ASSESSMENT OF BIOCHEMICAL MARKERS AND BEHAVIOR RESPONSE OF NON-TARGET FRESHWATER TELEOST *GAMBUSIA AFFINIS* TO CARBOFURAN TOXICITY

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(Received 11th Aug 2022; accepted 11th Nov 2022)

Abstract. The objective of this study is to evaluate the toxicity of a carbamate pesticide, carbofuran, on a non-target species *Gambusia affinis* females. Fish were exposed for 15 and 40 days to two concentrations 0.191 and 0.255 mg/L corresponding respectively ¼ and 1/3 of the LC_{50}) of carbofuran. After parturition, the frequency of malformations in offspring of treated females was evaluated. In addition, we studied the effect of acute toxicity of the carbofuran at 2, 4 and 6 hours on swimming activity, and on brain AChE response. A significant increase in enzymatic activity, and Malondialdehyde (MDA) concentration, along with decreased the activity of acetylcholinesterase (AchE), reduced glutathione (GSH) and total lipid contents in a time - and dose-dependent manner was observed in carbofuran group compared to controls. Acute exposure of *Gambusia* to carbofuran induced a decrease in swimming activity. This behavioral effect was significantly correlated with the decrease in the brain's AChE activity. Exposure of females to relatively low doses of carbofuran also led to a marked increase in the level of larvae skeletal alterations. Hence, the results proved that females strongly accumulate this pesticide during the reproductive cycle, and thus its effect can be transmissible to their offspring.
Keywords: *fish, carbamate pesticide, stress biomarkers, brain AChE response, swimming activity, deformities*

Introduction

Pesticides are wide-used chemicals in current agriculture activities and are considered one of the main factors involved in environmental pollution in the world (Hamed et al., 2021; Tissot et al., 2022). Aquatic environments are principally susceptible to pesticide pollution following the processes of leaching and runoff and treated water. Seasonal agricultural and weathering activities, including precipitation, photodegradation contribute to an uneven spatial and temporal distribution of pesticide concentrations in the natural water bodies (Amri et al., 2017; Bouzahouane et al., 2018). Despite their selectivity and specific mode of action, pesticides exert their harmfulness towards organisms involuntarily exposed, following the contamination of the environment and the food chain. They are cytotoxic, neurotoxic, embryotoxic, mutagenic, teratogenic, or carcinogenic. The genotoxic effects of these chemicals can go through activation of cell metabolism, and the formation of electrophilic intermediates able to interact with nucleic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2101_545559

acids, or through induction of oxidative stress, inhibition of the cell communication, formation of activated receptors, or others (Abdulelah et al., 2020; Archer et al., 2020; Kasonga et al., 2021).

Carbofuran (2,3-dihydro- 2,2-dimethyl-7-benzofuranyl-methyl carbamate) is a worldwide used chemical and a non- persistent spectrum systemic insecticide, nematicide and acaricide (Mishra et al., 2020). Water bodies can be contaminated with carbofuran due to its groundwater ubiquity index (GUS) of approximately 4.5, which increases their risk of reaching groundwater (Mishra et al., 2020). A set of recent works have been showed the impact of insecticides and in particular of carbofuran on growth (Mansano et al., 2016; Cheghib et al., 2020), on biochemical and hematological parameters of *Clarias batrachus* (Begum, 2008; Narra, 2016) and, on fish acetylcholinesterase in brain and muscle (Rendón-von Osten et al., 2005; Mahboob et al., 2014).

However, there is a lack of information on the genotoxic, teratogenic, and neurobehavioral effects of carbofuran on non-target organisms is available. In fish, nutritional imbalances involving deficiencies of tryptophan, essential fatty acids, and vitamins have been shown to result in skeletal malformations (Roy and Lall, 2007; Garcês et al., 2020; Liu et al., 2020). Environmental factors, like water temperature, heavy metal contamination, or hydrodynamic conditions have been reported to induce marked alterations during development (Sfakianakis et al., 2015; Mendes et al., 2021; Darvishi et al., 2022). Thus, the present study was aimed to assess the toxic effects of sub-chronic exposure to sub-lethal concentrations of carbofuran on the behavioral activity via measurement of swimming and acetylcholinesterase (AChE) activity, and the antioxidant activity evaluation via determination of the major antioxidant markers, including GSH, GST, CAT, and MDA.

Materials and Methods

Site description

Gambusia affinis, is a fish belonging to the family Poeciliidae, it is viviparous of small size varying from 2 to 6 cm. The species studied was fished in the wadi kherraza (36° 53' 60" N and 7° 46' 0.001" E located in Annaba (North-East, Algeria) this area is far from any industrial activity, housing and crop fields.

Biological material

The harvested fish are transported to the laboratory in a cooler. All our experiments were conducted in aquariums with a capacity of 70 liters whose water is dechlorinated and continuously filtered (500 ml/min) and under laboratory conditions, a photoperiod 12L: 12D, temperature 17.5 ± 0.73 C, pH 7.51 ± 0.33, dissolved oxygen 13.83± 4.23 and %, Salinity 239.5 ± 21.12 mg/L). A commercial prepared diet called TETRAMIN (composed of shrimp and dehydrated fish) was given to the experimental fishes.

Experimental protocol

A static nonrenewable toxicity bioassay was conducted according to a standard method APHA-AWWA-WEF (1998) to determine the lethal concentration. The fish were divided into three experimental batches of 60 fish per batch; a control batch composed of untreated fish, two batches treated with carbofuran (99.9% purity, Cluzeau Info Labo, France) dissolved in acetone (20 mg/ml). The carbofuran solution is homogenized in the

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aquarium 15 min before the introduction of the fish. The exposure times are 15 d and 40 d at two doses of 0.191 mg/L (¼ LC_{50}) and 0.255 mg/L (⅓ LC_{50}) according to tests previously performed using a battery of concentrations from 10 to 100 mg/L. After each exposure period, the hepatopancreas, and brains of the fishes are removed under ice, weighed and stored at -80°.

Preparation of homogenate and measurement of enzyme activities

Hepatopancreas and brains from each batch were pooled at 100 mg and homogenized in saccharose sodium phosphate buffer at pH 6.5. The sample homogenates were centrifuged at 5000 rpm for 15 minutes; the resulting supernatant was recovered for assay of total protein, lipids, malondialdehyde (MDA), reduced glutathione (GSH), GST activity, catalase (CAT), and acetylcholinesterase (AChE).

Metabolite analyses

Protein levels were quantified by the method of Bradford (1976) method using coomassie brilliant blue (BBC). Total lipid levels have been determined as previously described (Goldsworthy et al., 1972) using sulfo-phospho-vanillin assay. The absorbance was read in the dark at 530 nm. Lipid peroxidation was estimated by the method of Draper and Hadley (1990) based on the determination of thiobarbituric acid reactive species (TBARS), using 1, 1, 3, 3 tetra ethoxypropane as standard. The content of MDA was spectrophotometrically determined and expressed as nmol /mg protein.

Biomarkers analysis

The concentration of reduced glutathione (GSH) is quantified by the colorimetric method of Weckberker and Cori (1988), based on measurement of 2-nitro-5-mercapturic acid derived from the reduction of 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) by the SH thiol groups of glutathione measured at a wavelength of 412 nm. Glutathione S-transferase (GST) activity was measured by as described elsewhere (Habig et al, 1974) using a mixture solution of 0.1 M potassium phosphate buffer (pH 6.5) and 1 mM GSH, 1 mM CDNB. The enzymatic activity is displayed in nmol min-1 mg-1 protein. The catalase activity was determined as described by Aebi (1984). The absorbances were measured at 240 nm by the change in optical density following the hydrolysis of hydrogen peroxide (H_2O) by reacting 200 µL of H_2O and 20 µL of homogenate in phosphate buffer (100 mM, pH 7.4) for 1 min at an incubation temperature of 25°C. The enzymatic activity was given in nmoles of H_2O/ minute/ milligram of protein. Acetylcholinesterase (AchE) activity was evaluated as previously described (Ellman et al., 1961). through recording the sample absorbance after 5 min at 412 nm in the presence of 1mM acetylthiocholine, which would be hydrolyzed to acetic acid and thiocholine providing yellow product named TNB (5-thio-2 nitrobenzoic acid) in the presence of DTNB (5'-dithio-bis-2-nitrobenzoic acid). AChE activity was expressed in nmol min^{-1} mg^{-1} protein.

Measurement of swimming activity

The swimming activity of control and treated fish was determined by the method of Bretaud et al. (2001), Sousa and Nunes (2020). Visual cues were placed on the aquarium windows to determine 4 observation areas of 40x40x31cm to allow quantification of movements. Each observation involved groups of 5 naïve individuals (tested once). During 6 hours of exposure, fish movements were monitored every 2 hours for a period

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DOI: http://dx.doi.org/10.15666/aeer/2101_545559

of 15 min, with a positioning record every 30 seconds. During the exposure, fish movements were tracked and recorded using high-speed recording system with a camera (Uxsiya, Ref xetwbk62fg-02) and LED lights, which allowed us to accurately calculate the movements of the specimens. The movement activity is given by the number of individuals that moved from one area to another during the 15mn of observation. The concentrations tested and the controls were the same as for the neurochemical activity measurements. Each treatment was carried out five times.

Skeletal deformity

The estimation of skeletal malformations was carried out on aged fry. For the identification of skeletal malformations, the method improved by Staples (1964) based on the use of alizarin red was used. Fry from control and treated females were placed in absolute ethanol (99%) for 48h and then placed in a solution composed of ethanol (85%), KOH (1.5%) and alizarin red (0.0015%) for 5 days. The fry was macerated in an aqueous solution of KOH (1%) until total degradation of the tissues and their skeletons will be clearly visible. The specimens will be preserved in glycerol (50%) for microscopic observation.

Statistical analysis

The results were displayed as means and standard error. We used the two-criteria analysis of variance test "ANOVA" followed by the "Tukey" test to compare the means between them on the one hand and between those of the controls on the other hand. The variations are considered significant when, after this test, the significance has a probability greater than 95% ($p < 0.05$). All tests were performed using Statistica version 10.1 software.

Results

Effect of carbofuran on metabolic parameters (total lipids and MDA)

The results shown in *Figure 1* reveal that treatment of *Gambusia affinis* females with carbofuran induces a significant decrease in hepatopancreatic lipids. This variation was found to be dose and time-dependent ($p<0.001$). This decrease in lipid levels is associated with lipids peroxidation revealed by an increase in MDA levels after 15 days at LC_{50} ¼ and LC_{50} ⅓ respectively. The ANOVA test shows a highly significant dose and time effect ($p<0.001$) and a dose-time interaction ($p<0.001$).

Effect of carbofuran on biomarkers

Reduced glutathione level

Our results show a reduction in hepatopancreatic GSH content in females exposed for 15 and 40 days to both doses 0.191 mg/L and 0.255 mg/L, with reductions ranging from 27% to 33% after 15 days and 37% and 48% after 40 days of treatment in comparison with the control ($p<0.05$, $p<0.001$, *Figure 2A*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2101_545559

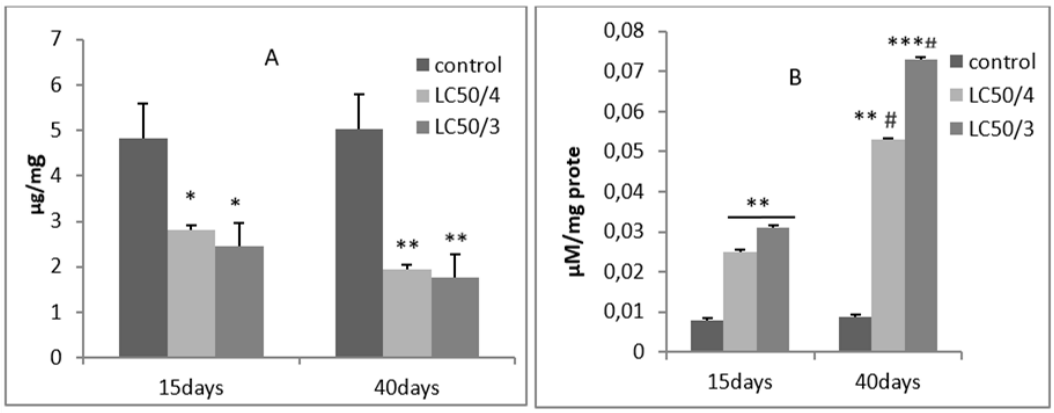

Figure 1. *Lipids (A) and malondialdehyde (B) content in Gambusia affinis hepatopancreas as response to carbofuran exposure. Values are expressed as means ± SEM (n = 5). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs control and # $p<0.05$ vs days 15*

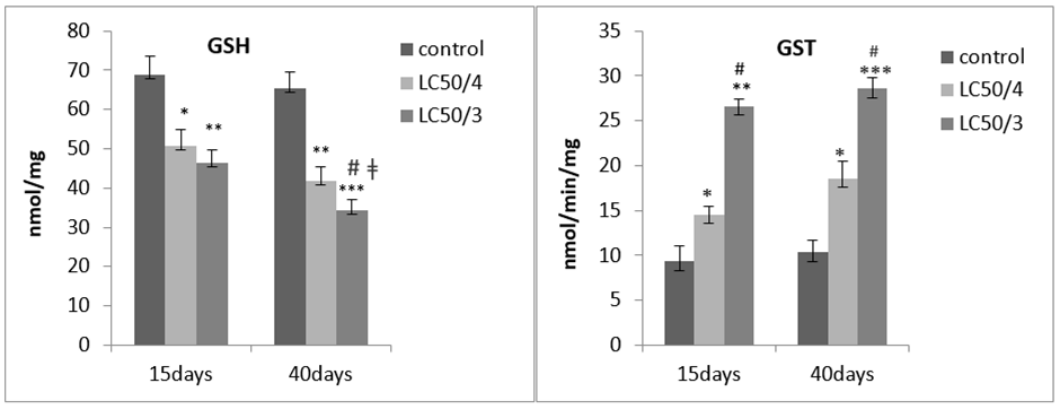

Figure 2. *GSH content (nmol/mg protein) and enzymatic activity of GST (nmoles C-DNB conjugate formed/min/mg protein) in Gambusia affinis. Values are given as the mean ± SEM of 5 specimens. Significant difference compared to the control group ($^{**}p < 0.01$, $^{*}p < 0.05$); Significant difference compared to the carbofuran LC50/3 treated group ($^{\#}p <0.05$) and ǂ $p< 0.05$vs 15 days treated group*

Glutathione S-transferase activity

According to *Figure 2*, the results reveal an increase in hepatopancreatic GST activity in treated females compared with a control. The GST activity increased significantly from day 15 for both two concentrations tested ($p < 0.0001$; *Figure 2B*). The highest activity was noticed at day 40 for 0.255 mg/L concentration. The ANOVA test reveals a highly significant time effect ($F_{4.39} = 189.7$ $p < 0.0001$), treatment effect ($F_{2,32} = 113.8$; $p < 0.0001$) and time/treatment interaction ($F_{9.48} = 21.31$, $p < 0.0001$).

Catalase activity

We found an increase in catalase activity in females treated with 0.191 mg/L and 0.255 mg/L of carbofuran compared to control females. Tukey's test showed a significant difference ($p<0.05$) for 0.191 mg/L after 40 days of treatment, while highest activity was recording at 0.255 mg/L after 15 and 40 days ($p < 0.01$, *Figure. 3A*).

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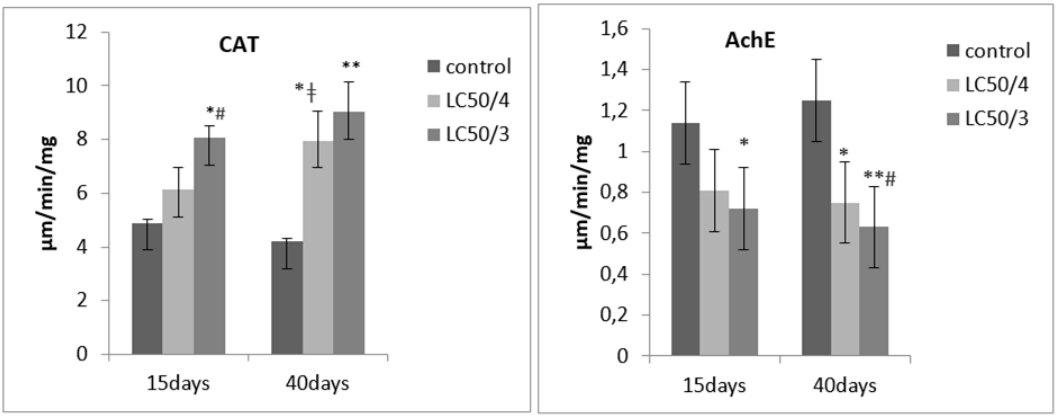

Figure 3. *Catalase (CAT) activity (µmol H_2O_2/min/mg protein) and acetylcholinesterase (AchE) (µmol/min/mg protein) in Gambusia affinis. Values are given as the mean ± SEM of 5 specimens. Significant difference compared to the control group ($^{**}p < 0.01$, $^{*}p < 0.05$); Significant difference compared to the carbofuran treated group $^{\#}p < 0.05$ and ǂ $p < 0.0$ vs 15 days treated groups*

Acetylcholinesterase activity

Brain acetylcholinesterase activity was strongly inhibited after exposure of females to 0.191 and 0.255 mg/L carbofuran compared to the control. This inhibition corresponded to a reduction of about 28.84% and 52.18%, respectively after 15 days and about 40.1% and 62.2% after 40 days of carbofuran exposure (*Figure 3B*).

Neurochemical and behavioral response to carbofuran

AChE activity and swimming activity were determined in the fish brain at different time (2, 4, and 6 hours) during the exposure period as shown in *Figure 4*. The activity of AChE was reduced as the concentration and duration of carbofuran exposure increased. The inhibition of AChE activity was increased from 26% to 49.1% with the dose of 0.191 mg/L after 6 hours of exposure. At the same periods, inhibition was more significantly increased from 42% to 60.3% with the highest concentration (0.255 mg/L). This variation in AChE was affected the swimming activity of treated females, where a reduction in swimming activity was recorded after 2 hours of exposure in females exposed to 0.191 mg/L and 0.255 mg/L. A decreased in swimming activity was a time and concentration-dependent (*Figure 4*). The ANOVA two-way test show a significant decrease after 4 h of exposure to 0.191 mg/L, 0.255 mg/L ($p<0.05$) and very significant after 6 h ($p<0.001$).

Effect of carbofuran on offspring deformities

The spinal deformities observed in the larvae are shown in *Figure 5*. Two types of spinal deformations were found, a kyphosis (dorsal curvature) and lordosis (Lordosis ventral curvature). The percentage of deformities represented in *Table 1*, show that the kyphosis is highest in the fry of females treated with both two concentrations of carbofuran (20% at 0.255 mg/L and 12.5% at 0.191 mg/L). Furthermore, comparison of the frequency of malformed fry shows that the highest concentration (0.255 mg/L) causes more deformities.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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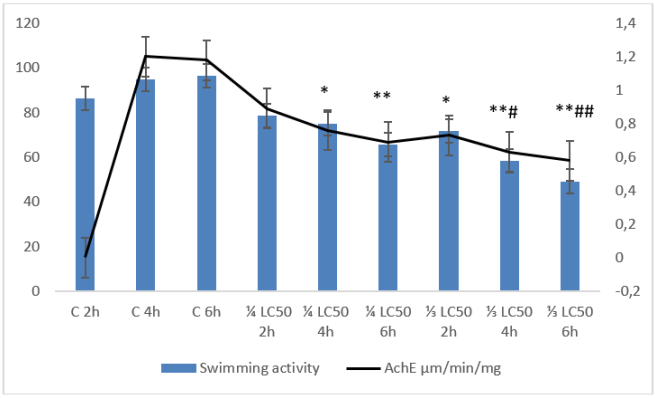

Figure 4. *Swimming and AchE activity of adult female Gambusia affinis after 2, 4 and 6 hours of exposure to carbofuran Values are given as the mean ± S.E.M of 5 observations. Significant difference compared to the control group ($^{*}p<0.05$, $^{**}p<0.01$); Significant difference compared to the treated group w ($^{\#}p<0.05$, $^{\#\#}p<0.01$)*

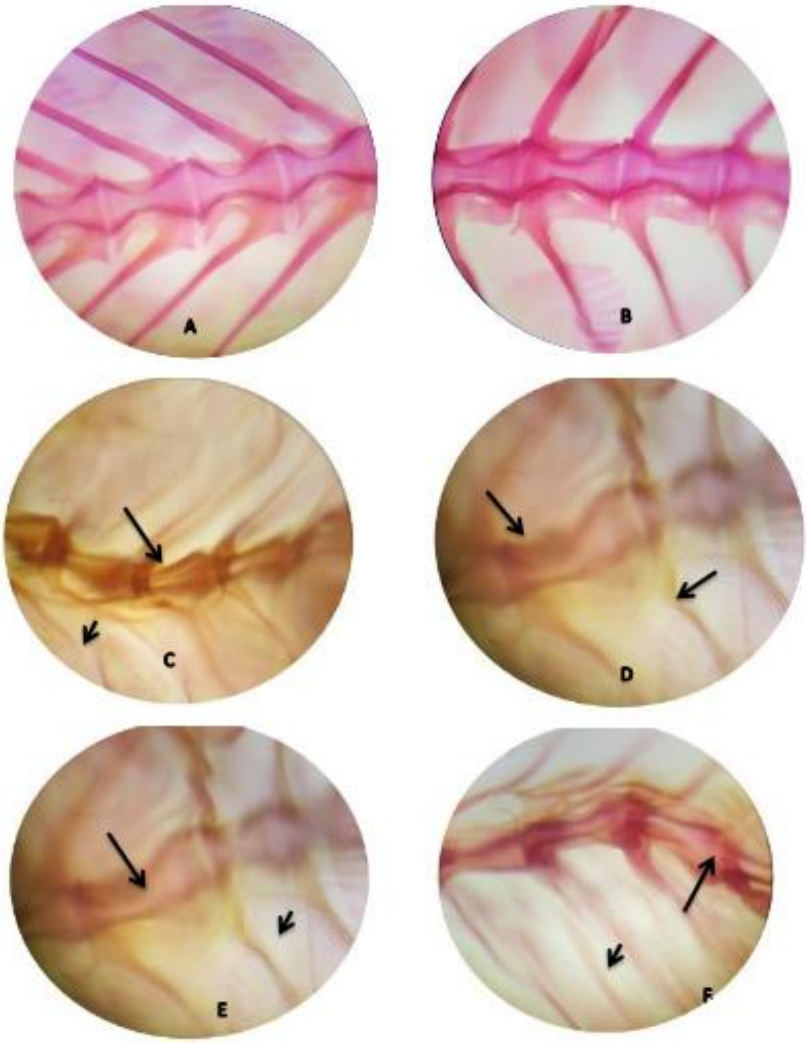

Figure 5. *Red alizarin stained slides of spine representation, shown deformities in juvenile Gambusia affinis exposed for to carbofuran. A and B Normal spines; C, D and E bifurcated neural and hemal spines (arrow). C and F broken hemal spine (small arrow). Manification 50X*

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DOI: http://dx.doi.org/10.15666/aeer/2101_545559

Table 1. *Spinal deformity incidences of juveniles from control and exposed females to 0.191, and 0.255.66 mg/L carbofuran*

	Control		**¼ LC_{50} 0.191 mg/l**		**⅓ LC_{50} 0.255 mg/l**	
Class of deformities	Kyphosis	Lordosis	Kyphosis	Lordosis	Kyphosis	Lordosis
% of deformities	0%	2.5%	12.5% *	7.50% *	21%*#	10%*#
Total deformities	**2.5%**		**20%***		**31%***	

Number of specimens examined = 30 in each group; * p< 005: significance from Control group; #P<0.05: significance from ¼ LC_{50} group

Discussion

In this work we tested two doses of carbofuran ⅓ and ¼ LC_{50}, a carbamate pesticide that can be extremely toxic to fish (Barbieri et al., 2016; Mendes et al., 2021). Our results reveal that exposure of *G. affinis* to carbofuran-induced concentrations and time-dependent variations in GSH levels and GST activity; we found a decrease in GSH levels in the hepatopancreas by both two concentration tested (⅓ and ¼ CL_{50}), starting at 15 days of exposure, this level continues to decrease until the end of the treatment. This result is similar with that observed by Akram et al. (2021) in fresh water bighead carp. A decrease of hepatic and renal glutathione (GSH) was also detected in the African catfish C*larias gariepinus* after its exposure to carbofuran (Hamed et al., 2017), and in liver, kidney, gills, gonads and muscles after 35 days of exposure (Ibrahim and Harabawy, 2014).

Glutathione S-transferases (GST) are belonged to the key antioxidant enzymes of the antioxidant defense system (Yang and Lee, 2015). Indeed, in phase II of biotransformation, GST enters mainly in detoxification process of xenobiotics, including herbicides and pesticides (Van der Oost et al., 2003; Higgins and Hayes, 2011) and endogenous chemicals able to conjugate with glutathione (Domingues et al., 2010). In this study, we noted an elevation in GST activity accompanied with a reduction in glutathione content. This adaptive response could be due to the solicitation of the phase II process of pesticide biotransformation in response to a substantial increase in ROS, as recently reported by Demirci and Güngördü (2020). Similar results were found in *Gambusia holbrooki* after 24 h and 96 h of exposure to acetamiprid and in *Oreochromis niloticus* exposed to 2-4- D (Oruc and Uner, 2004; Demirci, and Güngördü, 2020). Furthermore, Jiang et al. (2016) found that mRNA level of gstp1 correlates better with the activity of the GST enzyme, which hence can be controlled by the interaction of phase II products activating the regulation of antioxidant enzyme transcription and mainly that of GST.

The mechanisms of free radical detoxification are associated with a cascade of biochemical events involving the mixture of several enzymes such as CAT. CAT with superoxide dismutase SOD can be defined as the main defense line against the damaging effect caused by ROS overproduction and in particular the superoxide anion in response to chemical aggression (Hermes-Lima, 2004; Laouati et al., 2021). In addition, results showed a significant induction of CAT activity in *G. affinis* exposed to carbofuran, this increase is concentration-dependent. This response is likely explained by the catalytic activity of this enzyme to convert hydrogen peroxide H_2O_2 to water and oxygen (Amri et al., 2017). These results are in agreement with those of Nunes et al. (2004), reporting that

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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exposure to clofibrate and led to significant CAT activity in *Gambusia* and *Daphnia magna*. Other previous studies conducted on adult fish showed that exposure to 2,4-D and zinphosmethy lead to increase CAT activity (Oruç and Üner, 2000; Oruc et al., 2004; Atamaniuk et al., 2013).

The variations that were recorded during this study regarding biomarker responses clearly indicate that carbofuran induce an imbalance between antioxidants and pro oxidants, this is supported in our results by a marked decrease in total lipids and an increased level of MDA in fish exposed to different concentrations of carbofuran. Lipid peroxidation (LPO) is a potential parameter of oxidative stress damage (Ibrahim and Harabawy, 2014). MDA is the end product of lipids peroxidation, and a marker of oxidative stress and cell membrane components impairment (Kuder and Gundala, 2017). In this study, the MDA concentrations was increased in time-and concentration dependent manner. Consequently, the lipid tissue of *G. affinis* can highly accumulate the pesticide. Previous studies have also reported that *Clarias batrachus* and zebrafish *Danio rerio* exposed respectively to LC_{50} (7.66 mg/L) of carbofuran and 25 mg/L of 2,4-D induce malondialdehyde (MDA) accumulation (Begum, 2008; Li et al., 2017).

Acetylcholinesterase is a good indicator of neurotoxicity, at the neuromuscular and interneuronal junctions; the nerve impulse is transmitted through the release of acetylcholine. The inhibition of AChE by toxicants leads to a storage of chemical mediated in the synaptic spaces, maintaining a permanent transmission of the nerve impulse, which usually induces muscle tetany and death (Hernández-Moreno et al., 2011; Harabawy and Ibrahim, 2014). AChE activity is used in toxicology as organophosphates and carbamates toxicity marker (Fossi et al., 2001; Narra, 2016). In this study, we observed an inhibition of AChE activity of about 30% started from the 15^{th} day of treatment in *G. affinis* exposed to carbofuran, this inhibition is progressive until the end of treatment. Carbamate insecticides are specific inhibitors of AChE in several freshwater fish (Pessoa et al., 2011; Nunes et al., 2014; Ensibi et al., 2014). Similar results to ours have shown that treatment with organophosphate insecticides cause a decrease of AChE activity in the hemolymph after 24 h of exposure in the blue mussel *Mytilus edulis* (Rickwood and Galloway, 2004). In the same context Narra (2016), reported a decrease in AChE activity in the brain of Clarias batrachus after Sub-acute exposure $1/10^{th}$ of LC_{50} for 60 days to carbofuran.

Our findings also revealed that carbofuran appears to work in a time-and concentration dependent manner, as the inhibition of AChE rises with increasing carbofuran concentration and exposure time (30% at 0.191 mg/L on day 15 and 50% at 0.255 mg/L on day 40). Bretaud et al. (2001), revealed that the inhibition of AChE by carbofuran is concentration dependent, but several studies have reported that the inhibition of AChE activity is time dependent (Amblard et al., 1998; Legierse et al., 1999; Liu et al., 2013).

In this study, we tried to investigate the correlation between carbofuran AChE inhibition and locomotors behavior. Acute exposure to carbofuran causes a significant decrease in swimming activity after 4 h for both doses in *G. affinis*, this decrease is very significant after 6 h of exposure to the highest concentration, this disorder of swimming activity is accompanied by an inhibition of AChE activity, the activity of AChE was increased from 26% to 49.1% at 0.191 mg/L respectively after 2 h, 4 h and 6 h of exposure. At the same periods the inhibition rates are more important in the poisons treated with the highest dose 0.255 mg/L. These observations suggest the existence of a correlation between the concentration of carbofuran, AChE activity and swimming behavior. Similar results have been reported in *Carassius auratus* treated with various

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concentrations of carbofuran (Bretaud et al., 2001) and in *Gambusia yucatana* exposed to chlorpyriryfos, cargofuran and glyphosate (Rendón-von Osten et al., 2005). We found through the results obtained that the activity of the brain AChE in *G. affinis* is more sensitive to carbofuran compared to swimming activity. Indeed, the impact of carbofuran on swimming activity is only observed when AChE activity is inhibited at 44.2%. In this sense, a few works have reported that at concentrations near to lethal toxicity values, disturbances of locomotors behavior and swimming speed were observed in fish in response to exposure to carbofuran (Hernandez-Moreno et al., 2011; Pessoa et al., 2011; Campos-Garcia et al., 2015; Mendes et al., 2021).

Can the injurious effects observed after exposure *G. affinis* to carbofuran affect their offspring? Undeniably, the results obtained on the morphology of the juveniles of fish exposed to different concentrations of carbofuran clearly confirm this hypothesis. The effects of carbofuran can be transmitted to the offspring. Our results show an induction of spinal skeletal deformations (Kyphosis and Lordosis) in the larvae of *G. affinis* treated females compared to control and that the rate of these skeletal deformations is concentration-dependent.

In fish, the young stages are particularly sensitive to environmental contamination and can lead to the appearance of malformations, in particular deformations of the vertebral column (Messaoudi et al., 2009). Several studies have shown that skeletal deformation is strongly correlated with external stresses (Messaoudi et al., 2008). Thus, exposure to certain toxic, potentially teratogenic substances such as organophosphate herbicides and pesticides, carbamates, dioxin, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and heavy metals such as zinc and cadmium can alter the processes of bone formation and development and consequently lead to skeletal deformities (Koyama, 1996; Olsson et al., 1999; Cheng et al., 2000; Teraoka et al., 2002; Teh et al., 2002). These skeletal deformations are relatively well described in several fish species; however, the mechanisms of their induction are poorly studied. Some Studies have shown that these deformations are caused by an imbalance in phosphocalcic metabolism leading to decalcification of bones and resulting in their fragility (Muramoto,1981; Sato et al., 1983; Akiyama et al., 1986; Dabrowski et al., 1990; Kessabi et al., 2009).

Our results indicated an increase in the rate of skeletal deformations in larvae of the females treated with different concentrations of carbofuran. This suggests that carbofuran may have a potential negative effect on the molecular processes and mechanisms regulating the development and growth of bone tissue in fish. As a result, carbofuran could have teratogenic effects on *Gambusia affinis* larvae. Our results are confirmed by the work of Pawar and Katdre (1984) that showed a toxic and a teratogenic effect in *Microhyla ornat tadpoles* exposed to carbofuran, namely, body axis body curvatures, growth retardation and behavioral disturbances. In the same way Sassi et al. (2010) showed that cadmium treatment at high temperature (32°C) affects the embryos of *G. affinis* and causes an increase in frequency of Kyphosis and Lordosis.

In this study, the multi-marker approach at various levels of biological organization enabling us to understand the potential of *G. affinis* as a bioindicator species of pollution and gives a reliable prediction of the adverse effects of pesticides and emerging contaminants in aquatic organisms. In summary, it can be concluded that low dose exposure to carbofuran can induce oxidative stress in *G. affinis*. We observe increased activity of enzymatic antioxidants in response to carbofuran, justifying the adaptive capacities of this non-target species to environmental stresses. In this study, fish swimming activity and AChE activity are correlated and support a neurotoxic effect of

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DOI: http://dx.doi.org/10.15666/aeer/2101_545559

carbofuran however, AChE is more sensitive to the pesticide even at a low concentration. These effects may alter the health of aquatic organisms and suggest that carbofuran may be a potentially dangerous aggressor on non-target organisms.

Conclusions

The exposure of females of *Gambusia affinis* to carbofuran can affect their offspring, resulting in an increased incidence of spinal deformities. Therefore, the effects of carbamate become important in assessing the teratogenic effects on non-target organisms. Additionally, these results provide sufficient knowledge for further research studies on the impact of environmental factors responsible for bone deformations and in particular parameters related to phosphocalcic metabolism in fish.

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