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Toxicity of Agrochemicals to Common Hourglass Tree Frog (*Polypedates cruciger*) in Acute and Chronic Exposure

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ABSTRACT

Direct effect of four common agricultural pesticides viz., chlorpyrifos, dimethoate, glyphosate and propanil, on the survival, growth and development of malformations in common hourglass tree frog, *Polypedates cruciger* (Anura: Ranidae) was studied under laboratory conditions in acute and chronic exposure. Acute exposure to high concentrations was carried out to determine the LC_{50}. The 48 h LC_{50} of the pesticides were within the Pesticide Area Network specified limits, except for propanil. The percentage survival of the tadpoles under chronic exposure to ecologically relevant doses was lower (glyphosate 75%, dimethoate 77.5%, chlorpyrifos 80% & propanil 85%) than the control group (95.5%) and was significantly affected by the concentrations. Exposed tadpoles took more time to metamorphose and were significantly smaller in size than the control tadpoles. They also developed malformations at high frequencies (glyphosate = 69%, dimethoate = 64%, chlorpyrifos = 60%, propanil = 45%). Malformations were mainly kyphosis (hunched back), scoliosis (curvature), skin ulcers and edema. However, severe limb malformations were not observed in the study. Chlorpyrifos had a profound effect even at very low concentrations (0.05 ppm). This study provides the first empirical evidence of a comparative study on the effect of pesticides on an endemic amphibian species in Sri Lanka and underscores the importance of investigation the level of agricultural pesticides in freshwater ecosystems and their effect on non-target organisms.

Key Words: Agricultural pesticides; Amphibians; Survival; Growth; Malformation

INTRODUCTION

Widespread use of the pesticides in agriculture has attracted many ecologists to study the toxicity of these chemicals on non-target organisms. Among these, amphibians have been the focus of attention owing to reports on population declines and species extinctions from many parts of the world. Most amphibians live in two habitats and their sensitivity to changes in environment makes them good indicators of ecosystem's health. According to Global Amphibian Assessment nearly one third (1,856 species) of the world's amphibian species are threatened and 165 species may have already gone extinct (IUCN, 2006). Habitat loss and fragmentation, climatic changes, diseases and chemical contaminants have been identified as some of the main causes of amphibian decline worldwide (Blaustein & Wake, 1995). There is growing evidence that pesticides are responsible for amphibian declines (Cowman & Mazanti, 2000; Sparling *et al.*, 2001; Blaustein *et al.*, 2003).

The consequences of pesticides on amphibians can be a direct or an indirect effect at lethal or sub lethal level. The sub lethal effects of pesticides on amphibians include hampered growth, development and behavior leading to developmental and behavioral abnormalities, which in turn may alter susceptibility to predation and competition and decrease reproductive success (Bridges, 1999; Boone & Semlitsch, 2002; Relyea, 2005). Ouellet *et al.* (1997) reported hind limb deformities in wild caught green frogs, northern leopard frog, American toad and bullfrogs. Weakening of the immune system, making amphibians more susceptible to parasites and diseases has been observed by several authors including Chrsitin *et al.* (2003), Daszak *et al.* (2003) and Gendron *et al.* (2003). Exposure of other non-target organisms such as fish to chlorpyrifos and dimethoate damage liver functions and development of testis (Khan & Law, 2005). They also reported that glyphosate exposure disrupts sexual development with feminizing effect of male fish. Acute studies of these agrochemicals reported higher mortality in sensitive species like larval midges, microcustaceans, cladoceran (*Cerodaphnia* spp), amphipod (*Hyatella* spp.) (Moore *et al.*, 1998). Most of these studies have been carried out in the temperate regions, while significant attention has not been

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paid to the amphibian species in the tropics, where the highest diversity of amphibians is recorded.

Sri Lanka is a tropical country well known for its amphibian diversity with 103 species described so far (Manamendra-Arachchi & Pethiyagoda, 2006), which counts for 2% of the world's known anuran species (IUCN, 2006). The common hourglass tree frog, *Polypedates cruciger* (Anura: Ranidae) is a widely distributed, entirely arboreal endemic frog, found predominantly in the wet zone of Sri Lanka (Manamendra-Arachchi & Pethiyagoda, 2006). It generally occurs in secondary forest habitats but has extended its range close to human habitations, breeding in home gardens. They lay their eggs in a foam nest, which is then attached to almost any surface that overhangs a water body. Hence, the larvae are found in water storage tanks, semi-permanent pools and any stagnant water body including shallow waters of rice fields and other agricultural areas.

A large amount of agricultural pesticides are used in paddy, coconut and other crops in Sri Lanka and the run-off carries pesticides that might affect aquatic stages of amphibians directly, or indirectly by reducing the populations of prey insects of adults (Pethiyagoda & Manamendra-Arachchi, 1998). Here we report the direct effect of four commonly used pesticides in Sri Lanka, on the survival, growth and development of malformations in common hourglass tree frog under laboratory conditions.

MATERIALS AND METHODS

Study animals: Foamy egg masses of newly spawned *P. cruciger* were collected from ponds in the Department of Botany in Peradeniya University Park, Sri Lanka, during March 2006-May 2007 and were brought to the Parasitology Research Laboratory of the Department of Zoology, University of Peradeniya, Sri Lanka. Egg masses were attached to the wall of a glass trough containing dechlorinated tap water. Emergent tadpoles from four clutches (n=1520) were used in the study. Tadpoles were fed with ground commercial fish feed three times a day.

Test chemicals: Commercial formulations of four commonly used agricultural pesticides including two organophosphate insecticides, chlorpyrifos and dimethoate and two herbicides, glyphosate and propanil were selected. Active ingredient of these pesticides has been diluted in different solvents/surfactants (Table I). Commercially available chlorpyrifos (Lorsban EC 40® or Pattas®) was diluted in water (20-30 mL 10 L^{-1}, depending on the target pest) before applying to the field (4- 8 L ha^{-1}). Dimethoate (Dimethoate EC 40®) was dissolved in water again (20-40 mL 10 L^{-1}, depending on the target pest) and applied 4-8 L ha^{-1} depending on the extent of the pest attack. Commercially available product of glyphosate known as Roundup® or Glyphosate® was applied after dissolving in water (e.g., for coconut plantations 1.44 kg ha^{-1}). Propanil (3, 4 DPA®) is one of the most common herbicides used in paddy in the dry zone of Sri Lanka. Samples of commercial formulations of four pesticides were obtained from the Pesticide Registrar's Office (Peradeniya, Sri Lanka) and are referred to them based on the active ingredient (chlorpyrifos, dimethoate, glyphosae & propanil) throughout the manuscript.

Acute exposure to determine LC_{50} values: Lethal concentration, LC_{50} (concentration at which 50% of tadpoles die) of the four pesticides was determined by exposing five days post-hatch tadpoles (Gosner stage 25-26; Gosner, 1960) for 48 h. After a preliminary exposure to assess the range of pesticide concentration, a dilution series of 2.5, 2.0, 1.5, 1.0 and 0.5 ppm was used for chlorpyrifos and propanil and a slightly higher series; 25.0, 18.75, 15.00, 11.25, 9.50 ppm was used for dimethoate and glyphosate.

Serial doses were determined after preliminary exposures to find the best suitable concentration series for each chemical. The pesticides were diluted in dechlorinated tap water, which itself was control treatment. Of the four egg clutches, tadpoles from three clutches used to determine LC_{50}. Twenty tadpoles from each clutch were placed in a glass tank (15 × 15 × 25 cm) containing 2 L of the test solution and the mortality at 48 h after exposure was recorded.

Chronic exposure to pesticides at ecologically relevant concentrations: Five days post-hatch tadpoles (Gosner stage 25-26; Gosner, 1960) were exposed to ecologically relevant concentrations of pesticides in similar glass tanks (15 x15 x25 cm) containing 2 L of pesticide solution. The tadpoles were exposed to a concentration series of four pesticides similar to concentrations found in the field considering on the information obtained from the Pesticide Registrar's Office, Peradeniya Sri Lanka and literature. Concentration series of chlorpyrifos was prepared as 0.05, 0.10, 0.25 and 0.5 ppm according to the information given in Moore *et al.* (2002) and Mazanti *et al.* (2003). Concentration series of dimethoate, glyphosate and propanil was 0.25, 0.50, 0.75 and 1.00 ppm, which was chosen based on recent information on glyphosate concentrations in well water (up to 10 ppm; Navaratne & Devasurendra, unpublished results) and the information from Pesticide Registrar's Office. The exposure was carried out in a similar set of glass tanks containing 2 L of the respective solution. Tests were initiated with 20 tadpoles per tank, exposing them to above four different concentrations of each pesticide and a control containing dechlorinated tap water. The tadpoles were fed with commercial fish feed. Considering the rate of degradation of the chemicals in water and minimizing the stress due to extensive handling of tadpoles, the medium in the tanks was renewed weekly. Survival was recorded daily and the tadpoles were raised until metamorphosis. Snout-vent length (SVL) of the tadpoles was measured to the nearest 0.01 cm using a digital vernier caliper and the body weight to the nearest 0.001 g was recorded at metamorphosis using an analytical balance. Time required for the forelimb emergence of half the number of tadpoles in each treatment (TE_{50}) was recorded. Exposure was repeated using tadpoles from four different egg clutches separately. All the tanks were kept at room temperature with daytime temperature varying between 27-31^{o}C under a natural photoperiod of approximately 12:12 h.

Malformations were recorded at 10 days (Gosner stage 27), 30 days post-hatch (Gosner stage 31) and at metamorphosis. The percentage malformation was calculated as the number of malformed individuals at each stage divided by the number of surviving individuals at that particular stage. The dead malformed individuals were also included in the number of malformed individuals as some of the malformations cause direct mortality. At metamorphosis all the malformed individuals were anesthetized and preserved in 5% formalin. Laboratory rearing of the tadpoles and anesthetizing (using tricaine methane sulfonate MS-222) and killing of the malformed amphibians were carried out according to the protocols approved by the Canadian Council on Animal Care (1993). Malformations were categorized using "Field guide to malformations of frogs and toads" (Meteyer, 2000).

Statistical analysis: The LC_{50} values of the pesticides were calculated using EPA Probit Analysis Programme (Version 1.5; EPA, 2006) using the pooled data from three egg clutches. Results of the pesticide exposure at ecologically relevant doses were analyzed using MINITAB 14.0 for Windows. An *F*-test showed that at the 0.05 significance level, the variance of the data from four clutches could not be ruled out to be different and hence the data were pooled and analysed. Individual effect of concentration on the survival of the tadpoles was analysed using a chi-square test. A correlation analysis was performed to test the relationship of survival, growth parameters (body weight, SVL & TE_{50} values) and percentage malformations with the concentrations of each chemical. The effect of each pesticide on growth parameters was analysed using one-way ANOVA. The differences in growth parameters and malformations among the four pesticides were compared using an analysis of co-variance (ANCOVA).

RESULTS

Acute exposure and LC_{50}: A total of 480 five days post-hatch tadpoles (Gosner stage 25-26; Gosner, 1960) of *P. cruciger* were tested. LC_{50} values for chlorpyrifos, dimethoate, glyphosate, propanil were 1.21, 8.40, 14.99 and 2.21 ppm, respectively.

Chronic Exposure to Pesticides at Ecologically Relevant Concentrations

Survival of tadpoles and metamorphs: The percentage survival of the metamorphs after chronic exposure to ecologically relevant concentrations of the four pesticides was lower compared that of the control (95.5%). At the highest concentration of the pesticide (chlorpyrifos 0.5 ppm & 1.0 ppm for others) glyphosate caused the highest reduction in survival (75%) followed by dimethoate (77.5%) with chlorpyrifos and proponil having 80% and 85% survival, respectively (Table II). When the survival of the exposed tadpoles was compared with that of the control, a significant reduction was observed in 1.00 ppm concentration of glyphosate (75%, χ^2 = 9.760, *df* = 4, *p* = 0.001) and dimethoate (77.5 %, χ^2 = 21.715, *df* = 4, *p* = 0. 004). Chlorpyrifos exposed tadpoles experienced a significantly reduced survival compared to the control group even at low concentrations of 0.25 ppm (80%; χ^2 = 21.715, *df* = 4, *p* = 0. 05). Of the four pesticides, propanil had the least effect on survival of the tadpoles (Table II). While glyphosate recorded the most profound effect on the survival (75%) at 1.00 ppm, chlorpyrifos exposed tadpoles experienced high mortality even at 0.05-0.1 ppm concentrations. The reduction in survival was significantly dependent on increase in concentration, showing a negative correlation for all the pesticides (Pearson correlation, $p < 0.05$) except for chlorpyrifos (Pearson correlation coefficient r = -0.617, p = 0.267). Increase in chlorpyrifos concentration did not affect the reduction in the survival of tadpoles significantly as it was consistently high throughout the concentrations (Table II).

Growth of tadpoles: Pesticide exposed tadpoles were smaller in size than those of the control group. Growth of the tadpoles, assessed using SVL, body weight and TE_{50}, was impeded in the exposed groups compared to the control. A significant reduction in mean SVL and mean body weight in the four pesticides at all concentration levels was observed compared to the control (one way ANOVA, $p < 0.001$; Table III) with chlorpyrifos exposed group having the most profound effect. Both mean SVL and the mean body weight showed a negative correlation with concentration. However, a statistically significant correlation was observed only in dimethoate exposed group (Pearson correlation coefficient r = -0.996, $p < 0.001$). For body weight, a significant correlation was observed in both dimethoate and glyphosate treated groups (Pearson correlation coefficient r = -0.961, p = 0.009 & r = -0.912, p = 0.031, respectively) with increasing concentrations.

Tadpoles in the exposed groups took more time to develop. The average TE_{50} (time required for the forelimb emergence of half the number of tadpoles) value for *P. cruciger* was 57 days (range 36-78 days) in the control, while in the exposed groups it exceeded 100 days (range 105-147 days), which was significantly high compared to the control (ANCOVA, F = 10.91, *df* = 1, 14, p = 0.005). Of the four chemicals, chlorpyrifos (1.0 ppm) recorded the highest TE_{50} of 147 days, which was significantly high compared to the control tadpoles (One way ANOVA F = 884.95, *df* = 1,6; $p < 0.001$, Fig. 1). All pesticides caused significant lengthening of the development period compared to the control group at all four concentrations tested, except for dimethoate at 0.50 ppm (One way ANOVA, F= 4.48, *df* = 1,6; p = 0.079, Fig. 1) and 0.75 ppm (One way ANOVA, F= 0.00, *df* = 1,6; p = 0.675, Fig. 1), the lowest concentrations (0.25 ppm) of glyphosate (One way

Table I: Active ingredient, surfactant and commercial name of the four different pesticides used in the study

Active ingredient and strength	Surfactant/solvent	Trade name
Chlorpyrifos [O, O-Diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] 400g/L	Xylene	Lorsban 40 EC® or Pattas®
Dimethoate (O,O-Dimethyl phosporodithioate) 400 g/L	Water	Dimethoate 40EC®
Glyphosate [N-(Phosphonomethyl) glycine] 360 g/L	POEA	Round Up® or Glyphosate®
Propanil [N-(3,4-dichlorophenyl) propanamide] 360 g/L	Cyclohexanone & petroleum solvents	3, 4-DPA®

Table II: Percentage survival of *Polypedates cruciger* tadpoles at 10 and 30 days post-hatch stages and metamorphs, after the chronic exposure to the ecologically relevant doses of the four pesticides

Pesticide	Exposure level (ppm)	Percentage Survival (*p*)		
		10 days post-hatch tadpoles	30 days post-hatch tadpoles	Metamorphs
Chlorpyrifos	0.05	92.5 (p=0.088)	90.0 (p=0.096)	82.5 (p=0.053) [a]
	0.10	87.5 (p=0.056) [a]	82.5 (p=0.053) [a]	82.5 (p=0.053) [a]
	0.25	87.5 (p=0.056) [a]	80.0 (p=0.05) [b]	80.0 (p=0.05) [b]
	0.50	85.0 (p=0.051) [a]	80.0 (p=0.05) [b]	80.0 (p=0.05) [b]
Dimethoate	0.25	97.5 (p=1.000)	92.5 (p=0.352)	85.0 (p=0.058) [a]
	0.50	97.5 (p=1.000)	87.5 (p=0.087)	82.5 (p=0.053) [a]
	0.75	92.5 (p=0.088)	85.0 (p=0.058) [a]	82.5 (p=0.053) [a]
	1.00	87.5 (p=0.056) [a]	85.0 (p=0.058) [a]	77.5 (p=0.004) [c]
Glyphosate	0.25	100.0 (p=0.155)	92.5 (p=0.352)	92.5 (p=0.352)
	0.50	97.5 (p=1.000)	87.5 (0.057) [a]	85.0 (p=0.058) [a]
	0.75	95.0 (p=0.248)	82.5 (p=0.05) [b]	80.0 (p=0.05) [b]
	1.00	82.5 (p=0.05) [b]	80.0 (p=0.05) [b]	75.0 (p=0.001) [c]
Propanil	0.25	100.0 (p=0.155)	92.5 (p=0.352)	92.5 (p=0.352)
	0.50	100.0 (p=0.155)	92.5 (p=0.352)	92.5 (p=0.352)
	0.75	92.5 (p=0.088)	85.0 (p=0.058) [a]	85.0 (p=0.058) [a]
	1.00	85.0 (p=0.051) [a]	85.0 (p=0.058) [a]	85.0 (p=0.058) [a]
Control	0	97.5	95.8	95.5

[c]denotes significant differences at p<0.05, [b]denotes the difference is significant at p=0.05 and [a] denotes the difference is close to significant compared to the control. ppm - parts per million. The rest were not significant compared to the control

Table III: Mean snout-vent length (SVL) and mean body weight of *Polypedates cruciger* at metamorphosis after the chronic exposure to the ecologically relevant doses

Pesticide	Exposure Levels (ppm)	Mean SVL ± SD (cm)	*F*	Mean Weight ± SD (g)	*F*
Chlorpyrifos	0.05	1.36±0.060	192.24	0.614±0.039	63.22
	0.10	1.36±0.095	240.98	0.486±0.041	132.58
	0.25	1.30±0.266	213.65	0.527±0.112	125.74
	0.50	1.42±0.237	387.16	0.468±0.068	166.50
Dimethoate	0.25	1.68±0.024	67.16	0.785±0.083	15.26
	0.50	1.63±0.053	78.82	0.737±0.041	28.11
	0.75	1.54±0.074	95.66	0.708±0.030	37.30
	1.00	1.49±0.124	177.46	0.637±0.082	56.85
Glyphosate	0.25	1.67±0.022	79.67	0.768±0.040	27.94
	0.50	1.55±0.017	137.82	0.794±0.028	19.45
	0.75	1.57±0.104	112.39	0.752±0.046	30.70
	1.00	1.50±0.008	180.63	0.710±0.040	47.79
Propanil	0.25	1.68±0.009	91.51	0.747±0.013	31.31
	0.50	1.59±0.045	125.79	0.707±0.032	42.50
	0.75	1.61±0.005	146.99	0.677±0.042	59.53
	1.00	1.53±0.017	199.38	0.709±0.047	39.56
Control	0	2.06±0.028		0.946±0.077	

SD = Standard deviation; ppm = parts per million, All values differed from the controls at p< 0.001

ANOVA, F= 0.19, df = 1,6; p = 0.675, Fig. 1) and propanil (One way ANOVA, F = 0.00, df = 1,6; p = 0.948; Fig. 1).

Malformations of tadpoles and metamorphs: Tadpoles of *P. cruciger* exposed to pesticides developed malformations, while none of the tadpoles in the control group had any malformations. The percentage malformations in tadpoles and metamorphs exposed to pesticides varied at different pesticides and concentrations. Malformations were observed at high frequencies in 10 days post-hatch tadpoles (glyphosate = 69%, dimethoate = 64%, chlorpyrifos = 60%, propanil = 45%) at highest pesticide concentration (0.5 for chlorpyrifos & 1 ppm for others). Malformations observed were mainly in the spine, such as hunched back (kyphosis) and curvature (scoliosis), while edema and skin ulcers were also observed (Fig. 2). Of the total malformations, 47% were scoliosis, 29% were kyphosis and 20% were edema with only few cases having skin ulcers (4%). Edema caused shifting of the centre of gravity and twisting the body axis,

Fig. 1: Average TE_{50} (time required for the forelimb emergence of half the number of tadpoles) values of tadpoles of *Polypedates cruciger* exposed to chlorpyrifos, dimethoate, glyphosate and propanil at different concentrations (exposure levels are given as concentrations 1 = 0.05 ppm, 2 = 0.10 ppm, 3 = 0.25 ppm & 4 = 0.5 ppm for chlorpyrifos & 1 =0.5 ppm, 2 = 0.25 ppm, 3 = 0.75 ppm & 4 = 1.0 ppm for other pesticides),* denotes significant (p <0.05) compared to the control. Error bars represent the standard error of the mean

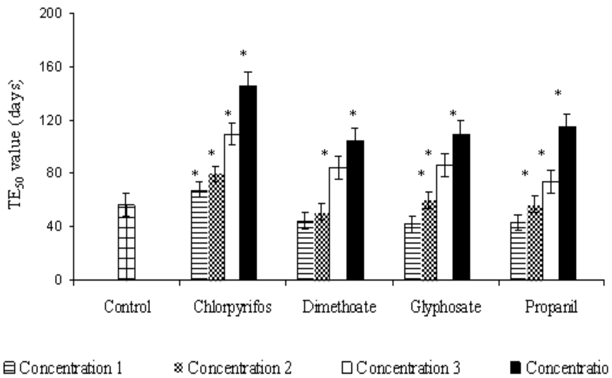
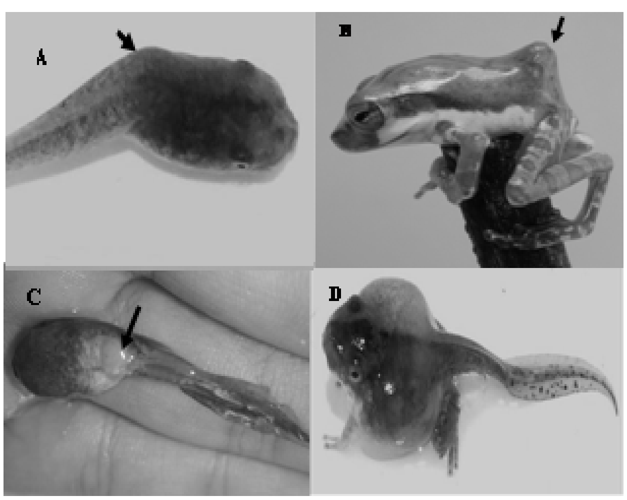


Fig. 2: Different types of malformations observed in pesticide exposure tadpoles and metamophs of *Polypedates cruciger*. Hunched back (khyphosis) in a (A) tadpole and (B) an adult frog (C) a tadpole with an ulcer on underside of the body (D) Late tadpole stage edema on both sides of the body



in turn affected their swimming behavior. Tadpoles affected with edema were seen swimming upside down, not being able to balance their body while swimming. These edemas were often fatal when rupture.

The malformation percentage of ten days post-hatch tadpoles was dependent on the type of pesticide (ANCOVA, $F = 34.68$, $df = 3, 11$; $p < 0.001$) and the concentration of pesticide (ANCOVA, $F_{1, 11} = 151.58$, $df = 1, 11$; $p < 0.001$). Glyphosate recorded the highest percentage of malformation (69%) compared to other pesticides in 1.00 ppm concentration where as chlorpyrifos had a consistently profound effect throughout the concentration series (Fig. 3). The concentration of pesticide was positively and significantly correlated with the percentage malformation in all the pesticides except for chlorpyrifos (Pearson correlation, dimethoate $r = 0.996$, $p < 0.001$; glyphosate $r = 0.985$, $p = 0.002$ & propanil $r = 0.994$, $p = 0.001$). The percentage malformation was consistently high even at 0.05 ppm of chlorpyrifos causing a higher percentage malformation (35%). However, some malformations disappeared with the age of the tadpole, especially those in the tail region as the tail was absorbed during metamorphosis.

DISCUSSION

The results indicate that chronic exposure to ecologically relevant concentrations of the four pesticides chlorpyrifos, dimethoate, glyphosate and propanil affects the survival, growth and development of common hourglass tree frog, *P. cruciger*. These four pesticides are extensively used to control weeds and pests attacking rice, tea, coconut and other crops in Sri Lanka. Recently, an analysis of water samples from a well with possible exposure to agricultural run off showed that water contained up to 10 ppm of glyphosate (Round up®; unpublished observations). Water of these wells is used for human consumption such as drinking, cooking, bathing and washing clothes, while such habitats provide excellent breeding sites for many frog species. *P. cruciger* attaches its foamy egg sacs on vegetation that hangs above the wells where the tadpoles fall into the water up on hatching and complete their larval development in the well.

Unlike other freshwater ecosystems, where pesticide run off is found, rice fields are subjected to direct application of the chemicals and hence the levels of these chemicals are expected to be much higher in rice fields. Rice fields are preferred habitats of amphibians and 18 species, including *P. cruciger*, have been found breeding in rice fields throughout Sri Lanka (Bambaradeniya, 2000). Hence, these results underscore the importance of investigation of the level of these agricultural pesticides found in freshwater ecosystems.

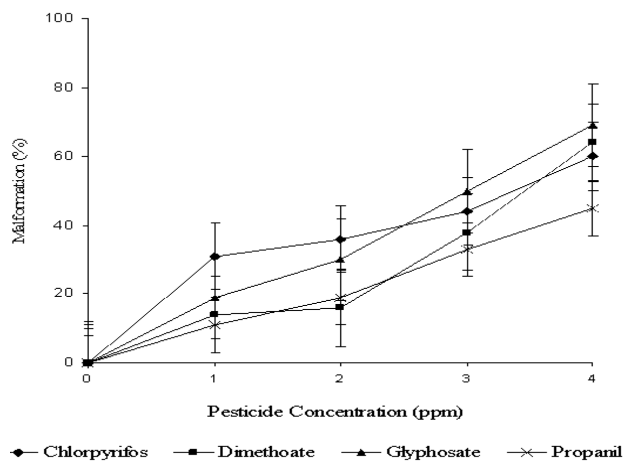
Although mortality has direct implications on population declines, reduced growth rate could also be important indirectly. A significant elongation of the growth period was common to all the exposed tadpoles of *P. cruciger* and the metamorphs were smaller in size compared to those in the controls. Previous studies have shown that a slower growing tadpole may take longer to metamorphose, or metamorphose at a much smaller size than normal, or may experience both (Diana *et al.*, 2000; Boone & Semlitsch, 2002). Tadpoles of European tree frog, (*Hyla arborea*) upon exposure to dimethoate (Mizgireuv *et al.*, 1984) and the Cope's grey tree frog, (*Hyla chrysoscelis*) exposed to chlorpyrifos and atrazine (Briston, 1998) have taken a longer period to metamorphose and were smaller in size. Tadpoles of wood frog, (*R. sylvatica*) exposed to glyphosate in a conifer plantation, showed a reduction in growth compared to unexposed frogs (Glaser, 1998).

The longer periods of metamorphosis and smaller size of adults can have many consequences in nature. Smaller size at metamorphosis affects the individual's reproduction (Hayes *et al*., 2006), survival (Shenoy *et al.,* 2009), immunocompetence (Carey *et al.,* 1999; Christen *et al.*, 2003) and ability to escape from predators and defending territories (Bridges, 1999). These might lead to increased indirect mortality and smaller size at metamorphosis makes them less competitive in foraging and other resource utilization, leading to reduced fitness than the larger individuals (Carey & Bryant, 1995). Nonetheless, delaying metamorphosis has a crucial impact on amphibian survival, especially for *P. cruciger,* as they often breed in temporary water bodies, particularly in agricultural and human altered habitats (Manamendra-Arachchi & Pethiyagoda, 2006), which may dry up before completion of metamorphosis. As Fernando (1995) suggested organisms in rapidly changing ecosystems, such as rice fields, rapid colonization, reproduction and growth are important for their survival.

Chronic exposure to the four pesticides not only affected survival and growth of tadpoles but also lead to development of malformations in *P. cruciger.* Malformations were mostly in the spine such as kyphosis (hunched back) and scoliosis (curvature). Also, most of the tadpoles that had malformations in the tail region metamorphosed into normal adults as the tail was absorbed. In addition to the axial malformations, *P. cruciger* also developed edemas, which were often fatal. However, exposed metamorphs did not develop severe limb malformation such as missing limbs or extra limbs. Tadpoles of *Rana sylvatica* exposed to glyphosate (Vision®) have shown a similar array of malformations, where newly metamorphosed frogs had dorsally curved, laterally curved or undulated (like a wave) axial skeleton together with skin blisters and abnormal tails (Glaser 1998). However, anurans collected from habitats with high agricultural contaminants exhibited severe limb deformities such as ectromely (missing limb), ectrodactyly (missing digit), polydactyly (extra digit), apody (missing foot) and polymely (extra limb; Ouellet *et al*., 1997). The contaminants were mainly composed of insecticides such as carbofuran, azinphos-methyl, herbicides such as glyphosate, MCPA, atrazine and fungicides like chlorothalonil and mancozeb.

The 48 h LC_{50} values for *P. cruciger,* for all the chemicals fall within PAN limits except for propanil. The LC_{50} value of *P. cruciger* for propanil (2.21 ppm) was lower than PAN reported values (10 – 100 ppm; Moore *et al.*, 1998; Kegley *et al.*, 2007), which shows that *P. cruciger* is more sensitive to acute exposure of high levels of propanil. Similarly, the LC_{50} values of tadpoles of the bog frog, *R.*

Fig. 3: Percentage incidences of malformations observed in 10 days post-hatch tadpoles of *Polypedates cruciger* exposed to chlorpyrifos, dimethoate, glyphosate and propanil at different concentrations. 0 = control, 1 - 4 denotes the concentrations (1 = 0.05 ppm, 2 = 0.1 ppm, 3 = 0.25 ppm & 4 = 0.5 ppm for chlorpyrifos & 1= 0.25 ppm, 2= 0.5 ppm, 3= 0.75 ppm & 4= 1.0 f ppm for other pesticides). Error bars represent the standard error of mean



limnocharis (8.13 ppm; Kegley *et al.,* 2007) and clawed toad *Xenopus leavis* (8.17 ppm; Moore *et al*., 1998) are also less than PAN reported values. Therefore, it is possible that amphibians in general are more sensitive to acute exposure of high levels of propanil than other non-target organisms. However, during chronic exposure at ecologically relevant low doses, propanil had the least effect on survival and development of malformations in *P. cruciger.* Conversely, the 48 h LC_{50} reported for *P. cruciger* for dimethoate (8.40 ppm) and glyphosate (14.99 ppm) were higher compared to the bog frog, which were 2.254 and 1.59 ppm, respectively (Kegley *et al.*, 2007), which shows the effects of the same chemical may vary in different species of amphibians. Thus, there is a need for species specific studies to fully understand the potentially harmful effect of different agrochemicals.

Glyphosate and propanil are herbicides. Herbicides are targeted to disrupt the photosynthetic pathway and therefore considered to have little effect on non-target animals. However, this study shows that these two herbicides, especially glyphosate, significantly affected the survival, growth and the development of malformations in *P. cruciger*. In the commercial formulation of glyphosate, the added surfactant (POEA) is more toxic than the active constituent, glyphosate (Wan *et al.*, 1989). Even though the current study did not isolate the impacts of glyphosate and the surfactant, laboratory studies have shown that glyphosate alone has a low toxicity, while the surfactant can be highly toxic to a variety of taxa including amphibians (Mann & Bidwell, 1999; Lajmanovich *et al*., 2003; Howe *et al.,* 2004). Glyphosate acts as an endocrine disruptor

(Kegley *et al.*, 2007) acting like hormones in the endocrine system and disrupt the physiologic function of endogenous hormones. Exposure to endocrine-disrupting chemicals at early stages alters normal hormonal signalling during embryonic development and also can permanently change adult reproductive system morphology and function, as well as reproductive behaviour. Even though propanil had the least toxic effect on common hourglass tree frog at ecologically relevant doses, it is known to affect the functions of the central nervous system and interferes with the normal development of the animal (Kegley *et al.*, 2007). Herbicides also appear to affect the morphology and function of organ systems of amphibians.

As Relyea and Hoverman (2006) suggest understanding and predicting the impacts of agrochemicals on non-target organisms is a challenging proposition. Since Amphibians are sensitive bioindicators, they are good models for evaluating the ecotoxicological effects, especially, in agricultural lands, where pesticide application is high. This study used concentrations of pesticides within the expected post-application levels in the field and showed they could have significant effects on amphibians at lethal and sublethal level. Even though we cannot extrapolate the findings of this study to a natural context uncritically, it highlights the importance of understanding the harmful effect of the pesticides and a great deal of insight into their direct and indirect effect on non-target species. Habitats of many endemic and endangered amphibian species are being converted to accommodate agricultural demand, often with heavy use of agrochemicals. Although traditional rice cultivation has been carried out in a sustainable manner over many millennia, modern rice cultivation depends heavily on chemical inputs, together with short term rice varieties, which has disrupted the balance of these ecosystems and hence poses a threat to the future sustainability of this ecosystem (Roger *et al.*, 1994). In addition to the direct exposure these insectivorous amphibians also feed on the pest insects of rice, which have got exposed to the chemicals.

In nutshell, these results support the hypothesis that the agrochemicals affect the survival, growth and development of *P. cruciger* and also cause malformations even at ecologically relevant doses. Widespread occurrence of malformations in local amphibian populations has not been studied in Sri Lanka, except for a brief report on field observations (Rajakaruna *et al.*, 2007) and a laboratory study on the possible involvement of a parasitic trematode induced malformations (Rajakaruna *et al.*, 2008). Further investigations into the prevalence of malformed amphibians in wild populations of Sri Lanka and the level of agricultural pollutants found in freshwater ecosystems and their effect on native amphibian are required.

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