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EFFECT OF POLYCHLORINATED BIPHENYLS AND MERCURY ON THE COMMON SNAPPING TURTLE (*CHELYDRA SERPENTINA SERPENTINA*)

A Dissertation in

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by

Jeanette L. Schnars

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The dissertation of Jeanette L. Schnars was reviewed and approved* by the following:

Jay R. Stauffer, Jr.
Distinguished Professor of Ichthyology
Dissertation Advisor
Chair of Committee

Hunter J. Carrick
Associate Professor of Aquatic Ecology

C. Andrew Cole
Assistant Professor of Landscape Architecture

C. Paola Ferreri
Associate Professor of Fishery Management

Charles H. Strauss
Director, School of Forest Resources

*Signatures are on file in the Graduate School

ABSTRACT

Snapping turtles are a good bioindicator of aquatic contaminants. Two contaminants of concern in aquatic environments are polychlorinated biphenyls (PCBs) and mercury. Snapping turtles are capable of surviving high levels of PCBs in their tissues. Females pass contaminants to their eggs, thereby exposing embryos prior to development. The effects of PCBs through maternal exposure on the developing embryo and neonate hatchling are poorly understood. My study addressed techniques to mimic maternal exposure, the change in PCBs from egg to hatchling, and the effects of PCBs on neonate hatchling respirations. My study also addressed estimating mercury levels in snapping turtles through a non-invasive technique. Snapping turtle eggs were collected, injected with oil or a low (1 ppm) or high (10 ppm) PCB solution, and incubated under low and high temperature and moisture treatments. Upon hatch, neonate hatchlings were measured in a respirometer for CO_2 production. In adult snapping turtles, the claw, liver, muscle, and adipose tissues were sampled and analyzed for mercury concentration. Hatching success in PCB injected eggs was 61% overall. A comparison of congener specific profiles from egg to hatchling indicated an increase in higher chlorinated congeners and a decrease in lower chlorinated congeners. The interaction of high PCB treatment and high incubation temperature resulted in a lower total PCB concentration in the hatchling. Data did not support the relationship of PCB treatment and CO_2. In modeling mercury contaminants in internal tissues, the claw alone was not a good indicator. The model to estimate mercury in the liver required the mercury in the claw and the weight of the turtle. Future contaminant studies should continue attempts with

egg injection techniques to mimic maternal transfer. Understanding the toxicokinetics of PCBs during embryonic development is still in its infancy. My study revealed that PCBs are mobile and dynamic during embryonic development. Although effects were not observed in respiration measurements, other physiological effects should continue to be examined. Contaminant monitoring should be limited to non-invasive techniques when possible. Predictive models should continue to be developed to limit impacts to stable populations and to infer contaminants in threatened or endangered populations of turtles.

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PREFACE

The following dissertation is a report of the effects of polychlorinated biphenyls and mercury on the common snapping turtles (*Chelydra serpentina serpentina*). The first two chapters focus on the introductory material and methodology that applies to the whole study. Chapters 3, 4, and 5 examine specific effects from the treatments outlined in the methodology, chapter 2. Although some of these chapters may appear to stand alone, I encourage the reader to read this dissertation in its entirety. Information presented in subsequent chapters is necessary for a full understanding of the methods and discussion in preceding chapters. Chapter 6 focuses on the effects of mercury, as opposed to polychlorinated biphenyls, and can be read as a complete study; however Chapter 1 would provide background material that would supplement the understanding of this study.

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Chapter 1

Introduction to the Common Snapping Turtle (*Chelydra serpentina serpentina*)

The common snapping turtle (*Chelydra serpentina serpentina*; Linnaeus, 1758) is a large freshwater turtle found throughout the eastern and midwestern United States and southern Canada. Their range nearly surrounds all of the Laurentian Great Lakes where they inhabit the soft muddy bottoms of ponds, lagoons, and tributaries and shallow waters. Snapping turtles have an omnivorous diet, consuming a range of organisms, including small insects, amphibians, reptiles, birds, small mammals, as well as algae and plant matter. Carrion is often consumed; however adults will also ambush live prey as well (Ernst et al., 1994).

Snapping turtles are an inconspicuous organism. They usually do not bask in the sun and rarely come onto land. During the nesting season (the month of June in Pennsylvania), it is common to observe female snapping turtles on land around dawn or dusk searching for a nesting site. Populations of snapping turtles are assumed to be abundant, although this assumption can be difficult to support due to limited observations on land and occasional difficulty in trapping. Other factors that negatively impact snapping turtle populations include overfishing (Pennsylvania fishing regulations allow 15 individuals per day or 30 individuals in one's possession), high predation rates on nests, high juvenile mortality, destruction of habitat, and contaminants in the water and their food source.

Contaminant levels have been of immense concern in the Great Lakes. Historically, contaminant studies have focused on the concentrations, what organisms carry specific contaminants, and in which tissues specific contaminants accumulate. Presently, studies are starting to focus on the underlying mechanisms contaminants may impose affecting the health of a population. Although contaminants are often found at sub-lethal concentrations in the organism, the synergistic effects of the contaminant with other natural stressor can result in fatalities. In studies using various species of tadpoles, interactions between pesticides and predatory stress were found to negatively impact tadpoles and increase fatalities when non-lethal doses of the pesticide were used (Relyea, 2005).

Many studies have identified the snapping turtle as a good bioindicator of aquatic contaminants (Meyers-Schöne and Walton, 1994; Bishop et al., 1998; Pagano et al., 1999, de Solla et al., 2001; Ashpole et al., 2004). Adipose, muscle, liver, blood, and eggs have been sampled to provide an estimate of various contaminant concentrations at a geographic location. At least 22 species of other freshwater and sea turtle species have been sampled in contaminant studies as well (Meyers-Shöne and Walton, 1994). Contaminant studies in sea turtles are more difficult because all sea turtle species are classified as endangered. Contaminant studies that have been conducted on sea turtles rely upon sampling tissue from deceased adult turtles or sampling eggs for chlorinated organic compounds (Meyers-Shöne and Walton, 1994). There are two families of sea turtles and four families of freshwater turtles all belonging to the order Testudines. Snapping turtles and sea turtles are similar in egg production, method of nesting, and they both spend the majority of their lives submersed in the water. In contrast, sea turtles are

much larger in size and lay a larger size clutch. Comparisons of contaminant studies within various freshwater species are possible, although habitat and feeding habits must be considered (Meyers-Shöne and Walton, 1994). Snapping turtles are an optimal model organism for contaminant studies because they are long-lived, have an omnivorous diet encompassing multiple trophic levels, and usually confine themselves to a specific pond or home range (Obbard and Brooks, 1991).

Two contaminants that have been the focus of numerous studies are polychlorinated biphenyls (PCBs) and mercury (Hg). The concentrations of these contaminants have regulated most of the game fish consumption advisories in the Great Lakes. Along with various species of fish, snapping turtles are also harvested for consumption. Consumption advisories for snapping turtles exist in New York and Ohio. Presently there is a consumption advisory in Pennsylvania for snapping turtle fat tissue; however a consumption advisory did not exist in Pennsylvania until the preliminary results of this study raised concerns regarding PCB and mercury concentrations. In northwestern Pennsylvania, there had been no other studies of contaminant levels in snapping turtle tissues.

During July to August 2002 I sampled turtles at three sites in Erie County, Pennsylvania as a preliminary means of determining contaminant levels in snapping turtles. Turtles were trapped in the lagoons on Presque Isle State Park, Siegel Marsh (PA Game Lands #218), and Lake Pleasant (Figure 1.1). Adult turtles collected were euthanized by decapitation (IACUC # 14569) and muscle, adipose, and liver tissues were sampled and analyzed for PCBs. Contaminant levels indicated high PCB levels in adipose tissue of snapping turtles from Presque Isle State Park (n=6), and lower levels of

PCBs in Siegel Marsh (*n*=2) and Lake Pleasant turtles (*n*=2) (Table 1.1). These data provided background PCB levels that assisted with the experimental design of my study.

Polychlorinated biphenyls include 209 unique congeners that differ from one another by the number and location of the chlorines on the two phenol rings. Once ingested, metabolism is the primary mechanism by which organisms eliminate PCB congeners (Bryan et al., 1987a). Generally, lower chlorinated congeners are more easily metabolized and excreted from the body than higher chlorinated congeners. Higher chlorinated congeners are more lipophilic than lower chlorinated congeners and are stored in lipid tissues rather than excreted (Bryan et al., 1987b).

Snapping turtles are capable of storing high concentrations of PCBs in lipid tissues without obvious gross morphological deformities (Meyers-Shöne and Walton, 1994), although these high concentrations might have detrimental effects on their physiology, such as the feminization of males. The snapping turtles in the Meyers-Shöne and Walton (1994) study were used as bioindicators, and most likely were adults that accumulated high contaminant concentrations over their lifetime. Polychlorinated biphenyls are of great concern because, although they are stored primarily in the lipid tissues, they also are found in high concentrations in the reproductive organs (Stone et al., 1980; Helwig and Hora, 1983; Bryan et al., 1987b), which could have direct effects on the production and health of offspring.

Embryos of oviparous organisms are exposed to contaminants by two pathways: 1) contaminant uptake from the surrounding environment and 2) transfer from female to offspring (maternal transfer; Hopkins et al., 2006). Either form of exposure may potentially lead to altered embryonic developmental patterns. The initial source of

contaminant exposure for most embryos is likely to be maternal transfer during, or prior to, egg development.

Female turtles annually pass organochlorine compounds to their developing eggs through the food they consume and by mobilizing contaminants that are stored in fat tissue. Therefore this is an important vector for the acquisition of contaminants in young turtles. The contaminant level in the eggs can be used as indicator of contaminant levels in the maternal tissues (Bishop et al., 1994, 1995, 1996, Pagano et al., 1999). Concentrations of PCBs in eggs are correlated with concentrations in maternal muscle, adipose, and liver tissues (Hebert et al., 1993; Pagano et al., 1999), and the pattern of contaminant accumulation observed in the liver is similar to that in the egg (Hebert et al., 1993). Older and therefore larger female turtles, which are capable of producing increased clutch size or mass, do not produce eggs with higher contaminant levels. Rather, contaminant levels in older females tend to reflect contaminant exposure over the last year during egg production (Bishop et al., 1994).

Contaminants such as PCBs can penetrate through an eggshell and accumulate in an embryo. This raises great concern about other potential bioaccumulation vectors. Contaminant exposure now becomes a problem for the individual prior to emergence from the nest; this includes the time periods of egg development, embryogenesis, and incubation. Exposure during these critical periods may eventually have greater impacts to both the individual and future populations. For example, not all contaminant related deformities are easily distinguished. Many physiological abnormalities do not result in morphological defects in hatchlings, but still may cause considerable dysfunction in adulthood. Organochlorides such as chlordane and Aroclor 1242 have significantly

affected hormone levels in embryos, as well as increasing aromatase levels (enzyme that converts testosterone to estradiol resulting in sex determination) (Willingham and Crews, 1999; 2000). These alterations in hormone productions lead to a significant increase in the percentage of females in the nest, despite what should be male biased incubation temperatures (Bergeron et al., 1994). Other physiological effects include increased growth rate and possible hyperthyroidism leading to early maturation (Willingham, 2001). Although an increased growth rate could produce larger hatchlings and increase survivorship, it is those individuals with initially high contaminant loads that would be more likely to survive to adulthood and reproduce. When these individuals reproduce the effects of high contaminant levels could result in an increase in defects in subsequent generation.

Chlorinated organic chemicals, such as PCBs, are deposited primarily in the lipid-rich yolk of the egg. At the time of deposition in the nest, the egg yolk contains over 95% of the total PCB contaminants (Bryan et al., 1987a), and the eggs from a single clutch do not significantly differ in PCB concentrations (Bishop et al., 1995). Contaminants, such as PCBs, are mobilized into the embryo during development, and their effects can be observed at low concentrations (Carey et al., 1998). As the PCBs are mobilized, the concentration in the snapping turtle embryos increases with incubation time, and the increase in total PCBs follows the trend of an increase in body mass (Bishop et al., 1995). deSolla and Fernie (2004) suspect that high concentrations of PCBs in eggs might be associated with changes in the reproduction and development of snapping turtles, but do not specify the type of changes. Exposure of contaminants prior to the critical stages of organogenesis, increases the probability of physiological

(hyperthyroidism, sex reversal) and morphological (dwarfed limbs, kinked tails, undeveloped appendages, shell deformities) deformities or fatalities prior to hatching. If the hatchling does emerge from the nest cavity, such abnormalities can compromise survival by interfering with efficiency of attempts by hatchlings to locate a home pond, increasing growth rate and possible hyperthyroidism, and acting as an endocrine disruptor (Sparling et al., 2000; Willingham, 2001).

Exposure prior to and during development can lead to changes that persist throughout the lifetime of the individual. Therefore, maternal transfer studies and evaluating the effects of the contaminant throughout development are critical in determining the effects of contaminants on reproductive adults and future populations. My study examined the change in PCB profiles from egg to hatchling and the effects of neonate hatchling PCB concentration on respiration. I also examined the ability to estimate mercury concentrations of internal tissues with non-invasive techniques. Such techniques reduce the need for euthanization in contaminant studies and allow for contaminant estimations on species with threatened or endangered status. These two contaminants are the focus of my study because of their history and long-term consequences in Great Lakes' species.

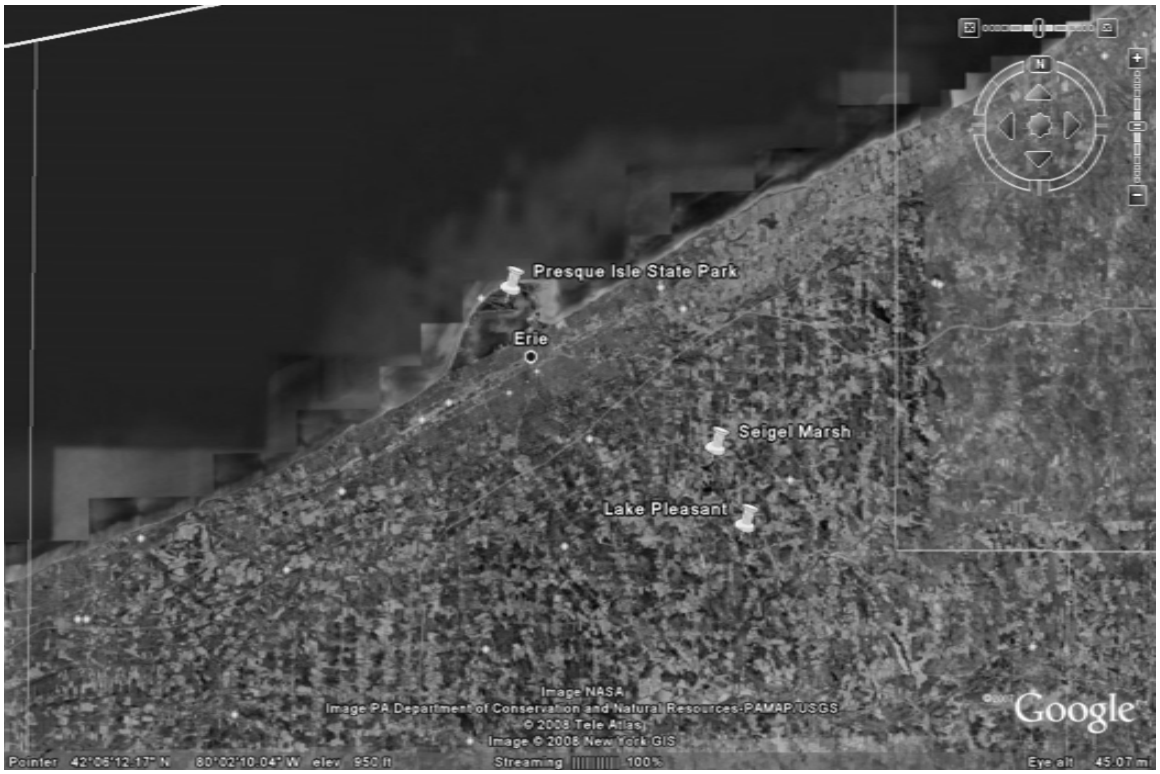

Figure 1.1 Sampling sites in Erie County, Pennsylvania to determine background polychlorinated biphenyl levels in snapping turtle adipose tissue ($^{©2007}$Google Earth).

Table 1.1 Polychlorinated biphenyl (PCB) analysis of adipose tissue of adult snapping turtles. Preliminary analysis from turtles sampled at Presque Isle State Park (PISP), Siegel Marsh (SM), and Lake Pleasant (LP). The range and mean (SD) indicate that Presque Isle State Park has snapping turtles with high PCB concentrations.

Sampling Site	***n***	**Range (ppm)**	**Mean**	**Weight Normalized Mean (ppm kg^{-1})**
PISP	6	18.66-207.14	91.41 (70.15)	8.90 (4.29)
SM	2	2.22-4.06	3.14 (1.30)	0.39 (0.04)
LP	2	0.64–7.81	4.23 (5.10)	0.54 (0.58)

Chapter 2

Methodology for Snapping Turtle (*Chelydra serpentina serpentina*) Studies

Site and Sampling

Wild populations of snapping turtles (*Chelydra serpentina serpentina*) were obtained through trapping at Siegel Marsh, Pennsylvania Game Lands #218, Erie County, PA (Figure 1.1) in June 2005. Turtles were trapped using turtle trap hoop nets (Nylon Net Company, Memphis, Tennessee). Traps were set perpendicular to shore in water less than 1 m deep. Preliminary sampling indicated that snapping turtles in Siegel Marsh had low organic contaminant loads (Chapter 1). Thus, maternal transfer of PCBs was assumed to be minimal at this site.

Upon removal from the trap, turtles were sexed and their pelvic region examined for the presence of eggs. Four gravid females were euthanized by decapitation (IACUC #15974 and #23499, IBC #18903) and necropsied in the laboratory. Eggs were removed immediately from the oviducts and placed in a container lined with aluminum foil, preventing possible PCB contamination of the eggs.

Three or four eggs per clutch (Table 2.1) were removed from the beginning, middle, and end of the oviducts to establish a baseline concentration of PCBs for the entire clutch. Female reptiles produce all their eggs simultaneously; therefore contaminant loads are uniform throughout a given clutch (Hall et al. 1979; Heinz et al. 1991). Concentrations of lipids and most chlorinated hydrocarbons do not vary significantly among eggs within the same clutch (Bishop et al., 1995); moreover,

organochlorinated compounds accumulate within lipids. In a study comparing the first 5 eggs, the last 5 eggs, and an arbitrary selection of the remaining eggs, it was concluded that PCBs did not significantly differ in eggs from the same clutch (Bishop et al., 1995). Therefore, concentrations from a composite sample of eggs should be representative of the entire clutch. Three eggs were harvested from females 3 and 4 (Table 2.1) as opposed to four eggs from a clutch to ensure enough eggs in each treatment. Each set of egg samples was stored in sterilized 250 ml glass specimen jars with metal screw-caps (Carolina Biological Supply Company, Burlington, North Carolina), frozen, and later sent for PCB analysis at the Environmental Research Center at the State University of New York at Oswego (Director, James Pagano, Chemistry Department, State University of New York at Oswego). Analysis was performed on a composite sample of the eggs from each clutch. Composite samples consisted of the blended contents of the three or four eggs from each clutch. A single PCB concentration was determined from analyzing these composited eggs, and was the representative PCB concentration for the entire clutch.

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. Eggs were arbitrarily treated with a low (1 ppm; n=27) or high (10 ppm; n=26) PCB solution, or oil (0 ppm; n=24) injection treatment (Table 2.2). Polychlorinated biphenyl solutions consisted of an Aroclor 1254:1260 (from the EPA Pesticide Repository) mixture in canola oil. The oil treatment contained only canola oil, which was used as the vehicle for the low and high PCB solutions. Eggs were injected with exclusively oil to account for fatalities that might occur from the injection procedure or presence of the canola oil. Thus, injection treatment was manipulated at the level of the

individual egg. The high PCB treatment was similar to that found in snapping turtle eggs at a highly contaminated USEPA superfund site in New York (J. Pagano, Department of Chemistry, State University of New York at Oswego, personal communication). Each egg was injected with a 100 μL of the treatment solution with a 0.3 cc insulin syringe (U-100 29G½ in., ultrafine permanently attached needle (Becton Dickinson & Company, Franklin Lakes, New Jersey)). The mixture was injected into the airspace of the egg using an aseptic technique. Injections were performed by two people simultaneously, thereby minimizing the time the eggs were exposed to air before injection. The injection site was sealed immediately with clear nail polish and the egg was labeled, using a Sharpie ultra fine point permanent marker, with a number and letter identifying mother and treatment.

Eggs were incubated in a controlled laboratory facility under different temperature regimes using a split-plot design (Figure 2.2). Nest boxes consisted of a 33 x 15 x 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 2-3 cm apart in each nest box and covered with glitter-grade vermiculite (www.bigappleherp.com). Once eggs were placed in the nest box, they were not individually handled until hatch. Handling of the egg can result in fatalities from the inner lining of the egg tearing away from the interior of the shell. Each box was weighed daily and watered to maintain the desired moisture content in the box. Three nest boxes in each incubator were incubated at low moisture and three nest boxes were incubated at high moisture levels (1:1 and 1:1.1 vermiculite to water [v/v], respectively). A total of 6 nest boxes were stacked in each of two Safety Hatch Reptile incubators

(www.bigappleherp.com). Boxes in each incubator were rotated daily to account for slight differences in temperature and humidity within the incubator. Eggs were incubated under one of two temperature treatments (24.5ºC or 30°C) until they hatched. Thus temperature was manipulated at the replication level of the incubator.

Table 2.1 Total number of eggs harvested per female snapping turtle collected at Siegel Marsh on date. Note the eggs 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.

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

Table 2.2 Set up of snapping turtle eggs in low (nest boxes 1-6) and high (nest boxes 7-12) temperature incubators. Each polychlorinated biphenyl treatment indicates the female the eggs were harvested from and the number of eggs from that female in parentheses. None of the eggs from female 1 were viable (n = 16).

		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 (2)	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**

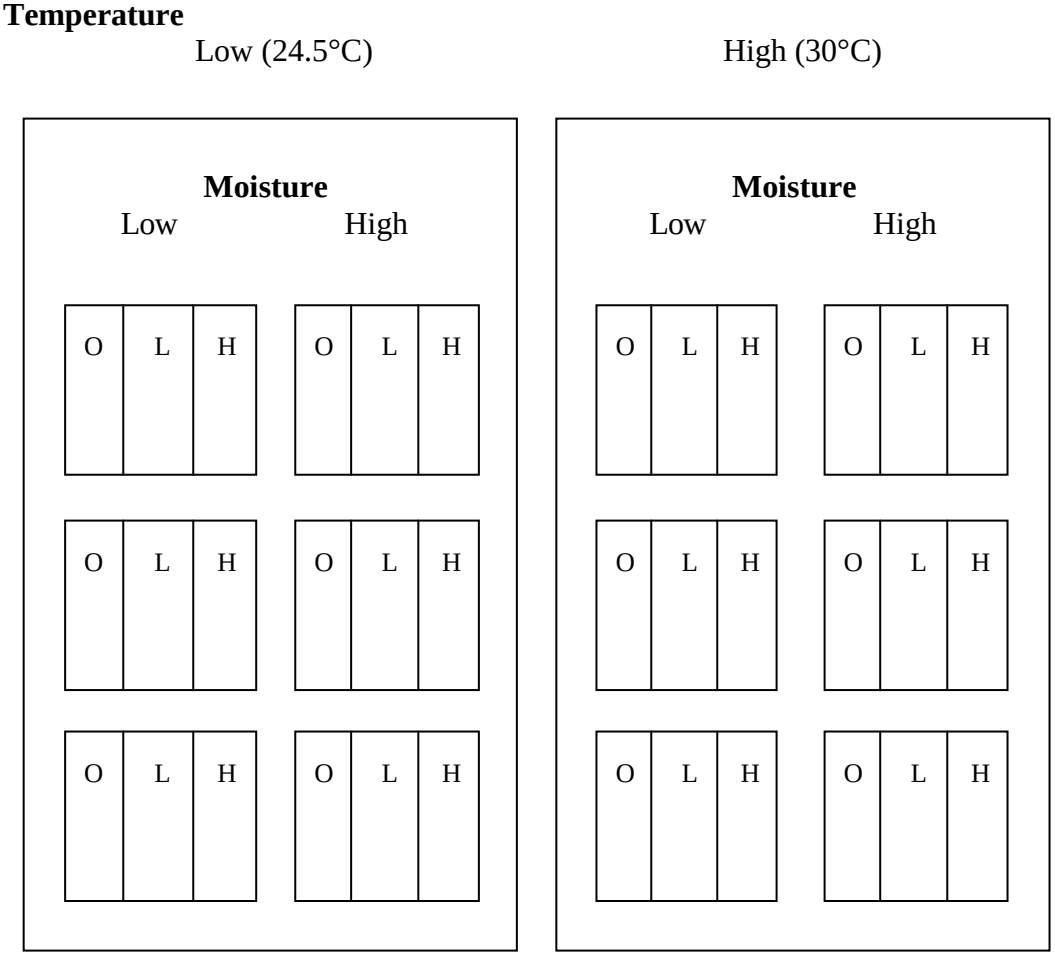

Figure 2.1 Split-plot design of nest boxes in low (24.5°C) and high (30°C) temperature incubators. Each nest box was treated as low (1:1 vermiculite:water) or high (1:1.1 vermiculite:water) moisture. Within each nest box were 6-8 snapping turtle eggs injected with a low (1 ppm) (L) or high (10 ppm) (H) polychlorinated biphenyl solution, or oil (0 ppm, canola oil only) (O).

Chapter 3

Hatching Success of PCB Injected Snapping Turtle (*Chelydra serpentina serpentina*) Eggs

Introduction

Contaminant research often relies on dosing individuals of a species and monitoring the effects, where experimental designs potentially have the advantage that the dose can be regulated and applied to numerous replicates. Controlled dosing of embryos in contaminant studies is difficult to accomplish under most circumstances (Muller et al., 2007). In reptiles, several methods have been developed to mimic the maternal transfer of contaminants. In some studies, contaminants have been applied topically to eggshells (Crews et al., 1991; Crain et al., 1998; Portelli et al., 1999; Sparling et al., 2006). This technique has limitations. It is often unknown, or poorly understood whether various contaminants can pass through calcareous eggshells such as those of reptiles and birds (Karasov et al., 2007); and if contaminants can travel by this pathway, it is often unclear to what degree (Russell et al., 1999; Muller et al., 2007). Topical treatment of alligator eggs found the transfer of chemicals across the eggshell was very low, varied between each egg, and did not correlate with the dose applied (Muller et al., 2007). Topical treatments to eggshells can negatively affect gas exchange and lead to increased mortality that is unrelated to contaminant effect (Deeming, 2004). These types of studies might better address the effects of contaminants in the soil surrounding the clutch than maternal transfer.

Other studies have tried to imitate maternal transfer using diet to expose gravid females to contaminants or injected contaminants into the eggs (Black et al., 1998; Rauschenberger et al., 2004; Muller et al., 2007). Female reptiles produce all their eggs simultaneously; therefore contaminant loads are similar in eggs within clutches (Hall et al., 1979; Heinz et al., 1991). Maternal metabolism and levels of contaminants in fat bodies also influence egg dose even when female dose is carefully controlled. Thus, genetic or phenotypic variability among females will increase variation in the maternal transfer of contaminants between clutches. It is therefore difficult to control the egg contaminant dose in replicate clutches using these techniques.

Injection of contaminants directly into eggs is another means of contaminant exposure and allows controlled doses and replication. This technique has been used with little success in reptiles, and several investigators have abandoned attempts at egg injections because of previous experiences with poor hatch rates, despite some studies sealing injection sites with Nexaband® topical adhesive (Abbot Laboratories, Abbot Park, IL, USA) to prevent bacterial infection (Muller et al., 2007). Successful use of egg injection has been particularly poor for reptiles with rigid, calcareous eggshells (e.g. turtles and crocodiles) (Grigg, 1987; Deeming, 2004). The parchment-like eggshells of lizards appear to be more tractable (Talent, 2002). One problem with this technique is distinguishing between mortality caused by the injection and mortality caused by the contaminants; furthermore, oil or clean injections are critical to evaluating results. For example, Talent et al. (2002) microinjected eggs of fence lizards with 17α ethinylestradiol. They reported very high egg mortality in contaminant treatments (especially high doses). Their eggs injected with solely the vehicle had good hatching

success (79.5%) indicating that mortality in contaminant treatments might not have been a result of the injection procedure.

The timing of egg injections also is critical. For example, injections in leghorn (*Gallus gallus*) and domestic chickens (*G. domesticus*) prior to organogenesis have higher mortalities than injections after organogenesis (Carlson and Duby, 1973; DeWitt et al., 2005a). If the objective is to understand maternal transfer and contaminant effects on the embryo, then egg injections prior to 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 (DeWitt et al., 2005b). 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 this study was to evaluate egg injection as a means of mimicking maternal transfer of PCBs to snapping turtle eggs. The benefit of egg injections, as opposed to other techniques, is that it permits replication of a controlled dose of contaminants to eggs. Snapping turtle 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.

Methods

Eggs were collected directly from female snapping turtle oviducts and injected with oil or a low or high PCB solution (Chapter 2). Eggs were incubated to full term

under different injection, temperature, and moisture treatments. After all viable eggs hatched, those that did not hatch were dissected and embryonic development was determined. Hatching success was determined by treatment. Contingency tables and Chi-squared analysis were used to evaluate the effects of female, temperature, moisture, and injection on hatching success. Significance was judged at α=0.05.

RESULTS

Thirty-seven (48%) of the 77 eggs hatched successfully. Hatchlings from successful eggs were free of any gross morphological deformities. Non-viable 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) were 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%.

Hatching success differed among treatments and females (Table 3.1). Hatching success of eggs from Female 4 (72%), was greater than that of eggs from Females 2 (57%) and 3 (55%), but, success was not significantly different among females (χ^2=1.46; $p > 0.05$). Hatching success of eggs in the low PCB treatment (71%) was higher than that of eggs in the oil (55%) and high PCB (55%) treatment, but PCB treatments did not significantly affect hatching success (χ^2=1.56; $p > 0.05$). Hatching success of eggs in the low moisture treatment (59%) did not differ from that of eggs in the high moisture treatment (62%) (χ^2=0.05; p>0.05). Hatching success of eggs in the low temperature

treatment (71%) was greater than that in the high temperature treatment (52%), but temperature did not have a significant influence on hatching success (χ^2=2.52; p>0.05).

Table 3.1 Hatching success of snapping turtle eggs with respect to female, injection solution, incubation temperature, and incubation moisture. Numbers for injection, temperature, and moisture factors do not include eggs from female 1.

Factor		***N***	**# Hatch**	**Hatching Success (%)**
Female	1	16	0	0
	2	21	12	57
	3	22	12	55
	4	18	13	72
Injection	Oil	20	11	55
	Low	21	15	71
	High	20	11	55
Temperature	Low	28	20	71
	High	33	17	52
Moisture	Low	32	19	59
	High	29	18	62

DISCUSSION

Overall hatching success in my study was 61%. This success rate was higher than in other studies using egg injections to mimic maternal transfer in chelonians and crocodilians (Muller et al., 2007). Chelonian and crocodilian eggs have rigid shells, and injection of such eggs is difficult once the shells harden. Higher success rates in this study may have resulted from eggs being harvested from necropsied females and efficiently completing the egg injections. As exposure time increased, eggs became more rigid and made a "popping" sound (J.L. Schnars, personal observation). Moreover, insertion of the needle though the outer calcareous layer was more difficult as the shells became more rigid. Thus, injection of eggs before they become fully hardened might explain why the success rate was similar to the rates observed by Talent (2002) with fence lizard eggs, which have pliable shells.

Failure of injected eggs to hatch could be the consequence of injecting nonviable eggs or of lethal effects of the procedure on viable eggs. The similar hatching success of eggs from Females 2, 3, and 4, and the lack of embryonic development suggest that the eggs from Female 1 were not viable prior to injection and could have been an unfertilized clutch. Dissection of unsuccessful eggs from Females 2, 3, and 4 also 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 had fatal consequences. Non-fatal cracks can occur in rigid shells such as those of crocodilians (Grigg, 1987; Deeming, 2004), but the puncture in the inner boundary or amorphous layer may be more detrimental. The calcareous outer shell was sealed post-injection; however, there is no means of ensuring that the amorphous layer

also resealed. This layer is thought to be the critical barrier in preventing fungal and bacterial infections (Kern and Ferguson, 1997), which are common causes of reptilian egg fatalities.

The volume of the injections also might have caused egg fatalities. High volumes of oil (1.0 μL/g egg) increased mortality in chicken eggs (DeWitt, 2005b). 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 (Ameenuddin and Sunde, 1984). Replacing the airspace in the egg with a high volume of an oil-vehicle injection could potentially create a hypoxic environment for the embryo (Henshel et al., 1997).

The timing of embryonic development on which injection occurs also influences hatching success. Injections before organogenesis result in higher mortalities in chickens (DeWitt, 2005a). In my study, snapping turtle eggs were injected immediately after removal from the female, before organogenesis, to accomplish the objective of investigating the influence of PCBs on embryonic development. Hatching success might have been higher had I waited until after organogenesis (Carlson and Duby, 1973; DeWitt et al., 2005a), but the potential to observe permanent effects of PCBs on the physiology and morphology of the individual would have been lost.

Hatching success was statistically independent of temperature and moisture. Hatching rates were higher in nest boxes held at lower incubation temperature (71%) than high incubation temperature (52%). Low temperatures might slow the rate of bacterial and fungal growth, which is a common problem when incubating eggs. Nests with high moisture levels produce hatchlings that are larger (Packard, 1991) and faster (Miller,

1993) than hatchlings from nests with low moisture levels. Hatching rates were similar in nest boxes in low and high moisture treatments (59% and 62%, respectively).

I did not observe a statistically significant influence of PCB treatment on hatching success (χ^2=1.56; p>0.05). The low PCB treatment had a 71% hatching success, whereas the high PCB treatment had a hatching success of 55%. In other studies, embryonic fatalities were more frequent when contaminant or hormone concentrations were high (Talent, 2002; Sparling, 2006). I did not observe gross morphological deformities in individuals that hatched successfully, suggesting that embryos might be able to tolerate high levels of total PCBs. Morphological abnormalities in hatchlings from other studies were related to total PCBs present in the egg. The mean of the egg at the specific location was 2.708±1119 ng/g wet weight, which was the sum concentration of PCB congeners 105, 118, 138, 153, 170 and 180 (Bishop et al., 1991). Moreover, PCB-caused disruption of the endocrine system also can affect reproductive development and function (Crews et al., 2000), and low doses (0.4 ng/10 g egg) can have significant effects on individuals, especially during critical stages of development. Willingham and Crews (1999; 2000) reported that Aroclor 1242 significantly affected hormone and enzyme levels responsible for sex determination in turtle embryos. Therefore, some abnormalities are difficult to detect and might not be recognizable until individuals become mature.

I think the success of the egg injection technique used in my study can be partially attributed to the decision to use eggs harvested directly from the female in a sterile lab, to inject eggs immediately after harvest, and the use of low incubation temperatures. All of these procedures minimized bacterial and fungal growth in eggs. I recommend

harvesting each egg from the female individually, and then cleaning and injecting them *before* harvesting the next egg. This technique should minimize the time eggs are exposed outside of the female and ensure injection while the shells are still pliable. In my opinion, minimizing exposure time to air is the critical step in the technique of egg injections that should result in better hatching successes. Egg injections in snapping turtle contaminant research have been abandoned because of low hatching success, but I believe my study has provided improvements to the method that will improve the success rate of the technique.

Chapter 4

Change in Congener Profile and the Effects of Incubation Temperature on PCBs in Developing Snapping Turtle (*Cheyldra serpentina serpentina*) Embryos

INTRODUCTION

Increased incubation temperature increases the embryonic growth of reptiles (Deeming and Ferguson, 1991). The growth rate during the first half of incubation at higher temperature is significantly faster compared to the growth rate at a lower temperature. Growth rate in the second half of incubation is similar and does not continue to increase, indicating temperature acclimation by the embryo (Birchard and Reiber, 1995). The effect of increasing incubation temperature in reptiles is that oxygen consumption over the egg surface and metabolism increases (Deeming, 2004; Whitehead, 1987; Leshem et al., 1991; Booth, 1998; Booth et al., 2000). Therefore, increasing incubation temperature would increase metabolism. An increase in growth rate indicates an increase in metabolic rate (Leshem et al., 1991; Birchard and Reiber, 1995). Thus, the temperature of the nest cavity should influence the embryo's metabolic rate. Incubation temperature also determines sex, although such studies use turtles and other reptiles with differing temperature regimes that determine the sex.

Nests with elevated moisture levels produce hatchlings that are larger (Packard, 1991) and faster (Miller, 1993). Larger and faster hatchlings imply a higher metabolic rate, and therefore potential to metabolize a higher concentration of contaminants more quickly. As metabolism is the primary mechanism for eliminating various PCB congeners (Bryan et al., 1987a), a change in metabolic rate may produce significant changes in both concentration and form of congeners accumulated. Contaminants can

also be acquired over the shell of the egg. Low doses of various organo-contaminants applied on the outer surface of red-eared slider turtle (*Trachemys scripta*) eggshells may be absorbed by the embryo, producing a significant increase in the percentage of females in clutches incubated at male biased temperatures (Willingham and Crews, 2000). As little as 0.2% of contaminants deposited on the outer eggshell may actually reach the embryo; however, this low concentration is sufficient to cause a considerable effect such as reversing the sex of the developing embryo (Crews et al., 1991).

The chemical and toxicological properties of individual PCB congeners differ. Therefore, it is important to consider how metabolism in the organism affects the accumulation and transfer of specific congeners (Pagano et al., 1999). I hypothesized that manipulation of nest temperature and moisture would increase metabolic rate, which would increase the metabolism of total PCB concentration and specific congeners. The objective of this study was to compare the total and congener-specific PCB profiles of egg and neonate hatchling and determine if incubation temperature and moisture influence these concentrations.

METHODS

Snapping turtle eggs were injected with oil or a low or high PCB solution and incubated until hatch (Chapter 2). When the hatchling began pipping from the egg, hatchling or egg and hatchling were removed immediately from the nest box and held in an individual labeled container. If more than one hatchling was present simultaneously in the same box, proper identification of PCB treatment could not be made and these hatchlings were excluded from further analysis of PCB effects.

Hatchlings were weighed, and their carapace length was measured using vernier calipers. Any gross morphological deformities (e.g., dwarfed limbs, kinked tails, undeveloped appendages, shell deformities) were recorded. These deformities have been previously documented in neonate hatchlings from areas with high organochlorides (Bishop et al., 1991). Hatchlings were euthanized via decapitation two days post-hatching, to ensure that the yolk sac had been completely absorbed, and individually frozen in 60 ml glass jars with metal screw-caps (Carolina Biological Supply Company, Burlington, North Carolina).

Because collected eggs are not free of contaminants, such as PCBs, composite egg samples were analyzed to determine background PCB concentrations existing in the egg prior to any treatment. Three or four eggs were arbitrarily collected from the first, middle, and last eggs harvested from the clutch. Total and congener specific PCBs were analyzed in composite egg samples and whole neonate hatchlings at The Environmental Research Center, State University of New York, Oswego, using gas chromatography, with electron capture detection (GC-ECD). Eggs from each clutch were blended together to create a composite sample, representing the entire clutch. Results of the PCB analysis for composite egg samples represented a baseline PCB concentration for each egg in the clutch. Injected treatment solutions were analyzed (May 2008) and error was calculated using the predicted and actual PCB concentration values.

Chemical Analysis

Congener-specific PCB analyses were conducted using the procedures in Pagano et al. (1999). The capillary column procedures and standards were based on those of the

Wadsworth Center for Laboratory and Research, New York State Department of Health (NYSDOH, Bush et al., 1982, Bush et al., 1985). The calibration standard was a 1:1:1:1 mixture of Aroclors 1221, 1016, 1254, 1260 from the United States Environmental Protection Agency (EPA) Pesticide Repository each at 200 pg/µL, Hexachlorobenzene (HCB) at 5 pg/µL, dichlorodiphenyldichloroethylene (DDE) at 10 pg/µL, and Mirex at 10 pg/µL. Congener assignments and weight percent distributions for the mixture of NYSDOH's Aroclor quantitation were established with a Hewlett-Packard (HP) Model 5890 II gas chromatograph with electron capture detector (GC ECD-Ni^{63}) and autosampler. Calibrations were completed every six samples, with a system blank and calibration check solutions analyzed during each analytical run.

Laboratory Quality Assurance/Quality Control is based on USEPA protocols (USEPA, 1997). Samples were analyzed in triplicate in order to assess method precision. A surrogate standard (analyte), decachlorobiphenyl (DCB) was utilized as an indicator of individual sample extraction proficiency. In addition, method spikes were conducted using the comprehensive congener distribution of the instrument calibration standard to validate method performance, and to determine if any congener-specific bias existed in the sample preparation.

Chromatographic data were collected by the HP ChemStation software and generated amount of each PCB congener (ng/g) in accordance to the appropriate International Union of Pure and Applied Chemistry (IUPAC) numbers. Homologue mole percent and average chlorines per biphenyl (Cl/bp) values were calculated.

Data Analysis

Because organic contaminants, such as PCBs, are lipophilic, an Analysis of Variance was performed comparing the percent lipid of the egg to the hatchlings from eggs injected with oil, and low and high PCB solutions. Total PCB concentrations of eggs and hatchlings were mass balanced to allow for direct comparisons of PCB concentrations between treatment groups. To obtain mass balanced numbers, the total PCB concentration (ng/g) of an individual hatchling was multiplied by the mass (g) of that individual. The result was a PCB concentration (ng) that was compared to the PCB concentrations of other individuals of varying mass. Using the mass balanced values, a t-test assuming unequal variances was performed to compare the PCB concentrations of eggs to the hatchlings from oil injected eggs, indicating if there was a difference due to embryogenesis. An Analysis of Variance was calculated using the mass balanced PCB concentrations of the eggs, and hatchlings from eggs injected with low and high PCB solutions to determine that the PCBs from the injected solutions were incorporated into the embryo. Using a Tukey test, significantly different treatments were determined. Significance was judged $\alpha=0.05$.

A t-test assuming unequal variances was performed to determine if embryogenesis influenced the number of Cl/bp resulting in the PCB analysis. As metabolism is the primary means of excreting contaminants, the Cl/bp may be influenced by the metabolism during embryogenesis. An Analysis of Variance was calculated to determine if there was a difference in the number of Cl/bp between eggs, and the hatchlings from eggs injected with the low and high PCB solution. These hatchlings were exposed to a PCB injection containing an elevated mixture of Cl/bp, therefore this

analysis would support the assumption that the PCB contaminants were incorporated into the embryo. Significance was judged at $\alpha=0.05$.

A PCB congener may have 1-9 chlorines bonded to the biphenyls. As higher chlorinated congeners are difficult to metabolize and excrete, homologue profiles are constructed to categorize the sample based on the number of chlorines (1-9) present on each congener. Homologue profiles for the nine chlorination levels in eggs and hatchlings from oil injected eggs were compared using a t-test assuming unequal variances to determine if embryogenesis resulted in a change of chlorination level. Significance was judged at $\alpha=0.05$.

RESULTS

None of the 16 eggs from Female #1 hatched and dissection of these eggs revealed no embryonic development, it was assumed that these eggs were not fertilized (Chapter 3). These eggs were distributed in six different nest boxes, both incubation temperatures, and subjected to all PCB treatments. These eggs were excluded from further analysis, leaving a total of 61 incubated eggs. Thirty-seven of the 61 eggs hatched successfully. All hatchlings eggs were free of gross morphological deformities (e.g., dwarfed limbs, kinked tails, undeveloped appendages, shell deformities). Twenty-six of the 37 hatchlings were analyzed for total and congener specific PCB concentrations. The hatchlings analyzed represented each interaction of the multiple treatments. The 24 eggs that did not successfully hatch were dissected revealing fungal infections throughout the interior and appeared dehydrated. Fungal infections can occur on fertile as well as unfertile eggs. No embryonic development was apparent.

Total and congener specific PCB concentrations

Treatment solutions injected into eggs were formulated to produce an oil (0 ppm or 0 ug), low (1 ppm or 1 ug), and high (10 ppm or 10 ug) PCB solution. Solutions were analyzed for actual values (May 2008) and were within 84% of the predicted concentration (Table 4.1). Percent lipid content of eggs and hatchlings from injection treatments did not differ significantly ($F_{0.05(1)3,24} = 2.21$, $p > 0.05$). One lipid sample from the high PCB treatment was lost due to a laboratory accident, and was not included in the statistical analysis. Mass balanced total PCB (ng) in composite egg samples were not significantly different from the mass balanced total PCB (ng) of hatchlings from oil injected eggs ($t_{0.05(1),3} = -0.42$, $p > 0.05$). The mass balanced PCB (ng) in egg composite samples, and hatchlings from eggs injected with low and high PCB solutions were significantly different ($F_{0.05(1)2,18}=8.14$, $p < 0.05$; Table 4.2). A Tukey test indicated that there was a significant difference between the egg composite samples and high PCB treatment hatchlings ($q=4.39$, $p<0.05$) and between the low PCB treatment hatchlings and the high PCB treatment hatchlings ($q= 4.96$, $p<0.05$). There was not a significant difference between the egg composite samples and the low PCB treatment hatchlings ($q=0.88$, $p>0.05$).

The mean number of chlorines per biphenyl did not differ significantly between eggs, oil, low, and high treatments ($F_{0.05(1)3,25}=2.47$, $p < 0.05$; Table 4.1). Congeners with IUPAC numbers 118, 153, 138+163+164, and 180 had the highest concentrations in all profiles (Figure 4.1A-D). Peaks were less pronounced in egg profiles (Figure 4.1A) than

in hatchlings in oil (Figure 4.1B), low (Figure 4.1C), or high (Figure 4.1D) PCB treatments.

Congener profiles differed between eggs and hatchlings in the oil treatment (Figure 4.2). Concentrations at 53% of the congeners increased, 23% decreased, and 23% did not change during incubation. Concentrations of congeners with low IUPAC numbers decreased or did not change during incubation, whereas concentrations of congeners with high IUPAC numbers increased during incubation. The PCB congeners were grouped into homologues, based on chlorination levels (1-9 chlorine/biphenyl), and the percent mole was calculated. Homologue profiles, grouped as nine chlorination categories, were compared from the eggs and hatchlings from oil injected eggs. Homologue profiles were not significantly different between eggs and hatchlings from oil injected eggs ($t_{0.05(1),16} = 0$, $p > 0.50$; Figure 4.3). The mole percent of homologues Cl 1 to Cl 4 tended to be higher in eggs than hatchlings, while in homologue Cl 5 to Cl 9 hatchlings were higher than eggs (Figure 4.3). Both egg and hatchlings from oil injected eggs had the highest PCB homologue mole percent at Cl 5 and Cl 6.

Effect of Incubation Temperature

Eggs incubated at high temperature hatched in 70±3.1 days, and eggs incubated at low temperature hatched in 105±2.6 days. The PCB treatment and incubation temperature interacted significantly to affect PCB concentrations in hatchlings ($t_{0.05(1)7}$= 2.83, p<0.05; Figure 4.4). Eggs incubated at high temperature and exposed to high PCB treatment resulted in a decrease in total PCBs in the neonate hatchling. Incubation temperature had no effect on PCB concentration of hatchlings in oil ($t_{0.05(1)6}$= 0.44,

$p>0.05$) and low PCB treatments ($t_{0.05(1)7}= 0.46$, $p>0.05$; Figure 4.4). Hatchlings from oil injected eggs were similar when comparing low and high incubation temperature (Figure 4.5A). Hatchlings from low PCB treatment were also similar when comparing low and high incubation temperature (Figure 4.5B). Low and high incubation temperature resulted in a difference in the congener profile of hatchlings in the high PCB treatment, with a change in 76% of the specific PCB congeners in the hatchling (Figure 4.5C). In the high PCB treatment, concentration of each congener was lower in hatchlings from eggs incubated at high temperature than from eggs incubated at low temperature (Figure 4.5C).

Homologue profiles did not differ among hatchlings in different temperature treatments, regardless of PCB treatment (Figure 4.6A-C). The proportion of homologues Cl 1 to 5 tended to be higher at low temperature than at high temperature, whereas the proportion of homologues Cl 7 to 9 tended to be higher at high temperature than at low temperature regardless of PCB treatment (Figure 4.6). Mean Cl/Bp did not differ between hatchlings from eggs in different temperatures in the oil (low temperature: 5.7, high temperature: 5.8) and low PCB treatments (low temperature: 5.9, high temperature: 5.9). For hatchlings in the high PCB treatment, average Cl/Bp was slightly higher at high temperature (6.2) than at low temperature (5.9).

Table 4.1 Actual values for injected treatment solutions. Mean (SD) total polychlorinated biphenyl (PCB) (ng) mass balanced and average chlorine/biphenyl (CL/Bp) values for snapping turtle eggs and hatchlings exposed to oil, low, and high polychlorinated biphenyl (PCB) treatments (SD).

		Injection Treatment		
	Composite Egg Sample	**Oil (0 ppm) Hatchling**	**Low PCB (1ppm) Hatchling**	**High PCB (10 ppm) Hatchling**
Predicted PCB Injection Solution (ppm)	N.A.	0	1	10
Actual PCB Injection Solution (ppm)	N.A.	0.0256	0.8392	8.9974
N	3	8	9	9
Total PCB (ng)	384.0 (166.2)	430.7 (162.2)	516.7 (158.3)	1044.5 (444.0)
Lipid (%)	3.5 (0.6)	2.9 (0.4)	2.8 (0.3)	2.8 (0.5)
Avg. Cl/bp	5.7 (0.1)	5.8 (0.1)	5.9 (0.2)	6.1 (0.4)

Table 4.2 Average values of polychlorinated biphenyl (PCB) (*n*) mass balanced for hatchlings incubated under the treatments in a split-plot experimental design. Treatments include incubation temperature, incubation moisture, and injection solution. Sample sizes ranged from 1-3.

		Injection Treatment		
Incubation Temperature	**Incubation Moisture**	**Oil (ng)**	**Low PCB (ng)**	**High PCB (ng)**
Low	**L**	527.7 (2)	476.5 (3)	1214.1 (2)
	H	395.1 (4)	602.5 (2)	1514.5 (2)
High	**L**	317.8 (1)	389.7 (2)	654.8 (3)
	H	468.3 (1)	634.8 (2)	921.8 (2)

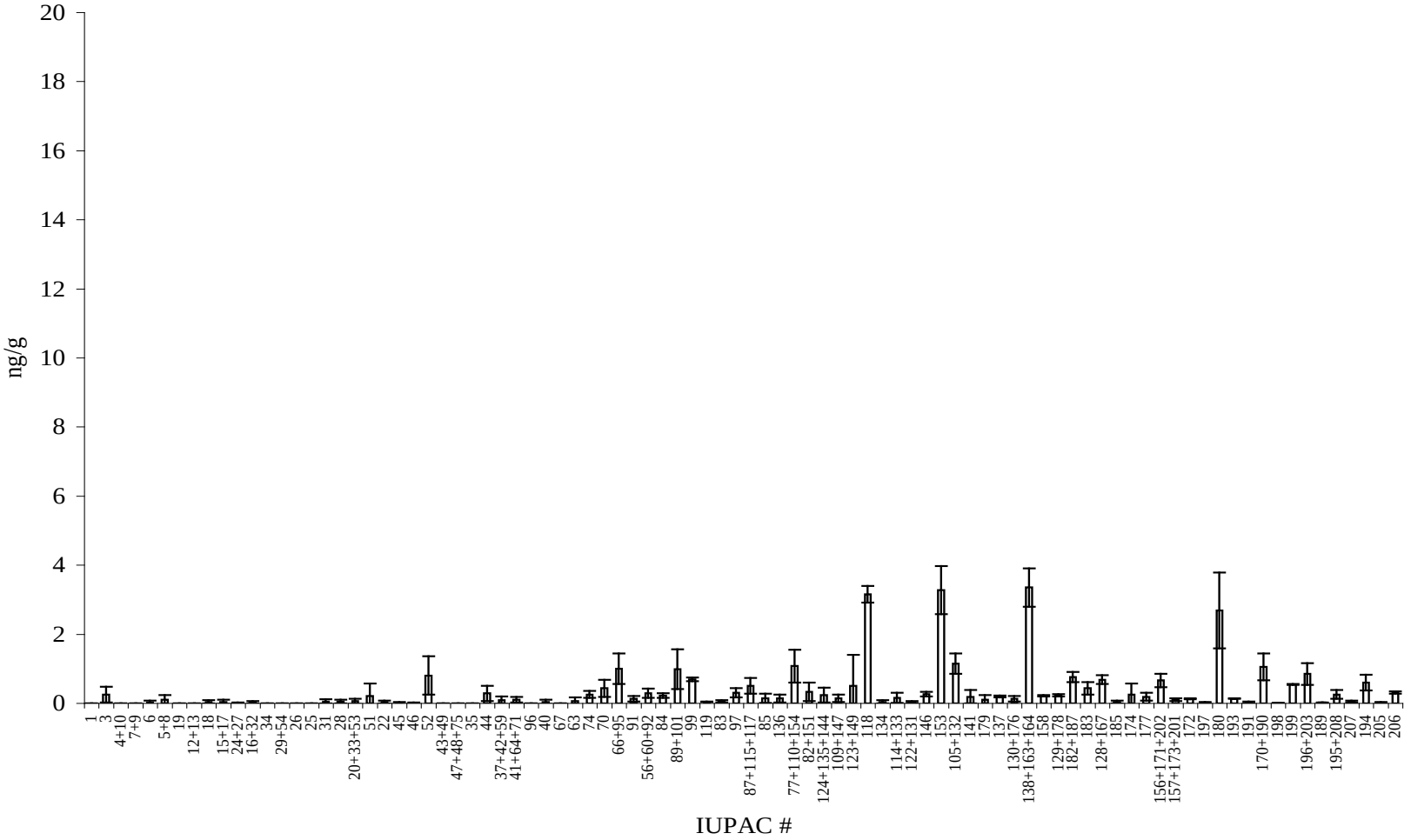

Figure 4.1A Congener specific polychlorinated biphenyl profile by International Union of Pure and Applied Chemistry (IUPAC #) of the mean values (SD) of the egg composite samples. Analysis was performed on whole eggs. The congener levels indicate baseline concentrations prior to treatment.

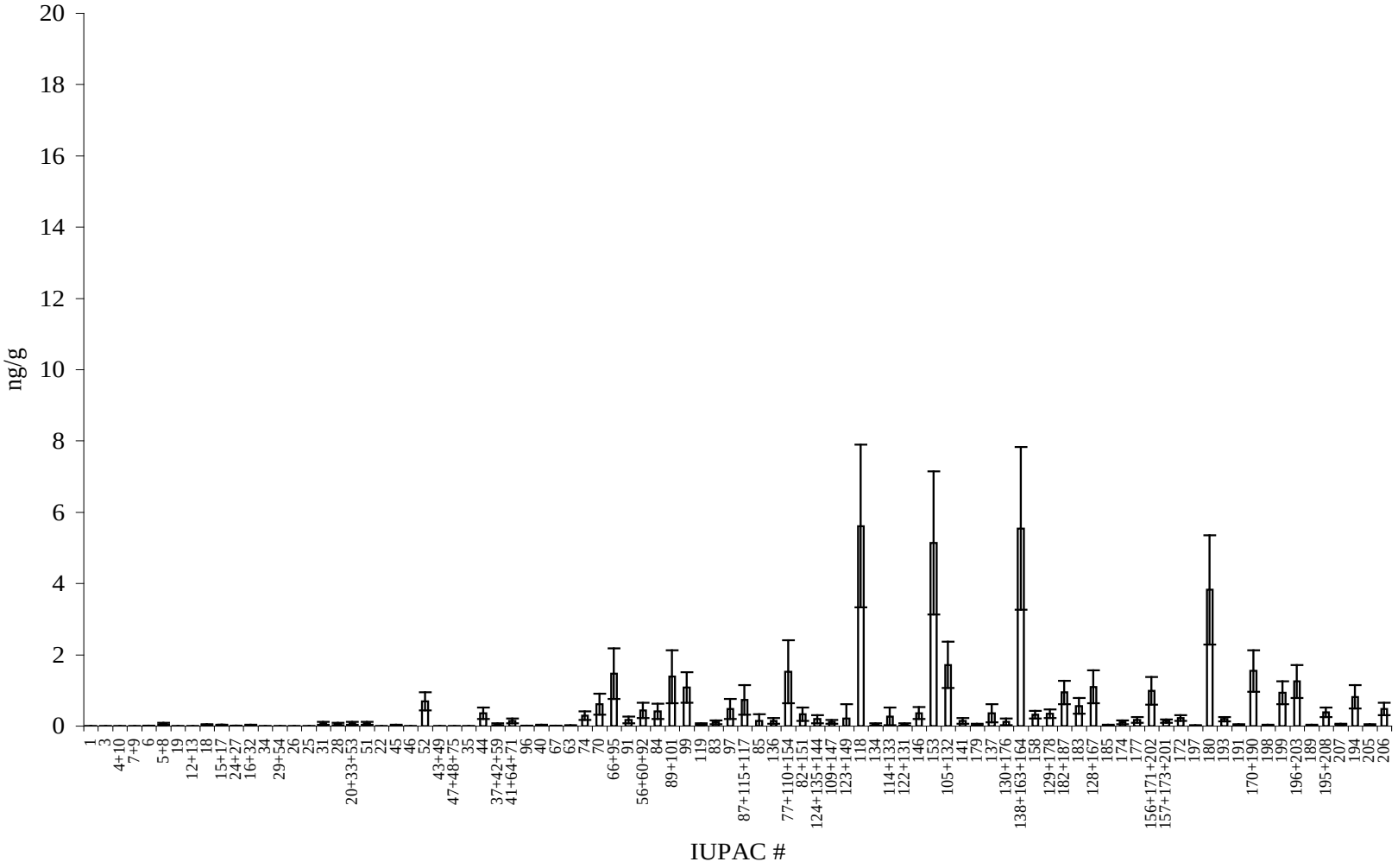

Figure 4.1B Congener specific polychlorinated biphenyl profile by International Union of Pure and Applied Chemistry (IUPAC #) of the mean values (SD) of the hatchlings from oil injected eggs. Analysis performed on whole hatchlings.

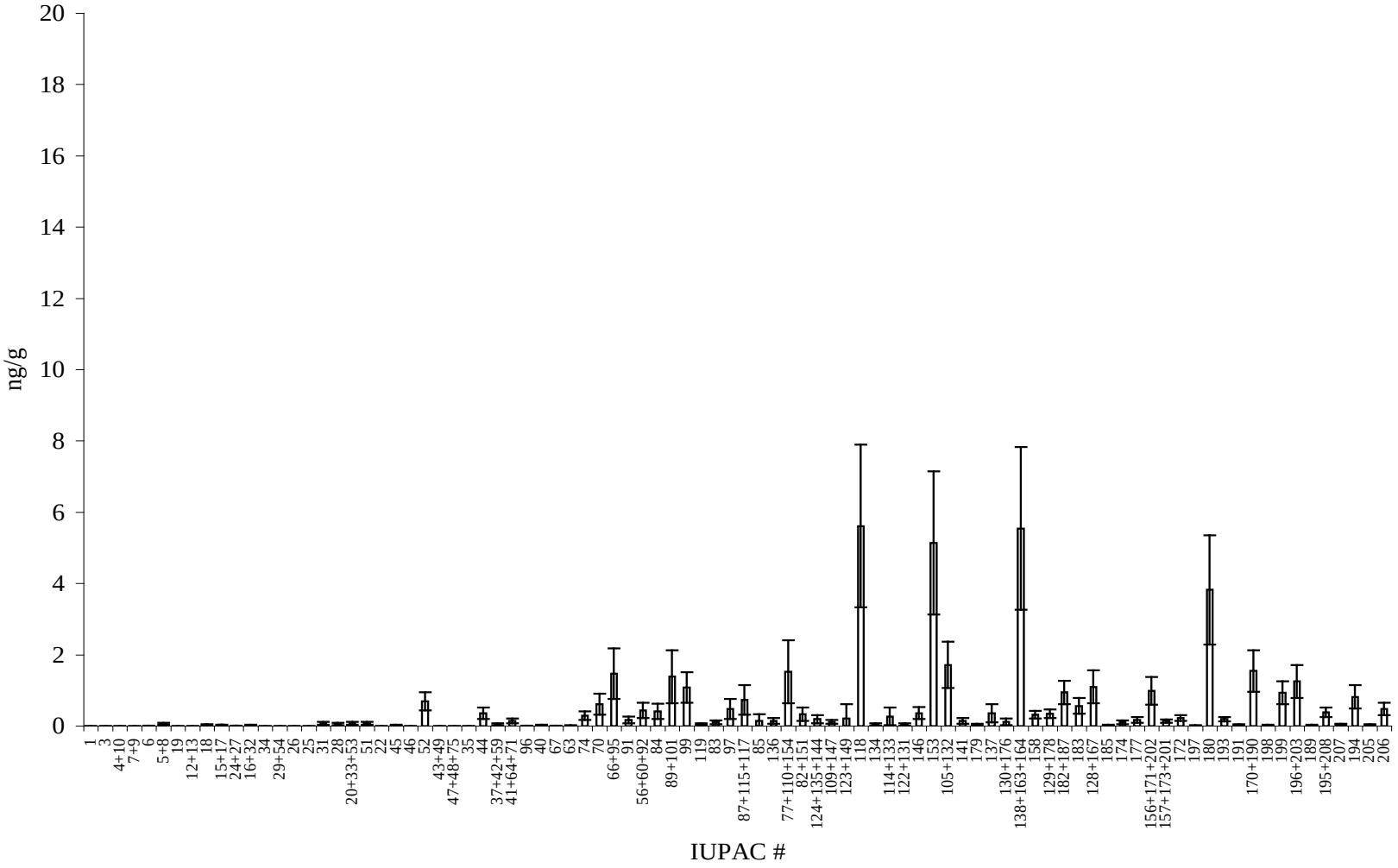

Figure 4.1C Profiles of the mean values (SD) by International Union of Pure and Applied Chemistry (IUPAC #) of low polychlorinated biphenyl treatment hatchlings. Analysis performed on whole hatchlings.

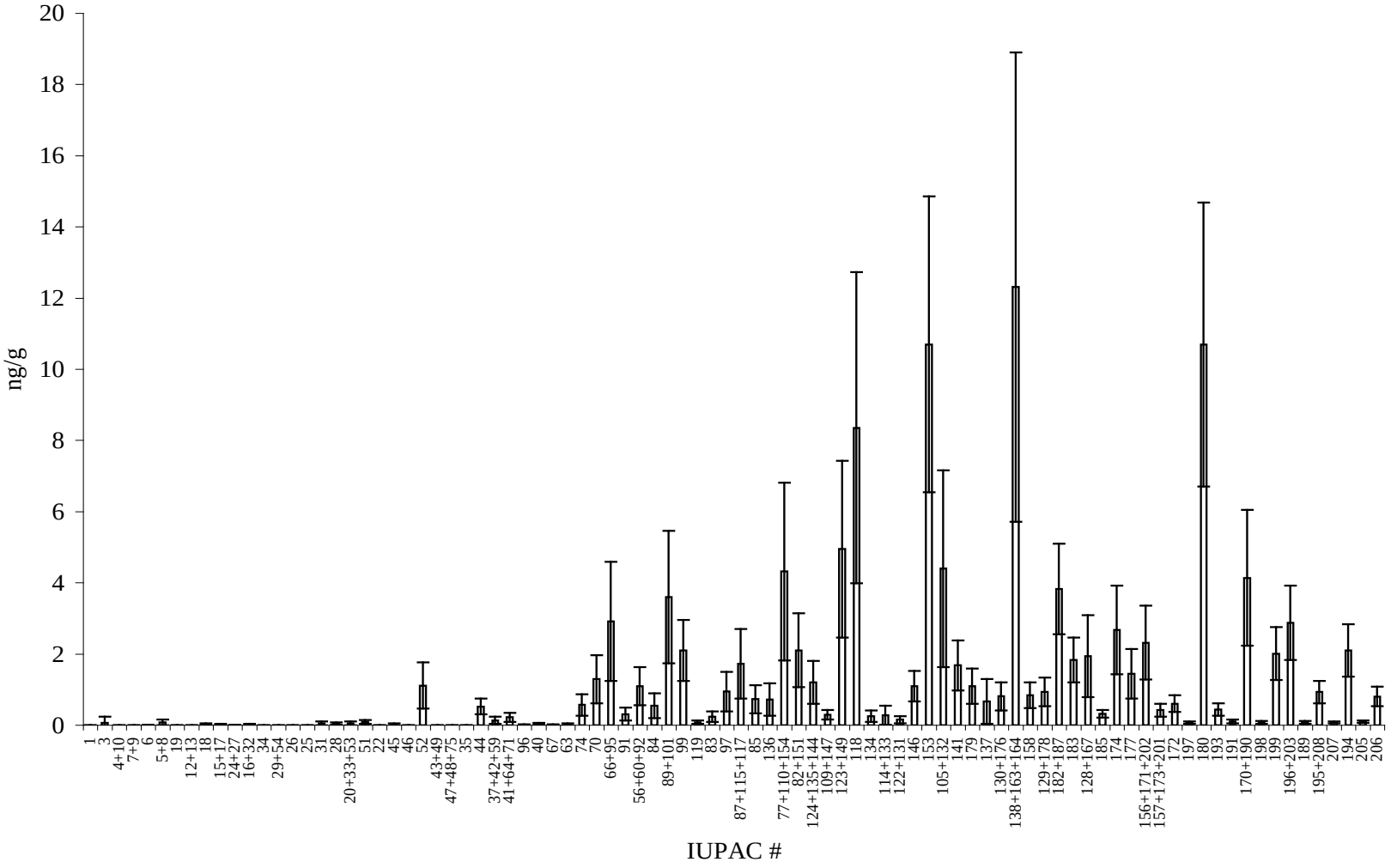

Figure 4.1D Profiles of the mean values (SD) by International Union of Pure and Applied Chemistry (IUPAC #) of high polychlorinated biphenyl treatment hatchling. Analysis performed on whole hatchlings.

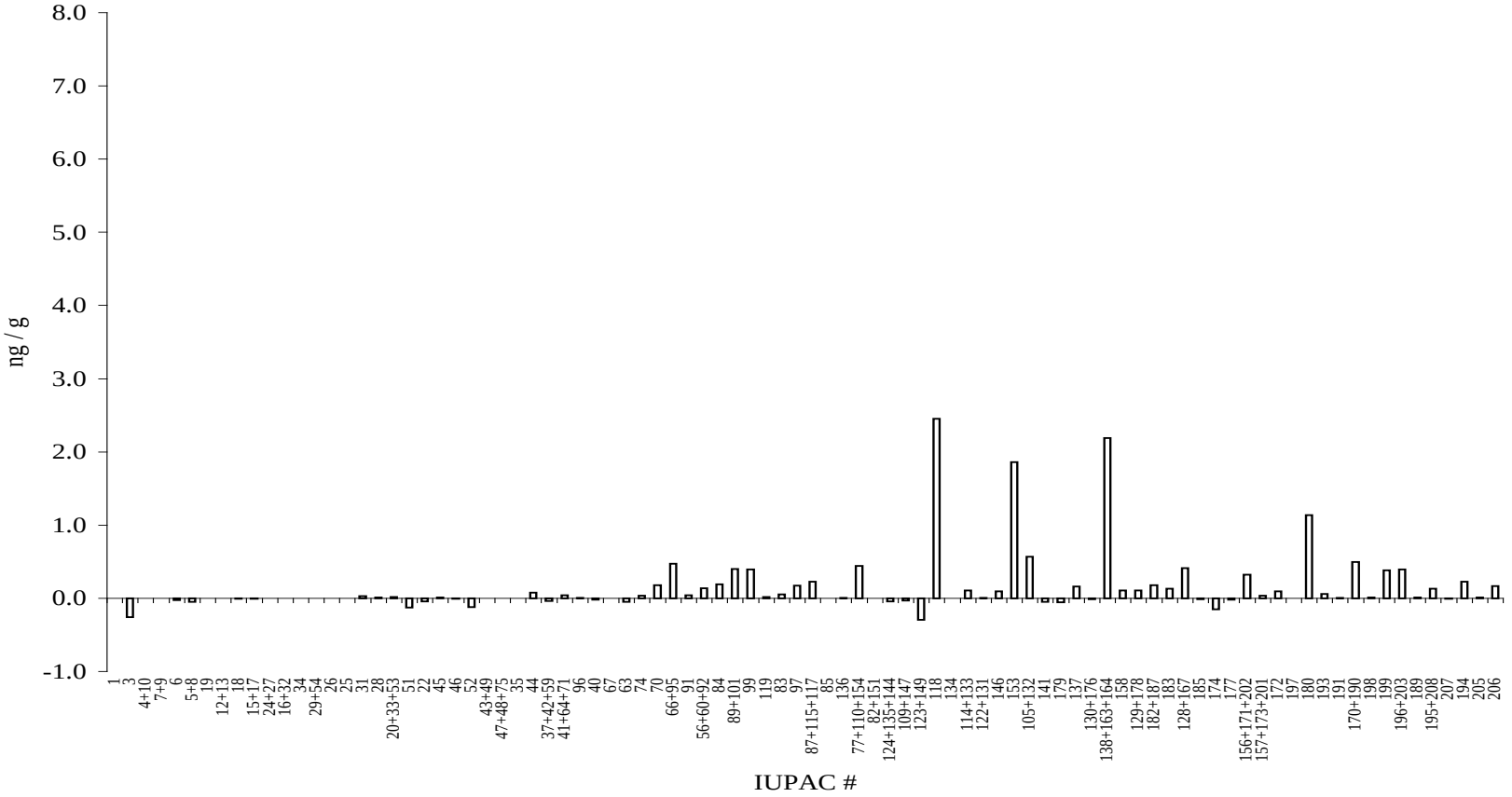

Figure 4.2 Profile of the mean difference between each polychlorinated biphenyl congener by International Union of Pure and Applied Chemistry (IUPAC#) found in egg composite samples and oil treatment hatchlings. Negative values indicate a decrease in the specific congener from egg to neonate hatchling, and positive values indicate an increase. Lower chlorinated congeners have remained the same or slightly decreased, while the higher chlorinated congeners increased.

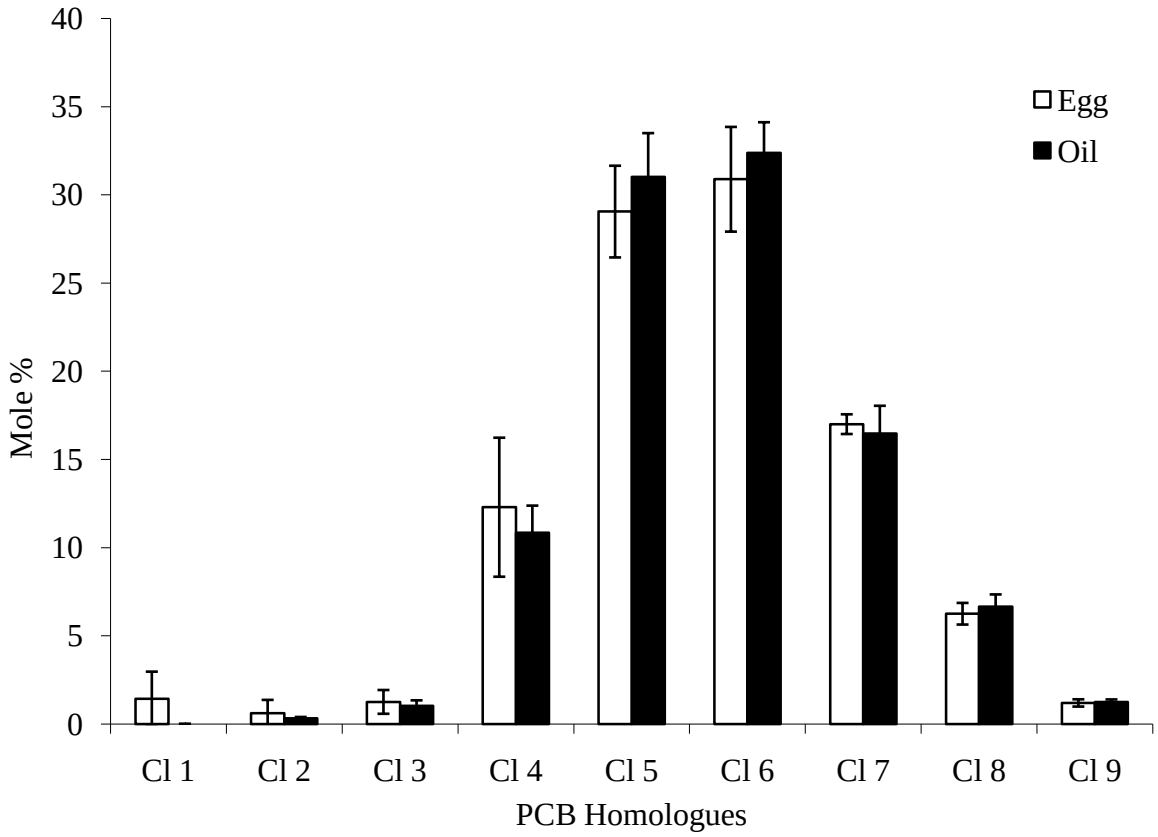

Figure 4.3 Polychlorinated biphenyl homologue mole % comparison of egg and hatchlings from oil injected eggs.

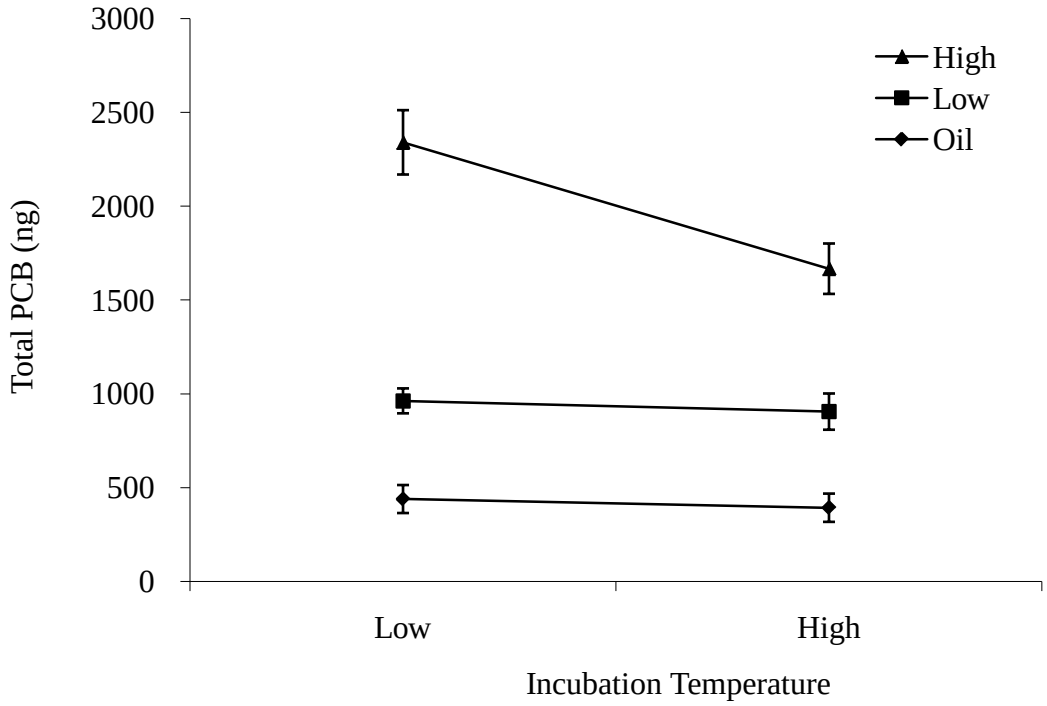

Figure 4.4 Temperature effect on the mass balanced mean total polychlorinated biphenyl (PCB) (ng). Oil and low polychlorinated biphenyl treatment show no interaction with temperature. High polychlorinated biphenyl treatment has a significant interaction when incubated under different temperatures ($t_{0.05(1),7}$ =2.83, p<0.05).

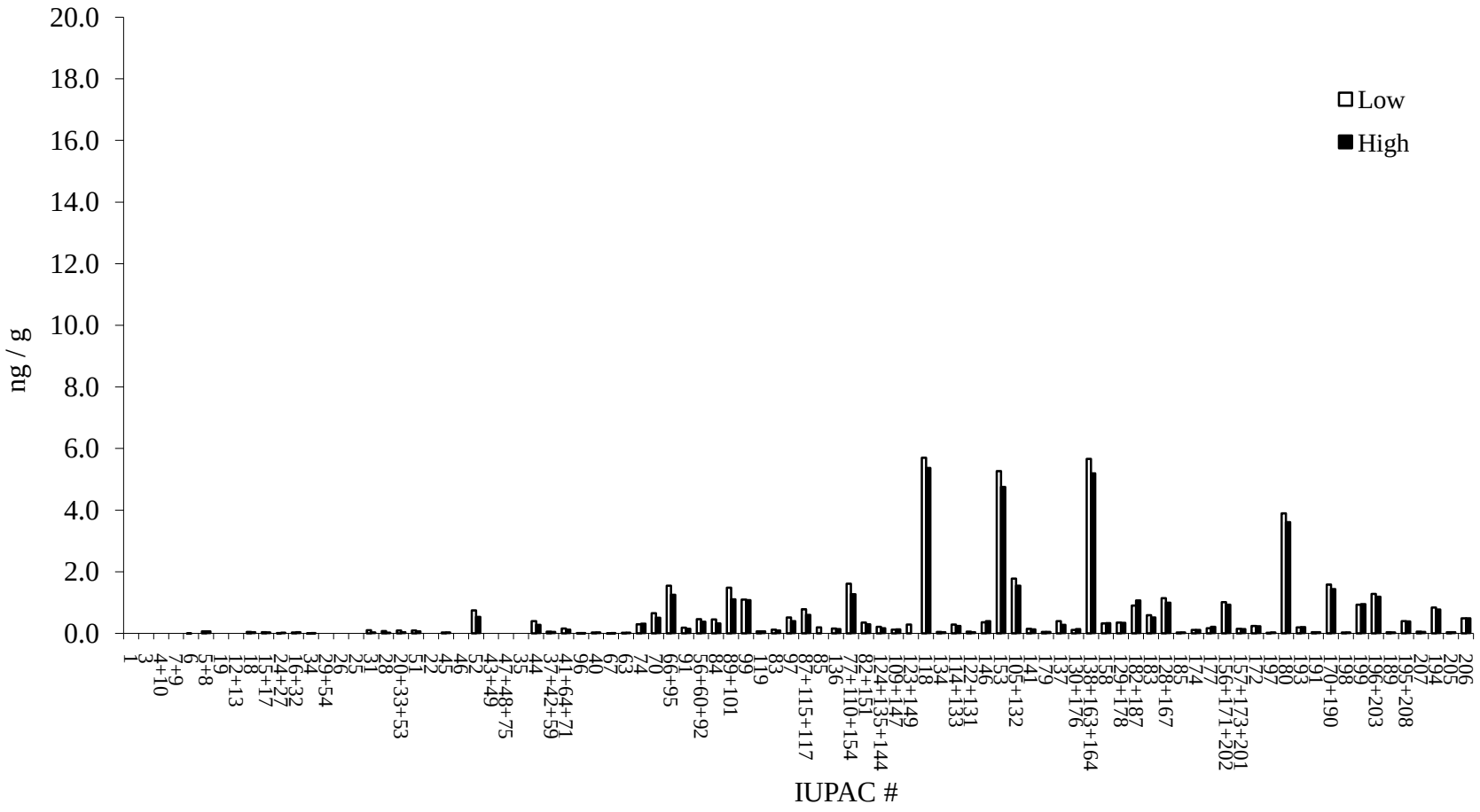

Figure 4.5A Congener specific polychlorinated biphenyl profile of hatchling from oil injected eggs comparing those incubated at low and high temperature. Congeners concentrations (ng/g) are lower when incubated at high temperature compared to those incubated at low temperature.

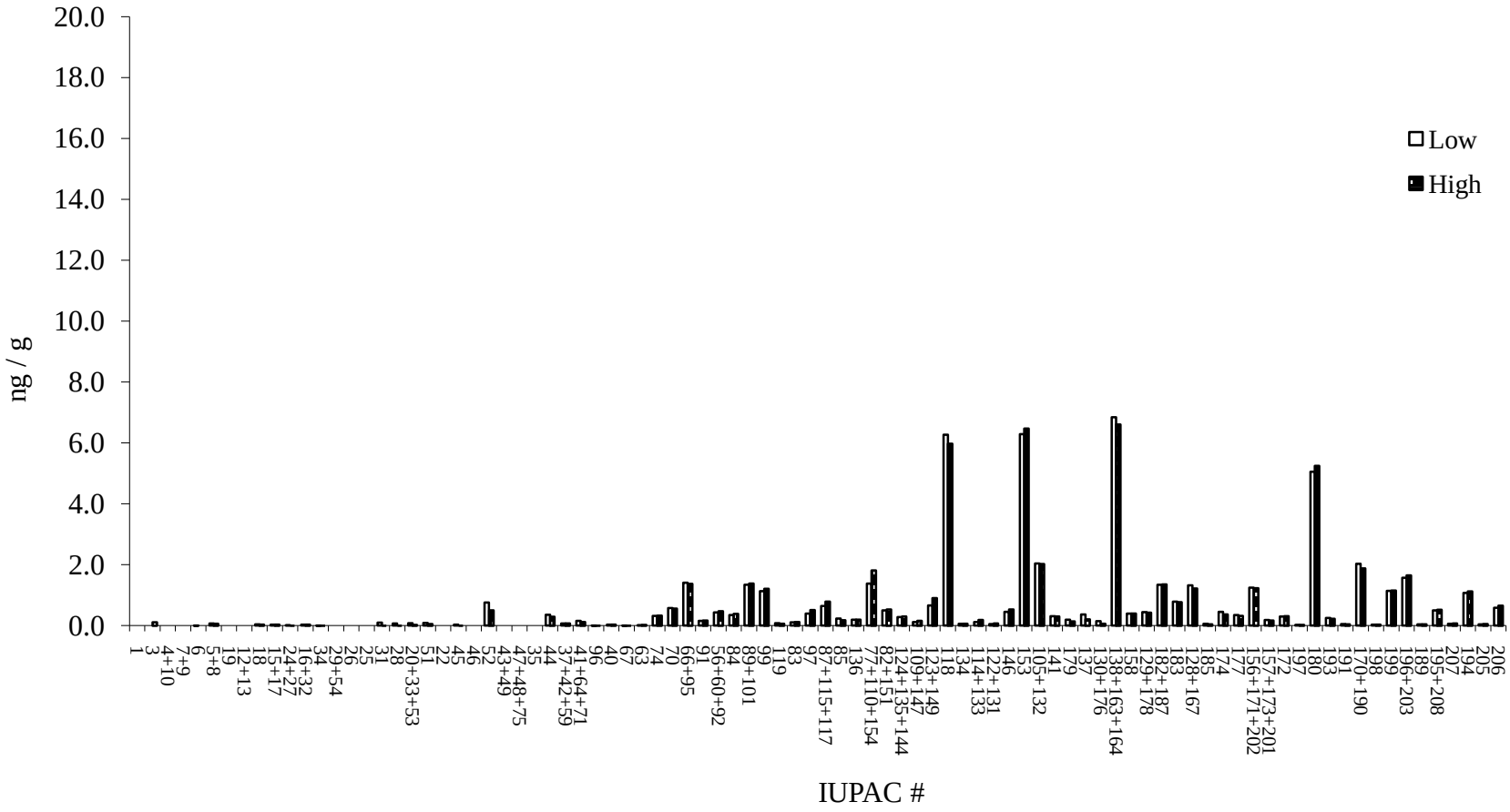

Figure 4.5B Congener specific polychlorinated biphenyl profile of low hatchling treatment comparing those incubated at low and high temperature. Congener concentrations (ng/g) are similar when incubated at high temperature or low temperature

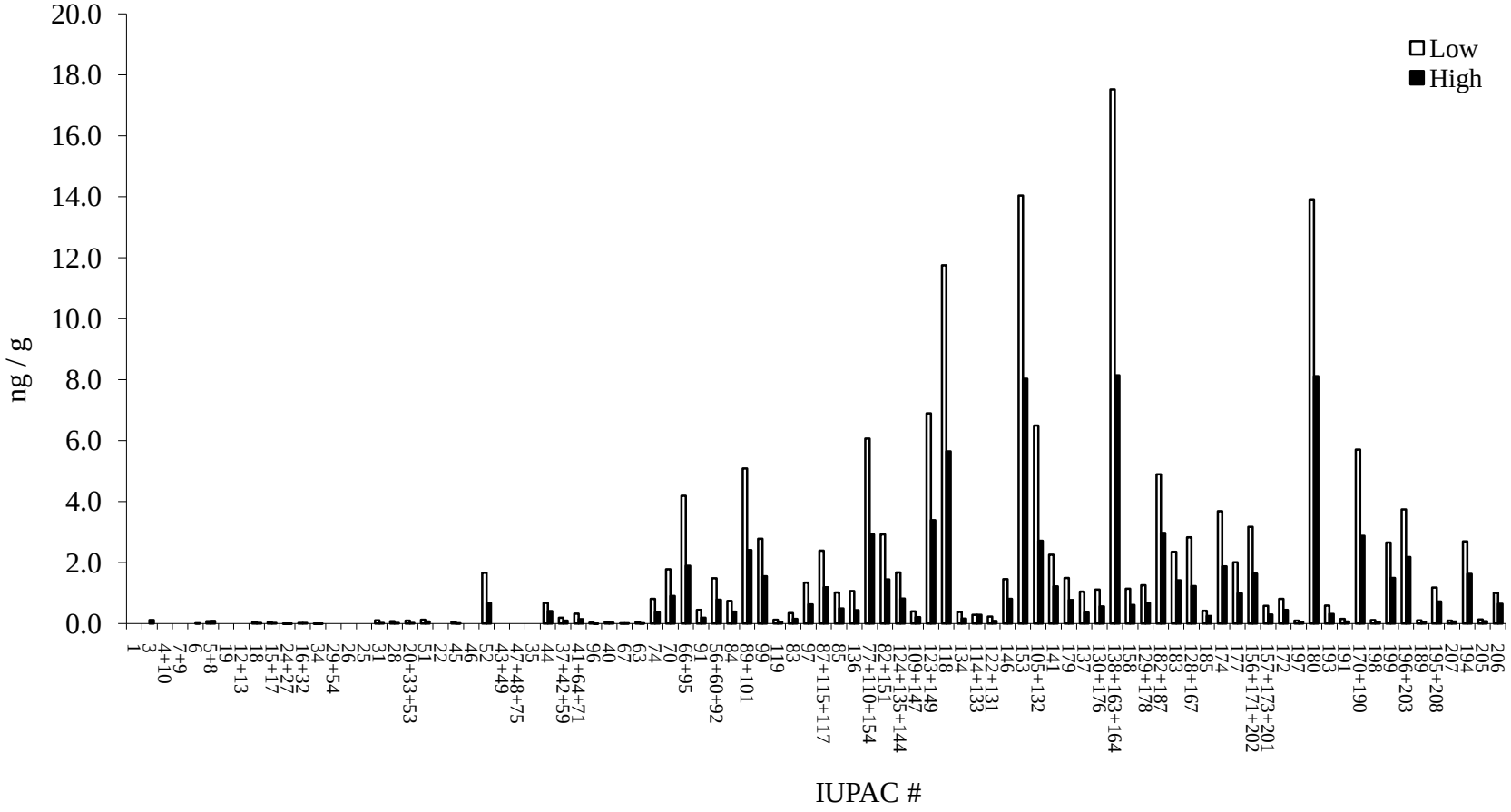

Figure 4.5C Congener specific polychlorinated biphenyl profile of high hatchling treatment comparing those incubated at low and high temperature. Congener concentrations (ng/g) are lower when incubated at high temperature compared to those incubated at low temperature.

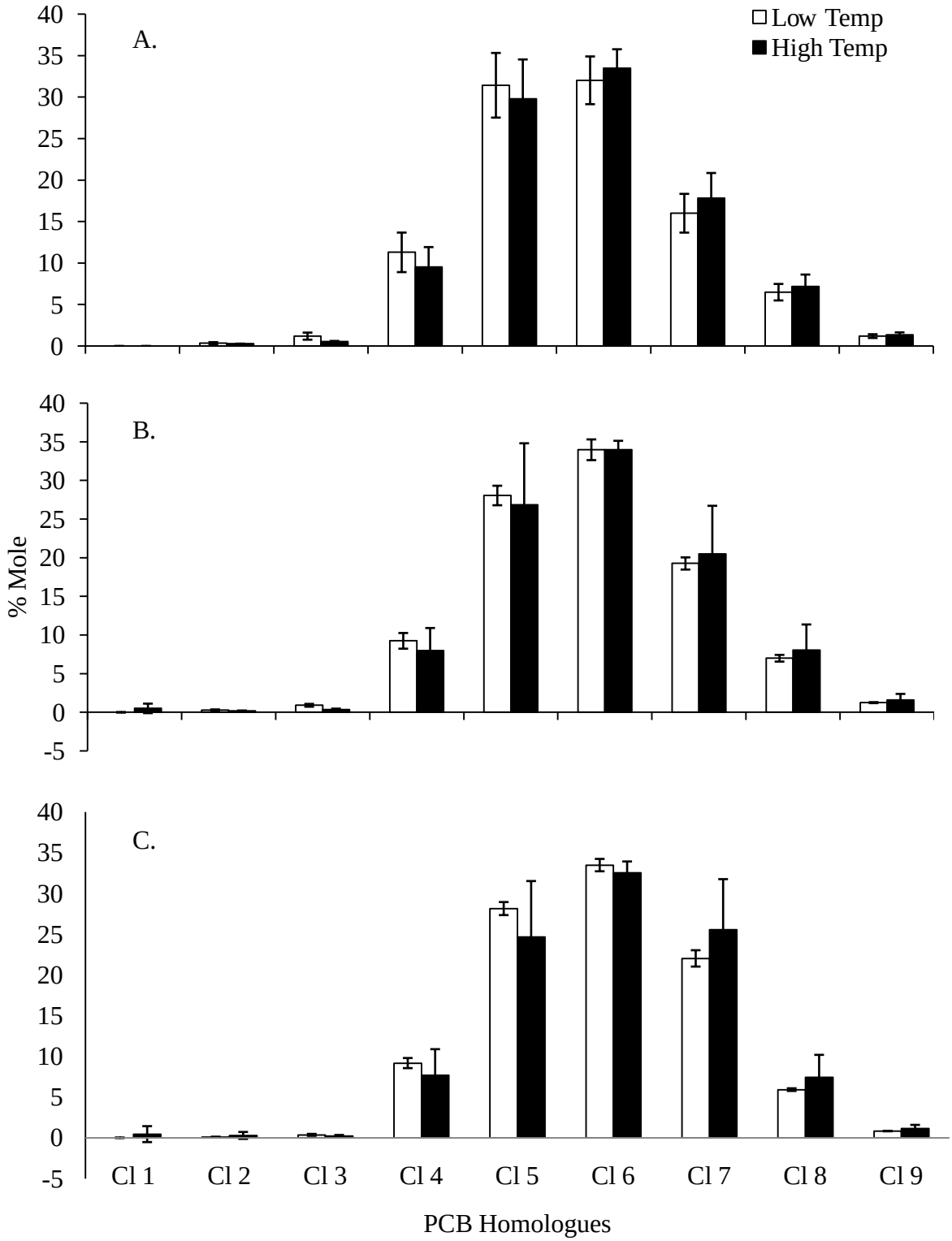

Figure 4.6 Polychlorinated biphenyl (PCB) homologue mole % comparison of high and low incubation temperature for hatchlings exposed to oil (A.) egg injection at a low (B.) or high (C.) PCB concentration.

DISCUSSION

This study addressed how incubation moisture, incubation temperature, and the embryonic development may affect the total and congener specific profile of neonate snapping turtle hatchlings. Although this study (Chapter 3) experienced favorable success for eggs exposed to an injection technique, sample size for specific moisture treatments was low and statistical analyses were unable to be conducted (Table 4.1). The two temperature treatments allowed for a larger sample to be compared and will be addressed in conjunction with the congener and homologue profiles.

Although there was no significance between the mass balanced total PCB in the egg samples and hatchlings from the oil injected eggs, there was a difference between the eggs and the hatchlings from PCB injected eggs, indicating the injected PCB solution was incorporated into the tissues of the developing embryo (Table 4.2). For a maternal contaminant exposure study utilizing the injection technique to be considered successful, not only does the embryo need to survive to the hatchling state, but the contaminants need to be found in the individual. Polychlorinated biphenyls are lipophilic. The percent of lipid tissue did not significantly differ between egg samples and hatchlings from all injection treatments; therefore this would not influence the PCB concentration in tissue samples (Table 4.2).

Total and congener specific PCB concentrations

To my knowledge, this result is the first published indication that changes in the congener-specific pattern of polychlorinated biphenyls (PCBs) could occur during

embryonic turtle development. Congener profiles differed when comparing the egg, oil, low PCB, and high PCB treatments (Figure 4.1A-D). Congener profiles changed during incubation (egg vs. oil treatment), such that concentrations of more than half of the congeners (53%) increased during incubation (Figure 4.1A-B). Polychlorinated biphenyl profiles of eggs peaked at highly chlorinated congeners 118, 153, 138+163+164, and 180. Dabrowska et al. (2006) observed a similar elevated pattern specific to these congeners in adult snapping turtles sampled in the Ohio Basin of Lake Erie. Profiles of hatchlings from oil injected eggs peaked at these same congeners, although concentrations were higher compared to the profile of the egg, even though eggs had not been injected. These congeners that increased from egg to hatchling, in the oil treatment, might be difficult to metabolize, or turtle tissues might have a high affinity for these congeners. The congener-specific differences in eggs and hatchlings from oil injected eggs show an increase in the higher chlorinated congeners and a decrease in the lower chlorinated congeners (Figure 4.2). Embryonic metabolism during incubation might break down lower chlorinated congeners, possibly liberating chlorines allowing the assemblage of higher chlorinated congeners. High chlorinated congeners are not easily metabolized (Bryan et al., 1987a), therefore they would store themselves in the lipophilic embryonic tissues.

The PCB homologue pattern between the egg and hatchlings from oil injected eggs was not significantly different (Figure 4.3). Eggs tended to have higher concentrations of homologues Cl 1-4 compared to the hatchlings from oil injected eggs, whereas hatchlings from oil injected eggs had higher concentrations of Cl 5-9 compared

to the egg. This result agrees with the congener-specific pattern of the eggs having more low-chlorinated congeners than the hatchlings (Figure 4.1A-B). Although not significant, the average Cl/bp was also lower in eggs than hatchlings from oil injected eggs (Table 4.2). Overall, eggs were enriched with Cl 5 and 6. This is similar to other studies that found turtle eggs with elevated levels of Cl 6 (Pagano et al., 1999; Dabrowska et al., 2006). Pagano et al. (1999) also determined the chlorination level (Cl/Bp) in maternal adipose tissue was higher than in the egg.

Differences in congener profiles, levels of chlorination among maternal adipose tissue, eggs, and hatchlings give strong indications that the changes in total PCB and chlorination levels might be the result of toxicokinetics or metabolic activity. In this study, congeners with low IUPAC numbers decreased during incubation, whereas concentrations of congeners with higher IUPAC numbers increased (Figure 4.2).

Effect of Incubation Temperature

It was hypothesized that temperature would increase embryonic metabolic rate thereby decreasing the total PCB concentration in the neonate hatchling. This hypothesis was partially supported. Polychlorinated biphenyl concentrations were significantly lower in hatchlings from eggs incubated at high than low temperature but only in the high PCB treatment. This result suggests that by elevating incubation temperatures, embryonic metabolism increases and results in a greater reduction of total PCBs. By increasing incubation temperature, the mean number of days to hatch decreased by 35 days and there was a greater decrease in total PCBs and in most congeners. The effect of

decreasing the temperature also supports the idea that the rate of metabolism is having a direct effect on the hatchling's PCB profile.

In the oil and low PCB treatments, hatchlings from eggs incubated at high temperatures have elevated levels of Cl 7-9 and similar average Cl/bp. In the high PCB treatment hatchlings incubated at high temperature have elevated levels of Cl 6-9 and the average chlorine/biphenyl is greater in the high temperature treatment. This result coincides with previous conclusions that as hatchlings metabolize lower chlorinated congeners, high chlorinated congeners increase.

The toxicokinetics of PCBs have not been investigated in the common snapping turtle. In this study I have examined the effect of PCB load and temperature on congener-specific PCB profiles and chlorination patterns to address how PCBs might be altered during embryonic development. My finding that specific congeners with elevated chlorination are higher in the neonate hatchling than in eggs raises concern about using eggs as a bioindicator of PCB contamination. Pagano et al. (1999) found that PCB chlorination levels were higher in maternal adipose and liver tissue than in eggs. Therefore, eggs might give a conservative estimate of levels of site contamination and might not signal detrimental effects to individual turtles or the population.

Chapter 5

Effects of PCBs on Carbon Dioxide Production in Neonate Snapping Turtle (*Chelydra serpentina serpentina*) Hatchlings

INTRODUCTION

Female snapping turtle reproduce annually, such that during egg production, organochloride contaminants are passed to their offspring through the food they consume during that time and by the mobilization of contaminants stored in their tissues (primarily lipid tissue)(Bishop et al., 1994). At the time of deposition in the nest, the egg yolk contains over 95% of the total PCB contaminants in the egg (Bryan et al., 1987a). During embryogenesis, organochlorine compounds move continually from the yolk to embryo and the increase in total PCBs follow the trend of an increase in body mass (Bishop et al., 1995). Embryonic organochlorine levels were found to peak several days prior to hatching during the incubation of several species of freshwater and sea turtles' nests (Bishop et al., 1995; Clark and Krynitsky, 1985), most likely due to absorption of the yolk sac. Levels of PCBs in snapping turtle hatchlings decreased 18 d post-hatch (Bishop et al., 1995). This decrease in PCB levels is probably a function of metabolism of this mass of energy needed within the first days of leaving the nest.

Sea turtle hatchlings enter what is termed a "frenzy" period after they emerge from the egg, which is a result of absorption of the yolk, giving the hatchling a period of high energy that aid them in evading predators until a safe feeding habitat is reached (Lutz and Musick, 1997). Freshwater turtle hatchlings also absorb their energy-rich yolk sac immediately prior to hatching giving them energy to leave the nest. Since this energy

is utilized rapidly, it may result in a quick metabolism of the yolk; hatchling PCB levels should subsequently decrease during this time. Owen and Wells (1976) confirmed the metabolic excretion of DDT compounds in red-eared sliders (*Trachemys scripta*) and midland painted turtles (*Chrysemys picta*). Not all congeners are capable of being metabolized. The chemical and toxicological properties of individual PCB congeners differ. Therefore, it is important to consider how metabolism in the organism affects the accumulation and transfer of specific congeners (Pagano et al., 1999). This study will further investigate embryonic metabolism of PCBs during incubation by comparing the carbon dioxide production of hatchlings during respiration.

Few studies have examined respiration in hatchlings, which indicates metabolic rate. Contaminants, such as PCBs can result in hyperthyroidism thereby influencing metabolic rates, and increasing growth rate leading to early maturation (Sparling et al., 2000; Willingham, 2001). Although an increased growth rate may also increase survivorship, the effects of the high contaminant levels these individuals carry could result in an increase in defects in subsequent generations. A recent study examined the ventilation pattern and response to CO_2 in respiration of sea turtle hatchlings (Price et al., 2007), but I am unaware of any studies investigating the effect of neonate freshwater turtle hatchlings respiration in response to their contaminant loads. The objective of my study was to examine the effects of CO_2 production in neonate snapping turtle hatchlings that were exposed to PCBs during embryogenesis. I also examined how incubation temperature and moisture, a proxy for habitat variation, might influence CO_2 production in the neonate hatchling.

METHODS

Egg Collection and Treatment

Eggs were collected from necropsied females in June 2005 and incubated under two temperature treatments and two moisture treatments until hatch (Chapter 1). During June 2006, eggs were collected directly from the nest cavity after females had completed laying their eggs at Siegel Marsh. Eggs were brought back to the lab and injected with oil or a low or high PCB solution following the same protocol as the 2005 season (Chapter 1). Eggs were incubated at low or high temperature, and all nest boxes maintained the same moisture level (1:1 vermiculite to water [v/v]) until hatch. To minimize the number of treatments, thereby allowing for a greater sample size, moisture was not a treatment during the 2006 season. The 2005 season had three viable clutches and the 2006 season had nine viable clutches. Upon hatch, hatchlings were placed into individual labeled containers.

Respirometry of Neonate Hatchlings

Several days prior to hatching, embryos absorb their yolk sac, rich in high-energy lipids, to survive the first days until they reach a water source and feeding habitat. To determine the maternal PCB effects on metabolic oxidation of the yolk sac, hatchling respiration was measured using the Qubit Systems Inc. Infrared Gas Analyzer (IRGA) (www.qubitsystems.com). Hatchlings were tested within 24 hours post emergence from the egg. Each hatchling was placed in a cylindrical respirometer chamber and covered to

exclude any light and ensure the animal was in a resting state. Previous observations of hatchlings showed that snapping turtle hatchlings remained stationary in a dark arena (Schnars, personal observation). There was air flow entering and exiting at each end of the chamber. The source of the air pumped into the chamber was from a 30 L air bag (Qubit Systems Inc.) filled with ambient air prior to testing. This ensured a constant concentration of O_2 and CO_2 being pumped into the animal chamber. Air flow into the chamber was controlled at a set rate of 400 mL/min. Ambient temperature and CO_2 production were monitored for a minimum of 2 min until they stabilized prior to the animal being placed in the chamber. Once stable, the animal was placed in the chamber and ambient temperature and CO_2 production were monitored for a minimum of 10 min and until increases in CO_2 leveled off. Once the CO_2 production had leveled off, the hatchling was removed from the chamber and weighed. Prior to testing the next hatchling, the respirometry apparatus was flushed out by continuing to run for a minimum of 20 min. The respirometer was calibrated daily to ensure precise readings. The CO_2 readings were normalized by hatchling weight and reported in mL g^{-1} hr^{-1}.

During each of the 2005 and 2006 seasons, 29 hatchlings were measured in the respirometer. Of the 29 respirations in hatchlings measured during the 2005 season, eight hatchlings were not measured for PCBs (ng). Not all hatchlings were able to be analyzed for PCB content due to available funding. Although a total of 26 hatchlings were analyzed for total PCB (ng), only 21 of those hatchlings produced successful results when tested in the respirometer. Respirometry measurements resulting in a stabilization or

decrease in CO_2 were excluded from further analysis due to an error in the CO_2 detection by the equipment.

Statistical Analysis

Changes in CO_2 production were tested for univariate normal distributions (SAS version 9.1, SAS Institute, Cary NC). The data were not normally distributed ($p<0.05$) and slightly leptokurtotic. Given the non-normal distribution, CO_2 production, as well as all independent variables in the experimental design was tested for multivariate normality. The data were determined to be multivariate normal, thus *a posteri* multiple regression techniques were used for further analysis. The model (CO_2 = PCB treatment, neonate weight, incubation temperature, ambient temperature during respirometry) was analyzed using stepwise regression to determine which independent variables explained most of variation seen in CO_2 production (SAS version 9.1, SAS Institute, Cary NC). Principle Component Analysis (PCA) clustering by PCB treatment and incubation temperature was run to examine possible grouping due to maternal female, PCB treatment, incubation temperature, incubation moisture, hatchling weight, PCB concentration of the hatchling, and CO_2 production, (SAS version 9.1, SAS Institute, Cary NC).

RESULTS

Mean hatchling weight was 9.4±0.09 g and 9.5±0.21 g for the 2005 and 2006 seasons, respectively. Hatchlings incubated at high temperature had a significantly

higher mean weight (9.61±0.09 g) compared to hatchlings incubated at low temperature (9.27±0.13 g) ($t_{0.05(1),27} = -2.29$, $p < 0.05$) in the 2005 season.

Analysis of the variables in the model using a stepwise regression, resulted in ambient temperature being significant but none of the other variables (CO_2 = PCB treatment ($F_{0.05(1),4,54}=0.50$, $p>0.05$), neonate weight ($F_{0.05(1),4,54}=1.45$, $p>0.05$), incubation temperature ($F_{0.05(1),4,54}=1.70$, $p>0.05$), and ambient temperature ($F_{0.05(1),4,54}=9.21$, $p<0.05$)) were significant. A partial regression with a residual plot indicated that as the concentration of the PCB treatment increased, the variance decreased. Examination of the regression model residuals revealed a pattern of decreasing variation with increasing PCB load; the coefficient of variation was used to examine this pattern further (Figure 5.1). The mean ambient temperature during respirometry was higher in 2006 (25.69±0.12 °C) compared to the 2005 (20.84±0.14 °C) season. Thus the two seasons were analyzed separately in further analysis.

The 2005 season included results of PCB analysis of eggs and hatchlings, and CO_2 production (Table 5.1). The difference in PCB (ng) was calculated by subtracting the hatchling PCB (ng) from the egg PCB (ng). The values from the composite egg samples (prior to PCB injection) were used as the value for the egg PCB (ng), therefore the egg PCB (ng) (Table 5.1) from the same maternal female would have identical values. All PCB treatments were included, therefore those injected with known amounts of PCB (low=1 ppm, high=10 ppm) would result in a positive value when calculating the difference. When examining the results from hatchlings with PCB and respiration data (N=21), the mean CO_2 (mL g^{-1} min^{-1}) production did not significantly differ between the

three injection treatments ($F_{0.05(1),2,18}$=2.26, p>0.05) (Figure 5.2) In comparing the difference between mass balanced hatchling PCB and mass balanced egg PCB (ng) and CO_2 (mL g^{-1} min^{-1}) production, there was a high amount of variance in hatchlings that had a low PCB (ng) level (Figure 5.3). Hatchlings with a higher PCB (ng) level produced less CO_2 and there was less variance (R^2=0.074).

A Principle Component Analysis (PCA) was performed incorporating maternal female, incubation temperature, incubation moisture, hatchling weight, PCB concentration of the hatchling, and CO_2 production of the hatchling to determine if these variables were influenced by injection treatment (Figure 5.4). Groupings by the injection treatment separated out when the x-axis was anchored by incubation moisture/hatchling weight/CO_2 production on one end and maternal female on the other end. The y-axis was anchored by CO_2 production on one end and hatchling weight/incubation temperature on the other end. Three clusters formed in respect to oil (0), low PCB (1), and high PCB (2) injection treatment. The oil treatment cluster was in the center of the graph, indicating no influence from those variables used as anchors on each axis (Figure 5.4).

Another PCA was performed incorporating maternal female, PCB treatment, incubation moisture, hatchling weight, PCB concentration of the hatchling, and CO_2 production of the hatchling to determine if these variables were influenced by incubation temperature (Figure 5.5). The PCB treatment separated out when the x-axis was anchored by maternal female/hatchling weight/ on one end and PCB treatment/PCB concentration of the hatchling on the other end. The y-axis was anchored by maternal female/PCB treatment on one end and CO_2 production/hatchling weight on the other end.

The low incubation temperature cluster was widespread, while the high incubation temperature cluster was more localized indicating a possible influence or interaction of high incubation temperature with the anchors found on the negative x-axis and negative y-axis (Figure 5.5).

The 2006 season included the CO_2 production and PCB treatment data. Mean CO_2 (mL g^{-1} min^{-1}) production did not significantly differ between PCB treatments ($F_{0.05(1),2,26}$=0.44, p>0.05) (Figure 5.6).

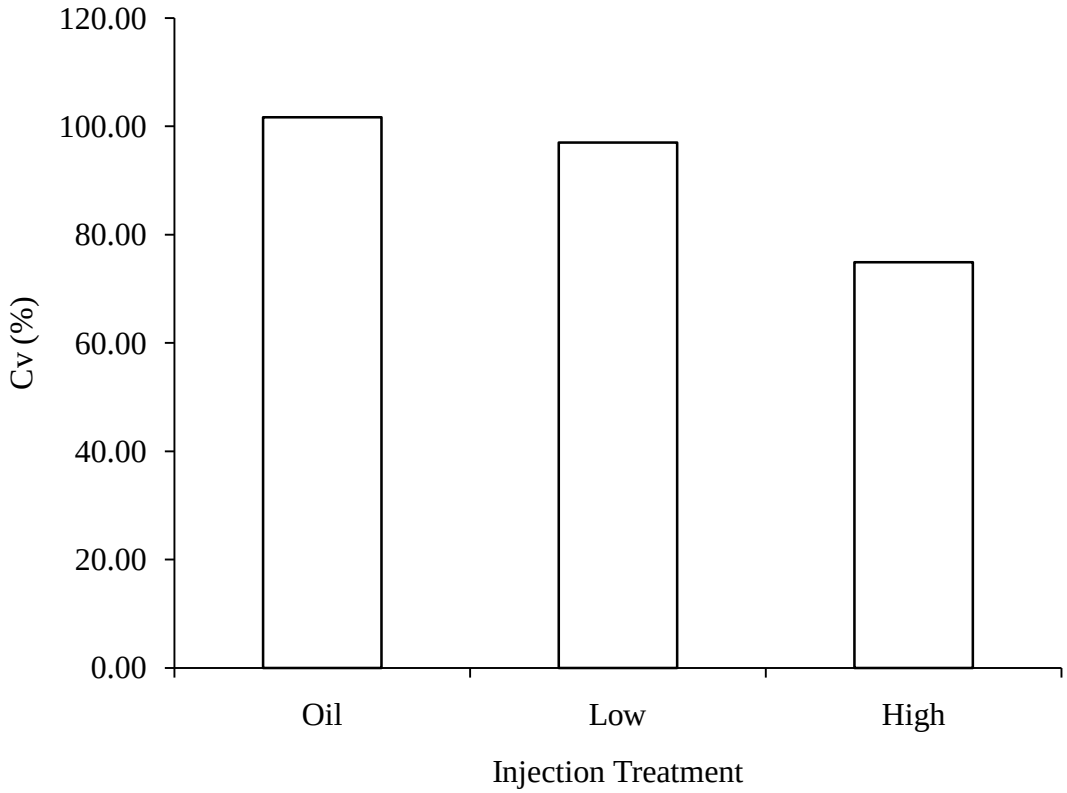

Figure 5.1 The coefficient of variation (%) of CO_2 (ml g^{-1} min^{-1}) for each injection treatment. As PCB concentration increased the variance decreased.

Table 5.1 Results from 2005 season identifying polychlorinated biphenyl (PCB) treatment, mass balance of PCB concentration in the egg prior to injection, mass balance of PCB concentration in neonate hatchling, and CO_2 produced in the respirometer. Hatchling PCB (ng) that was not included in sample analysis and respirometry testing that resulted in error is noted as not being available (N.A.).

Hatchling	Injection Treatment	Egg PCB (ng)	Hatchling PCB (ng)	Difference in PCB (ng)	CO_2 (ml g^{-1} min^{-1})
1	O	298.0	171.4	-126.6	0.0288
2	O	278.4	317.8	39.4	0.0411
3	O	298.0	513.9	215.9	0.0467
4	O	278.4	N.A.	N.A.	0.0589
5	O	575.5	709.5	134.0	0.0708
6	O	298.0	N.A.	N.A.	0.0818
7	O	278.4	345.8	67.4	0.1058
8	O	278.4	N.A.	N.A.	0.1207
9	O	278.4	384.2	105.8	0.1292
10	O	575.5	511.0	-64.5	0.2349
11	O	298.0	468.3	170.3	N.A.
12	L	298.0	N.A.	N.A.	0.0424
13	L	298.0	513.3	215.3	0.0416
14	L	298.0	N.A.	N.A.	0.0905
15	L	278.4	469.2	190.8	0.1036
16	L	298.0	418.4	120.4	0.0984
17	L	298.0	495.4	197.4	0.1269
18	L	575.5	N.A.	N.A.	0.1678
19	L	575.5	552.6	-22.9	0.1683
20	L	575.5	786.6	211.1	0.1851
21	L	575.5	N.A.	N.A.	0.2452
22	L	575.5	756.2	180.7	0.2470
23	L	575.5	284.0	-291.5	N.A.
24	L	278.4	407.7	129.3	N.A.
25	H	278.4	465	186.6	0.0230
26	H	278.4	699.5	421.1	0.0274
27	H	575.5	1625.3	1049.8	0.0561
28	H	278.4	874	595.6	0.0731
29	H	298.0	1457.7	1159.7	0.0682
30	H	575.5	1554.1	978.6	0.0856
31	H	298.0	502.2	204.2	0.1512
32	H	278.4	N.A.	N.A.	0.1734
33	H	278.4	997.3	718.9	N.A.
34	H	575.5	1144.0	568.5	N.A.

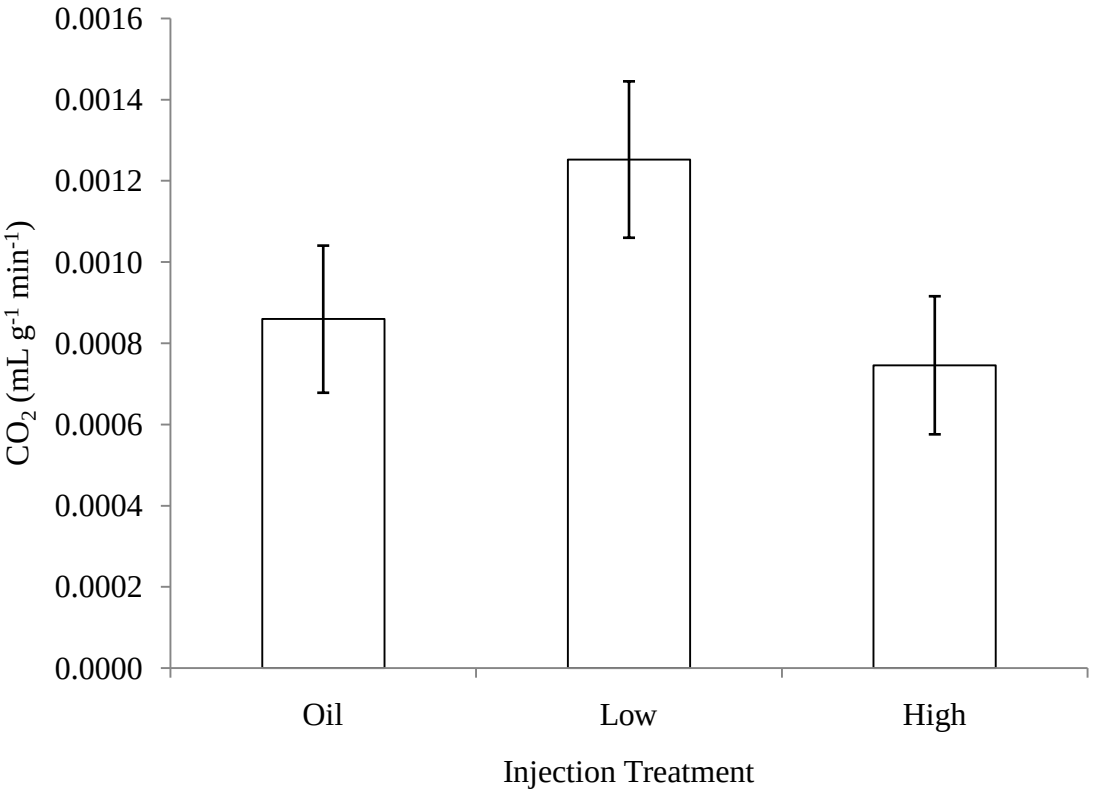

Figure 5.2 Mean CO_2 production of neonate hatchlings by polychlorinated biphenyl (PCB) treatment in the 2005 season. The CO_2 production between PCB treatments did not significantly differ ($F_{0.05(1),2,18} = 2.26$, $p>0.05$).

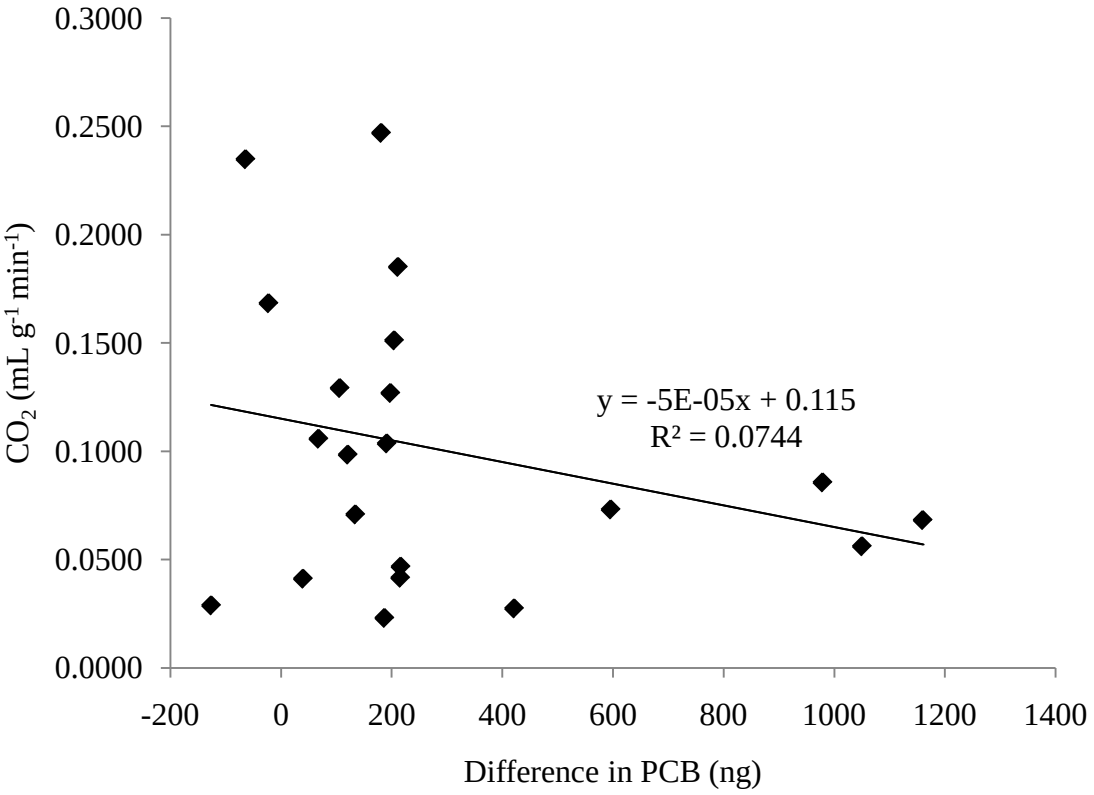

Figure 5.3 A comparison of the difference between the mass balanced hatchling polychlorinated biphenyl (PCB) (ng) and the mass balanced egg PCB (ng) and the CO_2 produced by the neonate hatchling. Hatchlings from all injection treatments are included. The trendline indicates that as the mass balanced PCB (ng) in the hatchling is elevated, CO_2 production decreases.

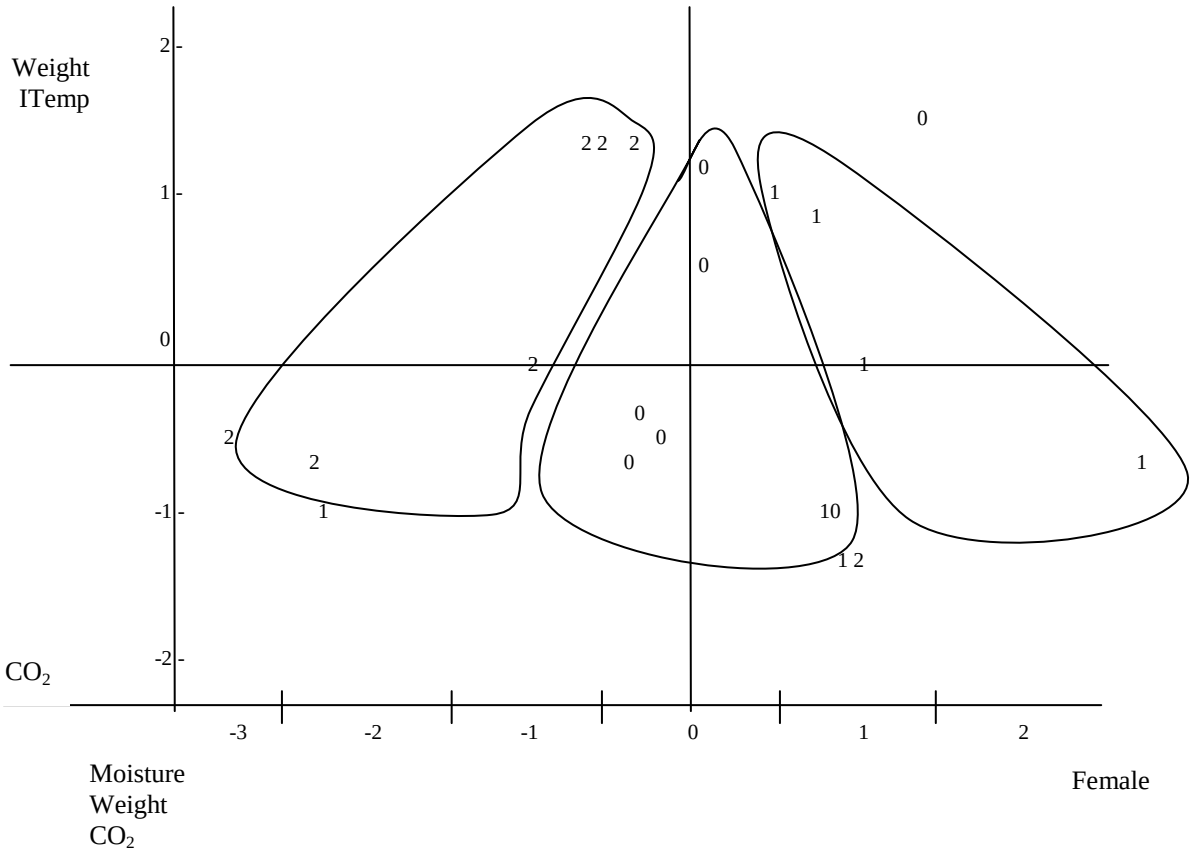

Figure 5.4 Principle component analysis incorporating maternal female, incubation temperature (ITemp), incubation moisture, hatchling weight, the polychlorinated biphenyl (PCB) concentration of the hatchling, and CO_2 production of the neonate hatchling. The symbol on the graph is injection treatment (0=Oil, 1=Low, 2=High). The x-axis is anchored by female (positive end) and moisture/hatchling weight/CO_2 (negative end). The y-axis is anchored by hatchling weight/incubation temperature (positive end) and CO_2 (negative end).

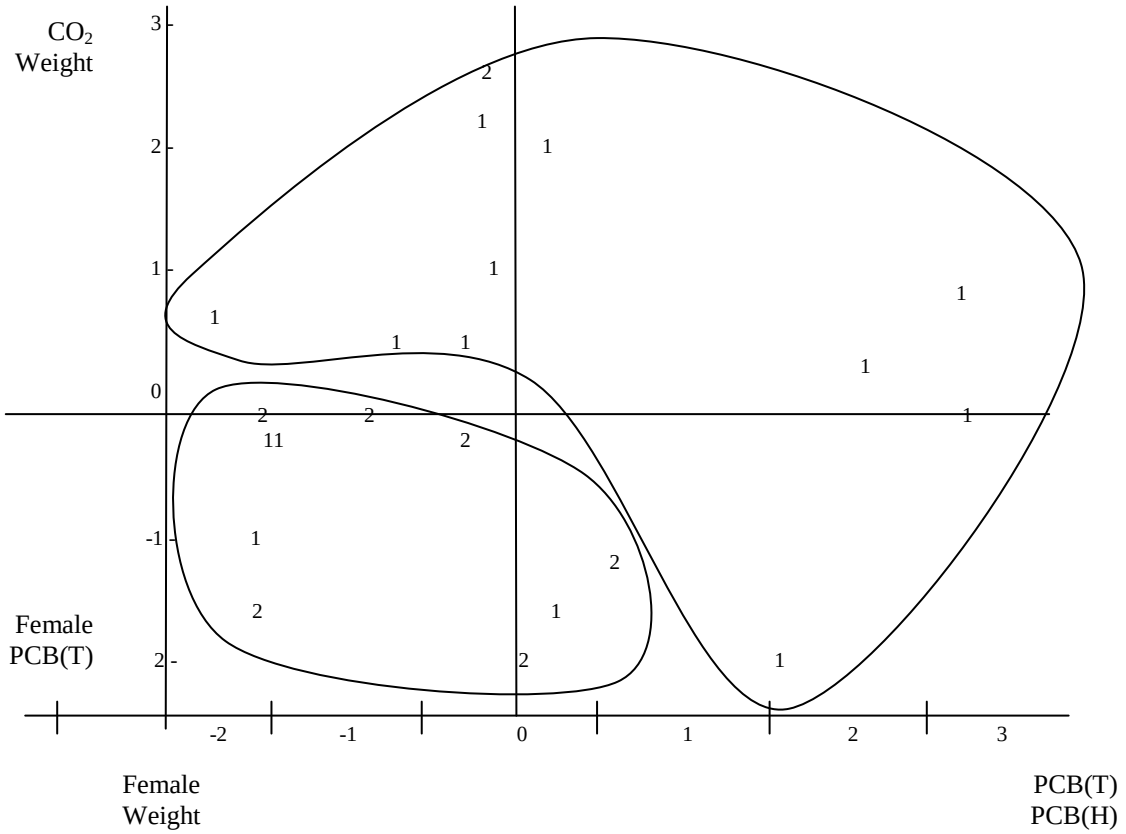

Figure 5.5 Principle component analysis incorporating maternal female, injection treatment (PCB(T)), incubation moisture, hatchling weight, the polychlorinated biphenyl concentration of the hatchling (PCB(H)), and CO_2 production of the neonate hatchling. The symbol on the graph is incubation temperature (1=Low, 2=High). The x-axis is anchored by PCB(T)/PCB(H) and female/hatchling weight, and the y-axis is anchored by CO_2/hatchling weight and female/PCB(T).

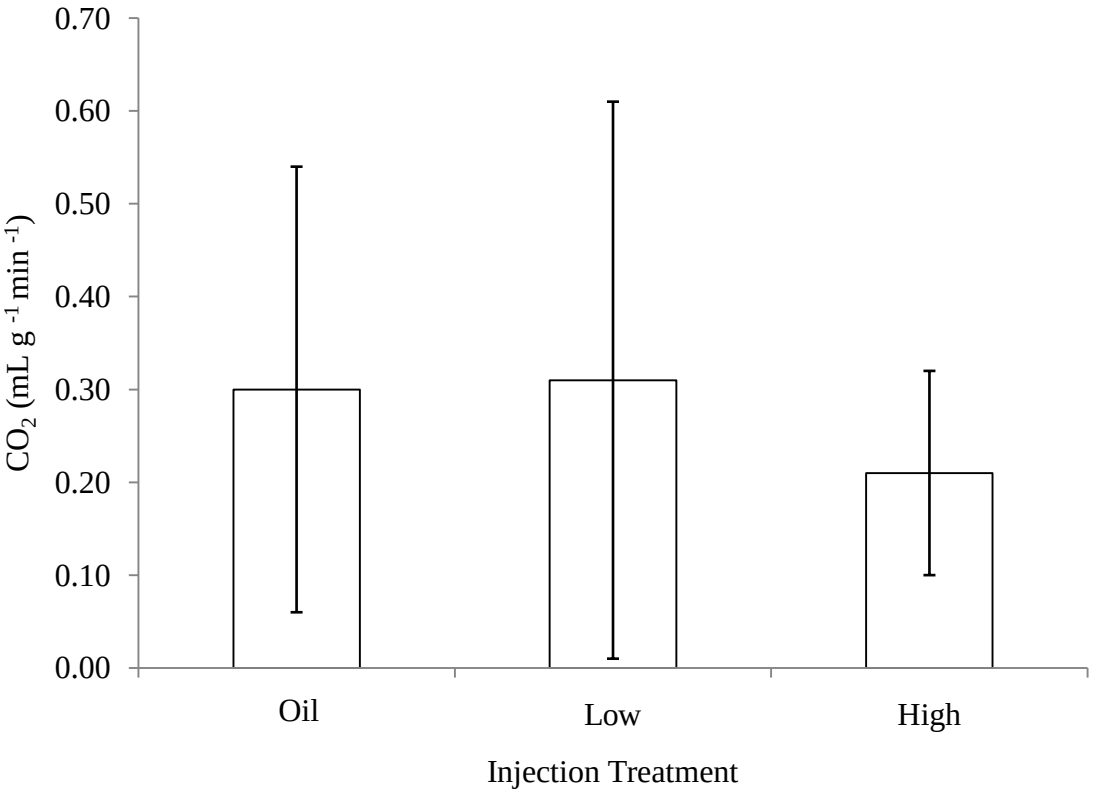

Figure 5.6 Mean CO_2 production of neonate hatchlings by injection treatment in the 2006 season. Mean CO_2 production did not significantly differ between injection treatments ($F_{0.05(1),2,26}=0.44$, $p>0.05$).

DISCUSSION

Few studies have examined respiration patterns in neonate sea turtle hatchlings (Price et al., 2006) or freshwater turtle hatchlings. There are currently no known studies on how respiration in neonate turtle hatchlings can be influenced by PCB contaminants. This study examined CO_2 production by neonate snapping turtle hatchlings at rest, in an effort to determine if PCB contaminants result in elevated CO_2 production. This could be used as a proxy for a contaminant induced elevation in metabolism.

Carbon dioxide production was significantly correlated to ambient temperature but not significantly correlated to PCB treatment, weight of the hatchling, or incubation temperature. Little is known regarding the effect of incubation temperature on embryonic metabolism in reptiles (Deeming and Ferguson, 1991). The heart rate of avian embryos decreases with a decrease in incubation temperature (Romanoff and Sochen 1936; Oppenheim and Levin, 1975; Bennett and Dawson, 1979; Deeming and Ferguson, 1991). Decreasing the ambient temperature in externally pipped embryos of the domestic quail (*Coturnix coturnix japonica*) and domestic duck (*Anas platyrhynchos*), resulted in a decrease in breathing frequency, and an increase in amplitude of each breath (Oppenheim and Levin, 1975; Nair and Dawes, 1980; Dawes, 1981; Nair et al., 1983; Spear and Dawes, 1983). This study did not specify if the CO_2 production was increasing or decreasing in concentration. In my study, increased ambient temperature resulted in an increase in CO_2 production (ml g^{-1} min^{-1}), thereby indicating an increase in metabolism.

The rate of CO_2 produced was not significantly correlated with the treatment variables used in this study. As the concentration of PCBs increased between treatments

the variance of the CO_2 production rate decreased (Figure 5.1). It is possible that if this study was conducted with more PCB treatments at higher concentrations, the variance may have continued to decrease and the effect of PCB treatment on the production rate of CO_2 would have been clearer. Although, it is important that toxicology studies reflect environmentally realistic values. In my study, the high PCB treatment was similar to that found in snapping turtle eggs at a highly contaminated USEPA superfund site in New York (J.J. Pagano, Director, Environmental Research Center, Department of Chemistry, State University of New York at Oswego, personal communication).

The treatments variables in this study all have the potential of influencing turtle metabolism (incubation temperature, incubation moisture, PCB treatment). By increasing or decreasing specific variables there is a direct effect, to some degree, to the individual's metabolic rate. In specific treatment combinations, one variable may be increasing metabolism while the other variable may be decreasing metabolism at some level. By increasing the concentration of the PCB treatment, the effect of the PCB treatment (signal) may be clearer and variance reduced (noise).

In Chapter 3, eggs that were incubated at high temperature and exposed to high PCB treatment resulted in a decrease in total PCBs in the neonate hatchling and a change in 76% of the specific PCB congeners in the hatchling. Reduction in total PCBs during embryogenesis might have influenced the production of CO_2 by the neonate hatchling. This change in the PCB congener profile implies that the developing hatchling is metabolizing specific PCB congeners (catabolism) and reassembling them into new congeners (anabolism). The respirometry data of neonate hatchlings may not show these

effects, as PCBs were metabolically active during embryonic development and respirometry is only a measurement of catabolism. In future studies, it may be more advantageous to measure the respiration of the egg through incubation to determine the effect of PCB treatment.

In the PCA the three PCB treatments in this study clustered when incorporating the multiple variables in this study (Figure 5.4). The high (2) PCB treatment cluster was related to increased incubation moisture, increased hatchling weight, decreased CO_2 production, and high incubation temperature. Increased incubation moisture and increased incubation temperature, as well as high PCB concentrations (increase of hyperthyroidism, possible increased growth rate) are factors that result in an elevated turtle weight (Chapter 1). Therefore this relationship is not surprising. This cluster is also related with decreased CO_2 (mL g^{-1} min^{-1}) production. This is explained by the relationship between the increasing body mass of an individual and the decrease in CO_2 production per gram. The oil (0) treatment clustered in the center of the graph and did not exhibit a relationship with any variable. No relationship to these variables was expected, as the purpose of the oil was to serve only as a vehicle for the low and high PCB injection treatments.

In the second PCA, the two incubation temperature treatments clustered using maternal female, PCB treatment, incubation moisture, hatchling weight, the PCB concentration of the hatchling, and CO_2 production of the hatchling as variables (Figure 5.5). Low incubation temperature was widespread and did not exhibit a relationship to any degree with the other variables; however high incubation temperature clustered by

high hatchling weight and low PCB treatment. By increasing incubation temperature, the growth rate of the embryo is increased and hatchlings are produced with increased weights (Chapter 1). The objective of this analysis was to determine if CO_2 production was influenced by a set of variables, including injection treatment or PCB (ng) levels in hatchlings. That was not apparent in this analysis.

During incubation, many abiotic factors have a direct effect on the development of snapping turtle embryos. This study simultaneously examined these variables to determine the combination of their effect on the rate of CO_2 produced in a PCB contaminated environment. Incorporating several variables may have resulted in one variable counteracting another. Elevated moisture levels in the nest produce larger (Packard, 1991) and faster (Miller, 1993) hatchlings. Such abiotic factors may negate the effects of PCB until higher levels of PCB are used as a treatment to outweigh their affects. Although I did not see a correlation between injection treatment and the rate of CO_2 production in this study, a relationship may exist at higher contaminant levels. Such studies may be unnecessary in hatchling studies given that the high PCB treatment used environmentally relevant concentrations.

Chapter 6

A Non-invasive Technique for Determining Mercury Concentrations in Snapping Turtle (*Chelydra serpentina serpentina*) Tissues

INTRODUCTION

Mercury is sequestered in tissues containing metal binding proteins such as those found in the liver and kidney, and can even penetrate the blood-brain barrier (Sparling et al., 2000). Mercury contamination is prevalent throughout the Great Lakes region due to fossil fuel incineration, which loads appreciable amounts of this metal into the atmosphere each year (Laws, 2000). Monitoring mercury levels in various tissues often results in invasive techniques requiring euthanization of the individual. Previous studies have attempted to find non-invasive techniques by sampling feathers, eggs, and scales that can estimate contaminant levels of internal tissues.

Other tissues such as bone, feathers, scales, scutes, carapace, blood, and eggs have also been examined for mercury storage (Heaton-Jones et al., 1997; Yanochko et al., 1997; Overmann and Krajicek, 1995; Sparling et al., 2000). In attempts to correlate mercury levels in various tissues, Overmann and Krajicek (1995) found that in snapping turtles there were significant correlations between mercury levels in liver versus blood, liver versus carapace, and bone versus carapace. Other studies have attempted correlations using scute scrapings, however no results were found to be published (University of Pennsylvania's School of Veterinary Medicine, Toxicology Lab, personal communication). Although you can collect blood and scute scrapings without euthanizing the individual, there are some drawbacks. Blood samples may only reflect

recent levels of mercury contamination and may be difficult to collect from such an aggressive animal. Carapace samples would be easier to collect, and reflect a historical concentration of mercury. Scute scrapings could be collected from live individuals, however samples could show high variation depending which scute is sampled and the region of the scute sampled. Such a sample may also be contaminated with water, sediment, or other macroinvertebrates that are commonly found on the carapace. Carapace samples have also been analyzed for mercury levels. The carapace samples were collected after the individual was euthanized and such a procedure may not be possible on a live turtle. The claw would be an alternative sample because it contains metal-binding proteins, would give historical mercury concentrations, and is easily collected with little stress on a live individual.

This study sampled the claw tissue of adult snapping turtles to determine if a correlation existed with the mercury concentration of internal tissues. A non-invasive sampling technique to determine mercury levels would be critical for populations in low numbers, or reptilian species with similar habitat and diet that are threatened and could not be euthanized for internal tissue collection.

METHODS

Adult snapping turtles were trapped during July-August 2002 in various locations in Presque Isle State Park and from impoundments within the inland Pennsylvania Lake Erie watershed. These inland impoundments include Eaton Reservoir (northeast region) and two sited near Crooked Creek (northwest region) (Figure 6.1). The inland

impoundments were chosen because they are dispersed throughout the watershed and receive runoff from large portions of the surrounding areas. Trapping was accomplished using baited nylon turtle hoop nets (1 meter dia., 4.0 cm sq. mesh, Nylon Net Co.). Traps were set perpendicular to shore in less than 1 meter of water and checked daily.

A total of 22 turtles were collected from the following sites: Eaton Reservoir (n= 6), Presque Isle State Park (n = 5), and two ponds near Crooked Creek (Camp Fitch, n = 8; Camp Notre Dame, n= 3). Turtles captured in the nets were weighed (kg), and the curved carapace length and curved carapace width were measured (cm). Turtles were transported back to the lab and euthanized by decapitation (IACUC #14569).

Turtles were necropsied, and liver, muscle, and adipose tissue samples as well as blood and claw samples were collected from each adult animal. Prior to each necropsy, the area and instruments were cleaned with hexane (research grade) to avoid sample contamination. Samples for mercury analysis were collected and stored in plastic jars. Elemental analyses were performed by the Toxicology Lab at the University of Pennsylvania's School of Veterinary Medicine using cold vapor atomic absorption (CVAA).

The Shapiro-Wilk test for normality confirmed the curved carapace length (W=0.9299, p>0.05), curved carapace width (W=0.9726, p>0.05), weight (W=0.9685, p>0.05), mercury levels in the claw (W=0.9069, p>0.05), and mercury levels in the liver (W=0.9737, p>0.05) were normally distributed. Mercury levels in the claw and liver were categorized in 0.500 mg/g intervals. Further statistical analyses were based on these categories. Using a multiple linear regression, samples of the various tissues were

analyzed to detect possible relationships between the toxicant levels. Significance was set at $\alpha=0.05$.

RESULTS

Mercury Analysis

Mercury levels were analyzed in claw, liver, muscle, and adipose tissue samples of turtles collected from four locations (two locations were near Crooked Creek) throughout Erie County. When analyzed for mercury, the claw, liver, and muscle tissue samples were above the detection limit (Figure 6.1); as the muscle tissue only had two samples above detection limits, the claw and liver mercury samples were used for statistical analysis. There was a significant difference among locations when comparing the total mercury in the claw ($F_{0.05(1),3,11}=4.22$, $p < 0.05$) and the liver ($F_{0.05(1),3,16}=8.61$, $p < 0.05$) tissues. Overall, mean mercury levels in the claw and in the liver were highest, while most muscle and fat had undetectable levels (Table 6.1). Mercury levels, which were normalized by weight, were highest in claw and liver samples at Eaton Reservoir in North East and lowest near Crooked Creek pond (Camp Notre Dame and Camp Fitch) (Table 6.2).

Using a stepwise linear regression technique, the mercury concentration in the claw alone was not a good indicator of mercury levels in the liver (Figure 6.2). The weight of the turtle ($t_{0.05(1),20}=3.96$, $p<0.05$) and the claw ($t_{0.05(1),14}=2.31$, $p<0.05$) were both significant and provided a better estimate of the mercury level of the liver (Table 6.3). Using a stepwise backward linear regression, the model was significant

($F_{0.05(1),2,18}$=10.28, p<0.05). A backward elimination linear regression was used rather than a forward linear regression because there were only four parameters considered for the model. A backward linear regression allowed all parameters to be considered, as a forward linear regression is used when many parameters are considered and only selects a subset of those parameters for analysis.

There was a positive linear relationship in weight versus mercury levels in the liver (R^2=0.2315) (Figure 6.3), CCL versus mercury levels in the liver (R^2=0.1468) (Figure 6.4), and in the CCW versus mercury levels in the liver (R^2=0.0863) (Figure 6.5). Using the Pearson correlation coefficients, the morphometric parameters turtle weight and CCL, turtle weight and CCW, and CCL and CCW were significant (Table 6.4) indicating an autocorrelation between the two variables. Weight had a higher level of significance than CCL and CCW and therefore was incorporated into the model (Table 6.3). The following equation (R^2 = 0.53, p < 0.05) (Table 6.5) estimates the concentration of mercury in the liver of snapping turtles in the Lake Erie Watershed:

Hg Liver (mg/kg) = -2.0014 + 0.4789 (weight (kg)) + 0.4867 (Hg claw (mg/kg))

Figure 6.1 Map of Erie County denoting sampling sites in Lake Erie Watershed. Samples were collected from Crooked Creek area (consists of the ponds at Camp Fitch and at Camp Notre Dame), Presque Isle State Park, and Eaton Reservoir in North East Township (©2007Google Earth).

Table 6.1 Mercury concentrations (mg/kg) in various snapping turtle tissues. The detection limit was 0.300 mg/kg, therefore values less than this were not reported and were not included in the calculation of the mean.

Tissue	*n* (above detection)	*n* (below detection)	Range	Mean	SD
Claw	15	7	<0.300-3.380	1.54	0.77
Liver	20	2	<0.300-4.670	1.61	1.08
Muscle	2	20	<0.300-0.374	0.34	0.05
Adipose	0	22	<0.300	N.A.	N.A.

Table 6.2 Mean (SD) morphometric measurements at Presque Isle State Park (PISP), Eaton Reservoir at North East (ERNE), and two sites near Crooked Creek (CC).

Location	Weight (kg)	CCL (cm)	CCW (cm)
PISP	24.20 (5.36)	39.58 (3.36)	37.66 (3.79)
ERNE	18.67 (7.84)	32.50 (4.42)	34.83 (3.99)
CC (Camp Notre Dame)	15.00 (6.56)	31.60 (5.89)	33.97 (4.76)
(Camp Fitch)	17.63 (5.53)	33.51 (4.10)	36.16 (4.78)

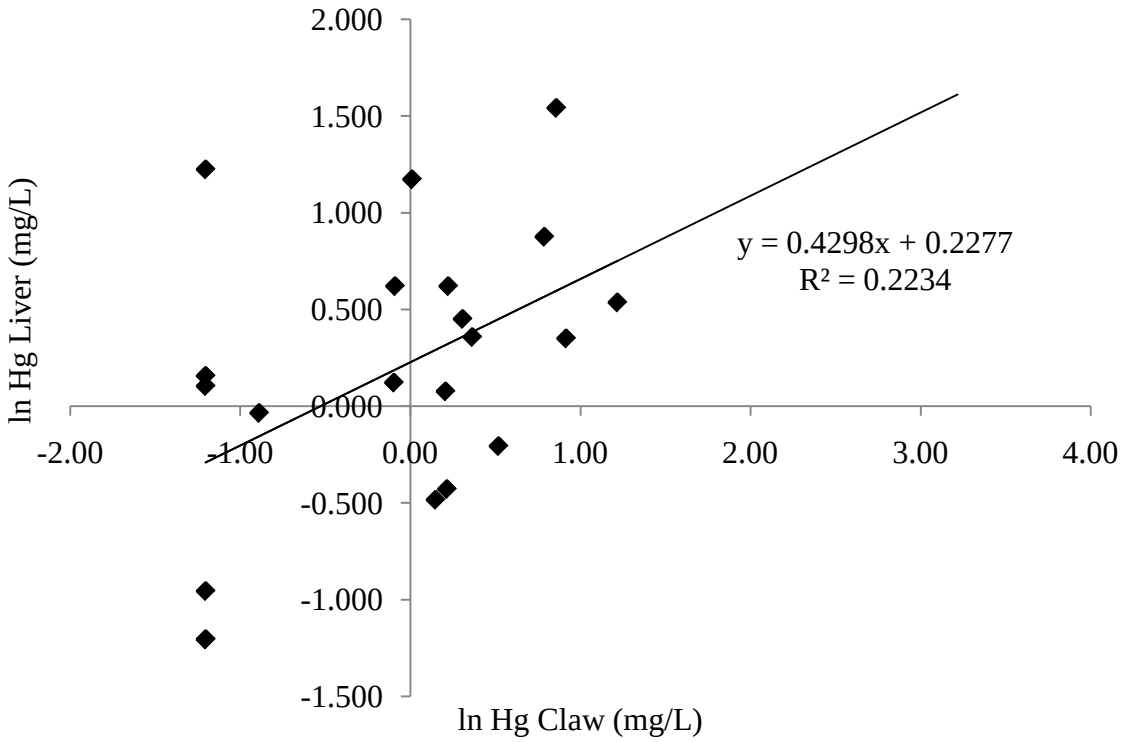

Figure 6.2 The relationship between curved carapace length (CCL)(cm) and the mercury (Hg) levels in the liver.

Table 6.3 Parameters utilizing morphometric measurements and mercury concentration in the claw to estimate mercury concentrations in the liver (dependent variable).

Variable	Parameter Estimate	St. Error	t-value	p-value
Intercept	14.77	6.47	2.28	*
Weight	1.46	0.37	3.98	***
CCL	-0.43	0.19	-2.32	*
CCW	-0.27	0.20	-1.36	>0.05
Hg Claw	0.22	0.21	1.06	>0.05

* Indicates significance

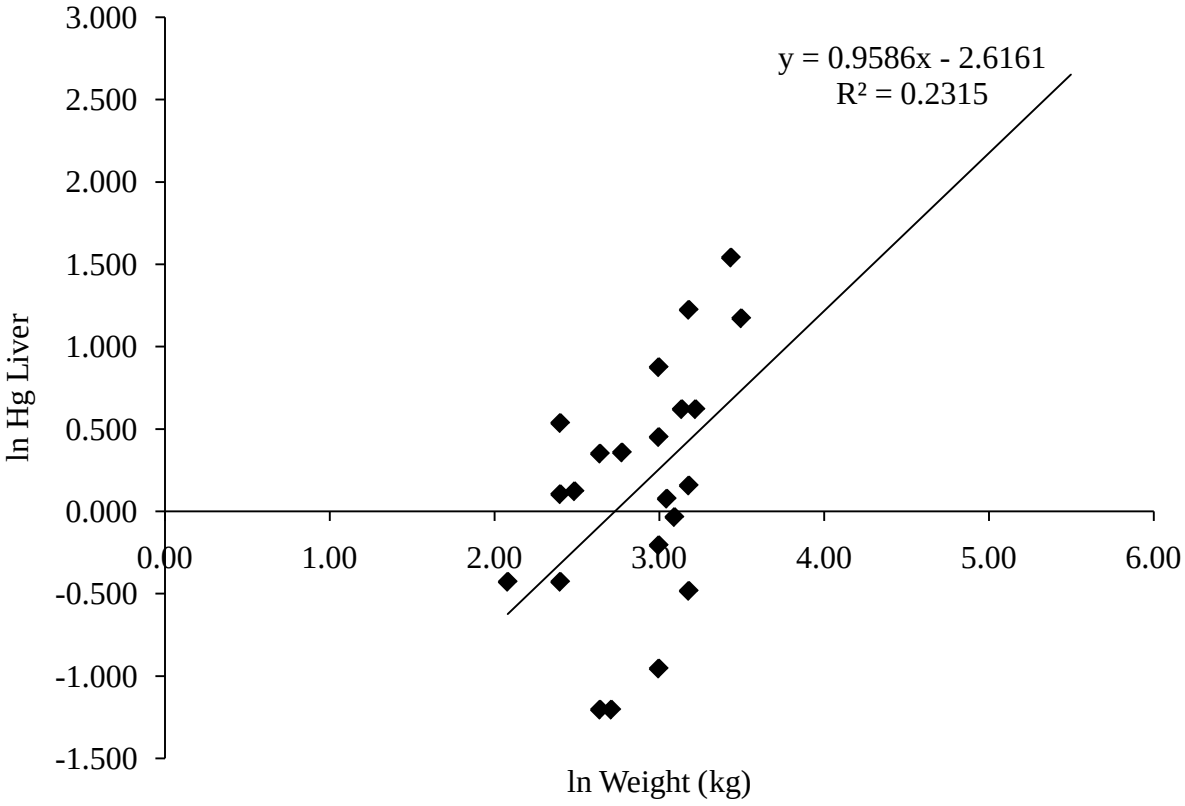

Figure 6.3 The relationship between the weight (kg) of the turtle and the mercury (Hg) levels in the liver.

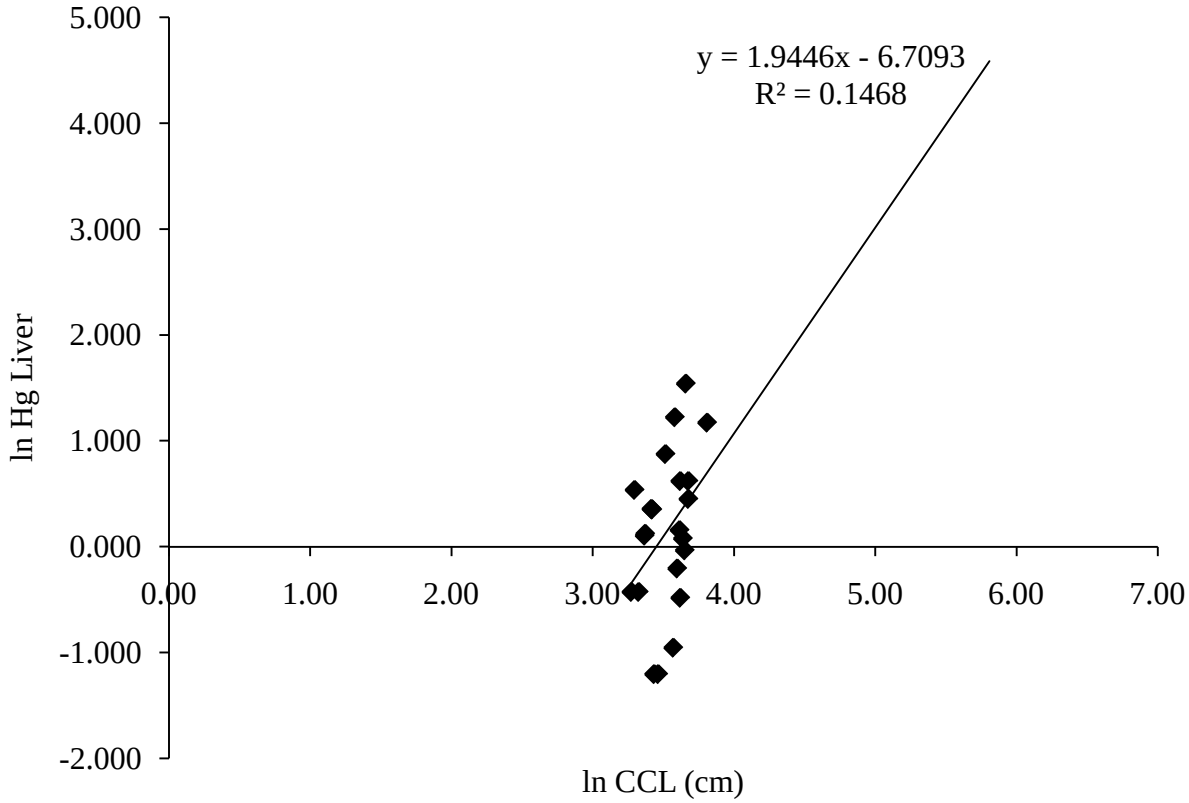

Figure 6.4 The relationship between curved carapace length (CCL)(cm) and the mercury (Hg) levels in the liver.

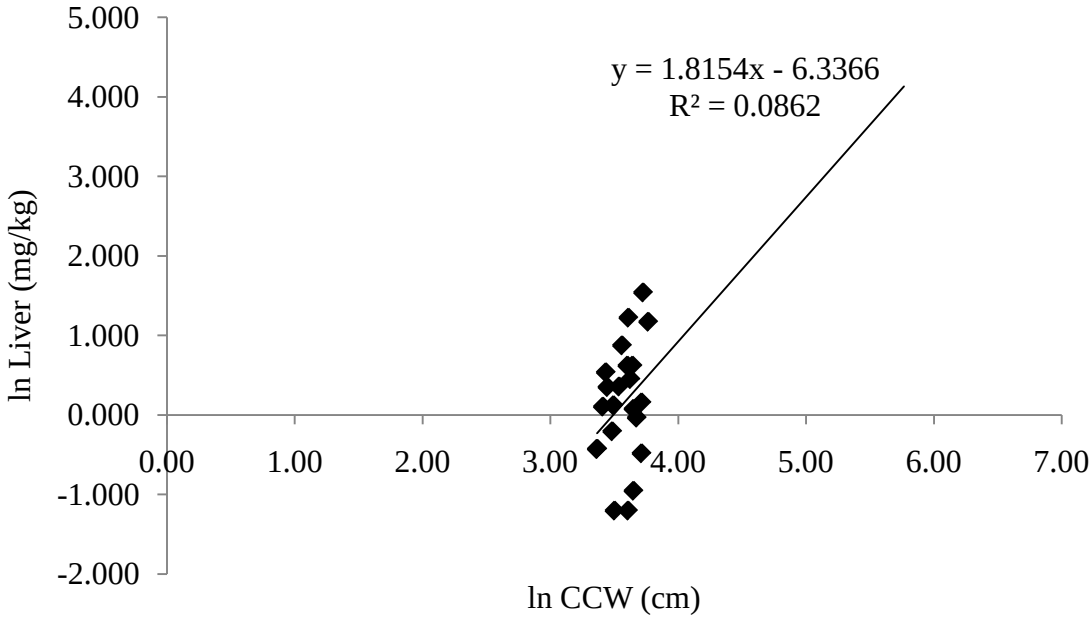

Figure 6.5 The relationship between curved carapace length (CCL)(cm) and the mercury (Hg) levels in the liver

Table 6.4 Correlation matrix using Pearson correlation coefficients (*p*-value) to determine an autocorrelation between measured parameters.

	CCL (cm)	**CCW (cm)**	**Weight (Kg)**	**Hg Claw**
CCL (cm)	1.00			
CCW (cm)	0.87 (****)	1.00		
Weight (Kg)	0.93 (****)	0.90 (****)	1.00	
Hg Claw	-0.14 (>0.05)	-0.22 (>0.05)	-.003 (>0.05)	1.00

***** Indicates significance

Table 6.5 Parameters utilizing turtle weight (kg) and mercury (Hg) concentration in the claw to estimate mercury (Hg) concentrations in the liver (dependent variable). Other morphometric measurements were excluded due to the autocorrelation with turtle weight ($F_{0.05(1),2,18}$=10.28, p<0.05), and the R^2 = 0.53.

Variable	**Parameter Estimate**	**St. Error**	**t-value**	**p-value**
Intercept	-2.00	1.28	-1.57	>0.05
Weight	0.48	0.12	3.96	***
Hg Claw	0.49	0.21	2.31	>0.05

***** Indicates significance

DISCUSSION

Mercury was found in snapping turtle tissues at all four sites in the Pennsylvania Lake Erie watershed. The significant difference in mercury levels among sites within the same watershed seems common in other studies (Golet and Haines, 2000; Meyers-Schöne et al., 1993; Heaton-Jones et al., 1997). The highest levels of mercury were found in claw and liver samples from turtles captured at Eaton Reservoir in North East (ERNE). Fish with elevated levels of mercury have also been sampled at ERNE in the past (Robert Wellington, Erie County Department of Health, personal communication). Mercury concentrations in tissue samples from Camp Notre Dame Pond (CNDP) were not significantly different from concentrations in samples collected at Presque Isle State Park (PISP). The CNDP is an isolated pond surrounded by cottages and facilities associated with a seasonal youth day-camp. The outflow of the pond goes directly into Lake Erie. At the present time the possible source of mercury is not known.

Turtles sampled from the Crooked Creek pond (CCP) site had the lowest mercury concentrations. Crooked Creek and its tributaries span most of the secluded western plot of the Lake Erie watershed in Pennsylvania. The pond where turtles were sampled is located near the downstream endpoint of the creek and can potentially act as a catch-basin for contaminants. Low mercury levels in the tissue samples can be a bioindicator for that area. Such an indicator is specific to the trophic levels encountered of those individual turtles. When analyzing two sympatric species of turtle for mercury, the difference in mercury concentrations was attributed to the differences in the diet (Sparling et al., 2000).

A model to estimate mercury levels using non-invasive techniques was developed in this study. I selected the claw as a non-invasive sampling tissue due to its composition of metal-binding proteins, and ease of collection. A stepwise multiple regression revealed that the claw alone is not a good indicator of mercury. External measurements including the turtle's weight and CCL were most significant in explaining the variation. These external characteristics also serve as a proxy for the age of the turtle or the time it has been exposed to the mercury in its environment.

Chapter 7

Conclusions on the Effect of Polychlorinated Biphenyls and Mercury on the Common Snapping Turtle (*Chelydra serpentina serpentina*)

This study examined the effects of PCBs and mercury in the common snapping turtle. Numerous studies in the Great Lakes have shown they are present in the aquatic fauna; thus consumption advisories have been issued for several species. Therefore understanding the effects and consequences of these contaminants on individuals and subsequent generations is critical. In this work, I demonstrated the use of improved contaminant delivery systems. This is important, because contaminant injection techniques in reptiles, provide the best imitation of maternal transfer, but have been abandoned due to poor hatching success. This study had favorable hatching success and strongly encourages the protocol outlined in Chapter 3. Those eggs that were injected and incubated to hatch provided insight into the toxicokinetics of PCBs during embryology. Congener specific profiles revealed a change in 76% of the congeners when eggs were treated with high PCBs and incubated at high temperature. There was an increase in higher chlorinated congeners and a decrease in lower chlorinated congeners. The treatment of abiotic factors, which are naturally experienced in the nest, demonstrated the interaction of eggs exposed to high PCBs and high temperatures which resulted in a lower total PCB concentration in the neonate hatchling. These results indicate that contaminants are not solely transferred from maternal tissue to offspring tissue, but rather they are dynamic and interact with metabolic processes during critical stages of development.

Although my study had multiple, simultaneous treatments that decreased sample size and increased the complexity of the experimental design, I would not discourage such treatments in future studies. Naturally occurring nests are influenced by these factors simultaneously as well, and therefore the interaction of these treatments needs to be considered.

One of the drawbacks of contaminant studies is that it often requires the euthanization of individuals. A portion of my study examined the claw as a non-invasive sample to estimate mercury concentration in internal tissues. With the incorporation of the turtle's weight into the model, the mercury concentration of the liver could be estimated. This model is important in regard to other turtle or reptilian species that have low populations. Especially if these reduced populations are a result of environmental contaminants.

Although stringent laws have reduced the introduction of contaminants into the environment, PCBs and mercury persist in snapping turtles, as well as other organisms. Knowing the concentrations of the contaminants present in organisms and how they behave in the organism is critical in understanding future populations.

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VITA
JEANETTE L. SCHNARS

640 Rankine Avenue
Erie, PA 16511
(814) 490-3267

Tom Ridge Environmental Center
301 Peninsula Drive, Suite #9
Erie, PA 16505

EDUCATION

2003-December 2008, The Pennsylvania State, PA; Ph.D. Wildlife & Fishery Science
2001, The State University of New York, College at Buffalo; M.A. Biology
1996, The Pennsylvania State University; B.S. Biology, Minor: Marine Science.

WORK EXPERIENCE

2007, October-present. Director of the Regional Science Consortium at The Tom Ridge Environmental Center at Presque Isle State Park.

2006, August-July 2007. Natural History Collections Manager. Tom Ridge Environmental Center / PA Sea Grant

2001, August-May 2006. Instructor of Biology. Penn State Erie

1996, December-August 1998. Sea Turtle Biological Science Technician. United States Geological Survey (GS-5) at Padre Island National Seashore

1996, Summer. Aquarist Trainee. Sea World of Ohio.

1994, June- April 1996. Aquarist and Mammology Intern & Assistant. The National Aquarium in Baltimore.

GRANTS & AWARDS

- Pennsylvania Sea Grant Applied Research Program 2005 - $15,000.
- Pennsylvania Department of Environmental Protection - $2,500
- Penn State Behrend-Sigma Xi Undergraduate Research and Creative Accomplishment Conference. Student Advisor 2002-2007), grants totaling over $15,000
- 1998 Recipient of the Department of the Interior Star Award for achievements made contributing to the Kemp's ridley Sea Turtle Project and Research

PRESENT RESEARCH PROJECTS

- The effects of PCBs in snapping turtles (*Chelydra serpentina*)
- Herpetofauna inventory and population assessment of Presque Isle State Park
- Telemetry of adult snapping turtles (*Chelydra serpentina*)
- Orientation cues of snapping turtle hatchlings

PROFESSIONAL SOCIETY MEMBERSHIPS

- βββ National Biological Honor Society
- Society of Environmental Toxicology and Chemistry (SETAC)
- International Association for Great Lakes Research (IAGLR)
- Ecological Society of America (ESA)

COMMITTEES & SERVICE

- Presque Isle Partnership, Board Member
- Presque Isle Partnership Environmental Research Committee, Chair