner and eroded microvilli of the oocyte membrane surface, more and bigger yolk granules than control, and chromatin free nucleus appeared in some oocytes (Fig. 10).

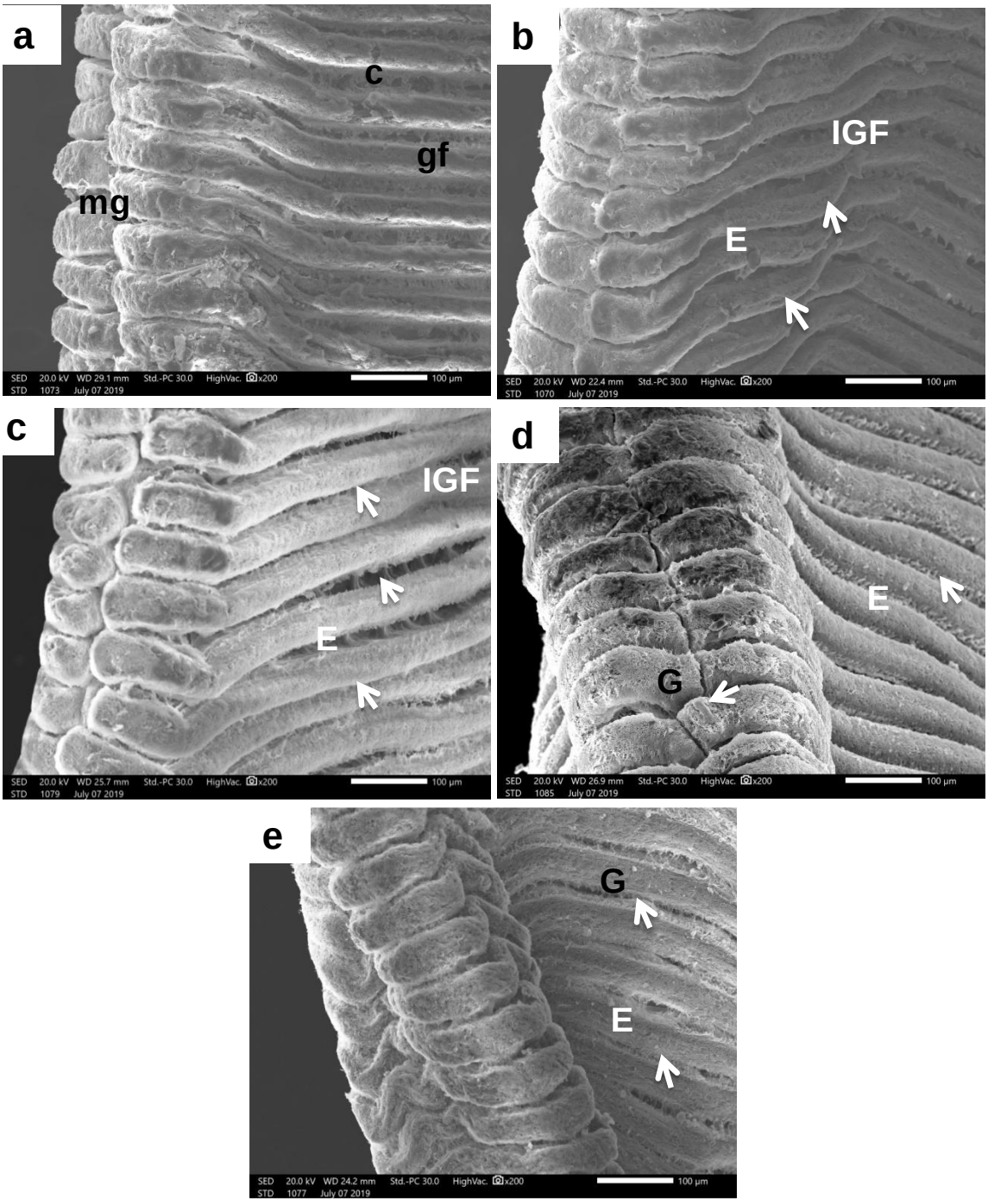

Fig. 2. Scanning electron micrographs of *C. nilotica* gills, showing the free end of gill lamellae edges, lateral aspect. **(a)** control clams, **(b & c)** 0.25 mg/l exposed clams and **(d & e)** 2 mg/l exposed clams after one and four weeks. MG, marginal groove, GF, Gill Filament; c, cilia; IGF, irregular gill filaments; E, erosion, G, gap.

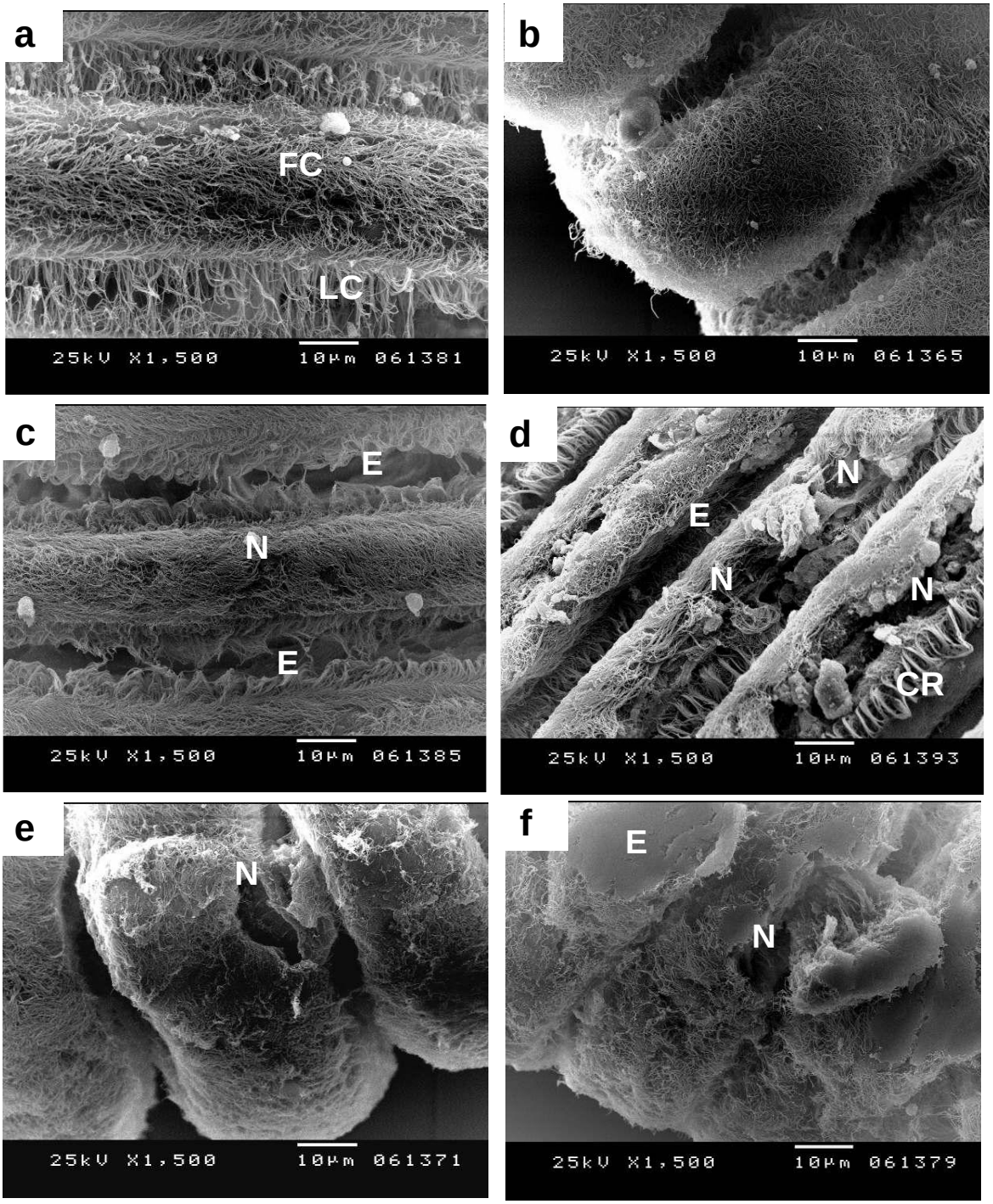

Fig. 3. Scanning electron micrographs of *C. nilotica* gills showing frontal and abfrontal aspects. **(a & b)** control clams, (**c & e**) 0.25 mg/l exposed clams and (**d & f**) 2 mg/l exposed clams. Fc, frontal cilia; LC, lateral cilia; CR, cirri, N, necrosis; E, erosion.

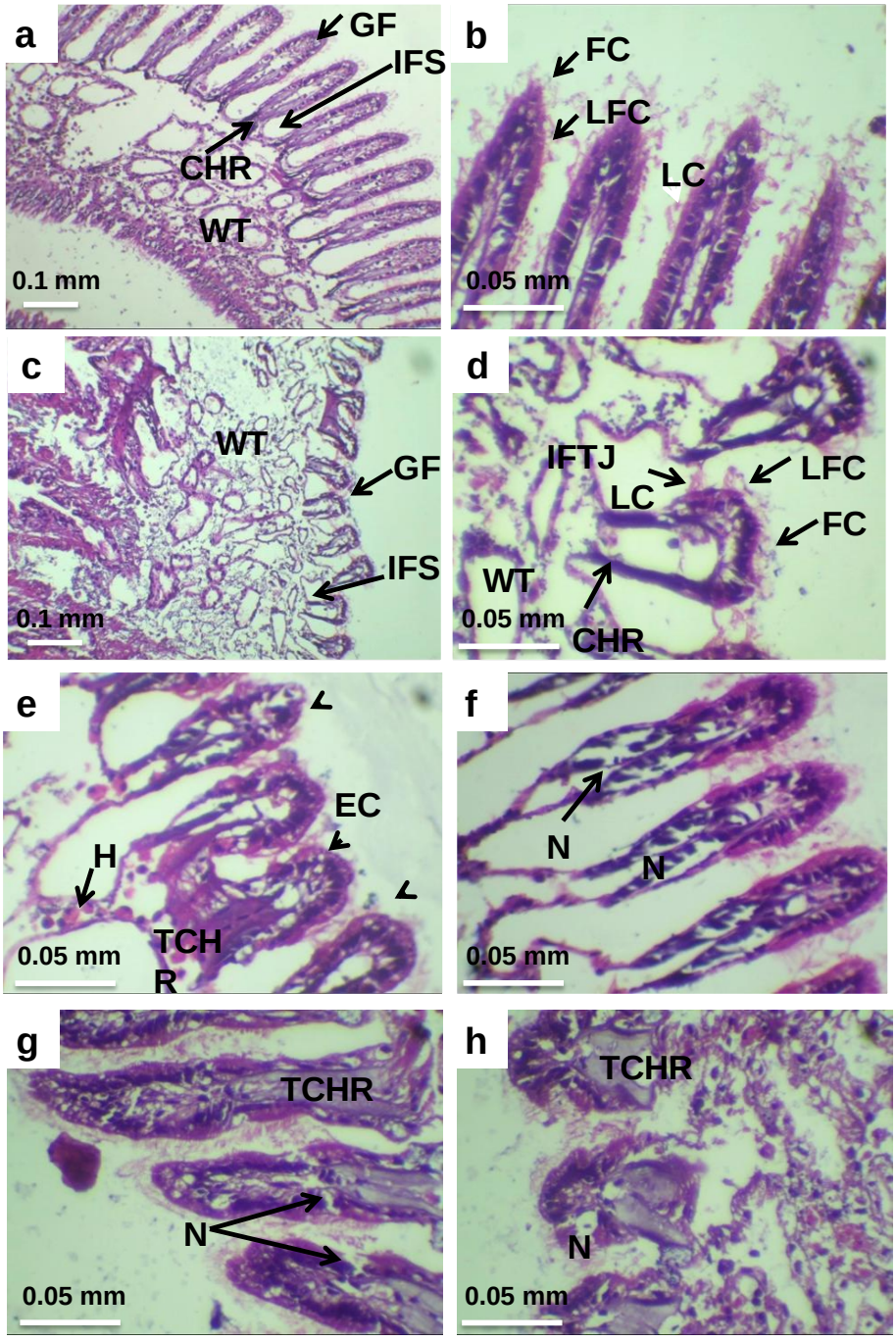

Fig. 4: Light micrographs through the gills of *C. nilotica*, stained with H & E after one week. (**a-d**) Control clams; (**e & f**) 0.25 mg/l BPA exposed clams; (**g & h**) 2 mg/l BPA exposed clams after one week. GF, gill fillament; IFS, inter-filamentar space; CHR: chitinious rod; WT, water tube; FC, frontal cilia; LFC, latero-frontal cilia; LC, lateral cilia; IFTJ: Inter- filamentar tissue junction; H, haemocyte; TCHR, thickened chitinious rod; EC: eroded cilia (arrows heads), N, necrosis.

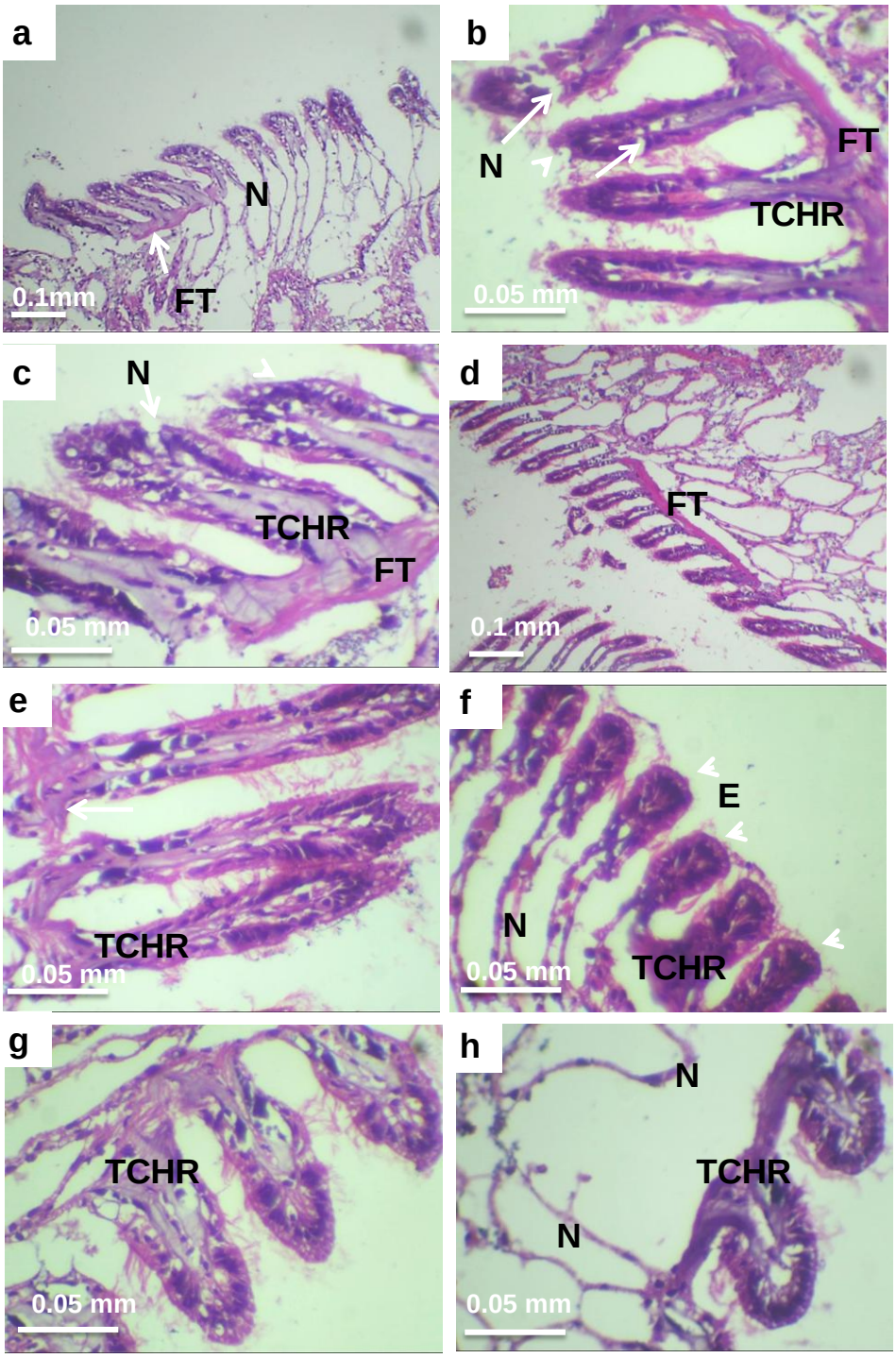

Fig. 5. Light micrographs through the gills of *C. nilotica***,** stained with H & E after four weeks showing (**a, b, c & d**) 0.25 mg/l exposed clams; (**e, f, g & h**) 2 mg mg/l exposed clams with thickened chitinious rod (TCHR); eroded cilia (E)**,** necrosis (N) and FT, fibrous tissue.

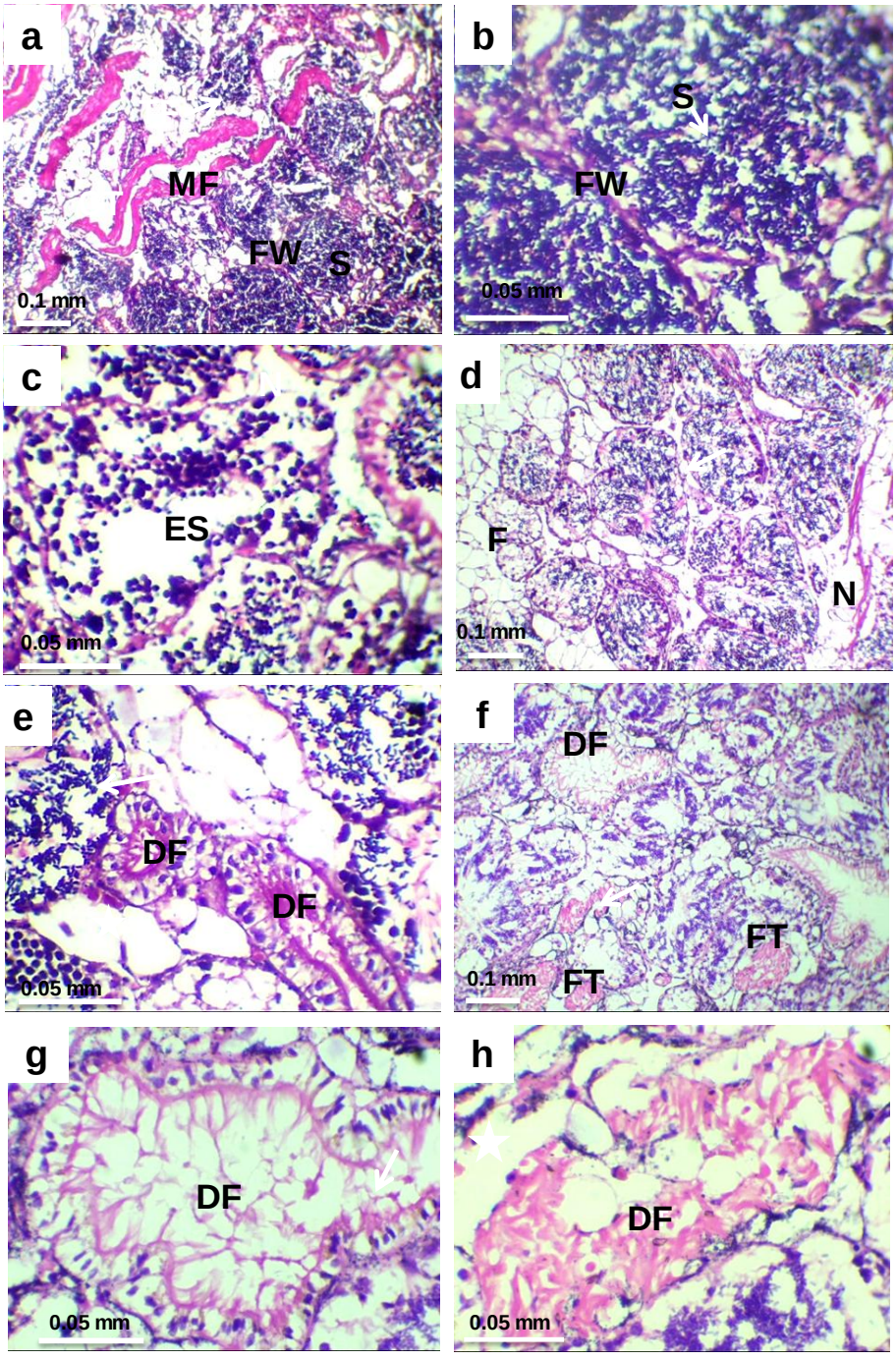

Fig. 6. Light micrographs through male gonads of *C. nilotica* stained with H & E. **(a & b)** control clams; **(c)** one week, **(d & e)** four weeks 0.25 mg/l BPA exposed clams; **(f)** one week, **(g & h)** four weeks 2 mg/l BPA exposed clams. TF, testicular follicle; MF, muscle fibres, CT, connective tissue; FW, follicular wall, S, sperms; ES, empty space; DF, deformed follicle; F, fusion; N, necrosis; FT, fibrous tissue.

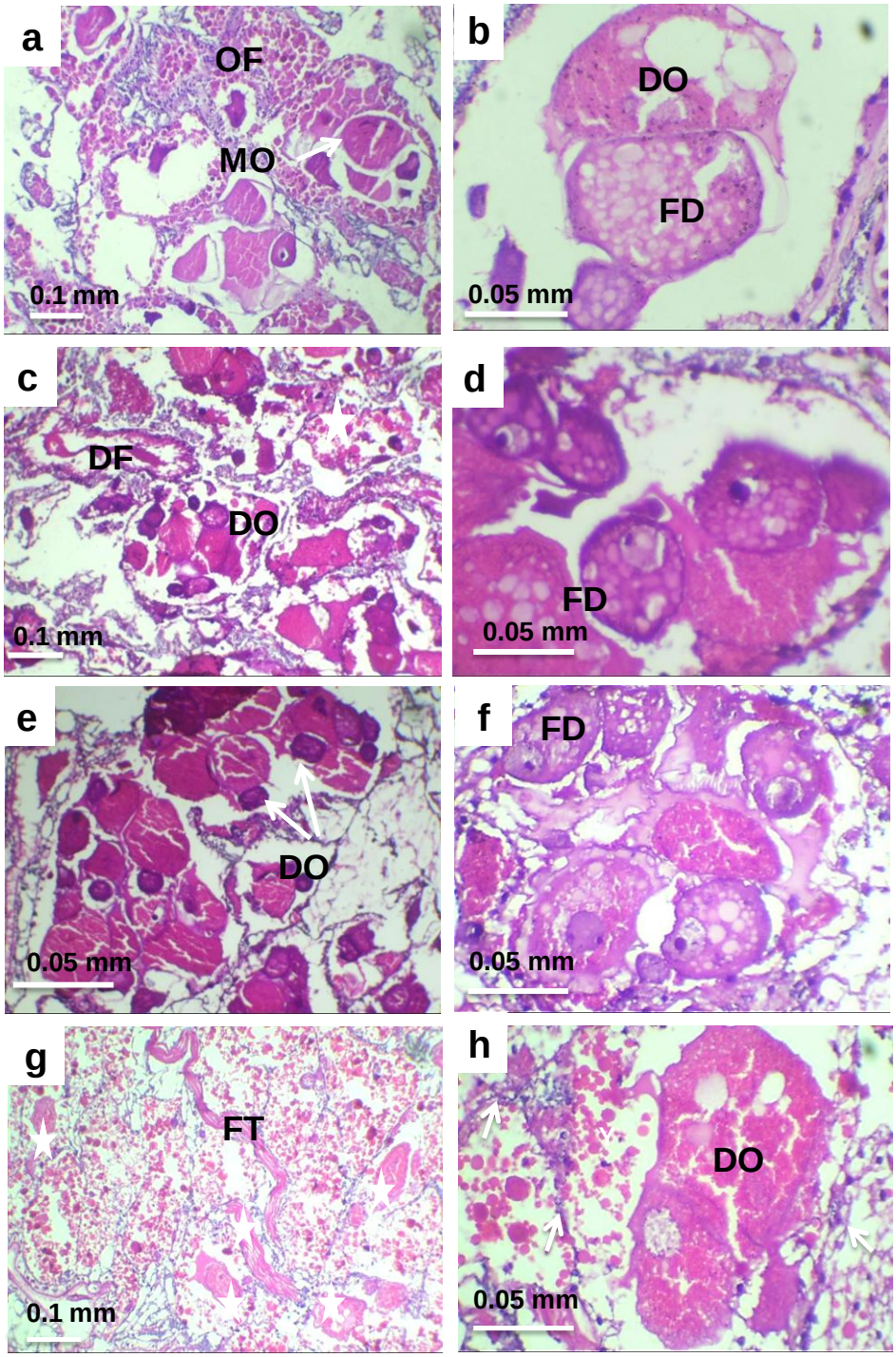

Fig. 7. Light micrographs through female gonads of *C. nilotica* stained with H & E. (**a**) Control clams; (**b)** one week, **(c & d**) four weeks 0.25 mg/l BPA exposed clams; (**e & f**) one week, **(g & h**) four weeks 2 mg/l BPA exposed clams. OF, ovarian follicle; MO, mature oocyte; FD, fat droplets; DO, deformed oocytes; DF, deformed follicle; FT, fibrous tissue; Y, yolk granules.

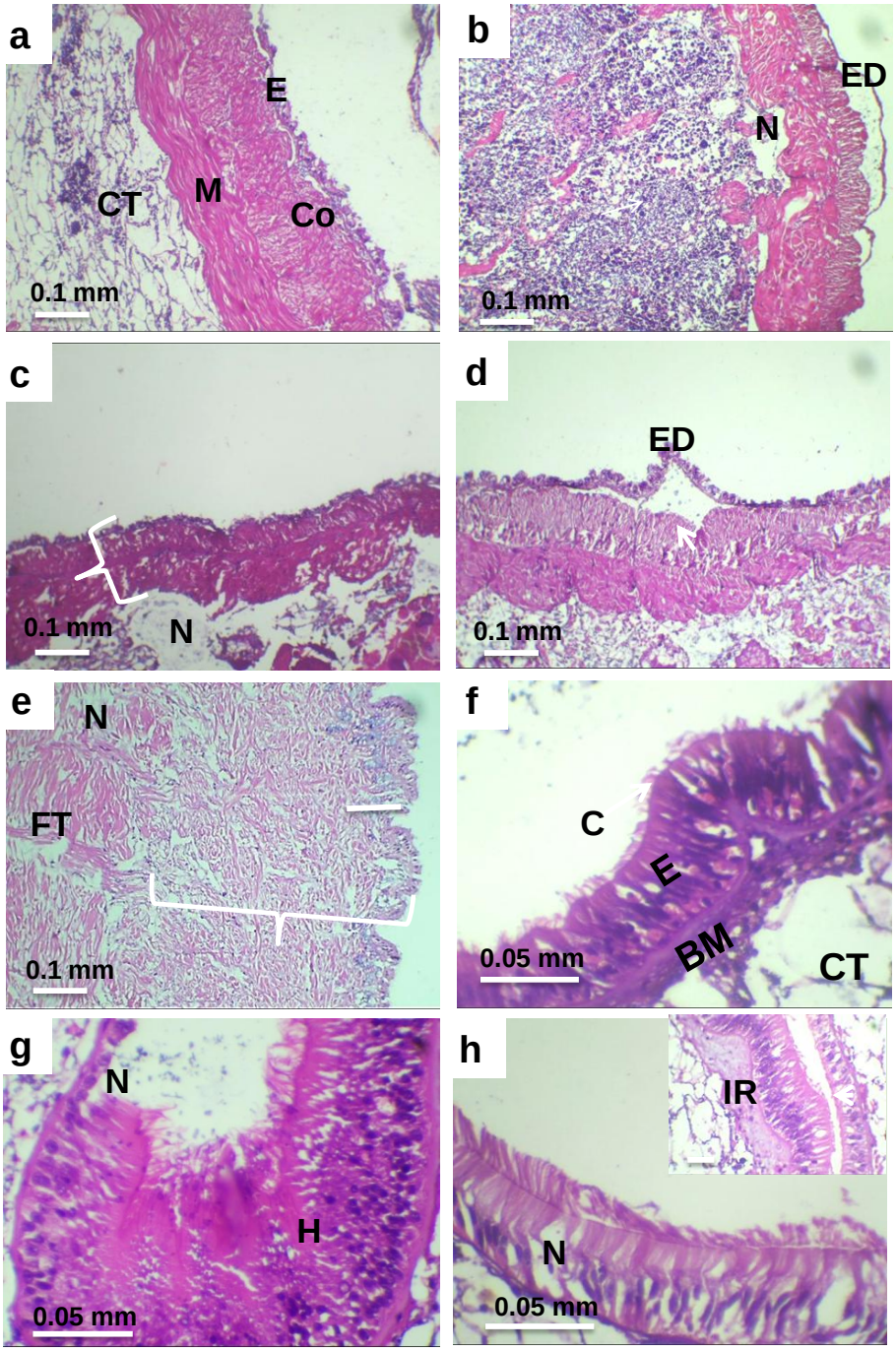

Fig. 8. Light micrographs through gonads wall and intestine of *C. nilotica* stained with H & E. (**a & f**) Control clams, (**b**) one week, (**c**) four weeks 0.25 mg/l BPA exposed clams, (**d**) one week, (**e**) four weeks 2 mg/l BPA exposed clams` gonads wall. **(g & h**) 0.25 and 2 mg/l BPA exposed clams after four weeks. E, epithelia; Co, concretion; M, muscles; CT, connective tissue; C, cilia; BM, basement membrane; FT, fibrous tissue; ED, epithelial detachment; N, necrosis; H, hyperplasia; IR, inflammatory response; brackets, thickness.

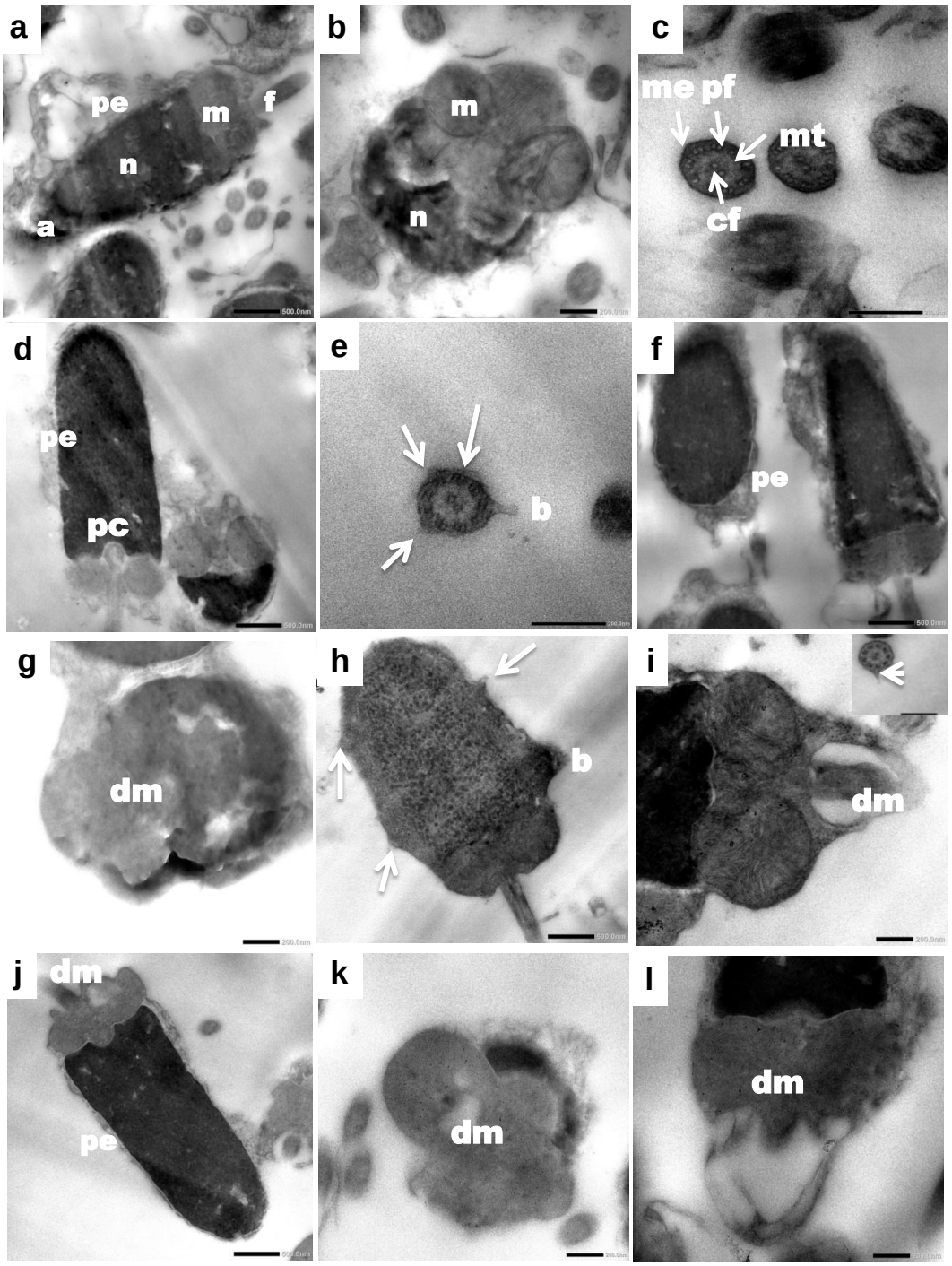

Fig. 9. Transmission electron micrographs of ***C. nilotica*** male gonads after one and four weeks. (**a, b & c**) Control clams; (**d, e, f, g, h & i**) 0.25 mg/l BPA exposed clams; (**j, k & l**) 2 mg/l exposed clams. pe, protoplasmic enzymes; n, nucleus; m, mitochondria; f, flagellum; ct, central tubules; pf, peripheral microtubules; cf, central microtubules; mt, matrix; me, membrane; pc, proximal centriole; b, blebbing (arrows); dm, deformed mitochondria; and stars, abnormalities.

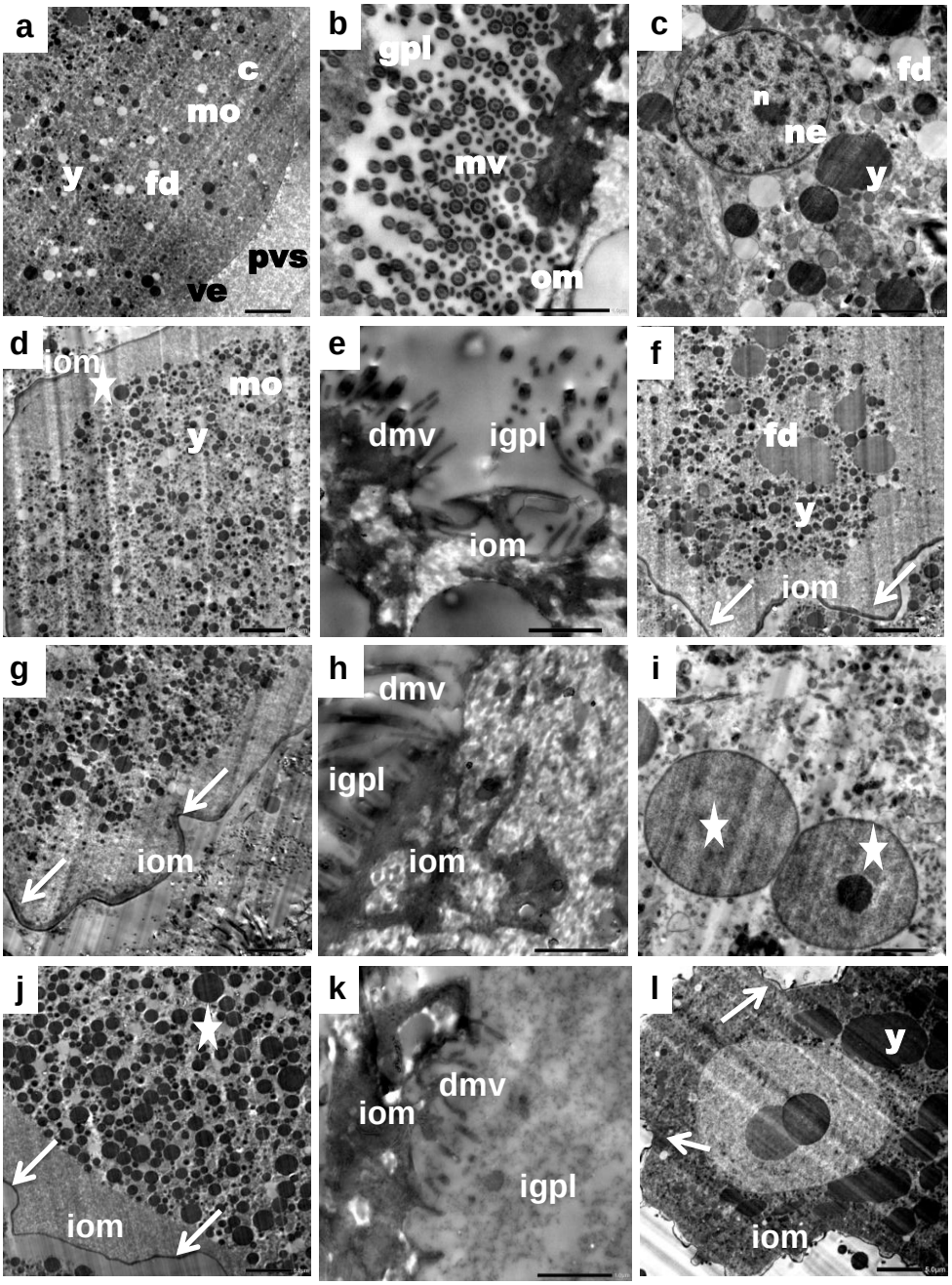

Fig. 10. Transmission electron micrographs of ***C. nilotica*** female gonads after one and four weeks. (**a, b & c**) control clams; (**d, e, f, g, h & i**) 0.25 mg/l BPA exposed clams; (**j, k & l**) 2 mg/l exposed clams. c, cytoplasm; y, yolk; mo, mature oocyte; fd, fat droplets; ve, vetilline envelope, pvs, prevetilline space; om, oocyte membrane; mv, microvilli, gpl, gelatinous protection layer; n, nucleus; ne, nucleolus; iom, irregular oocyte membrane (arrows); igpl, increased gelatinous protection layer; dmv, deformed microvilli and stars, defragmented chromatin.

Table 3. Semi-quantitative analysis of the histological alterations in gills and gonads (males and females) of the freshwater clam, *C. nilotica* after exposure to BPA for four weeks

Organ	Treatment	Necrosis	Hyperplasia	Fibrosis	Deformations	Haemocytes infiltration	Lipogenesis	Epithelial detachment	Cilia erosion
Gills	**Control**	-	-	-	-	+	-	-	-
	0.25 mg/l	+++	+	+	+++	++	-	-	+++
	2 mg/l	++++	-	++	++++	+	-	-	++++
Males' gonads	**Control**	-	-	-	-	-	-	-	-
	0.25 mg/l	-	-	+	+++	-	-	-	-
	2 mg/l	+++	-	++++	+++	-	-	-	-
Females' gonads	**Control**	+	-	-	-	-	+	-	-
	0.25 mg/l	++	-	-	+++	-	+++	-	-
	2 mg/l	++++	-	++	++++	-	++++	-	-
Gonads` wall	**Control**	-	-	-	-	-	-	-	-
	0.25 mg/l	++	+	-	++	-	-	++	-
	2 mg/l	+	+++	-	++++	-	-	+	-
Intestine	**Control**	-	-	-	-	-	-	-	-
	0.25 mg/l	+++	+	-	-	++	-	-	++
	2 mg/l	+++	-	-	-	++	-	-	+++

Note, $n \geq 5$ replicates for control and exposed clams. A scale was used as 0-4, where - represent no alterations and ++++ was the highest record of alterations.

DISCUSSION

The present study recorded the responses of the freshwater clam, *C. nilotica* under the effect of one of the most cosmopolitan environmental pollutant and hormonal disruptor, Bisphenol A. Hormonal disruption was coinciding with gonads` histopathology. In addition, gill topography abnormalities approved the toxicity of Bisphenol A with other organs other than gonads, which documented its effect on the clams` respiration and reproduction abilities also the total weight.

In the present study, total weight, shell and flesh weights of the exposed clams were increased (not significant) more than the control ones`. Similarly, Gust *et al*., (2014) recorded growth in the caged mud nails, *Potamopyrgus antipodarum* juveniles but not

significant between the sites contaminated with pharmaceutical contaminants. BPA and other hormonal disruptors were reported to cause weight gain and obesity for long period of exposure (Song *et al*., 2014). Estradiol exposure accelerated glycogenolysis and lipogenesis in the Pacific oyster, *Crassostrea gigas* (Mori *et al*., 1972). Also, 17β-Estradiol exposure caused increase in calcium concentrations and changes in the phosphorylation of signal transducers and transcription activators in *Mytilus* haemocytes as reported by Canesi *et al*. (2004). That may lead to modulation and changes in Ca concentrations/distribution in the shell or cause necrosis in the tissues as in the current data. However, condition index of exposed clams to BPA decreased with treatment and time of exposure. Sohoni *et al*. (2001) found decrease in somatic index of adult male fishes exposed to BPA (for 164 days). The authors explained this decrease because BPA has an inhibitory effect on somatic growth.

Exposure to different concentrations of BPA during this study resulted in decrease of testosterone concentrations. Bai and Acharya (2019) exposed quagga mussels, *Dreissena bugensis* to BPA for six weeks and found that testosterone was too low to be detectable in the mussels. The authors explained that testosterone might be released back to the water or esterified with fatty acids to stabilize the exogenous steroid inside its bodies. They rendered the decrease in testosterone levels to some factors, such as the activity of esterases or the availability of cofactors (i.e., fatty acids and acyl-CoA), that might regulate the equilibrium between free and esterified steroids. Andersen *et al*. (2001) explained that some hormonal disruptors (flutamide as antiandrogenic), can increase the metabolism of testosterone; i.e. androgenic hormone and compete with it for its receptors and decrease dramatically as in copepods. Besides, one steroid may bind to more than one sex steroid receptor of the gland. Consequently, this competitive binding could lead to that testosterone may be converted into estradiol by aromatase enzyme (Janer *et al*., 2005).

In the current study, 17β-estradiol concentrations significantly decreased under both treatment and time of exposure. These results were in harmony with Bai and Acharya (2019) who reported that naturally occurring estrogens were not detected in the mussel, *Dreissena bugensis* after 42 days of exposure to BPA. Therefore, 17β-estradiol either did not accumulate in the mussel or was esterified and stabilized in the tissue into a more stable form that was resistant to metabolism. Similarly, Singletone *et al*. (2006) interpreted the effect of BPA on estrogen as it disrupts the natural endocrine signaling through regulation of estrogen receptors target genes because it acts as an antagonist to them. So, it can down-regulate the expression of the natural estrogens and decrease their production. In addition, the effects of the steroids can be blocked by analogues (i.e. xenoestrogens (BPA)), known to act as receptor antagonists in the sea scallop, *Placopecten magellanicus* (Wang and Croll, 2003). In addition, Zhang *et al*. (2014) demonstrated that BPA down-regulated 17 b-hydroxysteroid dehydrogenases (17b-HSDs), which control metabolism of steroids and fatty acids in *Mytilus galloprovincialis* digestive gland. BPA also increased the expression of estrogen receptor (ER) and estrogen-related receptor (ERR) genes in the reproductive tissue of the gastropod snail, *Marisa cornuarietis* at high concentrations ($>10^{-5}$ M, Bannister *et al*., 2013).

In the present study, gills of *C. nilotica* clams showed different signs of deformations like necrosis and fibrosis, which varies in intensity under the effect of BPA exposure. These data agreed with Leonard *et al*. (2014) who reported that necrosis could

happen because BPA slows the metabolic processes of the cells, causing them to die as in the unionid mussel, *Lampsilis fasciola*. Another notable change caused to the gills of exposed groups was the appearance of fibrous tissue. Dublinowska *et al*., (2016) reported some dysfunctional gonads of the blue mussels, *Mytilus edulis* that contain fibrous tissue as a result of pollution effects. In the current work, irregular folds of gill filaments were detected. Kumar *et al*. (2012) reported irregularity in gill filaments of the freshwater bivalve, *Lamellidens marginalis*, treated with dimethoate (organophosphate insecticide, i.e. xenoestrogen) for 96 hrs. They rendered these changes in the filaments to serve as a barrier to prevent further entry of the contaminant material to damage other internal organs. Erosion or clumped cilia and the appearance of depressions on surface of some gill filaments were recorded after exposure to BPA in the present data. Similar gill atrophy was recorded by Shirdel and Kalbassi (2016) in the gills of the fish, *Salmo trutta caspius*, which exposed up to 100 µg/l of nonylphenol. Damage signs included curling and clubbing epithelial lifting of the lamella, shortening of the lamella, mucus cell hypertrophy and lamellar hyperplasia. The current pathological signs could be a result of direct toxicity of BPA on gills as what happened in vivo exposure of *Mytilus* sp. haemocytes to BPA which induced rapid changes in lysosomal membrane stability, and decreased the phosphorylation of CREB-like transcription factor (cAMP-responsive element binding protein) as discussed by Canesi *et al*. (2004)

During this study, exposure of clams to BPA caused reduction in sperms occupying the testicular follicles. Similarly, Oehlmann *et al*. (2000) found that exposure to BPA for 3 months resulted in reduction of sperm numbers of the male snails, *Potamopyrgus antipodarum* and *Nucella lapillus*. They concluded that BPA might initiate sexual arrest at the end of the breeding seasons. Another notable effect in the testis of the present exposed clams to BPA was the loss of sperm tails. This result was in accordance with Hatef *et al*. (2012), who reported adverse effects on sperm motility and velocity of the goldfish, *Carassius auratus L*. after BPA exposure. Several effects including spermatogenesis inhibition and reducing reproductive success have previously been reported in fishes treated with octylphenol or 17b-estradiol (Gronen *et al*., 1999; Kinnberg *et al*., 2003). They attributed these effects to inhibition of mitotic divisions of the spermatogonia as a result of the exposures to endocrine disrupting chemicals.

Blebbing of Sperm nucleus and flagella, besides mitochondrial abnormalities were detected in the present study. Anahara *et al*. (2006) reported change in cortactin (cellular protein) expression by the treatment of the exogenous hormonal disruptor (BPA). It was responsible for shaping the acrosome and the nucleus of the spermatozoans and its disruption caused their malformation. In addition, the damage effects of estradiol on the tissues due to the increase in nitric oxide release have been demonstrated in marine mussel, *Mytilus edulis* (Stefano *et al*., 2003). In addition, Chen *et al*. (2015) found that exposure to 0.228 µg/l BPA for continuous two generations of zebrafish resulted in decreased sperm density and motility. They mentioned that continuous exposure to low BPA levels reduced sperm density and motility through ROS (reactive oxygen species) production by the dysfunctional mitochondria of the spermatozoans. BPA can also cause disturbances of the microtubule organization that control flagella organization in vitro (Pfeiffer *et al*., 1997). In addition, BPA can induce morphological changes of the cells like cytoskeletal collapsing, membrane blebbing, and chromatin condensation or fragmentation, which are the morphological characterization of apoptosis (Lida *et al*.,

2003). Nakagawa and Tayama (2000) explained the toxicity of BPA on mitochondria as it inhibits oxygen consumption and affect respiration by interfering with NAD+-linked substrates (pyruvate plus malate) and/or with the FAD-linked substrate.

Histological observations in female gonads of exposed clams to BPA showed irregular shapes and sizes of the ovarian follicles with reduced number of mature oocytes. These results were parallel to Aarab *et al*. (2006), who found reduced size of the ovarian follicles and follicles were devoid of oocytes following exposure of *Mytilus edulis* mussels to BPA for 3 weeks. In the present study, some of the mature oocytes contained more yolk granules than the control clams. This was in accordance with Boulangé-Lecomte *et al*. (2017), who stated that levels of the egg yolk precursor protein vitellogenin increased gradually as females of the copepod, *Eurytemora affinis* mature and have been broadly accepted as a biomarker for endocrine disruption. Increased numbers of the early developmental stages of female oogonia (primary and secondary) more than mature ones was a sign of BPA exposure in the current work. Sohoni *et al*. (2001) reported that exposure to BPA slowed gonadal development and decreased ovulation in the female fathead minnows, *Pimephales promelas*. More lipid droplets were observed in the mature oocytes of exposed clams to BPA in the present study. Mori *et al*. (1972) recorded stimulation in lipidogenesis after estradiol exposure of the female Pacific oyster, *Crassostrea gigas*. They explained that it was due to regulation of some enzymes such as malate dehydrogenase and glucose-6-phosphate dehydrogenase. Moreover, a significant increase in expression of oestrogen receptor and oestrogen-related receptor genes was detected in the freshwater pulmonate, *Physa acuta* after exposure to BPA (5-96 hours, Morlase *et al*., 2018).

The intestinal epithelia showed hyperplastic response to the exposure of BPA, in the current study. Hyperplasia seemed to be a sign of exposure to BPA, in the Indian major carp, *Catla catla* gill mucous cells (Faheem *et al*., 2016). The authors explained this response as a defence mechanism of the animal against pollution. In addition, hyperplasia is a cell proliferation response to the increase in estrogen concentrations (exogenous estrogen-like compound, as in the current case). The muscular wall of the gonads showed changes in the thickness and integrity of muscle fibres with inflammation in the muscles around the intestine according to the concentration and time of exposure to BPA. Benjamin *et al*. (2016) reported similar pathological signs in the adductor muscles of the freshwater bivalve, *Corbicula fluminea* under the effect of BPA exposure. Osada and Nomura, (1990) detected stimulation of the prostaglandins synthesis in the Japanese scallop, *P. yessoensis*, when exposed to estradiol as a protective strategy against cells injury and inflammation.

Ultra-structurally, oocytes malformations in the lipid and yolk inclusions, vitelline layer and microvilli of exposed clams were detected in the present work. Estradiol exposure induced accumulation of vitellin in the gonads of the scallop, *P. yessoensis* (Osada *et al*., 2004). Estradiol exposure in the Pacific oyster, *C. gigas* accelerated glycogenolysis and lipidogenesis (Mori *et al*., 1972). Larsen *et al*. (2006) recorded significant increase in the concentrations of vitellogenin in the serum of male Atlantic cod, *Gadus morhua* after exposure to BPA. In addition, estrogen is a crucial component for oocytes` microvilli development and its decrease (see Table 2) can arrest/inhibit microvilli generation. Consequently, oocytes with less or short microvilli will be deprived from nutrients as discussed by Zachos *et al*. (2004).

CONCLUSION

Bisphenol A exposure affected clams' biometrics, impaired male and female reproductive organs by interfering with hormones and respiration by altering cell structure. This will compromise the animals` biological activities especially reproductive abilities and consequently affect freshwater resources.

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Arabic aummary

التاثيرات الحادة و المزمنة للبيسفينول أ علي الاضطرابات الهرمونية والتغيرات النسيجية في محار المياه العذبة ,(Cailliaud, 1827) *Caelatura nilotica*

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لوحظ ان تسجيل اضطرابات الغدد الصماء في التجارب المعملية وتأثيراتها على اللافقاريات اصبح نادرًا. لهذا تم تصميم هذه الدراسة للتحقيق في الآثار الخطرة للتركيزات المختلفة (0.25 و 2 ملجرام / لتر) من ثنائي الفينول أ (BPA) على محار المياه العذبة ، *Caelatura nilotica* لمدة 4 أسابيع. تم دراسة القياسات الحيوية والهرمونات الجنسية والتركيب النسيجي الشكل الخارجي للخياشيم والتركيب الدقيق للغدد التناسلية كأدوات أساسية للتقييم. وسجلت الدراسة زيادة في طول الصدفة ووزنها ووزن اللحم متوازيا مع التعرض والوقت. ومع ذلك ، انخفض مؤشر الحالة، التستوستيرون و 17β-estradiol بشكل ملحوظ توازيا مع التركيز والوقت ($P \leq 0.05$، ANOVA). كشف الفحص النسيجي عن علامات مرضية مثل النخر والالتهابات وتكون الأنسجة الليفية وزيادة و تضخم في الخلايا وتراكم قطرات الدهون في الخياشيم والغدد التناسلية والأمعاء. كما أظهر فحص المجهر الإلكتروني الماسح تآكل الأهداب وعدم انتظام وتكوين بؤر في الخياشيم. بالإضافة إلى ذلك ، كشف فحص المجهر الإلكتروني النافذ عن انخفاض في الإنزيمات البروتوبلازمية المحيطة بالحيوان المنوي وعلامات الموت المبرمج للخلايا في الاسواط وتحلل في الميتوكوندريا و الحيوانات المنوية. أيضا ، تم الكشف عن زيادة مح البيض ، قطرات الدهون ، الطبقة الجيلاتينية وانخفاض عدد microvilli في البويضات. لذا ، فإن BPA لا يسبب اضطرابًا في الهرمونات والأعضاء الجنسية فحسب، بل أيضًا سبب تغيرات بيومترية وتشوهات في الخياشيم في المحار محل الدراسة مما قد يتداخل مع تواجد و بيولوجية هذا الحيوان والمحافظة على البيئة المائية.