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Pesticide-Induced Alterations of Esterase and Antioxidant Enzymes of Aquatic Organisms *Oreochromis mossambicus* and *Xenopus laevis*

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Abstract

Pesticides are extensively utilized in modern farming to control pests and weeds, thereby ensuring high quality and quantity of crops. Aerial drifts and runoffs after rain transport these agrochemicals to aquatic bodies, where they adversely affect aquatic organisms. We carried out a study to assess the effects of carbaryl and dimethoate on esterase and antioxidant enzyme activities of tadpoles, adult frogs and juvenile fish. These organisms were exposed to sublethal contraptions of 2.9 ppm carbaryl and 4.8 ppm dimethoate for 96 hours. After the exposure period, the fish and frogs were sacrificed and post-mitochondrial fractions were prepared for enzymatic analysis. Acetylcholinesterase (AChE), carboxylesterase (CbE), superoxide dismutase, glutathione peroxidase and catalase were measured. Carbaryl and dimethoate inhibited the activities of acetylcholinesterase, carboxylesterase, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in juvenile fish, tadpoles and adult frogs. Inhibition of SOD, CAT and GPx suggests that the two pesticides caused oxidative stress in the aquatic organisms, while inhibition of AChE and CbE affected the normal transmission of nerve impulses. The results indicate that the two pesticides affect the well-being of the studied aquatic organisms.

Keywords

Carbaryl, Dimethoate, Frogs, Tadpoles, Fish, Antioxidant Enzymes

1. Introduction

Aquatic organisms like frogs and fish are indirectly affected, by human activities like farming which release toxic agrochemicals into the environment, which may

affect the well-being of aquatic organisms [1]. Farming activities involve the use of agrochemicals which include pesticides and fertilizers. Pesticides like carbamates and organophosphates are extensively used to control insect pests and weeds to ensure high quality and quantity yields of crops. Sharma *et al.* [2] and Popp *et al.* [3] also mentioned that pesticide use in agriculture ensures the production large volumes of quality crops, therefore, ensuring food security. While it is without a doubt that pesticides are essential for ensuring food security in different countries for their overgrowing nationals, pesticides have been shown to negatively affect the environment [4] [5]. Pesticides are toxic to aquatic and terrestrial organisms.

Ogeleka *et al.* [6] reported concentrations of the pesticides, propoxur, deltamethrin, atrazine, and furadan that caused the death of fish and earthworms in aquatic and terrestrial environments. Pesticides find their way to aquatic and terrestrial ecosystems through aerial drifts and runoffs after rains, where they exert harmful effects on aquatic bodies such as fish and frogs. The long-term impact of pesticidal chemicals is the reduction in populations of some of these organisms.

Reductions in populations of frogs and fish disturb the flow of energy in aquatic ecosystems as food chains are altered. In addition, reduction in the fish population impacts negatively on communities that live near aquatic bodies since fish is the main source of protein and fishing is a huge part of the economic activity and is a source of income for the people involved in fishing. Of importance is the role of frogs and fish in controlling disease-causing vectors like mosquitoes by feeding on these vectors [7] [8]. These aquatic animals feed on algae and therefore reduce or prevent algal blooms in water bodies. A reduction in the numbers undoubtedly negatively impacts the health of the aquatic ecosystem [9] [10].

Pesticides are mixed substances that are poisonous and efficient on target organisms [11]. They have numerous beneficial effects that include crop protection, preservation of food and materials and prevention of vector-borne diseases [12]. These pesticides are washed away into aquatic bodies and affect aquatic organisms like frogs and fish amongst others [13] [14]. Pesticides such as malathion, dimethoate and carbaryl are known to cause metabolic disturbance in non-target organisms, like fish [15]. Demetrio *et al.* [16] reported the toxicity and reproductive effects of chlorpyrifos and cypermethrin on *Daphnia magna*. The pesticide mixture decreases reproduction in exposed *Daphnia magna* [17]. Frogs are affected by water pollutants hence over the years, they have been used as bioindicators of aquatic pollution [18]. Endocrine disruptive effects of the herbicide linuron on amphibians have been reported [19]. The herbicide caused deformed hindlimbs and reduced fertility rates in exposed frogs [19].

Fish can be exploited as a bioindicator species for monitoring pollution in aquatic bodies and this can be done by monitoring their biochemical activities [20]. A biomarker is a change that can be observed at a molecular, cellular or

physiological level as a response to environmental exposure to a chemical [21]. The bream fish *Oreochromis mossambicus*, adult frog *Xenopus laevis* and Mababe dwarf puddle tadpoles (*Phrynobatrachus mababiensis*) were used in this study. They are good bioindicators for aquatic pollution as they are always underwater. This study sought to find the effects of dimethoate and carbaryl individually and as a mixture on esterase and antioxidant activity in fish, tadpoles and adult frogs to assess the adverse effects of the two commonly used insecticides in Zimbabwean farming.

2. Methods

2.1. Exposure and Tissue Processing

Adult frogs (*Xenopus laevis*) and tadpoles (*Phrynobatrachus mababiensis*) were obtained from a local breeder. *Xenopus laevis* frogs were fed on ground beef and the *P. mababiensis* tadpoles were fed on lettuce during the study. Juvenile bream fish *Oreochromis mossambicus* of about 2 cm in length were obtained from the NUST dam and were fed on fish flakes during the study. All the organisms used in the study were kept in the laboratory in glass tanks with tap water at room temperature and they were allowed to acclimatize for a week before being used in the experiments. For each organism, the exposures were carried out in quadruplicates. There were control groups for the fish, adult frogs and tadpoles. There were groups exposed to 2.9 ppm carbaryl, 4.8 ppm dimethoate and mixtures of 2.9 ppm carbaryl and 4.8 ppm dimethoate. The organisms were exposed for 96 hours with water and respective pesticide being changed after every 24 hours. Mortality was noted after every 24 hours. The dead organisms were identified by lack of movement and they were removed from the bowls.

2.2. Sacrificing of Organisms and Reparation of Post Mitochondrial Fraction (PMF)

Organisms were sacrificed after the period of exposure. The organisms were sacrificed following guidelines by the Institutional animal care and use committee (IACUC) [22]. Fish and tadpoles were put on ice until there was no movement, after which homogenates were prepared for enzyme assays. Frogs were put on ice until there was no movement and then they were put in a glass tank with CO_2 until the frogs were dead. Whole fish and tadpole samples were homogenized in potassium phosphate buffer at pH 7. Frogs were dissected then muscle and liver samples were obtained and homogenized in potassium phosphate buffer at pH 7. The homogenates were then centrifuged at 10,000 ×g for 15 minutes to collect post mitochondrial fraction. The supernatants were immediately stored at −80˚C until there were needed for enzyme activity analysis.

2.3. Protein Determination

Protein content in the post-mitochondrial fractions of the fish, frogs and tadpoles was determined using Lowry *et al.* [23] method. Bovine serum albumin

was used to make the standard curve. Two hundred microliters of the alkaline solution were added to 30 µl of sample solution into each well on a microtitre plate. The contents of the microtitre wells were allowed to stand for 10 minutes at room temperature. After this incubation period, 20 µl of Folin-Ciocalteau reagent was added into each well and immediately mixed and the reaction mixtures were allowed to stand for 30 minutes at room temperature. After the 30 minutes had elapsed, the absorbance was measured at 750 nm wavelength on a SpectraMax 190 plate reader.

2.4. Cholinesterase Activity

Cholinesterase activity was measured following the method of Kallander *et al.* [24]. The reaction mixture contained 110 µl of 0.01 M Tris-HCL buffer pH 8 and 20 µl of 3.2 mM 5.5 Dithio-bis-2 nitro benzoic acid (DTNB) was mixed in a microtitre plate. The homogenate (50 µl) was added to each well. Twenty microliters of 10 mM acetylthiocholine iodide were then added and an increase in absorbance was monitored for 3 minutes at a wavelength of 412 nm on a SpectraMax 190 plate reader. Each sample was analyzed in quadruplicates. Specific enzyme activity was calculated using an extinction coefficient of 14.15 $mM^{-1} \cdot cm^{-1}$.

2.5. Carboxylesterase Activity

Carboxylesterase activity was measured following the method of Mackness *et al.* [25]. In this assay, 20 ml of the assay reagent (0.027 M Tris/HCl buffer, pH 7.6 and 0.0096 M 4-nitrophenyl acetate were mixed in the ratio 2:1) Fifty microliters of sample and 200 µl of assay reagent were added into each well on a microtitre plate and increase in absorbance was monitored over 3 minutes. Specific enzyme activity was calculated using an extinction coefficient of 44.2 $mM^{-1} \cdot cm^{-1}$.

2.6. Superoxide Dismutase Activity

Superoxide dismutase activity was measured using the method of Yi *et al.* [26]. In this assay, 0.3 mM xanthine solution, 0.6 mM EDTA solution, 150 µM nitro blue tetrazolium, 400 mM sodium carbonate buffer, pH 10.2, 1 g/L Bovine serum albumin were mixed in the ratio of 4:2:2:1.2:0.6 respectively to make SODAR. Thereafter 2.45 ml of SODAR and 0.5 ml of SOD standard or post mitochondrial fraction of homogenate were put in test tubes and 50 µl xanthine oxidase to each tube at 30-second intervals. The tubes were then incubated at room temperature for 20 minutes. The reaction in each tube was terminated by adding 1 ml of 0.8 mM copper chloride. The absorbance was then measured at 560 nm using a UV-VIS spectrophotometer.

2.7. Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined using the method of Flohe and Gunzler [27]. For this method, 100 µl of 50 mM potassium phosphate buffer pH 7.0, 20 µl of 10 mM reduced glutathione, 10 µl of 20 mM sodium azide, 20 µl of

2.0 mM NADPH in 0.1% $NaHCO_3$, and 20 µl of 10 U/ml glutathione reductase were added into a microtitre plate. Thereafter 20 µl of homogenate was added. Ten microliters of 1.5 mM hydrogen peroxide were then added. A decrease in absorbance was monitored at 340 nm. Specific enzyme activity was calculated using an extinction coefficient of 43.6 $mM^{-1} \cdot cm^{-1}$.

2.8. Catalase Activity

Catalase activity was determined using the method by Claiborne [28]. In this assay, 2900 µl of 19 mM hydrogen peroxide in 400 mM sodium carbonate buffer pH 10.2 were added into a quartz cuvette and then 100 µl of homogenate was added and mixed. The decrease in absorbance was monitored over 30 seconds at 24 nm in a Shimadzu UV-VIS spectrophotometer. Specific enzyme activity was calculated using an extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$.

2.9. Statistical Analysis

One-way analysis of variance (ANOVA) in Tukey's multiple comparison test was used to assess statistical differences between exposed samples and unexposed samples and among exposed samples at 95% level of significance using the GraphPad Prism 7.04 software.

3. Results

3.1. Acetylcholinesterase Activity

Carbaryl, dimethoate and the mixture of the two pesticides decreased the activities of acetylcholinesterase (AChE) in exposed fish and frogs. Dimethoate caused the highest inhibitions in both fish and frogs (**Figure 1**). In tadpoles, acetylcholinesterase activity was increased by exposure to carbaryl and dimethoate individually and decreased when exposed to the pesticide mixture compared to enzyme activity of tadpoles exposed to the control (**Figure 1**). The observed activations of AChE activities in exposed tadpoles were not statistically different when compared to the enzymatic activities in tadpoles exposed to pesticide-free water.

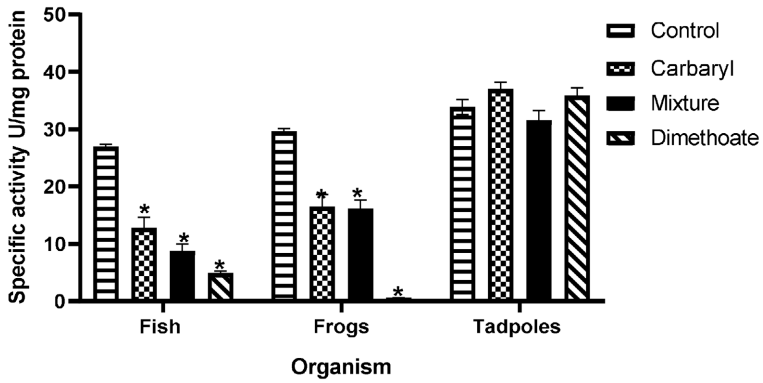

Figure 1. Effects of carbaryl and dimethoate on acetylcholinesterase in fish, frogs and tadpoles. Values represent the average of triplicate samples and these are expressed as means ± SD. The significance of the results was ascertained at $^*P < 0.05$.

3.2. Carboxylesterase Activity

Exposure of fish, frogs and tadpoles to carbaryl and dimethoate reduced their carboxylesterase activity (Figure 2). In fish, the decreases in enzyme activities were significantly different between the pesticide exposed fish and those exposed to control water at $p < 0.05$ (Figure 2). In frogs there was no statistical difference in carboxylesterase activities of frogs exposed to carbaryl and the enzyme activities of frogs exposed to dimethoate at $p < 0.05$. In tadpoles, carbaryl enhanced carboxylesterase activity compared to enzyme activity in tadpoles exposed to control water (Figure 2). Carboxylesterase activities in tadpoles exposed to dimethoate or the mixture of dimethoate and carbaryl were reduced compared to the same enzyme activities in tadpoles exposed to control water.

3.3. Superoxide Dismutase Activity

Carbaryl and dimethoate caused inhibitions of superoxide dismutase. The highest inhibitions were observed in carbaryl-exposed fish and frogs (Figure 3). The highest inhibitions in fish and frogs were 40% and 59% respectively whilst in tadpoles it was found to be in the tadpoles exposed to the carbaryl-dimethoate mixture at 64% (Figure 3). The lowest inhibition in fish was observed in fish exposed to the carbaryl-dimethoate mixture whilst the mixture caused the highest enzyme activity in tadpoles (Figure 3).

3.4. Glutathione Peroxidase Activity

Generally, glutathione peroxidase activities decreased in fish, adult frogs and in tadpoles compared to the same enzyme activities in organisms exposed to control water. In fish, the highest enzyme activity was observed in organisms exposed to the carbaryl and dimethoate mixture while in frogs and tadpoles the highest reductions were in organisms exposed to dimethoate (Figure 4). In frogs and tadpoles, there were no significant differences between organisms exposed to carbaryl and those exposed to control water (Figure 4).

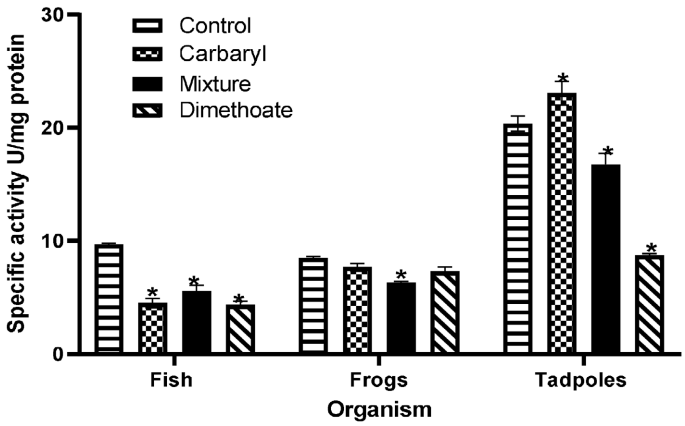

Figure 2. Effects of carbaryl and dimethoate on carboxylesterase in fish, frogs and tadpoles. Values represent the average of triplicate samples and these are expressed as means ± SD. The significance of the results was ascertained at *$p < 0.05$.

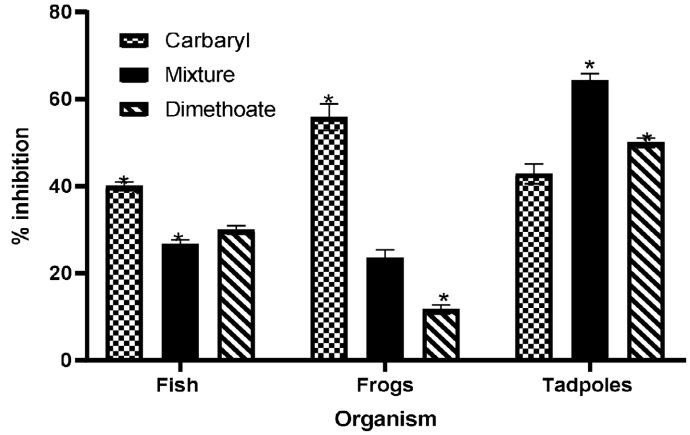

Figure 3. Effects of carbaryl and dimethoate on superoxide dismutase activity in fish, frogs and tadpoles. Values represent the average of triplicate samples and these are expressed as means ± SD. The significance of the results was ascertained at *p < 0.05.

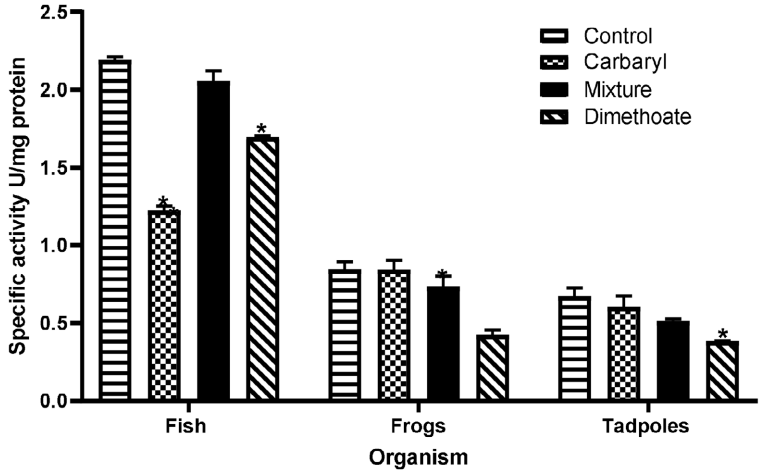

Figure 4. Effects of carbaryl and dimethoate on glutathione peroxidase activity in fish, frogs and tadpoles. Values represent the average of triplicate samples and these are expressed as means ± SD. The significance of the results was ascertained at *p < 0.05.

3.5. Catalase Activity

Catalase activity in fish and in frogs decreased in organisms exposed to the pesticides than in the same organisms exposed to control water. In fish, the highest inhibition was in organisms exposed to carbaryl while in frogs, the highest inhibition was in organisms exposed to dimethoate (**Figure 5**). In tadpoles, carbaryl and dimethoate caused activations of glutathione peroxidase activity (**Figure 5**). The highest activation of enzyme activity was in tadpoles exposed to dimethoate (**Figure 5**).

4. Discussion

Pesticides serve as the best method of choice for pest control in agriculture in Zimbabwe. Intensive use of pesticides in farming regions helps ensure maximum productivity and helps with food security. A study by Zinyemba *et al.* [29] showed

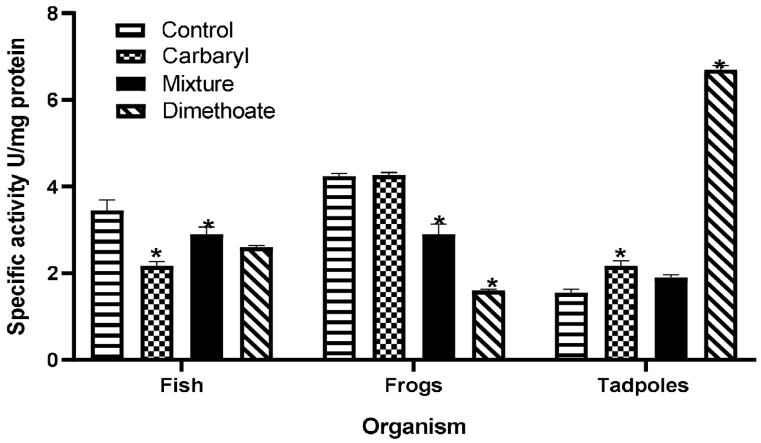

Figure 5. Effect of carbaryl and dimethoate on catalase activity in fish, frogs and tadpoles. Values represent the average of triplicate samples and these are expressed as means ± SD. The significance of the results was ascertained at *$p < 0.05$.

a significant increase in the use of pesticides by farmers in Zimbabwe over the past decades. These pesticides are washed by the surface runoffs into aquatic bodies all over the country, where they affect the organisms that reside in these water bodies. Like most countries, Zimbabwe has statutory instruments under the Environmental Management Act chapter 20:27 section 57, [30] that regulate water pollution.

Since agricultural pesticides are applied in the open environment, monitoring and enforcement of regulations on the use of pesticides pose significant challenges. This study assessed the effects of two pesticides, dimethoate and carbaryl, that are used extensively and are readily available over the counter in Zimbabwe. The two pesticides were shown to affect enzyme activity in fish, frogs and tadpoles. The extent of the enzyme alterations was pesticide, organism and age-dependent.

Cholinesterase activity in fish and frogs was inhibited by carbaryl and dimethoate (**Figure 1**). Dimethoate caused higher reductions of esterase activity than carbaryl, suggesting that dimethoate is a more potent cholinesterase inhibitor than carbaryl in the studied organisms. Frogs exposed to the carbaryl-dimethoate mixture had acetylcholinesterase activity higher than that of dimethoate but lower than that of carbaryl alone (**Figure 1**). This suggests an antagonistic interaction between the pesticides which impacted their overall effects on the enzymes of frogs when encountered as a mixture. Probably in the mixture solution, the two pesticides interfered with the other's mechanism of inhibition of acetylcholinesterase. Inhibition of AChE by carbaryl has been also reported in other organisms. Jeon *et al.* [31] reported inhibition of acetylcholinesterase (AChE) activity in the freshwater organism *Daphnia magna* exposed to carbaryl. A study by Olsvik *et al.* [32] showed inhibition of cholinesterase activity by pesticide residues in Aquafeeds on wild fish feeding on leftover pellets near fish farms.

While the studied pesticides caused inhibition of AChE activities in fish and adult frogs when compared to enzyme activities of the organisms exposed to the control water, a different trend was observed in tadpoles. Tadpoles exposed to

carbaryl and those exposed to dimethoate showed an increase in AChE activity when compared to the enzyme activity in tadpoles exposed to the control whilst the mixture of carbaryl and dimethoate caused a decrease in AChE activity compared to the enzyme activity of the tadpoles exposed to the control. However, the enzyme alterations in pesticide-exposed tadpoles were not statistically significant when compared to the enzyme activities of tadpoles exposed to the control at $p < 0.05$. The decrease in AChE activity observed in fish and frogs was expected as carbaryl and dimethoate belong to carbamates and organophosphate pesticide classes which are known inhibitors of AChE [33]. In the tadpoles on the other hand the AChE probably took the role of xenobiotic metabolizing enzymes and the organisms responded to exposure to the pesticides by increasing the synthesis of the enzymes as a protective measure. Attademo *et al.* [34] reported an increase in AChE activity in tadpoles (*Elachistocleis bicolor*) exposed to the herbicide Dicamba for 48 hours at a concentration of 0.0375 $mg \cdot L^{-1}$. However, the herbicide inhibited AChE activity at higher concentrations Attademo *et al.* [34]. A similar trend was shown by Van Meter *et al.* [14] who investigated the effects of atrazine on Southern leopard frogs (*Lithobates sphenocephala*). They reported an increase AChE activity in juvenile frogs exposed to atrazine.

Carboxylesterase activity in fish, frogs and tadpoles was generally reduced by exposure to the organophosphate dimethoate (**Figure 2**). Similar results were obtained by Cacciatore *et al.* [35] on freshwater snails *Planorbarius corneus* exposed to mixtures of the organophosphorus pesticides azinphos-methyl and chlorpyrifos. Carbaryl caused inhibition of carboxylesterase in fish and frogs whereas the same enzyme was activated in tadpoles (**Figure 2**). The mixture of carbaryl and dimethoate on frogs resulted in CbE activity which was lower than that observed in carbaryl and dimethoate individually, though the decrease was statistically insignificant at $p < 0.05$ (**Figure 2**). This was probably due to the combined individual inhibitions of carbaryl and dimethoate. In the current study, in tadpoles, carbaryl was observed to increase the CbE activity (**Figure 2**). This could be due to the tadpoles adapting to the presence of the pesticide and producing more CbE. Similar results were obtained by Ferrari *et al.* [36], who reported carbaryl-induced enzyme activity in juvenile rainbow trout (*Oncorhynchus mykiss*). Dimethoate inhibited CbE activity in fish and frogs. Similar findings were obtained by Andrés *et al.* [37] who reported inhibition of CbE activity in *Lysapsus limellum* frogs exposed to chlorpyrifos.

Carbaryl and dimethoate caused reductions in superoxide dismutase activities of exposed fish. In frogs and tadpoles, on the other hand, the same pesticides caused activation of SOD activity. This shows species' difference responses to pesticide exposure. Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to O_2- and to hydrogen peroxide (H_2O_2) [38]. Inhibition of SOD in fish observed in this study, which indicates oxidative stress, results in organisms being susceptible to reactive anion radicals, which may be generated during the metabolism of the pesticides. The superoxide anion radi-

cals generated during the metabolism of pesticides by living organisms can cause membrane lipid peroxidation and mutations leading to DNA damage [39]. Carbaryl, dimethoate and their mixtures induced SOD activities in exposed tadpoles. Similar results were obtained by Naz *et al.* [40] who reported induction of SOD activities in Labeo rohita fish exposed to endosulfan and chlorpyrifos. Oruc and Uner [41] also reported an increase in SOD activity in *O. niloticus* fish exposed to 2,4-D and azinphosmethyl.

Glutathione peroxidase activity was generally decreased in fish, frogs and tadpoles exposed to dimethoate and binary mixtures of carbaryl and dimethoate caused lower glutathione peroxidase activities in exposed fish, frogs and tadpoles than in organisms exposed to pesticide-free control water (**Figure 4**). Carbaryl caused a significantly lower GPx activity in pesticide-exposed fish than in fish exposed to the control water at $p < 0.05$. In frogs and tadpoles, the inhibition caused by carbaryl was not significantly different to that observed in organisms exposed to the pesticide. Inhibition of GPx activity after exposure to pesticides has been reported in other organisms. Matos *et al.* [42] reported inhibition of GPx activity in *Oreochromis niloticus* fish after exposure to carbaryl. A study by Oruc *et al.* [43] showed similar results where GPx activity was reduced in *Cyprinus carpio* fish exposed to 2,4-dichlorophenoxyacetic acid (2,4-D), azinphosmethyl and their mixtures.

Carbaryl and dimethoate reduced the activities of catalase in fish and frogs. A mixture of the two pesticides in fish caused lower inhibition of catalase activity than that caused by the added effects of the inhibitions caused by the individual pesticides. Catalase catalyzes the decomposition of hydrogen peroxide, produced by the dismutation of superoxide dismutase, to water and oxygen. Carbaryl and dimethoate affect catalase in other organisms. Matos *et al.* [42] reported reduced catalase activity in dimethoate exposed fish, *Oreochromis niloticus*. In the current study carbaryl and dimethoate caused activation of catalase activity in pesticide-exposed tadpoles (**Figure 5**). Similar results were obtained by Andrés *et al.* [37], who reported elevated CAT activity in *Lysapsus limellum* frogs exposed to chlorpyrifos. Activation of catalase activity was also reported in *Labeo rohita* fish after exposure to mixtures of pesticides [40]. The enhanced catalase activities suggest an adaptive mechanism by the tadpoles as means to cope with elevated levels of hydrogen peroxide generated by the increased activities of SOD in response to exposure to carbaryl and dimethoate.

5. Conclusion

Carbaryl and dimethoate caused inhibitions of esterases and the antioxidant enzymes SOD, catalase and GPx. Carbaryl and dimethoate exhibit different toxicological effects in different aquatic organisms. Tadpoles and adult frogs were affected differently by carbaryl and dimethoate. The alterations of enzymatic markers in pesticide-exposed fish and frogs observed indicated that the two pesticides affect the well-being of the aquatic organisms.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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