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Effect of mosquito larvicide Abate® on the developmental stages of the Asian common toad, *Bufo melanostictus*

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ABSTRACT

Exposure to chemical contaminants has been identified as a key factor causing widespread mortalities and malformations in amphibians world over. We examined the effect of exposure to commonly used mosquito larvicide, Abate® which has temephos as the active ingredient, on the Asian common toad, *Bufo melanostictus,* under laboratory conditions. The LC_{50} values of 16.56 ppm and 17.03 ppm were obtained when one-day old tadpoles (Gosner stage 21-24) and one-week old tadpoles (Gosner stage 25) were exposed to Abate for two weeks, respectively. Chronic exposure to Abate at effective field concentrations used in mosquito control programmes (WHO recommended doses) had a significant effect on the survival of egg stage (Gosner stages 12-14), and one day old tadpoles but not on one week old tadpoles. Tadpoles exposed to Abate grew larger and had a longer larval period and the difference in the duration of larval period was statistically significant. Exposed tadpoles also developed malformations such as rotation of bones, micromelia of the limb bones, hemimelia of femur, skin webbing in the hind limbs and ectrodactyly. Edemas were also observed in the exposed tadpoles. Exposure to all concentrations of Abate had a significant effect on the survival, growth and development of malformations in the early tadpole stages compared to the control group. The reduction in survival was not significant when older tadpoles of one week post-hatch were exposed, although they developed malformations and their larval period was prolonged significantly compared to the control group. Thus, the study shows that Abate is lethal to the early stages of the Asian common toad but the toxicity decreased as the tadpoles grow older.

Key words: temephos, amphibians, survival, growth, malformations

INTRODUCTION

Temephos is most frequently used as Abate® which is an emulsified formulation containing 44.6% temephos (O,O,O9,O9-tetramethyl O,O9-thiodi-*p*-phenylene phosphorothioate) and 55.4% inert ingredients (listed as petroleum distillates). Abate is an effective organophosphorus mosquito larvicide. It is hand sprayed to control mosquitoes breeding in isolated potholes and aquatic ecosystems particularly against *Anopheles* spp. in the Dry Zone of Sri Lanka. During epidemics or impending epidemics of malaria, Abate application is usually carried out at ten day intervals for effective larval control at an expected field level of 1.00 ppm as recommended by the WHO (World Health Organization) (personal communication, Regional Malaria Officers in Matale and Kurunegala districts). Advantages of temephos include effective toxicity to mosquito larvae, short term persistence in natural aqueous environments, and low toxicity to mammals, birds, and fish (Opong-Mensah, 1984; Tietz *et al*., 1991; Pierce *et al*., 1996). However, studies have shown that many other non-target organisms in freshwater ecosystems are affected by the larvicide (Gehrke, 1988; Ali *et al.*, 1992; Pierce *et al*., 2000; Hurst *et al.*, 2007) and its toxicity to amphibians, specifically on tadpoles raises concern (Sparling *et al*., 1997).

Evidence shows that commonly used chemicals such as agricultural pesticides have negative effects on local amphibians (Jayawardena *et al*., 2010; 2011). The present study examined the effect of Abate on eggs and two tadpole stages of the Asian common toad, *Bufo melanostictus*, as a non-target organism, under laboratory conditions. The Asian common toad is the most common anuran species in Sri Lanka with island-wide distribution (Pethiyagoda *et al*., 2006). It is associated with human-modified habitats and is listed as a species of least concern in the IUCN Red List of Threatened Species (IUCN, 2009). In the recent past, it has expanded its natural habitat range and established a higher relative dominance following habitat disturbances (Pethiyagoda *et al*., 2006). Water bodies in which it lays eggs are

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often exposed to direct insecticide applications as well as agricultural run-off. Sensitive life stages of many species of anurans are found in these aquatic systems and they may get exposed to these chemicals as non-target organisms.

MATERIALS AND METHODS

The commercially available product, Abate was used in the experiments which has temephos as the active ingredient. Abate samples were obtained from the Regional Malaria Officer at Matale.

Test animals
Eggs of *B. melanostictus* were collected from unpolluted ponds and tanks in the Peradeniya University Park and brought to the laboratory. Experiments were set up with three developmental stages of *B. melanostictus*; eggs (Gosner stage 12 - 14; Gosner 1960), one day post-hatch tadpoles (Gosner Stage 21 - 24) and one week post-hatch tadpoles (Gosner Stage 25). Eggs were allowed to hatch under laboratory conditions. Tadpoles were fed with commercial fish feed twice a day (~10% body weight per day). The debris and faeces that collected at the bottom of the rearing tanks were siphoned out. Water in the tanks was renewed completely once a week.

Determination of lethal concentrations
Acute toxicity of Abate to one day and one week post-hatch tadpoles was determined by exposing them to a series of high concentrations (5 , 10 , 20, 30 , and 40 ppm) for two weeks. Test solutions were prepared using dechlorinated tap water. A control experiment was set up without the chemical. Twenty tadpoles were kept in a glass tank (30×15×15 cm) containing 1800 ml of the test solution. Mortality was recorded daily for two weeks. The experiment was repeated with three egg clutches and the LC_{50}, LC_{10} and LC_{90} values of Abate were determined.

Chronic exposure at effective field concentrations
One day and one week post-hatch tadpoles of *B. melanostictus* were exposed to concentration series of 0.5, 1.0, and 1.5 ppm of Abate, closer to the effective field concentration (*i.e.* 1 ppm). Eggs were exposed to a lower concentration series of 0.0625, 0.125, 0.250, 0.5, and 1.0 ppm. Controls were prepared using only dechlorinated tap water. Twenty tadpoles (or eggs) were kept in each tank with 1800 ml of test solution. The medium in the tanks was renewed weekly.

Tadpoles were raised in the tanks until metamorphosis. Mortality was recorded daily. Newly metamorphosed toads were carefully observed for malformations and further examined under the dissecting microscope for malformations in the head region and limbs. The percentage of malformations was calculated taking the total number of malformed individuals divided by the initial number of individuals in a tank. The dead, malformed individuals were also included in the calculations. The newly metamorphosed toads with malformations were imaged using Magnus Live USB Viewer® and preserved in 70% alcohol after anesthetizing. Those without malformations were released into their original habitat after recovery. 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 (1999). Malformations were categorized using a field guide to malformations of frogs and toads (USGS, 2000).

Growth of the tadpoles was assessed by measuring the snout-to-vent length (SVL) to the nearest 0.1 cm using a vernier caliper and the body weight (to the nearest 0.01 g) at metamorphosis. Time required for the forelimb emergence of half the number of tadpoles (TE_{50}) was also recorded.

Data analysis
The LC values of Abate were calculated using EPA Probit Analysis Programme (Version 1.5; EPA 2006) using the pooled data from three egg clutches. Data on chronic exposure of Abate to effective field doses were analyzed using MINITAB 14.0 for Windows. The total number of individuals that survived at metamorphosis in the exposed and control groups were compared individually using a Chi square (χ^2) test. The effect on the growth (SVL, body weight and TE_{50}) of the tadpoles exposed to different concentrations of Abate at different age groups were tested using ANCOVA (analysis of co-variance). The number of malformations in each tadpole/ metamorph was counted and presented as a percentage.

RESULTS

Acute toxicity of Abate to the tadpoles of *B. melanostictus* is given as LC_{10}, LC_{50} and LC_{90} in

Table 1. The LC_{50} values for one day and one week post-hatch tadpoles of *B. melanostictus* were 16.56 ppm and 17.03 ppm, respectively (Table 1).

Chronic exposure at effective field concentrations

Survival

A total of 840 tadpoles from three egg clutches were used in the experiments. Chronic exposure of eggs and tadpoles to effective field concentrations of Abate showed a reduction in survival compared to the control group (Table 2).

Percentage survival at metamorphosis decreased with exposure to increasing concentrations in all the experimental groups. Individual comparisons showed that the reduction in survival was statistically significant at egg stage and one day post-hatch exposures (chi-squared test, $P<0.05$; Table 2). Significant reduction in survival up to 4% and 2% was observed in the egg stages exposed to Abate concentrations of 0.5 and 1.0 ppm, respectively (Table 2). However, when tadpoles at one week post-hatch were exposed there was no significant reduction in survival, inferring that age at exposure to Abate has a significant effect on the survival (ANCOVA, $F_{(2,10)} = 32.01$, $P < 0.001$).

Malformations

Tadpoles exposed to Abate developed malformations while none of the tadpoles in the control group had any malformations (Table 2). The percentage malformations in metamorphs varied depending on the age at exposure and the Abate concentration. Metamorphs had very high frequencies of malformations (up to 100%) when they were exposed to 1.0 ppm of Abate at the egg stage. Percentage malformations of tadpoles increased with increasing concentrations of Abate in all three groups (Table 2).

Table 1. Lethal concentrations (LC) of one day and one week post-hatch tadpoles of *B. melanostictus* exposed to Abate (n=300 per age group).

Age at exposure	LC_{10} (95% CI)/ppm	LC_{50} (95% CI)/ppm	LC_{90} (95% CI)/ppm
One day post-hatch tadpoles	9.21 (4.16 - 12.73)	16.56 (11.55 - 20.26)	29.75 (24.32 - 42.55)
One week post-hatch tadpoles	4.54 (1.63 - 6.88)	17.03 (12.15 - 31.82)	63.83 (33.45 - 399.36)

CI - Confidence interval; ppm- parts per million

Table 2. Survival and malformations in metamorphs of *B. melanostictus* following chronic exposure to effective field concentrations of Abate (n=60 /concentration).

Age at exposure	Concentration (ppm)	Survival (%)	χ^2	*p*	Malformations (%)
Egg stage	0.0	85	-	-	0
	0.0625	66	9.758	0.002	8
	0.125	20	84.718	0.000	25
	0.25	10	112.782	0.000	50
	0.5	4	132.827	0.000	50
	1.0	2	140.149	0.000	100
1 day post-hatch	0.0	90	-	-	0
	0.5	80	3.922	0.048	6
	1.0	80	3.922	0.048	10
	1.5	70	12.500	0.000	14
1 week post-hatch	0.0	85	-	-	0
	0.5	85	0.000	1.000	4
	1.0	75	3.125	0.077	7
	1.5	75	3.125	0.077	11

ppm - parts per million.

Different types of malformations were observed in the metamorphs and tadpoles. Among the malformations observed, rotation of the limb bones (Fig. 1a) was the most common type (Table 3). In addition, edemas (Fig. 1b), skin webbing in the limbs (Fig. 1c), hemimelia of femur (femur is short but foot is present; Figs. 1c & d), micromelia (proportionately short or small limb) of the fore limbs (Figs. 1d & e) and ectrodactyly (missing toes; Fig. 1f) were also observed in both one day and one week old tadpoles (Table 3). In most of the malformations, the limb malformations were bilaterally symmetrical.

Growth

Tadpoles exposed to Abate were larger in size at metamorphosis than those in the control group. The mean SVL and body weight of *B. melanostictus* at metamorphosis increased with increasing concentration (Table 4). Since the survival of the metamorphs exposed to Abate at egg stage was very low (2%), statistical comparisons were not carried out.

Figure 1. Malformations shown by the exposure groups. (a) Tadpole showing limb rotation in hind limbs. (b) Tadpole with edema on the trunk on the right side of the body. (c) Metamorph with skin webbing and hemimelia of femur. (d) Metamorph showing hemimelia of femur and micromelia of the fore limb, (e) Metamorph showing micromelia of the fore limbs. (f) Metamorph with ectrodactyly.

Table 3. Occurrence of malformations in tadpoles of *B. melanostictus* exposed to effective field concentrations of Abate. (n=660)

Type of malformation	**Percentage malformations***	
	One day old	**One week old**
Limb rotation	49 %	32 %
Hemimelia	4 %	16 %
Micromelia	9 %	32 %
Ectrodactyle	11%	9%
Skin webbing	2 %	16 %
Edema	36 %	28 %
Mean number of malformations per malformed metamorph ±SD	1.1±0.7	1.3±0.2

* Percentage of a particular type out of total malformed individuals

Table 4. Snout-vent length (SVL) and body weight of *B. melanostictus* at metamorphosis after chronic exposure to Abate at field concentrations. (n= 60 tadpoles / concentration)

Age at exposure	**Concentration (ppm)**	**Mean SVL ±SD (cm)**	**Mean body weight ±SD (g)**
Eggs	0.0625	8.9±0.05	0.06±0.03
	0.25	8.8±0.06	0.07±0.06
	0.5	8.9±0.06	0.07±0.07
	1.0	8.9±0.08	0.08±0.09
I day post-hatch	0.5	8.2±0.07	0.06±0.05
	1.0	8.5±0.06	0.07±0.03
	1.5	8.7±0.07	0.06±0.04
I week post-hatch	0.5	8.1±0.09	0.06±0.07
	1.0	7.9±0.11	0.06±0.08
	1.5	7.9±0.10	0.08±0.09
Control	0	7.2 ±0.04	0.05±0.03

Table 5. Time required for the forelimb emergence in half the number of tadpoles (TE_{50}) of *B. melanostictus* after chronic exposure to Abate at field concentrations. (n= 60 tadpoles /concentration)

Age at exposure	Concentration (ppm)	TE_{50} ±SD (days)	F	P
Control	0.0	73.0±3.4	-	-
Eggs	0.0625	86.2±6.6	15.78	0.004
	0.0125	96.4±10.6	22.11	0.002
	0.25	122.6±15.5	48.66	0.000
	0.5	137.4±10.1	81.42	0.000
	1.0	151.0±11.5	13.72	0.000
Control	0.0	65.0±7.2	-	-
One day post-hatch	0.5	75.0±7.9	2.61	0.182
	1.0	86.7±8.1	12.00	0.026
	1.5	106.0±9.1	37.08	0.004
Control	0.0	37.7±3.8	-	-
One week post-hatch	0.5	54.0±7.8	6.31	0.031
	1.0	69.7±6.5	1.90	0.002
	1.5	82.7±5.1	1.40	0.000

All the tadpoles exposed to Abate took more time to metamorphose than those in the control group. Time required for the forelimb emergence in half the number of tadpoles (TE_{50}) in all exposure groups was significantly increased with increasing concentration of Abate (Table 5). Age at exposure significantly affected the duration of the larval period in all the groups (ANCOVA, $F_{(2,10)} = 28.07$, $P < 0.001$).

DISCUSSION

Effect of temephos on non-target organisms has been tested on many aquatic (Brown *et al.*, 1996; 1998; Pierce *et al*., 2000; Hurst *et al*., 2007) and non-aquatic (Ferguson *et al*., 1985) species. Acute and chronic exposures to temephos adversely affected a range of reproductive indices, including gametogenesis in the leech, *Hirudinaria manillensis* (Hirudinidae) (Campbell *et al.,* 1992; Cikutovic *et al.,* 1993). Field toxicity tests that have been performed during routine larvicide applications using larvae of salt marsh crabs (*Aratus pesonii* and *Uca rapax*) and adult *Mysidopsis bahia* indicated hazardous effects on these non-target organisms (Pierce *et al.*, 2000). Brown *et al.* (1996; 1998) reported LC_{50} of temephos at 0.33 and 9.9 times the estimated field concentration on the estuarine shrimp *Leander tenuicornis* and pacific blue-eye, *Pseudomugil signifer* (Pisces), respectively. Acute toxicity of LC_{50} was 17 times the estimated field concentration to eggs and early stages of Asian common toad recorded in this study. This shows that these tadpoles are less sensitive to the chemical compared to the above two organisms.

Chronic exposure to effective field concentrations of Abate was toxic to the early tadpole stages of the Asian common toad while it was highly toxic to the egg stages. Exposure to Abate significantly reduced the survival, developed malformations and lengthened the larval period. Both concentration of the larvicide and age at exposure affected the survival and lengthening of tadpole stage significantly.

Exposure to organophosphorous pesticides can lead to adverse systemic effects in anurans, including effects on the nervous system and behaviour (Relyea, 2004), the reproductive system (Sheffield and Lochmiller, 2001), the immune system (Gilbertson *et al.,* 2003), and growth and development (Fordham *et al.*, 2001). There is an increased concern about the sublethal effects of organophosphorous compounds on human and animal health (Jaga and Dharmani, 2003). Integration of human and wildlife health research and modeling are necessary to assess the potential ecological risks of these pesticides because certain wildlife species can show effects that are more subtle or take longer to manifest in humans (Willens *et al.*, 2006). The widespread use of organophosphorous insecticides (due to their low toxicity to humans and low environmental persistence) coupled with the permeability to anuran skin and sensitivity to environmental toxicants make anurans important animal models for studying the impacts of insecticide contamination of the environment (Taylor *et al.*, 1999a,b).

Temephos reduced the activity levels of exposed green frog (*Rana clamitans*) tadpoles, whose levels of acetylcholinesterase (AChE) had increased with concentration of temephos while butyrylcholinesterase (BChE) showed a significant decrease (Sparling *et al.*, 1997). Among other non-target organisms, the larvivorous fish, *Melanotaenia duboulayi* showed a significant reduction in critical swimming speed which is very important for its survival, in response to exposure to temephos at a concentration ten times the effective field concentration of 0.33 mgL^{-1} (Hurst *et al.*, 2007).

Tadpoles of *B. melanostictus* exposed to Abate developed malformations. Observed malformations included rotation, bone bridging, skin webbing, micromelia, hemimelia, ectrodactyly, and edemas. All these malformations, except ectrodactyly and edemas fall in the category of distally complete, malformed limbs (USGS, 2000). Distally complete but malformed limbs have the complete complement of the bones but the limbs were still abnormal. Bones appearing twisted without bone bridging or skin webbing are categorized as rotational malformations. In the case of skin webbing, a band of skin crosses a joint. Webs of skin may have different degrees of tightness, thus, limiting the mobility at the joint to variable degrees. Limb malformations often lead to indirect mortality due to increased vulnerability to predation. Disturbed mobility makes them easy prey (Sessions and Ruth, 1990). In the present study it was observed that individuals with edemas often experienced imbalance and those with limbs rotated around their long axis were unable to maintain balance when swimming. Furthermore, metamorphs with malformations are unable to move on to land from the aquatic habitat after metamorphosis.

In nature, longer the larval life, higher the duration of exposure to aquatic predators and chemical pollutants that are either directly sprayed to water or added to water bodies by runoff. Moreover, the aquatic stages may be more susceptible to chemical pollutants due to the highly permeable and vascularized nature of the gills and skin. Delaying metamorphosis has another crucial impact on amphibian survival as they often breed in temporary water bodies, particularly in agricultural and human altered habitats (Manamendra-Arachchi and Pethiyagoda, 2006), which may dry up before completion of metamorphosis. Therefore, longer time spent as larvae may also increase risk of dying if ponds dry up in drier climates. Consequently, Abate, which is directly sprayed to water bodies, has the potential of posing a serious threat to the tadpoles through indirect mortality**.**

In Sri Lanka, during dry season pools are formed in the riverbeds below the dams that have been constructed across the rivers for hydroelectric power generation. These pools are important breeding sites for mosquitoes (Kusumawathie *et al*., 2006). To control mosquito larvae in these habitats, in addition to spraying of Abate, introduction of lavivorous guppy species, *Poecilia reticulata*, has been an efficacious method (Kusumawathie *et al*., 2003). It has been shown that application of *P*. *reticulata* was 2.67 times less costly than spaying Abate (Kusumawathie *et al*., 2008). When applying Abate all the aquatic systems are treated, irrespective of the presence or absence of larvae. On average, less than 20% of the pools are positive for anopheline larvae during any time of the year (Kusumawathie, 2006). Thus, the majority of pools are sprayed unnecessarily and this is not only a waste of chemicals and may lead to development of insecticide resistance in mosquitoes but also it is harmful to non-target organisms. Use of fish is more effective and less costly, but also it encourages community involvement (Kusumawathie *et al.*, 2008). It is important that the health authorities give due considerations to the use of environmentally friendly methods in routine mosquito control programmes. This will save the lives of non-target organisms like tadpoles. Insecticide spraying could be recommended only during or impeding outbreaks of mosquito diseases.

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