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Toxic effects of Carbofuran on Duttaphrynus Melanostictus Larvae

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ABSTRACT

This study investigated effects of the carbamate pesticide carbofuran on larvae of the Asian Common Toad *Duttaphrynus melanostictus*. Tadpoles were continuously exposed to four concentrations of carbofuran (50 - 500 μgl^{-1}) for 15 days during which mortality, growth, development, and swimming activity were monitored. In a separate trial, the liver and tail muscle tissues were examined for histopathological changes after one week of exposure to 250 and 500 μgl^{-1}. The study revealed that carbofuran at the tested concentrations induced significantly high levels of mortality in *D. melanostictus* tadpoles. Trends in mortality depicted a bell-shaped curve indicating a hormetic response. The $LC50_{1-15\ days}$ ranged from 1865 – 152 μgl^{-1}. Effects on growth, development and swimming were transient. A few tadpoles exposed to 250 μgl^{-1} and above showed swelling of the head region. Exposure to carbofuran also caused severe histological alterations in the tail muscle tissues, while the liver tissue was also affected, but to a lesser extent. Greater vacuolation in hepatocytes, sinusoidal dilations and the formation of bile plugs were observed in the liver of treated larvae whilst tail muscles were substantially disintegrated as a result of exposure to carbofuran. The observed harmful effects induced by carbofuran in the present study suggest that *D. melanostictus* populations may face detrimental consequences as a result of exposure to this pesticide.

Keywords: Carbamate, carbofuran, *Duttaphrynus melanostictus,* histopathology, pesticide, tadpole, toxicity

1. Introduction

A diversity of pesticides and their residues have been recorded in a wide variety of aquatic habitats (McConnell et al., 1998) exposing the inhabitants to these harmful substances. Amphibians, in comparison to other terrestrial vertebrates, are more susceptible to aquatic contaminants because of their aquatic larval stage, greater permeability of the skin and the presence of gills during the larval stage (Blaustein et al., 2003). Not surprisingly, pesticides have been identified as one of the major contributory factors for the worldwide decline of amphibian populations (Davidson et al., 2001). Many studies have confirmed that pesticides cause mortality, impairments in growth and development, and abnormalities in amphibian larvae (Eg. Bridges, 2000; Johansson et al., 2006; Relyea and Jones, 2009), while some have shown that histopathalogical alterations occur in important tissues (Colombo et al., 2005; Honrubia et al., 1993).

Sri Lanka, being an agricultural country and a unique refugium for amphibian species is particularly at risk from pesticide use. It is suspected that the intensified use of pesticides in recent times has contributed to the extinctions, range reductions, and population declines in the country's amphibian fauna (IUCN, 2006). Evidence for such effects is nevertheless

lacking. In this study we investigate the effects of a commonly applied carbamate pesticide, carbofuran, on the survival, growth, activity and histology of larvae of the Asian Common Toad *Duttaphrynus melanostictus* Schneider 1799. Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum carbamate widely used in Sri Lanka for pest control in agriculture (Tennakoon et al., 2009). Because of its high water solubility and low adsorption coefficient, carbofuran is present in surface runoff and has the potential to accumulate in lakes, streams and groundwater sources (Nicosia et al., 1991). The test species *D. melanostictus* is a bufonid that is frequently found in association with agricultural landscapes (Srinivasulu and Das, 2008). It is also distributed among other Asian countries making the results of this investigation widely applicable.

2. Materials and Method

2.1 Selection of pesticide concentrations

Since information on field levels of carbofuran is not available, test concentrations were based on preliminary laboratory observations and on concentrations used in similar exposure trials conducted in Sri Lanka (Pathiratne et al., 2008) and elsewhere (Bretaud et al., 2002, Stephenson et al., 1984). All test concentrations were prepared using a commercial preparation of carbofuran sold under the trade name Curaterr 3G purchased from Hayleys Agro Products Ltd, Colombo, Sri Lanka. The formulated product was used for the exposure trials to simulate field conditions.

Two separate exposure experiments were performed to investigate toxic effects. In the first, tadpoles were repeatedly exposed to four concentrations of carbofuran i.e. 50, 150, 250 and 500μgl^{-1} and monitored for 15 days to examine effects on larval survival, growth, development and swimming activity. In the second, tadpoles were exposed to the two highest concentrations of carbofuran i.e. 250 and 500μgl^{-1} and the liver and tail muscle tissues of larvae surviving a repeated exposure of seven days were examined for histopathological changes. A separate trial was necessary because observations of histological damage required the sacrificing of tadpoles.

2.2 Experimental procedure

D. melanostictus tadpoles of Gosner stages 24-25 were collected from three home garden ponds in widely separated areas within the city of Colombo. This ensured that the larvae collected were from different populations and that the larvae were from uncontaminated sources. The larvae were identified as those of *D. melanostictus* by their external morphology and teeth arrangement (Kirthisinghe, 1955). Glass tanks of 25 x 25 x 15 cm containing 2 L of tap water were used for the exposure trials following the methods of Sumanadasa et al., 2008 and Wijesinghe et al., 2010. The tap water was aged for 48 hrs for any chlorine to dissipate. Eighteen larvae (six from each clutch * 3 clutches) were randomly assigned to each tank. Tadpoles were staged according to a standard key (Gosner, 1960) under a dissecting microscope (NIKON SM5 610514, Tokyo, Japan), and snout-vent length of each tadpole was measured using a digital venier caliper (COMECTA, Electronic digital venier caliper, Barcelona, Spain). The relevant pesticide concentrations were then added to the tanks and mixed using a glass rod. Treatment (with pesticides) and control (without pesticides) tanks were maintained in triplicate. The larvae were fed once a day with fish food pellets (Blue Aqua pets, Godagama, Sri Lanka). Fifteen pellets (46 ± 4 mg, n=10) were added to each tank at the beginning of the experiment, with the amount of food being increased as tadpoles grew,

whilst adjustments were made depending on the number of surviving larvae. The tanks were aerated with a constant flow rate throughout the experimental period. In both trials water and relevant pesticide concentrations were renewed every three days and the experiments were conducted at ambient temperature (26 - 28°C) and under natural light conditions (approximately 12 hours light and 12 hours dark). Water quality parameters pH, temperature and dissolved oxygen were measured a day after each water change and pesticide renewal, using a HM-30v meter (TOA electronics Ltd, Tokyo, Japan) and a (HANNA HI 9142 Romania) DO meter. Light intensity above each tank was also measured using the Lux meter (S/N-LM2-1416 250A, USA). In the first trial, mortality in each tank was monitored daily and the tanks were examined for deformed or otherwise abnormal tadpoles. Body length measurements, staging (according to Gosner. 1960), and recording of swimming activity were conducted every three days during the 15 day experimental period. Swimming activity was assessed using a protocol described by Sumanadasa et al., 2008.

In the second trial live tadpoles were randomly caught from each of the two treatments and the control at the end of one week of exposure to carbofuran. The tadpoles were immediately fixed in Zenker fixative overnight, washed thoroughly in running tap water, dehydrated in series of ethanol and xylene, and embedded in Paraffin wax. Longitudinal sections (8µm) were obtained using a Rotary microtome (YAMATO, Tokyo, Japan) and stained with haemotoxyline and eosin following standard procedures (Dietrich et al., 2009).

3. Results and Discussion

3.1 Effects on survival

This investigation demonstrated that carbofuran at concentrations of 50 μgl^{-1} and above caused marked elevations in mortality in *D. melanostictus* tadpoles, with over one-third of the exposed larvae dying as a result of exposure. The Split-plot design repeated measures ANOVA and post-hoc tests were used to examine the variation in mortality with pesticide concentration and time. Mortality in the control tanks was low (less than 5 %), and mortality in all the tested concentrations was significantly higher (Treatment $F_{4,10} = 6.68$, $P<0.01$; Time $F_{12,120} = 7.2$, $P <0.001$; Treatment * Time, $F_{48,120} = 1.63$, $P < 0.05$). The lethal effects of carbofuran are mainly attributed to the direct inhibition of acetylcholine esterase activity at the central cholinergic synapses and neuromuscular junctions, which causes respiratory failure (Hayes et al., 1991) and ultimately death. The mechanism of action of carbofuran is mediated via carbamylation of the hydroxyl group of serine residue at the active site of acetylcholine-esterase (Hayes et al., 1991). Although mortality showed a dose-dependent trend (Pearson's correlation r = 0.834, P<0.05) at the three lower concentrations (50, 150 and 250 μgl^{-1}), mortality at the highest concentration of 500 μgl^{-1} (35 %) was markedly lower than that observed at 250 μgl^{-1} (63 %), giving a bell-shaped curve (Figure 1). This dose-response is known as hormesis (Calabrese and Baldwin, 2002) and has been also observed in other studies with pesticides (E.g. Samayawardhana et al., 1996; Storrs and Kiesecker, 2004). The hormetic response is reported to be more commonly observed when organisms are exposed to endocrine disruptors (Cavieres et al., 2002; Gupta et al., 1999; Salaberria et al., 2009), although the exact mechanisms are still not properly understood.

Due to the observed hermetic response mortality at 500 μgl^{-1} was disregarded when calculating the LC50 values. The Probit Analyses generated LC50 values for days 1, 2, 4, 6 and 15 were 1865, 693, 237, 193 and 152 μgl^{-1} respectively. With regard to relative

sensitivities shown by different taxa to carbofuran, larvae of *D. melanostictus* had a LC50 $_{4\ day}$ of 237 μgl^{-1} while that of the two fish species, Blue gill sun fish and Rainbow trout, were

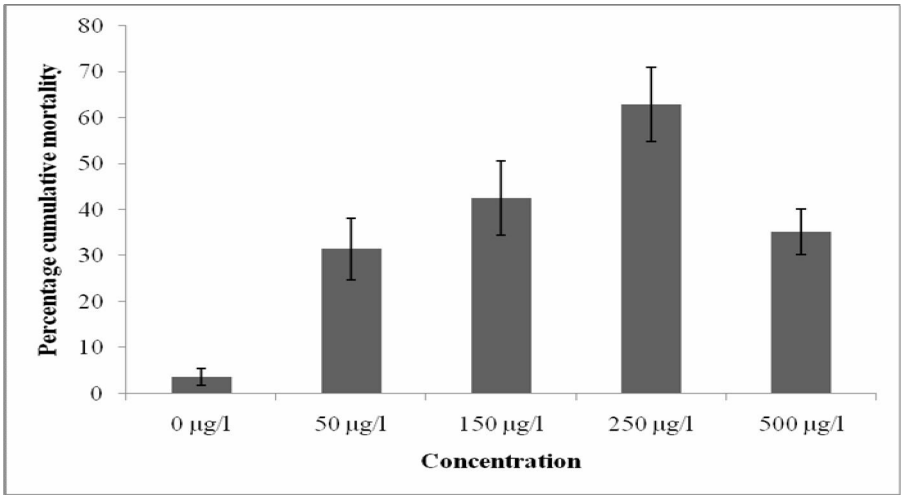


Figure 1: Mortality (± S.E) in *D. melanostictus* larvae, repeatedly exposed to four concentrations of carbofuran for 15 days. Note: The values represent means of three replicates. (all the tested concentrations had significantly higher (P<0.05) mortality levels than the controls).

240 and 380 μgl^{-1} respectively (EXTOXNET, 2001), indicating a similar sensitivity. Considering the sensitivity of *D. melanostictus* tadpoles to carbofuran in comparison with its sensitivity to other pesticides shows that, the tested carbamate is more toxic than the organophosphate pesticide chlorpyrifos (LC50 $_{4\ day}$ = of 2094 μgl^{-1}) (Wijesinghe et al. 2010), but that it is less toxic than the organochloride pesticide endosulfan (LC50 $_{4\ day}$ = of 123 μgl^{-1}) (Vardia et al., 1984).

3.2 Effects on growth and development

In contrast to mortality, the adverse effects of carbofuran on growth and development, where they occurred, were transient (Table 1). At the end of the first week the mean length of tadpoles exposed to 250 μgl^{-1} was 5.6 mm (± 0.5), while those in the controls were larger 6.5 mm (± 0.02). Nevertheless, after two weeks of exposure to 250 μgl^{-1}, the few surviving tadpoles were larger than those in the control. The Split-plot design repeated measures ANOVA and post-hoc tests were used to examine the variation in growth with pesticide concentration and time. These analyses revealed that there was no significant difference in growth with concentration although a significant difference was noted in growth patterns of tadpoles in different concentrations over time (Treatment $F_{4,10} = 0.86$, P=0.51; Time $F_{3,\ 30} = 614.06$, P <0.0001; Treatment * Time, $F_{12,30} = 2.72$, $P < 0.01$). There was no significant difference in the initial length of the tadpoles used for the experiment (One-Anova, $F_{4,\ 10} = 0.02$, P> 0.05). Although tadpoles in the control tanks developed at a faster rate than those in the treated tanks until about day 7, the pattern was reversed thereafter and no differences were evident in the growth stages of the treated and untreated tadpoles at the end of the trial. None of the larvae in the control or treatment tanks reached metamorphosis by the end of the experimental period. Impairment of growth and development generally occurs as a result of decreased feeding activity and the high rate of metabolism in tadpoles exposed to environmental stressors such as pesticides (Rowe et al., 1998). Growth retardations in

amphibian larvae have been observed with other carbamate pesticides such as carbaryl (Relyea, 2003) and with organophosphate pesticides like diazinon (Sumanadasa et al., 2008).

Table 1. Mean body length (± S.E) of *D. melanostictus* larvae continuously exposed to four concentrations of carbofuran over a period of two weeks. Means for each concentration were calculated using three replicates.

Concentration	Length (mm)					
	Day 1	Day 4	Day 7	Day 10	Day 13	Day 16
Control	3.43 ± 0.05	5.46 ±0.10	6.48 ± 0.02	7.72 ± 0.24	8.26 ± 0.15	9.22 ± 0.07
50 µg l^{-1}	3.42 ± 0.06	5.13 ± 0.23	6.07 ± 0.12	8.11 ± 0.43	8.81 ± 0.30	9.65 ± 0.25
150 µg l^{-1}	3.42 ± 0.06	4.84 ± 0.20	5.88 ± 0.41	8.20 ± 0.13	9.17 ± 0.47	9.85 ± 0.25
250 µg l^{-1}	3.43 ± 0.09	4.82 ± 0.28	5.62 ± 0.56	8.39 ± 0.32	9.60 ± 0.49	10.00 ± 0.43
500 µg l^{-1}	3.43 ± 0.05	5.18 ± 0.31	6.37 ± 0.77	7.78 ± 0.35	8.82 ± 0.35	9.28 ± 0.23

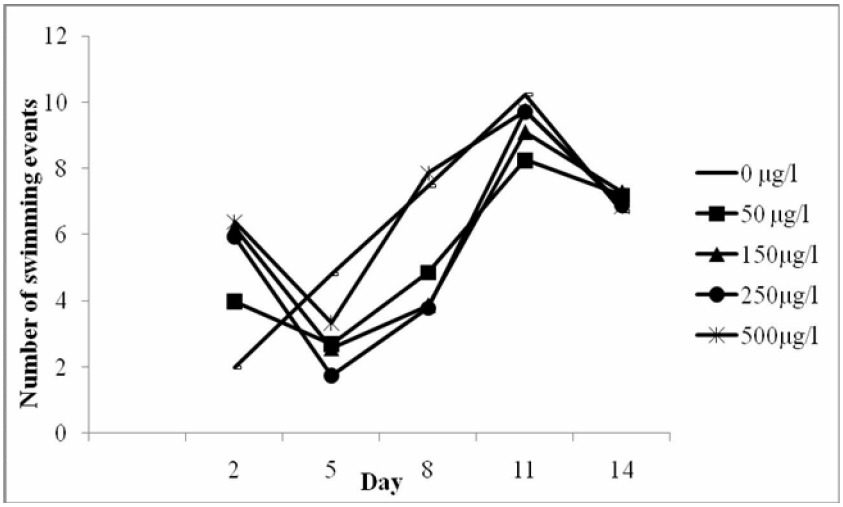
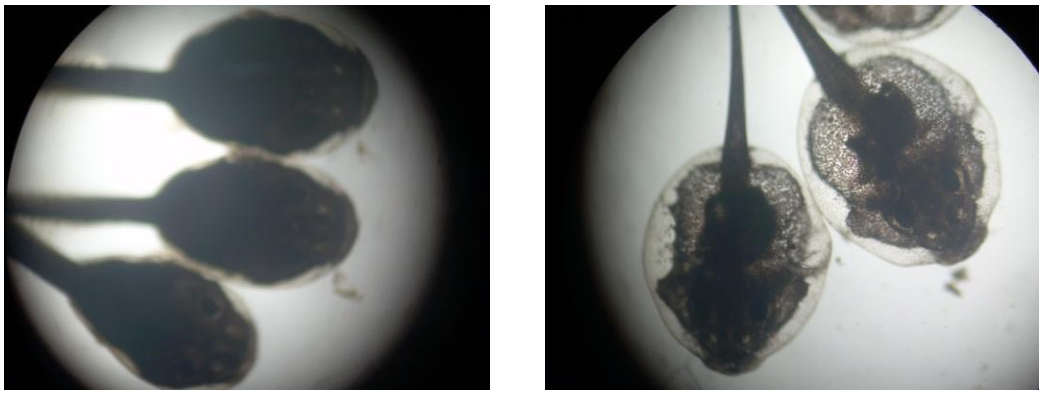


Figure 2: Effect of carbofuran on swimming activity of *D. melanostictus* larvae repeatedly exposed to four different concentrations of carbofuran during a two week experimental period.

3.2 Effects on swimming activity

Exposure to carbofuran initially caused an abnormally high level of activity in tadpoles. Activity subsequently decreased with continued exposure, and by the end of the trial (Day 14) the swimming intensities of treated and untreated tadpoles were nearly similar (Figure 2). The Split-plot design repeated measures ANOVA and post-hoc tests were used to examine

the variation in swimming activity with pesticide concentration and time. These analyses indicate that there was no significant difference in activity when only exposure levels were considered, although a significant difference was noted in activity with concentration across time (Treatment $F_{4,10} = 2.27$, P=0.13; Time $F_{3, 40} = 39.72$, $P < 0.0001$; Treatment * Time, $F_{16,40} = 2.7$, $P < 0.01$). This trend has been also noted in *Xenopus laevis* tadpoles during acute exposure to carbaryl (Zaga et al., 1998). Such behavioral changes could affect the long-term survival of the exposed species by making them more vulnerable to predation (Berrill et al., 1993). A morphological deformity that was noted in about 10 % of the tadpoles exposed to 250µgl^{-1} was swelling of the head and body region and the appearance of an enclosing vesicle (Figure 3).



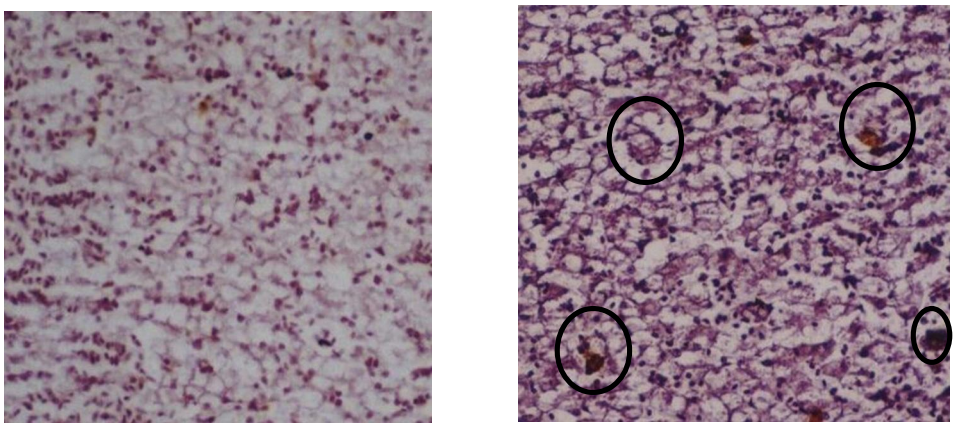
(i) (ii)

Figure 3: Abnormalities in larvae exposed to carbofuran. Note the swelling of the head and body region and the enclosing capsule in *D. melanostictus* tadpoles exposed to 250 µgl^{-1} of carbofuran as shown in (ii) and the normal head and body of tadpoles shown in (i).

3.3 Histopathological alterations in liver and tail muscle

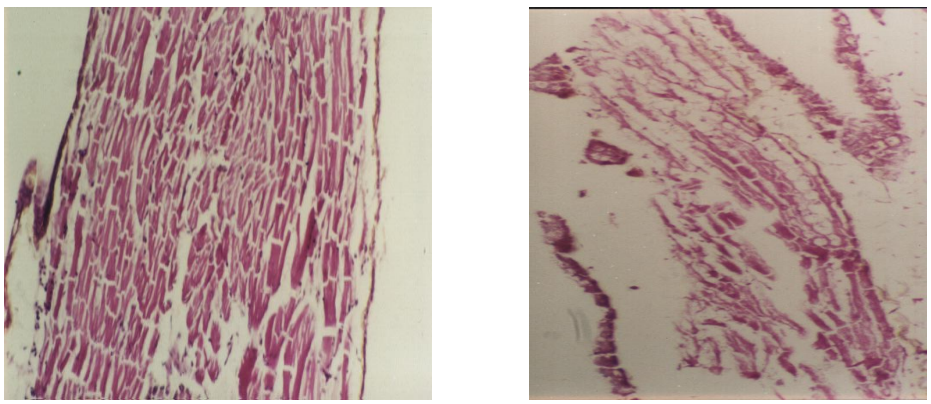
Examination of the liver and tail muscle tissues of the tadpoles revealed that exposure to 250 and 500 µgl^{-1} of carbofuran for one week induced histopathological alterations in liver and tail muscle tissues, although the degree of damage varied. No drastic structural changes were noted in the liver apart from greater vacuolation in hepatocytes, sinusoidal dilations and the formation of bile plugs in the treated larvae (Figure 4). In contrast to the liver, the muscle tissues of the tail in larvae exposed to 250 µgl^{-1} of carbofuran showed signs of severe muscle atrophy, where myotomes were substantially disintegrated (Figure 5). The myotomes of larvae not exposed to the pesticide were compact and well organized. Damage observed at 500 µgl^{-1} was much lower than that observed at 250µgl^{-1}. The liver is susceptible to chemical injury because it is the primary organ where the detoxification of xenobiotics occur (Sharkoori et al., 1990). The alterations in liver tissue recorded in the present study such as the increase in vacuolation, sinusoidal dialation and formation of bile plugs, has been also reported by Sakr et al. (2001) in fish. Sinusoidal dialation in the liver is attributed to the impairment of outflow of the hepatic veins (Tanaka and Wanless, 1998) while the formation of vacuoles in hepatocytes is due to the degeneration of cell membranes (Olurin et al., 2006) and an imbalance between the rate of synthesis and utilization of substances in cells (Gingerich, 1982). Bile plugs form due to the obstruction of bile tubes and the subsequent accumulation of bile in the liver. The observed muscle damage could be attributed to the inhibition of acetylcholine esterase with consequent muscular tetanic spasms. These changes

are similar to the tail skeletal muscle damage observed in *Xenopus laevis* embryos exposed to carbaryl (Bacchetta et al., 2007).



(i) (ii)

Figure 4: Effect of carbofuran on liver tissues (L.S.) of *Duttaphrynus melanostictus* larvae. (i) Normal liver tissue of control larvae and (ii) liver tissue of those exposed to 250 μgl^{-1} at the end of one week of repeated exposure showing cytoplasmic vacuolations and bile plugs. (encircled) (magnification X200)



(i) (ii)

Figure 5: Effect of carbofuran on tail muscle tissues (L.S.) of *Duttaphrynus melanostictus* larvae. (i) well compact myotomes of control larvae (M) (ii) acute muscle atrophy in larvae exposed to 250 μgl^{-1} of carbofuran at the end of one week of repeated exposure (magnification X200).

4. Conclusions

The present study provides empirical evidence for the negative impacts of a commonly used carbamate pesticide carbofuran on larvae of the toad species *Duttaphrynus melanostictus*. Continuous exposure to 50 to500 μgl^{-1} of carbofuran for 15 days caused marked reductions in survival, showing that this pesticide is toxic to the larvae of the tested species. In contrast to the typical linear dose-dependent curve exhibited by organisms exposed to pesticides, exposure to carbofuran triggered a bell-shaped response. Considering sublethal effects, it was

significant that the effect of carbofuran on growth, development and swimming activity was transient. Such transient effects of pesticides should, however, be interpreted with caution since these effects were observed in the few surviving tadpoles which probably exhibit an unusually high tolerance. With regard to histopathology, it was evident from the present study that carbofuran causes histopathological alterations in liver and tail muscles of *D. melanostictus* larvae. The tissue damage observed in larvae exposed to the mid concentration was greater than that at the higher concentration which is consistent with the hormetic response also observed for mortality. This study emphasizes the importance of carrying out toxicity tests to assess both lethal and sublethal damage induced by pesticides in non-target organisms such as amphibians that inhabit agricultural habitats.

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