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ORIGINAL ARTICLE

Effects of Pesticide Exposure on Embryonic Development and Hatchling Traits of Turtles

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Abstract Deltamethrin is a widespread environmental hormone with endocrine-disrupting properties, but its effect on embryonic development of reptiles is largely unexplored. We investigated the effects of deltamethrin on embryonic development and offspring traits in two turtle species, one with parchment-shelled eggs and the other with rigid-shelled eggs. Deltamethrin exposure during egg incubation did not affect hatching success and hatchling body size in either species. However, embryonic exposure to deltamethrin resulted in reduced hatchling locomotor performance in the red-eared slider turtle (*Trachemys scripta*) with parchment-shelled eggs, but not in the Chinese three-keeled pond turtle (*Chinemys reevesii*) with rigid-shelled eggs. These results suggest that parchment-shelled eggs are likely more vulnerable to deltamethrin than rigid-shelled eggs.

Keywords Parchment-shelled eggs, Rigid-shelled eggs, Red-eared slider turtle, *Trachemys scripta*, Chinese three-keeled pond turtle, *Chinemys reevesii*, Embryo, Environmental hormone

1. Introduction

Pyrethroids are widely applied to control insect pests of agriculture (particularly on crops), public health, home, and garden (Amweg *et al.*, 2005; Oros and Werner, 2005; U.S. Department of Agriculture, 2007). In spite of the relatively low mammalian toxicity and biodegradability (Leahay, 1985), environmental pollution and food safety problems are concomitant with the increase in pyrethroid pesticide application amounts. Moreover, pyrethroids have been found to occur in waters (0.04 to 24 μg/L) and animals (3 to 50ng/g) (Pawlisz *et al.*, 1998), and their biological effects have received increasing scientific attention (Mubarak *et al.*, 2006; Righi, *et al.*, 2009). Endocrine disruption by pesticides has raised concerns about their potential health hazard. The majority of pyrethroid pesticides belong to the "environmental hormones" group, and their long-term exposure can cause chronic disease and even have teratogenic, carcinogenic, and mutagenic effects (Sinha *et al.*, 2004).

Deltamethrin, an important synthetic pyrethroid and a highly effective pesticide, has been widely applied in public health and agricultural programs (Moretti *et al.*, 1997; McKinlay *et al.*, 2008). Deltamethrin has been reported to be ecotoxic to various animals such as copepods (Tidou *et al.*, 1992), mussels (Thybaud, 1990; Kontreczky *et al.*, 1997), freshwater fish (Delistraty, 2000; Datta and Kaviraj, 2003; Svobodova *et al.*, 2003), and the leopard frog (Bridges, 2000). Deltamethrin has already been reported to affect behavior (Kontreczky *et al.*, 1997; Lazarini *et al.*, 2001), growth (Datta and Kaviraj, 2003), reproduction (Presibella *et al.*, 2005), and the nervous system of animals (Aziz *et al.*, 2001). However, few studies have reported the effect of deltamethrin on the growth and development of reptiles, and especially embryos.

Reptile embryos are extremely sensitive to environmental conditions. The environment experienced by embryos can significantly influence the developmental rate and hatching success of embryos as well as the phenotype, functional performance, and fitness of offspring (Deeming 2004). Since most oviparous reptiles deposit their eggs in underground nests, embryonic

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development is affected by environmental contaminants, including pesticides (Deeming, 2004; Sparling *et al.*, 2006; Hopkins and Winne, 2006). Nonetheless, the effects of deltamethrin on embryonic development and offspring traits of reptiles remain unexplored.

In this study, we investigated the effect of deltamethrin on egg incubation in two turtle species, *Trachemys scripta* and *Chinemys reevesii*. *T. scripta* produces parchment-shelled eggs, whereas *C. reevesii* lays rigid-shelled eggs. Given the fact that the rigid shell is heavily mineralized and displays highly reduced permeability (liquid, vapor, and gas) than the parchment shell (Deeming and Thompson, 1991; Deeming and Unwin, 2004), we hypothesized that parchment-shelled eggs would be more vulnerable to deltamethrin than rigid-shelled eggs in turtles.

2. Materials and Methods

2.1 Egg collection and incubation Eggs of *T. scripta* and *C. reevesii* were purchased from a commercial supplier and transported to the laboratory. We assigned eggs in groups of 10 to plastic boxes ($16 \times 11.5 \times 4$ cm^3) containing a 2 cm layer of moist vermiculite (at water potentials of approximately –220 kPa). Eggs were incubated in an air-conditioned room under constant temperature of 28°C, which is the optimal temperature for egg incubation in this species and produces hatchlings with a balanced sex ratio (Du *et al.*, 2007). We weighed boxes with moist vermiculite to evaluate water evaporation once a week, and added water to maintain relatively constant vermiculite moisture. We changed the position of boxes daily to avoid any potential shelf effects from temperature gradients.

Eggs were assigned randomly to three deltamethrin treatment groups (0.1, 0.02, and 0.004 mg/L of deltamethrin dissolved in ethanol) and one control group (ethanol). Sample sizes were 174 (forty-two clutches) of *T. scripta*, and 170 (forty-two clutches) of *C. reevesii*. Sample sizes of *T. scripta* for the treatments of 0, 0.004, 0.02, and 0.1 mg/L were 42, 42, 45, and 45, respectively. Sample sizes of *C. reevesii* for the same treatments were 40, 43, 44, and 43, respectively. Eggs were spotted with 5 μL of different doses of deltamethrin (treatment) or ethanol (control) to the eggshell using a pipettor, which was applied once at approximately stage 17 of development (Crews and Bergeron, 1994; Wibbels *et al.*, 1994). The deltamethrin dosage is based on governmental limits of permissible levels in accordance with Chinese national standards for drinking water quality (GB 5749–2006) and Chinese national environmental quality standards for surface water (GB 3838–2002), in which the water quality standard limit value is 0.02 mg/L. Therefore, we selected the standard value as the dosage of the moderate concentration treatment group (0.02 mg/L). The dosages of the other two (high and low concentration) treatment groups were 5 and 1/5 times the standard value, respectively (0.1 and 0.004 mg/L).

2.2 Embryonic development and hatchling traits Hatching success was calculated as the percentage of successfully-hatched eggs. After hatching, each turtle was weighed and placed in a separate cup labeled with an individual number. Hatchling mass (0.001 g), carapace length, and width (0.01 mm) were measured. We examined each hatchling for morphological deformities (e.g. underdeveloped head and tail, carapace and plastron abnormalities, extra or missing scutes). The locomotor performance (swimming speed) of hatchlings was tested at 28 °C in the first week after hatching. We gently placed a hatchling turtle into a 1-m-long glassed sink. The sink was marked at 250 mm intervals, and filled with 10 cm height of water. We recorded the time that a turtle swam through the 1 m sink. Each hatchling was tested twice and the average swimming speed through the 1 m sink was calculated. The hatchlings were kept in cups individually at room temperature. An excess amount of commercial food were provided each day. Four months later, We dissected hatchlings to identify the sex of each individual by checking gonads. Female gonads are long and thin, whereas male gonads are short and well vascularized (Yntema, 1976; Crews *et al.*, 1991).

2.3 Statistical analysis Mixed-model ANOVA was used to test the effect of treatments on heart rate of embryos, with clutch as the random factor. Hatching success, morphological deformity and sex ratio were analyzed by using chi-square tests. Hatchling mass was compared using mixed-model ANCOVA with initial egg mass as the covariate, and clutch as the random factor. When significant differences were found among treatments, a posterior Tukey's Honestly Significant Difference (HSD) test for multiple comparisons of means was carried out (Zar, 1999). For all tests, differences at $\alpha = 0.05$ level of confidence were considered significant. All statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., Chicago. IL, USA).

3. Results

Deltamethrin exposure did not affect hatching success

in both species (*C. reevesii*: $X^2 = 4.40$, $df = 3$, $P = 0.22$; *T. scripta*: $X^2 = 0.81$, $df = 3$, $P = 0.85$) (Figure 1a). Deltamethrin exposure did not affect morphological deformities of hatchlings in *T. scripta* ($X^2 = 4.18$, $df = 3$, $P = 0.24$), nor in *C. reevesii* ($X^2 = 3.74$, $df = 3$, $P = 0.29$) (Figure 1b), nor hatchling size and mass in both species (Table 1). The sex ratio of hatchlings was not affected by deltamethrin exposure in both species (*C. reevesii*: $X^2 = 1.87$, $df = 3$, $P = 0.60$; *T. scripta*: $X^2 = 1.12$, $df = 3$, $P = 0.77$) (Figure 1c). However, deltamethrin exposure during embryonic development significantly decreased the swimming speed of *T. scripta* hatchlings, but not of *C. reevesii* hatchlings (Table 1).

4. Discussion

Animal embryos are extremely sensitive to pesticides. Some studies have shown that pesticides or herbicides might significantly affect hatching success of embryos in oviparous species. For example, treatments containing high concentrations of Glypro induce more frequent embryonic fatalities (Sparling *et al.*, 2006). Different concentrations of cypermethrin significantly affected hatching success of amphibians (Greulich *et al.*, 2003). Carbaryl significantly reduced survival of bullfrog (*Rana catesbeiana*) tadpoles in the laboratory (Puglis and boone, 2007). Our study indicated that deltamethrin exposure did not affect hatching success of turtle eggs. This result is inconsistent with other previous studies on other oviporous species. For example, Kenan *et al.* (2004) found that deltamethrin could significantly influence the hatching success of common carp (*Cyprinus carpio*) embryos and larvae. The relative insensitivity of the hatching success of turtle embryos to environmental contaminants has also been found in other species. For example, dichlorodiphenyldichloroethylene (DDE) failed to influence the hatching success in the marine turtle (*Chelonia mydas*) (Podreka *et al.*, 1998), and the toxicity of pesticides (chlorothalonil, S-metolachlor, metribuzin, and chlorpyrifos) did not influence the hatching success of the common snapping turtle (*Chelydra serpentina*) (Solla *et al.*, 2014).

Endocrine disruptor treatments during embryonic development could have profound impacts on the morphological deformity, offspring size, and offspring performance. For example, high application rate of the pesticide tefluthrin increased deformity rates of exposed snapping turtle embryos and hatchlings (Solla *et al.*, 2011); 17α-Ethinylestradiol, genistein, and fadrozole could induce malformations in zebrafish (Santos *et al.*, 2014); cadmium and EC50 caused lower hatchling weight in snail species (Coeurdassier *et al.*, 2003, Schirling *et al.*, 2006); Hopkins and Winne (2006) reported that high concentrations of carbaryl significantly reduced swimming performance of four species of natricine snakes. Our study indicated that exposure to deltamethrin did not affect hatchling morphology including carapace size and body mass, but reduced hatchling locomotor performance (average swimming speed) of *T. scripta*. This suggests that deltamethrin may have long-term

Table 1 Effect of deltamethrin exposure on hatchling morphometrics (hatchling mass, carapace length and width) and swimming speed of *Trachemys scripta* and *Chinemys reevesii*.

Traits	Species	Control	Deltamethrin			*P*-value
			0.004 mg/L	0.02 mg/L	0.1 mg/L	
Hatchling mass (g)	*T. scripta*	7.704 ± 0.121	8.224 ± 0.174	8.057 ± 0.169	7.957 ± 0.184	$F_{3,41} = 0.71$, $P = 0.55$
	C. reevesii	4.996 ± 0.120	5.165 ± 0.121	5.160 ± 0.104	5.083 ± 0.120	$F_{3,41} = 0.26$, $P = 0.86$
Carapace length (mm)	*T. scripta*	30.87 ± 0.27	31.64 ± 0.22	31.02 ± 0.36	30.83 ± 0.37	$F_{3,41} = 1.45$, $P = 0.23$
	C. reevesii	26.83 ± 0.36	27.04 ± 0.29	26.61 ± 0.36	26.72 ± 0.29	$F_{3,41} = 0.74$, $P = 0.53$
Carapace width (mm)	*T. scripta*	29.68 ± 0.31	30.52 ± 0.24	30.04 ± 0.34	29.50 ± 0.49	$F_{3,41} = 1.88$, $P = 0.14$
	C. reevesii	22.14 ± 0.27	21.95 ± 0.22	21.77 ± 0.31	21.87 ± 0.29	$F_{3,41} = 1.13$, $P = 0.34$
Average swimming speed (m/s)	*T. scripta*	0.082 ± 0.008^{a}	0.056 ± 0.005^{b}	0.047 ± 0.003^{b}	0.066 ± 0.007^{ab}	$F_{3,26} = 5.79$, $P = 0.002$
	C. reevesii	0.042 ± 0.003	0.050 ± 0.005	0.050 ± 0.003	0.050 ± 0.004	$F_{3,31} = 1.09$, $P = 0.47$

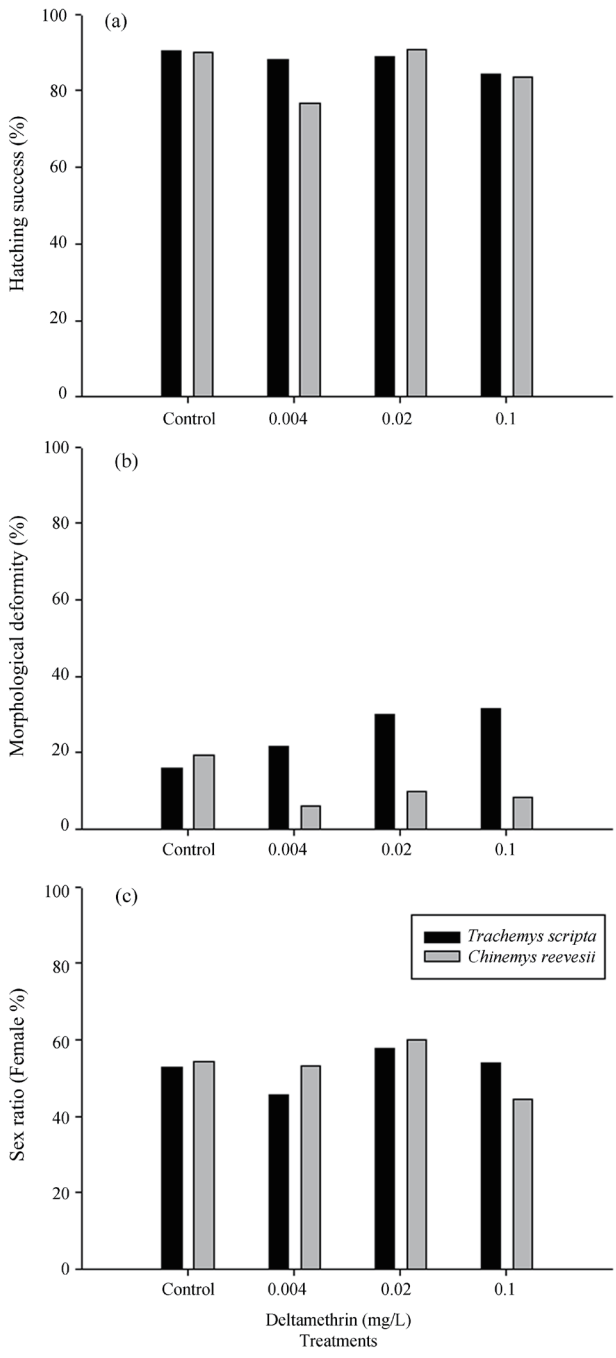

Figure 1 Effect of deltamethrin exposure on hatching success (a), morphological deformities (b) and Sex ratio (c) of *Trachemys scripta* and *Chinemys reevesii*. Sample sizes of *T. scripta* for the treatments of 0, 0.004, 0.02, and 0.1 mg/L were 42, 42, 45, and 45, respectively. Sample sizes of *C. reevesii* for the same treatments were 40, 43, 44, and 43, respectively.

effects on offspring fitness-related phenotypes, and therefore, presumably have a negative impact on offspring fitness. Such effects of environmental hormones on reptile embryos and offspring are largely unexplored and warrant further investigation.

The between-species difference in the effect of deltamethrin on embryonic development and offspring traits may be attributable to two aspects: species-specific sensitivity of embryos in response to deltamethrin and different types of eggshell (thickness and permeability). First, the sensitivity of reptile embryos to environmental stress differs among species. For example, reptile embryos have different optimal temperatures for developments (Du and Shine, 2015). Analogously, the response of reptile embryos to pesticides may also differ among species (Bishop *et al.*, 1998; Sparling *et al.*, 2006). Pesticide (e.g. atrazine) exposure could negatively affect embryonic development in *T. scripta* (Willingham, 2005), but not in the snapping turtle (*Chelydra serpentina*) (de Solla *et al.*, 2011). Second, parchment- and rigid-shelled eggs may have different responses to pesticides. Most reptiles lay eggs in beach sand or land soil, where the eggs are exposed to residual pesticides. Reptile eggs are able to absorb pesticides from soil because their eggshells are permeable and polyporous (Solla *et al.*, 2011). The shell of parchment-shelled eggs is thinner and more permeable to environmental substances like water, gas, and contaminants than that of rigid-shelled eggs (Deeming and Thompson, 1991; Oftedal, 2002; Belinsky *et al.*, 2004). As a result, the effect of pesticides on embryonic development and offspring traits is likely stronger in parchment-shelled eggs than in rigid-shelled eggs. In the two species we studied, the eggshell of *C. reevesii* (0.18 ± 0.02mm) is thicker than that of *T. scripta* (0.14 ± 0.01mm) (Kusuda *et al.*, 2013), and deltamethrin exposure during embryonic development affected functional performance of *T. scripta* hatchlings, but not *C. reevesii*. This result supports the hypothesis that parchment-shelled eggs are more vulnerable to deltamethrin than rigid-shelled eggs. Similarly, embryonic development in other turtle species having parchment-shelled eggs is also sensitive to pesticides (Solla *et al.*, 2011). Nonetheless, further verification of this hypothesis requires more data comparing parchment-shelled and rigid-shelled eggs across a wide range of reptile species.

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