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Environmental Toxicology and Chemistry, Vol. 30, No. 4, pp. 915–919, 2011

Printed in the USA
DOI: 10.1002/etc.455

AN EGG INJECTION TECHNIQUE TO EVALUATE THE EFFECT OF POLYCHLORINATED BIPHENYLS ON THE HATCHING SUCCESS OF THE SNAPPING TURTLE (*CHELYDRA SERPENTINA SERPENTINA*)

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(*Submitted* 10 *December* 2009; *Returned for Revision* 20 *January* 2010; *Accepted* 1 *November* 2010)

Abstract—Embryos of oviparous organisms are exposed to contaminants by two pathways: contaminant uptake from the surrounding environment, and the transfer from female to offspring (maternal transfer). The initial source of contaminant exposure for most embryos is likely to be maternal transfer; therefore, maternal transfer studies are critical in determining the effects of contaminants on future populations. Injection of contaminants directly into eggs is one route of experimental contaminant exposure that permits controlled doses and potential reliable replication. This technique, however, has been used in the past with little success in reptiles. The objective of the present study was to evaluate egg injection as a means of mimicking maternal transfer of polychlorinated biphenyls (PCBs) to snapping turtle eggs. Eggs from several clutches were injected with a PCB solution and incubated at several temperatures and moisture levels to measure interactive effects of injection, environmental condition, and contaminant load on hatching success. The injection technique allowed for application of consistent and specific doses among replicates. Overall hatching success in this study was 61% and was as high as 71% within specific treatments. Hatching success was much higher in this study than in other studies using egg injections to mimic maternal transfer in chelonians and crocodilians. Environ. Toxicol. Chem. 2011;30:915–919. © 2011 SETAC

Keywords—Polychlorinated biphenyls Chelonian eggs Injection techniques Organic contaminants

INTRODUCTION

Controlled dosing of embryos in contaminant studies is difficult to accomplish under most circumstances [1]. In reptiles, several methods have been developed to mimic the maternal transfer of contaminants. In some studies, contaminants have been applied topically to eggshells [2–5]. This technique has limitations. Whether various contaminants can pass through calcareous eggshells [6], and if so, to what degree [1,7], is unclear. For example, the transfer of chemicals across alligator (*Alligator mississippiensis*) eggshells has been shown to be very low, to vary between eggs, and to have low correlation with the dose applied [1]. Topical treatments also may negatively affect gas exchange across the eggshell and lead to increased mortality unrelated to contaminant effect [8]. Furthermore, topical treatment may be a more appropriate model of the effect of soil contaminants surrounding the clutch rather than maternal transfer.

Other studies have used diet to expose gravid females to contaminants in an attempt to model maternal transfer [1,9]. Female reptiles produce all of their eggs simultaneously; therefore, contaminant loads are similar in eggs within clutches [10,11]. Maternal metabolism and contaminant levels in fat bodies, however, influence egg dose even when female dose is carefully controlled. Thus, genetic or phenotypic variability among females increases variation in maternal transfer of contaminants between clutches. Controlling the egg contaminant dose in replicate clutches using these techniques is difficult.

Injection of contaminants directly into eggs permits controlled dosing and replication between clutches. This technique has been used with little success in reptile eggs. Several investigators no longer use the technique because of poor hatch rates, despite modifications designed to prevent bacterial infection (e.g., sealing injection sites with topical adhesives [1]). Egg injection has been particularly unsuccessful in reptiles with rigid, calcareous eggshells, such as turtles and crocodiles [8,12], although the parchment-like eggshells of lizards appear to be more tractable [13]. For example, Talent et al. [13] reported high mortality for microinjected 17α ethinylestradiol treatments in eggs of fence lizards (*Sceloporus occidentalis*); however, the treatment consisting of only the injection vehicle had good hatching success (79.5%), indicating that the high mortality observed in the contaminant treatments was unlikely to be the result of the injection procedure. The timing of egg injections is also critical. For example, injections in leghorn (*Gallus gallus*) and domestic chickens (*G. domesticus*) before organogenesis have higher mortalities than injections after organogenesis [14,15]. However, if the objective is to understand maternal transfer and contaminant effects on the embryo, then egg injections before organogenesis are essential. The volume of the injection also affects mortality rates. High-volume injections of the vehicle resulted in higher mortalities than low-volume injections or no injections [16]. Thus, interactive effects of the injection itself, timing and volume of injections, egg characteristics, and the contaminant all lead to an elevated risk of embryo mortality in egg injections.

The objective of the present study was to reevaluate egg injection techniques as a means of mimicking maternal transfer of polychlorinated biphenyls (PCBs) to rigid, calcareous snapping turtle (*Chelydra serpentina serpentina*) eggs. The benefit of egg injections, as opposed to other techniques, is that it

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Published online 28 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

permits replication of a controlled dose of contaminants to eggs. Snapping turtle eggs obtained from several females were injected with a PCB solution and incubated at two different temperatures and moisture levels to measure interactive effects of injection, environmental condition, and contaminant load on hatching success.

METHODS AND MATERIALS

Wild snapping turtles (*Chelydra serpentina serpentina*) were obtained from Siegel Marsh, Pennsylvania Game Lands 218 (Erie County, PA, USA) in June 2005, using turtle trap hoop nets (Nylon Net). Preliminary sampling indicated that snapping turtles in Siegel Marsh had low organic contaminant loads [17]. Thus, maternal transfer of PCBs was assumed to be minimal at this site.

On removal from the trap, turtles were sexed and their pelvic region examined for the presence of eggs. Female turtles were placed in identical plastic containers for live transport from Siegel Marsh to the laboratory at Penn State Erie. All females were processed in a similar manner within 10 h of capture. Four gravid females were euthanized by decapitation (Institutional Animal Care and Use Committee 15974 and 23499, Institutional Biosafety Committee 18903), and necropsies were performed in the laboratory. Eggs were removed immediately from the oviducts to permit injection before air hardening, and they were placed in a container lined with clean aluminum foil to prevent any extrinsic PCB contamination of the eggs. Eggs were of consistent size within and across clutches (average, 9.7 ± 0.05). Three to four eggs per clutch (Table 1) were removed from the beginning, middle, and end of the oviducts to establish a baseline concentration of PCBs for the entire clutch. Because of the concurrent formation of all eggs in a clutch, the baseline contaminate concentrations are not significantly different between individual eggs [10,11]. We wished to be conservative in establishing the baseline PCB concentration; thus we used a stratified sampling plan (beginning, middle, and end) to select eggs for a composite analysis. Although Bishop et al. [18] found that concentrations of lipids and most chlorinated hydrocarbons did not vary significantly among snapping turtle eggs within the same clutch, best analytical practices require a composite sample of the blended contents of three to four eggs to adequately represent the baseline PCB load for an entire clutch (J. Pagano, State University of New York at Oswego, personal communication). Each set of egg samples was stored in sterilized 250-ml glass specimen jars with metal screw-caps (Carolina Biological Supply) and frozen for subsequent analysis at the Environmental Research Center at the State University of New York at Oswego.

Table 1. Total number of eggs harvested per female snapping turtle collected at Siegel Marsh (PA, USA)[a]

Female	Total eggs collected	Eggs for composite	Eggs incubated
1	20	4	16
2	25	4	21
3	25	3	22
4	21	3	18

[a] Some eggs were collected from each female to use in a composite analysis to determine baseline polychlorinated biphenyl concentrations for the entire clutch. The remaining eggs were injected with oil or a low or high polychlorinated biphenyl solution and incubated at low or high temperature.

The remaining eggs ($n = 77$) were injected within 3 h of removal from the female. Injection sites on the eggshells were wiped with a dry cloth to remove blood or other fluids. Injection solutions consisted of a low (1 ppm) or high (10 ppm) concentration mixture of Aroclor 1254:1260 (U.S. Environmental Protection Agency [U.S. EPA] Pesticide Repository) in an injection vehicle (canola oil) and a solution of pure canola oil (0 ppm PCB). Eggs were arbitrarily selected for injection with the low PCB ($n = 27$), high PCB ($n = 26$), or oil ($n = 24$) treatment (Table 2). The high PCB treatment was similar to concentrations found in snapping turtle eggs at a highly contaminated U.S. EPA Superfund site in New York, USA (J. Pagano, personal communication). One hundred microliters (100 μl) of the appropriate treatment solution was injected into the airspace of each egg, using a new 0.3-cc insulin syringe and aseptic technique for each injection (Becton Dickinson). A new sterile syringe and needle were used for each injection. To insure eggs were treated before extensive air hardening, injections were performed by two people simultaneously. The injection site was immediately sealed with clear nail polish (Sally Hansen Hard as Nails); all eggs were processed with polish from the same bottle to insure consistency. This polish is guaranteed to be free of dibutyl phthalate, formaldehyde, and toluene; full chemical contents can be viewed at http://www.sallyhansen.com/product.cfm?product = 332. Each egg was labeled with a number and letter identifying the mother and treatment using a Sharpie ultrafine point permanent marker.

Eggs were placed in nest boxes consisting of a $33 \times 15 \times 13$-cm clear plastic box with a tight-fitting lid. Boxes were drilled (10-mm drill bit) with holes every 5 cm on all sides of the box to allow air circulation. Six to eight eggs were spaced approximately 2 to 3 cm apart in each nest box and covered with glitter-grade vermiculite. Individual eggs were not handled again until hatching to reduce mortality from extraembryonic membranes tearing away from the interior of the eggshell.

Eggs were incubated in a controlled laboratory facility under different temperature regimens, using a split-plot design. Each nest box ($n = 12$) contained six to eight snapping turtle eggs injected with a low (1 ppm), high (10 ppm), or control (0 ppm) treatment. Nest boxes were placed in either a low-temperature (24.5°C, $n = 6$) or high-temperature (30°C, $n = 6$) incubator (Safety Hatch Reptile incubators; Big Apple

Table 2. Allocation of treatments for snapping turtle eggs incubated in low and high temperature incubators[a]

		Injection treatment			
Incubator temperature	Nest box	Oil	Low	High	Total eggs
Low	1	3 (2)	4 (2)	2 (2)	6
	2	2 (2)	1 (2)	4 (2)	6
	3	4 (2)	2 (2)	1 (2)	6
	4	4 (2)	3 (2)	2 (2)	6
	5	2 (2)	2 (2)	3 (2)	6
	6	3 (2)	2 (2)	1 (2)	6
High	7	3 (2)	2 (2)	4 (2)	6
	8	4 (2)	3 (2)	4 (2)	6
	9	2 (2)	3 (2)	3 (2)	6
	10	3 (2)	2 (3)	1 (2)	7
	11	1 (2)	3 (2), 1 (2)	4 (2)	8
	12	1 (2)	4 (2)	2 (2), 3 (2)	8
	Total eggs	24	27	26	77

[a] Each polychlorinated biphenyl treatment indicates the female the eggs were harvested from, and the number of eggs from that female is given in parentheses. None of the eggs from female 1 were viable ($n = 14$).

Herpetological). Within each incubator, each nest box was treated as low moisture (1:1 vermiculite:water; volume:volume) or high moisture (1:1.1 vermiculite:water). Moisture levels were chosen based on the assumption that a 10% increase in moisture would produce a measurable effect. Nest boxes were weighed daily to measure evaporation, and water was added to maintain constant moisture levels. Boxes were rotated daily within incubators to account for potential regional differences in temperature and humidity.

Eggs in nest boxes were checked one to two times daily for hatchlings pipping through the egg shells. If pipping was observed, those eggs were removed and placed in individual containers with vermiculite to ensure proper identification of nesting female and PCB treatment. Hatchlings remained in containers until emergence from the egg was complete. On hatching, each hatchling was examined for gross morphological deformities, including underdeveloped limbs, kinked tails, and carapace and plastron abnormalities, including extra or missing scutes.

Individual treatments and all combinations of treatments were allocated equally across eggs from each female. The data were analyzed with a generalized linear model (SAS PROC GLM), using female of origin as a blocking factor to control for genetic variability. The response variable was the number of eggs hatched. Post-hoc pairwise comparisons (contrast coefficients) were used to identify differences between control and treatment levels.

RESULTS

Thirty-seven (48%) of the 77 eggs hatched successfully. Hatchlings from successful eggs were free of any gross morphological deformities. Nonviable eggs were dissected, and no embryonic development was found. Most unhatched eggs had fungal infections throughout the interior and appeared dehydrated. None of the eggs from female 1 ($n = 16$) was viable, and dissection of these eggs revealed no embryonic development. These eggs were excluded from further analysis, leaving a total of 61 incubated eggs, and an adjusted hatch rate of 61%.

An analysis of variance was used, even though assumptions of normality were violated for individual treatment combinations because of the small sample size. The standard deviation, and thus variance, for each treatment group was approximately equal (Table 3). Hatching success differed among treatments and females, although only the temperature treatment was statistically significant (Table 3). Hatching success was higher at the lower temperature ($p = 0.0265$). The PCB treatments did not significantly affect hatching success, although hatching success of eggs in the low PCB treatment (71%) appeared to be slightly higher than the control treatment (55%) and high PCB (55%) treatment.

DISCUSSION

The overall hatching success for the present study was 11% higher than in other studies using egg injections to mimic maternal transfer in chelonians and crocodilians [1,19]. Chelonian and crocodilian eggs have rigid shells, and injection is difficult once the shells harden. The higher success rates reported in this study may have resulted from eggs being harvested from females that had undergone necropsy and the timely completion of treatment injections. As the duration of air exposure increased, eggs became more rigid and made a popping sound on injection (J.L. Schnars, personal observation). Moreover, insertion of the needle though the outer calcareous layer became more difficult as the shells air hardened. Thus, injection of eggs before they become fully hardened might explain why the success rate was similar to the rates observed by Talent et al. [13] with the more pliable fence lizard eggs.

The failure of treated eggs to hatch could have been a consequence of either the injection of nonviable eggs or the lethal effects of the procedure on viable eggs. The hatching success for eggs from females 2, 3, and 4 was on average 61%;

Table 3. Analysis of variance for hatching success of snapping turtle eggs with respect to injection solution, incubation temperature, and incubation moisture[a]

Source	*df*	Sum of squares	Mean squares	*F*	*P*
Model	8	2.93	0.36	1.64	0.1375
Error	52	11.63	0.22		
Corrected total	60	14.56			
PCB	2	0.47	0.23	1.05	0.36
Temperature	1	1.16	1.17	5.22	*0.03*
Moisture	1	0.01	0.01	0.06	0.81
PCB × Temperature	2	0.24	0.12	0.54	0.58
PCB × Moisture	2	1.03	0.52	2.31	0.10
Post-hoc contrast comparisons					
Control vs PCB high and PCB low	1	0.3764	0.3764	1.68	0.2003
PCB high vs PCB low	1	0.1537	0.1535	0.69	0.4113
Temperature: high vs low	1	1.0064	1.0064	4.50	*0.0387*

Treatment	*n*	Mean	Standard Deviation
PCB control	20	0.50	0.51
PCB high	20	0.60	0.50
PCB low	21	0.71	0.43
Temp high	34	0.50	0.51
Temp low	27	0.74	0.45
Moisture high	28	0.64	0.49
Moisture low	33	0.58	0.50

[a] Data were blocked by female of origin to account for genetic variation (e.g., no eggs from female 1 were viable). Contrast coefficients for the main effects of PCB injection and temperature, as well as individual treatment means, are reported. Treatment means do not include eggs from female 1; significant values are italicized. *df* = degrees of freedom; PCB = polychlorinated biphenyl; *n* = number of eggs.

however, no egg from female 1 hatched, nor was any observed development seen in these eggs. Fertilization can occur several weeks before oviposition,and development is arrested at the late gastrula stage until oviposition [20]. This suggests that the eggs from female 1 were not viable (possibly unfertilized) before injection. Over the course of incubation, eggs were periodically candled to monitor development. If egg shells were observed to undergo a color change suggesting they were no longer viable (e.g., any change from white to either gray or pink), they were removed from the study and opened for microscopic inspection. Dissection of unsuccessful eggs from females 2, 3, and 4 revealed no noticeable embryonic development. Fatalities in these eggs might have been caused by several factors. Puncturing the inner layers of the eggshell during insertion of the needle might have fatal consequences. Nonfatal cracks can occur in rigid shells such as those of crocodilians [8], but the puncture in the inner boundary or amorphous layer may be more detrimental. The calcareous outer shell was sealed postinjection; however, ensuring that the amorphous layer also resealed is impossible. This layer is thought to be the critical barrier in preventing fungal and bacterial infections [21], which are common causes of reptilian egg mortality.

The volume of the injections also may have contributed to egg fatalities. High volumes of oil (1.0 μl/g egg) increased mortality in chicken eggs [16]. This study used a high injection volume (10 μl/g egg). All mortalities in studies using high-volume corn oil injection (100 μl) of chicken eggs occurred during the first week of incubation [22]. Replacing the airspace in an egg with a high volume of an oil-vehicle injection could potentially create a hypoxic environment for the embryo [23].

The timing of embryonic development during which injection occurs also influences hatching success. Injections before organogenesis result in higher mortalities in chickens [15]. In turtles, eggs diapause in the late gastrula stage of development while retained in the oviducts before oviposition. In the present study, snapping turtle eggs were injected immediately after removal from the female, and thus before organogenesis, to meet the objective of investigating the influence of PCBs on embryonic development. Hatching success might have been higher had treatment been postponed until after organogenesis [14,15], but the potential to observe permanent effects of PCBs on the physiology and morphology of the individual would have been reduced.

Hatching success was statistically independent of moisture but was influenced by temperature. Hatch rates were higher in nest boxes held at lower incubation temperature (71%) than in those with high incubation temperature (52%). Low temperatures might slow the rate of bacterial and fungal growth, which is a common problem when incubating eggs. Hatching rates for the present study were independent of moisture level, although prior work suggests that nests with high moisture levels usually produce larger [24] and faster [25] hatchlings than those from nests with low moisture levels.

Some studies suggest that embryonic fatalities should be more frequent when treatment injections contain high concentrations of contaminants or hormones [5,13]. No statistically significant effect of PCB treatment on either hatching success or gross morphological deformities as observed by Bishop et al. [26] was seen. This fact, coupled with the lack of any gross morphological deformities in individuals that did hatch, suggests that either snapping turtle embryos may tolerate high levels of total PCBs, or any morphological abnormalities present in this study were too subtle to detect. Polychlorinated biphenyl-caused disruption of the endocrine system can affect reproductive development and function [27]. Even low doses (0.4 ng/10 g egg) have been shown to have significant effects on individuals, especially during critical stages of development [27]. Willingham and Crews [28,29] reported that Aroclor 1242 significantly affected hormone and enzyme levels responsible for sex determination in turtle embryos. The present study did not quantify hormone or enzyme levels, nor did it determine sex ratio at hatch.

Egg injections have been largely abandoned for studies of contaminants in snapping turtle eggs because of low hatching success in previous studies. The egg injection methodology used in the present study significantly improved on the hatch rates reported for calcareous reptile eggs compared with other studies. We attribute this success to the decisions to use eggs harvested directly from the female in a sterile laboratory, to inject eggs immediately after harvest, and to use low incubation temperatures. We speculate that these factors worked in conjunction to minimize bacterial and fungal growth in eggs. To replicate these results, we recommend harvesting each egg individually from the female, then cleaning and injecting before harvesting the next egg. This minimizes the time eggs are exposed to air outside of the oviduct and ensures injection while the shells are still pliable; this is, in our opinion, the critical step resulting in improved hatching success. We advocate that the egg injection technique, with the modifications proposed here, should be reconsidered as the best experimental method to control treatment doses in studies meant to model the maternal transfer of contaminants to calcareous reptile eggs.

Acknowledgement—We thank Casey Bradshaw and Amy Smith for assistance with field work and Eric Obert for valuable discussion. This research was supported by a grant from Pennsylvania Sea Grant.

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