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Effect of Thiamethoxam and λ Cyhalothrin, Administered Individually and in Mixture on the Endocrine Function and Antioxidant Defense of Gonads of *Oreochromis niloticus*

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ABSTRACT

Endocrine disrupting chemicals are a serious concern all over the world. In this study, the endocrine disrupting effects of commonly used insecticides thiamethoxam and λ cyhalothrin were investigated by using *Oreochromis niloticus* as a model organism. The fish exposed to 1/20, 1/10 of 96-h LC_{50} value of thiamethoxam and λ cyhalothrin (477.29 mg/L, 2.901 µg/L), individually and in mixtures, for 7 and 15 days. Fish were then left to depurate in pesticide-free water for 7 days. Results showed that acetyhcholin-esterase, catalase and estradiol/testosterone levels decreased, while the amount of etoxyresorufin-O-deetylase (EROD), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione (GSH) and protein carbonil (PCO) increased in comparison to the control. After the recovery period, EROD, GST, malondialdehyde, estradiol/testosterone levels were found to be lower than the control. In the pesticide mixture group, the activity of antioxidant enzymes was highest and the level of hormones was lowered. The group of the mixture pesticide showed the highest lipid peroxidation and protein carbonylation than only thiamethoxam or λ cyhalothrin exposure. In conclusion, the mixture of the insecticides showed a more toxic effect on the gonad of tilapia compared to individual pesticide exposures. In addition, the depuration period of 7 days was not adequate to eliminate the toxic effects of these insecticides.

INTRODUCTION

Endocrine disrupting chemicals represent a long-standing concern for international area as they cause the alteration of the normal endocrine system by mimicking the activity; alter the synthesis or metabolism of steroid hormones leading to physiological disturbances (Zhou *et al*., 2009). λ cyhalothrin, a member of endocrine disrupting pyrethroid insecticides containing the α-cyano group, is widely used in the worldwide. It is also known that λ cyhalothrin is frequently used to control ectoparasites and biological vectors in fish farms (Moraes *et al*., 2013). The insecticide is generally recognized as a potent neurotoxicant and affects the central nervous system of organisms. Researchers reported that non target aquatic organisms have been reported to be extremely sensitive to the neurotoxic effects of λ cyhalothrin. Therefore, λ cyhalothrin is classified as restricted use pesticide in Extension Toxicology Network (Saravanan *et al*., 2009). Thiamethoxam, one of the neonicotinoid insecticides, is among the most profitable pesticides worldwide due to its widespread use to control pests to protect crop. It is known as a neurotoxin and acts as an agonist on insect nicotinic acetylcholine receptors of insects. Recent studies have suggested that thiamethoxam should be considered an endocrine disrupting chemical in bees (Baines *et al*., 2017). Also, thiamethoxam has been partially banned by European Commission due to its toxicity to honeybees (EFSA, 2018). Due to their systemic properties and low toxicity to mammalian, these pesticides which can be applied by spraying in agricultural areas such as rice fields are frequently found in water and sediments related to agricultural areas and cause to toxicity in non-target aquatic organisms such as fish (Danion *et al*., 2014). Also, researchers have reported that aquatic environment is threatened by various environmental pollutants (Ejilibe *et al*., 2019). The residues of λ cyhalothrin and thiamethoxam have detected 0.11-0.14 mg/L and 8.93-63.4 µg/L in water of agricultural watersheds (He *et al*., 2008; Moraes *et al*., 2013). Previous studies have reported that λ cyhalothrin induces neurotoxicity in fish during acute phase, leads to lipid peroxidation and changes antioxidant enzyme activities.Thiamethoxam inhibits superoxide dismutase (SOD) and catalase (CAT) enzyme activities activities as well as changes protein, carbohydrate and lipid metabolism (Kumar *et al*., 2012; Yan *et al*., 2015).

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Oreochromis niloticus plays an important role in food chains and they are used as bio-indicators to monitor pollution of aquatic system. Also, they have become valuable model for gene expression and gonad development studies (Pfennig *et al*., 2012). Fish are exposed to more than one pollutants in aquatic environment. Different pollutants may have synergistic or antagonistic effects when they are on the move. Therefore, in this study, it is thought that the investigation of endocrine disruption, antioxidant enzyme activities and oxidative damage of thiamethoxam and λ cyhalothrin, administered individually and in mixtures, on the gonad of *O. niloticus* will contribute to better understanding of the toxic effects of these pesticides. Furthermore, researchers have studied the effects of different depuration period on oxidative stress parameters of fish exposed to pesticides, because that knowledge of the prediction of recovery time is important for fish health. Also, it would be of indirect help to human health (Rios *et al*., 2014). Thus, we investigated the ability of *O. niloticus* to eliminate the toxic effects of these pesticides after depuration period.

MATERIALS AND METHODS

Chemicals

The commercial product of thiamethoxam (3-[(2-chloro-5-thiazolyl) methyl] tetrahydro-5-methyl-N-nitro-4H-1, 3, 5 oxadiazin-4-imine, (Actara, Sygenta, 25g/L, PubChem CID: 107646) and λ cyhalothrin (1-α (S*), 3α (Z)]- (±)-cyano (3-phenoxyphenyl) methyl-3- (2-chloro-3, 3, 3-trifluoro-1-propenyl)- 2, 2 dimethylcyclopropanecarboxylate, (Karate Zeon, Sygenta, 50g/L, PubChem CID: 71464055) were purchased from distributor company (Turkey). Other chemicals were obtained from Sigma Chemical Co. St. (USA) and Merck and Co. Inc (USA).

Test animals

O. niloticus (male, 31.87±1.24 g, 13.47±0.95 cm) were obtained from Cukurova University Fish Culture Farm. Fish were acclimatized to laboratory conditions for a month in 140 L glass aquaria containing dechlorinated and gently aerated tap water, at dissolved oxygen 7.40 ± 0.22 mg/ L, pH 8.01 ± 0.73, temperature 20 ± 2^{0}C, alkalinity 282 mg/L $CaCO_3$, and total hardness 188 mg/L $CaCO_3$ with a 12:12 light: dark photoperiod. Water was changed daily by transferring the fish to another aquarium (APHA, AWWA, WPCF, 1998). The stock and experimental fish kept in similar laboratory conditions. During acclimation and exposure periods, they were fed ad libitum once a day with commercial fish pellets (Pinar, Izmir, Turkey) and the remainder food was removed after feeding. Following the approval the Ethics Committee of Cukurova University, all animal maintenance and experimental procedures used were planned and conducted in accordance with the Guideline of APHA, AWWA, WPCF (1998).

For experimental design, a total 126 male fish were first divided into two groups; control and exposure groups, and then the exposure groups were divided into six subgroups viz. Group 1 (G1): 23.86 mg/L thiamethoxam (1/20 of the 96h LC_{50}), Group 2 (G2): 47.72 mg/L thiamethoxam (1/10 of the 96h LC_{50}), Group 3 (G3): 0.145 µg/L λ cyhalothrin (1/20 of the 96h LC_{50}), Group 4 (G4): 0.290 µg/L λ cyhalothrin (1/10 of the 96h LC_{50}), Group 5 (G5): 23.86 mg/L thiamethoxam+0.145 µg/L λ cyhalothrin, Group 6 (G6): 47.72 mg/L thiamethoxam+0.290 µg/L λ cyhalothrin under semi-static conditions (Piner and Uner, 2012; Kocamaz and Oruc, 2018). Thiamethoxam and λ cyhalothrin concentrations were calculated according to the active ingredient percentage present in the commercial formulation; and quantified at the beginning of each chemical renewal period by GC-ECD, showing recoveries >90% of the nominal value for each concentrations. Experimental fish were exposed to pesticides on day 7 and 15. Subsequently, fish were transferred into pesticide-free water for 7 days in order to determine the potential reversibility of pesticide toxicity. The experiments were combined from two replicated independent experiments. No mortality was observed during the experiments. At the end of the experiments, blood samples of fish were collected by severing the caudal peduncle. Following cervical decapitation, gonad tissues were collected, minced and homogenized (2.5% w/v) with ice-cold 1.17% KCl, 100 mM sodium phosphate buffer (pH 7.4) using a glass-teflon homogenizer and then were centrifuged at 10000 g for 30 minutes in an eppendorf centrifuge at 4ºC. Supernatants were used for biochemical analyses. All biochemical analyses were measured spectrophotometrically. Acetylcholinesterase (AChE) activity was determined using the method of Ellman *et al*. (1961). Ethoxyresorufin-O-deethylase (EROD) activity was measured according to the method of Klotz *et al*. (1984). SOD, CAT and glutathione S-transferase (GST) activities were determined according to the method of McCord and Fridovich (1969), Beutler (1984) and Habig *et al*. (1974), respectively. Glutathione (GSH), malondialdehyde (MDA), protein carbonil (PCO) levels and protein content were determined according to the method suggested by Beutler (1984), Ohkawa *et al*. (1979), Levine *et al*. (1990) and Lowry *et al*. (1951), respectively. Blood was centrifuged at 3600 rpm (Heittich, Universal 1200) for 5 min. Analyses of the concentrations of estradiol (Catalog# 03000079, Roche Diagnostics, IN) and testosterone (Catalog# 11776061, Roche Diagnostics, IN) in plasma samples of *O. niloticus* were measured using electrochemiluminescence immunoassay procedure using Roche Elecsys System.

Statistical analysis

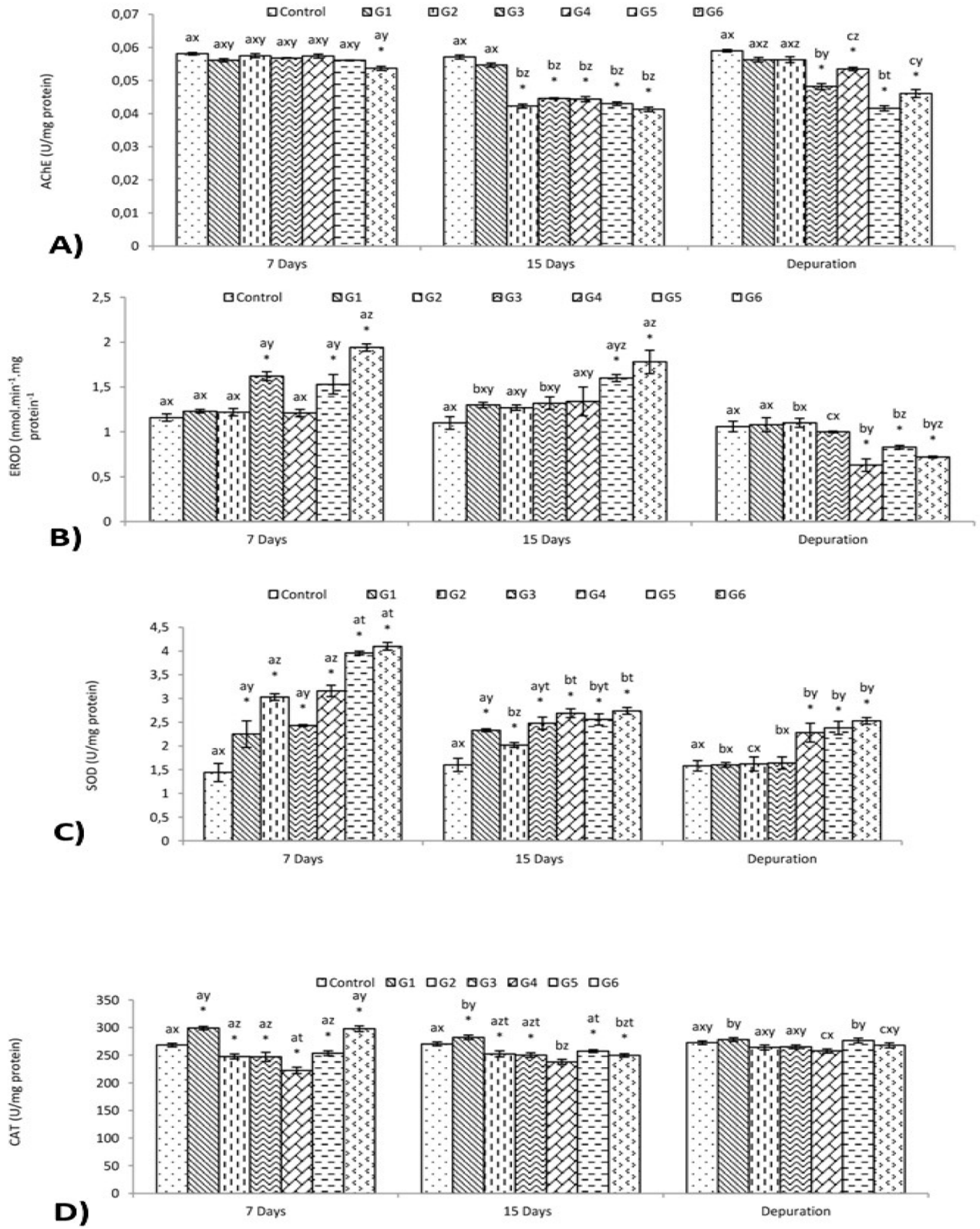
All data are reported as mean±standard error. Shapiro-Wilks test was applied to evaluate normality while Leven test was used to test the homogeneity of variance. To evaluate the differences between biochemical and hormone parameters measured, one-way analysis of variance (ANOVA) followed by Student Newman Keuls was performed to compare among treatments and exposure periods. Significance was accepted at $p<0.05$.

RESULTS AND DISCUSSION

AChE activity was reduced on 7th day in gonad tissue of *O. niloticus* exposed to G6 in comparison to the control and other exposure groups. AChE inhibition was determined to be 25.91%, 21.89%, 22.24%, 24.69% and 27.67% in G2, G3, G4, G5 and G6 on 15th day (Fig. 1A). Higher depletion of AChE activity was observed at G6 in comparison to the control and other exposure groups on 7th and 15th days. Also, AChE activity which exhibited a good concentration-time response relationship in all exposure groups except G1 compared to the control. Before, researchers had reported similar results by treating *Cyprinus carpio* with λ cyhalothrin and *Oncorhynchus mykiss* with neonicotinoid imidacloprid (Bibi *et al*., 2014; Topal *et al*., 2017). Also, Wang *et al*. (2012) reported that the full inhibition of AChE activity in *Carassius auratus* was observed after pesticide mixtures treatment. In this study, after depuration period, thiamethoxam individual treatments provided protection against toxicity, but fish were observed to be unable to overcome the stress of λ cyhalothrin and combined treatments. At the same time, combined treatment of thiamethoxam and λ cyhalothrin showed slightly additive effect on AChE activity after recovery. Decreased AChE activity on gonad of *O. niloticus* exposed chlorpyrifos was observed after recovery period (Ozcan-Oruc, 2010). The patterns of inhibition and recovery of AChE activity of exposed fish species can be a useful biomarker for better explanation of environmental effects of pesticides in fish. Thiamethoxam and λ cyhalothrin are known as a neurotoxin. In our study, the AChE inhibition by the λ cyhalothrin may be due to the hydrophobic interactions of pyrethroids with the hydrophobic aromatic surface region of AChE and the inhibition by the thiamethoxam may be a consequence of the agonistic effect of thiamethoxam on nAChR.

On 7th day, EROD activity increased up to 39.65%, 31.89 %, 67.24% at G3, G5 and G6, and on 15th day the activity increased about 45.45% and 61.81% at G5 and G6, respectively. Also, a higher induction of EROD activity was observed in the combined treatment of pesticides (G6) in comparison to the control and all exposed groups on 7th and 15th days (Fig. 1B). Treatments with only thiamethoxam or λ cyhalothrin showed similar tendency for the induction of EROD activity. EROD activity, a marker of receptor-mediated induction of CYP450-dependent monooxygenases by xenobiotics, is considered a highly sensitive biomarker of pollutant exposure in fish (Whyte *et al*., 2000). The main organ in which xenobiotics are detoxified in aquatic organisms is the liver. However, cytochrome P450 isoforms have been reported to be present in other tissues (Meyer *et al*., 2002). Smolowitz *et al*. (1991) found that 3, 3', 4, 4'- tetrachlorobiphenyl and 2, 3, 7, 8- tetrachlorodibenzofuran affect the EROD activity of liver, kidney, gill, testis, ovary and intestine in *Stenomus chrysaps*. In this study, EROD activity decreased after recovery period in G4, G5 and G6. Similarly, after the recovery period, decreased EROD activity in *O. mykiss* exposed different concentrations of pendimethaline was observed (Danion *et al*., 2014). The inhibition of EROD activity by the effect of pesticide mixtures after depuration period suggests that xenobiotic-mediated cellular protein synthesis is impaired.

SOD activity was determined to be 56.25%, 110.41%, 68.75%, 119.44%, 175.00%, 184.72% on 7th day, 45.62%, 26.25%, 55.00%, 68.12%, 60.00% and 71.25% on 15th day in G1, G2, G3, G4, G5 and G6, over control, respectively (Fig. 1C). After recovery period, the enzyme activity was still higher than the control at G4, G5 and G6 groups. The combined treatment of pesticides on 7th day caused a synergistic effect on SOD activity. Also, the highest SOD activity induction was found in combined treatment (G6) in all periods. Individual treatments of thiamethoxam and λ cyhalothrin showed similar results for SOD activity on 7th and 15th days. The antioxidant system containing enzymes such as SOD and CAT has an important role in protecting fish against free radicals produced under the conditions of oxidative stress caused by pesticide toxicity. Increased SOD activity suggested that superoxide radical production increased. Catalase activity increased up to 11.45 % and 10.98% at G1 and G6 on 7th day while it decreased in all other groups ascompared to the control. CAT activity decreased 6.60%, 7.43%, 12.00%, 4.75% and 7.59% in G2, G3, G4, G5 and G6 on 15th day compared to control, respectively (Fig. 1D). Interestingly, higher depletion of CAT activity was observed in group treated with λ cyhalothrin compared to control and all –other treated groups. After the recovery period, CAT activity was not changed. Similarly, increased SOD activity and decreased CAT activity were observed in *O. mykiss* exposed to λ cyhalothrin and in *D. rerio* exposed to thiamethoxam (Kutluyer *et al*., 2015; Yan *et al*., 2015). Pesticide toxicity has caused peroxidative damage in tissues and causing the excessive increase in the production of reactive oxygen species (ROS) in fish. In our study, the inhibition of CAT



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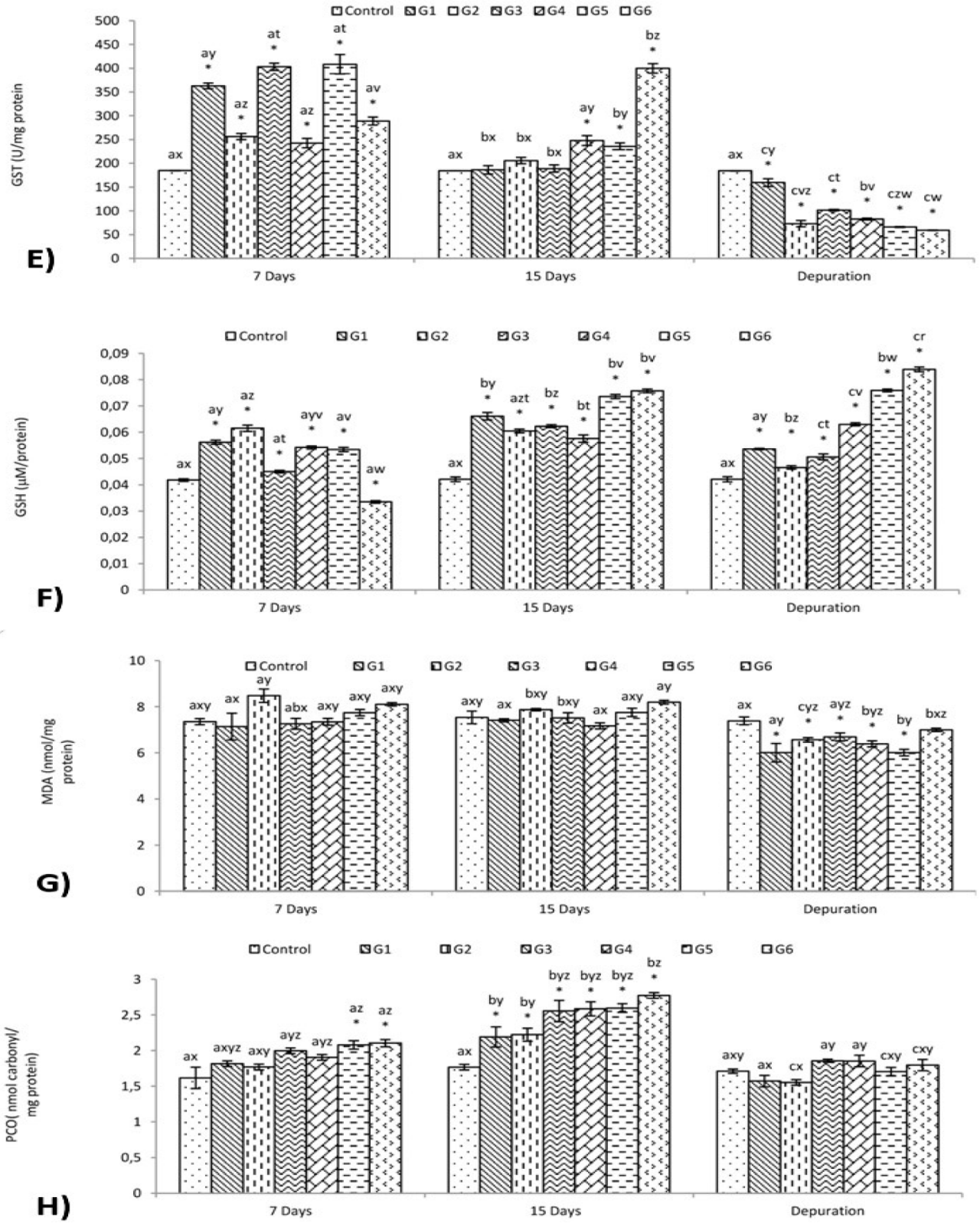

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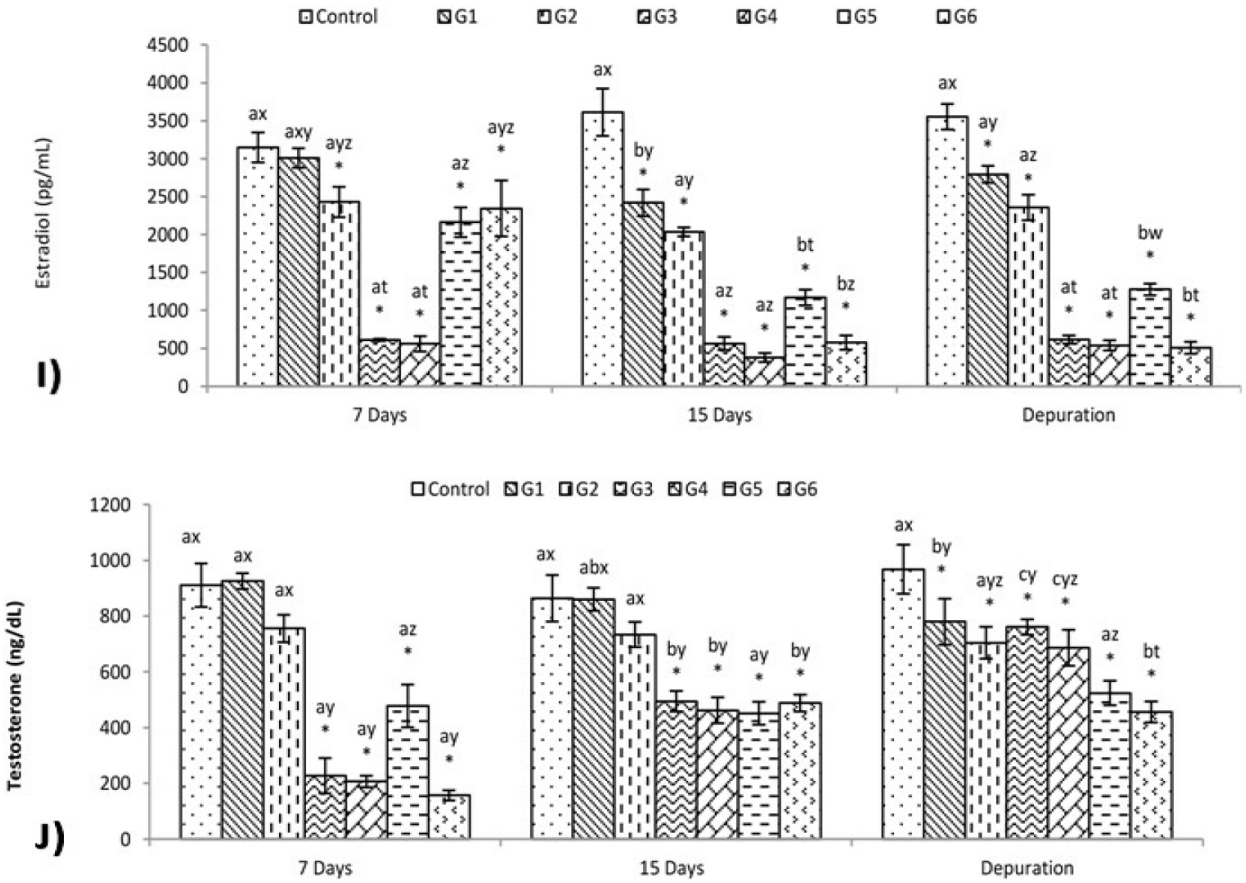

Fig. 1. A) AChE (U/mg protein); B) EROD (nmol. min^{-1}.mg protein^{-1}); C) SOD (U/mg protein); D) CAT (U/mg protein); E) GST (U/mg protein); F) GSH (µM/ protein); G) MDA (nmol/mg protein); H) PCO (nmol carbonyl/mg protein); I) estradiol (pmol/L) and J) testosterone (ng/dL) measured in *O. niloticus* exposed to hiamethoxam; λ cyhalothrin and their combined treatments on 7th, 15th days and depuration period. Values showen are means ± standard error. * groups with parameters significantly different from control. The letters of a-c show significant differences between periods and letters of x-w show indicate significant differences amoung exposure groups for same period (n=6, p<0.05). (G1:23.86 mg/L thiamethoxam, G2: 47.72 mg/L thiamethoxam, G3: 0.145 µg/L λ cyhalothrin, G4:0.290 µg/L λ cyhalothrin, G5: 23.86 mg/L thiamethoxam+0.145 µg/L λ cyhalothrin, G6: 47.72 mg/L thiamethoxam 0.290+ µg/L λ cyhalothrin).

activity may be according to the suppression of CAT activity by decreasing the SOD-CAT reaction rates due to the increased ROS.

GST activity increased up to 96.42%, 38.78%, 118.26%, 31.27%, 121.09%, 56.48% at G1, G2, G3, G4, G5 and G6 on 7th day, 34.43%, 27.87% and 116.71% in G4, G5 and G6 on 15th day, in compare to control, respectively (Fig. 1E). The combined treatment of pesticides caused slightly synergistic effect on GST activity on 15th day. The induction of GST after exposures to the pesticides is an indication of biotransformation of the pesticides and activation of the antioxidant defense system. After recovery period, GST activity significantly decreased in all groups compared to the control. Similarly, after recovery period, GST activity was decreased in liver and kidney of *Piaractus mesopotamicus* by the effect of λ cyhalothrin (Bacchetta *et al*., 2014). The decreased GST activity due to pesticide toxicity is an indication of the failure of detoxification mechanisms and the formation of oxidative stress. On 7th day, glutathione level decreased at G6 while it increased up to 34.44%, 47.12%, 7.65%, 29.66%, 27.75% in G1, G2, G3, G4 and G5 compared to the control, respectively. The GSH induction was determined to be 57.80%, 43.70%, 47.98%, 36.81%, 74.82% and 79.80% on 15th day and 27.31%, 10.68%, 20.19%, 49.64%, 80.28% and 99.28% after recovery period in G1, G2, G3, G4, G5 and G6 compared to the control, respectively (Fig. 1F). The highest GSH level was found in G6 on 15th day as it was after therecovery period. Narra *et al*. (2015) observed a significant increase

in GSH level in *Clarius batrachus* after treated with chlorpyrifos and monocrotophos. The increased amount of GSH is defined as one of the protective mechanisms in fish adapted to pesticide exposure. The induction of GSH level is considered to be indicative of the activation of pesticide elimination and biotransformation (Dey *et al*., 2016).

In this study, the differences in MDA level in control and treatment groups on 7th and 15th days were not statistically significant. On the other hand, after depuration period, the MDA level in G1, G2, G3, G4 and G5 decreased 18.70%, 11.12%, 9.41%, 13.54% and 18.60% compared to the control, respectively (Fig. 1G). Similarly, Oruc and Uner (2002) reported that MDA level was found to be equal of the control level in *C. carpio* after 2, 4-D and azinphosmethyl exposures, individually and in mixtures. Lipid peroxidation is the first step of cellular damage caused by pesticides. MDA is widely used as a biomarker for lipid peroxidation, which is considered as a valuable indicator of oxidative damage of tissues. In our study, decreased MDA level indicated that antioxidant defenses were able to scavenge reactive oxygen species (ROS) and prevent the oxidative damages in the gonad. PCO level increased up to 23.50%, 17.74%, 28.57%, 30.11% in G3, G4, G5, G6 on 7th day and 23.99%, 25.80%, 44.53%, 46.29%, 46.91% and 56.87% in G1, G2, G3, G4, G5 and G6 on 15th day, respectively (Fig. 1H). The highest PCO level was found in G6 compared to other exposed groups on 7th and 15th days. Also, thiamethoxam and λ cyhalothrin exposure groups showed similar result of PCO level on 7th and 15th days. Similarly, Xu *et al*. (2015) demonstrated that dichlorvos, deltamethrin and their mixtures induced the level of PCO. Elevated PCO level is owing to the deterioration of cellular protein metabolism due to excessive accumulation of ROS produced by xenobiotic-induced oxidative stress. In this study, PCO level remained at control level in all exposed groups, after depuration period. Toni *et al*. (2011) showed similar results in *C. carpio* exposed to tebuconazol. The presence of PCO in the control level suggested that the pro-oxidant effects of the pesticides were eliminated and that the antioxidant enzymes were more effective against the oxidative stresses.

Exposures to thiamethoxam and λ cyhalothrin, individually and in mixtures, resulted in a decrease in estradiol and testosterone levels. Estradiol level decreased 22.79%, 80.51%, 82.19%, 31.27% and 25.49% in G2, G3, G4, G5 and G6 on 7th day, 33.06%, 43.66%, 84.39%, 89.54%, 67.62% and 84.08% on 15th day, 21.39%, 33.71%, 82.69%, 84.85%, 64.05% and 85.70% in G1, G2, G3, G4, G5 and G6 after recovery period, compared to control, respectively (Fig. 1I). The most prominent decrease in estradiol level was found in G3 and G4 on 7th and 15th days. Testosterone level decreased 75.08%, 77.27%, 47.53%,82.76% on 7th day, 42.82%, 46.52%, 47.80%, 43.51% on 15th day in G3, G4, G5 and G6, compared to control, respectively (Fig. 1J). Interestingly, λ cyhalothrin and treatment with the mixture of the pesticides showed similar tendency in testosterone level on 7th and 15th days compared to the control and thiamethoxam exposed groups. At the end of the recovery period, testosterone level was still decreasing, and fish were unable to overcome the stress of the pesticides Similarly, Singh and Singh (2008) reported that cypermethrin caused a decrease in estradiol and 11-ketotestosterone level in *Heteropneustes fossilis*. The reduction of steroid hormone levels bypesticide toxicity implies that the biosynthetic capacity of the gonads is changed indirectly by the inhibition of aromatization activity responsible for estrogen synthesis or by the suppression of gonadotropin secretion.

CONCLUSION

Treatments withthiamethoxam and λ cyhalothrin induced CYP450 activity which is used as an early warning signal for the existence of natural or synthetic pollutants. Induction of AChE activity in *O. niloticus* after pesticide exposures showed the irregularity of the antioxidant defense system. Increased SOD activity and decreased CAT activity suggested the increase in free radical formation and decrease in the reaction rate of SOD-CAT due to pesticides toxicity. Increased GST activity-GSH level, as well as the presence of MDA in the control, showed the increase in pesticides detoxification and activation of antioxidant defense system. Also, increased PCO level showed that oxidative damage has started. Decreased estradiol and testosterone levels showed that thiamethoxam and λ cyhalothrin caused to dysfunction of steroid hormone synthesis and metabolism. In conclusion, results of this study showed that treatment with the mixture of the pesticides caused higher depletion or induction on biochemical parameters in gonad tissue and combined treatment of pesticides develop synergistic effects which were a more toxic compared to individual pesticide exposures.

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Statement of conflict of interest

The author declares that they have no conflict of interests.

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