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Effect of sub-lethal concentrations of urea on the development of endemic arboreal tree frog, *Polypedatus cruciger* (Amphibia: Racophoridae)

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Abstract Amphibian decline has become a worldwide problem today. Climate change, UV radiation, diseases, contaminants, and fertilizers could be considered as the main reasons. The present study was carried out to investigate the effect of urea on the survival, growth, behaviour and morphology of the endemic arboreal tree frog *Polypedatus cruciger* (Anura: Ranidae). First, the Lethal Concentration 50 (LC50) of urea on 10 days old post-hatched tadpoles was determined. After the estimation of LC50, the tadpoles were exposed to three sub-lethal concentrations of urea, namely, 7.0 g/L, 7.5 g/L and 8.2 g/L. The effects were investigated from the age of 10 days until they became adults. The impact of sub-lethal concentrations of urea for the survivals, snout-vent length and weight showed significant differences ($p<0.05$), and a strong negative correlation existed when compared to the control. The tadpoles were very sensitive to urea as they showed several behavioural changes such as reduction in feeding, growth retardation and inability to grow up to the adult stage. The observed external morphological changes under the laboratory conditions included edema, abdominal lesions, colour change, tail deformations and rupturing of tadpoles. The impact of urea on the amphibians in the field may be severe due to the synergistic effects of different pollutants. This may adversely affect amphibians, as their life stages are associated with the agroecosystems and may cause a severe decline in amphibian fauna in Sri Lanka.

Keywords: Amphibian fauna, Life stages, Pollutants, Synergistic effect, Tadpoles

1 Introduction

Amphibians are the first vertebrate group with shell-less eggs and the only vertebrate group with an aquatic larval stage and terrestrial adult phase (Blaustein *et al*. 2003). Amphibians are important vertebrates in ecological interactions, including food chains and webs. The tadpoles in almost all amphibian species entirely depend on the aquatic

environment, and the adults are terrestrial. The amphibians are more susceptible to many environmental toxicants due to their bi-phasic life history and semi-permeable skin (Hall and Henry 1992). The contaminants, including agro-chemicals and other numerous industrial and household effluents, affect the life stages of amphibians (Blaustein *et al*. 1997, Bonin *et al*. 1997), and they show different kinds of abnormalities when they are exposed to those contaminants. Therefore, amphibians can be considered bioindicators of the environment. They are one of the non-target organisms that many ecologists use to study the toxicity of commonly used pesticides in agriculture (Jayawardana *et al*. 2010). Moreover, amphibians are highly susceptible to global decline and extinction threats due to their enormous sensitivity to environmental changes. According to the global amphibian assessment, nearly one-third of the world's amphibian species (nearly 1856) are threatened, and 165 species may have gone extinct (IUCN 2006). There are several reasons for the decline of amphibians. Some are climate change, habitat loss and fragmentation, diseases, and aquatic pollution (Blaustein and Wake 1995). Apart from all these reasons, aquatic pollution caused by chemicals has become a more significant problem worldwide. The sub-lethal effects of pesticides on amphibians include hampered growth and development, decreased reproductive success and behavioural abnormalities, which may also alter susceptibility to predation and competition (Bridges 1999, Boone and Semlitsch 2002).

Chemical fertilizers are considered the primary anthropogenic source of nitrogen in the environment (Likens *et al*. 1997). In freshwater ecosystems, pollution from fertilizers, especially those with nitrogen, is becoming a severe problem (Vidal *et al*. 2000, Haygarth and Jarvis 2002). Excess nitrogen in water bodies can harm the aquatic stages of organisms (Oritiz *et al*. 2004). Urea fertilizer, which has 46% nitrogen, is considered the most important, widely used agro-fertilizer in all cultivation systems. The breakdown of urea occurs within a few days to a week into ammonium ions. After several weeks, it degraded to nitrate (Rose 2018). The environmental effects of urea relate to the degradation of urea into ammonia or other nitrogen products (Bobmanuel *et al*. 2006, Capkin *et al*. 2010). In natural conditions, ammonia occurs as unionized (NH_3) and ionized (NH_4^+) forms in water and can be a hazardous toxicant to aquatic species (EI-shafai *et al*. 2004). The excess amount of nitrogen may be essentially toxic to aquatic organisms. Ammonia toxicity depends principally upon the presence of ammonia, which can readily diffuse across the gill membrane due to its lipid solubility and lack of charge. In contrast, the ionized form cannot readily pass through the hydrophobic micropores in the gill membrane (Sheehan and Lewis 1986). The increased nitrate levels in ground and surface water are major sources of severe environmental stress to aquatic organisms because nitrate is known to be highly toxic to amphibians (Baker and Waights 1993, Baker and Waights 1994). The increased concentrations of nitrate in water cause one of the most prevalent environmental problems responsible for water quality (Wetzel 2001). Amphibians prefer to lay eggs in stagnant water bodies associated with agricultural lands. When the agrochemicals and fertilizers accumulate in those stagnant water bodies, it causes enormous stress on the amphibians, resulting in a decline.

Sri Lanka is a tropical country well known for its amphibian diversity, with around 103 species (Meegaskumbura *et al.* 2002, Manamendra-Arachchi and Pethiyagoda 2006). It accounts for 2% of the world's known anuran species (IUCN 2006). The common hourglass tree frog, *Polypedatus cruciger* (Anura: Ranidae), is widely distributed and an entirely arboreal endemic frog, which can be found mainly in the wet zone of Sri Lanka (Manamendra-Arachchi and Pethiyagoda 2006). It can be seen in association with human residents, wet zone forest areas and breeding in gardens. Also, it forms a kind of "bubble nest". About 240-300 eggs (2.00 mm in diameter) are deposited within a nest, hanging over stagnant water bodies and pools with shallow water (Manamendra-Arachchi and Pethiyagoda 2006). The foamy nest is a secretion from the anal region of the female frog. When the tadpoles are hatched, they fall into the water and spend the larval stage as free swimming in water (Manamendra-Arachchi and Pethiyagoda 2006). The present research was conducted to study the toxicity of urea to the development of common hourglass tree frogs and how the different concentrations affect their development.

2 Material and Methods

2.1 Experimental organisms

The foamy egg masses of *Polypedatus cruciger* were collected from the premises of the Department of Zoology, University of Ruhuna, Matara, Sri Lanka and transported to the laboratory. The egg masses were hung above 250 L fiberglass tanks filled with de-chlorinated tap water and floating *Hydrilla* sp. The hatched tadpoles were allowed to drop into the rearing tanks. Zooplankton collected from the selected ponds in the Department of Zoology, University of Ruhuna were added to rearing tanks as food source to newly hatched tadpoles twice daily. The pond water (6 L at a time) was filtered using the zooplankton net, and the number of zooplankton in 10 mL of pond water sample was counted under the compound light microscope. Then, the density of zooplankton was extrapolated from a 10 mL sample to a 6 L volume. These zooplankton were directly added to the fiberglass tanks without any debris. Commercial fish feed (Sri Lanka) was provided to the tadpoles at the end of the external gill stage. In addition to *Hydrilla* plants, floating objects such as small branches of trees were put into the rearing tanks.

2.2 Obtaining purified urea

The urea fertilizer ('Kunlun Urea', China) used in this research was 99% pure with less impurities (Ratnayake and Navaratna 2014). Some other chemicals (1%) were mixed with this fertilizer, like biuret and inorganic salt. A recrystallization process was applied to remove those impurities. The granulated urea sample (about 30 g) was added to 100 mL of distilled water in a 150 mL beaker. This solution was heated using a Bunsen burner (up to 100^{o}C) with constant stirring until the urea was completely

dissolved. After that, the solution was cooled to room temperature and placed in an ice bath to promote crystallization. The urea crystals were filtered using a Whatman filter paper. Finally, these crystals were rinsed quickly with a small amount of cold distilled water, and allowed to dry. For preparing the sub-lethal urea concentrations, this purified urea was used.

2.3 Acute exposure to determine LC_{50} values

The Lethal Concentration 50 (LC_{50}) (the concentration at which 50% of tadpoles die) of urea was determined by exposing 10 days old post-hatched tadpoles for 48 hours. The age of the tadpoles was determined from the first day of hatching as a tadpole. The stages of the tadpoles were determined by observing them under a compound light microscope each day (external gill stage and internal gill stage). After preliminary exposure to assess the range of urea concentrations, a series of 8.30, 8.70, and 9.20, 9.50 g/L was considered. Urea was diluted in de-chlorinated tap water. Fifteen tadpoles were placed in each experimental glass tank of 4.5 L (15 X 15 X 20 cm^3) containing 1.0 L of the test solution. After 48 hours, the number of survivors was recorded (Schuytema and Nebeker 1999a).

2.4 Chronic exposure to urea at ecologically relevant concentrations

10 days old post-hatched tadpoles (n=120) were exposed to urea in experimental glass tanks (15 X 15 X 20 cm^3) containing 1.0 L of urea solution and control containing 1.0 L of de-chlorinated tap water. A sub-lethal concentration series of 7.0, 7.5, and 8.2 g/L were selected to study the following biological parameters from the internal gill stage (10 days old) of tadpoles until they complete the aquatic phase of their life cycle. Every concentration was repeated three times. The tests of length measurements and wet weight measurements were conducted using 10 tadpoles in each experimental tank and the control. The tadpoles were fed with commercial fish feed. The medium of the tanks was renewed once a week. The tanks were checked, and the dead tadpoles were removed daily. Moreover, the morphological changes and behavioural changes were recorded. The number of survivors was recorded in both control and three sub-lethal concentrations of urea throughout the life cycle from the internal gill stage to the end of the aquatic phase.

The snout-vent length (S-V length) of the tadpoles was measured after blotting the tadpoles for five seconds using tissue papers, and the snout-vent length of the tadpoles was measured using SCHEER-Tumico Vernier calliper (China). The wet weight of the tadpoles was measured by using RADWAG Analytical Balance (Poland) after blotting the tadpoles using tissue papers. This was done from the internal gill stage up to metamorphosis. The measurements were taken every 10^{th} day of the life cycle (Jayawardana *et al*. 2010). Behavioural changes like abnormalities in swimming, activeness, feeding and equilibrium were observed in the control and experimental groups separately. This was continued throughout the developmental stages of tadpoles. The external morphological changes (abnormalities/malformations) of the

developmental stages of tadpoles were reported daily in all study groups. The exact time required for the major morphological changes in the life cycle of *P. cruciger* was recorded in the control and the experimental groups separately.

2.5 Histological analysis

The dead tadpoles were fixed in Bouin's fluid (7-10 mL) for 7-10 days in the glass vials, and washed with 70% Alcohol several times until the disappearance of yellow colour. In order to dehydrate, samples were kept for 48 hours in 70% Alcohol and 90% Alcohol, respectively and finally transferred to 100% Alcohol three times. After that, the samples were transferred to vials with chloroform, and wax pellets were added. Then, the wax blocks were prepared, and finally, 0.5 mm thin sections were obtained using the Thermo-Scientific microtome (England). These sections were double stained with H&E and observed under the compound light microscope. The photographs of the deformed regions were taken using the Olympus Digital microscope (Japan).

2.6 Statistical Analysis

The statistical program SPSS (version 25) was used for data analysis. Using the SPSS program, the LC_{50} (the concentration at which 50% of tadpoles die) of urea was obtained. The survival, snout-vent length, and weight data were not normally distributed ($p<0.05$), so, non-parametric one-way ANOVA (Kruskal-Wallis test) was applied for each analysis. A non-parametric Mann-Whitney U test was applied to check the significant differences among the groups.

3 Results

3.1 Life cycle of *Polypedatus cruciger* under the laboratory condition

The egg masses of *P. cruciger* contained nearly 240-300 eggs each, white in colour and around 2.0 mm in diameter. The foamy egg masses were yellow to brownish in colour and sticky. From the egg mass, tadpoles were hatched within 5-8 days. The newly hatched tadpoles had a yellow yolk sac used for nourishment in the first few days. The hatchlings could not swim and generally attached to the tank's walls or floating objects. After feeding on the yolk sac, they started to feed on zooplanktons. Generally, the tadpoles swim slowly at the bottom of the tank, and the external gill stage lasts 9 to 11 days. The internal gill stage started between 10-12 days. In the final days of the internal gill stage, the tadpoles could feed on larger food particles like commercial fish feed. They gain the ability to detect these large food pellets quickly. The tadpoles started breathing air after 36-38 days. Usually, they swam quickly to the water surface and rapidly move to the bottom after taking the air.

Table 1. Time duration needed (in days) for morphological and behavioural changes in the life cycle of *Polypedatus cruciger* under the selected sub-lethal concentrations of urea (treatment 1 = 7.0 g/L, treatment 2 = 7.5 g/L, and treatment 3 = 8.2 g/L; the average time spent for each change is mentioned outside the parenthesis and general time duration needed for each physiological change is shown within the parenthesis).

Morphological or behavioural change	Time duration (days)				
	Normal condition	Control	Urea concentrations		
			Treatment 1	Treatment 2	Treatment 3
1. Hatching out from egg mass	7 (5-8)	7 (5-8)	7 (5-8)	7 (5-8)	7 (5-8)
2. Feeding on yolk sac	9 (8-10)	9 (9-10)	10 (8-10)	10 (8-10)	11 (8-11)
3. Start swimming	9 (8-10)	10 (10-12)	11 (10-12)	12 (10-12)	11 (10-12)
4. Disappearance of external gills	9 (9-11)	10 (10-11)	10 (10-11)	12 (10-13)	13 (10-14)
5. Appearance of internal gills	10 (10-12)	11 (10-12)	11 (10-12)	13 (10-12)	14 (11-14)
6. Begin to feed on zooplankton	12 (11-13)	11 (11-13)	12 (11-13)	12 (11-13)	14 (13-16)
7. Feed on commercial fish feed	16 (15-18)	16 (15-18)	25 (24-30)	28 (27-30)	25 (24-30)
8. Start air breathing	30 (30-34)	30 (30-34)	35 (30-36)	35 (30-36)	37 (36-38)
9. Appearance of hind limb bud	38 (36-38)	38 (36-38)	69 (68-70)	72 (70-72)	69 (69-70)
10. Appearance of toe joints	44 (42-45)	50 (50-52)	All dead	All dead	All dead
11. Appearance of real long tail fin	42 (43-44)	50 (48-50)	All dead	All dead	All dead
12. Appearance of distinct elongated body	44 (44-45)	52 (52-55)	All dead	All dead	All dead
13. Formation of skin pigments and patches on limbs	49 (47-50)	54 (53-56)	All dead	All dead	All dead
14. Appearance of distinct head region	54 (53-54)	60 (60-62)	All dead	All dead	All dead
15. Appearance of fore limbs	58 (57-60)	63 (63-65)	All dead	All dead	All dead
16. Protruding eyes	59 (58-60)	65 (65-67)	All dead	All dead	All dead
17. Stop feeding completely	60 (58-61)	68 (66-68)	All dead	All dead	All dead
18. Become an Adult frog	62 (60-62)	70 (68-70)	All dead	All dead	All dead

The hind limb buds protruding from the cloaca region appeared from 36-38 days. Gradually, the hind limb buds grew, and the body length increased. Also, the toes became prominent in size with distinct webbing. The fore legs were visible after the emergence of hind legs, and the forelimbs were shorter than the hind limbs. Both fore and hind limbs bore green colour pigmentation. The dorsal and ventral fins were well-developed in the elongated tail. When it was nearly 60 days, the tadpoles ultimately stopped feeding, and they looked like young adult frogs with elongated tails. In the 9th week of the life cycle, they completed the metamorphosis, shed the tail, and became tiny froglets. They were greenish and showed locomotion as adult frogs. The time needed for morphological and behavioural changes in the life cycle of *Polypedatus cruciger* is as follows in Table 1.

3.2 Behavioural changes

Tadpoles in urea-introduced tanks (all three treatment groups) avoided feeding, were immobilised, and stayed near the bottom of the tank. Most of the tadpoles had severe edema and exhibited upside-down swimming. They were unable to balance their bodies during swimming. Finally, these edemas ruptured, and the tadpoles were dead.

3.3 Survival of tadpoles

Figure 1 shows the relationship between the number of survivors and the age of tadpoles (post-hatched days) in control and three sub-lethal urea concentrations.

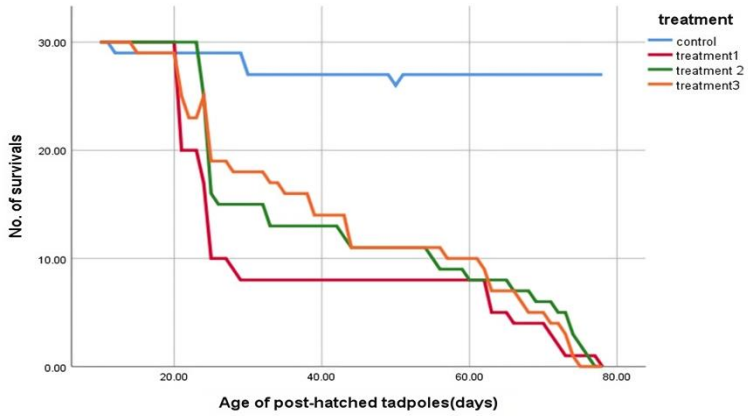

Fig 1. Number of survivals in three different concentrations of urea (treatment 1 = 7.0 g/L, treatment 2 = 7.5 g/L, and treatment 3 = 8.2 g/L) and control group throughout the study period.

According to the statistical analysis, the survival data were statistically significant ($p<0.001$). There was a significant difference between control and all three treatments ($p<0.001$), as well as between treatment 1 and treatment 2 ($p<0.001$) and treatment 1

and treatment 3 (p=0.002). The data was not statistically significant between treatment 2 and treatment 3 (p=0.735). Survival of the tadpoles exposed to urea had shown a significant negative correlation with the concentrations (Spearman's-rho correlation coefficient r = -0.297, p<0.001).

3.4 Growth of tadpoles

Snout to vent length (S-V length)

The relationship between the S-V length of tadpoles with their age (post-hatched days) in control and three sub-lethal urea concentrations of urea is shown below in Figure 2.

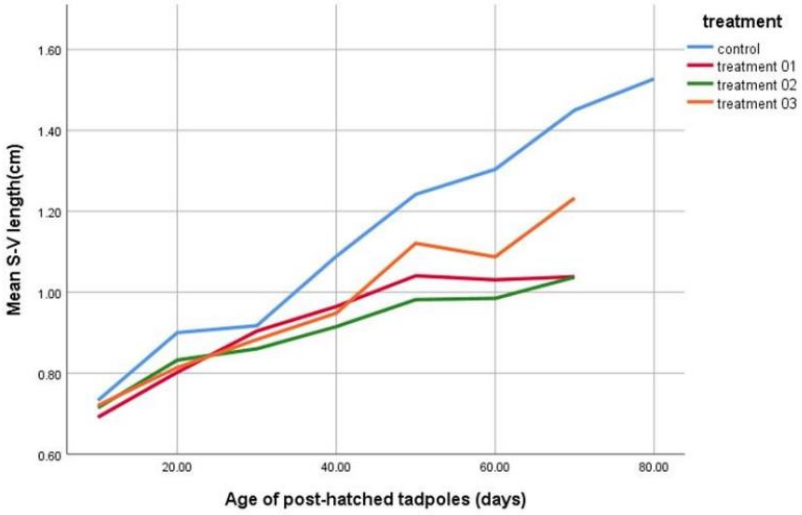

Fig 2. Mean S-V length of tadpoles in three sub-lethal concentrations of urea (treatment 1 = 7.0 g/L, treatment 2 = 7.5 g/L, and treatment 3 = 8.2 g/L) and control group throughout the study period

According to the statistical analysis, the S-V length data were statistically significant (p<0.001). There was a significant difference between control and all treatments (p<0.001). The obtained results were not statistically significant between treatment 1 and treatment 2 (p=0.506), treatment 1 and treatment 3 (p=0.321) and treatment 2 and treatment 3 (p=0.593). The S-V length of the tadpoles exposed to urea had a significant negative correlation with the concentrations (Spearman's-rho correlation coefficient r = -0.405, p<0.001).

Wet weight

Figure 3 shows the relationship between the wet weight of tadpoles and their age (post-hatched days) in control and three sub-lethal urea concentrations.

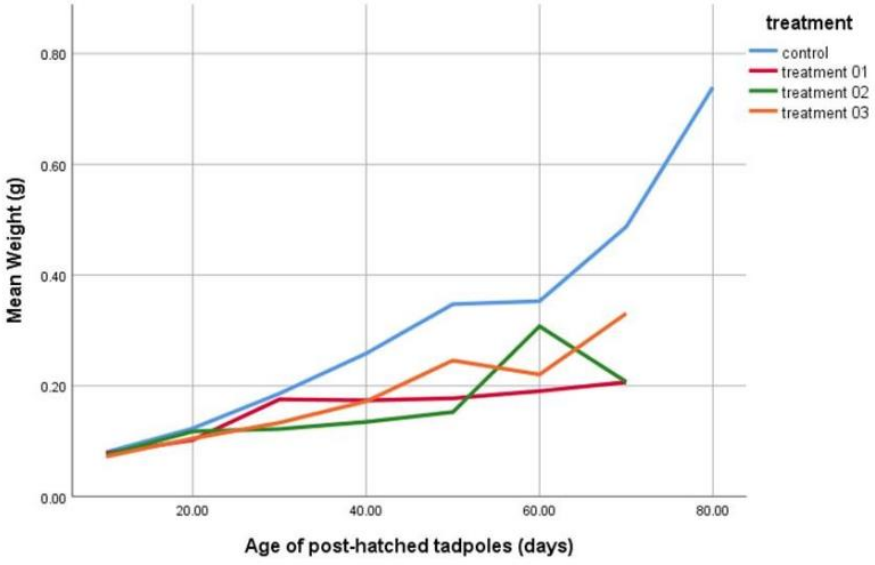

Fig 3. Mean weight of tadpoles in three sub-lethal concentrations of urea (treatment 1 = 7.0 g/L, treatment 2 = 7.5 g/L, and treatment 3 = 8.2 g/L) and control group throughout the study period

According to the obtained data, the weight data were statistically significant. There was a significant difference between control and all treatments ($p<0.001$). The obtained results were not statistically significant between treatment 1 and treatment 2 ($p=0.693$), treatment 1 and treatment 3 ($p=0.936$) and treatment 2 and treatment 3 ($p=0.825$). Weights of tadpoles showed a significantly negative correlation with the concentrations of urea (Spearman's–rho correlation coefficient $r = -0.436$, $p <0.001$)

3.5 Morphological changes

External morphological changes

The observed external morphological changes were as follows (Figure 4).

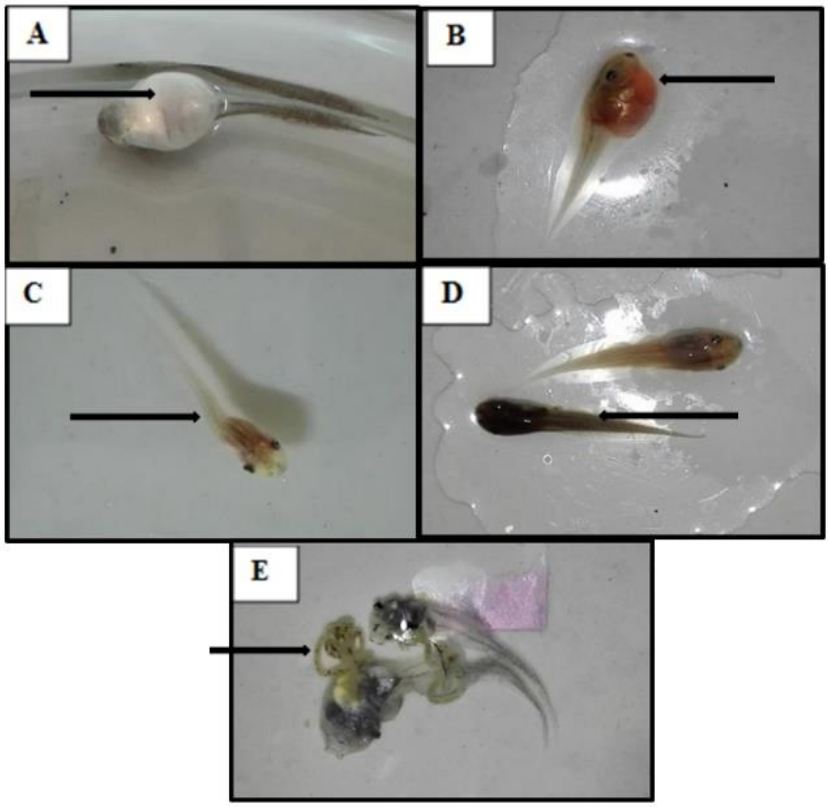

Fig 4. External deformations of tadpoles.
A: Severe edema in the abdominal region (50 days old), B: Abdominal lesions (25 days old), C: tail deformation in a tadpole (28 days old), D: Colour change (compare to the control 15 days old), E: rupturing of tadpoles (20 days old)

Internal morphological changes

Dead tadpoles were in the treatment groups, and the control was fixed to get histological sections. The observations are given in Table 2. The deformations in the optic and heart regions were as follows in Figures 5 and 6.

Table 2. Comparison of the changes in internal regions of tadpoles under selected sub-lethal concentrations of urea (treatment 1 = 7.0 g/L, treatment 2 = 7.5 g/L, and treatment 3 = 8.2 g/L)

Region	Control	Treatment 1	Treatment 2	Treatment 3
Optic region	No change	No change	No change	Swelling in optic tissues
Heart region	No change	No change	Edema in heart cells	Edema in heart cells

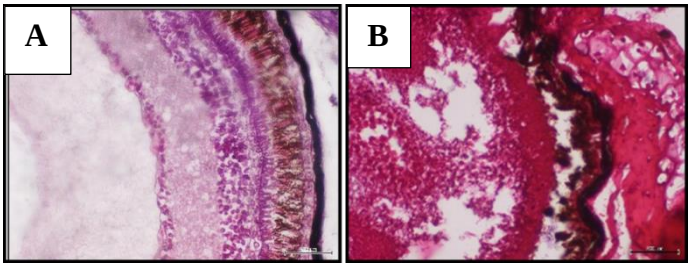

Fig 5. Internal deformations in optic region A) T.S. through optic region (Control), B) T.S. through optic region (treatment 3)

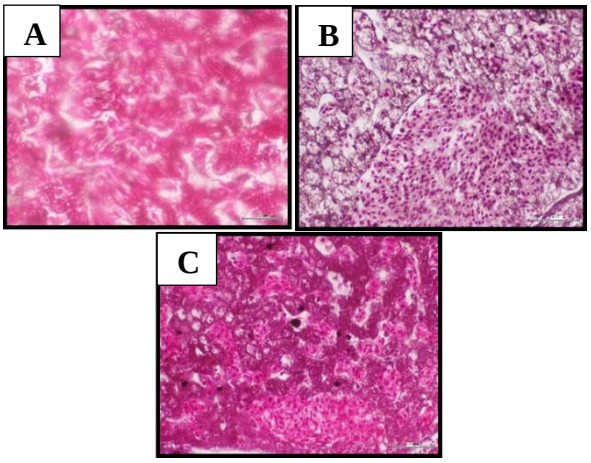

Fig 6. Internal deformations in heart region, A: t.s. through heart region (control) B: t.s. through heart region (treatment 2), C: t.s. through heart region (treatment 3)

4 Discussion

The common hourglass tree frog has distinct characteristics such as longer and stout limbs, protruding eyes and colourful patch patterns on the skin. Under natural conditions, this frog needs 60-62 days to complete their life cycle. The adult frog's skin in the head region is very tightly attached to the skull (Manamendra-Arachchi and Pethiyagoda 2006). The adult male is 50.0-59.8 mm, and female frogs are 72.0-90.0 mm long and have webbed feet. The skin colour range of adults is from greenish brown, dark brown to yellowish orange. The skin has a cross-like colour band from the inter-optical cup region to the middle region of the body. Adult frogs are associated with human habitats, some as permanent dwellers. They rest in shady places and bathrooms in human dwellings in the daytime. This frog can sometimes be seen in tropical

rainforests in Sri Lanka, especially during the wet seasons (Manamendra- Arachchi and Pethiyagoda 2006).

The bubble nests usually attached to the objects above the stagnant water bodies contain 240-300 eggs. After the hatching, which takes about 5-7 days, tadpoles fall into the water. The larval stages are entirely aquatic (Manamendra-Arachchi and Pethiyagoda 2006). The newly emerged tadpoles are unable to swim. So, they attach to the walls of the rearing tanks and rest immobile without swimming. The tadpoles emerging from egg mass feed on the yolk sac in the first few days. In the first few days, they have external gills to absorb dissolved oxygen from water. Later (from 10-11 days), the external gills disappear, and the internal gills appear for internal respiration. In the external gill stage, the tadpoles feed totally on the yolk sac. However, in the internal gill stage, the tadpoles can feed on zooplankton and commercial fish feed.

With the age of the tadpoles, they quickly detect food and feed on them. Until they become adults, the internal gills act as their respiratory organs. The first sign of air-breathing can be identified when the tadpoles swim to the water's surface frequently to absorb oxygen in the air. With time passing, the growth of the tadpoles occurs. The snout-vent length and the body weight increased continuously. The emergence of the hind leg buds is the first indication of being an adult frog. Normally, these leg buds appear together and grow simultaneously to powerful hind legs. The development of the joints occurs after that. After the emergence of hind legs, the fore legs emerge. These fore legs appear one after the other, which may be either right or left. Hind legs are longer than fore legs. At this stage, the tadpole is morphologically like a young frog, having an elongated, transparent tail. It is near to moult into the adult stage. At this stage, the pigmentation occurs in the tadpole's body, with green bands in the fore and hind legs. The resorption of the tail causes a tadpole to become an adult frog.

Most amphibians prefer stagnant water bodies for breeding and nesting. However, these water bodies could be polluted with environmental contaminants, including pesticides, heavy metals, nitrogen fertilizers, industrial chemicals, and salts (Blaustein and Wake 1995). These chemical contaminants can cause morphological deformations and alter physiological conditions, especially in the larval stages of amphibians. Subsequently, this might affect their survival in nature as adults. At present, fertilizers are widely used to increase crop harvesting. Compared to pesticides, the effects of fertilizers on non-target organisms are less reported. Against this background, it is much more important to study the effects of urea, one of the widely used fertilizers in Sri Lanka, on the larval stages of selected amphibian species.

According to the present study, the LC_{50} for urea for tadpoles of *P. cruciger* was 9.218 g/L, as shown by the probit analysis. Therefore, three concentrations below this concentration were used to study the toxicity of urea on *P. cruciger* tadpoles.

According to the results, the survival of tadpoles was significantly reduced in urea-exposed groups compared to the control. Also, there was a strong negative relationship (correlation) between urea concentrations and the number of survivals, and when the urea concentration is increased, the number of survivals decreased. The tadpoles exposed to urea were immobilized at the bottom of the experimental tank and not feeding. Hatch *et al*. (2001) also have recorded altering feeding behaviour of recently

metamorphosed frogs *Rana cascadae* and *Bufo boreas* when exposed to urea. Recent experimental studies demonstrated that in the presence of nitrogenous fertilizers, the larvae of some species reduce feeding activity, swim less vigorously, display less equilibrium, develop malformations of the body, and die (Marco *et al*. 1999). When *Rana temporaria* were exposed to elevated levels of NH_4^+, the mortality increased (Schutema and Nebeker (b) 1999). According to Jofre and Karasov (1999), ammonia has been shown to affect the survival and development of anuran larvae. So, the increased mortality is obvious when exposed to urea. Nitrogen fertilizers such as urea fertilizer can increase ammonia concentration in water (Palanivelu *et al*. 2005, Bobmanuel *et al*. 2006). According to Rouse *et al*. (1999), ammonia is the most toxic, followed by nitrite and nitrate. In this study, the life cycle of the tadpoles exposed to urea has ceased from the hind bud stage due to the toxicity. There was no development after the hind leg bud emergence. According to the study, no tadpoles could complete their metamorphosis and become adult frogs.

In the last few days of their life cycle, most tadpoles had severe edema in the abdominal region and could not balance their body when swimming. Upside-down swimming was common in the last few days of the life cycle of most tadpoles. The edemas were fatal after rupturing the abdominal region. Some tadpoles had lesions in the abdominal region, while some had deformations in the tail. The tadpoles exposed to urea have distinct colour changes compared to the control group. The urea-exposed tadpoles had a much darker body colour (blackish brown), while the tadpoles in the control group were yellowish. Other than the above external morphological changes, there were internal morphological changes, which can be seen in the histological analysis. The transverse sections of the tadpole's optic region in the highest concentration of urea (treatment 3) show significant swelling compared to the control group. Also, in the transverse sections of tadpoles in treatment 2 and treatment 3 through the heart region, there was edema in the cells of the heart region, which were filled with red blood cells. The most common deformations are cardiac and abdominal edema, bent tails and dorsal fin curvature.

The snout-vent length and the body weight of the tadpoles were taken as indicators of growth. According to the results, there was a significant difference in snout-vent length and body weight of urea-exposed tadpoles of *P. cruciger* compared to the control group. Jofre and Karasov (1999) recorded a decreased tadpole survival and growth in green frogs (*Rana clamitans*) with a concentration of 0.6 mg/L NH_3. Ammonia is toxic to aquatic organisms, including fish, by affecting ionic regulation (Paley *et al*. 1993). Also, ammonium ions may cause gill damage and stress in aquatic organisms (Smart 1978, Todgham *et al*. 2001, Wicks *et al*. 2002). When the urea concentration increases, the pH of the medium increases, which might be due to ammonia accumulation. Also, when the urea concentration increases, the number of survivors decreases. Ammonia is a basic compound. When the pH value increases, it causes stress in aquatic organisms. With the elevated ammonia and high pH in the external environment, the tadpoles cannot excrete their metabolic by-products. So, the toxicants accumulate in their internal organs, resulting in the death of tadpoles.

Nitrogen toxicity affects them with reduced growth rate, inhibiting metamorphose and reducing snout-vent length and weight (Macro and Ortiz-Santaliestra 2009).

Nitrogen pollution is a huge problem worldwide, and it causes unknown consequences for the amphibian population (Rouse *et al*. 1999). In the field, urea fertilizer causes hypoxia and changes in behavioural patterns in aquatic organisms like fish (Kind *et al*. 2002). Further, it may be a major reason for the disappearance of sensitive species from aquatic habitats. Naturally occurring ammonia plays a vital role in the aquatic environment. The aquatic animals excrete urea as the metabolic by-product. However, the excess ammonia in water has become a more significant problem for these aquatic animals. Some researchers have shown frogs' avoidance behaviour in zones with elevated ammonia concentration (Mantifel 2006).

Nitrogen contamination in stagnant water bodies is known to significantly affect amphibians worldwide (Bogardi *et al*. 1991). The amphibian larvae depend on aquatic habitats to go through the larval development. So, the natural habitats of *P. cruciger* larvae face severe threats due to various pollutants in water bodies (Balangoda *et al*. 2018). Pesticides, insecticides, hormones, and fertilizers protect crops and increase yields. So, the synergistic effect may be more harmful than the single compound. Long-term exposure to ammonium and nitrate resulting from the dissociation of this compound in water is known to alter the developmental and behavioural functions of anurans (Hecnar 1995). Malformed individuals are more susceptible to predation, have low reproductive success, and have weak immune and endocrine systems (Ouellet *et al*. 1997, Boone and Semlitsch 2002), considerably reducing their survival chances in the wild. As the skin of amphibians is semi-permeable, the osmotic flow of water is proportional to the osmotic gradient ((Rouse *et al*. 1999). Also, the excess urea causes methohemoglutanemia (Rouse *et al*. 1999). When ingested nitrate, it may be reduced to nitrite in the large intestine, where it enters the blood. In the blood, nitrites react with haemoglobin. Then, the ferrous ion oxidizes to a ferric ion and produces methaemoglobin. Methaemoglobin cannot bind or transport oxygen, thus causing tissue hypoxia and leading to death (Huey and Beitinger 1980).

The urea fertilizer in Sri Lanka is mainly applied to the paddy fields. As a nitrogen (N) fertilizer, urea is commonly used in the agricultural sector worldwide (Ghosh and Bhat 1998, Guo *et al*. 2004). From this, up to 30% of urea is absorbed by the plant, and the rest is flown to nearby water bodies due to the extreme solubility of urea in water. According to the annual report (2016) of the Department of Agriculture, Sri Lanka, urea imported to Sri Lanka in 2015 was 460,021 metric tons for agricultural purposes and 224,814 metric tons used for paddy cultivation. Nearly 100 Kg of urea is applied to the paddy (to one hectare) in one season (Yala/Maha). For paddy and almost all crops, including leafy vegetables and coconut, urea fertilizer is the major nitrogen source. In 20 ^{0}C in 1 L, 1079 g of urea is dissolved. Urea increases the harvest and improves plant growth. Excess nitrogen in water bodies can seriously harm amphibian aquatic stages (Ortiz *et al*. 2004). Without efficient usage, synergism caused by the run-off of agrochemicals may adversely affect the non-target organisms.

5 Conclusions

According to the results of the present study, urea fertilizer harmfully affects the various developmental activities of the life cycle of *P. cruciger*. No tadpole was moulted into an adult frog when exposed to urea fertilizer. Also, the growth retardation occurs due to exposure to urea fertilizer. This is obvious when comparing the snout-vent length and the weight of the tadpoles with the control group. Also, when the urea concentration increases, their development decreases. There was a strong negative correlation between urea concentration, weight and snout-vent length. Also, the survival of tadpoles was reduced when they were exposed to urea. Not only that, but this fertilizer also caused several deformations throughout the life cycle of common hourglass tree frogs. It can be concluded that urea, widely used as the main nitrogen source for crops, may adversely affect the life stages of *P. cruciger*, *even* at sub-lethal levels.

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