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Original Article

Effects of penconazole and copper nanoparticle fungicides on redbelly tilapia, *Coptodon zillii* (Gervais, 1848): Reproductive outcomes

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Abstract: Several effective fungicides have recently been applied, but they can harm ecosystems and non-target organisms. The findings of this study should be helpful to evaluate the reproductive response of redbelly tilapia, *Coptodon zillii*, of both sexes upon exposure to 0.8 and 1.6 μg/L penconazole and 7.5 and 15 mg/L copper nanoparticle as fungicides for three months. The gonadosomatic index of males increased in the penconazole groups in parallel with testosterone. A significant increase was observed in estradiol and progesterone of penconazole and copper nanoparticle groups. In testicular homogenate, there was a significant decrease in superoxide radical in penconazole (I) and penconazole (II) groups and CAT of penconazole (I) and copper nanoparticle (I) groups, along with a significant increase in nitric oxide (NO) of Cu-NP (II) group. In ovarian homogenate, a significant increase in NO of penconazole (I) group and lipid peroxides of copper nanoparticle (I) group, along with decrease in CAT of penconazole (II) and copper nanoparticle (I) groups and SOD of penconazole (II) and copper nanoparticle (I) groups were found. The histopathological examination indicated gross deteriorations in the gonads of fish exposed to the fungicides except in the copper nanoparticle (II) group. These findings suggested the reproductive burden in *C. zillii* following exposure to the investigated fungicides by disrupting gonadal sex hormones and inducing redox imbalance and cytopathological abnormalities. It is recommended to reduce the flow of these materials to aquatic areas particularly the natural fisheries and artificial hatcheries.

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Introduction

Penconazole (PEN) is a commonly used triazole fungicide for foliar pathogen control in the horticultural, agricultural, and forest industries, but it is poisonous to a variety of aquatic organisms, including fishes (Kenyon et al., 1997; Icoglu et al., 2018). Its environmental persistence, bioaccumulation through the food chain, and resistance to degradation draw much attention to its health risk as an aquatic toxicant (Güdücü et al., 2011; Cabizza et al., 2012). Triazole fungicide intoxication affects not only challenge fish's reproductive ability during their entire life cycle but also their progenies, implying that it has implications for the health of fish populations on a large scale (Liao et al., 2014; Liu et al., 2014; Dong et al., 2018). Penconazole, along with other members of the triazole family, has a negative effect on fish reproductive biology on several levels, including spermatogenesis, estrogen/androgen balance, reproductive performance, sexual differentiation, apoptosis, and testicular and ovarian histoarchitecture, in addition to their ill effects on human health (Li et al., 2012; Shen et al., 2013; Liao et al., 2014; Bhat et al., 2015; Chu et al., 2016; Dong et al., 2018). These outcomes are most likely caused by disruption of the hypothalamic-pituitary-gonadal (HPG) axis, transcript levels of steroidogenic enzymes and androgen and estrogen receptors, redox homeostatic status, and cytohistological features (Skolness et al., 2013; Dong et al., 2018).

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Nanoparticles (NPs), on the other hand, are used in the fishery and aquaculture industries for a variety of direct and indirect purposes. Direct applications include the feeding industry and animal welfare, such as fish disease control, while indirect applications include water and wastewater treatment, fishpond sterilisation, and commercial fish packaging (Khosravi-Katuli et al., 2017). The use of nanoparticles (NPs) in biomedical sciences and engineering has gradually expanded over the last decade (Kaida, 2004; Wang et al., 2007). Despite its many applications, NPs toxicity has drawn much attention from scientists due to its unique properties, such as a large surface-to-volume ratio and high reactivity toward chemical and biological molecules (Wani et al., 2011). Cu-NPs and soluble $CuSO_4$ have reproductive effects in *Clarias batrachus in vivo* and *in vitro*, even at low concentrations (Murugananthkumar et al., 2016). On the other hand, female mice and rats showed oxidative stress and apoptosis-mediated ovarian dysfunction, as well as a disturbance in sexual hormonal balance (Yang et al., 2017; Zhang et al., 2018). However, it is still unknown how Cu-NP exposure affects fish female reproductive performance, especially when complex interrelationships of neuro-hormonal circuits are involved.

The tilapia fish's ability to spread across African waters appears to be due in large part to their diet flexibility and ability to withstand harsh aquaculture conditions (Lowe-McConnell, 2000). In addition, their high fecundity, combined with their rapid growth rate, allows them to breed and establish in areas outside of their native range (Martin et al., 2010). The purpose of this research was to find out more about the effects of PEN and Cu-NP on *C. zillii* reproductive function in both sexes.

Materials and Methods

Determination of 96 hrs lethal concentration (LC_{50}) of PEN: The LC_{50} of PEN was determined according to Turner (1965). The pre-acclimatized *C. zillii* specimens were held in six glass aquaria (25 L capacity), each containing six fish. Dead fish were recorded every 12 h and removed. The acute toxicity test was conducted according to Turner (1965). Each group of fish was subjected to daily renewed single doses of PEN at 10, 12, 14, 16, 18, and 20 µg/L. The LC_{50} for 96 hrs exposure was calculated according to:

$$LC_{50} = \text{Largest dose} - \sum \frac{A \times B}{N}$$

Where A is the dose difference between two successive doses, B is the mean of dead fish between two successive doses, and N is the total number of fish per group. Fish were exposed to 1/10 LC_{50} (1.6 µg/L) and 1/20 LC_{50} (0.8 µg/L) of PEN for three months. The LC_{50} of Cu-NPs was 150 mg/L (1/10 LC_{50} = 15 mg/L and 1/20 LC_{50} = 7.5 mg/L) (Abdel-Khalek et al., 2015) (Suplemntary 1).

Fish maintenance: In 160 L rectangular tanks containing dechlorinated tap water, 180 adults *C. zillii* of both sexes with average body weights of 30±5 g and average lengths of 14±4 cm were preserved. The water parameters were maintained as conductivity = 2000 µs/cm; pH ≈ 7.5; oxygen saturation = 90-95%; temperature = 25°C; and photoperiod = 12:12 light: dark. The fish were fed 3% of their body weight in commercial feed pellets (40% crude protein, 4.22% fat, 5.88% crude fiber, 10.30% ash, and 10.03% moisture) twice a day for 21 days, and the water was changed every two days.

Experimental groups and sampling: Fish were randomly assigned to five groups with three replicates of 12 fish each at the end of the acclimatization period. The control group received no treatment, the PEN (I) group received 0.8 µg/L PEN, the PEN (II) group received 1.6 µg/L PEN, the Cu-NP (I) group received 7.5 mg/L Cu-NP, and the Cu-NP (II) group received 15 mg/L Cu-NP for three months. Air stones connected to an air compressor were used to aerate the tanks. The fish were independently sampled after three months of exposure. The gonad weights, body weights (BW), and standard lengths (SL) were all measured. Five males and five females from each group were gathered at the end of the experiment and anaesthetised with 0.02% benzocaine solution. Blood

samples were taken from the caudal vein based on Blazer et al. (2000), then centrifuged at 300 rpm for 10 min, and the obtained serum was stored at -20°C until analysis of testosterone, estrogen (E2) and progesterone (P4). Testes and ovaries were dissected out and preserved for the later histological examination. To prepare 10% w/v homogenates of testis and ovary tissues were homogenized in phosphate buffer (pH 7.4) using a homogenizer (IKA Yellow line DI 18 Disperser, Germany). The homogenates were centrifuged at 8.000 rpm for 15 minutes, and the supernatants were kept frozen at -20°C for the subsequent biochemical assays.

Reproductive studies: The percentages of condition factor K % (Cren, 1951), gonadosomatic index GSI% (Solomon; Ramnarine, 2007), fecundity (Sturm, 1978), and hatchability (Ali, 2001) were computed as follows: K % = (W/L3) ×100

GSI % = (Gonad weight / Total body weight) ×100

Fecundity = (Average number of ripe eggs × Ovary weight)/ Sample weight

Hatchability % = (Number of hatched eggs / Total number of eggs) ×100

Estimating gonadal sex hormones: T enzyme-linked immunosorbent assay kits (catalogue number: 11-TESHU-E01, ALPCO, Salem, NH, USA) were used to measure testosterone (T) according to the manufacturer's instructions. The concentrations were determined using a standard curve built from calibrator concentrations and optical densities. E2 and progesterone (P4) (catalogue numbers BC-1111 and BC-1113, respectively) were also measured using E2 enzyme immunoassay test kits from BioCheck in Foster City, California, following the manufacturer's instructions.

Estimation of oxidant/antioxidant parameters: Catalase (CAT) was evaluated according to Aebi (1984), superoxide dismutase (SOD) was determined according to (Misra; Fridovich, 1972) Lipid peroxides (LPO) were estimated according to Ohkawa et al. (1979). Nitric oxide (NO) was measured as nitrite concentration using the method of Ding et al. (1988). Superoxide radical ($O_2^{\cdot-}$) was measured according to Podczasy and Wei (1988).

Histopathological examination: After the experiment, testes and ovaries were dissected and fixed in 10% neutral formalin. The specimens were then washed in 70% ethyl alcohol three times (24 h each time). Serial sections of 6-8 µm thickness were prepared according to normal histological techniques for staining with the hematoxylin and eosin method (Levison, 1997). The sections were examined by an Olympus BX51 microscope and the photographs were taken by an Olympus DP72 camera adapted onto the microscope.

Statistical analysis: The statistics package for social sciences SPSS (V 21) program was used to analyze the results. To find the significant difference between means, the Duncan post-test was chosen from the Post Hoc window. The significance of probability values between 0.05 and 0.01 (both included) was determined.

Results

Effects on the reproductive performance: There were no significant changes in K among all experimental groups (Table 1). The condition factor was reduced in the treated groups compared to the control one. Fish exposed to Cu-NP (II) showed no obvious change compared to the control. Regarding PEN I, PEN II, and Cu-NP (I) treatments, they did not show any significant difference. In males, the GSI of PEN (I) and PEN (II) groups were significantly higher than that of the control group. However, there was no significant change in the GSI of Cu-NP (I) and Cu-NP (II) groups compared to the control group. The GSI of Cu-NP (I) and Cu-NP (II) groups were significantly lower than that of PEN (I) group, while the GSI of PEN (II) group was significantly higher than that of the Cu-NP (I) group. The comparisons between the GSI of PEN (I) group and that of PEN (II) group, the GSI of PEN (II) group and that of Cu-NP (II) group, and the GSI of Cu-NP (I) group and that of Cu-NP (II) group did not reveal a significant difference. There were no significant differences in the GSI, fecundity, and hatchability percentage of female *C. zillii* among all the experimental groups.

Table 1. Effects of penconazole and copper nanoparticles on the condition factor percentage (K %), gonadosomatic index percentage (GSI %), fecundity, and hatchability percentage of *Coptodon zillii*.

Parameter		Group					
		Control	0.8 µg/L PEN	1.6 µg/L PEN	7.5 mg/L Cu-NP (I)	15 mg/L Cu-NP (II)	*P*-value
K %		1.88±0.11	1.48±0.01	1.66±0.046	1.55±0.008	1.89±0.23	0.068
GSI %	Male	0.325±0.10^{c}	0.76±0.07^{ab}	0.41±0.084^{c}	0.53±0.04^{bc}	0.53±0.047^{bc}	0.000
	Female	1.52±0.45	3.634±0.39	2.73±0.121	3.17±0.92	3.17±0.92	0.431
Fecundity		1171±498	817.6±92.9	991.3±238.5	520.1±61.9	944±182	0.533
Hatchability %		98.23±0.64	72.30±6.86	71.76±5.84	85.11±10.45	81.94±4.06	0.161

$^{a-c}$ letters indicate significant differences at $P<0.05$ (one-way ANOVA followed by Duncan post-test).

Table 2. Effects of PEN and Cu-NPs on serum sex hormone levels.

Parameter	Group					
	Control	0.8µg/L PEN (I)	1.6µg/L PEN (II)	7.5mg/L Cu-NP (I)	15mg/L Cu-NP (II)	*P*-value
T level (µg/L)	14.12±2.82^{c}	30.32±3.77^{b}	56.67±8.17^{a}	29.90±4.43^{b}	13.8±2.5^{c}	0.000
E2 level (ng/L)	66.4±5.2^{c}	44.5±6.9^{c}	857.8±40.8^{a}	94.2±3.8^{c}	220.55±58.23^{b}	0.000
P4 level (µg/L)	0.240±0.075^{c}	0.03±0.009^{c}	1.12±0.160^{a}	0.180±0.037^{c}	0.540±0.051^{b}	0.000

$^{a-c}$ Different letters indicate significant differences at $P<0.05$ (one-way ANOVA followed by Duncan post-test).

Effects of the different treatments on the serum levels of the gonadal sex hormones: There were significant increases in the serum testosterone levels (Table 2) of PEN (I), PEN (II), and Cu-NP (I) groups compared with that of the control group. The serum testosterone levels of PEN (I) and Cu-NP (I) groups were significantly lower than those of the PEN (II) group. On the other hand, the serum testosterone level of Cu-NP (II) group did not significantly change compared to that of the control group. Compared to the control group, E2 levels in PEN (II) and Cu-NP (II) groups significantly increased, while those in PEN (I) and Cu-NP (I) groups exhibited insignificant differences. E2 level in PEN (II) group was significantly higher than that in Cu-NP (II) group. There was an insignificant difference in E2 level between PEN (I) and Cu-NP (I) groups. E2 levels in PEN (I) and Cu-NP (I) groups were significantly lower than those in PEN (II) and Cu-NP (II) groups.

Regarding P4, PEN (II) and Cu-NP (II) groups exhibited a significant increase in their levels compared to the control group. In contrast, a comparison between P4 level in PEN (I) and Cu-NP (I) groups and that in the control group showed no significant difference. P4 level in PEN (II) group was significantly higher than that in Cu-NP (II) group. P4 levels in PEN (I), Cu-NP (I) groups were significantly lower than those in PEN (II) and Cu-NP (II) groups. P4 level in PEN (II) and Cu-NP (II) groups exhibited a significant elevation compared to the control one.

Effects on the gonadal oxidant/antioxidant balance: Table 3 shows the changes in the redox homeostatic parameters in both testis and ovary of *C. zillii* exposed to either PEN or Cu-NP. Concerning the oxidant /antioxidant parameters in the testis, O_2^- levels of PEN (I) and PEN (II) groups were significantly lower than those of the control group. However, no significant difference in O_2^- levels were found between the Cu-NP (I) and Cu-NP (II) groups (and the control group). Cu-NP (I) and Cu-NP (II) groups had slightly higher O_2^- levels than the PEN (II) group but were not substantially different from the PEN (I) group. There was no significant difference in O_2^- levels between the PEN (I), Cu-NP (I), and Cu-NP (II) groups. When compared to the control group, the NO level of the Cu-NP (II) group was significantly higher, whereas the other experimental groups showed no significant difference. There were no significant changes in LPO levels and SOD activities among the experimental groups. Compared with the control

Table 3. Effects of PEN and Cu-NPs on the gonadal oxidant/antioxidant balance.

Treatment / Parameters	Control	0.8µg/L PEN (I)	1.6µg/L PEN (II)	7.5mg/L Cu-NP (I)	15 mg/L Cu-NP (II)	*P-value*
Testis						
O_2^- level (nmol/mg protein)	0.07±0.01^{a}	0.05±0.003^{bc}	0.034±0.01^{c}	0.061±0.003^{ab}	0.069±0.001^{ab}	*0.007*
NO level (nmol/mg protein)	2.37±0.30^{bc}	2.78±0.17^{ab}	1.923±0.42^{c}	2.59±0.063^{abc}	3.17±0.234^{a}	*0.036*
LPO level (nmol/mg protein)	0.11±0.030	0.074±0.019	0.139± 0.095	0.116±0.033	0.073±0.032	*0.801*
CAT activity (U/mg protein)	0.06±0.01^{a}	0.038±0.003^{b}	0.02±0.005^{b}	0.077±0.010^{a}	0.084±0.010^{a}	*0.002*
SOD activity (U/mg protein)	3.58±0.69	4.301±1.160	2.380±0.013	5.028±0.58	5.574±0.889	*0.134*
Ovary						
O_2^- level (nmol/mg protein)	0.36±0.05	0.24±0.074	0.31± 0.0322	0.411±0.0336	0.290±0.023	*0.159*
NO level (nmol/mg protein)	2.58±0.08^{b}	5.442±1.450^{a}	2.24±0.22^{b}	2.55±0.265^{b}	2.943±0.824^{b}	*0.027*
LPO level (nmol/mg protein)	0.47±0.06^{bc}	0.418±0.072^{c}	0.59±0.07^{bc}	0.733±0.005^{a}	0.657±0.097^{ab}	*0.044*
CAT activity (U/mg protein)	0.05±0.01^{a}	0.04±0.021^{ab}	0.02±0.003^{bc}	0.021±0.001^{c}	0.050±0.003^{a}	*0.028*
SOD activity (U/mg protein)	4.64±0.60^{a}	5.287±0.678^{a}	2.49±0.35^{b}	2.245±0.001^{b}	5.42±1.10^{a}	*0.015*

group, the CAT activity of PEN (I) and PEN (II) groups significantly increased, and that of Cu-NP (I) and Cu-NP (II) groups showed no significant difference. A significant reduction in CAT activity was observed following exposure to PEN at doses of 0.8 and 1.6 µg/L compared with exposure to copper Nano formulated fungicide at 7.5 and 15 mg/L. No significant differences were found when comparing the CAT activity of PEN (I) group with that of PEN (II) group, and Cu-NP (I) group with that of the Cu-NP (II) group. Concerning the oxidant/antioxidant parameters in the ovary, the comparison between the O_2^- levels of all experimental groups revealed a lack of significant difference.

NO level in *C. zillii* exposed to PEN at a dose of 0.8 µg/L was significantly higher than the other experimental groups. The comparison among NO levels in Cu-NP (I), Cu-NP (II) and PEN (II) groups, and also between these groups one side and the control one on the other side revealed the absence of significant difference. LPO levels of Cu-NP (I) and Cu-NP (II) groups were significantly higher than that of the control group. LPO level of the PEN (I) group was significantly lower than that of Cu-NP (I) group, while the LPO level of the PEN (II) group was significantly lower than that of Cu-NP (II) group. There was no significant difference in LPO level between either PEN (I) or PEN (II) groups and the control group. The comparison between LPO levels of PEN (I) and PEN (II) and between Cu-NP (I) and Cu-NP (II) showed the absence of significant difference. Compared to the control group, the CAT activities in PEN (II) and Cu-NP (I) groups showed significant decreases, while those in PEN (I) and Cu-NP (II) exhibited insignificant differences. A significant decrease in CAT activity was observed in Cu-NP (I) group when compared with Cu-NP (II) group, while a comparison between CAT activity of PEN (I) group and that of PEN (II) group revealed the insignificant difference. CAT activity of PEN (I) group was significantly higher than that of Cu-NP (I) group while, CAT activity of PEN (II) group was significantly lower than that of Cu-NP (II) group. SOD activity was significantly decreased in Cu-NP (I) and PEN (II) groups compared to the control group. There was no significant difference in SOD activity of PEN (I) and Cu-NP (II) groups compared to the control group. SOD activity of PEN (I) group was significantly higher than that of PEN (II) group while, SOD activity of Cu-NP (I) group was significantly lower than that of Cu-NP (II) group. A significant increase in SOD activity of PEN (I) group was found when compared with that of Cu-NP (I) group while, a significant decrease in SOD activity of PEN (II) group was found when compared with that of Cu-NP (II) group.

Effects of different treatments on the histopathological feature of gonads: The histopathological alterations in the testes of the various experimental groups are shown in Figure 1A-E. The histoarchitecture of the control group's testes was normal, with capsules, seminiferous tubules, and

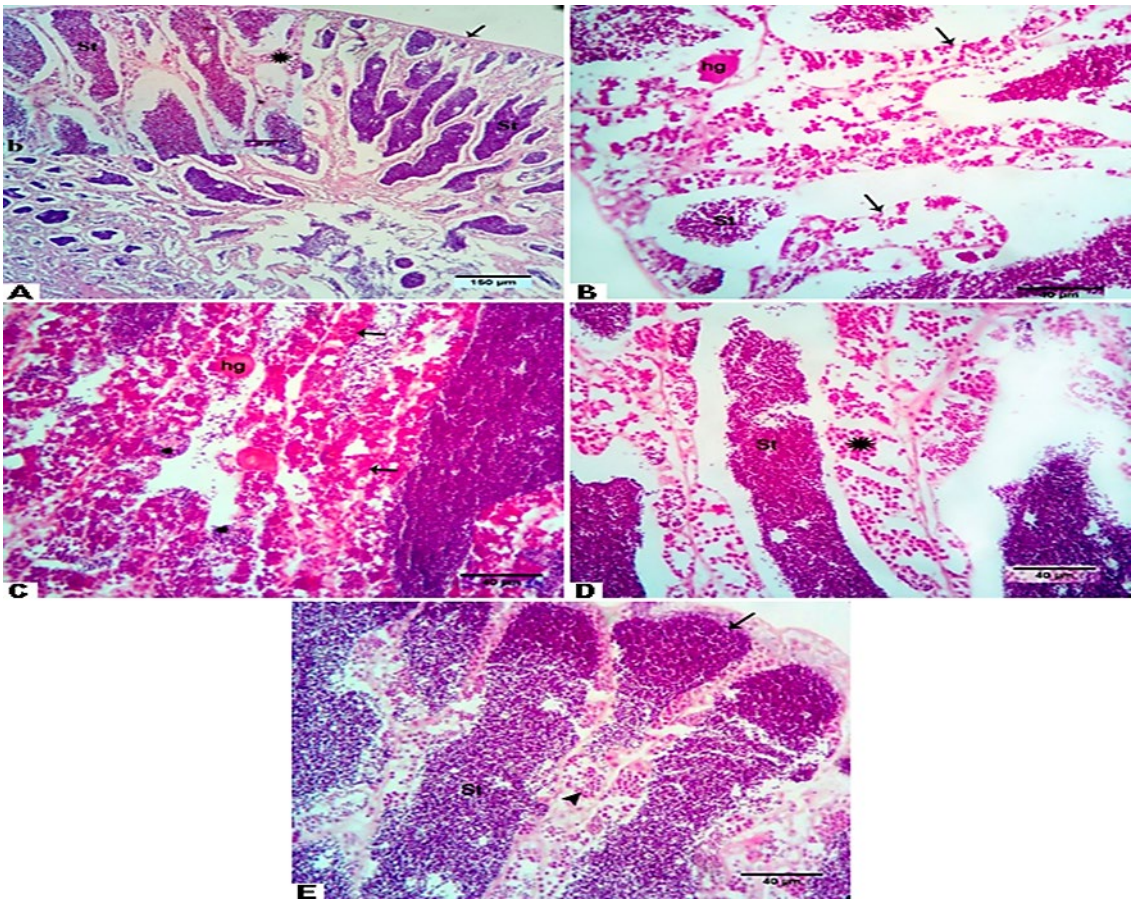

Figure 1. Photomicrographs of histological changes in testes of *Coptodon zillii* fish (H&E stain) exposed to different concentrations of penconazole and copper nanoparticles. A: Testes of control group showing normal histological features of testis with normal seminiferous tubules, normal capsule (arrow), normal spermatozoa (St), and normal spermatogonia and spermatocytes (star): a: bar = 150 mμ, b: bar = 40 mμ. B: Testes of PEN (I) group showing gross degenerative changes in the seminiferous tubules (arrow), decrease in the number of spermatozoa (St), and hemorrhage (hg): bar = 40 mμ. C: Testes of PEN (II) group showing congestion in the seminiferous tubules (arrow), hemorrhage (gh), and leucocyte infiltration (star): bar = 40 mμ. D: Testes of Cu-NP (I) group showing normal spermatozoa (St), spermatogonia and spermatocytes (star), and decrease in the number of spermatozoa and increase in the number of spermatocytes and spermatogonia relative to the control. Bar = 40 mμ. E: Testes of Cu-NP (II) group showing normal spermatozoa (St), spermatogonia and spermatocytes (arrowhead) with leucocytes infiltration at the margins (arrow): bar = 40 mμ.

germ cells (Fig. 1A). In the testes of the PEN (I), however, gross degenerative changes in the seminiferous tubules, a decrease in the number of spermatozoa, and hemorrhage were discovered (Fig. 1B). In the experiments of the PEN (II) group, signs of inflammatory responses appeared in the form of congestion in the seminiferous tubules, hemorrhage, and leukocyte infiltration (Fig. 1C). The testes of the Cu-NP (I) group displayed normal spermatozoa, spermatogonia, and spermatocytes; at the same time, there was a marked decrease in the number of spermatozoa relative to spermatocytes and spermatogonia (Fig. 1D). Slight histopathological changes were observed in the Cu-NP (II) testes group, including leukocyte infiltration at the margins while spermatozoa, spermatogonia, and spermatocytes maintained their normal appearance (Fig. 1E).

The histopathological changes in the ovary of the various experimental groups are shown in Figure 2A. The structure in the control group was found to be normal. The vitellogenic oocyte/vitelline membrane appeared normal and intact to the outside, with easily distinguishable zona radiata and follicular epithelium. The size of the follicles increased proportionally at each stage of development. The follicular layers were not fully formed in the primary stage, but they were visible. The follicles were smaller in diameter at this point in their growth. The ooplasm was filled with

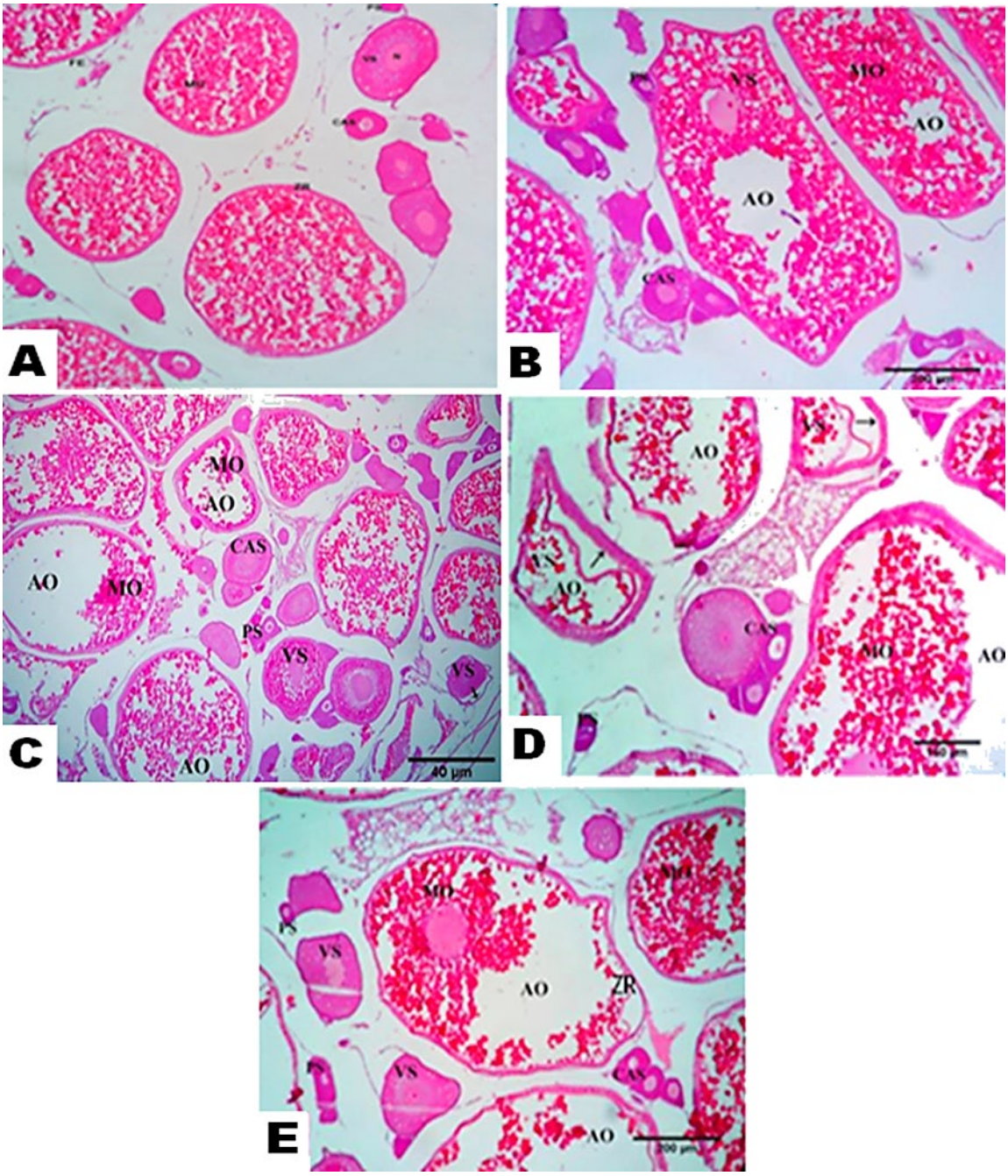

Figure 1. Photomicrographs of histological changes in ovary of *Coptodon zillii* fish (H.E stain) exposed to different concentrations of penconazole and copper nanoparticles. A: Ovary of control group showing; normal primary stage (PS), cortical alveoli stage (CAS), vitellogenic stage (VS) and mature oocyte (MO), follicular epithelium (FE), zona radiate (ZR), nucleolus (NO) and nucleus (N), bar = 150 µm. B: Ovary of PEN (I) group showing; decreased primary stage (PS) and cortical alveoli stage (CAS), atretic vitellogenic and mature oocyte stages (AO), bar= 200 µm. C: Ovary of PEN (II) group showing; degeneration (atretic) of all mature oocyte stage (AO) with membrane detachment of vitellogenic stage (arrow), bar = 40 µm. D: Ovary of Cu-NP (I) group showing; Normal primary stage (PS) and cortical alveoli stage (CAS), atretic vitellogenic and mature oocyte stages (AO) with membrane detachment (arrow), bar= 150 µm. E: Ovary of Cu-NP (II) group showing normal primary stage (PS) and cortical alveoli stage (CAS), atretic vitellogenic and mature oocyte stages (AO) with membrane detachment with mild degenerative changes and slight vitellogenic droplets (arrow), degenerated zona radiate (ZR), bar= 200 µm.

granular structures called cortical alveoli during the cortical alveolus stage. At this stage, the zona radiata began to form, and follicular epithelial cells appeared to form on the exterior. The next stage of development was the vitellogenic stage, and vitellogenesis was observed in the developing follicles. At the final stage of oocyte development, i.e., the mature stage, marked vitellogenesis was observed in the matured oocyte.

After being exposed to various concentrations of PEN and Cu-NP, structural changes in the ovaries were observed. Microscopic examination found mild ovarian changes after three months of Cu-NP exposure. The ovaries of the PEN (I) group (Fig. 2B) showed a decrease in the primary and cortical alveoli stages, as well as elongated ovarian follicles in the atretic vitellogenic and mature oocyte stages. Degeneration in (atretic) of all mature oocyte stages was observed in the ovaries of the PEN (II) group (Figure 2C), along with membrane detachment of the vitellogenic stage. The Cu-NP (I) group's ovaries (Fig. 2D) revealed zona radiata detachment from the oocyte membrane in vitellogenic and mature oocytes. This condition, however, was not seen in the primary or cortical alveoli stages. Similarly, oocytes in the primary and cortical alveolar stages appeared normal in the Cu-NP (II) group's ovaries (Fig. 2E).

Discussions

According to the results, exposure to PEN and Cu-NP caused an increase in testosterone production, indicating a possible disruption in the HPG axis. Although testosterone levels in the PEN and Cu-NP groups were higher, there were obvious negative changes in the spermatogenesis process, which could decrease fertility. Adaptive changes in metabolic pathways to cope with contaminant stress were caused by the PEN and Cu-NP challenges, as were organ and dose-specific changes in the oxidant/antioxidant profile to reduce oxidative stress. The biochemical measurements are supported by histopathological alterations in the testicular and ovarian histoarchitecture, which indicate a disruption in the cellular microenvironment and functionality. These findings should be useful in evaluating the potential ecological risk of fungicides, particularly in linking changes in endocrine function indicators to negative consequences in the entire organism. The GSI significantly increased following exposure of males to PEN at doses of 0.8 and 1.6 μg/L in the current study, similar to *Pimephales promelas* exposed to propiconazole (PCZ) and *Danio rerio* exposed to CLT (Baudiffier et al., 2013; Skolness et al., 2013). Histomorphometric analysis of the testes challenged with other members of triazole fungicide showed enlargement of the interstitial space, duplication of Leydig cell mass, and distension of seminiferous tubules. The observed increase in GSI under the effect of PEN might be due to the stimulation of the HPG axis leading to hypertrophy of the testicular tissues. The current findings of GSI disagreed with the observations of other dangerous materials such as DDT and chlorpyrifos (Mlambo et al., 2009). Gonadosomatic index is a sensitive marker, and its decrease indicates decreased hypothalamic, pituitary, or gonadal activity (Kime, 1999; Rahman et al., 2020). In this study, the significant increase in testosterone levels in the PEN (I), PEN (II), and Cu-NP (I) groups corresponds to previous reports on the effects of other triazole family members and soluble and NP forms of copper on other fish species (Wu et al., 2014; Murugananthkumar et al., 2016). The significant increase in E2 level in the PEN (II) group compared to the baseline level is similar to that seen in zebra fish (*Danio rerio*) after exposure to difenoconazole (Dong et al., 2018).

Cu-NP stimulated basic hormonal functions in *Coptodon zillii* exposed to 15 mg/L Cu-NP, as confirmed by an *in vitro* study on cultured porcine ovarian granulosa cells, suggesting the stimulatory effects of Cu-NP on their basic hormonal functions, taking into account differences in response in relation to Cu-NP shape, which play a role in modifying its activity and selectivity (Sirotkin et al., 2020). Compensatory responses include up-regulation of steroidogenic enzymes, transcription factors, Sertoli cell genes androgen receptor, and testicular expression of the follicle-stimulating hormone gene, as well as the proliferation of gonadal tissues, may be responsible for the current results. These adaptive mechanisms may be important in overcoming PEN and Cu-NP gonadotoxicity and also in preventing gonadal sex hormone overproduction. Nonetheless, despite a significant increase in testosterone levels in the PEN and Cu-NP groups. The apparent disruption in the spermatogenesis process based on the histopathological section of the current study should

be kept in mind.

The significant decreases in CAT activity in the testis of PEN (I) and PEN (II) groups in the present work are in harmony with that reported in rainbow trout (*Oncorhynchus mykiss*) exposed to sublethal concentrations of PCZ for 20 and 30 days (Li et al., 2010b) and in goodeid fish (*Chapalichthys pardalis*) exposed to Ag-NP for 21 days (Valerio-Garcia et al., 2017). The decrease in CAT activity may be interpreted as a sign that the redox-sensitive condition improves. However, this conclusion will not be cutting-edge until other enzymatic and non-enzymatic antioxidants are estimated, reflecting the logic behind the recommendation that total antioxidant capacity analysis is the most relevant investigation for evaluating oxidant/ antioxidant capacity, rather than the simple sum of observable antioxidants, takes into account the cumulative synergistic action of all antioxidants present in the sample and provides an integrated parameter (Ghiselli et al., 2000).

The reduction in testicular O_2^- levels in the PEN (I) and PEN (II) groups compared to the control group may represent an improvement in the antioxidant defensive mechanism; it is not necessary to look at the levels of studied antioxidants because the testis is armed with a wide range of enzymatic and non-enzymatic antioxidants. Triazole fungicides cause low-intensity oxidative stress, which causes a compensatory increase in the antioxidant potential to counteract the negative effects of ROS (Husak et al., 2017). They also cause low-intensity oxidative stress, which causes a compensatory increase in the antioxidant potential to counteract the negative effects of ROS. Increased NO levels in Cu-NP (II) testicular homogenate compared to control and PEN (II) groups, as well as PEN (I) ovarian homogenate compared to other groups, could be attributed to increased inducible NO synthase (Majewski et al., 2020). Through its interaction with the superoxide radical to create highly reactive peroxynitrite, NO is a potent mediator of cellular injury (Pacher et al., 2007), highlighting its possible role in gonadotoxicity. The ability of Cu-NP to induce lipid peroxidation in the ovary is inconsistent with that observed in the liver and gills of juvenile *Epinephelus coioides* (Wang et al., 2014). This may be due to the catalytic production of ROS on the NP surface (Srikanth et al., 2016). Following endocytosis, the release of copper from Cu-NP causes damage to macromolecules, especially polyunsaturated fatty acids, due to an excess of reactive oxygen species (ROS) produced by Fenton-type reactions (Heinlaan et al., 2008). The present study's findings of substantial inhibition of CAT activity in the testis of PEN (I) and PEN (II) groups, as well as SOD in the ovary of PEN (II), corroborate previous findings in *Cyprinus carpio* acutely exposed to various concentrations of tebuconazole (Li et al., 2010a)**.** According to Li et al. (2010a), under triazole fungicides, excessive reactive oxidant production has been shown to suppress enzymatic antioxidants, shifting the oxidant/antioxidant balance to the oxidant side.

As shown in this study, increased T levels may have a negative feedback effect on GnRH, FSH, and LH secretion, resulting in spermatogenesis inhibition. In PEN (II) group, the fungicide evoked inflammatory responses in testis as manifested in this study by congestion in the seminiferous tubules, haemorrhage, and leucocyte infiltration. The activation of nuclear transcription factors may promote the release and formation of various inflammatory chemokines due to increased ROS production and decreased in antioxidant defence mechanisms. Chemokines play a role in leukocyte migration during intoxication (Bautista, 2002; Reinke et al., 2000). Increased leukocyte infiltration in the testes exposed to PEN is likely due to upregulated expression of various chemokine system constituents. The separation of the zona radiata from the oocyte membrane in vitellogenic and mature oocytes after exposure to Cu-NP (I) is consistent with previous findings in zebrafish (*Danio rerio)* challenged with gold NP (Dayal et al., 2016). One of the explanatory factors involved in this cytological modification following Cu-NP bioaccumulation in the ovarian tissue could be oxidative stress-induced cell cycle arrest and induction of the apoptotic cascade (Yang et al., 2017; Zhang et al., 2018; Naeemi et al., 2020). The adverse

histopathological changes in the ovary of PEN (I) and PEN (II) groups are most likely due to the down-regulation of vitellogenin and chorion genes involved in ovary development and protein synthesis.

Conclusion

By disrupting the sex hormonal balance and causing gonadal redox potential disturbance and cytopathological lesions, male and female reproductive biomarkers of *C. zillii* deteriorated following exposure to PEN and Cu-NP fungicides. These biomarkers appear to be a reasonable basis for their utility, not only in identifying exposures to fungicides but also in predicting harmful effects at the level of the whole organism and, perhaps, through linkage to suitable models and populations. However, further studies are highly recommended to evaluate the physiological status of *C. zillii* in contaminated aquaculture under an actual field condition as a more complex mixture of pollutants in the environment might interfere with the outcome.

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Suplemntart 1. Determination of 96 hrs lethal concentration (LC50) of PEN

PEN (µg /L)	Number of fish	Number of dead fish	A	B	
0	6	0	0	0	0
12	6	0	12	0	0
14	5	1	2	0.5	1
16	3	3	2	2	4
18	1	5	2	4	8
20	0	6	2	5.5	11
			Sum		24
LC_{50} = 20 - (24/6) = 16 µg/L					