Sri Lanka J. Aquat. Sci. ***22(1)*** *(2017): 29-37*

Survival and cholinesterase activity of Asian common toad *(Duttaphrynus melanostictus)* tadpoles following short term exposure to a carbosulfan-based pesticide

H. M. T. R. Samarakoon and A. Pathiratne*

Department of Zoology and Environmental Management, University of Kelaniya, Kelaniya, GQ 11600, Sri Lanka

*****Corresponding author: asoka@kln.ac.lk

Abstract Carbosulfan, a carbamate pesticide widely used in agriculture especially in the Asian region has received less attention in ecological impact assessments particularly to non-target amphibians. In this study, short term effects of a carbosulfan-based pesticide (Marshal®) on survival and cholinesterase (ChE) activity of tadpoles of *Duttaphrynus melanostictus*, a non-target amphibian in tropical Asia were evaluated. Based on the concentration-toxicity response patterns, estimated median lethal concentration (LC_{50}) and median ChE inhibitory concentration (IC_{50}) for 96 h were 24 and 2.1 mg/L carbosulfan respectively. For the ChE inhibition for 96 h, the lowest observable effect concentration and no observable effect concentrations of carbosulfan were 0.6 and 0.3 mg/L respectively. Upon transfer to pesticide-free water by 14 days, ChE activities of the tadpoles were restored to normal levels. Since the tadpoles exposed to 0.3 mg/L carbosulfan have not exhibited lethality, morphological deformities, abnormal behavior or statistically significant ChE depression, it is unlikely that the carbosulfan-based pesticide could induce neurotoxic effects for *D. melanostictus* tadpoles at the concentration of ≤ 0.3 mg/L of active ingredient under short term exposure.

Keywords: carbamate pesticide; carbosulfan; cholinesterase; tadpole

INTRODUCTION

Among the multitude of factors which have been implicated as contributing to global amphibian species decline, agricultural practices and agrochemicals have gained much scientific attention (Beebee and Griffiths 2005; Mann et al. 2009; Bruhl et al. 2011; Wagner et al. 2013; Lanctot et al. 2014). The larval stages of amphibians are especially vulnerable to agrochemical pollution because they are entirely gill breathing animals in the first life stages and can be found in different freshwater habitats including temporary ponds and pools closer to agricultural areas. Ecological impacts of commercial pesticide formulations to non-target amphibians are needed to be assessed as they are being exposed to the end user products in the natural habitats (Puglis and Boone 2011).

Carbosulfan, a widely used carbamate pesticide for agricultural pest management. Carbosulfan belongs to the benzofuranyl methyl carbamate group of pesticides. Its toxicity is mediated by the inhibition of the enzyme acetylcholinesterase, the key enzyme in cholinergic transmission in the nervous system (Fukota 1990). Even though limited toxicological data relevant to carbosulfan are available for several fish species (Yi et al. 2006; Chandrasekera and Pathiratne 2007; Capkin et al. 2010; Nwani et al. 2010; Altinok et al. 2012), to our knowledge, no scientific studies were found in the global literature on toxicity of carbosulfan or a carbosulfan-based pesticide to amphibians. Sri Lanka is considered as a global amphibian hotspot; 103 species of anurans and caecilians have been described so far in Sri Lanka, which accounts for 2% of the world's known anuran species (Pethiyagoda and Manamenda-Arachchi 1998). Although no data are yet available on population trends of Sri Lankan amphibians, concerns have been raised that amphibian larval stages are particularly at a risk and may be vulnerable to adverse effects of the agricultural pesticides and may undergo range reductions or population declines in the future (Jayawardena et al. 2011). Asian common toad, *Duttaphrynus melanostictus* is a widely distributed and abundant bufonid in tropical Asia. It is found in disturbed lowland habitats especially in human dominated agricultural and urban areas (IUCN 2004). *D. melanostictus* is currently categorized under "least concern" in the Global Amphibian Assessment (IUCN 2004). The present study evaluated potential short term toxicity of a widely used carbosulfan-based pesticide

formulation (Marshal®) on tadpoles of *D. melanostictus*, a non-target amphibian in agricultural landscapes using survival and ChE activity as endpoints.

MATERIALS AND METHODS

Tadpoles

Egg strands of newly spawned *D. melanostictus* were collected from a small freshwater pond located in Mawanella, Sri Lanka. (7°10'36.69"N; 80°30'0.46"E). The collection site is located in a nonagricultural area where there are no past records of any domestic pesticide use. The egg stands were transported immediately to the laboratory and were allowed to hatch in aerated pond water in glass aquaria. Emerged tadpoles were raised in glass tanks with aged tap water under continuous aeration until they developed to a free swimming feeding stage. The tadpoles were fed with ground commercial fish food pellets daily at 10% of the body mass. Feces and debris on the bottom of the tank were siphoned out, and the water levels were replenished daily with aged tap water. During the acclimation period, temperature, pH and dissolved oxygen (DO) levels in rearing water were measured using multi-parameter water quality checker (MPS-556, YSI, USA) and ranged between 26.1–29.6°C, 6.8–7.6 and 4.7–5.6 mg/L respectively. Developmental stages were determined as described by Gosner (1960). Pesticide exposure started when the tadpoles reach the Gosner stage 25. Few tadpoles that were reared in the laboratory until completion of full metamorphosis, confirmed that the collected egg strands belonged to *D. melanostictus.* All applicable international guidelines for the laboratory maintenance of animals and use of animals were followed in this study.

Pesticide

A commercial formulation of carbosulfan (Marshal®: active ingredient: carbosulfan, 200 g/L, a soluble concentrate from FMC Corporation, USA, marketed by Innovative Pesticides Marketing Pvt Ltd., Colombo, Sri Lanka) was used for exposure studies.

Pesticide exposure

Based on the active ingredient, stock solutions of the carbosulfan were freshly prepared by diluting appropriate amounts of the commercial formulation with aged tap water followed by further dilutions with aged tap water to obtain required test concentrations. Expected carbosulfan concentration in the stock solutions were analytically verified by Gas chromatography/Mass Spectrometry by the Industrial Technology Institute, Colombo, Sri Lanka. In general, measured concentrations of carbosulfan in the stock solutions showed less than 10% deviations from the nominal concentrations. Toxicity tests were conducted with a series of nominal concentrations of carbosulfan as the active ingredient (0, 0.3, 0.6, 1.2, 1.8, 2.4, 5, 10, 20, 25, 30, 40 and 50 mg/L) in glass tanks (15 × 25 × 30 cm) containing 4 L of exposure medium using triplicates with 30 tadpoles per replicate. Randomly selected tadpoles at Gosner stage 25 were used for the pesticide exposure studies. The tadpoles in the tanks with only aged tap water served as controls. Exposure media were renewed daily for four days. The tadpoles were not fed during the exposure period. Survival, behaviour and morphological abnormalities of the tadpoles were recorded daily for 96 hours. Dead tadpoles were removed from the tanks as soon as noticed. After 96 hours, twelve tadpoles from each concentration (4 tadpoles from each tank) were euthanized with benzocaine (Sigma-Aldrich) and immediately stored at -80°C until they were used in ChE assay. Remaining tadpoles were transferred separately to the tanks containing only aged tap water and maintained with continuous aeration for a period of 14 days for recovery studies. Water in the recovery tanks was renewed once in two days. During the recovery phase, the tadpoles were fed with ground fish food pellets (10% of the body weight) two hours before the renewal of water. After 14 days in aged tap water, developmental stages of the tadpoles were noted and twelve tadpoles from each pesticide pre-treatment and controls were euthanized with benzocaine and stored in -80°C for the ChE assay. Temperature, pH and DO levels in each aquaria were monitored daily during the pesticide exposure phase (temperature 26.9 – 28.5°C; pH 6.9 – 7.2; DO 4.8 – 5.3 mg/L) and once in two days in the post exposure phase (temperature 26.5 – 28.0°C; pH 7.1 – 7.5; DO 4.8 – 5.1 mg/L) and found to be within favorable limits for tadpoles.

ChE assay

The chemicals for the ChE assay were purchased from Sigma-Aldrich, USA. ChE activity in the whole body was assessed as described by Liu et al. (2011) with modifications in the original procedure of Ellman et al. (1961) specifically for the tadpoles. The tadpoles which were stored at -80°C were allowed to thaw in ice prior to the assay. They were wiped dry with a paper towel and the actual weights of the tadpole tissues were recorded to the nearest 0.001g using an analytical

balance. The ChE source was prepared by homogenizing tadpoles in ice cold 0.1 M sodium phosphate buffer at pH 8.0 (20 mg composite sample: 1 mL of buffer) by the tissue homogenizer (Ultra-Turrax T25, Janke & Kunkel, IKA Labortechnik). The homogenates were centrifuged at 2000 g for 5 min at 4°C (Hettich Zentrifugen) and the supernatant was used as the enzyme source. In the assay, 400 µL supernatant was added to a 350 µL of 0.1 M phosphate buffer solution (pH 8.0) in a glass cuvette, and mixed with 25 µL of 0.01 M 5,5-dithiobis-2-nitrobenzoic acid. For the control blank, a 400 µL phosphate buffer was added instead of the enzyme source. The mixture was placed in a computer controlled spectrophotometer (UV-Visible Spectrometer, Cintra 10*e*, Australia) and the changes in absorbance of reaction solution were recorded at 412 nm for 3 min. During the scanning process, 5 µL of freshly prepared acetylthiocholine iodide was added to the cuvette at the 0.5 min and mixed with a plastic stirring rod and the mixture was scanned until 3 min. The assays were carried out in duplicates. The absorbance values were corrected for non-enzymatic reaction and the ChE activity was determined based on linear activity range using the extinction coefficient (Ellman et al. 1961) of the 5 thio-2-nitrobenzoate ion (13,600 M/cm) and was expressed in nmoles per minute per mg body weight.

Data analysis

Median lethal concentration (LC_{50}) of carbosulfan for 48, 72 and 96 h and median ChE inhibitory concentration (IC_{50}) of carbosulfan for 96 h were determined by Probit analysis (Finney 1971). Significant differences among controls and pesticide exposed groups with respect to the data on ChE activity were tested by one way analysis of variance (ANOVA) (Zar 1999). Where there were significant differences, mean values were compared from the controls by the Dunnett's test and each other by the Tukey's test respectively. The results were considered as significantly different if $p \leq 0.05$.

RESULTS

The control *D. melanostictus* tadpoles and the tadpoles exposed to the pesticide at 0.3 mg/L carbosulfan displayed active swimming patterns in the water column whereas the tadpoles exposed to ≥ 0.6 mg/L carbosulfan displayed uncontrolled rapid movements initially and later loss of coordination in swimming, rotating swiftly in a spiral orbit and erratic swimming patterns. Majority of tadpoles exposed to ≥ 5 mg/L of carbosulfan lied down on the bottom of the aquaria motionlessly for longer times. These tadpoles died in a rapid rate during the course of the exposure. The tadpoles exposed to 0.3 mg/L of carbosulfan did not exhibit mortalities or morphological deformities during the 96 h exposure. Morphological deformities (abnormal swellings and tail bending) were seen in the tadpoles exposed to ≥ 0.6 mg/L of carbosulfan. The frequency of tadpoles with tail bending was more common. Estimated 48 h, 72 h and 96 h LC_{50} values based on carbosulfan concentration and mortality responses of *D. melanostictus* tadpoles are presented in Table 1. With the increase in exposure time, the LC_{50} value is slightly decreased but time-specific LC_{50} values are not significantly different from each other as the respective 95% confidence limits overlap.

Table 1 Estimated median lethal concentrations (LC_{50}) of carbosulfan* for 48, 72 and 96 h exposure of *D. melanostictus* tadpoles.

Exposure Period	LC_{50} (mg/L)	Standard Deviation (mg/L)	95% Confidence Limits (mg/L)
48 h	28.5	1.5	20.1 - 32.6
72 h	25.9	1.1	24.0 - 28.4
96 h	24.0	0.9	22.2 - 25.9

*Based on the active ingredient in the pesticide formulation

After 96 h pesticide exposure, the survival and ChE inhibition patterns of the tadpoles (Figure 1) show concentration dependent increase in ChE activity inhibition with the decrease in tadpole survival. Carbosulfan concentration dependent ChE inhibition pattern in the tadpoles is presented in Table 2. Based on statistical analysis of ChE activities in the tadpoles exposed to different exposure concentrations, "no-observed-effect concentration" (NOEC) and "lowest-observed-effect concentration" (LOEC) for the 96 h ChE activity inhibition in *D. melanostictus* tadpoles were found as 0.3 and 0.6 mg/L carbosulfan respectively. Based on the carbosulfan induced ChE inhibition pattern, the 96 h-IC_{50} value of carbosulfan (with concentration 95% confidence limits) was estimated as 2.1 mg/L (1.8-2.5 mg/L). The maximum level of ChE activity inhibition reached at 5 mg/L carbosulfan concentration with 95% tadpole survival. Upon transferred to pesticide free water (aged tap water) for 14 days, mortality of the *D. melanostiscus*

tadpoles pre-exposed to the pesticide was increased further but ChE activities of the survived tadpoles were restored to the control levels (Table 2). At the end of 14 days recovery in aged tap water, the control tadpoles have reached Gosner 32 to 39 development (Figure 2). Some tadpoles pre-exposed to 0.3 mg/L carbosulfan have also reached the Gosner stage 32 to 39 similar to the controls. None of the survived tadpoles pre-exposed to ≥ 0.6 mg/L carbosulfan had reached the Gosner stage 39.

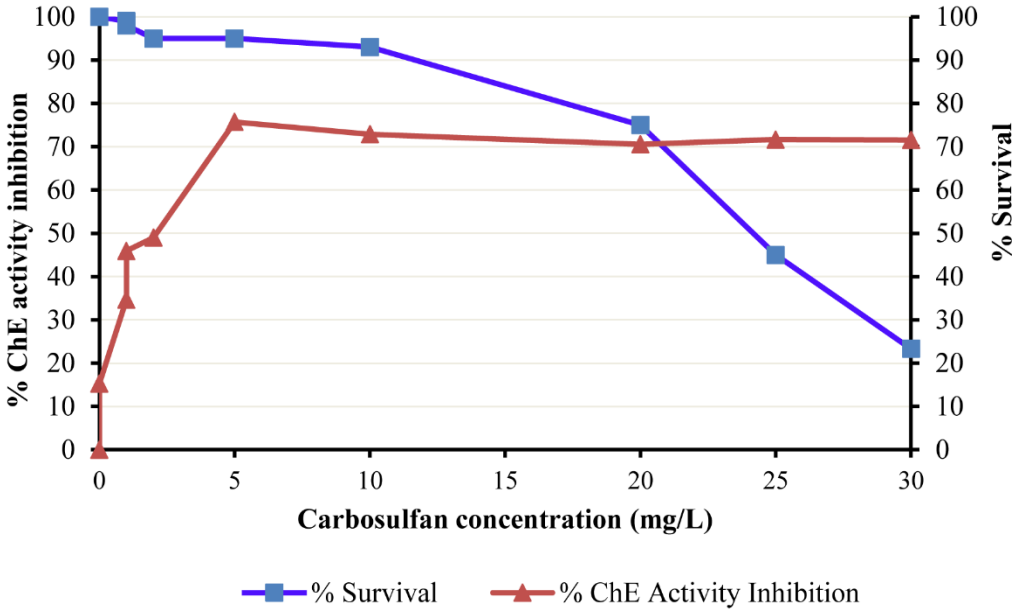

Fig. 1 Survival and cholinesterase activity inhibition patterns in the of *D. melanostictus* tadpoles following 96 hour exposure to different concentrations of carbosulfan-based pesticide

Table 2 Cholinesterase **(**ChE) activities of the tadpoles of *D. melanostictus* following 96 h exposure to different concentrations of carbosulfan-based pesticide and 14 days post exposure to pesticide free aged tap water

	ChE activity (nmol/min/mg)	
Carbosulfan (mg/L)	At 4 days exposure	At 14 days post exposure
control	0.98 ± 0.05^{a}	1.14 ± 0.08^{a}
0.3	1.10 ± 0.06^{a}	1.02 ± 0.08^{a}
0.6	0.83 ± 0.03^{b*}(15%)	1.41 ± 0.04^{a}
1.2	0.64 ± 0.05^{c*}(35%)	1.16 ± 0.08^{a}
1.8	0.53 ± 0.03^{c*}(46%)	1.27 ± 0.13^{a}
2.4	0.49 ± 0.02^{c*}(50%)	1.16 ± 0.07^{a}
5	0.24 ± 0.05^{d*}(76%)	NM
10	0.26 ± 0.06^{d*}(73%)	NM
20	0.28 ± 0.05^{d*}(71%)	NM
25	0.27 ± 0.02^{d*}(72%)	NM
30	0.27 ± 0.03^{d*}(72%)	NM

Data are presented as mean ± SEM (n = 3 composite samples with 4 tadpoles for each composite sample). The numbers within parentheses indicate % inhibition of ChE activity. In a column, data not followed by same superscript are significantly different from each other (ANOVA, Tukey's Test, $P < 0.05$). The data with the asterisk indicate the significant difference from the control (Dunnett's test, $P < 0.05$). NM: not measured due to inadequate sample size

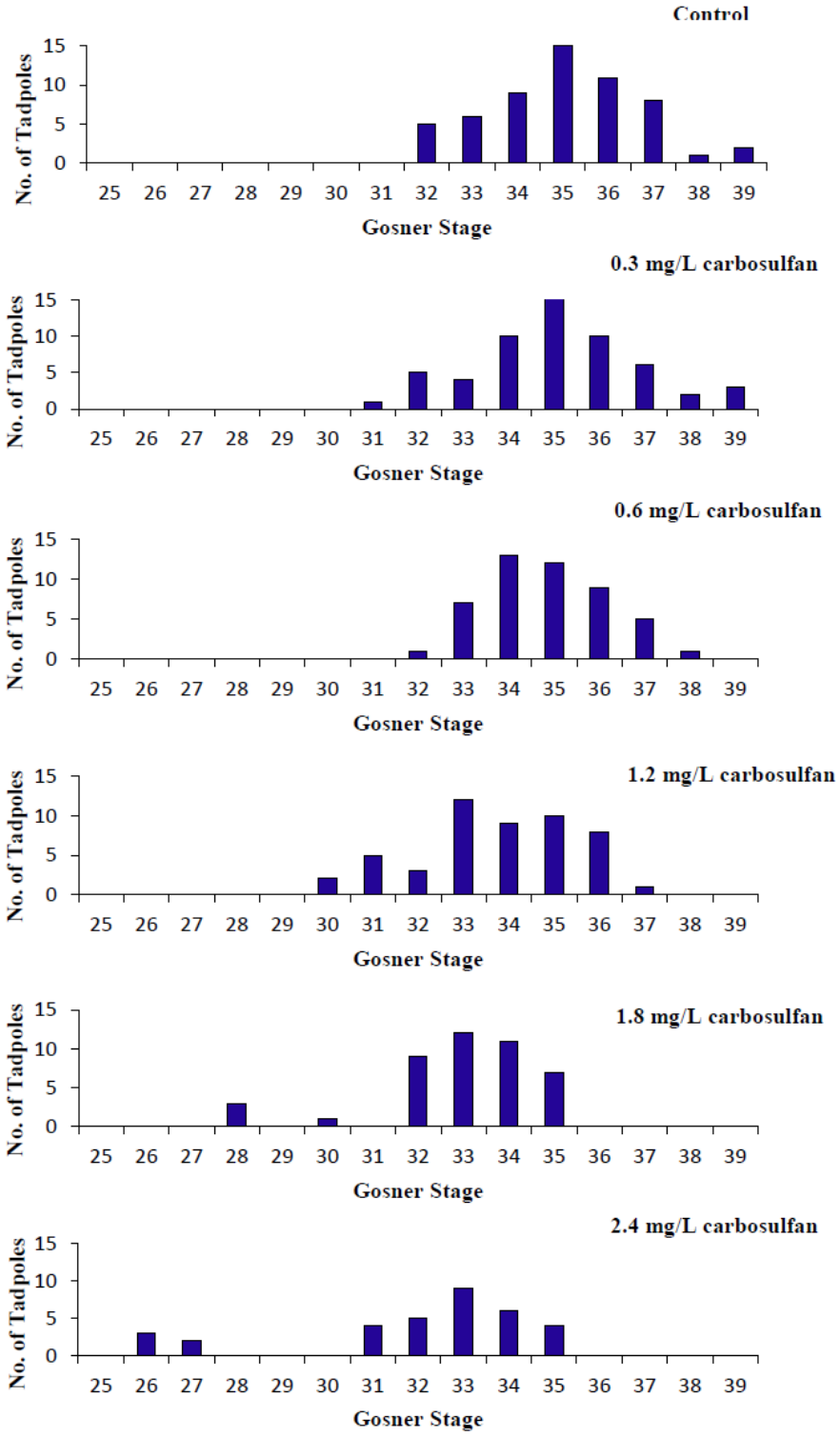

Fig. 2 Developmental stages of the tadpoles of *D. melanostictus* pre-exposed to selected concentrations of carbosulfan-based pesticide (for 96 hours) after 14 days of recovery in aged tap water

DISCUSSION

Carbosulfan is a procarbamate pesticide and its toxicity is mediated by the inhibition of the enzyme acetylcholinesterase, the key enzyme in cholinergic transmission in the nervous system (Fukota 1990). In aquatic environments, carbosulfan can be hydrolyzed to carbofuran which is also a potent cholinesterase inhibitor. However, both chemicals have low persistence in water and medium persistence in soil solutions in tropical irrigated rice fields (De Melo Plese et al. 2005). Based upon the stipulated acute toxicity criteria by USEPA (2004), carbosulfan could be considered as 'slightly toxic' to the tadpoles of *D. melanostiscus* as the 96 h-LC50 value of carbosulfan estimated in this study was 24 mg/L (Table 1) which is within the range of 10-100 mg/L. Cholinesterase enzyme levels showed that majority of the tadpoles (95%) exposed to 5 mg/L carbosulfan could tolerate the highest levels of ChE activity inhibition (76%) without associated mortality by 96 h (Table 2). While the mortality rate was increased further at higher concentrations (≥20 mg/L), ChE inhibition in the remaining tadpoles was in the range of 71-73%. Although there is some controversy in the literature regarding the extent of cholinesterase inhibition required to cause death in aquatic animals, the most estimates fall within the range 70–85% (Chandrasekera and Pathiratne 2007). Current study with the tadpoles of *D. melanostiscus* exposed to carbosulfan also agrees with this range of cholinesterase inhibition. It is likely that some carbamates are released from the carbamate-ChE complex to continue normal ChE activity with time as carbamates form relatively a weak bond with ChE enzyme (Fukuto 1990). In the present study, upon transferred to pesticide free water (aged tap water) for 14 days, mortality of the *D. melanostiscus* tadpoles pre-exposed to the pesticide was increased further but ChE activities of the survived tadpoles were restored to the control levels. Complete restoration of the whole body ChE activities by 14 days of recovery period may be attributed to the release of ChE enzyme active site from carbosulfan inhibition, in addition to the denovo synthesis of new enzymes in the body at normal rates.

During the 96 h exposure, abnormal behavioural patterns exhibited by the *D. melanostictus* tadpoles exposed to ≥ 0.6 mg/L carbosulfan indicate neurotoxic effects of carbosulfan associated with ChE activity inhibition. Morphological deformities observed in the *D. melanostictus* tadpoles (tail bending and abnormal swellings) could also contribute to the abnormal behaviour displayed by the intoxicated tadpoles. Tail bending due to the curvature in the spinal cord (scoliosis) especially could affect swimming patterns in the tadpoles (Alvarez et al. 1995). Similar malformations have been reported earlier in *D. melanostiscus* tadpoles after exposure to some organophosphate insecticides and herbicides (Jayawardena et al. 2011; Wijesinghe et al. 2011). These deformed and abnormally behaved tadpoles could be highly vulnerable in the wild to increased predation due to reduced ability to evade predators (Relyea 2005; Yadav et al. 2013). In the present study, the lowest tested concentration of carbosulfan which induced deformities in the *D. melanostiscus* tadpoles after 96 h exposure was 0.6 mg/L which is the estimated LOEC for body ChE inhibition indicating the association between the development of deformities and the ChE inhibition. Permanent contraction of the paraxial musculature in the amphibian larvae could contribute to the twisting of spinal column and tail leading to scoliosis condition (Alvarez et al. 1995). Carbosulfan induced ChE inhibition may have caused increased accumulation of acetylcholine in the synapses which could have induced constant muscle contraction in the paraxial musculature eventually leading to deformities in the tail and trunk causing observed scoliosis condition in *D. melanostiscus* tadpoles.

At the end of 14 days recovery in aged tap water, the survived tadpoles pre-exposed to ≥ 0.6 mg/L carbosulfan had not reached the advanced developmental stages (Gosner stage 39) seen in the comparable controls (Figure 2). Although pesticide induced mortality of tadpoles has direct implications on amphibian population decline, prolong period of development could also affect the populations indirectly. It is likely that pesticide exposed tadpoles are at a disadvantage under natural conditions and may face threats and indirect mortality as a consequence of delayed development. Delayed development and metamorphosis due to exposure to other pesticides have also been reported earlier for *D. melanostiscus* (Jayawardena et al. 2011; Wijesinghe et al. 2011) and other amphibian species (Svartz et al. 2012; Saka et al. 2013). Among other factors, increased energetic costs involved in abnormal behaviour could have led to prolong developmental period of these tadpoles. Delayed metamorphosis of the individuals can have many consequences in the natural environment. *D. melanostictus* often breed in temporary habitats, particularly in agricultural areas and human altered habitats (IUCN 2004) where water may dry up before

completion of the metamorphosis which could lead to increased mortality. Furthermore longer development period increases the chances of exposure to predators and parasites in the environment leading to increased mortality (Relyea 2005; Yadav et al. 2013).

In Sri Lanka, large amounts of agrochemicals are used in cultivation especially in rice culture. These substances are typically washed with the surface runoff into surrounding freshwater ecosystems exposing aquatic organisms to the agrochemicals. Moreover, concentrations of agrochemicals in rice fields are expected to be much higher as agrochemicals are directly applied to the rice fields. The application of carbosulfan has become widespread among Sri Lankan farmers with the recent banning of several pesticides including chlopyrifos. However reliable reports on field concentrations of pesticides in Sri Lankan freshwater ecosystems are not available. According to the recommended maximum application rate of carbosulfan for rice pest management (0.32 kg/ha), if carbosulfan was directly applied at this recommended rate, predicted highest concentration of carbosulfan in 10 cm depth of water just after the application would be 0.32 mg/L. The present study found that laboratory exposure of the *D. melanostictus* tadpoles to 0.3 mg/L carbosulfan had no significant effect on survival, and ChE activity under short term exposure conditions. Hence it is unlikely that the tadpoles of *D. melanostictus* would be greatly impacted at the predicted ecologically realistic environmental concentrations. In fact, the predicted field concentration based on recommended application rates is at a low range, but this situation cannot be always expected because local farmers tend to apply pesticides repeatedly at concentrations well above the recommended levels which may reach threshold levels of toxicity to the tadpoles. Recent studies confirmed the mutagenic potential of carbosulfan to some fish species (Nwani et al. 2010; Altinok et al. 2012). Further studies on potential DNA integrity alterations associated with carbosulfan exposure will help to understand the risk of having heritable toxic effects that may adversely affect the future generations of amphibians.

In conclusion, the present study demonstrated that the short term exposure of the *D. melanostiscus* tadpoles to carbosulfan concentrations at $\geq$ 0.6 mg/L resulted in behavioural abnormalities, morphological deformities, and depression of ChE activity in a concentration dependent manner. Based on the concentration-toxicity response patterns, 96 h median ChE inhibitory concentration of carbosulfan is estimated as 2.1 mg/L whereas the 96 h LOEC and NOEC values for inhibition of ChE activity were 0.6 and 0.3 mg/L carbosulfan respectively. As the tadpoles exposed to 0.3 mg/L carbosulfan did not exhibit lethality, morphological deformities, abnormal behavior or statistically significant ChE depression, it is unlikely that the carbosulfan-based pesticide could induce adverse neurotoxic impacts for *D. melanostictus* tadpoles at the concentration of $\leq$ 0.3 mg/L of active ingredient under short term exposure.

REFERENCES

Altinok I., E. Capkin and H. Boran 2012. Mutagenic, genotoxic and enzyme inhibitory effects of carbosulfan in rainbow trout *Oncorhynchus mykiss*. Pesticide Biochemistry and Physiology 102: 61-67. doi: 10.1016/j.pestbp.2011.10.011

Alvarez R., M.P. Honrubia and M.P. Herr'aez 1995. Skeletal malformations induced by the insecticides ZZ-Aphox_ and Folidol_ during larval development of *Rana perezi*. Archives of Environmental Contamination and Toxicology 28: 349–356. doi:10.1007/BF00213113

Beebee T.J.C. and R.A. Griffiths 2005. The amphibian decline crisis: A watershed for conservation biology? Biological Conservation 125: 271–285. doi: 10.1016/j.biocon.2005.04.009

Bruhl C.A., Z. Pieper and B. Weber 2011. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. Environmental Toxicology and Chemistry 30(11): 2465–2472. doi:10.1002/etc.650

Capkin E., E. Terzi, H. Boran, I. Yandi and I. Altinok 2010. Effects of some pesticides on the vital organs of juvenile rainbow trout (*Oncorhynchus mykiss*). Tissue Cell 42: 376-382. doi: 10.1016/j.tice.2010.10.001

Chandrasekara L.W.H.U. and A. Pathiratne 2007. Body size-related differences in the inhibition of brain acetylcholinesterase activity in juvenile Nile tilapia (*Oreochromis niloticus*) by chlorpyrifos and carbosulfan. Ecotoxicology and Environmental Safety 67: 109–119. doi: 10.1016/j.ecoenv.2006.04.002

De Melo Plese L.P., L.C. Paraiba, L.L. Foloni and L.R.P. Trevizan 2005. Kinetics of carbosulfan hydrolysis to carbofuran and the subsequent degradation to this last compound in irrigated rice fields. Chemosphere 60: 149–156. doi:10.1016/j.chemosphere.2005.02.049

Ellman G.L., K.D. Courtney, V. Andres Jr. and R.M. Featherstone 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88-95. doi:10.1016/0006-2952(61)90145-9

Finney D.J. 1971. Probit Analysis. Cambridge University Press, London.

Fukuto T.R. 1990. Mechanism of action of organophosphorous and carbamate insecticides. Environmental Health Perspectives 87: 245-254.

Gosner K.L. 1960. A Simplified table for staging anuran embryos and larvae with notes in identification. Herpetologica 16(3): 183-190.

IUCN 2004. *Duttaphrynus melanostictus*. The IUCN Red List of Threatened Species. Version 2015.2. Available at www.iucnredlist.org. Accessed 07 July 2015.

Jayawardena U.A., A.N. Navaratne, P.H. Amarasinghe and R.S. Rajakaruna 2011. Acute and chronic toxicity of four commonly used agricultural pesticides on the Asian common toad, *Bufo melanostictus* Schneider. Journal of National Science Foundation Sri Lanka 39: 267 276. doi: http://doi.org/10.4038/jnsfsr.v39i3.3631

Lanctôt C., L. Navarro-Martín, C. Robertson, B. Park, P. Jackman, B.D. Pauli and V.L. Trudeau 2014. Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: Agriculturally relevant exposures to Roundup WeatherMax® andVision® under laboratory conditions. Aquatic Toxicology 154: 291–303. doi: 10.1016/j.aquatox.2014.05.025

Liu W., C. Wang, T. Wang, G.M. Fellers, B. Lai and Y. Kam 2011. Impacts of herbicide butachlor on the larvae of a paddy field breeding frog *(Fejervarya limnocharis)* in subtropical Taiwan. Ecotoxicology 20: 377-384. doi: 10.1007/s10646-010-0589-6

Mann R.M., R.V. Hyne, C.B. Choung and S.P. Wilson 2009. Amphibians and agricultural chemicals: Review of the risks in a complex environment. Environmental Pollution 157: 2903–2927. Doi: 10.1016/j.envpol.2009.05.015

Nwani C.D., W.S. Lakra, N.S. Nagpure, R. Kumar, B. Kushwaha and S.K. Srivastava 2010. Mutagenic and genotoxic effects of carbosulfan in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food and Chemical Toxicology 48: 202-208. doi:http://dx.doi.org/10.1016/j.fct.2009.09.041

Pethiyagoda R. and K. Manamendra-Arachchi 1998. Evaluating Sri Lanka's Amphibian diversity. Occasional papers No. 2, Wild Life Heritage Trust, Colombo.

Puglis H.J. and M.D. Boone 2011. Effects of Technical-Grade Active Ingredient vs. Commercial Formulation of Seven Pesticides in the Presence or Absence of UV Radiation on Survival of Green Frog Tadpoles. Archives of Environmental Contamination and Toxicology 60: 145–155. doi:10.1007/s00244-010-9528-z

Relyea R.A. 2005. The lethal impacts of roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology 48: 351-357. doi:10.1007/s00244-004-0086-0

Saka M., N. Tada and Y. Kamata 2013. Application of an amphibian *(Silurana tropicalis)* metamorphosis assay to the testing of the chronic toxicity of three rice paddy herbicides: Simetryn, mefenacet, and thiobencarb. Ecotoxicology and Environmental Safety 92: 135-143. doi: 10.1016/j.ecoenv.2013.03.023

Svartz G.V., J. Herkovits and C.S. Pérez-Coll 2012. Sublethal effects of atrazine on embryo-larval development of *Rhinella arenarum* (Anura: Bufonodae). Ecotoxicology 21: 1251-1259. doi:10.1007/s10646-012-0880-9

USEPA 2004. Overview of the Ecological Risk Assessment Process in the office of pesticide programmes. Office of Prevention, Pesticides, and Toxic substances. Office of Pesticide Programmes, Washington, DC

Wagner N., W. Reichenbecher, H. Teichmann, B. Tappeser and S. Lotters 2013. Questions concerning the potential impact of glyphosate-based herbicides on amphibians. Environmental Toxicology and Chemistry 32(8): 1688–1700. doi: 10.1002/etc.2268

Wijesinghe M.R., M.G.D.K. Bandara, W.D. Ratnasooriya and G.P. Lakraj 2011. Chlorpyrifos induced toxicity in *Duttaphrynus melanostictus* (Schneider 1799) larvae. Archives of Environmental Contamination and Toxicology 60: 690-696. doi:10.1007/s00244-010-9577-3

Yadav S.S., S. Giri, U. Singha, F. Boro and A. Giri 2013. Toxic and genotoxic effects of Roundup

on tadpoles of the Indian skittering frog *(Euflictis cyanophlyctis)* in the presence and absence of predator stress. Aquatic Toxicology 132-133: 1-8. 10.1016/j.aquatox.2013.01.016

Yi M.Q., H.X. Liu, X.Y. Shi, P. Liang and X.W. Gao 2006. Inhibitory effects of four carbamate insecticides on acetylcholinesterase of male and female *Carassius auratus* in vitro. Comparative Biochemistry and Physiology Part C 143: 113-116. doi:10.1016/j.cbpc.2005.12.008

Zar J.H. 1999. Biostatistical Analysis. Prentice Hall, New Jersey.