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RESEARCH PAPER

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The Effects of Bisphenol A on Oxidative Stress, Antioxidant Defence, Histopathological Alterations and Lysozyme Activity in Narrow-Clawed Crayfish (*Pontastacus leptodactylus*)

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Abstract

This study aimed to investigate of bisphenol A (BPA) on histopathological tissue (hepatopancreas and gills), immune ability (lysozyme activity), oxidative stress and antioxidant defence in crayfish (Pontastacus leptodactylus). Crayfish were exposed to BPA to various concentrations (0 (control), and 10, 50, 100 µg/L) for 5 and 20 days in triplicates. Histopathological findings showed moderate to severe hyperemia and inflammatory cell infiltrations, irregular epithelial cell arrangements, degeneration, necrosis and sloughing at the cells, collapse of the hepatopancreas tubules and hyperemia, oedema and sloughing at the epithelial cells of gills. In addition to swelling of gills, accumulation of hemocytes in the hemocoelic space of the gill lamellae relating to dosage were noticed. Antioxidant-related enzyme activities which superoxide dismutase (SOD), Glutathione-S-transferase GST, and glutathione reductase (GR) activity in 10, 50, 100 µg/L BPA group were significantly lower than control at 5 days ($p<0.05$). Lysozyme activity (LSZ) was no significantly enhanced according to control ($p>0.05$). These results suggest that the alterations in the antioxidant enzymes and the histological structure of the hepatopancreas, and gill tissue of crayfish can be used as potential biomarkers for risk assessment in aquatic ecosystem.

Introduction

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl) propane) is an industrial chemical that has been used to harden plastics in bottles, CD, beverage cans and medical supplies used in daily lives and in various sectors (Geens et al., 2012; Michalowicz, 2014). With an annual production of approximately 5 million tons, BPA is one of the chemicals with the highest production volume in the world, and approximately half a million tons of it has the risk of leaking into the environment, so aquatic orgamisms can be exposed to this chemical (Vandenberg et al., 2009; Zhang et al., 2020). BPA is reported to act as a teratogen and endocrine distrupter in vertebrate animals (Flint et al. 2012). Teratogenic effects of BPA were found at 1- 10 mg/L, while endocrine and pleiotropic effects have been observed at lower concentrations (within the mg/L range; Flint et al. 2012).

Fish and crustaceans are known as bioindicators of environmental pollution (Borković et al., 2008; Farombi et al., 2007). BPA contamination has been found in many countries of the world. The concentrations of BPA, ranged from 0.28 to 25.2 mg/ kg in fish sampled from Taiwan (Lee et al., 2015), 0.56 mg/kg in muscles of fish obtained from the coast of Italy (Mita et al., 2011) and

0.83-19.25 mg/kg in marine fish from Hong Kong (Wong et al. 2017). Previous studies in aquatic animals have shown that BPA can influence the nervous system, behaviour, morphology, growth, and sexual differentiation as well as biochemical variables (Crain et al. 2007; Lam et al. 2011; Akram et al. 2021; Ribeiro et al. 2021; Minaz et al. 2022a-2022b). In the ecotoxicological studies, antioxidant enzymes and oxidative stress parameters are used to determine the toxic effects. There are many studies showing an induction of oxidative stress by BPA in fish (Makinwa and Uadia, 2021). However, limited studies exist about the toxicity of BPA in *A. leptodactylus.*

Water pollution causes histopathological changes in the gills that are considered to be the most appropriate indicators in aquatic animals (Kang et al., 2007; Fernandes et al., 2008; Karlsson, 1983). Histology is a useful tool to indicate the degree of pollution, particularly for sublethal and chronic effects (Bard, 2000; Devia et al., 2015; Faheem et al., 2016). *Pontastacus leptodactylus (*syn: *Astacus leptodactylus)* is an ecologically and economically important species. Plus, it has the potential to be used for ecotoxicological and environmental monitoring studies due to their feeding habits (Sepici-Dinçel et al. 2013). A short-term (96 h) application of BPA has significant negative effects at sublethal concentrations (12-48 mg/L) on crayfish in terms of several biochemical markers (Uçkun, 2021). However, very little information is available about the effects of BPA in narrow-clawed crayfish. This is the first study to examine the histopathological alterations in hepatopancreas and gill tissues and oxidative stress, antioxidant defence and lysozyme activity of crayfish exposed to acute and chronic sublethal exposure (5- 20 days) between 10-100 µg/ L BPA.

Material and Methods

Chemical and Stock Solutions

BPA doses (Sigma Aldrich, Inc. St. Louis, MO, USA; purity>99%) were prepared by dissolving in 100% acetone (analytical grade, Sigma Chemicals, Inc. St. Louis, MO, USA) in glass vials, sealed with parafilm and stored at 4°C until use. Selected doses of BPA in rearing water (10, 50, and 100 µg/L BPA) were adjusted using the stock solutions. A one to four day half-life of BPA reported by Chen et al. (2017) for lakes was taken into account to keep a constant BPA concentrations by the treatment groups over the study period. Accordingly, experimental tanks were thoroughly cleaned every 2 days, water was renewed with dechlorinated municipal water and then freshly prepared BPA stock solutions were added (Çetinkaya, 2010).

Animals and BPA Exposure Protocols

This study was carried out at the crayfish experiment unit of Eğirdir Faculty of Fisheries, Isparta University of Applied Sciences. *P. leptodactylus* (Eschscholtz, 1823) used in the experiment were provided from a fishermen cooperative in September 2020 from Eğirdir Lake, Isparta, Turkey. All experimental methods were conformed to the Regulation (EC) 710/2009 at the European Union level (Segner et al., 2019). Crayfish weighing an average of 35±0.6 g were

Table 1. Effect of different levels of BPA in the antioxidant enzymes of *P. Leptodactylus*

	Treatment	MDA (µmol/mg protein)	CAT (µmol/mg protein minute)	SOD (Unit/mg protein)	GST (Unit/mg protein)	GR (Unit/mg protein)
5 days	Control	0,81±0,01^{a}	0,06±0,05^{ab}	5,92±0,16^{a}	1,08±0,14^{a}	1,40±0,02^{a}
	10	0,55±0,08^{b}	0,04±0,00^{b}	4,46±0,15^{c}	0,37±0,03^{b}	0,94±0,02^{b}
	50	0,49±0,08^{b}	0,16±0,04^{a}	5,52±0,10^{ab}	0,32±0,16^{b}	0,23±0,04^{c}
	100	0,56±0,11^{b}	0,17±0,04^{a}	5,22±0,20^{b}	0,23±0,15^{b}	0,22±0,03^{c}
ANOVA		0.011	0.007	0.001	0.002	0.001
Linear		*0.058*	0.002	0.761	0.004	0.001
Quadratic		*0.075*	0.174	0.905	*0.059*	0.001
20 days	Control	0,25±0,01	0,12±0,02^{b}	5,31±0,45^{b}	ND	ND
	10	0,35±0,11	0,28±0,03^{a}	5,75±0,19^{b}		
	50	0,35±0,02	0,16±0,02^{ab}	5,60±0,09^{b}		
	100	0,13±0,02	0,14±0,05^{b}	6,84±0,24^{a}		
ANOVA		0.105	0.037	0.011	-	-
Linear		0.376	0.344	0.008	-	-
Quadratic		0.025	0.717	*0.068*	-	-

*All data were expressed as mean ± SD (n=3), (P<0.05) indicates statistically significant differences between the control and exposure. Significant differences were analysis of variance using SPSS package program.

adapted for two weeks to the experimental conditions. After adaptation, crayfish were exposed to BPA sub-lethal concentrations (0 (control), 10, 50 and 100, BPA µg/L) for 5 and 20 days. Each treatment dose was tested in triplicated tanks. Each tank with a volume of 80 L was stocked with 10 crayfish. The water used in the experiment was treated according to American Society for Testing and Materials guidelines (ASTM, 2014). Water parameters such as pH, conductivity, temperature, and dissolved oxygen water were measured using YSI Pro Plus Multi Parameter (YSI Incorporated, Ohio, USA) and averaged as 7.57±0.22; 22.18±0,15 S/cm; 25.15±0,18 °C, and 7.29±0,05 mg/L respectively. The photoperiod was kept constant at 12-h light and 12-h dark. Ten pieces of pipes were placed in each tank as a shelter for crayfish. Aeration was also supplied for all tanks by air blower. The crayfish were fed daily with potato and fish pellet feed over the experiment periods (Diler, 2013; Nane et al., 2021). Individuals that died during the experiment were daily removed from the tanks (a total of six crayfish died during the acut and chronic sublethal exposures).

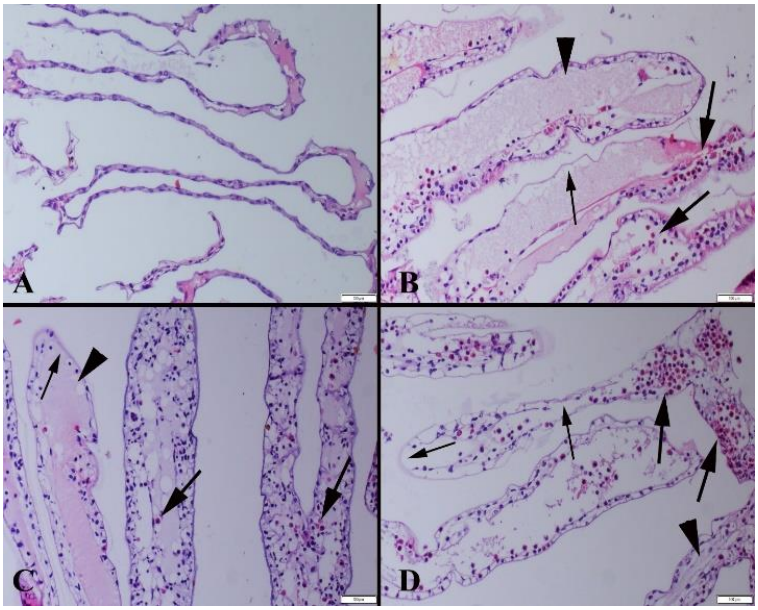

Figure 1. Histopathological appearance of the gills in 5 days. (A) Normal gill histoarchitecture in a control group, (B) moderate histopathological changes in groups exposed to 10 µg/L BPA, (C) moderate pathological findings in groups exposed to 50 µg/L BPA, (D) severe changes in groups exposed to 100 µg/L BPA. Hyperemia and hemocytic infiltrations (thick arrows), oedema (arrow heads) and sloughing in necrotic epithelial cells (thin arrows). HE, 100µm.

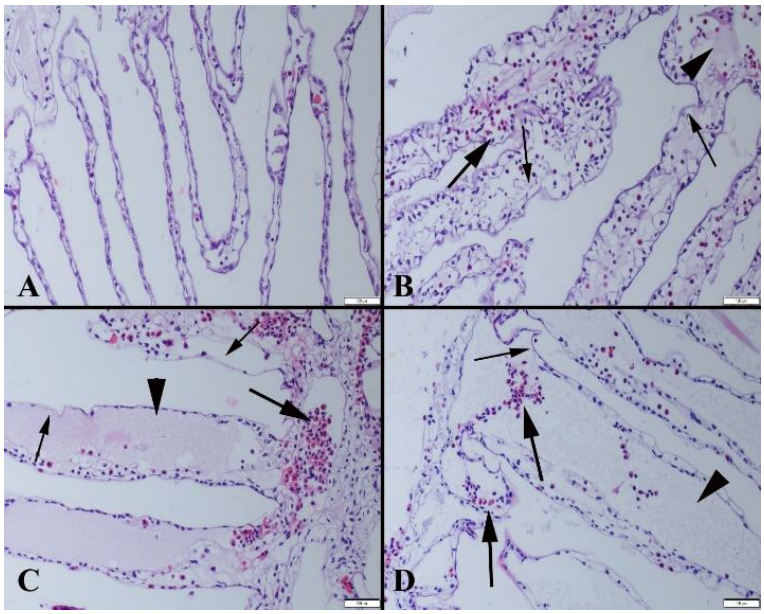

Figure 2. Histopathological appearance of the gills in 20 days. (A) Normal gill histoarchitecture in a control group, (B) moderate histopathological changes in groups exposed to 10 µg/L BPA, (C) severe pathological findings in groups exposed to 50 µg/L BPA, (D) severe changes in groups exposed to100 µg/L BPA. Hyperemia and hemocytic infiltrations (thick arrows), oedema (arrow heads) and sloughing in necrotic epithelial cells (thin arrows). HE, 100µm.

Histological Examinations

At the end of the study, 36 crayfish were anaesthetised in an ice bath and dissected between the thorax and abdomen on the ventral side (Leksrisawat et al., 2010). The gill and hepatopancreas tissues from the crayfish were collected and fixed in 10% neutral formalin (Formaldehyde solution/Sigma Aldrich, Inc. St. Louis, MO, USA; 37 wt. % in H2O) for histopathological examination. The samples were processed by an automatic tissue processing equipment and 5 µm sections were taken (Leica RM 2155, Leica Microsystems Nussloch, Germany). After one-night drying, all sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Bancroft and Stevens, 1977).

Lysozyme (LSZ) Activity

At the end of the study, the hemolymph was sampled from the base of the secondary walking legs using disposable insulin syringe needles under ice anaesthesia. The hemolymph was centrifuged immediately at 5.000 g for 10 min at 4°C to separate the serum. LSZ activity was determined according to Lie et al. (1989) using a lysoplate assay (Ellis, 1996; Grinde, 1989).

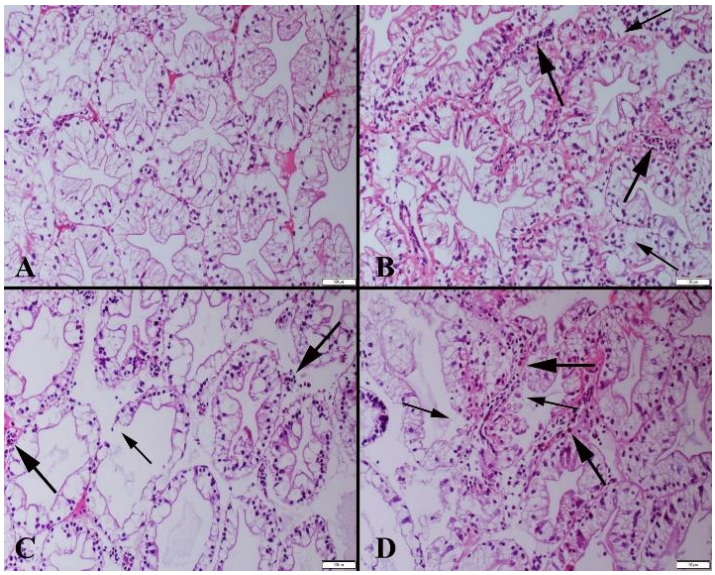

Figure 3. Microscopical appearance of the hepatopancreases in 5 days. (A) Normal tissue histoarchitecture in a control group, (B) moderate histopathological changes in groups exposed to 10 µg/L BPA, (C) moderate pathological findings in groups exposed to 50 µg/L BPA, (D) severe changes in groups exposed to 100 µg/L BPA. Inflammatory cell infiltrations (thick arrows) and necrosis and sloughing in epithelial cells (thin arrows). HE, 100µm.

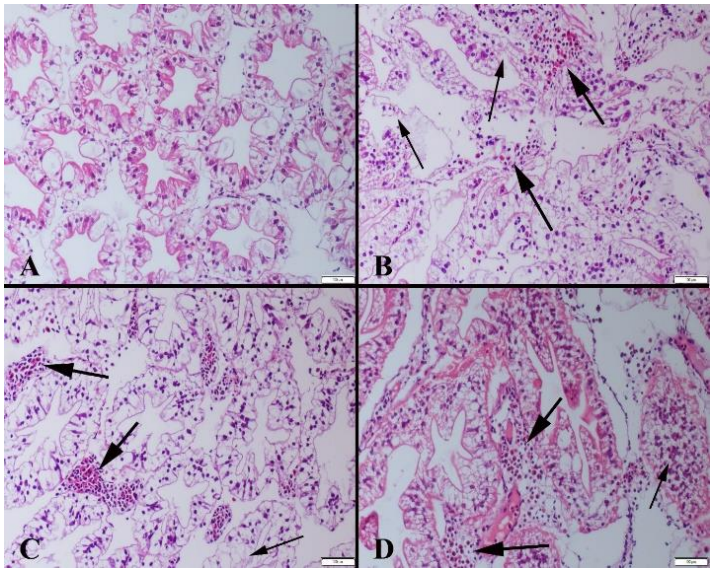

Figure 4. Microscopical appearance of the hepatopancreases exposed 20 days. (A) Normal tissue histoarchitecture in a control group, (B) moderate histopathological changes in groups exposed to 10 µg/L BPA, (C) severe pathological findings in groups exposed to 50 µg/L BPA, (D) severe changes in groups exposed to 100 µg/L BPA. Inflammatory cell infiltrations (thick arrows) and necrosis and sloughing in epithelial cells (thin arrows). HE, 100µm.

Sample Preparation and Biochemical Assays

After BPA exposure, the muscles and hepatopancreas samples were collected from all crayfish. The tissue samples were homogenized with a Potter-Elvenjem glass-glass homogenizer in fragmented ice. They were centrifuged at 1500 g for 15 min at +4°C and the supernatants were used for assays (Leksrisawat et al., 2010).

Malondialdehit (MDA) Level, Superoxide Dismutase (SOD), Catalase (CAT) Activity Assay

The level of MDA as a sign of lipid peroxidation was established according to the procedure of Ohkawa et al. (1979). On the basis of the reaction with thiobarbituric acid (TBA/Merck, Inc. St. Louis, MO, USA; purity>98%), SOD and CAT were assayed according to the Marklund and Marklund (1974) and Aebi (1984), respectively. The MDA level was expressed as mol/mg protein.

Glutathione Reductase (GR) and Glutathione S-Transferase (GST) Activity

The GR activity was determined according to the BioAssay Technology brand kit in Unit/mg protein. The GST measurements were made using BioAssay Technology brand kit. Results were calculated as Unit/mg protein.

Statistical Analysis

The data were evaluated using the SPSS package program and Microsoft Excel 2016. One way analysis of variance (ANOVA) was used to test the effects of BPA concentrations on the variables and the differences between the group averages were dicriminated by the Duncan test multiple comparison test at a significance level of P<0.05. The effects of BPA doses on the variables obtained from crayfish were also tested with polynomial contrasts using the statistical software (JMP v.8) to

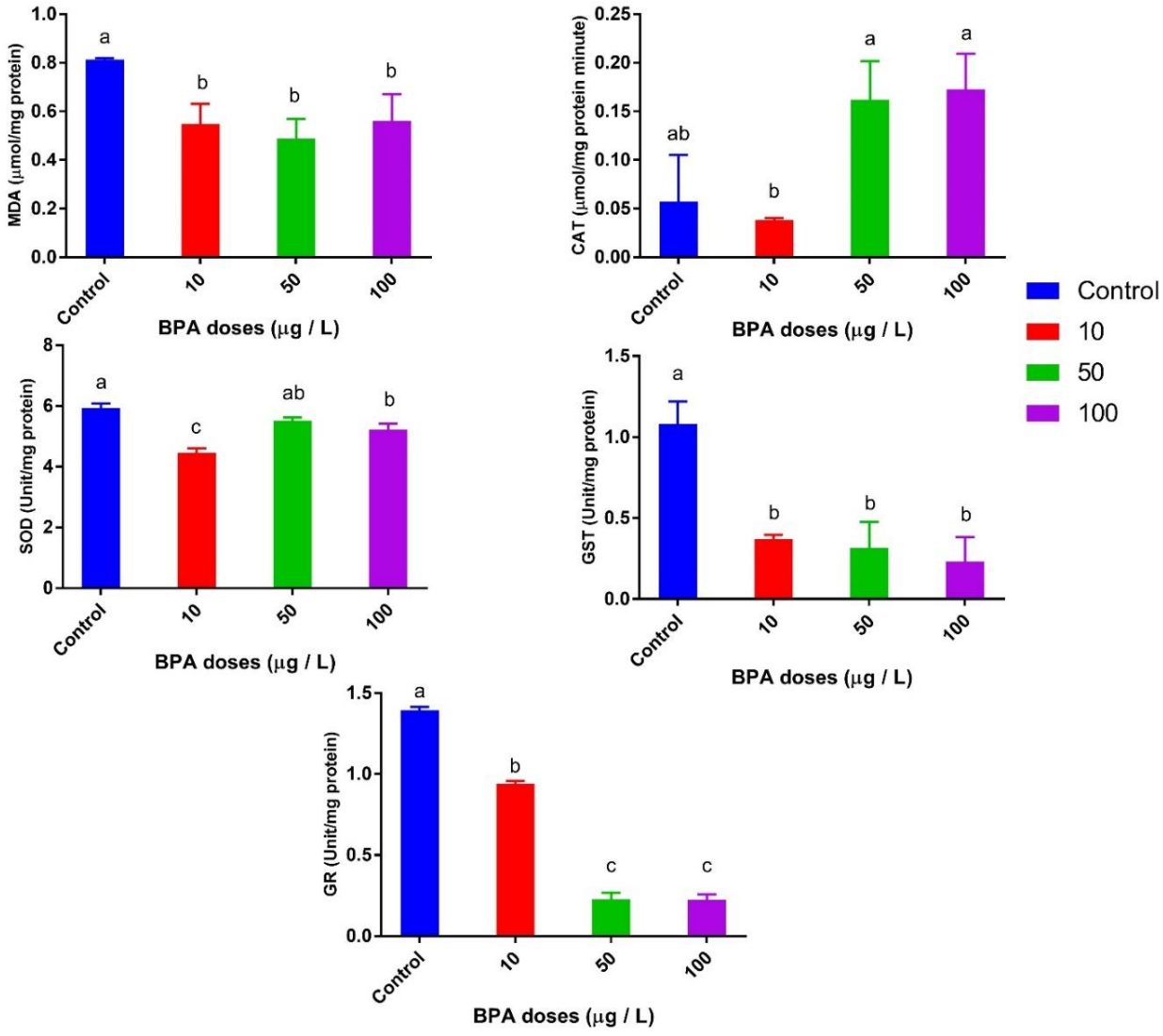

Figure 5. The oxidadative stress and antioxidase activity in muscles of crayfish after BPA exposure for 5 days. Different letters on the bar show statistical sigificance (p<0.05)

detect dose-related trend responses. Significant treatment effects were considered at P≤0.05 whereas the level of P≤0.10 was considered a significant trend.

Results

The Histopathological Effect of BPA on Gills

At the histopathological examination, slight to moderate changes were observed in 10 and 50 µg/L BPA exposed groups in both 5- and 20-days. Severe histopathological changes were determined in 100 µg/L BPA exposed groups both on days 5- and 20. Histopathological findings were slight to severe hyperemia, oedema and sloughing at the epithelial cells. In addition to swelling of gills, accumulation of hemocytes in the hemocoelic space of the gill tissue was noticed relating to dosage (Figure 1-2).

Hepatopancreas Findings

The result showed moderate to severe hyperemia and inflammatory cell infiltrations, irregular epithelial cell arrangements, degeneration, necrosis and sloughing at the cells, collapse of the hepatopancreas tubules. The severity of the lesions increased with BPA dosages in both exposure durations (i.e., 5- and 20-days) (Figure 3-4).

LSZ Activity

LSZ activity did not significantly change at various concentrations (10, 50, 100 µg/L BPA) compared with the control in crayfish regardless of exposure durations (P>0.05).

The Oxidative Stress and Antioxidant Activity

When the changes of MDA, CAT and SOD enzyme activity values were examined in crayfish samples exposed to increasing BPA concentrations for 5 days, it was found that MDA values significantly decreased in all BPA groups compared to the control (P_{ANOVA}: 0.011). The influence of increasing BPA concentrations on MDA was also a significant decreasing quadratic trend (P_L and P_Q = 0.058 and 0.075, respectively) (Table 1). According to ANOVA, CAT values of crayfish exposed to 10µg/L were similar to the control group but significantly lower than those exposed to 50 and 100 µg/L BPA. However, a closer look at the effects of increasing BPA doses on CAT levels reveals a significant linear increase (P_L: 0.002). It was determined that the SOD values decreased in all

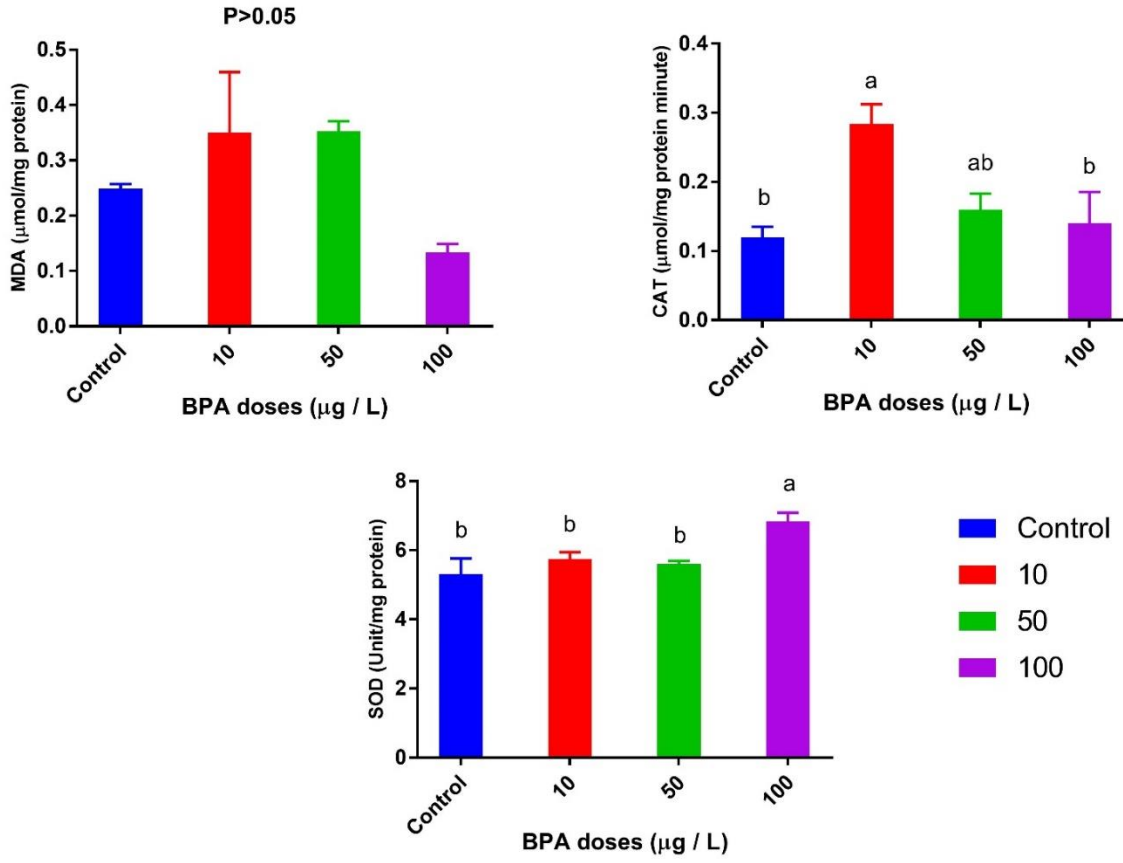

Figure 6. The oxidadative stress and antioxidase activity in muscles of crayfish after BPA exposure for 20 days. Different letters on the bar show statistical sigificance (p<0.05)

BPA groups, except 50 µg/L, compared to the control ($p<0.05$) (Figure 5). The GST and GR enzyme activity values were significantly decreased in all BPA groups compared to the control ($P<0.05$). Both variables were linearly decreased with the BPA concentrations ($P_L<0.05$).

According to ANOVA results, MDA values of crayfish exposed to various BPA levels for 20 days were comparable among the treatments ($p>0.05$) but polynomial contrast analysis revealed that MDA concentrations were quadratically affected by the BPA doses (P_Q: 0.025). There was no significant dose-dependent effect in CAT values on day 20 ($P>0.05$) while 10 µg/L group was significantly higher than the control and 100 µg/L group ($P<0.05$) (Table 1) The SOD values were the highest in the 100 µg/L group with significant differences ($P<0.05$) (Table 1, Figure 6) but actually the dose-dependent trend of SOD was a significant linear increase (P_L: 0.008).

Discussion

The use of products containing BPA has negative effects not only on living organisms, but also on the ecosystem and the environment (Michałowicz, 2014; Poormoosavi et al., 2018). BPA has been determined in fresh water and drinking water at different concentrations from µg/L to mg/L. (Flint et al., 2012; Crain et al., 2007). In previous studies, concentrations of BPA were recorded up to 17.2 mg/L and 12 µg/L in wastewaters in Japan and The United States, respectively (Yamamoto et al., 2001; Kolpin et al., 2002). Exposure to BPA has resulted in adverse effects on reproduction, embryonic development and genetic aberrations in wildlife, including annelids (both aquatic and terrestrial), molluscs, crustaceans, insects, fish, and amphibians (Oehlmann et al., 2009). Contaminated aquatic environments have negative effects on the benthivorous feeding fish species. For instance, BPA was found in all serum samples of catfish (*Clarias gariepinus* and *Clarias nigrodigitatus*) caught from ponds, rivers and lagoons in south western Nigeria at concentrations ranging from 0.05±0.01µg/L- 2.76±0.97µg/L (Makinwa and Uadia, 2021). BPA was ranges from 960-2700 µg/L in aquatic invertebrates. However the LC_{50} value of BPA for *A. leptodactylus* was determined as 96.45 mg/L (Uçkun, 2021).

Biologically, cellular antioxidant defence systems are damaged by chemical pollutants. However, antioxidant levels in living organisms increase to repair the damage caused by oxidative stress. Thus, the antioxidant enzymes in living organisms are evaluated as antioxidant status and bioindicator of oxidative stress (Livingstone, 2003; Valavanidis et al., 2006). The inhibition of these antioxidase activities causes the accumulation of reactive oxygen species (ROS). Since the accumulation of ROS will negatively affect hemocyte function, it must be rapidly eliminated. Elimination of ROS is provided by a defence mechanism consisting of antioxidant enzymes such as SOD, CAT, GR and GST (Chitra and Sajitha, 2014). Uçkun (2021) found that a 96 h of exposure to BPA had increased (SOD), (GST) and (MDA) in *A. leptodactylus.* However, and conversely, in the present study MDA, GST and GR values linearly decreased with increasing BPA concentrations in rearing water after a 5-day exposure duration. There was an increasing trend CAT values in parallel with BPA concentrations. The resulting antioxidant levels were sufficient to neutralize free radicals, thereby reducing MDA (Qu et al., 2014). Our study supports the findings of Uçkun (2021), who claimed that *A. leptodactylus* had higher tolerance to BPA. We also noticed that the SOD, CAT, and MDA levels displayed different trends on day 20 in comparison to those on day 5. For instance, a linear decrease in CAT values with BPA levels on day 5 changed to a heterogeneous trend with with the highest level in the 10 µg/L group on day 20. Moreover, a heterogeneous response in SOD values on day 5 changed to a linear increase with BPA levels on day 20. Similarly, in a previous study by Peng et al. (2018), the toxic effects of different concentrations of BPA on the crabs (*Charybdis japonica*) reflected a fluctuating trend in SOD and CAT activities with the extension of exposure durations. In another study, *P. clarkii* exposed to 225 µg/L BPA for one week showed an increasing ROS concentrations, which led to an inhibition of the activities of antioxidant enzymes such as SOD, POD and CAT (Zhang et al., 2020). Although a similar trend was observed in GST and GR activities but not in CAT and SOD on day 5 in the present study, an inverse response was the case on day 20 for SOD. The discrapencies can result from several factors such as differences in species, BPA concentrations applied and water temperature.

Gills are generally considered a good indicator of surrounding water quality (Camargo and Martinez, 2007). In this study, slight to moderate changes in 10 and 50 µg/L BPA exposed groups and severe histopathological changes in 100 µg/L BPA exposed group were determined on days 5- and 20. Histopathological findings were slight to severe hyperemia, oedema and sloughing at the epithelial cells. In addition to swelling of gills, accumulation of hemocytes in the hemocoelic space of the gill tissue was noticed depending on the BPA doses. Nane et al., (2021) demonstrated the histopathological effects of BPS (a BPA analogue) on gills in goldfish (*Carassius auratus*). Fish were exposed to BPS at different concentrations (0, 100 and 500 µg/L) for a duration of 21-days. That study found hyperemia, oedema, epithelial desquamation and necrosis of in the gills of fish exposed to BPS, being consistent with the present investigation. In another study, El Shaer et al., (2013) revealed that gills from BPA exposed fish showed several histopathological lesions and, hyperplasia in the epithelial cell.

The hepatopancreas secretes digestive enzymes and plays a leading role in digesting nutrients (Yan et al., 2005). In addition, this organ is sensitive to harmful substances (Yongxu et al., 2000). The histopathological

examination of the hepatopancreases in the current study showed moderate to severe hyperemia and inflammatory cell infiltrations, irregular epithelial cell arrangements, degeneration, necrosis and sloughing at the cells, collapse of the hepatopancreas tubules in crayfish. The severity of the lesions increased related to the dosage both on days 5- and 20. Zhang et al., (2020) observed the tissue damage of the hepatopancreas, the expansion in the lumen space and vacuoles after acute sublethal exposure to BPA in *P. clarkii.* In another study, the endoplasmic reticulum swelled and decreased numerically in hepatopancreatic cells upon exposure of *Charybdis japonica* to BPA (Peng et al., 2018). EDCs tamoxifen can damage walls of hepatopancreatic tubules in crab, *Portunus trituberculatus* (Liu et al., 2019).

Lysozyme is very important for the defence mechanism of animals by taking part in many immune responses (Möck and Peters 1990; Saurabh and Sahoo, 2008). However, in this study, no significant changes in LSZ activity for 5 and 20 days of exposure to relatively low concentration of BPA (10, 50, 100 µg/L) were observed. In a similar study, 225 µg/L BPA exposure depressed the LSZ activities in the hepatopancreas of *P. clarkii* (Zhang et al., 2020).

In conclusion, the toxic effects of sublethal concentrations (µg/L) BPA were examined for the first time in short and long-term exposure periods in *P. leptodactylus.* Exposition of crayfish to variying concentrations of BPA for 5 days disrupted the activities of antioxidant-related enzymes (SOD and GST, GR) being consistent with the results of histological findings. Therefore, it could be recommended to monitoring studies on BPA contamination for aquatic organisms and human health.

Ethical Statement

Not applicable

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Author Contribution

ÖD took part in designing the research, collecting data and writing the manuscript. ÖÖ, İDN and MN analysed all data of the study statistically and writing the manuscript. MM has edited the graphics and figures of the article. KA prepared their field studies and references. In addition, ÖÖ, İDN, MN, MM and KA took part on establishment of experimental design and on fixation of tissues. RA undertook the antioxidant activity analysis part. ÖÖ has directed the histopathological analysis department. All authors took part in a part of the article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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