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RESEARCH ARTICLE

Effects of diazinon on survival and growth of two amphibian larvae

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Abstract: Amphibian populations are declining globally at an alarming rate and evidence suggests that pesticides may be a principal cause. The present study investigated the effects of diazinon on the survival and growth of larvae of two amphibians, *Bufo melanostictus* (Asian common toad) and the Sri Lankan endemic *Polypedates cruciger* (Common hourglass frog).

Larvae were laboratory bred from egg clutches collected from ponds and wells in home gardens in the Gampaha and Colombo districts. Two separate trials were conducted using gill stage hatchlings (Gosner stages 20-22) of each species. The larvae were repeatedly exposed to 4 µg/L, 400 µg/L and 10 mg/L of diazinon for seven days. Results showed that exposure to 10 mg/L of diazinon caused significant elevations ($p<0.05$) in larval mortality in both *B. melanostictus* and *P. cruciger* as compared to the controls. No significant increases in mortality were noted at 4 µg/L and 400 µg/L. The trends in mortality were significant and positive for both species. Growth retardation was also noted at the highest dose of 10 mg/L, these larvae being significantly smaller than those in the controls ($p<0.05$). Larval activity was also seriously impaired at the highest dose.

Keywords: Amphibian, *Bufo melanostictus*, diazinon, mortality, organophosphate pesticide, *Polypedates cruciger*, tadpoles

INTRODUCTION

The widespread application of pesticides has attracted the attention of ecologists due to the impacts of these chemicals on natural communities. A diversity of pesticides and their residues are present in a variety of aquatic habitats[1]. While pesticides have the potential to affect many types of aquatic organisms, amphibian larvae are especially sensitive because of their permeable skin and gills[2]. Not surprisingly, pesticides have been identified as one of the major causes of amphibian declines worldwide [3-6].

Sri Lanka, with about 2% of the world's amphibian fauna, has been recognized as a global amphibian hotspot. The island supports more than a hundred species, of which 88 are found nowhere else in the world[7]. The majority of these amphibians, especially the endemics, are restricted to the southwestern rainforests that are mostly surrounded by agricultural plantations. Although no data are yet available on population trends of Sri Lankan amphibians, it is clear that many species have undergone range reductions or population declines in the last decade. It is of significance that about 70 % of the island's amphibians are currently facing the threat of extinction[7]. The expansion of agriculture and horticultural industries in the country and the accompanying increase in the use of pesticides [8] have been implicated as probable causes for the threatened status of many of these amphibian species[7] . Despite the widely held belief that pesticides are responsible for amphibian declines in Sri Lanka, there is a dearth of empirical evidence to justify these claims.

To understand the role of pesticides in causing amphibian decline, it is necessary to first study their direct toxicity through empirical studies [9-11]. The present study was aimed at providing evidence for the potential toxic impacts of a widely used organophosphate pesticide, diazinon, on the growth and survival of the larvae of two amphibian species, the common toad *Bufo melanostictus* and the endemic common hourglass frog *Polypedates cruciger*. Apart from a preliminary study on the effect of chlorpyrifos on *Rana* sp.[12], there are no reports of investigations on the effects of pesticides on endemic amphibian species in Sri Lanka.

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MATERIALS AND METHODS

In February and March 2006, six egg masses, three from each of the two species *B. melanostictus* and *P. cruciger*, were collected from ponds and wells in home gardens, in Delgoda and Malabe in the Districts of Gampaha and Colombo. The egg masses were identified as belonging to the study species by their external morphology as described by Kirthisinghe (1955)[13]. The egg masses were left to hatch under natural light conditions (approximately 12 h day light).

Three concentrations of diazinon, 4 µg/L, 400 µg/L, and 10 mg/L, were selected for the exposure trials. This selection was based on experiments conducted elsewhere [14]. As the application dose of diazinon is in the range of 1000 mg/L (as per instructions), it was also considered necessary to test toxicity at a high dose. Commercial grade diazinon purchased from Baurs Ltd. Colombo was used to prepare the selected test concentrations of pesticide. All experiments were conducted at ambient temperature and under natural light conditions. Separate sets of experiments were conducted for the two species. Glass tanks of 25x25x15 cm containing 2 liters of tap water were used for the exposures. Tap water was initially collected into buckets and left for 24 h to allow chlorine levels to reduce. Preliminary investigations revealed that tadpoles of *B. melanostictus* and *P. cruciger* survived well in this water. Subsequently, eighteen hatchlings of a given species (6 from each clutch x 3 clutches) corresponding to Gosner stages 20-22[15] were randomly assigned to each tank. The relevant pesticide concentrations were then added and the water was mixed using a glass rod. Each treatment and the controls (without pesticides) were maintained in triplicate. The water was changed and pesticide concentrations were renewed once (after three days) during the 7-d trial period. Previous studies conducted with pesticides following a similar methodology, but with water renewal being carried out every five days[16], has shown less than 25% deviations in pesticide levels The larvae were fed once a day on fish food pellets (Qualitypets Aquatics, Ja-Ela). Fifty pellets (0.048 ± 0.005 g) were added daily to each tank. The temperature and pH were measured in each tank using a thermometer (Brannan, Lloyd's register company, North Carolina, U.K.) and pH meter (TOA HMV30v, Tokyo) The water temperature varied between 27.3 °C and 28.0 °C and pH between 7.2 and 7.9 with no statistical difference in water temperature or pH being detected between and within the treatments and replicates.

Larval mortality was monitored daily. The body length (from the tip of the snout to anus) was measured at the end of the 7-d exposure using a vernier caliper (Dialmax Spi 2000, Switzerland). The activity levels of the larvae were also recorded on the 7th day after the initial exposure. For this, two lines (one vertical and the other horizontal along the mid lines) were drawn on a white sheet of paper and each glass tank was placed on this paper. The tanks were observed from above and the numbers of crossings by the tadpoles were counted for 10 min.

To assess the effect of diazinon on mortality, a one-way ANOVA was performed using total percentage mortality (square-root transformed), length or activity at the end of the exposure period as the dependent variable and the pesticide concentration as the categorical variable. The activity levels of the larvae were calculated by dividing the total number of crossings observed per tank by the number of larvae in the tank at the time of observation. In all cases, the post-hoc HSD Tukey tests were used for pair-wise comparisons. Dose-dependency was examined with the Pearson's correlation test using the total mortality values of the two species.

RESULTS

The results show that diazinon causes marked increases in the levels of larval mortality in both species of amphibians (Figure 1). A highly significant elevation in larval mortality (11 % in *B. melanostictus* and 10 % in *P. cruciger*) was evident at the highest tested dose of 10 mg/L in both amphibian species (*B. melanostictus* $F = 5.71$, $p<0.01$; *P. cruciger* $F = 11.48$, $p<0.01$). The mortality levels also correlated positively with the magnitude of the dose (*B. melanostictus* $r = 0.99$, $p<0.001$; *P. cruciger*: $r = 0.95$, $p<0.05$). No larval deaths occurred in the controls or in larvae treated with 4 µg/L of diazinon in both species during the 7-day exposure period. A concentration of 400 µg/L was not detrimental to the larvae of *B. melanostictus* but resulted in low levels of mortality (3.4%) in *P. cruciger*.

In addition to mortality, diazinon also caused growth retardation in larvae of both amphibian species. The length of tadpoles treated with 10 mg/L were significantly lower than those in the control tanks (one-way ANOVA: *B. melanostictus* $F = 4.66$, $p<0.05$; *P. cruciger* $F = 16.69$, $p<0.001$). No significant reductions in length were evident in larvae exposed to 4 µg/L and 400 µg/L. Results also showed that there was a significant reduction in larval activity in both *B. melanostictus* and *P. cruciger* by the seventh day (one-way ANOVA: *B. melanostictus* $F = 99.18$, $p<0.05$; *P. cruciger* $F = 151.58$, $p<0.05$). The impairment of activity was severe with a 40% reduction in larval activity being noted for *B. melanostictus* treated with 10 mg/L of diazinon as compared to the control. The retardation of activity was even greater for *P. cruciger*

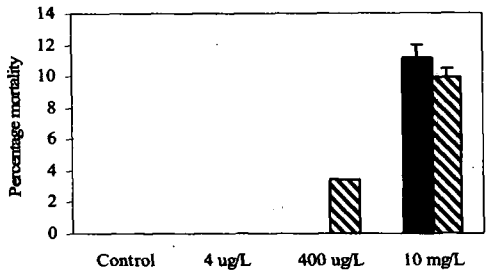

Figure 1: Mean percentage mortality (±S.E) in gill-stage hatchlings of *B. melanostictus* (solid bars) and *P. cruciger* (hatched bars) exposed to diazinon for seven days. Values are based on three replicates (n=18 per replicate). Absence of bars indicates zero mortality.

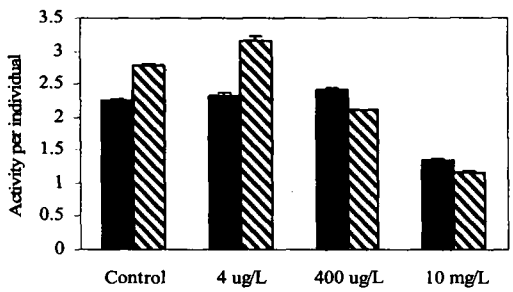

Figure 3: Mean activity levels of gill-stage hatchlings of *B. melanostictus* (solid bars) and *P. cruciger* (hatched bars) after exposure to diazinon for seven days. Values are based on three replicates (n=18 per replicate).

with a 60% reduction as compared to the controls. No significant alteration in activity was noted at the lower doses of 4 and 400 µg/L.

DISCUSSION

The present study provides clear empirical evidence for the adverse effects of a commonly used organophosphate pesticide on the larval stages of two amphibian species in Sri Lanka indicating that such agrochemicals may at least in part contribute to the recorded declines of these species in the country. It was evident that short term exposure to 10 mg/L of diazinon caused significant elevations in larval mortality in *B. melanostictus* and *P. cruciger*. The toxic effects of diazinon are mainly due to the metabolite diazoxon that is formed in animals. Diazoxon is a potent enzyme inhibitor capable of killing tadpoles directly by inhibiting acetylecholinesterase and numerous other important enzymes with molecular structures that are similar to it [17]. Both the tested species suffered near-similar toxicities at the highest dose of 10 mg/L. However, interspecific differences in diazinon toxicity have been noted in other studies. A LC50 value of

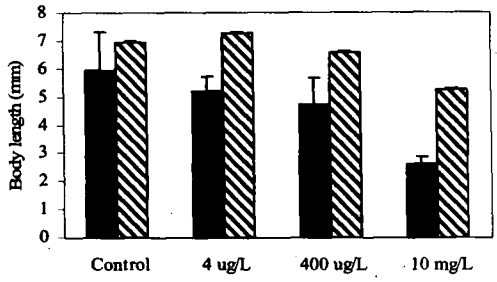

Figure 2: Mean body length (±S.E) of gill-stage hatchlings of B. *melanostictus* (solid bars) and *P. cruciger* (hatched bars) exposed to diazinon for seven days. Values are based on three replicates (n=18 per replicate).

14 mg/L for diazinon has been reported for *Bufo bufo* [18], while a LC50 value of 5 µg/L has been obtained for the same pesticide in the case of *Rana clamitans* [19]. Such interspecific differences in pesticide toxicity have been attributed to factors such as the variation in the age and duration of the exposure [20,21]. Interspecific differences in susceptibility to pesticides could be also due to differential rates of absorption through the skin and variation in the ability to detoxify chemicals [22]. No LC50 values were calculated in this study since the range of concentrations tested did not cause 50% mortality in the larvae of the two studied species.

Diazinon also impaired the growth of the larvae of the two study species. Growth retardation in organisms exposed to pesticides could be due to decreased activity [22] ultimately affecting food intake of the exposed animals. Additionally, the higher rate of metabolism displayed by animals exposed to environmental stressors such as pesticides may also result in reduced weight gain [23]. A smaller size is related to lower survival and fecundity and a lesser ability to compete for food [24]. This implies that exposure to pesticides might ultimately result in repeated failure to recruit juveniles to populations thereby facilitating their extinction. The slight increase in body size and activity in *P. cruciger* larvae exposed to 4 µg/L as compared to the control, is probably due to a growth stimulatory effect induced by some contaminants at low doses [25].

The magnitude of the impact of pesticide exposure on an amphibian species depends greatly on its biological attributes. Bufonids and some aquatic ranids that breed in shallow ponds or streams may be exposed to agrochemicals during both the egg and larval stage. On the other hand, rhacophorids that use arboreal habitats to breed may be exposed to aquatic pollutants only during their aquatic larval phase. Totally terrestrial species such as the philotids would be far less affected by aquatic contaminants. In addition to the direct effects

on amphibian larvae the widespread application of pesticides poses a direct threat to adults, as they traverse across agricultural landscapes in search of breeding sites. Pesticides may also severely affect adults by drastically reducing the availability of their arthropod prey [14].

This study has shown that diazinon causes larval death and the impairment of growth and activity in the two tested amphibian species at a dosage of 10 mg/L. This is indeed alarming given that the recommended dose for spraying diazinon in Sri Lanka is in the range of 1000 mg/L. This finding further emphasizes the importance of considering both lethal and sub lethal effects of pesticides on non-target organisms like amphibians that are integral components of natural ecosystems. This is of particular concern in Sri Lanka where pesticides are repeatedly applied at concentrations well above the recommended levels. While the protection of amphibians depends on a global commitment to control the use of toxic chemicals, well-targeted research can also make a significant contribution towards amphibian conservation. This is particularly relevant to a country like Sri Lanka which has an agriculturally based economy and a wide diversity of amphibians.

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