ABSTRACT

Title of Document:	COMPARATIVE SUB-LETHAL EFFECTS OF POLYBROMINATED DIPHENYL ETHERS FOLLOWING SIMULATED MATERNAL TRANSFER AND DIETARY EXPOSURE IN TWO SPECIES OF TURTLE
	Karen Marie Eisenreich, Ph.D., 2011
Directed By:	Associate Professor Christopher Rowe, Marine Estuarine and Environmental Science

Polybrominated diphenyl ethers (PBDEs) are contaminants of concern as their concentrations have been increasing in the environment in recent years. This project sought to determine the effects of embryonic and dietary exposure to two PBDE congeners (BDE-47 and BDE-99) on a suite of endpoints including development, growth, metabolic rate, behavior and thyroid function of embryonic, hatchling and juvenile red-eared slider turtles (*Trachemys scripta elegans*) and snapping turtles (*Chelydra serpentina*). Topical egg dosing was employed for embryonic exposures; transfer efficiencies across the red-eared slider eggshell were 25.82 % and 9.87 % for BDE-47 and -99 respectively whereas they were 31.30 % and 12.53 % across the snapping turtle eggshell. These transfer efficiencies were taken into account when topically dosing eggs in a subsequent exposure-response study of embryonic exposure to BDE-47. Sodium perchlorate was included as a positive control for thyroid

disruption in the embryonic exposure study. Embryonic exposure to five concentrations of BDE-47 (target exposure range from 40 ng/g - 1000 ng/g ww) led to patterns of elevated standard metabolic rate in hatchlings of both species and increased liver weights in snapping turtles. No impacts were found on incubation time, hatching success or total glandular thyroxine (T_4) of the hatchlings. Embryonically exposed red-eared slider juveniles displayed delayed righting response behavior and both species showed patterns of reduced thyroid size and T_4 following exposure. Sodium perchlorate had significant impacts on survival, incubation time, volume of the external yolk and T_4 in the red-eared slider hatchlings. In snapping turtles, sodium perchlorate exposures led to impacts on hatching success, standard metabolic rate, liver and thyroid sizes, and T_4. A separate study of dietary exposure to BDE-47 and BDE-99 (2055 ng/g and 1425 ng/g respectively) over a six month period in both species revealed altered behavior and decreased T_4 in red-eared sliders and elevated standard metabolic rate in snapping turtles. Embryonic and dietary exposures to BDE-47 and -99 can elicit a suite of impacts potentially related to thyroid system function and are cause for concern, but the observed species specific differences in response require further investigation.

COMPARATIVE SUB-LETHAL EFFECTS OF POLYBROMINATED DIPHENYL
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EXPOSURE IN TWO SPECIES OF TURTLE

By

Karen Marie Eisenreich

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

Advisory Committee:
Associate Professor Christopher Rowe, Chair
Research Associate Professor Andrew Heyes
Associate Professor Carys Mitchelmore
Professor Mary Ann Ottinger
Adjunct Professor Barnett Rattner

Acknowledgements

I sincerely want to thank my advisor, Chris Rowe, for his guidance, mentorship and support over my time at the Chesapeake Biological Laboratory. I am incredibly thankful for his support to pursue my own interests within the world of reptiles and toxicology. These opportunities have not only helped me become a confident and successful scientist, but have prepared me for a successful transition into my next endeavor.

I am very grateful for the support of my other committee members both financially and scientifically. I thank Dr. Barnett Rattner for encouraging me to pursue and expand my scientific education through my Ph.D., and for his scientific advice and support throughout this process. A large portion of my research would not have been able to be completed without the support of Dr. Mary Ann Ottinger. She provided laboratory space, knowledge, personnel, financial support and encouragement to push through the trials and tribulations of research. I thank Dr. Andrew Heyes for the laboratory space and analytical support he provided for a me to conduct the chemical analysis of my samples, an imperative part of my research. I would also like to thank Dr. Carys Mitchelmore for providing laboratory support, as well as having willingness to provide advice and interest in my research.

I would like to thank all those who helped with the seemingly endless turtle care with special thanks to my three REU students, Adam Obaza, Jennifer Barkman, and Kim Hohlweg for their hard work and diligence with my research. I cannot acknowledge Ashley Sides enough for her assistance and most importantly her friendship. She provided endless hours of hard work helping to care for my turtles,

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Chapter 1: Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of organic compounds used as flame retardants and historically employed in a wide variety of consumer products including flame-resistant polystyrene and polyurethane foams, treated textiles, electronics and plastics (de Wit 2002; Alaee et al. 2003). Their widespread use as an additive chemical and global distribution has resulted in PBDEs being ubiquitous in marine and freshwater systems (de Wit et al. 2002; Hale et al. 2003; Law et al. 2003). PBDEs have been readily identified in environmental matrices and human tissues and have rapidly spurred concern worldwide in scientific and regulatory communities (e.g., Betts 2001).

PBDEs have been commercially produced in three distinct formulations: Penta-bromodiphenyl ether (penta-BDE; dominated by congeners having 4 or 5 bromine [Br] atoms; see Figure 1-1), octa-bromodiphenyl ether (octa-BDE; dominated by congeners having 7 or 8 Br atoms; see Figure 1-1), and deca-bromodiphenyl ether (deca-BDE; primarily composed of the fully brominated BDE-209; see Figure 1-1). Deca-BDE is the most commonly employed group of PBDEs worldwide, accounting for approximately 80% of total use, but has traditionally been considered not to be bioavailable due to its large size (959 g/mol) and log K_{OW} (10; Hardy 2002) despite its very high concentrations in sediments. However, deca-BDE can be assimilated and metabolized by several organisms, producing lesser-brominated congeners such as penta-BDEs (Kierkegaard et al. 1999; Stapleton et al. 2004a, 2004b and 2006; Chen et al. 2007, 2008; Chen and Hale 2010) that may be much more toxic and hormonally active than the parent compounds (Meerts et al. 2001). Penta-BDE comprised about 12 % of PBDEs used worldwide, although the United States was responsible for 98% of its use (Hale et al. 2003).

Due to the increasing environmental concentrations of PBDEs, in particular the lower brominated mixtures, and the well documented bioaccumulative and toxic properties, both the Penta-BDE and Octa-BDE are no longer on the market in Europe and North America (Directive 2003/11/EC; Tullo 2003; Ward et al. 2008). Deca-BDE is largely unregulated, however, and although it was banned in 2008 in Europe from use in electronic equipment it remains in use in the U.S. Use in the U.S. will soon be voluntarily phased out of production and use by 2012 due to an increasing body of evidence demonstrating that BDE-209 can be assimilated and metabolized in the environment to form the more toxic lower-brominated congeners (Kierkegaard et al. 1999; Stapleton et al. 2004a, 2006; Van den Steen et al. 2007; BSEF 2010). Even with regulations in place for PBDEs, environmental concentrations are continuing to increase and continue to pose a threat to wildlife health.

PBDEs are globally distributed in freshwater, estuarine, and marine systems (de Wit 2002). Recent studies demonstrate that BDEs are accumulated by aquatic animals and magnified through food webs much like the well-studied PCBs (Hale et al. 2003; Stapleton and Baker 2003; Burreau et al. 2004), ultimately reaching relatively high concentrations in higher trophic level consumers, including humans (Boon et al. 2002; Law et al. 2003; Sormo et al. 2006). The propensity for bioaccumulation of BDEs is due in part to their lipophilicity. The octanol-water coefficients (K_{OW}) of lesser-brominated congeners such as tetra- and penta-BDEs are within the range of values in which accumulation potential is maximal (log K_{OW} = 5.9 - 7.0 for tetra- and penta-BDEs; Darnerud et al. 2001). On the other hand, higher brominated forms (octa- and deca-BDEs) display higher values of K_{OW} (log K_{OW} = 8.4 - 10; Darnerud et al. 2001) reflecting

their large molecular size which impedes diffusion and reduces bioaccumulation potential through steric hindrance.

Studies suggest that PBDEs may act as endocrine disrupting compounds (EDCs), potentially affecting thyroid hormone homeostasis (McDonald 2002; Branchi et al. 2003; Darnerud 2003; Legler and Brouwer 2003; Fernie et al. 2005a; Skarman et al. 2005; Mikula and Svobodová 2006; Costa and Giordano 2007; Talsness 2008). Of most concern are the PBDEs that comprise the penta-BDE mixture as they tend to have a greater potential to be bioaccumulated and are more commonly found in biological matrices, even though they are no longer in commercial use. PBDEs can directly affect thyroid function via hyperplasia and tumor formation in thyroid tissues (NTP 1986; Darnerud 2003) as well as operate through hormonal pathways to affect thyroid function.

As a result of structural similarity to the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3; Figure 1-2), PBDEs may compete with T_4 for binding sites on the transport protein transthyretin, reducing transport of T_4 to sites of activity (Meerts et al. 1998 and 2000; Hallgren and Darnerud 2002; Morgado et al. 2007). PBDEs may also bind to thyroid hormone receptors, reducing conversion of T_4 to T_3 at the target site (Marsh et al. 1998). In addition, increased metabolism of T_4 has been observed in the presence of PBDEs, especially the lower brominated congeners such as BDE-47 and BDE-99 (Zhou et al. 2001 and 2002; Hallgren and Darnerud 2002; Richardson et al. 2008). Reductions of plasma T_4 and increases in hepatic enzymatic levels due increased metabolism of T_4 indicate that increased hepatic glucuronidation of T_4 is taking place, subsequently leading to increased biliary elimination of T_4 as T_4-glucuronide (Zhou et al. 2001 and 2002). Thus, PBDEs appear to operate not only to reduce the amount of T_4

transported in the blood, but also to reduce the activity at the target site. Reduction in thyroid hormone activity at the target site can lead to a decrease in thyroid hormone mediated regulation of cellular proteins and membrane pumps, thereby affecting metabolism, growth, development, and behavior.

Many studies have demonstrated inverse relationships between plasma thyroid hormone concentrations and whole body or treatment concentrations of penta-BDEs (Fernie et al. 2005a; Costa and Giordano 2007; Talsness 2008). In addition to changes in thyroid hormone homeostasis, exposure to PBDEs can lead to substantial developmental and neurotoxic effects. Several studies have verified that PBDEs can cause developmental and behavioral effects including reduced motor skills, learning abilities, memory and hyperactivity in rats and mice (Eriksson et al. 1998, 1999, 2002; Viberg et al. 2003; Branchi et al. 2002, 2003, 2005; Kuriyama et al. 2005; Costa and Giordano 2007; Driscoll et al. 2009). Changes in development and behavior after exposure to penta-BDEs have also been documented in a variety of non-mammalian vertebrates. Juvenile zebrafish (*Danio rerio*) exposed to BDE-47 through their diet exhibited early impacts to growth after exposure as well as reduced activity and swim distance that were negatively correlated with tissue concentrations of BDE-47 while *Rana pipiens* tadpoles exposed to a commercial mixture of penta-BDEs showed reduced growth and delayed development (Chen et al. 2010; Chou et al. 2010; Coyle and Karasov 2010). In addition, adult American kestrels (*Falco sparverius*) exposed through their diet to a penta-BDE technical mixture exhibited changes in reproductive courtship behavior (Fernie et al. 2008; Marteinson et al. 2010). Embryonic exposures to PBDEs in birds have also led to impacts on growth, development, immunological parameters, and reproductive behaviors

(Fernie et al. 2005a, 2005b, 2006; McKernan et al. 2009; Marteinson et al. 2010). When

considered in an environmental context, such cognitive and behavioral deficits might be

predicted to reduce fitness of affected individuals thus potentially leading to population

level effects (see Figure 1-3).

A significant gap in the understanding of the influence of PBDEs on thyroid

dynamics exists in the role that altered thyroid activity may have in modifying processes

other than neurological development (Darnerud 2003; see Figure 1-3). While the thyroid

plays a major role in neurological development, it also tightly regulates growth and

metabolic processes. The role of thyroid homeostasis in regulation of metabolism

suggests that significant alteration of the thyroid system may have long-reaching

consequences by modifying bioenergetic processes. Metabolic rate governs

developmental and growth patterns by determining the proportion of energy in yolk that

can be allocated to tissue formation after respiratory demands are met as well as the rate

at which this occurs. Thus, cellular and sub-cellular irregularities, such as modified

thyroid dynamics, can lead to overall metabolic anomalies which can therefore affect

bioenergetic processes regulating survival, energy storage, development, and growth

during embryonic and juvenile growth periods (Rowe et al. 1998, 2001a, and 2001b;

Congdon et al. 2001; Steyermark 2002; see Figure 1-3). Contaminant-induced reductions

in metabolic rates (Newman and Unger 2003) may modify rates of conversion of energy

stores to somatic tissues, influencing rates of differentiation and extending the embryonic

period, increasing the risks of mortality due to nest predators or microclimatic variations.

Conversely, a sustained elevation in metabolic rate can reflect inefficiency in metabolism

translating to reductions in growth per unit energy metabolized (Rowe et al. 2001a,

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2001b). Either an increase or decrease in metabolic rate via modified thyroid function or

induction of damage repair mechanisms (Calow 1991) is potentially deleterious to the

affected individuals.

A lack of information on PBDE effects on a variety of long-lived species presents

a critical data gap hindering understanding of the importance of chronic accumulation

and effects of PBDEs in many natural systems. Thus, development of models that

represent the types of processes that occur under natural exposure regimes is critical for

evaluating long-term ecological implications of PBDEs. Many vertebrates having long

lifespans, delayed maturation, and a high trophic position accumulate contaminants over

exceptionally long periods of time. Over an extended pre-reproductive period, high body

burdens of BDEs may accumulate and, upon maturation and reproduction, the

accumulated compounds may be transferred to developing offspring via milk and

placenta (mammals) or yolk (oviparous vertebrates), ultimately producing tissue burdens

that may result in toxic effects. Thus, to examine environmental risks of PBDEs, models

should reflect species that possess life history traits that confer them with a high potential

for bioaccumulation and maternal transfer (e.g., Rowe 2008).

The common snapping turtle (*Chelydra serpentina*) and the red-eared slider

(*Trachemys scripta elegans*) both possess life history and ecological traits that make them

exceptionally well suited to studies of persistent, bioaccumulative compounds. The

snapping turtle does not reach sexual maturity until 11 to 16 years of age (Congdon et al.

1994) and the red-eared slider 2 to 10 years (Cagle 1950) after hatching with average

clutch size ranging from 26 to 55 eggs for the snapping turtles (Congdon et al. 2008) and

from 6 to 12 for the red-eared sliders (Gibbons and Greene 1990). In addition, as

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juveniles, both species have carnivorous diets while as adults the snapping turtles remain carnivorous and the red-eared sliders switch to a more omnivorous diet, consuming high amounts of protein when available (Gibbons 1990; Parmenter and Avery 1990; Spotila and Bell 2008). These characteristics may lead to accumulation of persistent lipophilic compounds for long periods prior to reproduction, potentially resulting in maternal transfer of high concentrations to offspring. While the snapping turtle and red-eared slider have different phylogeny belonging to different families (Chelydridae and Emydidae respectively) they and other turtles are known to accumulate PBDEs (de Solla et al. 2007, 2008; Moss et al. 2009; van de Merwe et al. 2010), but resultant effects on embryonic, hatchling and juvenile health and fitness are unknown.

To date there have not been controlled laboratory studies conducted to determine specific effects of PBDE exposure in embryonic or juvenile turtles. However, other research studying contaminant effects on turtles have focused on both environmental (typically snapping turtles) and laboratory embryonic exposures (typically red-eared sliders) to various contaminants and resultant hatchling effects (Bishop et al. 1991, 1998; Crews et al. 1995; Willingham and Crews 1999, 2000; Willingham et al. 2000; Bell et al. 2006). The likelihood of adverse effects in wild populations seems great for turtles that can accumulate high concentrations of PBDEs. This is due to their high trophic status and propensity to accumulate and subsequently transfer persistent lipophilic contaminants to developing offspring [for example; (Stone et al. 1980; Burger and Garber 1995; Pagano et al. 1999; de Solla et al. 2007; Kelly et al. 2008)] asserting the importance of using turtles as models for PBDE-induced hormone disruption.

The widespread occurrence of PBDEs in the environment, their bioaccumulative properties, propensity for maternal transfer to offspring, and potential to elicit endocrine effects on developmental processes suggests that significant ecological ramifications of PBDEs could occur. Most laboratory models currently in use (laboratory rats, mice and fish) do not capture environmentally-relevant exposures representative of long-lived organisms that accumulate PBDEs over decades of exposure. Therefore long-lived model species such as turtles, having life spans approaching those of humans and many other vertebrates, provide excellent sentinel species for use in examining potential risks to environmental quality.

While there is strong evidence that PBDEs can disrupt the thyroid system, the exact mechanism by which the disruption occurs is less clear. This is not the case with perchlorate, another contaminant of concern, which is a known thyroid inhibitor with the mechanism of disruption well characterized. Increasingly, perchlorate and its salts have become contaminants of concern due to their stability and persistence in ground and surface water as well as widespread use in explosives, pyrotechnics, rocket fuel, missiles and some fertilizers (Urbansky, 1998; Sridhar et al. 1999). The extensive use of perchlorate in the aerospace, defense and chemical industries has led to significant contamination of water, soil, sediment and biota (vegetation, aquatic insects, fish, amphibians, and mammals) throughout the United States (Urbansky, 1998; Smith et al. 2001, 2004; Mayer 2006).

Perchlorate salts, such as ammonium perchlorate (NH_4ClO_4) and sodium perchlorate ($NaClO_4$), are highly soluble in water and once released into an aquatic system they readily dissociate producing the salt and perchlorate ions (Urbansky, 1998).

In vertebrates, ionic perchlorate competitively inhibits the thyroid gland from taking up iodide, ultimately reducing thyroid hormone production leading to reduction of glandular thyroxine as well serum concentrations of thyroxine (T_4) and triiodothyronine (T_3; Stanbury and Wyngaarden 1952; Saito et al. 1983; Wolff 1998). Alterations of thyroid hormone homeostatis have serious implications for animal health because the hormones play a role in regulating growth, embryonic and neurological development, and metabolism of lipids and proteins (Clark 2000).

In this dissertation the impacts of controlled embryonic exposure to BDE-47 and perchlorate on hatchlings, embryonic exposure to BDE-47 on juveniles as well as dietary exposure to BDE-47 and BDE-99 in juveniles of red-eared sliders and snapping turtles were assessed. The overall goal was to provide a comprehensive examination of the effects of common PBDEs based upon a suite of developmental, physiological, endocrinological and behavioral traits in order to make stronger inferences regarding the multiple processes that may be altered by PBDEs and the overall effects on offspring performance and quality. Specifically, the individual studies addressed impacts of BDE-47 and perchlorate on thyroid-mediated processes including embryonic and hatchling development and metabolic efficiency, as well as direct measurements of thyroid function, behavior, metabolic efficiency, growth, and development in juveniles. In addition, an initial, key project for elucidating these impacts was an assessment of the relative transfer efficiency of the two PBDE congeners among the two species. Data from these studies were also intended to assess the relative utility of the two turtle species as sensitive models for examining long-term effects of embryonic exposure to BDE-47 and dietary exposure of juveniles to BDE-47 and BDE-99.

Figures

BDE-47
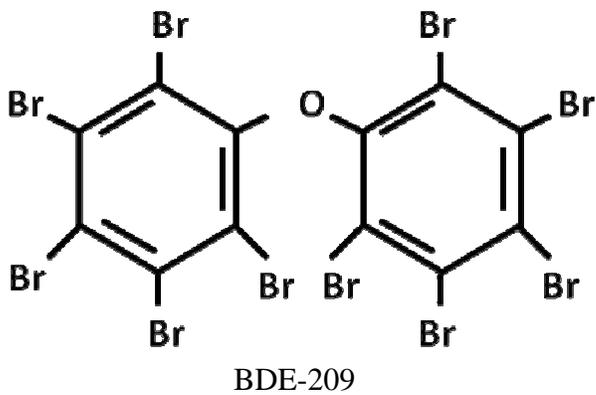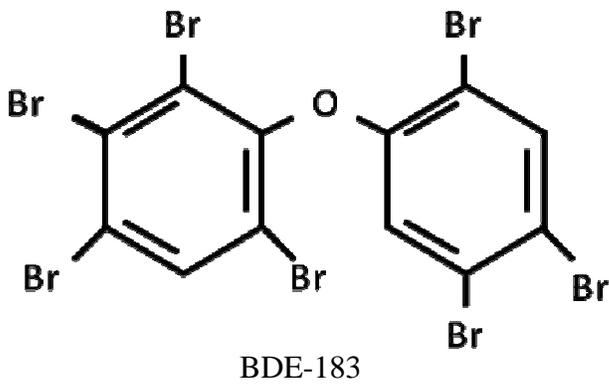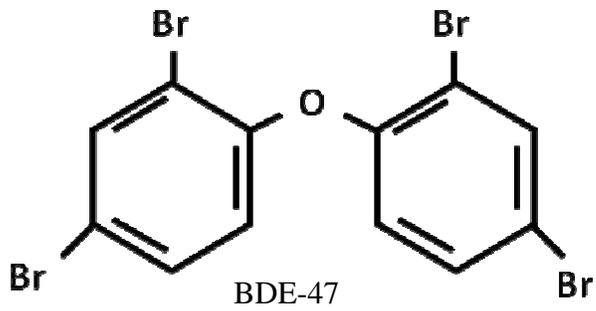
BDE-183

BDE-209

Figure 1.1. Examples of PBDE congeners representing the penta-BDE (BDE-47), octa-BDE (BDE-183), and deca-BDE (BDE-209) mixtures.
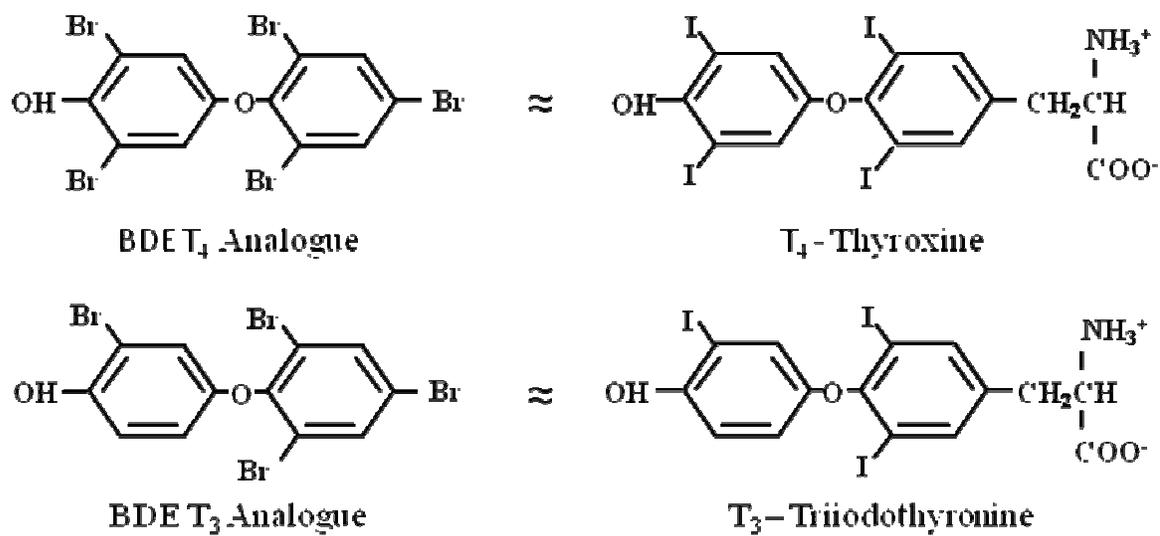
Figure 1.2. Structural similarities between potential BDE metabolites and thyroid hormones, thyroxine and triiodothyronine.
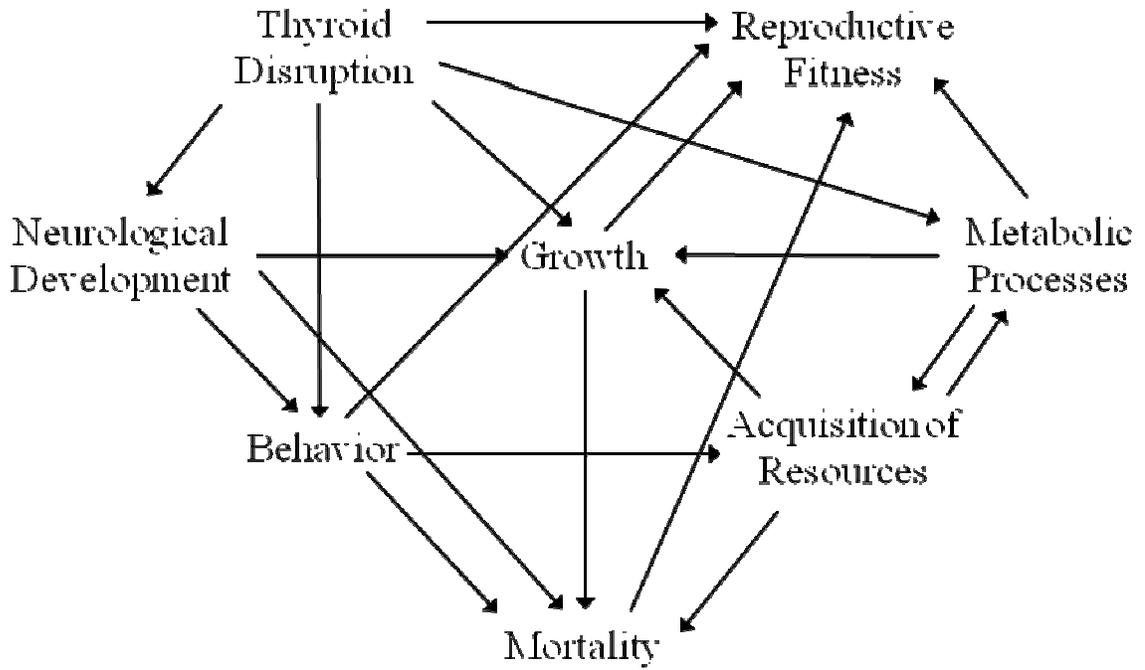
Figure 1.3. Diagram illustrating connections between disruption of the thyroid system and other developmental, physiological and behavioral traits potentially negatively impacted by exposure to PBDEs.

Chapter 2: Embryonic exposure to polybrominated diphenyl ethers BDE-47 and BDE-99 in red-eared sliders (*Trachemys scripta elegans*) and snapping turtles (*Chelydra serpentina*) via topical dosing of eggs

Abstract

Polybrominated diphenyl ethers (PBDEs) are a class of flame retardants that are bioaccumulative, persistent and toxic. These characteristics make PBDEs contaminants of concern, thus requiring the development of models to determine the effects resulting from exposure to the compounds. An important mechanism of exposure is through maternal transfer, which has been documented in reptiles in natural habitats. Simulating maternal exposure in a controlled and replicated manner to examine exposure-response relationships in turtles is challenging, as the rigid eggshell provides a barrier to delivery of contaminants to the embryo. However, topical dosing studies in which compounds were applied to the egg surface and allowed to diffuse into the egg have been shown to be an effective method of exposing embryos to some contaminants. This study demonstrates transfer of two PBDE congeners (BDE-47 and BDE-99), across the eggshell into the egg contents of the red-eared slider (*Trachemys scripta elegans*) and snapping turtle (*Chelydra serpentina*). After 22 days of exposure to either BDE-47 or BDE-99 it was found that BDE-47 had higher transfer efficiency than BDE-99 in the red-eared sliders (25.82 % $\pm$ 1.86 % vs. 9.87 % $\pm$ 1.08 %) and snapping turtles (31.30 % $\pm$ 1.57 % vs. 12.53 % $\pm$ 1.37 %) with transfer being greatest in the snapping turtles for both compounds. It appears that BDE-99 was debrominated to BDE-47 in red-eared slider

eggs, but not in snapping turtle eggs. This was evidenced by increased BDE-47

concentrations in the egg contents of the red-eared slider eggs topically dosed with only

BDE-99. The efficacy of topical dosing for administering desired embryonic exposures

is clearly affected by the chemical properties of the individual compounds, and was more

successful for BDE-47 in both species.

Introduction

Polybrominated diphenyl ethers (PBDEs) are compounds that are added to a

variety of products to reduce flammability, but due to their extensive use, as well as their

bioaccumulative and toxic properties the two lower brominated penta-BDE and octa-

BDE mixtures have been banned from use and production (Directive 2003/11/EC; Tullo

2003; Ward et al. 2008). The only mixture currently remaining in use is the deca-BDE

mixture which is primarily composed of the fully brominated BDE-209. This mixture,

however, is scheduled to be phased out of use and production due to similar concerns of

bioaccumulative properties and toxicity (BSEF 2010). While use of PBDEs has declined

they are still widely being detected in aquatic and wildlife samples. The lower

brominated congeners, including BDE-47, -99, -100 and -153, are most widely detected

and usually in the greatest quantity with this potentially attributed to metabolism of the

higher brominated congeners through debromination (Law et al. 2003; Hites 2004;

Letcher et al. 2010). There is evidence in several different species including fish, rats,

and birds that reductive debromination is a potential pathway for metabolism of PBDEs

(Stapleton et al. 2004a, 2004b, 2004c; Huwe and Smith 2007; van den Steen et al. 2007;

Browne et al. 2009; McKernan et al. 2010; Noyes et al. 2010; Roberts et al. 2011). The

environmental persistence of PBDEs as well as metabolism to the more bioaccumulative and toxic congeners suggests that they are likely to remain contaminants of concern despite the recently imposed restrictions on use. Therefore, there is a need for additional animal models that can provide information on potential effects resulting from major pathways of exposure to bioaccumulative and persistent compounds such as PBDEs.

The common snapping turtle (*Chelydra serpentina*) and the red-eared slider (*Trachemys scripta elegans*) possess life history and ecological traits that make them exceptionally well suited to studies of persistent, bioaccumulative compounds. The snapping turtle does not reach sexual maturity until 11 to 16 years of age (Congdon et al. 1994) while the red-eared slider can require up to 10 years to reach maturity (Cagle 1950), providing both species the potential to accumulate persistent lipophilic compounds for long periods prior to reproduction. This chronic bioaccumulation can result in the maternal transfer of those compounds to their offspring during embryogenesis. Several studies have documented maternal transfer of contaminants to eggs in turtles as well as resultant effects on embryonic, hatchling and juvenile health and development (Bishop et al. 1991, 1998; Bell et al. 2006; de Solla et al. 2008; Kelly et al. 2008; Eisenreich et al. 2009). Exposure and effects of contaminants resulting from maternal transfer are important to characterize since the embryonic stages of development represent a sensitive and key life stage that, if altered, could have impacts of hatchling and juvenile health and development.

Quantifying impacts of specific contaminants on embryos is difficult in environmental settings as natural habitats often contain complex chemical mixtures. To overcome such complexities, controlled exposures in replicated laboratory studies are

required. In some cases, exposures of captive adults to contaminants and subsequent assessment of their offspring has been used to provide a more controlled but natural maternal transfer. Typically these types of studies use species with short reproductive cycles or are conducted where there is access to captive populations or colonies of the organisms (Hammerschmidt et al. 2002; Rauschenberger et al. 2004; Fernie et al. 2005b, 2006; Albers et al. 2007). However, individual variations in physiological traits among adults can bring about considerable variability in transfer of contaminants to offspring, making establishment of exposure-response relationships tenuous.

Alternatives to natural maternal transfer studies that can eliminate potential clutch affects have been to provide controlled doses to the eggs directly via injection or to employ topical egg dosing techniques in which the compound is applied directly to the egg surface and allowed to passively diffuse through the shell. Egg injections have been successfully employed in avian embryonic exposure studies with various contaminants (e.g., Hill and Hoffman 1990; Hoffman et al. 1996; Fernie et al. 2005b, 2006; Heinz et al. 2009; McKernan et al. 2009, 2010). In contrast, injection of reptilian eggs has frequently resulted in very high embryonic mortality rates ranging from approximately 44 % - 79 % (Gutzke and Bull 1986; Bull et al. 1988; Crews et al. 1991; Muller et al. 2007a), suggesting that the reptilian egg is far more sensitive to the physical damage associated with injection relative to the avian egg. Schnars and colleagues (2011) reported the highest hatching success after injection at 61 % in snapping turtle eggs harvested directly from the oviducts of adult females, which is much lower than studies employing topical dosing techniques. Compared to reptile egg injection techniques, topical dosing methods do not have such a negative impact on hatching success as shown in several studies

reporting greater than 85% hatching success of the controls (Wibbels and Crews 1991; Muller et al. 2007a, 2007b; Chapter 3).

While topical dosing has been shown to be less detrimental to reptile embryos than egg injections, only a few studies have verified the percentage of the topically applied dose (transfer efficiency) that ultimately is incorporated into the egg contents, an issue that needs to be addressed to determine exposure and effect relationships (see Muller et al. 2007b). This study sought to quantify transfer of two polybrominated diphenyl ethers of different molecular weights and log K_{OWs} (BDE-47 and BDE-99, 485.8 g/mol and 6.81 and 564.7 g/mol and 7.32 respectively) from the eggshell into the egg contents of red-eared sliders and snapping turtles to validate the method for embryonic exposure and effect studies of PBDEs. In addition, the concentrations of the two congeners on the eggshells and in the egg contents analyzed with and without the chorioallantoic membrane were determined in an attempt to determine the location of the topically applied dose and to determine species and congener differences.

Methods

Red-eared sliders and snapping turtle eggs were collected from Concordia Turtle Farm in Wildsville, LA. All eggs for both species were laid on May 23rd with 41 eggs from 15 red-eared slider clutches and 53 eggs from seven snapping turtle clutches used in this study. Upon collection, each egg was given a number, written on the upper surface of the egg in number 2 pencil, in order to identify each individual. Eggs were also weighed, measured for diameter (snapping turtle eggs) and length and width (red-eared slider eggs) after which they were immediately placed in shallow bins containing damp

vermiculite, mixed with water in a 1:1 ratio. The eggs were maintained at a cool temperature (approximately 18 ºC) until June 12th when incubation at 26 ºC, a temperature known to produce only males (Yntema 1976; Wibbles et al. 1991), began. Moisture and humidity were maintained by misting the eggs and nest substrate with water at two to three day intervals. Previous research has found that within-clutch variation in contaminant concentrations in the egg contents is very low (Bishop et al. 1994) thus, prior to incubation two eggs from each clutch were randomly selected and egg contents homogenized for contaminant analysis of background concentrations of PBDEs. All procedures were approved by the University of Maryland Center for Environmental Science Institutional Animal Care and Use Committee (S-CBL-07-04).

Dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburg, PA, USA) was used as a vehicle for the topical application of BDE-47 and -99 to the upper surface of the eggshells for both species, as it has been widely employed in reptile topical dosing studies and repeatedly shown to have very little toxicity to reptilian embryos (Crews et al. 1991; Wibbels and Crews 1992; Wibbels et al. 1993; Bergeron et al. 1994; Crain et al. 1997; Prodreka et al. 1998; Willingham and Crews 1999, 2000; Willingham et al. 2000; Gale et al. 2002). Working stock solutions for both BDE congeners were prepared by adding neat BDE-47 or -99 (Accustandard Inc., New Haven, CT, USA) to DMSO followed by further dilution of the working stock in DMSO to prepare the topical dosing solutions for both congeners and species to achieve a target embryonic exposure of 40 ng/g for each egg. The concentrations of the doses for each species were calculated based on a 20 % transfer rate of BDE-47 and -99 across the eggshell and chorioallantoic membrane into the embryo as determined in a previously conducted pilot study

(Eisenreich, unpublished data). Doses for each species were further adjusted for the average egg mass (red-eared sliders-11.56 g and snapping turtles-14.45 g). All dosing solutions were analytically verified (see below). Prior to the start of incubation, eggs from each clutch were randomly assigned to either the BDE-47 or -99 treatments for both species. Initial sample sizes for dosed eggs were 20 and 21 red-eared slider eggs and 27 and 26 snapping turtle eggs topically dosed with BDE-47 and -99 respectively.

Solutions were topically applied to the vascularized upper surface of the eggshell in 5 µl volumes over a period of 8 days to avoid an acutely toxic embryonic exposure starting on the first day of incubation for a total applied solution volume of 40 µl . The range in egg masses for the red-eared sliders and snappers was wide (7.93 g -16.26 g and 10.72 g -18.55 g respectively) thus the dose applied to each egg was adjusted for individual egg mass to prevent drastically under- or over-exposing the embryos of eggs in a given treatment. To account for the variation in mass, the eggs were categorized into one gram mass classes and dosing solution volumes were adjusted accordingly for each class. The dosing solution was then administered each day in 5 µl volumes until the entire dose was applied. For the largest egg mass class, 8 days of dosing were required to administer the entire dose resulting in a total applied volume of 40 µl, and all smaller mass classes received an equal volume, but of a more dilute BDE solution.

After 8 days of dosing, eggs were incubated for 14 days after which they were frozen (approximately 55 days from hatching) for analysis of BDE-47 and -99 on the eggshell and in the contents (yolk and albumin). Prior to extraction, eggs were thawed and contents removed from the shell. For 10 eggs from each treatment and species the chorioallantoic membrane was extracted along with the shell and for an additional 10

eggs the chorioallantoic membrane was extracted along with the egg contents. This was done to estimate the concentration of the two BDE congeners in the chorioallantoic membrane, as the membrane alone did not contain enough tissue mass to extract on its own for each egg.

Total PBDEs (34 congeners) were analyzed in two homogenized eggs collected prior to incubation for background concentrations of PBDEs, representing each clutch of eggs from each species. In addition, eggshells and contents were analyzed for BDE-47 and -99 for estimation of transfer efficiency of the two congeners across the eggshell and chorioallantoic membrane for both species. Individual egg contents were homogenized and the eggshells cut into small pieces using a small surgical scissors after which water from the samples was removed by adding sodium sulfate and grinding to further homogenize the samples using a ceramic mortar and pestle. All samples were then extracted using accelerated solvent extraction (ASE 300; Dionex) with dichlormethane. Samples were packed atop deactivated alumina in stainless steel extraction cells to remove potential interfering lipids and other polar compounds. Prior to extraction, ^{13}C-BDE-15 and ^{13}C-BDE-118, surrogate standards were added to each extraction cell for calculation of analyte recoveries. Extracts were then concentrated and subjected to purification using deactivated Florisil® column chromatography for removal of nonpolar interferences. A sodium sulfate blank sample was run concurrently with each set of extractions as a measure of quality assurance for measurement of any laboratory contamination during the extraction and purification procedures.

Dosing solutions were diluted in hexane with BDE-47 and -99 concentrations directly determined along with all extracted egg contents and shell sample BDE

concentrations using a gas chromatograph (Agilent 6890N) coupled to a mass-selective

detector (Agilent 5973N) operated in negative chemical ionization mode. Prior to

analysis, ^{13}C-CDE-86 (2,2',3,4,5-pentachlordiphenyl ether) and ^{13}C-BDE-209 were

added as internal standards to all samples and calibration standards. All BDE standards

were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), Wellington

Labs (Guelph, Ontario, Cananda), and Accustandard (New Haven, CT, USA) or received

from the U.S. National Institute of Standards and Technology (NIST; Githersburg, MD,

USA). The programmable temperature vaporization (PTV) injector was used in pulsed

splitless mode with 5 µl injections and a 15 m DB-5MS column (J&W Scientific,

Folsom, CA, USA) having an inner diameter of 0.25 mm and 0.1 µm film thickness.

Instrument program specifications follow those methods routinely used in our laboratory

(see Klosterhaus and Baker 2010). The mass fragments *m/z* -79 and -81 were monitored

for di- to octa-BDEs, -487 and -409 for the nona-BDEs and BDE-209, -318 and -316 for

[13]C-CDE-86, and -495 and -415 for ^{13}C-BDE-209 for quantitative and qualitative ions,

respectively.

Three times the analyte mass in the laboratory blanks divided by the mass of the

sample extracted was determined to be the method detection limit (MDL) for all analytes.

Mean recoveries (± 1 standard error) for BDE surrogate standards ^{13}C-BDE-15 and [13]C-

BDE-118 in red-eared slider (n=15) and snapping turtle (n=7) eggs used for background

concentrations were 120.67 % ± 4.48 % and 118.50 % ± 5.33 % as well as 117.53 % ±

6.72 % and 102.35 % ± 6.32 % respectively, all of which were abnormally high. Mean

recoveries for eggshells were 86.98 % ± 2.51 % and 95.59 % ± 2.61 % for the red-eared

slider and 86.83 % ± 2.28 % and 92.82 % ± 2.10 % for the snapping turtle. Lastly,

recoveries for the red-eared slider contents of dosed eggs were 89.17 % ± 1.97 % and 99.36 % ± 2.21 % as well as 86.14 % ± 1.94 % and 95.34 % ± 2.17 % for the snapping turtle dosed egg contents.

All data were analyzed using Minitab® (Minitab Inc., version 15). BDE-47 and -99 concentrations on the eggshell and in the contents of eggs for both species as well as the transfer efficiencies defined as the percent ng in the egg contents were analyzed using analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. Analysis of the chorioallantoic membrane BDE-47 and -99 content was completed using 2 sample t-tests to comparing differences in membrane contributions to egg content and shell concentrations for each species and congener. Statistical significance was judged based upon a type I error rate of $\alpha \square 0.05$. Prior to statistical analyses, data were tested and verified that they met the assumptions of the statistical model using Levene's test for homogeneity of variance and Shapiro–Wilk (W) statistic for normality in distribution employing log transformations as necessary with the exception of BDE-47 and -99 content in the shell for which the data were rank transformed (Conover and Iman 1981).

Results

Background Concentrations and Dosing Solutions

Background concentrations of total PBDEs detected in the eggs were extremely low with the snapping turtle eggs having a greater number of detectable congeners and higher concentrations than the red-eared slider eggs. A total of 15 clutches of red-eared slider eggs were analyzed for background concentrations with total PBDE (mean ± SE) concentration of 0.324 ± 0.073 ng/g wet weight (ww). Only BDE congeners -100, -153,

and -154 were detected in all red-eared slider eggs. Additional congeners detected were -183 in 12 eggs, -99 in three eggs while -47, -196, -197, -198/203, and -204 were detected in one egg. In the seven clutches of snapping turtle eggs analyzed for background PBDEs, total PBDEs was 4.614 ± 1.124 ng/g ww with congeners -47, -99, -100, -153, -154, and -155/85 detected in all eggs. Congeners -28/33 and -183 were detected in two eggs while -138 was only detected in one egg. The single red-eared slider egg that contained BDE-47 had a concentration of 0.137 ng/g ww whereas the mean background concentration of BDE-47 in snapping turtle eggs was 1.354 ± 0.338 ng/g ww, 10 times higher than contained in the single red-eared slider egg. Similarly, mean BDE-99 in the three red-eared slider eggs it was detected in was 0.069 ± 0.002 ng/g ww compared to 0.769 ± 0.386 ng/g ww in the snapping turtle eggs. Overall, the background concentrations of both BDE-47 and -99 in egg contents for both species were much lower than the target concentrations in the contents of dosed eggs (40 ng/g).

Red-eared slider BDE-47 and -99 dosing concentrations were analytically verified to be 63.67 ng/µl and 70.39 ng/µl respectively, lower than the calculated concentrations (77.07 ng/µl) needed to achieve the target 40 ng/g in the egg contents over the 8 day dosing period. BDE-47 and -99 dosing concentrations for the snapping turtle were verified to be 104.31 ng/µl and 111.10 ng/µl respectively, higher than the calculated concentrations (96.33 ng/µl). There were no other PBDE congeners detected in the dosing solutions.

BDE-47 and BDE-99 in Egg Contents and Eggshells

Overall, regardless of dose, the egg contents contained higher concentrations of BDE-47 than BDE-99 in both species. Specifically, BDE-47 concentrations in egg contents of snapping turtle eggs dosed with BDE-47 were significantly greater than BDE-47 in egg contents of red-eared slider egg contents ($p<0.0001$; Figure 2-1). In addition, BDE-47 concentrations in snapping turtle eggs dosed with BDE-47 were significantly greater than the BDE-99 in red-eared slider and snapping turtle eggs dosed with BDE-99 (all comparisons $p<0.0001$; Figure 2-1). Similarly, BDE-47 concentrations in red-eared slider eggs dosed with BDE-47 were significantly greater than BDE-99 concentrations in contents of eggs from both the red-eared slider and snapping turtle dosed with BDE-99 ($p<0.0001$ and $p=0.0069$ respectively; Figure 2-1). While not significant, BDE-99 concentrations in red-eared slider egg contents seemed lower than BDE-99 in snapping turtle egg contents in eggs dosed with BDE-99 ($p=0.0546$; Figure 2-1).

Concentrations of BDE-47 and -99 on the eggshells of both species were reversed from differences found in the egg contents. Across species and congeners, BDE-47 concentrations were lowest on red-eared slider eggshells and significantly lower than BDE-47 dosed snapping turtle eggs ($p=0.0412$; Figure 2-1) and BDE-99 dosed eggs of both species (both comparisons $p<0.0001$; Figure 2-1). Likewise, snapping turtle eggs dosed with BDE-47 had significantly lower concentrations than BDE-99 on eggshells of both species dosed with BDE-99 (both comparisons $p<0.0001$; Figure 2-1). Finally, concentrations of BDE-99 on red-eared slider eggshells were significantly lower than BDE-99 on eggshells of snapping turtles dosed with the same congener ($p=0.0004$; Figure 2-1).

Transfer Efficiency Comparisons

Transfer efficiency, calculated as the percent of administered compound detected in the egg contents was compared across treatments and species. Percent transfer of BDE-47 across the eggshell was similar for both the red-eared sliders and snapping turtles as was the case for the transfer of BDE-99 (BDE-47: 25.82 ± 1.86 % vs. 31.30 ± 1.57 %; BDE-99: 9.87 ± 1.08 % vs. 12.53 ± 1.37 %, slider vs. snapping turtle respectively). However, transfer of BDE-47 was lower in the red-eared sliders than the snapping turtles, but not statistically significant ($p=0.0536$; Figure 2-2). In contrast, there was significantly greater transfer of BDE-47 over the eggshell than BDE-99 in the red-eared sliders ($p<0.0001$), and in the snapping turtles ($p<0.0001$; Figure 2-2).

Impacts of the Chorioallantoic Membrane

The chorioallantoic membrane did not appear to act as an accumulator of BDE-47 or BDE-99 or a barrier to transfer from the shell to the egg contents of either congener. Only red-eared slider eggs dosed with BDE-47 showed a significant difference in concentrations when comparing samples of egg contents with the chorioallantoic membrane included to the samples without the chorioallantoic membrane (443.93 ± 39.70 ng/g ww and 536.73 ± 63.90 ng/g ww, respectively). The contents analyzed with the chorioallantoic membrane included in eggs dosed with BDE-47 had significantly lower concentrations of BDE-47 than contents without the chorioallantoic membrane possibly indicating that the membrane is diluting the BDE-47 concentrations in the egg contents ($p=0.042$). Similar comparisons were made with the eggshells and it was found

that snapping turtle eggshells dosed with BDE-47 and-99 had significantly lower concentrations of both congeners when the chorioallantoic membrane was analyzed with the shell compared to the shell alone ($p=0.002$ and $p=0.007$ respectively). This finding also suggests dilution of BDE-47 and BDE-99 in the tissue when the chorioallantoic membrane was added.

Total Dose Accounting

The entire eggshell was analyzed for BDE concentrations, while 3 g egg aliquots of egg contents were analyzed. To gain an estimation of the total egg content mass, the shell mass was subtracted from the total egg mass and this was used to convert the BDE concentrations in the egg contents to total ng of BDE in the contents (ng/content). With this estimation of total ng of BDE in or on the egg, it was determined that for both compounds and species, less than 45 % of the total dose topically applied to the eggs could be accounted for. Specifically, only 30.63 % $\pm$ 2.43 % of the total dose of BDE-47 could be accounted for in the red-eared slider eggs and shells. This was not significantly different from the 28.44 % $\pm$ 1.81 % accounted for of the total BDE-99 dose applied to red-eared slider eggs ($p=0.7685$). However, the percent of BDE-47 accounted for in the red-eared slider eggs was significantly less than the amount accounted for in both BDE-47 and -99 dosed snapping turtle eggs (37.16 % $\pm$ 1.29 %, $p=0.0293$; 43.75 % $\pm$ 1.92 %, $p<0.0001$). The percent of BDE-99 accounted for in red-eared slider eggs was also significantly less than the two congeners in snapping turtle eggs ($p=0.0013$ and $p<0.0001$). There was no statistical difference in the percent of BDE-47 and -99

accounted for in snapping turtles eggs topically dosed with the two congeners (p=0.1842).

The red-eared sliders dosed with BDE-99 had the lowest percentage of the total dose accounted for. There were also substantial concentrations of BDE-47 detected in the egg contents of these eggs dosed with BDE-99 (2.699 ± 0.650 ng/g ww). The BDE-47 detected cannot be accounted for by the background concentrations as BDE-47 was non-detectable in the contents of eggs analyzed for background concentrations. In addition, the BDE-99 concentrations in the egg contents showed a positive and significant relationship with the concentrations of BDE-47, although only 25.7 % of variation was explained by the model (p=0.022; r^2=0.257; Figure 2-3). In contrast, the BDE-47 concentrations in the egg contents of snapping turtles dosed with BDE-99 were lower than the concentrations recorded in the background eggs (0.722 ± 0.093 ng/g ww vs. 1.354 ± 0.338 ng/g ww) and there was no relationship in BDE-99 and BDE-47 concentrations in the egg contents (p=0.366).

Discussion

Studies using topical egg dosing techniques for exposing reptile embryos to chemicals have often either not quantified transfer across the eggshell into the egg contents, or have done so in too few samples to achieve good characterization of variability. However, topical dosing techniques serve as a viable option for controlled embryonic exposure studies of dose-response relationships so long as exposure is verified. While it is known that a low percentage of the total compound placed on top of the egg is transferred to the contents, the total dosing concentration applied can be

calibrated to achieve an amount in the egg contents at concentrations found in environmental samples and/or those suspected to cause adverse effects (Podreka et al. 1998; Portelli et al. 1999; Gale et al. 2002).

Egg injections are a common embryonic exposure technique employed in studies of avian species, but these techniques have resulted in high incidences of embryonic mortality in crocodilian or chelonian eggs (Gutzke and Bull 1986; Bull et al. 1988; Crews et al. 1991; Muller et al. 2007a). Mortality in reptilian eggs following injection is likely due to fungal or bacterial infections as well as physical damage to the inner layers of the eggshell that may not re-seal following injection (reviewed in Muller et al. 2007b). Schnars et al. (2011) employed an injection technique in snapping turtle eggs that resulted in the highest reported hatching success after injection (61 %) when eggs were injected shortly following removal from the oviducts of the female. While this technique improved hatching success relative to other injection studies with reptile eggs, it is still much lower than hatching success (typically above 85 %) achieved when using topical dosing techniques (see Chapter 3). In addition, the technique employed by Schnars and co-workers (2011) requires the sacrifice of adult female snapping turtles, which can be problematic due to the large number of females that could be required for statistically adequate sample sizes.

Findings from this study indicate that PBDE congeners -47 and -99 are transferred across the eggshell and chorioallantoic membrane into the egg contents following our protocol. Transfer efficiency was higher for BDE-47 than BDE-99 in both species, but generally low (BDE-47: slider-25.85 %, snapping turtle-31.30 %; BDE-99: slider-9.87 %, snapping turtle-12.53 %). These transfer efficiencies are within the range reported for

the few other reptilian topical dosing studies in which transfer of other compounds was quantified. Gale and co-workers (2002) reported 4 % of total 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) and 10 % of total PCB-126 topically applied to the egg crossing the eggshell into the egg contents over a 16 day period during the middle of incubation in red-eared slider eggs. A shorter time period for transfer (72 hours) resulted in 1.6-20 % transfer of *p,p*-DDE across the eggshell of snapping turtles (Portelli et al. 1999), whereas a longer time period (49 days) resulted in approximately 33 % transfer of *p,p*-DDE across the eggshell of the green sea turtle (*Chelonia mydas*; Podreka et al. 1998). Similarly, less than 2 % transfer occurred in alligator (*Alligator mississippiensis*) eggs topically exposed to DDE, dieldrin and chlordane for 14 days (Muller et al. 2007a). Interestingly, in several species of birds, eggs injected with a penta-BDE mixture (DE-71) that includes both BDE-47 and -99 only 18.8-29.6 % of the total injected amount was absorbed into the egg contents by date of pipping (26 days of incubation; McKernan et al. 2010).

Transfer efficiency was higher for BDE-47 than for BDE-99 in both species, but there also appeared to be a difference in transfer between the two species. Although not statistically different, the observed pattern of greater transfer efficiency of BDE-47 compared to BDE-99 is likely due to the greater molecular size of BDE-99 (564.7 g/mol vs. 485.8 g/mol) or larger log K_{OW} (7.32 vs. 6.81) preventing it from being as readily transferred through the eggshell pores. Lower absorption of the higher brominated congeners was also described by McKernan and co-workers (2010) who injected chicken, mallard and American kestrel eggs with either a penta-BDE mixture (DE-71) or an octa-BDE mixture (DE-79) and found significantly less DE-79 in the egg contents. In

addition, they observed preferential uptake of the lower brominated congeners across the incubation period in the eggs injected with DE-71 (McKernan et al. 2010).

Differences in transfer of the same congener between species are less easily explained. The eggshells and chorioallantoic membranes of both species have similar compositions with eggshells having similar distributions and sizes of pores (described in Packard et al. 1982), thus it is unlikely that species differences in shells or chorioallantoic membranes would explain transfer efficiency differences. The red-eared slider egg, due to its oval shape, has a larger surface area to spread out the multiple topical doses compared to the round snapping turtle egg suggesting potential for higher transfer across the eggshell of the red-eared slider. However, it is unclear why transfer was higher across the snapping turtle egg, especially for BDE-47.

The chorioallantoic membrane did not seem to retain either BDE-47 or BDE-99. In this study, chorioallantoic membranes were analyzed either with the eggshell or egg contents to obtain an estimate of how much BDE may be retained in the membrane. Egg contents analyzed without the chorioallantoic membrane had slightly higher concentrations of the two BDE congeners than egg contents analyzed with the membrane for both species. This suggests that inclusion of the chorioallantoic membrane may have diluted the concentrations of the congeners in the egg contents. Of the prior studies that measured transfer across the eggshell after topical application, none reported concentrations in the membrane or indicate if the membrane was analyzed with shell or egg contents. However, McKernan and colleagues (2010) analyzed pooled air cell membranes collected from chicken eggs injected with DE-71 and determined that 4.3 % of the injected dose was associated with the membrane. They also estimated that an

additional 32 % of the administered dose could potentially be associated with the inner membrane of the chicken egg (McKernan et al. 2010). These observations suggest that a large concentration of the injected dose remains associated with the membranes. However, results from the present study suggest otherwise for PBDEs retention in the turtle chorioallantoic membrane.

For both congeners in both species, less than 45 % of dose applied to the egg was accounted for. There are several possible explanations for the loss of the remaining 55 % of the dose. It was observed that during the eight days of dosing that the DMSO absorbed moisture from the air in the incubation chamber. This absorption of water caused the total volume of liquid on the egg to increase and occasionally roll off the egg. In these instances, a portion of the dose was potentially lost from the eggshell. In addition, it is possible over the 22 days of incubation that the PBDE remaining on the eggshell volatilized. A third possibility for PBDE loss from the eggshell is due to handling of the egg and eggshell for storage and analysis, potentially removing a portion of the dried dose off the eggshell.

An additional mode of loss, metabolism of the parent compound, cannot be ruled out as a potential mechanism of loss in red-eared slider eggs. While it was not within the scope of this study to characterize metabolites of BDE-47 and -99 such as methoxylated or hydroxylated compounds, it was observed that egg contents of red-eared sliders (but not snapping turtles) that had been dosed only with BDE-99 contained a significant concentration of BDE-47 above the background concentration, suggestive of reductive debromination of BDE-99 to BDE-47 *in ovo*. McKernan et al. (2010) reported the presence of several PBDE congeners in the egg contents of several species of birds that

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were injected with DE-71 but were not detected in the dosing solution or control eggs. While early embryonic development processes of red-eared sliders and snapping turtles are similar (Yntema 1968; Greenbaum 2002), there are likely species differences in enzymatic systems potentially responsible for differential capacities for debromination, as has been shown in fish exposed to BDE-99 (Browne et al. 2009; Roberts et al. 2011). While several studies have shown debromination of BDE-99 to BDE-47 in carp, other species such as Chinook salmon and rainbow trout do not possess the same route of metabolism (Stapleton et al. 2004a, 2004b; Browne et al. 2009; Roberts et al. 2011; Noyes et al. 2010). Deiodinase enzymes (DIs), which are responsible for removal of iodine atoms from thyroid hormones resulting in activation or deactivation of the hormones, have been implicated as the primary enzymatic system for debromination of BDE-99 to BDE-47 in fish, particularly in carp (Stapleton et al. 2004c; Benedict et al. 2007; Browne et al. 2009; Noyes et al. 2010; Roberts et al. 2011). There is preliminary data suggesting higher DI activity in red-eared sliders than in snapping turtles providing evidence for a greater capacity for reductive debromination in red-eared sliders (Hugenberger 1997). Glutathione-s-transferase (GST) which is responsible for phase II conjugation processes through dehalogenation reactions, have also been reported to debrominate PBDEs (Hakk and Letcher 2003), but are less likely to be involved in debromination in fish (Noyes et al. 2010; Roberts et al. 2011). While it is possible that there could be differences in the enzymatic systems of the embryonic red-eared slider and snapping turtle that would result in differential debromination capacities, further study is required to specifically assess the extent of debromination in the two species and the mechanisms by which it occurs.

Conclusion

Topical dosing techniques used in this study were successful in achieving target concentrations of BDE-47 in the egg contents of both red-eared sliders and snapping turtle. However, topical dosing of BDE-99 resulted in lower than predicted concentrations in the egg contents, likely due to the larger molecular size of the compound relative to BDE-47. BDE-99 concentrations were lower in the red-eared sliders compared to snapping turtles, perhaps due to metabolism of the parent compound by the red-eared slider embryos. It cannot be assumed that the dose topically applied to the shell is equal to the exposure to the embryo. Natural maternal transfer of contaminants occurs through different timing and mechanisms from embryonic exposure through the use of topical dosing techniques, but a controlled study employing replicated concentrations through natural maternal transfer would be extremely difficult to achieve in turtles. While topical dosing studies cannot replicate the timing and mechanisms of exposure that would naturally occur via maternal transfer, such studies can provide for controlled and repeatable exposures necessary for establishing dose-response relationships for studies of contaminant effects on embryonic turtles.
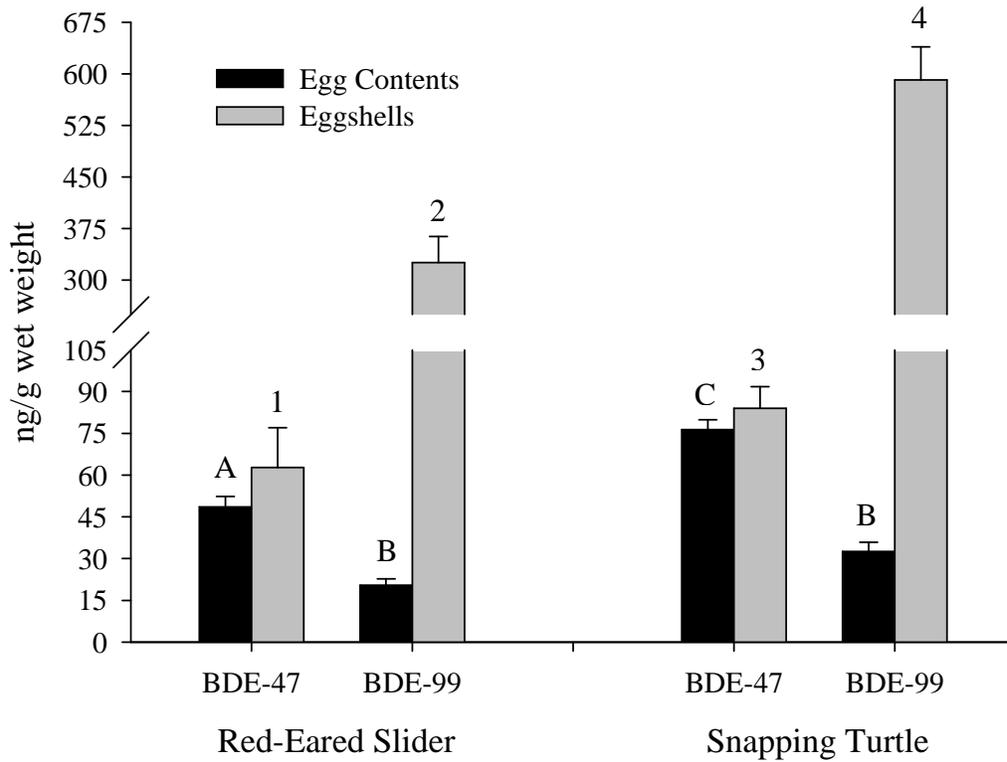
Figure 2-1. Concentrations of BDE-47 and BDE-99 (ng/g ww) in egg contents and shells of red-eared sliders and snapping turtles (mean ± 1 SE; n=20). Comparisons were done across the two congeners and species for the contents and eggshells separately. Different letters signify significant differences within the egg contents and different numbers indicate significance within the eggshells.
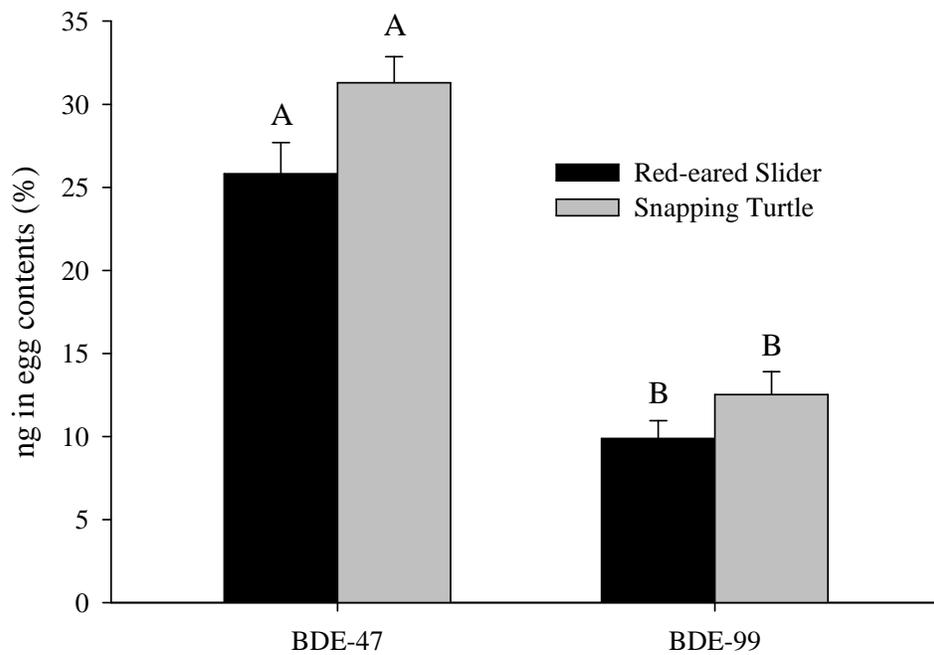
Figure 2-2. Percent transfer of BDE-47 and BDE-99 across the eggshell into the egg contents represented by the ng in the egg contents as a percentage of the total ng topically applied to each egg (mean ± 1 SE; n=20). Different letters signify significant differences.

$$[\text{BDE-47}]_{\text{Contents}} = -0.2071 + 0.143*[\text{BDE-99}]_{\text{Contents}}$$
$$R^2 = 0.257, p=0.0225$$
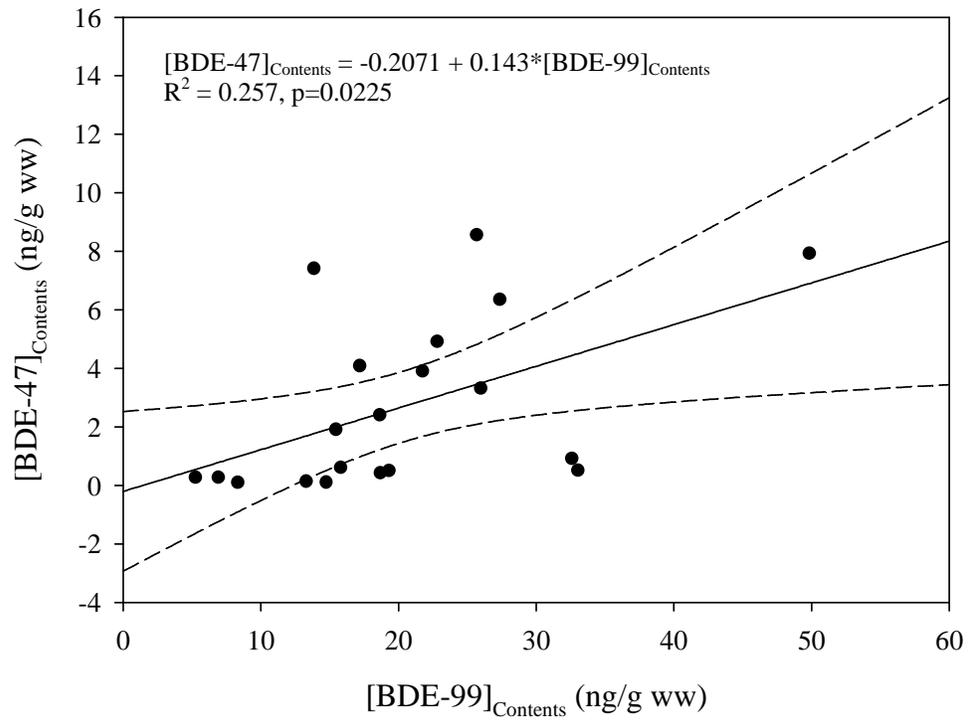
Figure 2-3. Relationship between BDE-99 concentrations (ng/g ww) in red-eared slider eggs topically dosed with BDE-99 and BDE-47 concentrations (ng/g ww) in the same egg contents. Dotted lines indicate 95 % confidence interval (n=20).

Chapter 3: Effects of embryonic exposure to BDE-47 in hatchling and juvenile red-eared sliders (*Trachemys scripta elegans*) and common snapping turtles (*Chelydra serpentina*)

Abstract

Red-eared slider (*Trachemys scripta elegans*) and snapping turtle (*Chelydra serpentina*) embryos were exposed *in ovo* to five concentrations of BDE-47 (40 ng/g-1000 ng/g) to assess impacts on embryonic, hatchling and juvenile development, growth, metabolism, behavior, and impacts on glandular thyroxine (T_4). Topical application of BDE-47 led to higher concentrations 6 months post exposure (MPE) in snapping turtle whole body tissues compared to red-eared sliders and in both species the whole body concentrations increased with increased applied dose. Hatching success, incubation time, hatchling size, and external yolk size at hatching were not affected by the treatments in either species. No significant differences in standard metabolic rate (SMR) were detected for either species at 3 MPE, but there were patterns of elevated SMR in both species suggestive of a biological response. Liver weights were significantly increased in exposed snapping turtles while there were no differences on thyroid weights or total glandular T_4 in hatchlings of either species. While growth was unaffected in the juveniles, masses of snapping turtles were significantly reduced in the treated animals compared to the controls following overwintering. Other measurements of development and health of juveniles such as size at necropsy (16 MPE), standard metabolic rate, and liver size did not suggest impacts on either species. While behavioral response to the presence of a simulated predator was not affected in either species, the righting response (time to

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recover from an inverted position) was delayed in exposed red-eared sliders. There were also patterns suggesting reduced thyroid size and glandular T_4, but statistical differences were not found in these data for either species. These results suggest that embryonic exposure to BDE-47, at the levels used in this study, may have subtle impacts on the thyroid endocrine system, metabolism, and neurological development. However, the observed patterns require further investigation to determine their potential significance to overall health or performance.

Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of persistent, mobile, and bioaccumulative compounds that are used as additive flame retardants in a wide variety of commercial products. Their widespread use and global distribution has resulted in PBDEs being ubiquitous in marine and freshwater systems (de Wit 2002; Hale et al. 2003; Law et al. 2003). Although produced in many forms, PBDE congeners most commonly detected in wildlife are those of the penta-BDE technical mixtures including BDEs -47, -99, -100 and -153 (Law et al. 2003; Hites 2004). Due to the increasing environmental concentrations of these common PBDEs, as well as their well documented bioaccumulative and toxic properties, the penta-BDE mixtures are no longer marketed in Europe and North America (Directive 2003/11/EC; Tullo 2003; Ward et al. 2008). Despite the recently imposed restrictions on use, however, the environmental persistence of these compounds and continued leaching from consumer products suggests that PBDEs are likely to remain contaminants of concern over the foreseeable future.

Based upon studies to date, the thyroid endocrine system appears to be particularly sensitive to PBDEs. Penta-BDEs, through impacts on several different processes resulting in reduced hormone concentrations, can impact thyroid mediated processes such as neurological development, growth, metabolic processes, and behavior. PBDEs have the potential to compete with thyroxine (T_4) for transthyretin binding (Meerts et al. 1998, 2000), compete with binding at thyroid hormone receptors (Marsh et al. 1998), and increase metabolism of T_4 leading to increased excretion (Zhou et al. 2001, 2002; Richardson et al. 2008). These impacts to the thyroid system can have downstream impacts on development, growth and behavior as has been evidenced in a variety of studies of rats and mice orally exposed to penta-BDE mixtures and single congeners (reviewed in Costa and Giordono 2007). Non-mammalian studies have also documented changes in behavior in response to PBDE exposure as well as impacts on growth and development. For example, locomotion behavior and growth were impacted by dietary exposure to BDE-47 in juvenile zebrafish (*Danio rerio*; Chou et al. 2010; Chen et al. 2010). As well, survival, growth and development in *Rana pipiens* tadpoles (Coyle and Karasov 2010), and reproductive behaviors in adult American kestrels (*Falco sparverius*) were impacted by dietary exposure to penta-BDE (Fernie et al. 2008).

With the majority of effects of PBDE exposure having been documented in dietary exposure studies, there has been relatively little research focused on embryonic exposures and effects. Embryonic stages of development are typically highly sensitive to contaminants and, with evidence of maternal transfer of PBDEs to embryos (de Solla et al. 2007), it is important to understand potential effects during the embryonic period. Furthermore, very few studies have addressed how maternal exposure effects might carry

over from early developmental stages to potentially impact growth, development and behavior in later life stages. One study focusing on embryonic exposures indicated impacts on pipping and hatching success in American kestrels (McKernan et al. 2009). An additional study of American kestrels, using a combination of embryonic and oral exposures, provided evidence of effects on immune and thyroid function as well as growth in hatchlings (Fernie et al. 2005a, 2005b, 2006). There is only one study that examined long-term effects on reproduction resulting from an embryonic exposure to PBDEs; Marteinson et al. (2010) reported that embryonic exposure of American kestrels had lasting impacts on reproductive success and behaviors of adults.

To date there have been no controlled laboratory studies of effects of embryonic exposure to PBDEs in reptiles. Very little information regarding PBDE exposure in reptilian models is readily available and even less information on the potential effects of those exposures exists. de Solla and colleagues (2007) have shown that common snapping turtles (*Chelydra serpentina*) maternally transferred PBDEs to their eggs under natural conditions, but the effects of those concentrations as well as the potential for additive effects from other contaminants, such as PCBs which were also present in the eggs, are unknown.

The red-eared slider (*Trachemys scripta elegans*) has routinely been used as reptilian model species to address laboratory conducted embryonic exposures while the snapping turtle has been used as a model to study natural maternal transfer of many classes of contaminants in wild populations. In this study, we assessed the impacts of controlled embryonic exposure to BDE-47 on hatchlings and juveniles of red-eared sliders and snapping turtles. Specifically, we sought to elucidate impacts to thyroid

mediated processes including embryonic and hatchling development and metabolic

efficiency, as well as direct measurements of thyroid function, behavior, metabolic

efficiency, growth, and development in juveniles over a period of 16 months post

exposure. Data from this study were also intended to assess the relative utility of the two

turtle species as sensitive models for examining long-term effects of embryonic exposure

to BDE-47.

Methods

Egg Collection

Twenty-three clutches of red-eared slider eggs (laid on May 21st) and seven

clutches of snapping turtle eggs (laid on May 27th) were collected and purchased from

Concordia Turtle Farm in Wildsville, LA and RR Aquafarms LLC in Preston, MD

respectively. At the point of collection, eggs were weighed, measured for diameter

(snapping turtle eggs) and length and width (red-eared slider eggs) and given a unique

identifying number written on the upper surface of the egg in number two pencil. The

eggs were immediately placed in shallow bins with lids and damp substrate (vermiculite

mixed with water in a 1:1 mass ratio), and maintained at a cool temperature

(approximately 18 ℃) until June 1st when incubation at 26 ℃, a temperature known to

produce only males (Yntema 1976; Wibbles et al. 1991) began. Moisture and humidity

were maintained by misting the eggs and nest substrate with water at 2-3 day intervals.

Previous research has found that within-clutch variation in contaminant concentrations in

the egg contents is very low (Bishop et al. 1994) thus, prior to incubation two eggs from

each clutch were randomly selected and egg contents homogenized for contaminant

analysis of background concentrations of PBDEs. All procedures were approved by the University of Maryland Center for Environmental Science Institutional Animal Care and Use Committee (S-CBL-07-04).

Embryonic Exposure Protocol

Dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburg, PA, USA) was used as a vehicle for the topical application of BDE-47 to the upper surface of the eggshells for both species as it has been widely employed in reptile topical dosing studies and repeatedly shown to have very little toxicity to reptilian embryos (Crews et al. 1991; Wibbels and Crews 1992; Wibbels et al. 1993; Bergeron et al. 1994; Crain et al. 1997; Prodreka et al. 1998; Willingham and Crews 1999, 2000; Willingham et al. 2000; Gale et al. 2002). One working stock solution for the two species was prepared by adding neat BDE-47 (Accustandard Inc., New Haven, CT, USA) to DMSO followed by further dilution of the working stock in DMSO to prepare five different concentrations for each species to be used for topical dosing. The dosing concentrations for each species were calculated based on a 20 % transfer rate of BDE-47 across the eggshell and membrane into the embryo as determined in a previous pilot study in our laboratory (Chapter 2). In addition, doses for each species were further adjusted for the average egg mass (red-eared sliders-9.79 g and snapping turtles-11.89 g). All dosing solutions were analytically verified (see below). Prior to the start of incubation, eggs from each clutch were randomly assigned to one of eight treatment groups). Treatment groups consisted of a control, vehicle control, positive control for thyroid disruption (perchlorate; see Chapter

4), and five concentrations of BDE-47 for each species (see Table 3-1; n=23 for all treatments in both species).

Egg sizes of the red-eared sliders and snappers varied greatly (7.61 g -12.77 g and 9.99 g -13.70 g respectively) requiring that the dose for each egg be adjusted by individual egg mass in order to prevent substantial under or over exposing the embryos of eggs in the same treatment. To account for the variation in mass, eggs were categorized into one gram mass classes and dosing solution volumes were adjusted accordingly for each class. To avoid an acutely toxic embryonic exposure and to ensure solubility of BDE-47 in DMSO, dosing solution were topically applied to the vascularized upper surface of the eggshell daily over multiple days in 5 µl volumes until the entire target dose had been applied. The largest egg size class required 14 days of dosing in order to administer the total required dose, resulting in a total administered volume of 70 µl. Smaller mass classes received an equal volume of solution (70 µl), but proportionally less BDE-47 solution and increased pure DMSO. True controls were handled identically to the treated eggs, although no solutions were applied, while vehicle controls received only DMSO in the same volume as BDE-47 treated eggs. Survival was monitored throughout the incubation period by candling and any eggs showing arrested development were removed and dissected to determine embryonic stage at mortality and to document any gross deformities.

Hatchling Maintenance

Eggs were inspected daily after approximately 50 days of incubation, and eggs showing signs of hatching (sloughing of outer shell layers) or pipping (initial cracking of

the eggshell) were subsequently transferred to individual 0.5 L polyethylene cups containing a bed of damp sphagnum moss. Within 24 hours after complete emergence from the shell, hatchlings were measured to determine wet weight and examined for morphological abnormalities. In addition, the length, width and height of the external yolk sac were measured using digital calipers to calculate an external yolk sac volume as an indication of overall health. Time to hatch was measured as the number of incubation days from the start of incubation until complete emergence from the shell occurred and hatching success was measured as a proportion of individuals that successfully pipped and hatched.

Approximately 6 months post-exposure (MPE), 7 to 10 individuals of each species from each treatment were euthanized by inhalation of isoflorane (NLS Animal Health, Owings Mills, MD, USA) followed by decapitation. At the time of necropsy, livers and thyroids were collected and weighed for calculation of organ mass indices ([(liver or thyroid gland weight / body weight)*100]) and preserved for chemical and hormone analyses. Ten to 13 hatchlings from each treatment were not sacrificed and were maintained to assess effects of embryonic exposure on juvenile development for an additional 10 months.

Juvenile Maintenance

Individuals used to assess juvenile traits were maintained in 0.5 L polyethylene cups filled with approximately 1-3 cm of well water. They were held at 20 ℃ for two weeks and the temperature was lowered by 5 ℃ every two weeks thereafter until a final temperature of 5 ℃ was reached to simulate natural over-wintering conditions

experienced by temperate species. Approximately 9 MPE the temperature was increased

by 5 ºC every two weeks until a final temperature of 20 ºC was reached. At this time

turtles were placed individually into 4.8 L flow-through tanks providing a continuous

source of fresh well water (approximately 3 cm depth). The water turnover rate per tank

was approximately 230 mL/min. Turtles were fed Fluker's aquatic turtle diet (Port Allen,

LA, USA) *ad libitum* every 2 days and tanks were cleaned every 3 weeks coinciding with

size and growth measurements. Approximately 16 MPE all juveniles were euthanized in

the same manner as had been used with the hatchlings.

Biological Endpoints

Size/Growth and Standard Metabolic Rate

Wet weight was measured at hatching and every 2 weeks thereafter until

overwintering began. Following overwintering, juveniles were measured every 3 weeks.

Juvenile growth rate (k) was measured for each 3 week measurement period ($k=ln(W_2)-ln(W_1)/(t)$; where W_2 is size at end of the 3 week period, W_1 is size at the start of

measurements after overwintering, and t is the number of days between measurements).

Microrespirometry (MicroOxy-max^{TM}; Columbus Instruments, Columbus, OH)

was used to measure standard metabolic rate ("SMR", μl O_2 consumed/min) of a subset

of 10 to 13 hatchlings at 3 MPE (prior to over-wintering) for all treatments and a subset

of 9 to 11 juveniles at 12, 13 and 14 MPE from the control, DMSO and 3 highest BDE-47

treatments. The MicroOxy- max^{TM} was calibrated against a standard oxygen, carbon

dioxide, and nitrogen mixture. The turtles were acclimated and fasted for 24 hrs prior to

being placed into the respiration chambers. Immediately following acclimation,

respiration measurements were taken over a period of approximately 18 hrs to ensure a minimum of 8 measurements were obtained for each individual for calculation of mean rate of oxygen consumption over the assay period (Nagle et al. 2001). Wet weight and carapace length measurements were made following the micro-respirometry trials.

   Behavioral Assays

   Potential changes to neurological development or function were assessed using two behavioral assays on juveniles. Assays were repeated in three consecutive months (12, 13 and 14 MPE) with all individuals from the three highest treatments as well as those from the untreated and vehicle controls (n=7-14). The first behavioral assay tested the ability of juveniles to recognize and respond to a visual stimulus. The technique was adapted from Winkelman (1996) where a simulated avian predator was used to cast a shadow on or near the test animal. Individuals were placed in a 38 L aquarium with approximately 2-5 cm of water, covered on all sides and lighted by an overhead unidirectional light source. Individuals were allowed to acclimate for 20 minutes after which an avian-shaped silhouette was passed through the light path in a standardized fashion to cast a shadow over the turtle. The type of response was recorded (0-response did not occur or 1-response occurred) and categorized over three trials per individual. Responses were categorized as moving away from the shadow, flinch, head bob, no response, any combination of responses, or movement not listed.

   The second behavioral response assessed was the righting response in which the time required for the individual to invert itself from a ventrally-exposed position was measured (Steyermark and Spotila 2001). Using replicated tests on representative animals (as above), individuals were placed on their back on a packed sand substrate and

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the time to initiate righting, time to compete righting, and any other movements were recorded. Measurements took place at 20 °C under indirect light with observations made from behind a blind.

Glandular Thyroxine

For glandular T_4 measurements, thyroids were removed from individuals at necropsy, snap frozen in liquid nitrogen, and stored in -80 ℃ conditions for future analysis by enzyme immunoassay (EIA). Digestion of the thyroid glands followed methods of McNabb et al. 2004a and detailed in Chapter 4. Whole glands were digested for 24 hours at 37 ℃ in a mixture of 99.88 mM TRIS, 5.00 mM L-Glutathione reduced, 2.50 mM 6-n-propyl-2-thiouracil, and 1 % Triton X-100 and stored at -20 ℃ until time of assay.

As detailed in Chapter 4, competitive EIA kits, purchased from Calbiotech (Spring Valley, CA), were used to measure total glandular T_4. The kit has a sensitivity of 4 ng/ml using standards ranging from 20-250 ng/ml in human serum. An identical standard curve was prepared using L-thyroxine (Sigma-Aldrich) in 0.5 M HCl, 75 % ethanol and diluted in 1 % sheep serum (Innovative Research in Southfield, MI) to ensure turtle glandular T_4 could be accurately assayed using this kit. The standard curve prepared in sheep serum was also used to verify parallelism in each assay.

To prepare the digested samples for assay, 20 µl of 1.0 M perchloric acid was added to 100 µl of each sample and incubated for 40 min at room temperature, spun at 5000 rpm and diluted in 1 % sheep serum. Red-eared slider hatchling samples were diluted from 1:5 to 1:40, while the juvenile samples were diluted from 1:10 to 1:100. The

snapping turtle hatchling samples were diluted from 1:5 to 1:10 and juvenile samples from 1:10 to 1:100. Final sample and standard volumes were 25 µl for the assay.

As reported in Chapter 4, intra-assay variation was measured as the coefficient of variation for both species and was minimal (2.87 % $\pm$ 0.11 and 2.80 % $\pm$ 0.15 for snapping turtles and red-eared sliders respectively; $\pm$ 1 SE as a percent of the mean). Similarly, inter-assay variation was satisfactory (7.94 % $\pm$ 1.63 and 6.33 % $\pm$ 0.90 for snapping turtles, n=34, and red-eared sliders, n=24, respectively; $\pm$ 1 SE as a percent of the mean). Accuracy was determined to be 5.84 % $\pm$ 0.51 (mean $\pm$ 1 SE) for a snapping turtle thyroid composite that was charcoal stripped and spiked with 10 µg/ml T_4 and diluted 1:100 with a recovery of 101.51 ng/ml $\pm$ 0.91 (mean recovery $\pm$ 1 SE). Background interference for the charcoal stripped and composited snapping turtle thyroids was determined to be 15.46 ng/ml $\pm$ 0.30 (mean $\pm$ 1 SE) and samples were corrected accordingly. Finally, kit cross-reactivity was <1 % with *d-* and *l-* triiodothryonine.

Chemical Analyses

Chemical analyses on eggs, hatchlings, juveniles and food for background and post-treatment concentrations of total PBDEs were conducted. Briefly, for all biota samples, 3 g of homogenized wet tissue were ground with 10 times sodium sulfate (by mass) using a ceramic motar and pestel to remove water. Two eggs from each clutch were homogenized for background PBDE concentrations, while individual whole bodies of hatchlings and juveniles were homogenized by first freezing the body (without the carapace or plastron) in liquid nitrogen immediately followed by crushing of the frozen

tissue using a steel hammer. PBDEs from the tissue and food samples were extracted

using accelerated solvent extraction (ASE 300; Dionex) with dichloromethane. The

samples were packed on top of 18 g deactivated alumina in 35 ml stainless steel

extraction cells to remove lipids and other polar compounds that could cause interference.

Each sample was spiked directly in each extraction cell with two surrogate standards,

^{13}C-BDE-15 and ^{13}C-BDE-118 prior to extraction for calculation of analyte recoveries.

Recovered extracts were concentrated and removal of non-polar interferences was

completed using deactivated Florisil® column chromatography. With each set of

extractions a sodium sulfate blank was extracted to assess any laboratory contamination

during the extraction process as a measure of quality assurance. In addition, replicated

samples were analyzed providing information on precision and variance in extraction

processes.

Analytical methods used were consistent with those routinely used in our

laboratory as outlined by Klosterhaus and Baker (2010).   Briefly, 34 PBDE congeners

were quantified using a gas chromatograph (Agilent 6890N) coupled to a mass

spectrometer (Agilent 5973N) operated in negative chemical ionization mode. The

programmable temperature vaporization (PTV) injector was used in pulsed splitless mode

(5 µl injections). A 15 m DB-5MS column (J&W Scientific, Folsom, CA, USA) with an

inner diameter of 0.25 mm and 0.1 µm film thickness was used. Two slightly different

oven temperature programs, injector and detector temperatures as well as inlet and

column flows were used due to method improvements.   For the egg and food samples the

oven temperature program consisted of an initial hold at 40 °C for 1 min, a 20 °C/min

ramp to 250 °C with no hold, a 1.5 °C/min ramp to 260 °C held for 1 min, and a 25

°C/min ramp to 320 °C and held for 20 min. The injector and detector temperatures were 45 °C and 320 °C, respectively, while inlet and column flow were 100 ml/min and 1.5 ml/min, respectively. However, for the hatchling and juvenile whole body analyses the oven temperature program consisted of an initial hold at 80 °C for 2 min, a 20 °C/min ramp to 250 °C with no hold, a 1.5 °C/min ramp to 260 °C with no hold, and a 25 °C/min ramp to 300 °C and held for 27 min. The injector and detector temperatures were 80 °C and 280 °C, respectively. Inlet and column flow were 100 ml/min and 1.3 ml/min, respectively. Prior to instrumental analysis, ^{13}C-CDE-86 (2,2',3,4,5-pentachlorodiphenyl ether) and ^{13}C-BDE-209 (decabromodiphenyl ether) were added as internal standards to all samples and calibration standards. Mass fragments *m/z* -79 and -81 were monitored for di- to octa-BDEs, -487 and -409 for the nona-BDEs and BDE-209, -318 and -316 for ^{13}C-CDE-86, and -495 and -415 for ^{13}C-BDE-209 for quantitative and qualitative ions, respectively. BDE standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), Wellington Labs (Guelph, Ontario, Cananda), and Accustandard (New Haven, CT, USA) or received from the U.S. National Institute of Standards and Technology (NIST; Githersburg, MD, USA).

Three times the analyte mass in the laboratory blanks divided by the mass of the sample extracted was the method detection limit (MDL). Mean recoveries (± 1 standard error) for BDE surrogate standards ^{13}C-BDE-15 and ^{13}C-BDE-118 in red-eared slider and snapping turtle eggs were 76.21 % ± 1.81 % and 81.73 % ± 2.60 % as well as 78.76 % ± 1.45 % and 77.74 % ± 1.62 % respectively. Mean recoveries of surrogate standards in red-eared slider hatchlings were 66.84 % ± 1.54 % and 83.35 % ± 2.46 % while for the juveniles, recoveries were 73.46 % ± 2.61 % and 82.00 % ± 2.29 %. In the snapping

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turtle hatchlings mean recoveries of surrogate standards were 61.77 % ± 3.81 % and

70.38 % ± 3.72 % and recoveries in the juveniles were 89.46 % ± 4.88 % and 85.24 % ±

3.60 %.

Statistical Analyses

All data were analyzed using Minitab[®] (Minitab Inc., version 15). Dosing

solution concentrations and tissue concentrations were analyzed using analysis of

variance (ANOVA) followed by Tukey's pairwise comparisons. Binary logistic

regression was used to analyze hatching success while other hatchling endpoints

including size at hatch and necropsy, time to hatch, volume of external yolk, standard

metabolic rate (SMR), organ to body weight ratios and total glandular thyroxine (T_4)

were analyzed using ANOVA. Juvenile measurements of growth, final size, SMR,

righting time, organ to body weight ratios, and total glandular T_4 were analyzed using

ANOVA while measures of predator avoidance and the number of respondents in the

righting response and were analyzed using contingency analysis followed by Bonferroni

correction to conduct pairwise comparisons. When analyzing growth data of the

juveniles, initial size was defined as the size immediately following overwintering.

Standard metabolic rate (oxygen consumption as µl O_2/min) varies allometrically with

body size, and thus was corrected for mass following methods employed by Manyin and

Rowe (2006). Statistical significance was judged based upon a type I error rate of $\alpha<0.05$

and $\alpha\square0.017$ for all contingency table analyses that were subject to the Bonferroni

correction. Prior to statistical analyses, data were tested to verify that they met the

assumptions of the statistical models using Levene's test for homogeneity of variance and

Shapiro–Wilk (*W*) statistic for normality in distribution. Log or square root

transformations were employed as necessary. As there were no statistical differences

found between the true control and vehicle control (DMSO) eggs for any endpoints, these

two treatments were combined to create a single control group. Variation within the

continuous data for each endpoint was calculated as the coefficient of variation presented

as percent variation.

Results

BDE-47 in Dosing Solutions, Eggs, Whole Body Tissue and Food

Background concentrations of total PBDEs in the red-eared sliders and snapping

turtle eggs were 0.135 ± 0.022 ng/g wet weight (ng/g ww; n=23) and 1.13 ± 0.724 ng/g

ww respectively (n=7). In the red-eared slider eggs, no single PBDE congener was

consistently detected in all eggs. PBDE congeners -100 and -153 were detected in more

than 50 % of the eggs (19 and 17 eggs respectively) with congeners -183, -99, and -47

detected in 10, 9 and 8 eggs respectively. BDE-154 was detected infrequently (3 eggs)

and BDE-49 and -71 (co-elutants) were only found in 1 egg. In the snapping turtle eggs,

PBDE congeners -100, -153, and -154 were detected in all 7 eggs whereas BDE-99 and

co-eluting congeners -49 and -71 were found in 3 eggs. Less commonly detected were

BDE-47 and -183 which were found in 2 eggs while BDE-75 and co-eluting congeners -

28 and -33 were detected only in 1 egg. The average concentration of BDE-47 in red-

eared slider eggs was 0.058 ± 0.006 ng/g ww in contrast to 0.238 ± 0107 ng/g ww found

in the snapping turtle eggs. However, BDE-47 was above detection levels in only 35 %

of the red-eared slider eggs and 29 % of the snapping turtle eggs.

Although whole body concentrations of BDE-47 in red-eared slider hatchlings at 6 months post-exposure (MPE) increased with increasing concentration, the only significant differences in body burden detected among treatments was between the highest (1000 ng/g) and the lowest (40 ng/g) treatment (p=0.0175). However, all BDE-47 treatments except the lowest were significantly higher than the controls (see Table 3-1). Whole body concentrations of BDE-47 in the snapping turtles at 6 MPE were significantly greater in all treatments from the control with the exception of the lowest (40 ng/g) treatment. Concentrations increased with increasing dose in the snapping turtle whole body tissue with the exception of the highest (1000 ng/g) treatment which was statistically lower than the 700 ng/g treatment (p=0.0043) and equal to the two middle treatments (see Table 3-1). Concentrations of BDE-47 in the whole body tissue of animals in the second highest (700 ng/g) treatment were significantly higher than all other treatments. Whole body concentrations between species of the same treatment groups were not significantly different from each other with the exception of the second highest (700 ng/g) treatment and controls of the red-eared slider being significantly lower than the same treatments of the snapping turtle (p=0.0333 and p=0.0324 respectively).

Concentrations of BDE-47 in whole body tissue of red-eared sliders approximately 16 MPE were below detection limits in all treatments. Total PBDE concentrations for all individuals were very low (mean: 1.833 ± 0.404 ng/g ww; minimum-maximum: 0.0347-6.210 ng/g ww) with PBDE congeners -100, -154, and -153 dominating the total concentration. Snapping turtle juvenile (16 MPE) BDE-47 concentrations were not significantly different among treatments with the exception of the second highest (700 ng/g) treatment being significantly greater than the highest (1000

ng/g; p=0.0276), lowest (40 ng/g; p=0.0028) and control (p=0.0018) treatments (see

Table 3-1). However, BDE-47 concentrations were generally low for all treatments

(mean: 0.289 ± 0.215 ng/g ww; range: 0.0062-4.547 ng/g ww). The highest individual

concentration (4.547 ng/g ww) from the second highest treatment (700 ng/g) was the only

juvenile snapping turtle with a whole body concentration over 0.50 ng/g ww, and the next

2 highest concentrations also belonged to individuals in the 700 ng/g treatment. The total

PBDE concentration in the diet for both species of juveniles was 3.146 ng/g ww with a

BDE-47 concentration of 0.216 ng/g ww.

Hatchlings

Hatching Success, Incubation Time, and External Yolk Size

Topical egg dosing did not affect hatching success, incubation time or external

yolk size at hatching in either species (Table 3-2). The control treatments had hatching

success above 90 % while the lowest BDE-47 (40 ng/g) treatment in the red-eared sliders

and the middle (400 ng/g) treatment in the snapping turtles showed less than 80 %

hatching success. However there was no overall exposure-dependent relationship in

hatching success for either species. The number of incubation days did not differ among

treatments for the red-eared sliders with incubation lasting from a 79 to 90 days with the

mean falling between 83 and 84 days of incubation for all treatments (Table 3-2). The

average number of incubation days for all red-eared sliders regardless of treatment was

83.75 ± 0.212. Incubation for the snapping turtles lasted from of 73 to 86 days with mean

falling between 79 and 81 days for all treatments with no significant differences among

treatments (Table 3-2). The average number of incubation days for all snapping turtles

was 80.01 ± 0.217. There was no significant difference in external yolk volume among treatments in the red-eared sliders with an average size, regardless of treatment, of 1170 ± 62.99 mm^3, much larger than the average external yolk size across all treatments in the snapping turtle (380.5 ± 18.75 mm^3). Similarly, there were no significant differences in external yolk size among treatments in the snapping turtle.

Size at Hatching and 6 MPE and Standard Metabolic Rate at 4 MPE

Size measured as wet weight was not significantly different among treatments for either the red-eared sliders or snapping turtles at hatching or 6 MPE (Table 3-2). There were no significant differences in SMR measured after 4 MPE among the BDE-47 treatments for either species (red-eared slider: p=0.091; snapping turtle: p=0.088; Table 3-2). Despite lack of statistical differences based upon a critical value of p=0.05, inspection of the data reveals elevated SMR in several individuals from the BDE-47 treatments in both species (Figure 3-1). There were individuals from all treatments with the exception of the lowest (40 ng/g) treatment that showed elevated SMR measurements above the highest SMR observed in the control treatment for the red-eared sliders (Figure 3-1). A similar pattern can be seen for the snapping turtles, although there was one individual in the control treatment with an unusually high value making the pattern less clear. The high variation and the low sample size and power in the SMR data precluded detecting significant differences among the treatments for both species, yet the observed pattern and nearly significant overall p-values for both species is suggestive of a subtle biological response (Tables 3-3 and 3-4).

Organ Indices and Total Glandular T₄

Analysis of the red-eared slider liver mass to body mass ratio (HSI) revealed no significant differences or consistent patterns among treatments. In contrast, snapping turtle HSI was significantly greater in the lowest (40 ng/g) and the two highest treatments (700 ng/g and 1000 ng/g) compared to the control (p=0.0124, 0.0147, and 0.0485 respectively). All individuals from the middle (400 ng/g) treatment had HSIs above the mean of the control group suggesting that these individuals also may have responded to the BDE-47 treatment to a lesser degree (Figure 3-2). Statistical analysis of the thyroid mass to body mass ratio (TSI) suggested no significant differences among treatments in either species (Table 3-2). However, when all thyroid mass data were pooled over the BDE-47 treatments and compared to the control, the red-eared sliders exposed to BDE-47 had a significantly larger TSI than the controls (p=0.017). Wide variation in individual measurements of glandular T_4 as well as low sample size and power precluded detecting significant difference among treatments in either species (Tables 3-2, 3-3 and 3-4).

Juveniles

Size, Growth and Standard Metabolic Rates

Sizes following over-wintering (10 MPE) or at time of necropsy (16 MPE), measured as wet weight, were not significantly different among treatments of the red-eared sliders. In contrast, size following over-wintering (10 MPE) in the snapping turtles was significantly different among treatments (p=0.038; Table 3-5). Specifically, the 220 ng/g treatment had significantly lower mass than the controls (p=0.050) while mean sizes in other treatments (400 ng/g, 700 ng/g and 1000 ng/g) were less than controls, but the

differences were not significant. The majority of individuals in the latter two treatments (700 ng/g and 1000 ng/g) had lower masses than the mean mass of the individuals from the control treatments (Figure 3-3). These differences in size were no longer evident 6 months later (16 MPE). Growth rate did not differ among treatments for any of the eight measurement periods for either species. In general, the snapping turtles had greater growth rates than the red-eared sliders with growth rate peaking at approximately 12 MPH for the snapping turtles (Figure 3-4). Growth rate was relatively constant across measurement periods for the red-eared sliders (Figure 3-4).

Standard metabolic rates of juvenile red-eared sliders did not significantly differ among treatments at any time point (12, 13, or 14 MPE) and remained relatively stable over this 3 month period, corresponding with stable growth rates. Snapping turtle SMR was significantly increased at 13 MPE (p=0.007) with the 700 ng/g treatment exceeding the control treatment (p=0.0138; Table 3-6). This increase may be the result of anomalously high SMRs (approximately 3x higher) in two individuals of the 700 ng/g treatment for that assay period.

Behavior

There was no evidence of effects of BDE-47 on predator avoidance behavior in either species. However, there did appear to be some impacts of BDE-47 on righting response behavior in both species. Several individuals of both species did not make any movements when placed on their back, and were therefore classified as non-respondents. Pooled across the three assay periods (12, 13, and 14 MPE), red-eared sliders exposed to the two highest BDE-47 treatments (700 ng/g and 1000 ng/g) had a significantly higher proportion of non-respondent individuals compared to the controls (p=0.013). In

contrast, snapping turtles in the control treatment had a higher proportion of non-respondent individuals compared to all other treatments (p<0.0001). In the control treatment, 80 % of snapping turtles responded compared to 97 %, 93 % and 92 % in the 400 ng/g, 700 ng/g and 1000 ng/g treatments, respectively.

When all data were pooled across assay periods for the red-eared slider individuals that did attempt a righting response, individuals exposed to the highest concentration (1000 ng/g) displayed a significantly greater righting time than the control treatment (p=0.0121). This difference was also found in first assay period (12 MPE; p=0.027; Table 3-6), but not in the second (13 MPE) or third (14 MPE) assay periods. When righting times of respondent snapping turtles were analyzed from all time periods combined, those individuals exposed to the second to highest concentration (700 ng/g) had a significantly reduced time to right compared to the controls (p=0.0214). This overall difference was also evident in the first assay period (12 MPE; p=0.0047; Table 3-6) while no differences found in righting times among treatments in the second or third assay periods (13 and 14 MPE).

Organ Indices and Glandular Thyroxine

There were no differences in the HSI among treatments of either species. While there were no significant differences in the TSI among treatments for the red-eared slider, all the BDE-47 treatment means were below that of the control. In addition, each treatment contained only two to four individuals having TSIs above the mean TSI of the control, possibly driving the insignificant differences (Figure 3-5). The TSI for the highest (1000 ng/g) treatment in the snapping turtles was significantly lower than the two treatments below it (400 ng/g, p=0.0246; 700 ng/g, p=0.0191) as well as the control

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(p=0.0167; Table 3-5). There were no significant differences in total glandular T_4 among treatments in either species. However, there does appear to be a pattern of decreased glandular T_4 in the BDE-47 treatments compared to the controls for both species, but individual variation and low sample size as well as low power precluded detection of differences (Figure 3-6; Tables 3-7 and 3-8).

Discussion

The purpose of this study was to determine if embryonic exposure to BDE-47 would affect embryonic and hatchling growth and development and/or lead to latent impacts on juvenile traits in red-eared slider and snapping turtles. Exposure of embryos via a topical exposure along with egg injection methods typically used in avian embryonic studies have been used extensively to evaluate embryonic toxicity to a variety of compounds, although their biological similarity to true maternal transfer is limited. Natural maternal transfer occurs during vitellogenesis as lipophilic contaminants bioaccumulated by the mother are transferred to the egg. However, in laboratory exposures, the injected or topically administered contaminants are incorporated into the yolk or albumin long after vitellogenesis has ceased, leading to non-natural timing of initial exposure. Although there are differences in the mechanisms by which the embryos are exposed between natural maternal transfer and laboratory embryonic exposures through injections or topical dosing, the laboratory methods allow for a controlled setting to replicate a range of exposures over several clutches of eggs.

In this study topical dosing appeared to be an effective tool for exposing embryos to BDE-47 and assessing effects resulting from multiple exposure concentrations. Doses applied to the eggs over a 16-day period were calculated and verified to attain BDE-47

concentrations in the embryo ranging from 40 ng/g to a 1000 ng/g thus all eggs were

exposed to appropriate concentrations under the assumptions of 20 % transfer efficiency

of BDE-47 across the eggshell (Chapter 2). Further, hatchling body burdens of BDE-47

at 6 MPE suggest that the topical dosing technique was effective in exposing embryos to

different and increasing concentrations.

Hatchling whole body concentrations measured at 6 MPE were elevated

compared to background concentrations and with no additional source of PBDEs

available. However, reduced BDE-47 concentrations in red-eared sliders relative to

snapping turtles at 6 MPE suggest inter-species differences in either transfer efficiency or

metabolism of the compound. Eggshell structure for both species is characterized by a

calcareous layer with abundant and well defined pores attached to a membrane of about

the same thickness (Packard et al. 1982). Previous research examining the transfer

efficiency of BDE-47 across the eggshell and membrane of both red-eared sliders and

snapping turtles suggests that there is reduced transfer efficiency across the red-eared

slider shell and membrane (Chapter 2). This supports the findings that that the red-eared

slider hatchlings contain lower concentrations of BDE-47.

There could also be species differences in embryonic and hatchling metabolism of

BDE-47. For example, embryonic red-eared sliders appeared to have the ability to

debrominate BDE-99 to BDE-47 while the snapping turtles did not show the same

capability (Chapter 2), suggesting that red-eared sliders may have the ability to

metabolize BDE-47 where the snapping turtles cannot metabolize any PBDEs. Species

differences in embryonic metabolism of PBDEs have been suggested in American

kestrels and chickens where the former had significantly reduced pipping and hatching

success in response to a penta-BDE while the chickens did not. These differences were attributed to kestrels having a lower ability to metabolize PBDEs compared to other species, possibly due to lower activity of some of their metabolic enzymes in addition to their kidneys and livers being less developed during incubation compared to the chickens (Walker 1980; McKernan et al. 2009). Differential metabolism and elimination of PBDE congeners has also been shown in rats and mice as well as different species of fish in more advanced life stages than studied here (Haak and Letcher 2003). These studies suggest that there could be metabolic differences between the two turtle species, although this would require additional studies to specifically assess embryonic and hatchling metabolism of PBDEs. Species differences in uptake, metabolism and elimination could also explain why concentrations of BDE-47 were detectable in the juvenile snapping turtles at 16 MPE but not the red-eared sliders.

Despite accumulating and retaining BDE-47 after embryonic exposure in both species, there was a general lack of significant responses by hatchlings even though penta-BDE compounds have been shown to have minor impacts after embryonic exposure in American kestrels (McKernan et al. 2009, Fernie et al. 2005a, 2005b, 2006). Hatchling endpoints that appeared to be impacted, although to a limited degree, were SMR and organ size. Although not statistically different, there appeared to be an increase in SMR for individuals of both species exposed to BDE-47, but a dose-dependent response was not evident. Based upon the sample sizes and the variability among observations within treatments the power to detect differences at $\alpha=0.05$ was as low as 0.1934 for the red-eared slider SMR and 0.0782 for the snapping turtle (Tables 3-9 and 3-10). The treatments which resulted in elevated SMRs were the same treatments that had

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significantly elevated whole body concentrations of BDE-47 (6 MPE). These potential

elevations in energy expenditure for maintenance can represent costs associated with

metabolism and elimination mechanisms for dealing with the contaminant exposure as

seen in several other studies (Calow 1991; Rowe et al. 1998; Hopkins et al. 1999; Manyin

and Rowe 2006).

Increased liver weights have been documented in rats and mice exposed to penta-

BDE mixtures with exposure times much longer and concentrations much higher than

used in this study (Dunnick and Nyska 2009; Albina et al. 2010; Bruchajzer et al. 2010).

Increased hepatic enzyme activity in the liver associated with the metabolism of PBDEs

can lead to liver toxicity and increased liver size (Zhou et al. 2001; Sanders et al. 2005;

Stapleton et al. 2009; Bruchajzer et al. 2010). Although not measured in this study,

increased hepatic enzyme activity may in part explain the increased liver sizes in the

snapping turtles exposed to BDE-47. In addition, increased liver size and enzyme

activity would be expected to elevate metabolic energetic requirements, increasing SMR

as we observed.

The thyroid mass of the hatchling red-eared sliders was the only other hatchling

endpoint potentially impacted by the embryonic exposure. There was no dose-dependent

pattern in thyroid size and no significant differences were found among the treatments,

but when the BDE-47 treatment data were pooled across all doses and compared to the

control, those exposed to BDE-47 had a significantly greater thyroid size. This suggests

an overall effect of BDE-47 on the thyroid at low concentrations. It is possible that while

the effect occurred at low concentrations there was not a great enough difference in the

exposure treatments to have greater impacts at higher exposures. A larger sample size

would be needed to overcome individual variation as well as the low power when looking at individual treatment comparisons as well as a wider range in exposure concentrations (Tables 3-6 and 3-7). While thyroid size was increased, there was no effect of BDE-47 on the total glandular T_4 concentrations for the red-eared sliders even when the BDE-47 treatment data were pooled. It is well documented that exposure to the lower brominated PBDEs can have impacts on thyroid hormone concentrations with impacts on T_4 binding to transthyretin, binding to the thyroid hormone receptors, as well as in increased metabolism of T_4 (Meerts et al. 1998, 2000; Marsh et al. 1998; Zhou et al. 2001, 2002; Hallgren and Darnerud 2002; Morgado et al. 2007; Richardson et al. 2008; Talsness 2008). However, these studies focused on responses in rats and mice and thus may not be applicable to investigations of reptiles. *In ovo* exposures to lower brominated mixtures of PBDEs in American kestrels resulted in no morphological changes to the thyroid (Fernie et al. 2005a, McKernan et al. 2009), and while there were no significant differences in plasma T_4 concentrations, these T_4 concentrations were negatively correlated with BDE-47 concentrations (Fernie et al. 2005a). The lack of effects on the glandular T_4 concentrations in the current study, but potential impacts on thyroid weight in the red-eared slider and no impacts in the snapping turtle suggest that thyroid function may not be sensitive to embryonic exposure to BDE-47 at concentrations used in this study.

There was an overall lack of significant effects from the embryonic exposure of BDE-47 in juveniles ranging from 7 to 14 months post-hatch, although subtle effects did appear to be present. While hatchling snapping turtles exposed to BDE-47 displayed lower size after overwintering (10 MPE) compared to controls, suggesting increased use of energy from internal yolk stores over the hatchling and over-wintering period,

differences in size (growth) were not sustained throughout the ensuing juvenile period. This apparent "catch-up growth" may reflect the *ad libitum* diet provided to the juveniles, supplying surplus energy to compensate for deficits that the (non-feeding) hatchlings had experienced. Under more natural conditions in which food may be a limiting natural variable, size differences between exposed and unexposed individuals might be sustained later in life.

There were potential long-term impacts of embryonic exposure on the righting response behavior. In snapping turtles there were more non-respondents in the control treatment compared to the BDE-47 treatments and in the first assay control animals had an increased time to right compared to the animals in the 700 ng/g BDE-47 treatment. These results may be an artifact of the variation within the data as the coefficient of variation was over 100 % for the snapping turtles for all treatments with the exception of the control in that first assay period (Table 3-9). In addition, time to right was affected only in the first assay period and showed no consistent pattern in the data across the 3 assay periods. Furthermore, results from a dietary exposure study (Chapter 5) using eggs and turtles from the same clutches incubated and housed under the same conditions indicated no differences in the righting response endpoints in the snapping turtles. In contrast the red-eared sliders exposed to BDE-47 showed an increased frequency of non-response relative to controls, suggesting that neurological development in the embryonic and hatchling stages could have been impacted. Although the control treatment of the snapping turtle suggests an opposite response from the red-eared slider, it has clearly been shown in several studies with rats and mice that BDE-47 and other lower brominated PBDEs have impacts on neurological development resulting in behavioral

changes and deficits (reviewed in Costa and Giordano 2007, Branchi et al. 2002, 2003; Viberg et al. 2003). In addition, male American kestrels showed alterations to reproductive behaviors one year following direct maternal exposure to a penta-BDE mixture, indicating long-term effects on behavior (Marteinson et al. 2010).

While there were no significant differences in total glandular T_4 in either species there were discernable patterns of decreased T_4 in BDE-47 treated individuals for both species (Figure 3-5). At $\alpha=0.05$ the power for detection of significant differences in total glandular T_4 for both species was below 0.2 requiring a larger sample size for the patterns seen in T_4 to be significant (Table 3-8). For the snapping turtle there was also a significant decrease in thyroid gland size in the highest treatment, and red-eared sliders displayed a similar pattern, however differences were not statistically significant (Figure 3-5). Potential impacts to the thyroid system resulting from embryonic exposure to BDE-47 might explain the minor changes and patterns observed in juvenile size, SMR and behavior as these processes are regulated by thyroid activity.

Conclusion

Although there were limited observed impacts resulting from embryonic exposure to BDE-47 at the concentrations tested in this study, results are mixed and their significance to long term health or fitness is questionable. It is clear that the exposure methodology did provide measureable concentrations to the embryos up to 6 MPE, but exposure induced few toxicological responses. Several studies have documented turtles maternally exposed to multiple organic contaminants such as PCBs, pesticides, polycyclic aromatic hydrocarbons, and PBDEs have had impacts to several biological

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endpoints such as sexual development, hatching success, morphological development, metabolic efficiency, and survival (Bishop et al. 1998; de Solla et al. 2002, 2008; Bell et al. 2006; Eisenreich et al. 2009). Embryonic development may therefore be more sensitive to natural maternal transfer and more obvious indications of toxicity from PBDEs may become evident if exposure took place through a natural process. However, in natural populations, exposure is rarely limited to a single compound and cannot be controlled, thus, the method of embryonic exposure employed in this study is an important option for attempting to gain some insights into effects resulting from embryonic exposures to specific concentrations of compounds.

Tables

Table 3-1. Concentrations of BDE-47 dosing solutions and BDE-47 in hatchling (6 MPE) and juvenile (16 MPE) whole body tissues of the red-eared slider and snapping turtle.

BDE-47 Dose ng/g egg	Red-eared Slider				Snapping Turtle			
	Nominal Dose (ng/µl)	Measured Dose (ng/µl)	Hatchling Whole Body (6 MPE)*	Juvenile Whole Body (16 MPE)*	Nominal Dose (ng/µl)	Measured Dose (ng/µl)	Hatchling Whole Body (6 MPE)*	Juvenile Whole Body (16 MPE)*
Control		1002 ± 45.47	0.073 ± 0.019^A	ND	1000	1002 ± 45.47	0.661 ± 0.198^A	0.014 ± 0.0058^A
40	32.63	41.56 ± 0.449^A	0.393 ± 0.179^{AB}	ND	39.63	52.52 ± 0976^A	2.766 ± 0.034^{AB}	ND^A
220	179.48	199.09 ± 7.031^B	0.739 ± 0.128^{BC}	ND	217.98	188.40 ± 14.24^B	7.108 ± 2.790^{BC}	0.108 ± 0.067^{AB}
400	326.33	269.74 ± 7.545^C	2.316 ± 1.587^{BC}	ND	396.33	400.58 ± 28.19^C	15.264 ± 2.002^C	0.063 ± 0.044^{AB}
700	571.08	542.58 ± 28.88^D	2.142 ± 0.520^{BC}	ND	693.58	674.96 ± 83.70^D	33.905 ± 6.321^D	1.748 ± 1.401^B
1000	815.85	665.28 ± 45.47^D	4.373 ± 1.751^{BC}	ND	990.83	999.89 ± 10.34^E	12.461 ± 1.963^C	0.066 ± 0.057^A

*Concentration units of whole body analyses are ng/g animal wet weight.
The stock concentration was 1002 ± 45.47 ng/µl which was used to prepare treatment solutions. Values are means $\pm$ 1SE.   n=3 for all treatments and measurements. Different superscripts represent significant differences following multiple comparisons tests ($p<0.05$). MPE=months post exposure; ND=below detection limit.

Table 3-2. Biological responses of red-eared slider and snapping turtle hatchlings to BDE-47 exposures.

		BDE-47 Dose (ng/g egg)				
	Control	40	220	400	700	1000
Red-eared Slider						
Hatching Success	42/46 (91.3%)	18/23 (78.3%)	19/23 (82.6%)	20/23 (87.0%)	20/23 (87.0%)	20/23 (87.0%)
Incubation Time (d)	83.98±0.410 (42)	83.56±0.627 (18)	83.89±0.567 (19)	83.40±0.387 (20)	83.45±0.705 (20)	83.95±0.484 (20)
Vol. of External Yolk (mm^3)	1148±125.1 (42)	900.7±185.5 (18)	1383±158.0 (19)	1090±138.8 (20)	1227±156.1 (20)	1279±169.4 (20)
Size at Hatch (2 MPE; g)	8.719±0.127 (42)	8.417±0.157 (18)	8.563±0.229 (19)	8.595±0.171 (20)	8.475±0.240 (20)	8.350±0.155 (20)
Size at Necropsy (6 MPE; g)	7.500±0.203 (18)	7.357±0.284 (7)	7.256±0.302 (9)	7.280±0.270 (10)	6.850±0.217 (10)	6.911±0.210 (9)
SMR (4 MPE; µl O_2/min/g)	0.458±0.019 (31)	0.515±0.024 (10)	0.843±0.262 (11)	0.637±0.074 (10)	0.523±0.038 (10)	0.518±0.094 (10)
HSI (6 MPE; %)	4.900±0.167 (18)	4.966±0.251 (7)	4.855±0.175 (9)	5.142±0.195 (10)	4.789±0.167 (10)	5.046±0.118 (9)
TSI (6 MPE; %)	0.033±0.0021 (18)	0.036±0.0051 (7)	0.041±0.0039 (9)	0.036±0.0039 (10)	0.041±0.0058 (10)	0.045±0.0043 (9)
Total Glandular T_4 (6 MPE; ng/ml)	1079±216.7 (18)	851.3±160.0 (7)	1567±224.2 (8)	1737±458.3 (10)	970.6±155.3 (10)	1472±227.4 (9)
Snapping Turtle						
Hatching Success	43/46 (93.5%)	20/23 (87.0%)	23/23 (100%)	18/23 (78.3%)	20/23 (87.0%)	19/23 (82.6%)
Incubation Time (d)	79.91±0.432 (43)	80.10±0.507 (20)	79.91±0.412 (23)	80.53±0.607 (18)	79.95±0.672 (20)	80.11±0.648 (19)
Vol. of External Yolk (mm^3)	431.6±40.55 (43)	352.4±40.60 (20)	310.0±23.11 (23)	397.1±59.46 (18)	373.0±59.26 (20)	372.0±42.08 (19)
Size at Hatch (2 MPE; g)	10.42±0.088 (43)	10.33±0.117 (20)	10.33±0.105 (23)	10.43±0.104 (18)	10.40±0.122 (20)	10.32±0.106 (19)
Size at Necropsy (6 MPE; g)	9.965±0.093 (20)	10.01±0.219 (10)	10.19±0.243 (10)	10.20±0.226 (7)	10.19±0.148 (10)	10.07±0.191 (9)
SMR (4 MPE; µl O_2/min/g)	0.429±0.025 (31)	0.447±0.032 (10)	0.546±0.042 (13)	0.504±0.045 (10)	0.472±0.028 (10)	0.402±0.020 (10)
HSI (6 MPE; %)	1.900±0.057 (20)	2.152±0.053 (10)*	2.013±0.040 (10)	2.099±0.039 (7)	2.148±0.040 (10)*	2.123±0.64 (9)*
TSI (6 MPE; %)	0.013±0.0013 (20)	0.014±0.0016 (10)	0.011±0.0010 (10)	0.014±0.0022 (7)	0.012±0.0012 (10)	0.011±0.0008 (9)
Total Glandular T_4 (6 MPE; ng/ml)	381.4±58.11 (20)	588.4±104.4 (10)	387.7±79.38 (10)	529.9±206.2 (7)	572.8±107.0 (10)	469.7±97.29 (9)

Values are means ± standard error with sample sizes listed below each mean in parentheses. * indicates significant difference from control only after multiple comparison tests ($p<0.05$).
SMR=standard metabolic rate; MPE=months post exposure; HSI=hepatosomatic index; TSI=thyrosomatic index.

Table 3-3. Coefficient of variation calculated as a percentage for each continuous data hatchling endpoint.

		BDE-47 Dose (ng/g egg)				
	Control	40	220	400	700	1000
Red-eared Slider						
Incubation Time (d)	3.16 %	3.19 %	2.94 %	2.07 %	3.78 %	2.58 %
Volume of External Yolk (mm^3)	70.60 %	87.37 %	49.78 %	56.93 %	56.91 %	59.22 %
Size at Hatch (2 MPE; g)	9.43 %	7.93 %	11.68 %	8.92 %	12.64 %	8.30 %
Size at Necropsy (6 MPE; g)	4.19 %	6.91 %	7.55 %	5.86 %	4.59 %	5.68 %
SMR (4 MPE; $\mu l\ O_2/min/g$)	22.88 %	14.67 %	102.92 %	36.87 %	23.00 %	57.51 %
HSI (6 MPE; %)	14.44 %	13.37 %	10.80 %	11.98 %	11.00 %	7.01 %
TSI (6 MPE; %)	26.45 %	37.44 %	28.08 %	33.78 %	44.55 %	28.76 %
Total Glandular T_4 (6 MPE; ng/ml)	85.21 %	49.72 %	40.47 %	83.44 %	50.58 %	46.34 %
Snapping Turtle						
Incubation Time (d)	3.55 %	2.83 %	2.47 %	3.11 %	3.77 %	3.53 %
Volume of External Yolk (mm^3)	61.60 %	51.53 %	35.75 %	63.52 %	71.06 %	49.31 %
Size at Hatch (2 MPE; g)	5.52 %	5.06 %	4.89 %	4.11 %	5.26 %	4.49 %
Size at Necropsy (6 MPE; g)	4.19 %	6.91 %	7.55 %	5.86 %	4.59 %	5.68 %
SMR (4 MPE; $\mu l\ O_2/min/g$)	32.01 %	22.42 %	27.76 %	28.49 %	18.87 %	16.04 %
HSI (6 MPE; %)	13.38 %	7.76 %	6.28 %	4.96 %	5.90 %	9.01 %
TSI (6 MPE; %)	46.84 %	36.13 %	27.83 %	42.30 %	30.19 %	22.00 %
Total Glandular T_4 (6 MPE; ng/ml)	68.14 %	56.09 %	64.75 %	102.98 %	59.07 %	62.14 %

Table 3-4. Observed power and sample size needed to achieve a power of 0.8 for hatchling endpoints.

	Sample Size	Observed Power	Sample Size Needed for a Power of 0.8
Red-eared Slider			
Incubation Time (d)	18-42	0.0706	484
Volume of External Yolk (mm^3)	18-42	0.2639	62
Size at Hatch (2 MPE; g)	18-42	0.1349	135
Size at Necropsy (6 MPE; g)	7-18	0.1546	41
SMR (4 MPE; $\mu l\ O_2/min/g$)	10-31	0.3704	24
HSI (6 MPE; %)	7-18	0.6799	9
TSI (6 MPE; %)	7-18	0.2060	30
Total Glandular T_4 (6 MPE; ng/ml)	7-18	0.2185	28
Snapping Turtle			
Incubation Time (d)	18-43	0.0843	284
Volume of External Yolk (mm^3)	18-43	0.1987	89
Size at Hatch (2 MPE; g)	18-43	0.0634	686
Size at Necropsy (6 MPE; g)	7-20	0.0746	147
SMR (4 MPE; $\mu l\ O_2/min/g$)	10-31	0.4002	22
HSI (6 MPE; %)	7-20	0.3176	19
TSI (6 MPE; %)	7-20	0.1113	64
Total Glandular T_4 (6 MPE; ng/ml)	7-20	0.1115	64

Table 3-5. Biological responses of red-eared slider and snapping turtle juveniles to embryonic BDE-47 exposures.

	BDE-47 Dose (ng/g egg)					
	Control	40	220	400	700	1000
Red-eared Slider						
Size after overwintering (10 MPE; g)	7.605±0.128 (19)	6.833±0.166 (9)	7.400±0.323 (10)	7.310±0.294 (10)	7.660±0.243 (10)	7.130±0.229 (10)
Size at Necropsy (16 MPE; g)	10.71±0.407 (16)	11.45±1.007 (8)	11.54±0.930 (8)	11.09±0.722 (10)	10.96±0.940 (8)	9.860±0.776 (10)
HSI (16 MPE; %)	5.642±0.290 (16)	5.070±0.355 (8)	5.601±0.263 (8)	5.737±0.284 (10)	4.908±0.371 (8)	5.169±0.279 (10)
TSI (16 MPE; %)	0.032±0.0027 (16)	0.027±0.0048 (8)	0.025±0.0038 (8)	0.024±0.0026 (10)	0.028±0.0046 (8)	0.032±0.0037 (10)
Total Glandular T_4 (16 MPE; ng/ml)	5353±579.8 (3)	4156±907.4 (5)	4233±826.3 (4)	3762±1070 (5)	2024±379.0 (4)	3956±1258 (5)
Snapping Turtle						
Size after overwintering (10 MPE; g)	10.70±0.127 (23)	10.45±0.160 (10)	10.11±0.156* (13)	10.31±0.238 (10)	10.36±0.173 (10)	10.09±0.172 (10)
Size at Necropsy (16 MPE; g)	26.37±2.20 (23)	23.68±2.74 (10)	29.80±2.63 (13)	25.14±2.52 (10)	24.68±3.14 (10)	29.47±2.63 (10)
HSI (16 MPE; %)	4.835±0.317 (23)	4.276±0.378 (10)	5.544±0.373 (13)	4.990±0.391 (10)	4.957±0.515 (10)	5.320±0.391 (10)
TSI (16 MPE; %)	0.014±0.0008 (23) [A]	0.013±0.0013 (10) [AB]	0.012±0.0014 (13) [AB]	0.015±0.0011 (10) [A]	0.015±0.0018 (10) [A]	0.0093±0.006 (10) [B]
Total Glandular T_4 (16 MPE; ng/ml)	4198±651.7 (12)	3377±490.6 (5)	3176±793.9 (7)	2453±334.2 (5)	3325±1044 (5)	4300±616.4 (5)

Values are means ± standard error with sample sizes listed below each mean in parentheses. Different superscripts represent significant differences following multiple comparisons tests (p<0.05). * indicates significant difference from control only after multiple comparison tests (p<0.05). MPE=months post exposure; HSI=hepatosomatic index; TSI=thyrosomatic index.

Table 3-6. Standard metabolic rate and times to right of juvenile red-eared sliders and snapping turtles at three different time periods embryonically exposed to BDE-47.

BDE-47 Dose (ng/g egg)	SMR (μl O_2/min/g)		Time to Right (s)	
	Red-eared Sliders	Snapping Turtle	Red-eared Sliders	Snapping Turtle
Control				
Assay 1 (12 MPE)	0.636±0.029 (20)	0.743±0.032 (23)	189.3±35.81 (11)	199.9±43.14 (20)
Assay 2 (13 MPE)	0.801±0.054 (20)	0.937±0.045 (23)	298.2±53.31 (11)	228.4±42.54 (22)
Assay 3 (14 MPE)	0.789±0.055 (20)	0.766±0.036 (22)	287.9±51.65 (14)	125.6±27.23 (19)
400				
Assay 1 (12 MPE)	0.739±0.048 (10)	0.871±0.089 (10)	309.8±70.68 (6)	72.67±25.15 (10)
Assay 2 (13 MPE)	0.889±0.044 (10)	0.944±0.069 (10)	376.1±49.24 (9)	94.27±33.79 (10)
Assay 3 (14 MPE)	0.857±0.032 (10)	0.774±0.029 (10)	273.6±91.35 (6)	189.9±59.24 (10)
700				
Assay 1 (12 MPE)	0.697±0.055 (10)	0.715±0.041 (10)	288.8±91.07 (5)	36.32±21.09* (8)
Assay 2 (13 MPE)	0.761±0.074 (10)	1.284±0.159* (10)	389.8±96.37 (5)	94.52±35.07 (10)
Assay 3 (14 MPE)	0.692±0.041 (10)	0.747±0.049 (10)	351.2±145.9 (4)	126.1±47.02 (10)
1000				
Assay 1 (12 MPE)	0.734±0.038 (9)	0.776±0.057 (10)	495.1±75.88 (6)	78.20±38.60 (10)
Assay 2 (13 MPE)	1.055±0.136 (9)	1.305±0.176 (10)	412.2±85.45 (5)	100.7±26.70 (10)
Assay 3 (14 MPE)	0.901±0.046 (9)	0.792±0.032 (10)	451.6±103.9 (5)	210.5±72.45 (9)

Values are means ± standard error with sample sizes listed after each mean in parentheses.
* indicates significant difference from control only after multiple comparison tests
($p < 0.05$).   MPE=months post exposure; SMR=standard metabolic rate.

Table 3-7. Coefficient of variation calculated as a percentage for each continuous data juvenile endpoint.

		BDE-47 Dose (ng/g egg)				
	Control	40	220	400	700	1000
Red-eared Slider						
Size after overwintering (10 MPE; g)	7.34 %	7.28 %	13.81 %	12.71 %	10.02 %	10.14 %
Size at Necropsy (16 MPE; g)	15.20 %	24.87 %	22.81 %	20.59 %	24.25 %	24.90 %
HSI (16 MPE; %)	20.54 %	19.79 %	13.30 %	15.64 %	21.36 %	17.07 %
TSI (16 MPE; %)	33.08 %	49.81 %	42.69 %	35.37 %	47.26 %	36.70 %
Total Glandular T_4 (16 MPE; ng/ml)	18.76 %	48.82 %	39.04 %	63.61 %	37.46 %	71.10 %
Snapping Turtle						
Size after overwintering (10 MPE; g)	5.71 %	4.84 %	5.57 %	11.93 %	5.29 %	5.38 %
Size at Necropsy (16 MPE; g)	40.07 %	33.05 %	31.78 %	31.75 %	40.24 %	28.22 %
HSI (16 MPE; %)	31.44 %	27.95 %	24.25 %	24.78 %	32.86 %	23.23 %
TSI (16 MPE; %)	26.88 %	31.69 %	40.36 %	25.12 %	37.09 %	21.41 %
Total Glandular T_4 (16 MPE; ng/ml)	53.78 %	32.49 %	66.13 %	30.47 %	70.21 %	32.06 %

Table 3-8. Observed power and sample size needed to achieve a power of 0.8 for juvenile endpoints.

	Sample Size	Observed Power	Sample Size Needed for a Power of 0.8
Red-eared Slider			
Size after overwintering (10 MPE; g)	9-19	0.3357	24
Size at Necropsy (16 MPE; g)	8-16	0.1489	50
HSI (16 MPE; %)	8-16	0.1846	39
TSI (16 MPE; %)	8-16	0.1591	46
Total Glandular T_4 (16 MPE; ng/ml)	3-5	0.1987	11
Snapping Turtle			
Size after overwintering (10 MPE; g)	10-23	0.7043	28
Size at Necropsy (16 MPE; g)	10-23	0.1538	61
HSI (16 MPE; %)	10-23	0.2705	33
TSI (16 MPE; %)	10-23	1.00	NA
Total Glandular T_4 (16 MPE; ng/ml)	5-12	0.0628	181

Table 3-9. Coefficient of variation calculated as a percentage for juvenile standard metabolic rate (SMR) and time to right for each of the three assay periods.

	SMR (µl O_2/min/g)		Time to Right (s)	
	Red-eared Sliders	Snapping Turtle	Red-eared Sliders	Snapping Turtle
BDE-47 Dose (ng/g egg)				
Control				
Assay 1 (12 MPE)	20.50 %	20.70 %	62.74 %	96.53 %
Assay 2 (13 MPE)	38.64 %	23.09 %	59.30 %	87.37 %
Assay 3 (14 MPE)	30.95 %	22.23 %	67.12 %	94.51 %
400				
Assay 1 (12 MPE)	20.63 %	30.59 %	55.89 %	109.46 %
Assay 2 (13 MPE)	15.74 %	23.08 %	39.28 %	113.35 %
Assay 3 (14 MPE)	11.95 %	12.01 %	81.78 %	98.65 %
700				
Assay 1 (12 MPE)	24.80 %	17.96 %	70.52 %	164.20 %
Assay 2 (13 MPE)	30.79 %	39.19 %	55.28 %	117.32 %
Assay 3 (14 MPE)	18.73 %	20.62 %	83.09 %	117.88 %
1000				
Assay 1 (12 MPE)	15.66 %	23.29 %	40.48 %	156.09 %
Assay 2 (13 MPE)	30.33 %	42.73 %	46.35 %	83.86 %
Assay 3 (14 MPE)	15.30 %	12.59 %	51.44 %	103.26 %

Table 3-10. Observed power and sample size needed to achieve a power of 0.8 for juvenile standard metabolic rate (SMR) and time to right for each of the three assay periods.

	Red-eared Slider			Snapping Turtle		
	Sample Size	Observed Power	Sample Size Needed for a Power of 0.8	Sample Size	Observed Power	Sample Size Needed for a Power of 0.8
SMR (μl O2/min/g)						
Assay 1 (12 MPE)	9-20	0.1934	45	10-23	0.2740	31
Assay 2 (13 MPE)	9-20	0.4049	21	10-23	0.3676	27
Assay 3 (14 MPE)	9-20	0.7431	11	10-22	0.0782	217
Time to Right (s)						
Assay 1 (12 MPE)	5-11	0.3777	12	8-20	0.3437	21
Assay 2 (13 MPE)	5-11	0.1049	52	10-22	0.2819	33
Assay 3 (14 MPE)	4-14	0.1147	34	9-19	0.1242	79

Figures
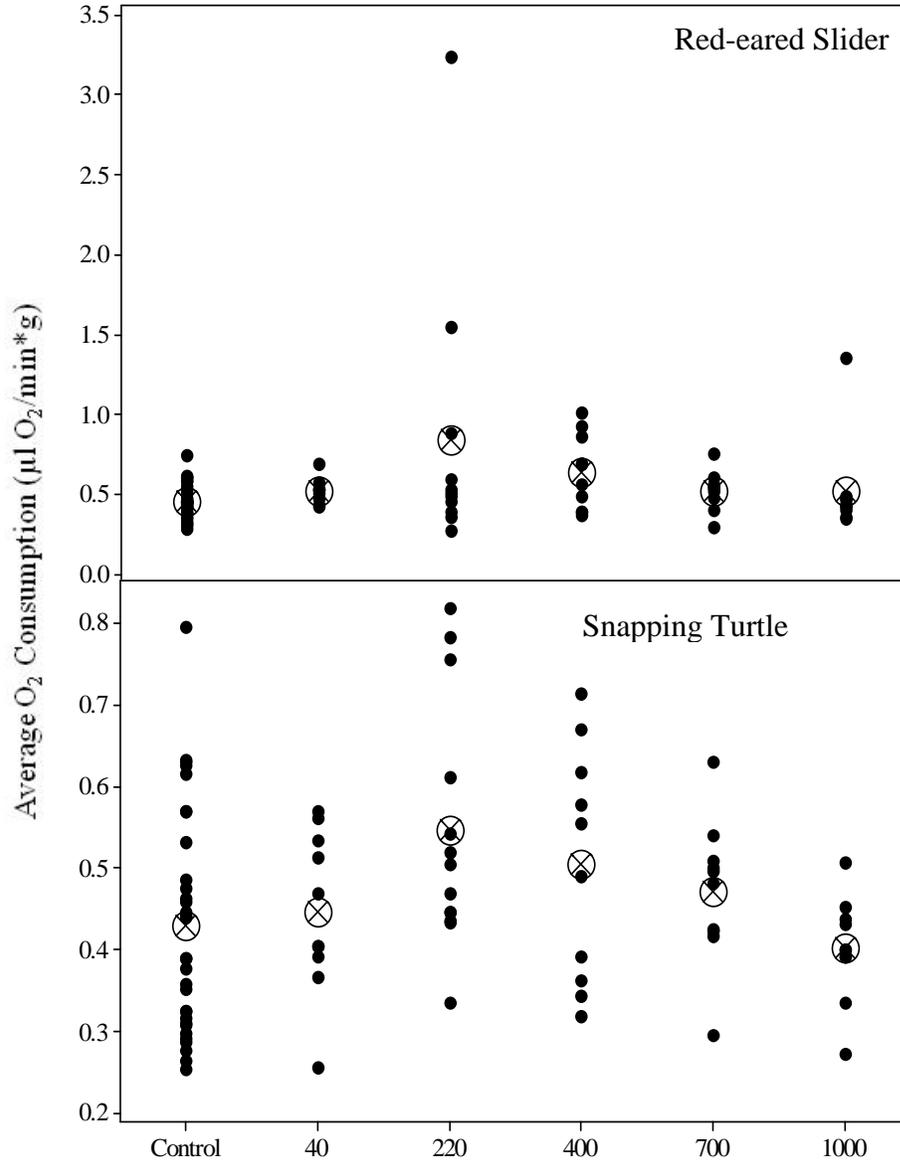
Figure 3-1. Hatchling red-eared slider and snapping standard metabolic rate measured 4 months post exposure for BDE-47 (ng/g egg) embryonic treatments. The crosshairs represent treatment means.
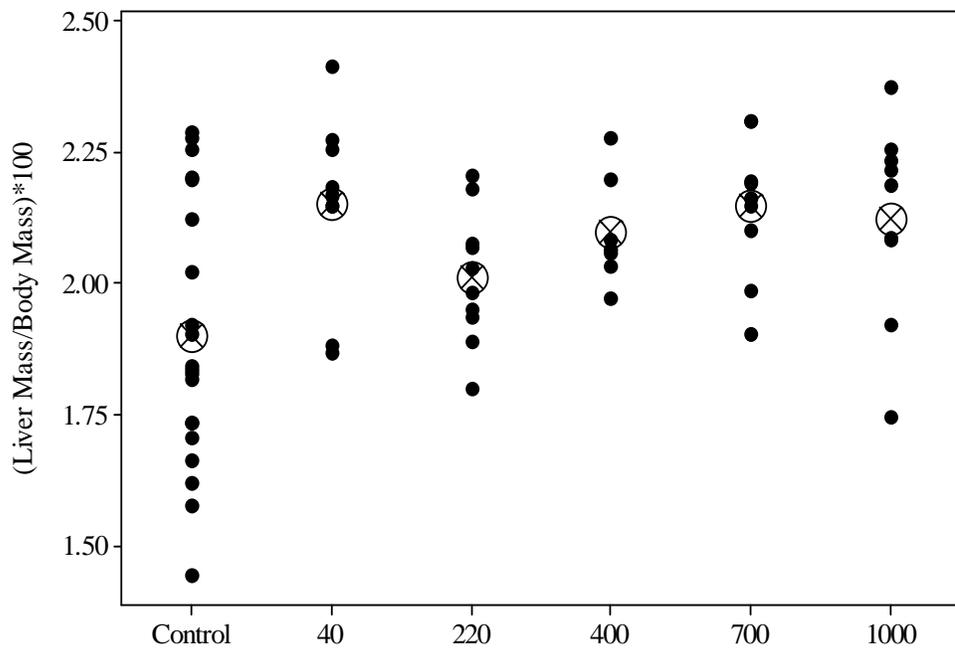
Figure 3-2. Snapping turtle hatchling hepatosomatic index (HSI) measured 6 months post exposure for BDE-47 (ng/g egg) embryonic treatments. The crosshairs represent treatment means.
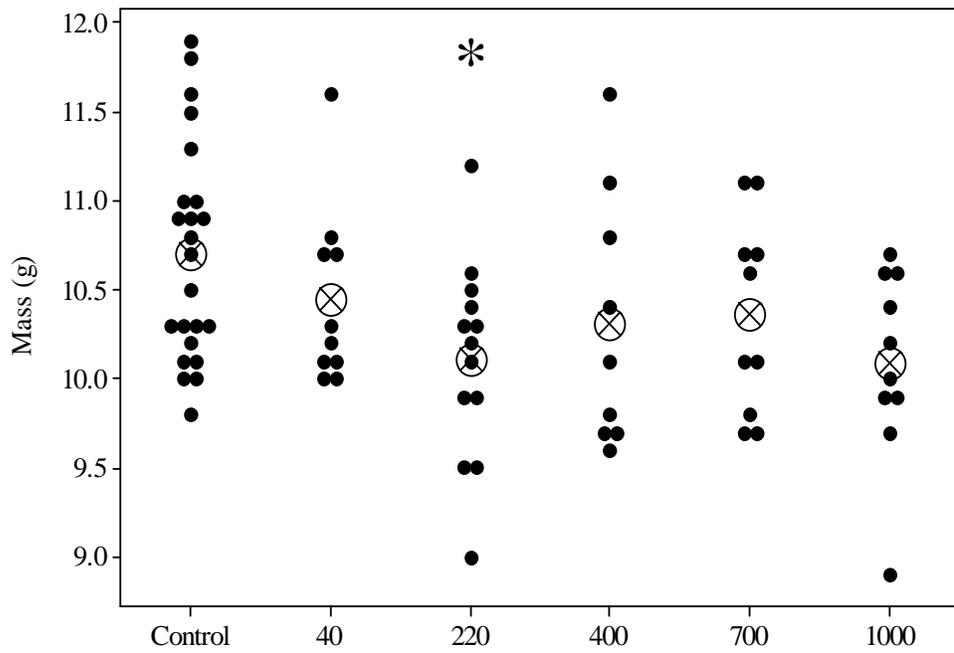
Figure 3-3. Snapping turtle mass immediately following over-wintering (10 months post exposure) for BDE-47 (ng/g egg) embryonic treatments. The crosshairs represent treatment means and * indicates significant difference from control after multiple comparison tests (p<0.05).
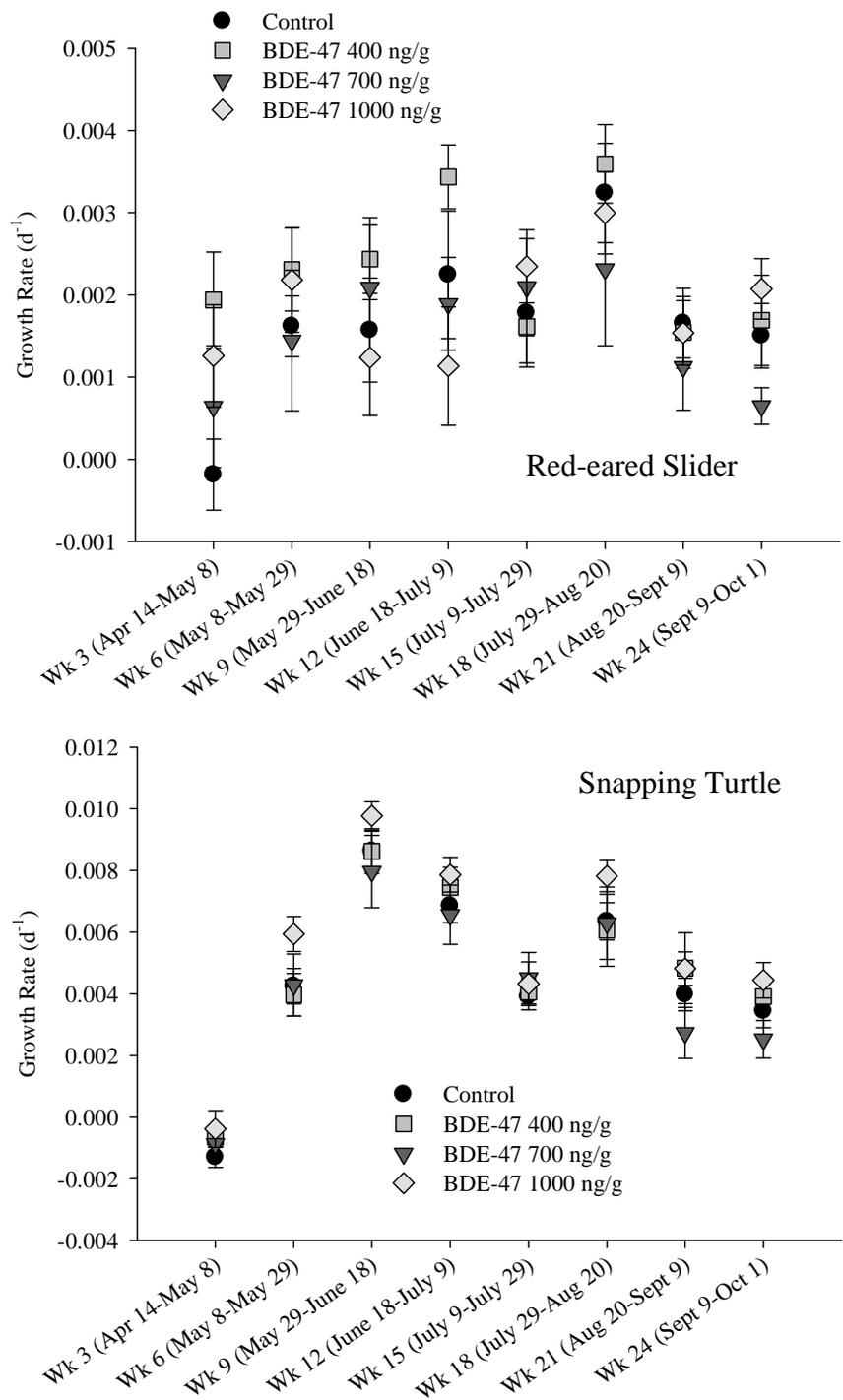
Figure 3-4. Growth rate (d^{-1}) across the juvenile period for the red-eared slider (top) and snapping turtle (bottom). Symbols represent the mean ± 1 SE.
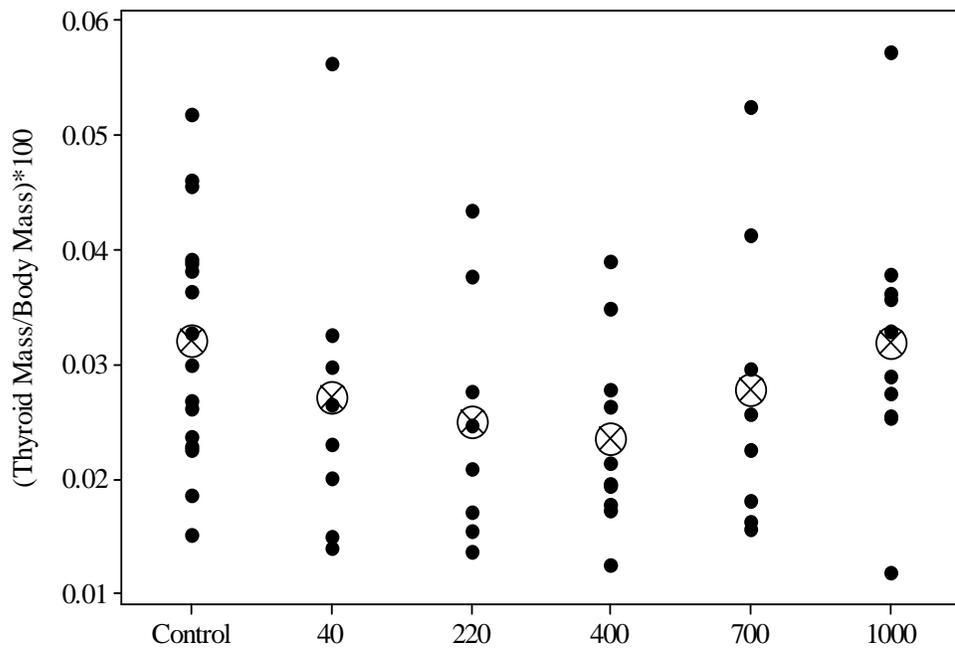
Figure 3-5. Red-eared slider juvenile thyrosomatic index (TSI) 16 months post exposure for BDE-47 (ng/g egg) embryonic treatments. The crosshairs represent treatment means.
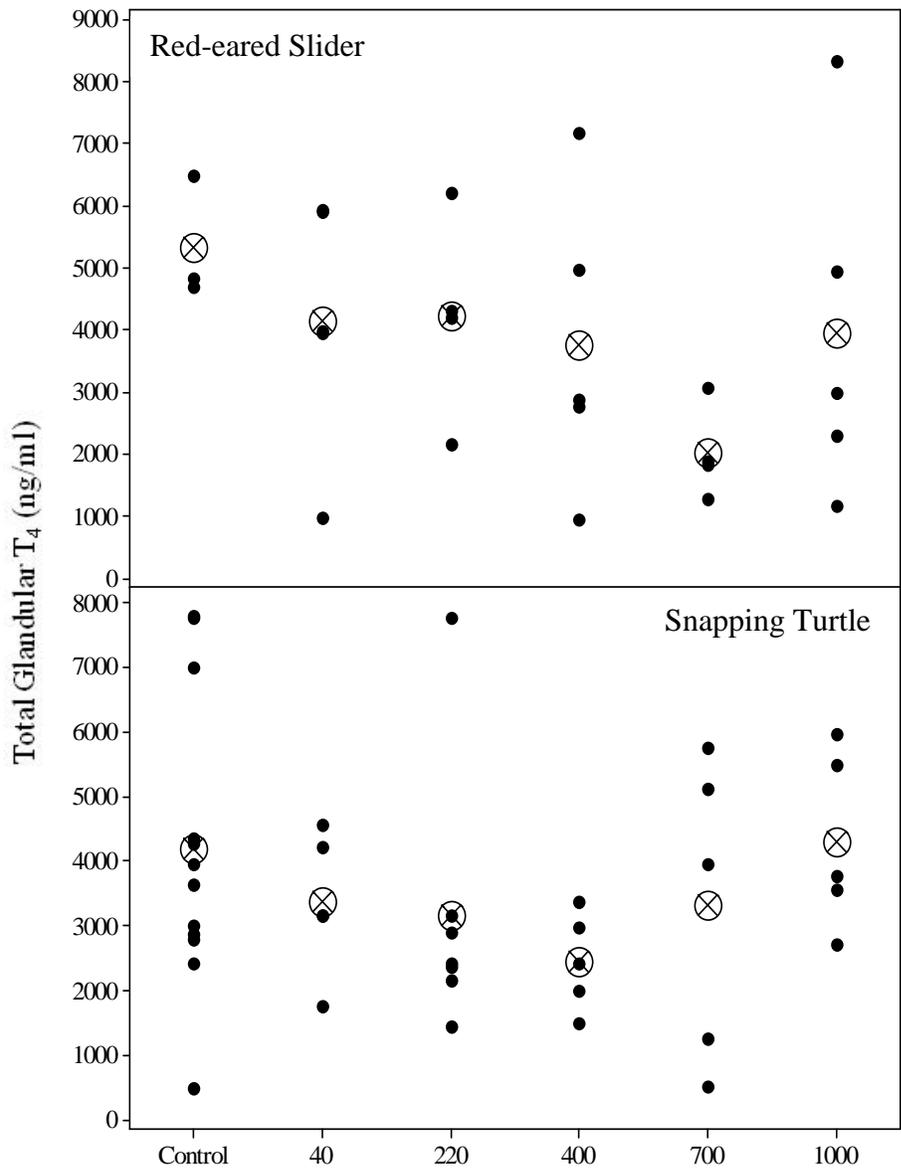
Figure 3-6. Juvenile red-eared slider and snapping turtle total glandular T_4 16 months post exposure for BDE-47 (ng/g egg) embryonic treatments. The crosshairs represent treatment means.

Chapter 4: Comparative effects of *in ovo* exposure to sodium perchlorate on the development, growth, metabolism, and thyroid function in the common snapping turtle (*Chelydra serpentina*) and red-eared slider (*Trachemys scripta elegans*)

Abstract

Perchlorate is a surface and groundwater contaminant frequently present in areas associated with munitions and rocket manufacturing and use. It is known to be a thyroid-inhibiting compound, preventing uptake of iodide by the thyroid gland, ultimately reducing thyroid hormone production. Thyroid hormones play key roles in regulating growth and development, and thus perchlorate exposure during the embryonic period may impact embryonic traits that ultimately influence performance or survival. In this study, eggs of red-eared sliders (*Trachemys scripta*) and snapping turtles (*Chelydra serpentina*) were exposed to a total of 200 µg/g and 177 µg/g respectively of perchlorate as $NaClO_4$ early in the incubation period to determine impacts on glandular thyroxine concentrations, embryonic growth and development, and metabolic rates of hatchlings for a period of two months post-hatching. *In ovo* perchlorate exposure resulted in increased incubation time, external yolk size, and hatchling mortality while decreasing hatchling total glandular thyroxine concentrations in the red-eared sliders. In the snapping turtles, hatching success and standard metabolic rates were reduced, liver and thyroid sizes were increased, and hatchling total glandular thyroxine concentrations were reduced after exposure to perchlorate. While both species were negatively affected by exposure to

perchlorate, impacts on red-eared sliders were most severe, suggesting that the slider may be a more sensitive sentinel species for studying effects of perchlorate exposure to turtles.

Introduction

Increasingly, perchlorate and its salts have become contaminants of concern due to their stability and persistence in ground and surface water as well as widespread use in explosives, pyrotechnics, rocket fuel, missiles and some fertilizers (Urbansky, 1998; Sridhar et al. 1999). The extensive use of perchlorate in the aerospace, defense and chemical industries has led to significant contamination of water, soil, sediment and biota (vegetation, aquatic insects, fish, amphibians, and mammals) throughout the United States (Urbansky, 1998; Smith et al. 2001, 2004; Mayer et al. 2006).

Perchlorate salts, such as ammonium perchlorate (NH_4ClO_4) and sodium perchlorate ($NaClO_4$), are highly soluble in water and once released into an aquatic system they readily dissociate producing the salt and perchlorate ions (Urbansky, 1998). In vertebrates, ionic perchlorate competitively inhibits the thyroid gland from taking up iodide, ultimately reducing thyroid hormone production leading to reduction of glandular thyroxine as well serum concentrations of thyroxine (T_4) and triiodothyronine (T_3; Stanbury and Wyngaarden 1952; Saito et al. 1983; Wolff 1998). Alterations of thyroid hormone homeostatis have serious implications for animal health because the hormones play a role in regulating growth, embryonic and neurological development, and metabolism of lipids and proteins (Clark 2000).

The majority of studies that have evaluated the effects of perchlorate on vertebrates focused on amphibians, birds and mammals, whereas to our knowledge there

has been only one study that has examined effects on reptiles (Redick-Harris 2006). Redick-Harris (2006) studied the impacts of perchlorate on embryonic development of fence lizards (*Sceloporus sp.*) by exposing the eggs to perchlorate in the incubation substrate. At extremely high concentrations of perchlorate in the substrate (1585 and 15,852 µg ClO_4^-/g substrate), exposure resulted in embryonic mortality and decreased total embryonic oxygen consumption. At lower concentrations, mortality and oxygen consumption were not significantly altered, but duration of embryonic development was increased and whole body T_4 was significantly reduced (158 µg ClO_4^-/g substrate).

Like the fence lizard, freshwater turtles nest in habitats potentially contaminated by perchlorate. Turtle embryos are quite sensitive to water soluble, lower molecular weight contaminants (such as perchlorate) since the eggs are laid in moist soil, thus being in direct contact with contaminants present in soil and water. This study focused on two species of freshwater turtle, the common snapping turtle (*Chelydra serpentina*) and red-eared slider (*Trachemys scripta*), both of which have flexible, porous shells allowing for absorption of water from the nest substrate (Packard et al. 1982), thus exposing embryos to water-soluble contaminants that diffuse across the eggshell.

The primary purpose of this study was to determine whether thyroid processes can be disrupted by perchlorate exposure during the embryonic stages of freshwater turtles, and whether there are subsequent implications for the health and survival of the hatchlings. Secondly, this study compared species-specific response to perchlorate to determine which species may be best suited for use as a sensitive model species for impacts of perchlorate on embryonic and hatchling traits. To these ends, we exposed eggs of snapping turtles and red-eared sliders a concentration of perchlorate predicted to

disrupt thyroid function and quantified effects on endocrinological, developmental, and physiological functions in hatchlings.

Methods

Egg Collection

Red-eared slider eggs were collected from Concordia Turtle Farm in Wildsville, LA. All slider eggs were laid on May 21st. Snapping turtle eggs were purchased from RR Aquafarms LLC in Preston, MD. All snapping turtle eggs were laid on May 27th. Upon laying eggs were measured for diameter and wet weight, given a unique identifying number written on the upper surface of the eggshell with a number two pencil, and immediately placed in shallow bins half submerged in vermiculite mixed with water in a 1:1 mass ratio. One egg from each of 23 different red-eared slider clutches was randomly assigned to either the perchlorate treatment (n=23) or to the control group (n=23) while only seven different snapping turtle clutches were needed with three to five eggs from each clutch randomly selected for each treatment (n=23 for each treatment). Eggs were kept at approximately 18 ºC until June 1st (Day 1) when incubation temperatures were raised to 26 °C, a temperature known to produce only males in both species (Yntema 1976; Wibbles et al., 1991). Moisture and humidity were maintained by misting the eggs and nest substrate with water at 2-3 day intervals.

Dosing

The dosing protocol was established for use in a larger study to determine sublethal effects of polybrominated diphenyl ether embryonic exposure on turtles (Chapter 2). One of the key endpoints in the larger study was thyroid function, thus perchlorate was chosen for use as a positive control for this endpoint, due to its known effects on thyroid function (Stanbury and Wyngaarden 1952; Saito et al. 1983; Wolff 1998). Sodium perchlorate was purchased from Sigma ($\geq$98 % purity) and prepared in 5 ml of nanopure water. All eggs (n=23) were topically dosed with a total of 70 µl over 14 days with verified concentrations of 28 mg/ml for red-eared sliders and 30 mg/ml for snapping turtles. The dosing solutions were analyzed by Summit Environmental Technologies, Inc. (Cuyahoga Falls, OH) using ion chromatography and United States Environmental Protection Agency methods for determination of perchlorate in drinking water (Hautman et al. 1999) with a limit of quantitation of 0.5 mg/L. The eggs were dosed over 14 days to avoid an acutely toxic dose of perchlorate reaching the embryo in addition to allowing for use of a smaller volume of solution per day to prevent the solution from rolling off the surface of the eggshell. Concentrations were adjusted based on the average egg mass for both species due to egg size differences (sliders 9.79 g, snapping turtles 11.89 g). Exposure regime was based on a study of fence lizards in which eggs were exposed to perchlorate-laced substrate, where exposure to 195 µg $NaClO_4^-$/g of substrate was observed to reduce whole body total T_4, yet did not significantly elevate mortality relative to controls (Redick-Harris 2006). Control eggs (n=23) were handled in the same manner as the perchlorate treated eggs (n=23) but received no applications of fluid to the surface of the egg.

Hatchlings

Each week, during incubation, random eggs from each treatment were candled to determine viability and developmental stage. Eggs showing signs of arrested development or mortality were removed and dissected to identify the stage of development at which mortality occurred and to document any gross deformities. Approximately 50 days after the start of incubation we began inspecting eggs daily and removing individuals that had pipped (e.g., split the egg shell but had not yet emerged). These individuals were held on moist sphagnum moss until having emerged from the shell. Hatching of red-eared slider controls occurred from August 20th to August 26th (91 to 97 days of incubation) whereas hatching of the perchlorate treated eggs occurred from August 22nd to September 27th (93 to 98 days of incubation). Hatching of snapping turtle controls occurred from August 12th to August 24th (77 to 89 days of incubation) whereas the perchlorate treated snapping turtle eggs occurred from August 14th to August 27th (79 to 92 days of incubation).

Hatching success was measured as a proportion of individuals that successfully pipped and hatched. Since all eggs for each species were collected and incubated on the same days, time to hatch was measured as the number of incubation days from the start of incubation until complete hatch occurred. Upon hatching the turtles were measured for size and weight (carapace length-CL, carapace width-CW, plastron length-PL), and examined for gross abnormalities of the eyes, jaws, limbs, tail, bridge, and scutes. Finally, the volume of external yolk at hatch was also used as a measurement of overall

health. The length, width, and height of the residual, external yolk were measured using digital calipers to estimate the volume of the external yolk present at hatching.

Metabolic Rate and Organosomatic Indices

Resting metabolic rate (20 °C) was measured as oxygen consumption of resting and fasted animals, four weeks post-hatch, at 2.5 h intervals over a period of approximately 18 h by microrespirometry (MicroOxy-max^{TM}; Columbus Instruments, Columbus, OH) following a 24 h acclimation period (Nagle et al. 2001). The MicroOxy-max^{TM} was calibrated against a standard oxygen, carbon dioxide, and nitrogen mixture. After metabolic measurements were made, approximately two months post-hatch, individuals were weighed, measured, and euthanized by inhalation of isoflurane (NLS Animal Health, Owings Mills, MD, USA) followed by decapitation. Livers and thyroids were removed and weighed to calculate organosomatic indices.   We calculated hepatosomatic and thyrosomatic indices (HSI and TSI) as [(liver or thyroid gland weight / body weight)*100]. Individuals that died prior to measurements of metabolic rate were excluded. All procedures were approved by the University of Maryland Center for Environmental Science Institutional Animal Care and Use Committee (S-CBL-07-04).

Glandular Thyroxine

Upon being euthanized thyroid glands were removed from each turtle, snap frozen in liquid nitrogen and stored in −80 ºC conditions until analysis of thyroxine (T_4) content. Thyroid glands were digested according to McNabb et al. (2004a). Briefly, glands were

incubated in 350 µl of a digestion mixture (99.88 mM TRIS, 5.00 mM L-Glutathione reduced, 2.50 mM 6-n-propyl-2-thiouracil, and 1 % Triton X-100) containing 25 mg pronase (protease from *Streptomyces griseus*) for 24 hours at 37 ºC. L-Glutathione reduced, 6-n-propyl-2-thiouracil and Triton X-100 were supplied by Sigma-Aldrich and the pronase by EMD chemicals. After digestion, samples were stored at -20 ºC until later dilution and analysis by enzyme immunoassay (EIA).

Total glandular T_4 concentrations were measured using a competitive EIA kit purchased from Calbiotech (Spring Valley, CA) with a standard curve range of 20-250 ng/ml. This kit has a sensitivity of 4 ng/ml using standards prepared in human serum. To ensure that glandular T_4 could be accurately measured using this system, standard curves were also prepared in dilution buffer. T_4 standards were prepared by dilutions from a stock solution containing 0.5 mg/ml *l*-Thyroxine (Sigma-Aldrich) in 0.5 M HCl, 75 % ethanol. Further T_4 working stock solutions were prepared in 50 mM Tris buffered saline (0.9 % NaCl). The final standard solutions were prepared in the same concentrations as the serum standards (0 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, 150 ng/ml and 250 ng/ml) provided in the kit, in 1 % sheep serum (Innovative Research in Southfield, MI) and 50 mM Tris buffered saline, 0.9 % NaCl. Standard curves prepared in sheep serum were run along with the standard curve supplied by the manufacturer to verify parallelism for each assay.

Twenty microliters of 1.0 M perchloric acid was added to 100 µl of digested sample and incubated at room temperature for 40 minutes to precipitate proteins. The samples were spun at 5000 rpm for 10 min and then diluted in 1 % sheep serum to run for assay. Perchlorate samples from both species were diluted 1:5 while red-eared slider

controls were diluted from 1:5 to 1:40 and all snapping turtle controls were diluted 1:5 in 1 % sheep serum buffer (50 mM Tris buffered saline, 0.9 % NaCl). Final assay volumes were 25 µl for all samples and standards.

The EIA was validated for use on thyroid extracts of both species. Samples were run in duplicate to determine intra-assay variation using the coefficient of variation for both species (2.87 % ± 0.11 % and 2.80 % ± 0.15 %) for snapping turtles and red-eared sliders respectively; ± 1 SE as a percent of the mean). One snapping turtle thyroid sample digested, treated with perchloric acid and diluted 1:100 was run in each assay as a measure of inter-assay variation for both species (7.94 % ± 1.63 % and 6.33 % ± 0.90 %) for snapping turtles and red-eared sliders respectively; ± 1 SE as a percent of the mean) for 34 snapping turtle and 24 red-eared slider replicates. A composite of snapping turtle thyroids digested, charcoal stripped, precipitated with perchloric acid, spiked with 10 µg/ml T_4 standard and diluted 1:100 was run in each assay along with the 10 µg/ml spiking standard diluted 1:100 to determine accuracy (5.84 % ± 0.51 %; mean ± 1 SE). Recovery in the spiked samples was also determined (101.51 ng/ml ± 0.91; mean recovery ± 1 SE). Finally, a composite of snapping turtle thyroids digested, charcoal stripped, precipitated with perchloric acid and diluted 1:10 was run as a blank to determine background interference (15.46 ng/ml ± 0.30; mean ± 1SE). Each sample was corrected for background interference in each assay. Cross-reactivity of the kit is <1 % with *d-* and *l-*triiodothryonine.

Statistics

Contingency tables were used to analyze hatching success and post-hatching mortality for both species. One-way analysis of variance (ANOVA) was used to analyze all other data with the exception of the red-eared slider thyrosomatic index (TSI) where an unequal variance t-test was used because variances were heterogeneous regardless of transformation (Ruxton, 2006). Statistical significance was judged based upon a Type I error rate of $\alpha \square 0.05$. The rate of oxygen consumption ($\mu l\ O_2$/min.), which varies allometrically with body size, was corrected for mass following methods employed by Manyin and Rowe (2006). These data were log transformed to meet assumptions of normality and homogeneity of variances. Data for time to hatching and total glandular T_4 concentrations in red-eared sliders were rank-transformed (Conover and Iman, 1981). Data for organosomatic indices for both species and the total T_4 concentrations in the snapping turtles were log transformed.

Results

Hatching success was reduced in the perchlorate exposure treatment for both species (snapping turtle: control-100 %, perchlorate-73.9 %; red-eared slider: control-95.7 %, perchlorate-78.3 %). This reduction was statistically significant for the snapping turtles (p=0.009), but not for the red-eared sliders (p=0.080; Table 4-1). Of the snapping turtles that hatched, only two from the perchlorate treatment died within two month post-hatch duration of the study. In contrast, five red-eared sliders exposed to perchlorate died within two months after hatching, which was significantly greater than mortality in controls (p=0.008; Table 4-1). The number of incubation days prior to hatching was

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significantly greater in the perchlorate treated red-eared sliders relative to controls (p<0.0001), but this was not the case for snapping turtles (p=0.196).

For perchlorate-exposed snapping turtles, hatchling sizes did not differ from controls, whereas carapace width of exposed sliders was significantly reduced relative to controls (p=0.050).   The volume of the external yolk did not vary significantly between treatments for the snapping turtles, but was significantly larger in the red-eared slider hatchlings exposed to perchlorate and on observation took a longer time to be absorbed (p≤0.001; Table 4-1).

Standard metabolic rates were significantly decreased in the snapping turtle hatchlings exposed to perchlorate relative to controls (p=0.025; Figure 4-1). However, standard metabolic rates did not differ between treatments for red-eared sliders (p=0.987; Figure 4-1). Standard metabolic rates in the controls differed between species such that red-eared sliders had significantly lower standard metabolic rates than snapping turtles (p≤0.0001), indicating species-specific differences in respiration, despite correction for size differences.   Hepatosomatic index (HSI) was significantly larger in the snapping turtle hatchlings exposed to perchlorate compared to the control (p=0.012; Table 4-1). This was not the case in the red-eared sliders (p=0.657). Thyrosomatic index (TSI) for the snapping turtles exposed to perchlorate was significantly larger than the controls (p=0.001); however, there was no significant difference in red-eared sliders (p=0.180; Table 4-1). Both species exposed to perchlorate had significantly reduced total glandular T_4 concentrations compared to the controls. This was true for the total glandular T_4 concentrations either when corrected or uncorrected for thyroid mass (p≤0.0001 for both species; Figure 4-2).

Discussion

Embryonic exposure to perchlorate had significant impacts on survival and indicators of physiological and thyroid health in both snapping turtles and red-eared slider. However, the only response common to both species was a decrease in glandular thyroxine (T_4) concentrations. It is well known that perchlorate inhibits iodide uptake into the thyroid, preventing the synthesis of thyroid hormones (Stanbury and Wyngaarden, 1952; Wolff, 1998). The significant decrease in glandular T_4 concentrations in both species was therefore expected.

It is known that thyroid hormones have important roles in regulation of several processes in vertebrates including growth, embryonic development, metabolism of lipids and proteins, and neurological development (Clark, 2000). Thus the perchlorate-driven decrease in total glandular T_4 concentrations may explain the physiological disruptions that were found in embryonic development and hatchling health. In this study, both the control and perchlorate-exposed snapping turtle hatchlings were able to successfully utilize their external residual yolk sacs by two weeks after hatching. In contrast, red-eared sliders exposed to perchlorate not only had significantly larger yolk sacs at hatching than the controls, but were unable to completely utilize their external yolk after hatching.

Although the snapping turtles exposed to perchlorate absorbed their yolk sac, they also displayed an increased hepatosomatic index, suggesting that they may not have been able to metabolize the yolk for energy and rather may have allocated the energy to lipid stores in the liver (however we did not quantify hepatic lipid content). Hepatic lipidosis

is not an uncommon response of vertebrates to exposure to contaminants; in fact, several studies have documented increased lipid content in livers in response to PCBs (Heaton et al. 1995; Kuzyk et al. 2003). Although the mechanism of toxicity of perchlorate is different from that of PCBs, both inhibit thyroid function (Webb and McNabb 2008; McNabb and Fox, 2003; Klaassen and Hood, 2001) linking impacts on the liver to thyroid function and contaminant exposure.

The reduced glandular T_4 concentrations in the perchlorate treated snapping turtles led to hypothyroidism as evidenced by the significantly increased thyroid size which potentially led to systemic downstream effects. For example, the lack of T_4 present in the hatchlings could have led to a decrease in metabolism measured by the standard metabolic rate since thyroid hormones control metabolic processes. Thus, when thyroid function is compromised, metabolism of lipids and proteins for energy could be impacted (Clark, 2000), leading to increased liver size found in the snapping turtles.

In contrast to the snapping turtles, the red-eared sliders did not show increased thyroid or liver size, nor did they have a reduced standard metabolic rate when exposed to the same concentration of perchlorate that induced these effects in snapping turtles. However, embryonic perchlorate exposure reduced glandular T_4 concentrations in red-eared sliders. Similar to our results whole body T_4 concentrations were significantly decreased in fence lizards at a similar embryonic exposure concentration (Redick-Harris, 2006). Other studies have examined exposure of juvenile and adult vertebrates to perchlorate in drinking water and reported reduced T_4 concentrations (McNabb et al. 2004a; Goleman et al. 2002; Tietge et al. 2005). However, it is difficult to interpret our

results in comparison to these studies of other species due to differences in routes of exposure and life stages of the exposed individuals.

Despite reduced glandular T_4 in the red-eared slider, they responded differently to hypothyroidism than the snapping turtles. Unlike snapping turtles, red-eared sliders showed a marked response to perchlorate in that they possessed significantly larger external yolk reserves at hatching as well as significantly increased mortality after hatching relative to unexposed individuals. Redick-Harris (2006) exposed western fence lizards eggs to perchlorate in the incubation substrate and found that at an exposure of 1,950 and 19,500 µg $NaClO_4$/g substrate hatching success was significantly impaired. In contrast, we observed reduced hatching success of red-eared sliders at a much lower concentration (200 µg $NaClO_4$/g egg weight). However, we applied perchlorate directly to the surface of the egg whereas exposures in the study by Redick-Harris (2006) were more indirect (via incubation substrate), perhaps accounting for greater toxicity in our study. Embryonic exposure to perchlorate appeared to have greater lethal impacts on hatchling red-eared sliders compared to the snapping turtle suggesting it to be a more sensitive species under embryonic exposure conditions. On inspection of red-eared slider individuals at hatching it was clear that the individuals exposed to perchlorate were more severely impacted than the snapping turtles. For example, in the five cases of red-eared sliders that did not survive to two months post hatch, their external yolk sacs had not been fully absorbed prior to death. Compared to the snapping turtles, some red-eared sliders were not able to utilize their energy stores from their yolk, resulting in mortality.

Based on the significantly larger size of the external yolk it was expected that the standard metabolic rate of the red-eared sliders from the perchlorate treatment would

have been significantly lower than the controls, however, this was not the case. This can be explained by the generally low standard metabolic rate of the controls. The control red-eared sliders had a significantly lower standard metabolic rate compared to the snapping turtle controls and since hatchling turtles in general have a low standard metabolic rate immediately following hatching there was not enough difference in the oxygen consumption of the perchlorate and control treatments of the red-eared sliders to elicit a significant difference (Figure 4-1). It is possible with a larger sample size the separation between treatments would have been more pronounced.

This is the first study to quantify impacts of perchlorate on embryonic and hatchling traits of turtles. Turtle embryos can easily be exposed to perchlorate due to their porous egg shells and presence of nests in moist soils, providing for diffusion of dissolved perchlorate into the egg during incubation. The common snapping turtle and red-eared slider could potentially be useful reptilian models for identifying effects of embryonic exposure to perchlorate. Our results demonstrate that, even at a single concentration, both red-eared sliders and snapping turtles are affected lethally and sublethally by perchlorate exposure *in ovo*. In addition, the red-eared slider was more sensitive to an *in ovo* perchlorate exposure as evidenced by its greater lethal response. With little information available for impacts of perchlorate on reptiles, additional work is required to further determine turtle embryonic and hatchling sensitivity to perchlorate. In particular, further studies of species-specific responses across a range of environmentally relevant concentrations and during different portions of the embryonic period would provide information for assessing the risks of perchlorate exposure to turtles under a

variety of conditions similar to those that the animals may experience in contaminated

habitats.

Tables

Table 4-1. Endpoint measures of embryonic exposure to perchlorate in both species. Mean ± standard error followed by the minimum and maximum points and the sample size. * indicate significant difference from the control of the same species (significance at p≤0.05).

Endpoint	Common Snapping Turtle		Red-eared Slider	
	Control	Perchlorate	Control	Perchlorate
Hatching Success (%)	100 % (23/23)	73.91 %* (17/23)	95.65 % (22/23)	78.26 % (18/23)
Post Hatching Mortality (%)	0 % (0/23)	11.76 % (2/17)	0 % (0/22)	27.78 %* (5/18)
Incubation Length (Days)	79.83±0.57 73-85 n=23	81.06±0.77 75-88 n=17	83.55±0.47 80-88 n=22	96.53±2.35* 83-118 n=18
External Yolk Volume (mm^3)	439.06±41.86 136.72-921.26 n=23	548.02±68.07 193.86-1313.86 n=17	1207.36±138.15 103.37-2329.07 n=22	2415.29±242.46* 621.55-4627.96 n=18
Carapace Width (mm)	27.60±0.18 26.37-29.21 n=23	27.77±0.30 25.86-30.00 n=17	26.58±0.33 23.99-30.17 n=22	25.42±0.49* 22.22-28.34 n=18
HSI (%)	1.82±0.086 1.44-2.56 n=10	2.42±0.20* 1.75-4.78 n=17	4.77±0.22 3.85-5.88 n=10	5.23±0.40 2.78-7.48 n=16
TSI (%)	0.014±.0025 0.0075-0.035 n=10	0.028±0.0044* 0.0067-0.078 n=17	0.034±0.0021 0.024-0.044 n=10	0.044±0.0068 0.017-0.094 n=16
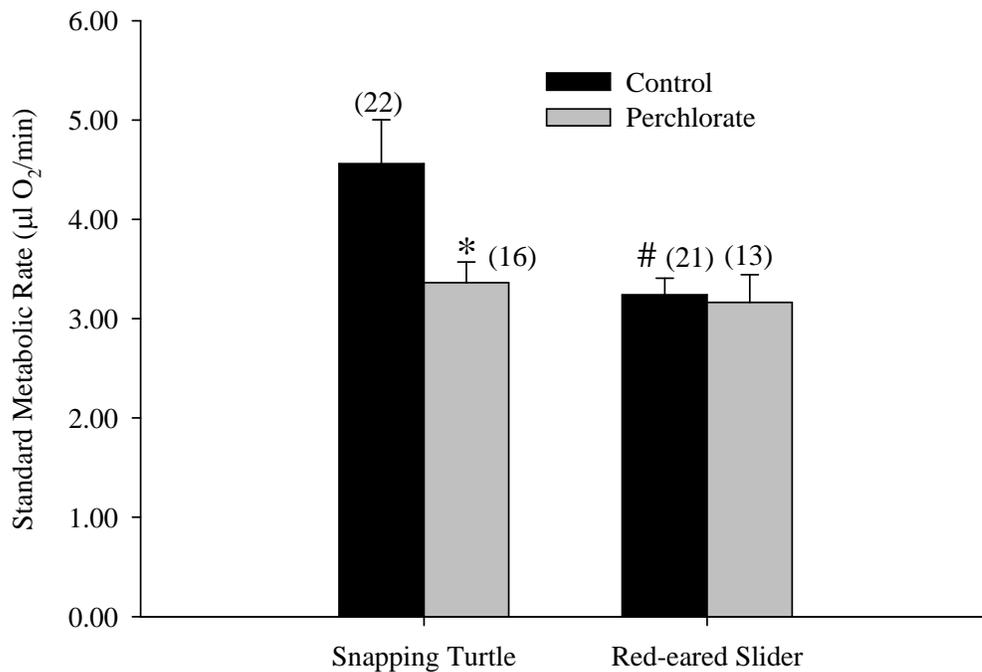
Figure 4-1: Standard metabolic rate corrected for mass. * Indicates significant differences from the control of the same species. # Indicates significant differences between the same treatments of the two species. Significance at $p \leq 0.05$; mean $\pm$ 1 SE. Sample sizes in parentheses.
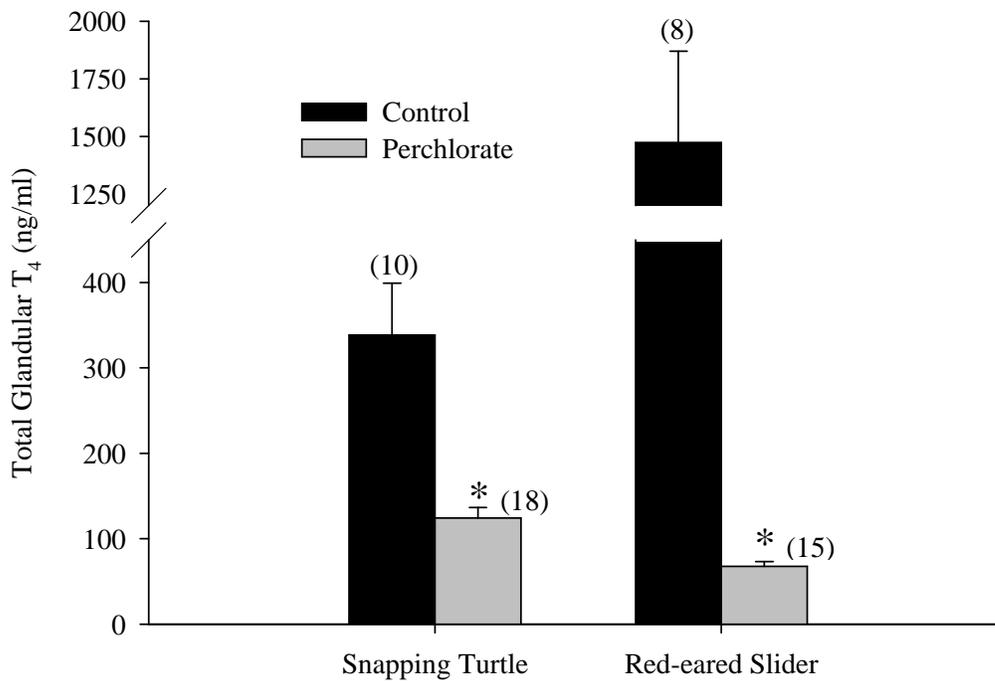
Figure 4-2: Total Glandular Thyroxine (T_4) (ng/ml). * Indicates significant differences from the control of the same species. Significance at $p \leq 0.05$; mean $\pm$ 1 SE. Sample sizes in parentheses.

Chapter 5: Dietary exposure of BDE-47 and BDE-99 and effects on behavior, bioenergetics and thyroid function in juvenile red-eared sliders (*Trachemys scripta elegans*) and common snapping turtles (*Chelydra serpentina*)

Abstract

In this study, red-eared sliders (*Trachemys scripta elegans*) and snapping turtles (*Chelydra serpentina*) were fed food dosed with BDE-47 or BDE-99 for six months beginning approximately nine months post-hatch. During the exposure period, measurements of growth, bioenergetics and behavior were made while thyroid function and accumulation were quantified post-exposure. Whole body concentrations of both congeners were lower in red-eared sliders compared to the snapping turtles after six months of exposure. BDE-47 and -99 did not impact growth in either species but snapping turtles receiving a diet containing BDE-47 had significantly elevated standard metabolic rates after three and four months of exposure. Behavior was modified in red-eared sliders exposed to both congeners. When exposed to BDE-47 there was increased activity in response to a simulated predator in the red-eared sliders. In contrast, when they were exposed to BDE-99, the red-eared sliders were slower to right themselves after having been inverted. Snapping turtle behavior was not affected by dietary exposure to either congener. No apparent effect on liver or thyroid masses was observed in either species, but total glandular T_4 concentrations were significantly reduced in the red-eared sliders exposed to BDE-47. These results demonstrate that dietary exposure to BDE-47

and -99 can elicit a suite of responses and effects in two species of turtles, but the red-eared slider appeared to be a more sensitive species across endpoints than the snapping turtle. This suggests that the snapping turtle may not be the best surrogate for relating concentrations to effects.

Introduction

Polybrominated diphenyl ethers (PBDEs) are flame retardants that have been historically used in a wide variety of consumer products including flame-resistant polystyrene and polyurethane foams, treated textiles, electronics and plastics (de Wit 2002; Alaee et al. 2003). PBDEs have been commercially produced in three distinct formulations including the lower brominated Penta-bromodiphenyl ether, Octa-bromodiphenyl ether, and the Deca-bromodiphenyl ether primarily containing the fully brominated congener BDE-209. As a result of their widespread use and global distribution of consumer products incorporating these compounds, PBDEs are ubiquitous in marine and freshwater organisms (Hale et al. 2001, 2003; Law et al., 2003; de Solla et al. 2007; Chen and Hale 2010). Due to the increasing environmental concentrations of PBDEs, in particular the lower brominated mixtures, in addition to the well documented bioaccumulative and toxic properties, both the Penta-BDE and Octa-BDE are no longer on the market in Europe and North America (Directive 2003/11/EC; Tullo 2003; Ward et al. 2008). Deca-BDE is still largely unregulated, however, and although it was banned in 2008 in Europe from use in electronic equipment it remains in use in the U.S. Use in the U.S. will soon be voluntarily phased out of production and use by 2012 due to the increasing evidence that BDE-209 can be assimilated and metabolized in the environment

to form the more toxic lower-brominated congeners (Kierkegaard et al. 1999; Stapleton et al. 2004c, 2006; Van den Steen et al. 2007; BSEF 2010). Even with regulations in place for PBDEs, environmental concentrations are continuing to increase and continue to pose a threat to wildlife health.

Of most concern are the PBDEs that make up the Penta-BDE mixture as they tend to have a greater potential to be bioaccumulated and are more commonly found in biological matrices even though they are no longer in use. Impacts of penta-BDEs on developmental and behavioral processes have been documented in a wide variety of vertebrate species. Of particular concern are indications that PBDEs may disrupt the thyroid system potentially affecting thyroid hormone homeostasis by competing with thyroxine (T_4) for binding sites on the transport protein transthyretin, binding to the thyroid hormone receptors, as well as increasing metabolism of T_4 leading higher excretion (Meerts et al. 1998 and 2000; Marsh et al. 1998; Zhou et al. 2001, 2002; Hallgren and Darnerud 2002; Morgado et al. 2007; Richardson et al. 2008). Many studies have demonstrated inverse relationships between plasma thyroid hormone concentrations and whole body or treatment concentrations of Penta-BDEs (Fernie et al. 2005a; Costa and Giordano 2007; Talsness 2008).

In addition to changes in thyroid hormone homeostasis, exposure to PBDEs can lead to substantial developmental and neurotoxic effects. Several studies have verified that PBDEs can cause developmental and behavioral effects including reduced motor skills, learning abilities, memory and hyperactivity in rats and mice (Eriksson et al. 1998, 1999, 2002; Viberg et al. 2003; Branchi et al. 2002, 2003, 2005; Kuriyama et al. 2005; Costa and Giordano 2007; Driscoll et al. 2009). Changes in development and behavior

after exposure to Penta-BDEs have also been documented in a variety of non-mammalian vertebrates. Juvenile zebrafish (*Danio rerio*) exposed to BDE-47 through their diet exhibited early impacts to growth after exposure as well as reduced activity and swim distance that were negatively correlated with tissue level of BDE-47 while *Rana pipiens* tadpoles exposed to a commercial mixture of Penta-BDEs showed reduced growth and delayed development (Chen et al. 2010; Chou et al. 2010; Coyle and Karasov 2010). In addition, adult American kestrels (*Falco sparverius*) exposed through their diet to a Penta-BDE technical mixture exhibited changes in reproductive courtship behavior (Fernie et al. 2008).

A lack of information on PBDE effects on a variety of long-lived species presents a critical data gap hindering understanding of the importance of chronic accumulation and effects of PBDEs in many natural systems. Development of models that represent the types of processes that occur under natural exposure regimes is critical for evaluating ecological implications of PBDEs. Vertebrates having long life spans, delayed maturation, and high trophic status may be particularly useful for assessing ecological effects of PBDEs due to their propensity to accumulate persistent lipophilic contaminants (e.g., Rowe 2008). Turtles in particular possess these traits and, while they are known to accumulate PBDEs (de Solla et al. 2007, 2008; Moss et al. 2009; van de Merwe et al. 2010), resultant effects on juvenile health and fitness are unknown.

There have not been controlled laboratory studies conducted to determine specific effects of PBDE exposure in juvenile turtles. A pair of studies has examined environmental levels of a suite of co-occurring persistent organic pollutants, including PBDEs in snapping turtle (*Chelydra serpentina*) eggs and effects on hatching success and

incidence of deformities (de Solla et al. 2007, 2008). In these studies, however, it was determined that concentrations of PBDEs in eggs were on average 45.6 times lower than polychlorinated biphenyl (PCB) concentrations, but PBDE concentrations were increased compared to other typically measured organochlorine compounds such as mirex and dichlorodiphenyldichloroethylene (DDE) complicating determination of direct causal links between the contaminants measured and impacts to hatching success and deformities (de Solla et al. 2008).

In addition, other research studying contaminant effects on turtles, typically using red-eared sliders (*Trachemys scripta elegans*), have focused on both environmental and laboratory embryonic exposures to various contaminants and resultant hatchling effects, none of which focus on PBDEs (Bishop et al. 1991, 1998; Bell et al. 2006; Crews et al. 1995; Willingham and Crews 1999, 2000; Willingham et al. 2000). Only one study, to the authors' knowledge, carried out a laboratory dietary study looking at multiple developmental, metabolic and behavioral endpoints in juvenile snapping turtles, again not focusing on PBDEs but PCBs (Eisenreich et al. 2009).

In this study we exposed juvenile red-eared sliders and snapping turtles to two PBDE congeners in the diet to determine the impacts of dietary exposure on survival, growth, metabolism, behavior, and thyroid function over a six month feeding period. Using two species as models, we also examined species-specific differences in response to exposures to evaluate their relative utilities as bio-indicators of ecological impacts of PBDEs.

Methods

Eggs Collection

Thirteen clutches of red-eared slider eggs (laid on May 21st) and four clutches of snapping turtle eggs (laid on May 27th) were collected and purchased from Concordia Turtle Farm in Wildsville, LA and RR Aquafarms LLC in Preston, MD respectively. Care and incubation were described in Chapter 3. Briefly, measurements of size, both weight and diameter (snapping turtle eggs) or length and width (red-eared slider eggs) were taken after each egg was given an unique identifying number written on the upper surface of the egg in number two pencil. Eggs were maintained in a damp substrate (vermiculite mixed with water in a 1:1 mass ratio) with incubation beginning on June 1st at 26 ºC, a temperature known to produce only males (Yntema 1976; Wibbles et al. 1991). Two eggs from each clutch were randomly selected and homogenized for contaminant analysis of background concentrations of PBDEs in the egg contents as within-clutch variation of contaminant concentrations have been found to be low (Bishop et al. 1994). All procedures were approved by the University of Maryland Center for Environmental Science Institutional Animal Care and Use Committee (S-CBL-07-04).

Hatchlings and Juvenile Husbandry

Approximately 50 days after incubation, daily monitoring for hatching occurred. At first signs of hatching, initial crack in the eggshell, individual eggs were transferred to a plastic cup with a bed of wet sphagnum moss. After full emergence from the eggs, wet mass, carapace length and width, and plastron length were measured. In addition to size

measurements, each individual was checked for any possible deformities and those with classified deformities were eliminated from the study. Approximately 2 months post-hatch (MPH) hatchlings were transferred from moss to 1-3 cm of water and maintained at 20 ℃ in a constant temperature room for 2 weeks followed by a reduction in temperature of 5 ℃ increments in two week time periods until a final over-wintering temperature of 5 ℃ was reached to simulate more natural over-wintering conditions and environmental stresses. Approximately 6 MPH the temperature was raised at 5 ℃ increments every two weeks until a temperature of 20 ℃ was reached (7 MPH). Individuals were then moved to a flow through housing system providing a constant fresh water source with details provided in Chapter 3. Turtles were acclimated to their housing conditions and food (prepared control food described below) following feeding with prepared treatment food every other day *ad libitum*. Approximately 14 MPH and after 6 months of dietary exposure all juveniles were euthanized by inhalation of isoflorane (NLS Animal Health, Owings Mills, MD, USA) followed by decapitation. At the time of necropsy, livers and thyroids were collected and weighed for calculation of organ mass indices ([(liver or thyroid gland weight / body weight)*100]) as well preserved for chemical and hormone analyses.

Food Preparation

Control food was prepared by mixing Fluker's aquatic turtle diet (40 % of wet mass; Port Allen, LA, USA) ground in a blender with Gel Fish Food (17 % of wet mass; Aquatic Eco-Systems Inc. Apopka, FL, USA), deionized water (43 % of wet mass) and 10 ml cod liver oil. BDE-47 and BDE-99 treated food were prepared by dissolving neat

forms of both congeners (Accustandard Inc., New Haven, CT, USA) in 10 ml cod liver oil. The BDE solutions were then added drop wise to the dry mixture of aquatic turtle diet and Gel Fish Food. The dry mixture was then mixed with an electric hand mixer to homogenize the dry contents after which deionized water was added to and mixed. The bulk food for all treatments was then formed into 5.0 ± 0.1 g pellets and frozen at -20 ºC until needed for feeding.

Biological Endpoints

Growth and Standard Metabolic Rate

Juveniles were measured and weighed every three weeks immediately following overwintering. Juvenile growth rate (k) was measured for each three week measurement period ($k=\ln(W_2)-\ln(W_1)/(t)$, where W_2 is size at end of the 3 week period, W_1 is size at the previous 3 week period, and t is the number of days between measurements.

Microrespirometry (MicroOxy-max[TM]; Columbus Instruments, Columbus, OH) was used to measure standard metabolic rate ("SMR": µl O_2 consumed/min) of fasted, resting juveniles after 2, 3 and 4 months of dietary exposure. However, during the 2-month measurement for the snapping turtles, the respirometer failed, thus precluding use of those data. Respiration measurements were taken according to methods of Chapter 3 and Nagle et al. (2001) and the MicroOxy-max[TM] was calibrated against a standard oxygen, carbon dioxide, and nitrogen mixture. Wet weight and carapace length measurements were made following the trials to account for the impact of size on the amount of O_2 consumed by each individual.

Behavior

Behavioral assays commenced after one month of dietary exposure using individuals of all three treatments (n=15) repeated for months 3 and 4 of exposure as described in Chapter 3. Briefly, the response to a visual stimulus was used to test the ability of the juveniles to recognize and respond to a visual stimulus. The technique was adapted from one employed by Winkelman (1996), in which a simulated avian predator was used to cast a shadow on or near the test animal. Individuals were placed in an aquarium covered on all sides and lighted from above by a unidirectional light source. A shadow of an avian predator was casted over the turtle with the response recorded, characterized, and categorized over three trials per individual. Responses were categorized as moving away from the shadow, flinch, head bob, no response, any combination of responses, or movement not listed.

The second behavioral assay conducted ("righting response") was the time required for the individual to right itself from a ventrally-exposed position was measured (Steyermark and Spotila 2001). Using replicated tests on representative animals (as above), individuals were placed on their back with movements and time to right recorded.

Glandular Thyroxine

For glandular Thyroxine (T_4) measurements, thyroids were removed from individuals and snap frozen in liquid nitrogen and stored in -80 ℃ conditions for future analysis by enzyme immunoassay (EIA). Digestion of the thyroid glands followed methods of McNabb et al. (2004a) and detailed in Chapter 4. Whole snapping turtle (n=13) and red-eared slider (n=12) glands were digested for 24 hours at 37 ℃ in a

mixture of 99.88 mM TRIS, 5.00 mM L-Glutathione reduced, 2.50 mM 6-n-propyl-2-

thiouracil, and 1 % Triton X-100 and stored at -20 ºC until time of assay.

As detailed in Chapter 1, a competitive EIA kit purchased from Calbiotech

(Spring Valley, CA) was used to measure total glandular T_4. The kit has a sensitivity of

4ng/ml using standards ranging from 20-250 ng/ml in human serum. An identical

standard curve was prepared using L-thyroxine (Sigma-Aldrich) in 0.5 M HCl, 75 %

ethanol and diluted in 1 % sheep serum (Innovative Research in Southfield, MI) to ensure

turtle glandular T_4 could be accurately assayed using this kit. The standard curve

prepared in sheep serum was also used to verify parallelism in each assay. After

digestion and perchloric acid treatment, Red-eared slider and snapping turtle juvenile

samples were diluted 1:10 to 1:100 with final sample and standard volumes of 25 µl used

for the assay.

As reported in Chapters 3 and 4, intra-assay variation was measured as coefficient

of variation for both species and was minimal (2.87 % $\pm$ 0.11 and 2.80 % $\pm$ 0.15 for

snapping turtles and red-eared sliders respectively; $\pm$ 1 SE as a percent of the mean).

Similarly, inter-assay variation was satisfactory (7.94 % $\pm$ 1.63 and 6.33 % $\pm$ 0.90 for

snapping turtles, n=34, and red-eared sliders, n=24, respectively; $\pm$ 1 SE as a percent of

the mean). Accuracy was determined to be 5.84 % $\pm$ 0.51 (mean $\pm$ 1 SE) for a snapping

turtle thyroid composite that was charcoal stripped and spiked with 10µg/ml T_4 standard

and diluted 1:100 with a recovery of 101.51 ng/ml $\pm$ 0.91 (mean recovery $\pm$ 1 SE).

Background interference for the charcoal stripped and composited snapping turtle

thyroids was determined to be 15.46 ng/ml $\pm$ 0.30 (mean $\pm$ 1 SE) and samples were

corrected accordingly. Finally, kit cross-reactivity was <1 % with *d-* and *l-*
triiodothryonine.

Analytical Methods

Chemical analyses on eggs, juveniles and food for background and post-treatment
concentrations of total PBDEs were conducted. Extraction details and analytical
procedures are described in detail in Chapter 3. Briefly, 3 g of homogenized wet tissue
(egg composite, whole body tissue, or food samples) was dried using sodium sulfate.
PBDEs from the tissue and food samples were extracted using accelerated solvent
extraction (ASE 300; Dionex) with dichloromethane.   Each sample was spiked directly
with ^{13}C-BDE-15 and ^{13}C-BDE-118 (surrogate standards) prior to extraction for
calculation of analyte recoveries. Recovered extracts were concentrated and removal of
nonpolar interferences was done using deactivated Florisil® column chromatography.

Thirty-four BDE congeners were quantified using a gas chromatograph (Agilent
6890N) coupled to a mass spectrometer (Agilent 5973N) operated in negative chemical
ionization mode. The programmable temperature vaporization (PTV) injector was used
in pulsed splitless mode (5 µl injections). A 15 m DB-5MS column (J&W Scientific,
Folsom, CA, USA) with an inner diameter of 0.25 mm and 0.1 µm film thickness was
used. Two slightly different oven temperature programs, injector and detector
temperatures as well as inlet and column flows were used due to improvements on
methods as detailed in Chapter 3. The egg and food samples were run using one program
and juvenile whole body analyses using the second program with slight differences in
initial hold temperature and time, the final hold temperature and time, and the injector
and detector temperatures. Inlet and column flow were 100 ml/min and 1.3 ml/min,

respectively. Prior to instrumental analysis, ^{13}C-CDE-86 (2,2',3,4,5-pentachlorodiphenyl ether) and ^{13}C-BDE-209 (decabromodiphenyl ether) were added as internal standards to all samples and calibration standards. The mass fragments *m/z* -79 and -81 were monitored for di- to octa-BDEs, -487 and -409 for the nona-BDEs and BDE-209, -318 and -316 for ^{13}C-CDE-86, and -495 and -415 for ^{13}C-BDE-209 for quantitative and qualitative ions, respectively. BDE standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), Wellington Labs (Guelph, Ontario, Cananda), and Accustandard (New Haven, CT, USA) or received from the U.S. National Institute of Standards and Technology (NIST; Githersburg, MD, USA).

Three times the analyte mass in the laboratory blanks divided by the mass of the sample extracted was the method detection limit (MDL). Mean recoveries (± 1 standard error) for BDE surrogate standards ^{13}C-BDE-15 and ^{13}C-BDE-118 in red-eared slider and snapping turtle eggs were 76.21 % ± 1.81 % and 81.73 % ± 2.60 % as well as 78.76 % ± 1.45 % and 77.74 % ± 1.62 % respectively. Mean recoveries of surrogate standards in red-eared slider juveniles were 73.46 % ± 2.61 % and 82.00 % ± 2.29 and 89.46 % ± 4.88 % and 85.24 % ± 3.60 % in snapping turtle juveniles.

Statistics

Data were analyzed using Minitab® (Minitab Inc., version 15). Whole body concentrations of BDE-47 and BDE-99, size, growth rate, standard metabolic rate (SMR), times to right, organ to body mass ratios, and total glandular T_4 were compared using analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. Data from both behavioral assays, response to the shadow of a predator, and participation in

the righting response were compared using contingency analysis with a Bonferroni

correction to conduct pairwise comparisons. Prior to conducting statistical analyses,

continuous data were checked for homogeneity of variances using Levene's test and

normality in distribution using Shapiro–Wilk (W) statistic followed by log, square root or

rank transformations as necessary. Statistical significance was judged based upon a type I

error rate of $\alpha \square 0.05$ for all ANOVA analyses and $\alpha \square 0.017$ for all contingency table

analyses that were subject to the Bonferroni correction. Due to the allometric

relationship between metabolic rate and body size, oxygen consumption was corrected

for mass and analyzed following methods employed by Manyin and Rowe (2006).

Results

Egg, Food and Whole Body PBDEs

Background concentrations of total PBDEs in the red-eared sliders and snapping

turtle eggs were 0.166 ± 0.027 ng/g wet weight (ng/g ww) and 0.144 ± 0.035 ng/g ww,

respectively. PBDE congeners -47, -99, -100, -153, -154, and -183 were regularly

detected in red-eared slider eggs whereas -100, -153, -154 and -156 were detected in

snapping turtle eggs. The average concentrations of BDE-47 and BDE-99 in red-eared

slider eggs were 0.064 ± 0.008 ng/g ww and 0.098 ± 0.014 ng/g ww respectively.

Congeners BDE-47 and BDE-99 were below detection limits in the snapping turtle eggs.

Low concentrations of BDE-47 and BDE-99 were found in the control food while

the concentration of BDE-47 and -99, in their respective treatment food, were 2055 ng/g

ww and 1425 ng/g ww (Table 5-1). BDE-47 and -99 whole body tissue concentrations,

reported on a per animal basis, for the control treatments were non-detectable or

extremely low for both species (Table 5-1). Red-eared sliders receiving a diet of BDE-47 or BDE-99 treated food had whole body concentrations significantly lower than that of the snapping turtles exposed to the same diet ($p=0.010$ and $p=0.039$; Table 5-1). Across treatments, red-eared sliders had significantly lower whole body concentrations of BDE-47 relative to BDE-99 ($P=0.005$), however, no significant difference was found between treatments in the snapping turtles ($P=0.279$; Table 5-1).

Growth and Standard Metabolic Rate

There were no significant differences in size immediately following overwintering (7 MPH), measured as both mass and carapace length, among treatments in either species (Table 5-2). In addition, mass and carapace length were not statistically different among treatments in either species at the end of the exposure period (Table 5-2). Growth rate did not differ among treatments for any of the eight measurement periods for either species. Growth rate peaked at approximately 10 MPH (third measurement period) for the snapping turtles and at approximately 10 to 11 MPH (between the third and fifth measurement periods) for the red-eared sliders (Figure 5-1).

Standard metabolic rates measured within two, three, and four months of exposure did not significantly differ among the treatments in any of the measurement periods for the red-eared slider (Figure 5-2). In general, SMR for the red-eared sliders was higher for the first two months of measurements and then decreased during the third month corresponding with the decrease in growth rate (Figures 5-1 and 5-2). At three months of exposure, SMR was significantly elevated in the snapping turtles exposed to BDE-47 compared to those receiving control food and BDE-99 treated food ($p=0.014$;

Figure 5-3). However, at four months of exposure, SMR was only significantly increased in the snapping turtles exposed to BDE-47 compared to the control (p=0.019; Figure 5-3).

Behavior

Predator Avoidance

Predator avoidance response by red-eared sliders was impacted by exposure to BDE-47. When all responses were pooled (e.g., regardless of the specific type of response) across the three assay time periods, red-eared sliders exposed to BDE-47 displayed increased activity in response to a simulated predator relative to both the control and BDE-99 exposed treatments (p=0.003 and p=0.011 respectively). When examined by specific reaction (head bob, flinch, or move away), those exposed to BDE-47 had significantly greater head bob and flinch responses compared to both control and BDE-99 exposed treatments (head bob: p=0.007 and p=0.019; flinch: p=0.002 and p=0.014) when all three assay period were combined.

When data were examined by the specific assay period, responses were not significant until the third month of dietary exposure (i.e., second assay period). Red-eared sliders exposed to BDE-47 showed an increased active response to the shadow compared to the control treatment (p=0.002) and the BDE-99 exposed group prior to Bonferroni correction (p=0.029). When the reactions were grouped by specific reaction type, red-eared sliders exposed to BDE-47 showed increased head bob and flinch reactions compared to the control treatment (p=0.003 and p=0.007). The head bob reaction was also significantly greater when compared to those individuals exposed to BDE-99, but only prior to correction (p=0.034). Similar patterns in increased activity

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were found during the third assay period, during four months of exposure, but were not significant. In contrast to the red-eared sliders, snapping turtle behaviors were not influenced by exposure to either BDE-47 or -99 (p-values ranged from p=0.052 to p=0.514).

Righting Response

Several red-eared sliders did not make any movements or attempts to right when placed on their back and thus were classified as non-respondents in the righting response. Pooled data across the three assay periods (2, 3, and 4 months of exposure) indicated that significantly more red-eared sliders exposed to BDE-99 were non-respondent than individuals in the control treatment (p<0.0001).

Significantly more individuals in the BDE-99 treatment were non-respondent relative to controls during the first (2 months of exposure; p=0.030) and second (3 months of exposure; p=0.005) assay periods. Similar, yet not statistically significant patterns of response were found following the fourth month of exposure (p=0.057). As in the predator avoidance behavioral assay, numbers of non-respondent snapping turtles in the righting response assay were not affected by treatment across assay periods.

For red-eared sliders and snapping turtles that attempted a righting response, there were no significant differences in times to right for either species among treatments for any assay time period. Although not statistically significant, there appeared to be a pattern of increased time to right for red-eared sliders exposed to both BDE-47 and -99 in each righting response assay. Furthermore, high numbers of non-respondent individuals in the BDE-99 treatment resulted in a low sample size for analysis of time to right. While the smaller sample size precluded detection of significant differences among treatments

during the first assay period (2 months of exposure), a higher percentage of red-eared sliders in both the BDE-47 and -99 treatments did not completely right themselves within the 10 minute maximum compared to individuals in the control treatment (Figure 5-4a). In the second assay period (3 months of exposure) there was only one red-eared slider from the BDE-99 treatment with righting times less than the mean for controls (Figure 5-4b). In the third assay period (4 months of exposure) the data show a similar case with only two red-eared sliders from the BDE-99 treatment with a righting time below the mean of the control treatment. Otherwise, red-eared sliders exposed to BDE-99 had much longer righting times than controls (Figure 5-4c). There was no suggestion of a pattern in righting times related to exposure to either compound in snapping turtles (Figures 5-5a, 5b, and 5c).

Organ Indices and Glandular Thyroxine

Liver mass to body mass ratios among treatments for both species were not statistically different with only a slight increase in the mean ratio in the snapping turtles exposed to BDE-47 and BDE-99 (Figure 5-6). Analysis of the thyroid mass to body mass ratio yielded very slight and insignificant differences among treatments of both species (Figure 5-7).

Total glandular T_4 concentrations were significantly reduced in the red-eared sliders exposed to BDE-47 (p=0.034), whereas individuals exposed to BDE-99 responded with more subtle changes. The majority of individuals from the red-eared slider BDE-99 treatment had T_4 concentrations below that of the mean concentration of the control treatment. The wide range in values precluded detecting significance differences

between the BDE-99 and control treatments (Figure 5-8). No significant differences among treatments were detected for total glandular T_4 concentrations in snapping turtles.

Discussion

Based upon the suite of sub-lethal endpoints measured in this study, it appears that red-eared sliders were much more sensitive to dietary exposure to BDE-47 and -99 compared to snapping turtles, despite greater accumulation of both congeners by the snapping turtles. Behavioral traits, organ mass ratios, and total glandular T_4 concentrations of red-eared sliders were influenced by exposures, whereas for snapping turtles, only metabolic rate appeared to have been altered. To the authors' knowledge, this study is the first controlled comparison of species-specific bioaccumulation of and responses to dietary contaminant exposures in juvenile turtles. There is evidence from field studies, however, of differential accumulation (but not evidence of biological effects) of the two PBDE congeners examined here among species of adult turtles occupying the same habitat; Moss et al. (2009) measured PBDEs in plasma of adult Cumberland sliders (*Trachemys scripta troosti*) and the common musk turtle (*Sternotherus odoratus*) showing higher concentrations of both BDE-47 and -99 in the common musk turtle. These differences are likely due to natural dietary preferences of the two species.

Egg, Food and Whole Body PBDEs

Background concentrations of BDE-47 and -99 in eggs collected from turtle farms used in this study for both species as well as the whole body concentrations of the control treatments were well below or very near the concentrations measured in various species of turtle eggs and plasma samples collected from the environment (de Solla et al. 2007; Moss et al. 2009; Swarthout et al. 2010; van de Merwe et al. 2010). In these studies, concentrations of PBDEs in eggs or turtles did not correlate to any developmental measurements or other indicators of health taken suggesting that the background concentrations in the eggs used in this study would not compromise the measurements of development, behavior and endocrine disruption (de Solla et al. 2008; Swarthout et al. 2010; van de Merwe et al. 2010). While the dosing concentrations of BDE-47 and -99 used in this study were relatively high in their food source, these concentrations have been observed in small fish as well as in surface sediments in areas where turtles could potentially be exposed (Hale et al. 2001; Xia et al. 2008).

While this study did not focus specifically on the dynamics of uptake, elimination, and metabolism of BDE-47 and -99, the differences in concentrations that we observed in whole body tissue of both species after a total of six months of dietary exposure indicate differences in bioaccumulation parameters as both species were fed identical diets. The red-eared sliders had lower concentrations of both congeners compared to the snapping turtle which could indicate species differences in bioavailability of the compounds, uptake over the intestines, metabolism of the compounds and elimination. This difference could be due to higher growth rates in the snapping turtle relative to the red-eared slider, which suggests a higher level of food consumption rate with resultant increased potential

for accumulation. As adults, snapping turtles would likely bioaccumulate to a greater extent than the red-eared sliders as they typically have a carnivorous diet while adult red-eared sliders are typically omnivorous (Parmenter and Avery 1990; Spotila and Bell 2008). However, as juveniles, the diets of both species overlap considerably, as juvenile red-eared sliders consume a larger proportion of protein than they do as adults (Parmenter and Avery 1990). These dietary differences could account for differences in environmental bioaccumulation; however, in this study both species received the same diet indicating differences in parameters such as uptake across the intestines and metabolism of the compounds. Differential metabolism and elimination of PBDE congeners has been shown in rats and mice as well as different species of fish suggesting that there could be differences in the two turtle species, but would require additional studies to specifically address uptake and metabolism of PBDEs (Haak and Letcher 2003).

While the red-eared slider accumulated less of both congeners than the snapping turtle, both species accumulated less BDE-47 than BDE-99 even with the lower concentration of BDE-99 provided in the food. Typically, greater accumulation and assimilation of BDE-47 relative to BDE-99 has been observed in field and laboratory studies of vertebrates (Stapleton et al. 2004b; de Solla et al. 2007; Swarthout et al. 2010; Munschy et al. 2011). However, one study (Fernie et al. 2006) of American kestrels exposed to penta-BDE congeners showed higher concentrations of BDE-99 and -100 than BDE-47 despite the kestrels being exposed to higher concentrations of BDE-47. It was suggested that the American kestrels had a higher rate of metabolism and elimination of BDE-47 than for BDE-99 and -100 as well as that debromination of BDE-99 to BDE-47

was not significant (Fernie et al. 2006). Further study would be required to determine if both snapping turtles and red-eared sliders have a higher rate of metabolism and elimination for BDE-47 than for BDE-99 for similar reasons as was for the American kestrels.

Growth and Standard Metabolic Rate

Neither growth rates nor final sizes of either species were impacted by the dietary exposure to either BDE-47 or -99. The turtles were fed *ad libitum* thus they were likely able to consume enough energy and nutrients to compensate for any subtle changes that may have otherwise affected size and growth under more natural feeding regimes. While growth was not a sensitive endpoint in this study, studies of zebrafish exposed to BDE-47 and tadpoles exposed to a penta-BDE mixture showed reductions in growth and delays in development (Coyle et al. 2010; Chen et al. 2010).

While size and growth were not impacted by BDE-47 or -99 exposure, standard metabolic rate was significantly increased in the snapping turtles exposed to BDE-47. This elevation in energy expenditure for maintenance potentially represents the costs associated with metabolism and elimination mechanisms for dealing with the contaminant exposure (Calow 1991; Rowe et al. 1998; Hopkins et al. 1999; Manyin and Rowe 2006). An elevated standard metabolic rate would result in less energy available for basic energetic requirements, such as energetic needs for activity and growth. However as noted, this study employed an *ad libitum* feeding regime which may have provided energetic surpluses ample to compensate for elevated energetic expenditures resulting from the contaminant. If food was a limited resource as may occur in a more

normal environmental situation, the individual snapping turtles may not be able to consume enough energy to compensate for the increased standard metabolic rate and thus would allocate energy to deal with the contaminant at the expense of needs for growth and activity.

Behavior

There have been multiple studies that have documented effects of PBDEs on animal behaviors (reviewed in Costa and Giordano 2007; Fernie et al. 2008; Marteinson et al. 2010). However, only the red-eared sliders showed impacts of BDE-47 and -99 on behaviors in this study. In the predator avoidance assay red-eared sliders exposed to both PBDEs showed increased activity in response to the shadow of a predator. Developmental neurotoxicity in the form of behavioral alterations has been commonly documented as hyperactivity in response to PBDE exposure (see Costa and Giordano 2007). Contrary to the hyperactivity documented in the predator avoidance assay, red-eared sliders exposed to BDE-99 showed decreased activity in the righting response assay. This could be due to other neurological functions that can be altered by exposure to PBDEs including impacts to neurobehavioral development such as learning abilities and memory (Branchi et al. 2002, 2003; Viberg et al. 2003). Alterations to behavior such as those found in this study have potential connections to effects on the population level. For example, both the behavior assays conducted in this study are measures of predator avoidance and are essential for individual survival and necessary to maintain recruitment of juveniles into the population.

Organ Indices and Glandular Thyroxine

The liver mass to body mass ratio was slightly increased in the snapping turtles exposed to BDE-47 and -99, although the increases were not significant. The liver is primarily responsible for the metabolism of contaminants and hepatic enzyme activity involved in metabolism has been observed to increase after exposure to PBDEs (Zhou et al. 2001; Sanders et al. 2005; Stapleton et al. 2009; Bruchajzer et al. 2010). These increases in enzyme activity can lead to liver toxicity with one outcome being an increase in liver size (Dunnick and Nyska 2009; Albina et al. 2010; Bruchajzer et al. 2010). This suggests that the slight increase in liver size of the snapping turtles may be due to increased hepatic enzyme activity although it was not measured in this study. The observed increased standard metabolic rate in the snapping turtles also may suggest increased hepatic metabolic activity since elevated enzymatic activity in the liver would increase the total metabolic expenditures of the individual.

This study examined total glandular T_4 levels as it has been shown to be a more sensitive indicator of impacts on the thyroid system in birds (McNabb et al. 2004a, 2004b) than other physiological metrics such as plasma concentrations of T_4. The red-eared sliders exposed to BDE-47 had significantly reduced levels of total glandular T_4 compared to the controls. Those exposed to BDE-99 also showed lowered glandular T_4 levels, although they were not statistically significant. Total glandular T_4 in snapping turtles was not affected by exposure suggesting that they are less sensitive to PBDE exposure than the red-eared sliders at the concentrations used in this study. There are multiple studies that document decreases in plasma T_4 in response to PBDE exposure with impacts on T_4 binding to transthyretin, binding to the thyroid hormone receptors, as

well as in increased metabolism of T_4 (Meerts et al. 1998 and 2000; Marsh et al. 1998; Zhou et al. 2001, 2002; Hallgren and Darnerud 2002; Morgado et al. 2007; Richardson et al. 2008; Talsness 2008). Yet very few studies of non-mammalian vertebrates have examined PBDE exposure and respective T_4 concentrations. Fernie et al. (2005a) carried out a post-hatch exposure in American kestrels that found lower plasma T_4 concentrations in response to PBDEs, but McKernan et al. (2009) did not find alterations to glandular T_4 content after an *in ovo* exposure to PBDEs in the chicken, mallard (*Anas platyrhynchos*) or American kestrel. These differences suggest that the timing of exposure can be important.

Conclusion

This study demonstrates that dietary exposure to BDE-47 and -99 can elicit a suite of impacts potentially related to thyroid system function. Changes to the thyroid system can result in impairment of behavior, metabolism, and growth, several of which have been documented in this study. Total glandular T_4 levels and behavior were impacted in the red-eared slider while only metabolism was impacted in the snapping turtle even though the snapping turtle accumulated greater concentrations of both BDE-47 and -99. Thus, the red-eared slider was the more sensitive of the two species to dietary exposure of these two PBDEs at the tested concentrations. While the snapping turtle has been suggested to be a valuable indicator of accumulation of persistent organic pollutants in natural systems, this species may not be the best surrogate for relating concentrations to effects. Although this is the first study to look at the effects of a juvenile dietary exposure of PBDEs in turtles, additional work is required to further determine the effects

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of PBDEs on the thyroid system of turtles and the precise physiological mechanisms of

effects.

Tables

Table 5-1. BDE-47 and BDE-99 concentrations in all food treatments (ng/g wet weight) and whole body measurements after 6 months of exposure (ng/g animal wet weight ± 1 SE).

	Control		Treatment	
	BDE-47	BDE-99	BDE-47	BDE-99
Food[A]	0.53	0.33	2055	1425
Red-eared Slider[B]	ND	ND	2.32±0.685*[#]	22.33±7.69[#]
Snapping Turtle[B]	0.085±0.059	0.010±0.001	43.63±26.98	72.32±18.24

[A]Units of food concentrations are ng/g wet weight.
[B]Units of whole body concentrations are ng/g animal wet weight (n=3).
*Indicate significant differences within species BDE treatments.
[#]Indicate significant differences between the BDE treatments within species.

Table 5-2. Wet weight and carapace length (CL) immediately following overwintering (7 MPH) and at necropsy (14 MPH) for both the red-eared sliders and snapping turtles. Values are means ± 1 SE. MPH = months post hatch.

| Dietary Treatment	Red-eared Slider				Snapping Turtle			
	Size Post-overwintering (7 MPH)		Size at Necropsy (14 MPH)		Size Post-overwintering (7 MPH)		Size at Necropsy (14 MPH)	
	Wet Weight (g)	CL (mm)	Wet Weight (g)	CL (mm)	Wet Weight (g)	CL (mm)	Wet Weight (g)	CL (mm)
Control	7.85 ± 0.234	32.94 ± 0.307	12.01 ± 0.428	38.23 ± 0.640	10.09 ± 0.151	30.54 ± 0.178	31.72 ± 2.090	44.95 ± 1.070
BDE-47	7.34 ± 0.198	32.09 ± 0.327	11.39 ± 0.534	37.03 ± 0.684	10.01 ± 0.143	30.39 ± 0.146	34.13 ± 2.020	46.36 ± 0.984
BDE-99	7.54 ± 0.240	32.72 ± 0.307	12.01 ± 0.428	38.16 ± 0.610	10.08 ± 0.166	30.41 ± 0.158	33.64 ± 2.041	45.73 ± 0.987
	p=0.286	p=0.193	p=0.445	p=0.345	p=0.922	p=0.784	p=0.684	p=0.618
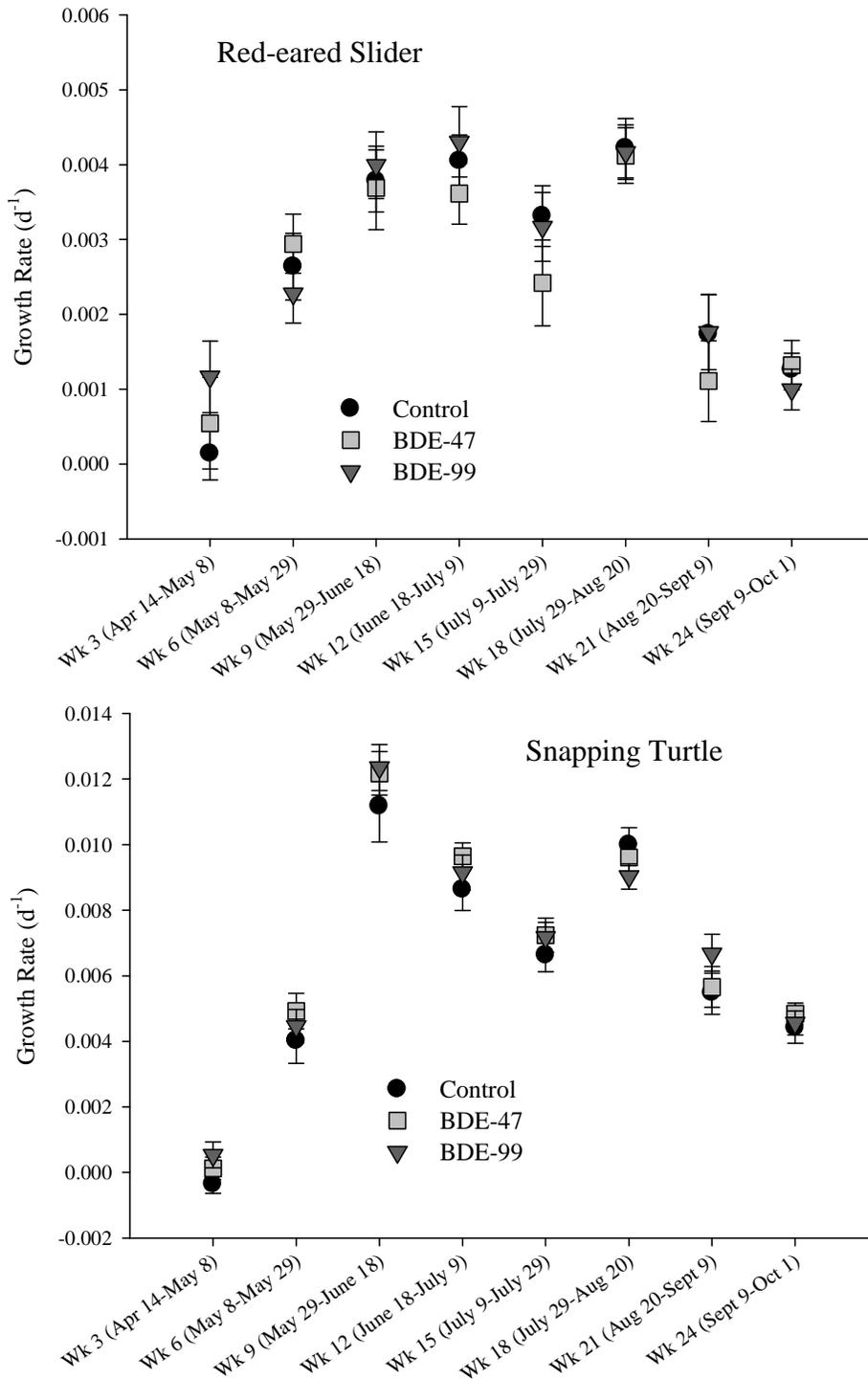
Figure 5-1. Growth rate (d^{-1}) across the six month exposure period for the red-eared slider (top) and snapping turtle (bottom). Symbols represent the mean $\pm$ 1 SE.

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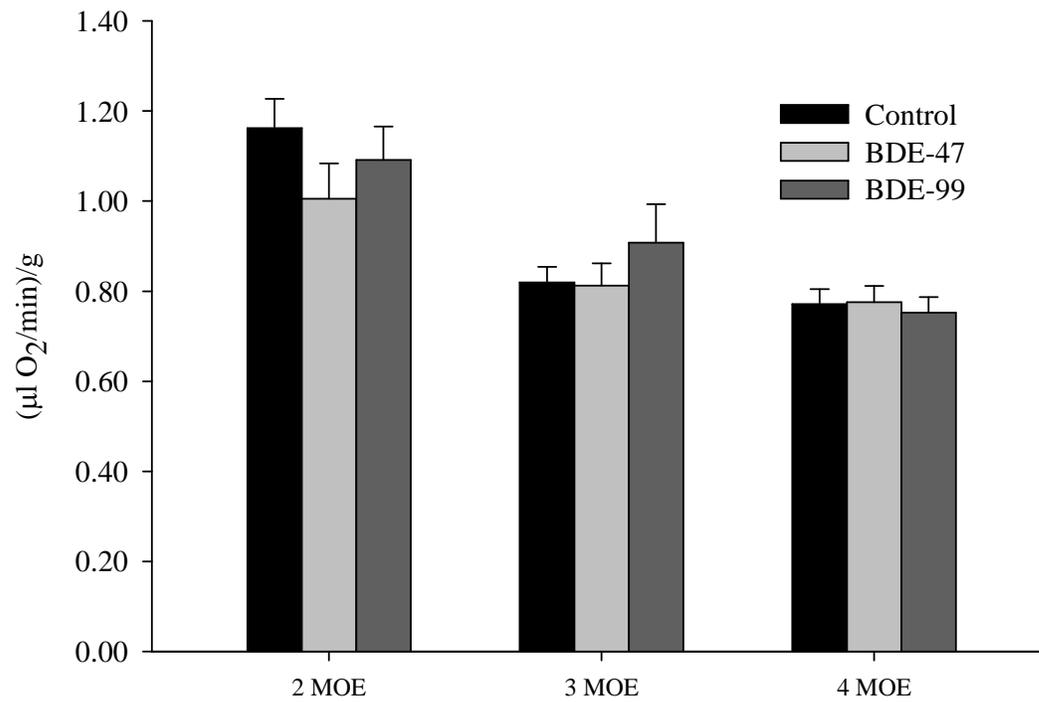
Figure 5-2. Standard metabolic rate of red-eared sliders after two, three, and four months of dietary exposure (mean ± 1 SE). MOE = months of exposure; n=15.
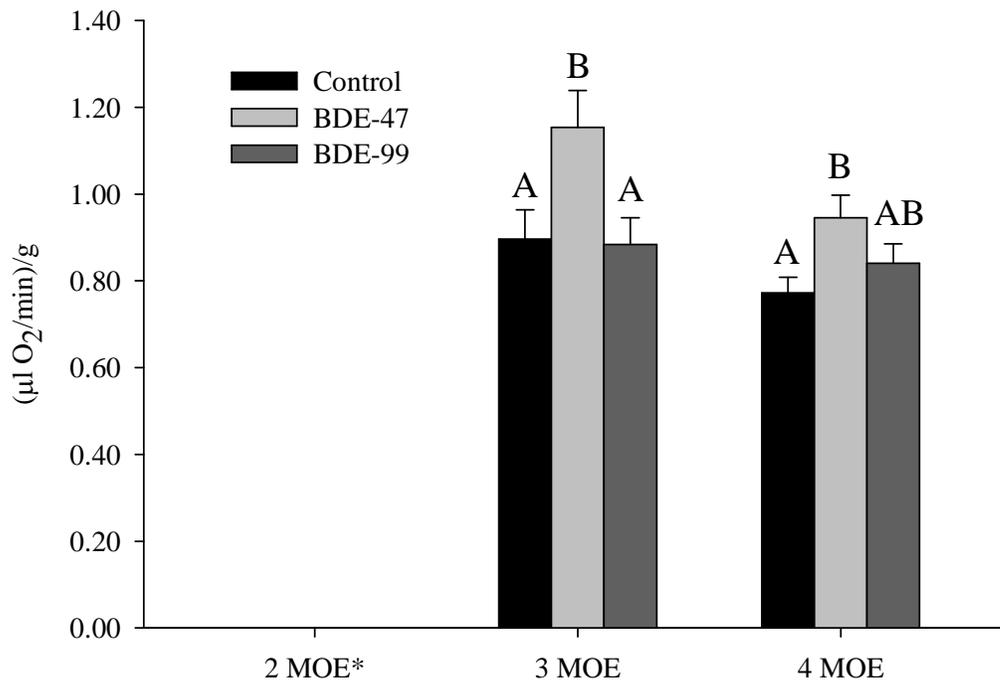
Figure 5-3. Standard metabolic rate of snapping turtles after two, three, and four months of dietary exposure (mean ± 1 SE). MOE = months of exposure; n=15. *Respirometer failed during measurements. Different letters signify significant differences within month of measurement (p<0.05).
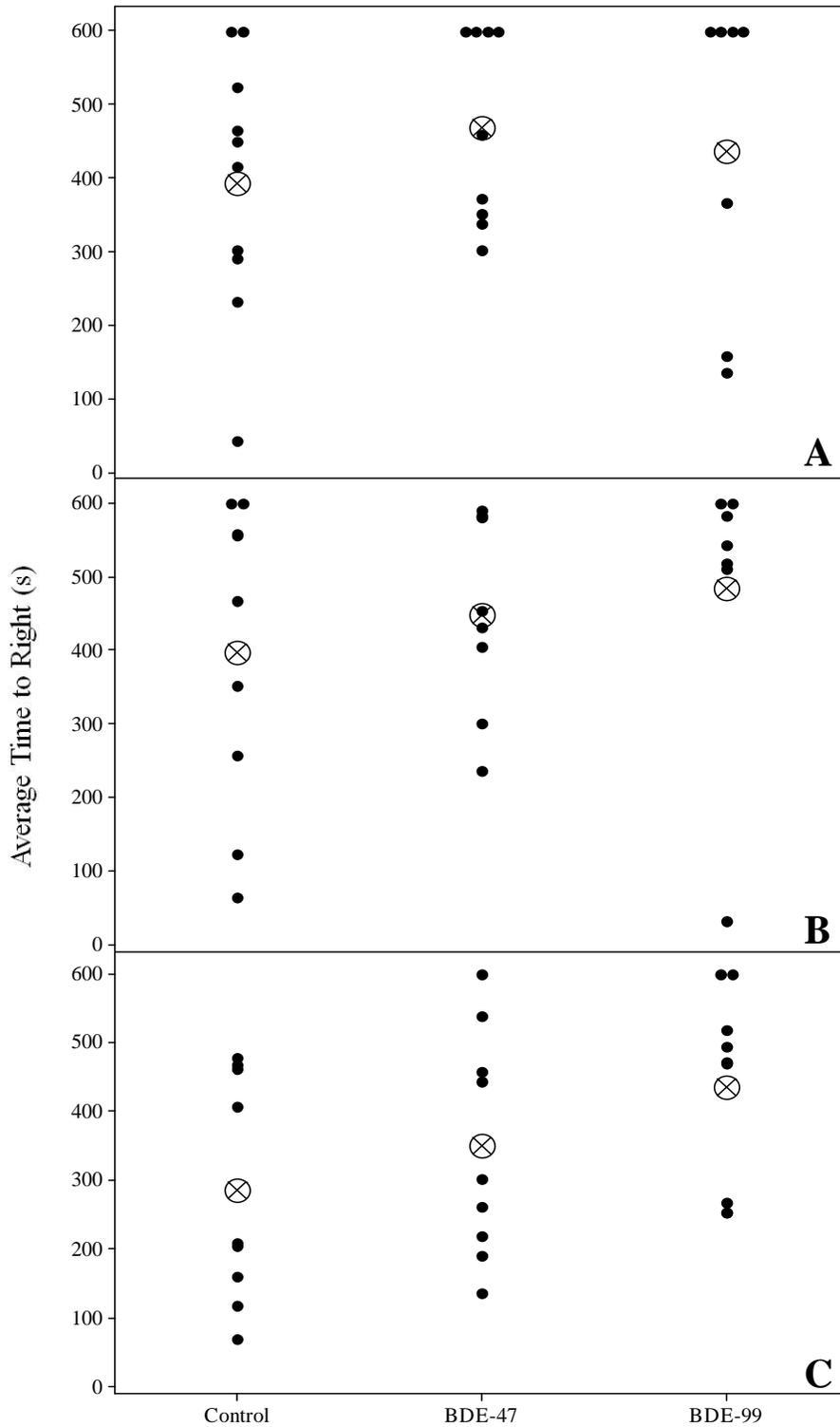
Figures 5-4A, 4B, 4C. Time to completely right from ventrally exposed position for the red-eared slider with 600 s assigned to any individual making attempts to right, but never fully righted. Crosshairs in a circle represent the mean. A=two months of exposure. B=three months of exposure. C=four months of exposure.
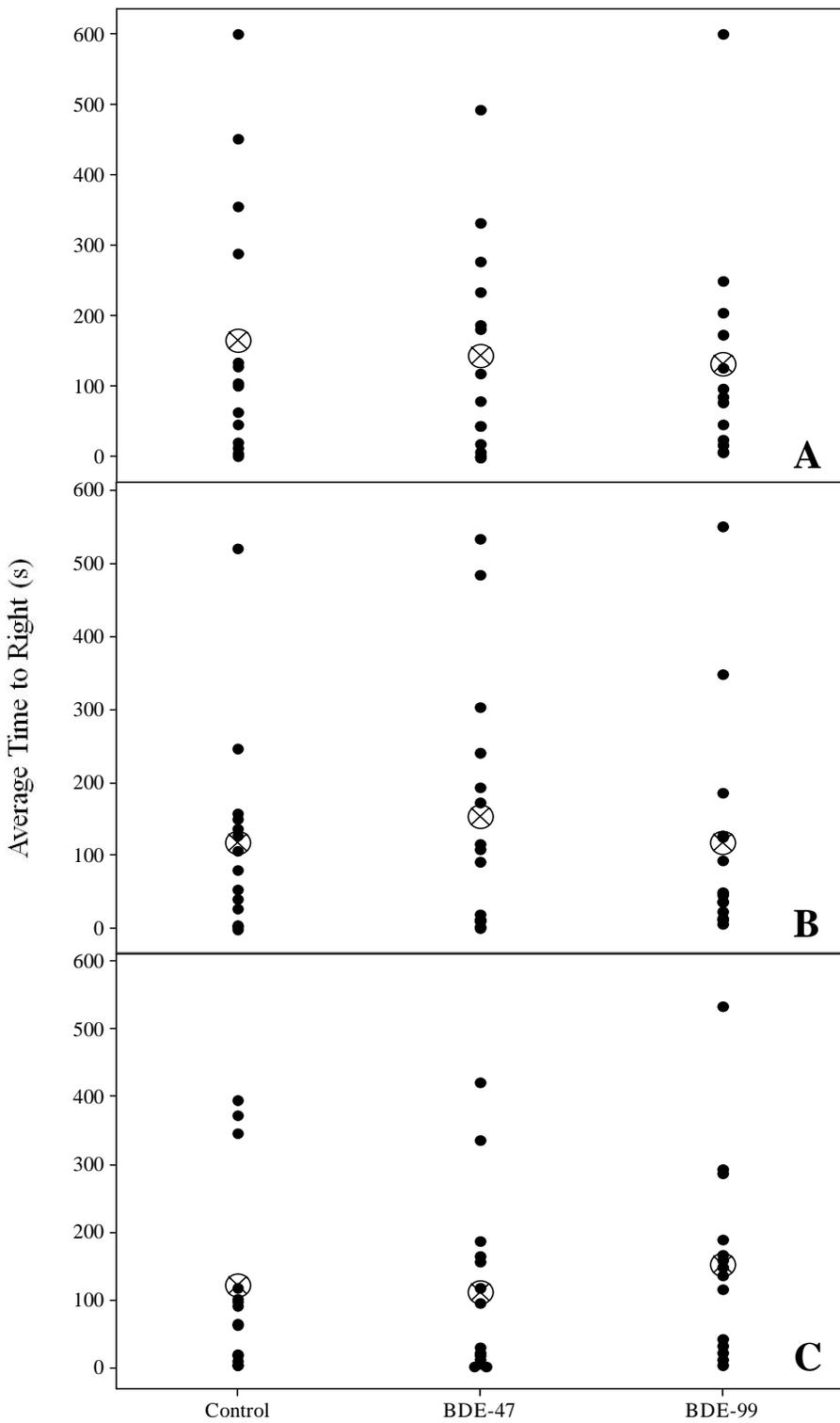
Figures 5-5A, 5B, 5C. Time to completely right from ventrally exposed position for the snapping turtle with 600 s assigned to any individual making attempts to right, but never fully righted. Crosshairs in a circle represent the mean. A=two months of exposure. B=three months of exposure. C=four months of exposure.
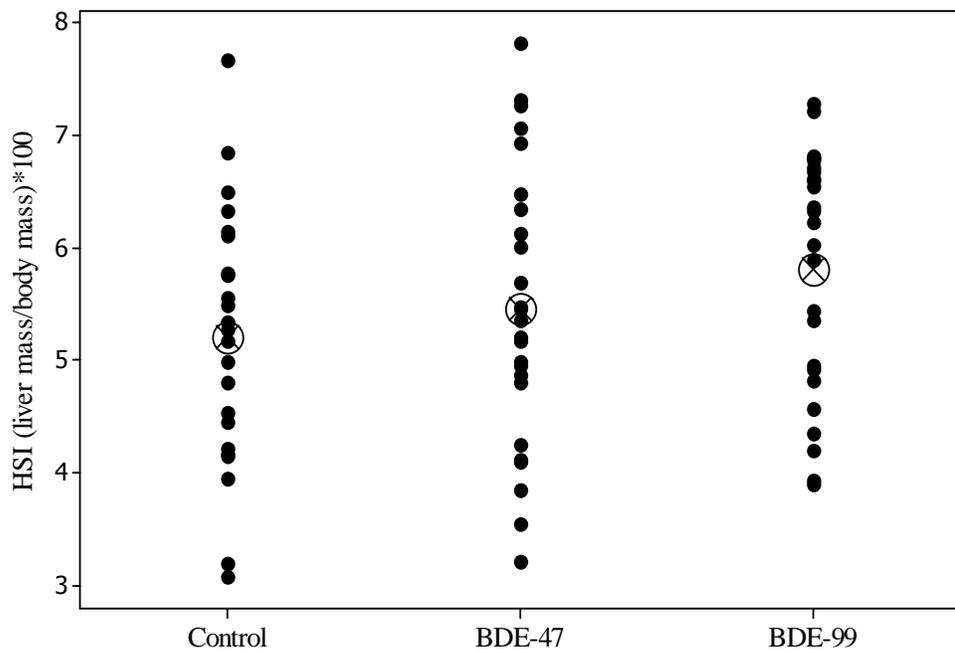
Figure 5-6. Liver mass to body mass ratio of snapping turtles. Crosshairs in circle represent the mean (Control n=24; BDE-47 and -99 n=25).
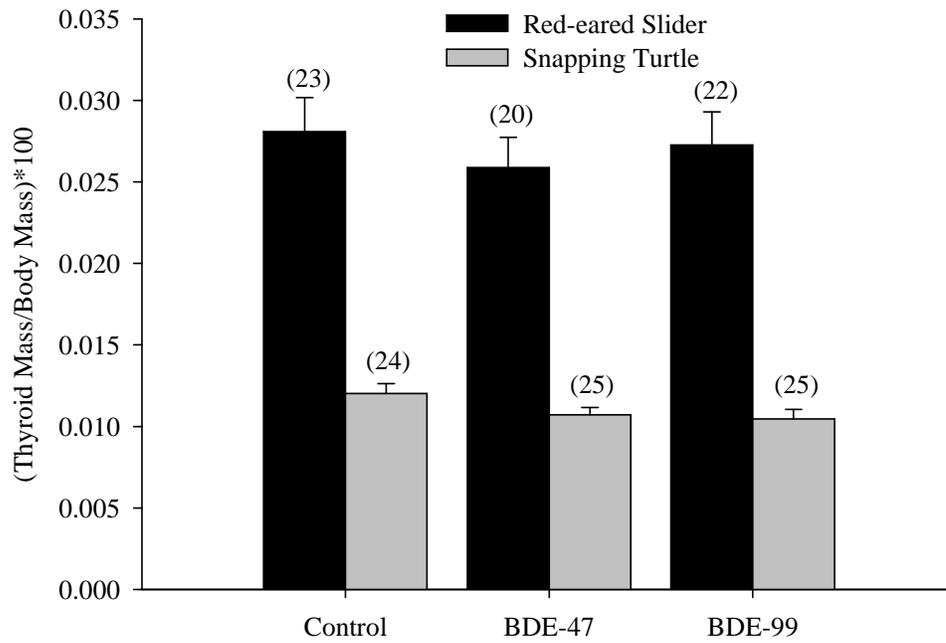
Figure 5-7. Thyroid mass to body mass ratio for both red-eared sliders and snapping turtles (mean ± 1 SE). Sample size noted in parentheses.
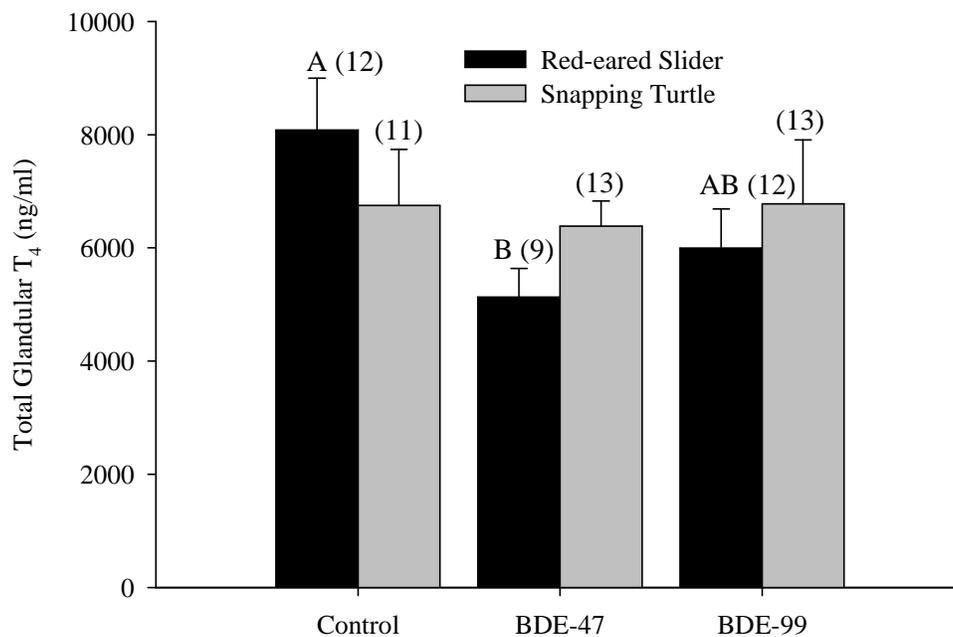
Figure 5-8. Total Glandular T_4 concentrations in red-eared sliders and snapping turtles after 6 months of exposure to BDE-47 and BDE-99 (mean ± 1 SE). Different letters signify significant differences ($p < 0.05$). Sample sizes noted in parentheses.

Chapter 6: Overall Conclusions

Although the production of polybrominated diphenyl ethers (PBDEs) have either been discontinued or are in the process of being phased out, there remain serious concerns about the effects of PBDEs that are likely to persist in the environment. As existing products containing PBDEs continue to degrade with age, release of the PBDEs to the environment will continue. In addition, it has been well documented that PBDEs can be debrominated to the lower and more toxic congeners providing a secondary source of the PBDEs of most concern such as those found in the penta-BDE mixture.

Research that has focused on the biological effects of PBDEs has typically been conducted using rats and mice. Limited studies have been conducted in fish and birds, even fewer in amphibians, and until now, no research on the effects of PBDEs has addressed reptiles even though it has been shown that adult turtles and their eggs contain PBDEs. Turtles are useful models for studying effects of bioaccumulative and persistent compounds as they are likely to be exposed through multiple pathways including through maternal transfer and their diet. While the research presented here has shown impacts from both embryonic and dietary exposure (Chapters 3 and 5), more information is still needed to elucidate mechanisms of toxicity in turtles.

Embryonic developmental stages represent sensitive and key life stages that, if altered, could have lasting impacts of hatchling and juvenile health and development. Since it has been demonstrated that adult turtles can maternally transfer PBDEs to their eggs in nature, controlled laboratory methods of embryonic exposure are critical for elucidation of resulting effects. The topical dosing method for embryonic exposure of BDE-47 and -99 used in the studies presented here was effective in exposing embryos to

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both compounds (Chapters 2 and 3). However, it is clear that the extent of exposure is dependent upon the individual chemical properties of the compounds. For example, perchlorate was used in the topical dosing study (Chapter 4) as a positive control for thyroid effects and the results indicated that it passed readily through the eggshell and embryonic effects were very clear. Perchlorate is a relatively small, water soluble compound (molecular weight 98.95 g/mol) thus it easily passed through the eggshell. However, compounds of a larger size and lower water solubility are likely to less readily pass through the egg shell.

Results from these studies (Chapter 2) demonstrate that BDE-99 transfers across the eggshell approximately 60 % less efficiently than BDE-47 in both red-eared sliders and snapping turtles. This difference is likely due to the larger size of BDE-99 and potentially the larger log K_{OW} (molecular weight 564.7 g/mol; log K_{OW} 7.32) and an additional bromine ion in its structure compared to BDE-47 (molecular weight 485.8 g/mol; log K_{OW} 6.81). In order to achieve target embryonic exposure concentrations via topical dosing, these differences in transfer across the eggshell must be clearly understood so that the dosing protocol may be designed to account for differences in transfer among compounds. This is the case even when working with different congeners of the same class of compounds since a difference of only 79 g/mol between BDE-99 and BDE-47 had a large influence on transfer across the eggshell. These differences in molecular size indicate that topical dosing may not be successful for larger compounds. For example, it is unlikely that BDE-209 would be able to cross the eggshell due to its much larger size (molecular weight 959 g/mol) than BDE-99.

However, there currently is no better alternative to topical dosing for controlled studies of embryonic exposure in reptiles. For such studies in birds, injections of the compounds directly into the egg air cell have been successfully used. However, studies employing this technique using turtle eggs have resulted in high embryonic mortality due to the injection process alone. When survival has been the highest (61 %) using injection methods, the adult females were sacrificed in order to collect eggs directly from the oviducts and for injection before the eggshells hardened. However, this method could not be used regularly for toxicity testing in turtles as it would not be sustainable for wild turtle populations as the testing organisms would have to be taken from the environment.

Transfer efficiency across the eggshell was not only different between the two compounds, but also different between the red-eared sliders and snapping turtles. Transfer was greatest for BDE-47 across the snapping turtle eggshell and lowest for BDE-99 across the eggshell of the red-eared slider. In relation to the low transfer, concentrations were lowest in the red-eared slider egg contents and can partly be accounted for by evidence of debromination of BDE-99 to BDE-47. Measureable concentrations of BDE-47 were found in the red-eared slider eggs topically dosed with BDE-99, but the same pattern was not observed in the snapping turtle eggs. This suggests that the red-eared sliders have the capacity to debrominate BDE-99 while snapping turtles do not. There is further evidence in the feeding study (Chapter 5) of potential metabolic capabilities of the red-eared sliders where there were lower concentrations of BDE-47 and -99 in whole body tissue of the red-eared sliders compared to the snapping turtles following six months of dietary exposure. Several factors could play a part in the reduced concentrations found in the red-eared sliders, including less

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accumulation or lower uptake over the intestines, but metabolism of the PBDEs followed by excretion are also likely important. Further research is necessary to directly assess these potential differences in PBDE metabolism between the two species and elucidate the exact mechanisms leading to lower whole body concentration. While debromination has been shown to occur in several fish species, this is the first set of studies to suggest metabolic and debromination capabilities and species-specific differences in turtles.

In addition to evidence for species differences in metabolism of PBDEs, this research also indicated species sensitivity differences in response to embryonic and dietary exposures (Chapters 3, 4 and 5). Embryonic development was impacted in both species exposed *in ovo* to perchlorate, but the red-eared slider appeared to be more sensitive to the exposure (Chapter 4). Similarly, the red-eared sliders appeared to be impacted to a greater extent than the snapping turtles when exposed to BDE-47 and -99 through their diet even though the snapping turtle accumulated greater concentrations of both compounds (Chapter 5). While the snapping turtle has been shown to be a valuable indicator of accumulation and maternal transfer of persistent organic pollutants in natural systems, this species may not be the best surrogate for relating concentrations to effects. Species sensitivity differences clearly have to be taken into account when choosing a model species for exposure and effect studies.

In general, this research has shown that exposure to BDE-47 and -99 can elicit a suite of impacts potentially related to thyroid system function in both red-eared sliders and snapping turtles. Although there were limited observed impacts resulting from embryonic exposure to BDE-47, results were mixed and their significance to long term health or fitness is unknown (Chapter 3). Behavior and metabolism which were impacted

by dietary exposure to BDE-47 and BDE-99 in both species are directly related to thyroid

system function (Chapter 5). It appears, based on the results of this research, that BDE-

47 and BDE-99 can cause thyroid toxicity and alter neurological development or function

which in turn can impair metabolism and behavior. Future studies are needed to

elucidate specific mechanisms by which multiple effects are induced by exposure to

PBDEs.

Appendix 1

Latent Mortality of Juvenile Snapping Turtles from the Upper Hudson River, New York, USA Exposed Maternally and Via the Diet to Polychlorinated Biphenyls (PCBs)

Karen M. Eisenreich, Shannon M. Kelly, Christopher L. Rowe*
University of Maryland Center for Environmental Science,
Chesapeake Biological Laboratory,
PO Box 38, Solomons, MD, 20688

Preface

The methods developed for the following paper provided the basis for examining hatchling and juvenile effects to PBDEs presented within the chapters of this dissertation. Due to the structural similarities between PCBs and PBDEs and the latent affects of PCBs on snapping turtle juveniles it was determined that it would be important to examine potential for latent effects of PBDEs as well as impacts to a dietary exposure. In addition, power analyses were carried out using data from the PCB study to determine sample sizes for the PBDE study. For these reasons the following paper was presented in its entirety as an appendix to the PBDE studies.

Abstract:

We conducted a factorial experiment to compare sublethal and lethal responses of juvenile snapping turtles exposed maternally and/or through the diet to polychlorinated biphenyls (PCBs) over 14 months post-hatching. Maternal exposure did not affect embryonic development or hatching success. Thyrosomatic indices were not influenced by treatments, although hepatosomatic indices were lower in animals having been exposed to PCBs maternally relative to those having been exposed both maternally and via the diet. Dietary PCB exposure reduced metabolic rates of juveniles in two of three assays conducted. Approximately eight months after hatching, high rates of mortality began to emerge in individuals having been exposed maternally to PCBs, and mortality rate correlated with $[PCB]_{Total}$ in eggs. Prior to death, individuals that died experienced lower growth rates than those that survived, suggesting chronic effects prior to death. By 14 months post-hatching, only 40 % of juveniles derived from females in the contaminated area had survived, compared to 90 % from the reference area. Such latent effects of maternally-derived contaminants suggest that assessments of environmental impacts based upon shorter-term studies may provide very conservative estimates of the severity of effects, as they cannot capture responses that may emerge later in the life cycle.

Introduction:

Snapping turtles (*Chelydra serpentina*) have widely served as biomonitors as they have the propensity to bioaccumulate contaminants and transfer lipophilic contaminants to their eggs (*1 - 6*). Thus, eggs provide useful indicators for establishing the presence of

contaminants in local systems that contain species that are prone to maternal transfer. As well as serving as biomonitors, snapping turtles have been employed as models to examine effects on development that may result from maternal transfer of contaminants in species of long life span and high trophic position (*7*). Effects included decreased hatching success and increased incidence of deformities in hatchlings (*8 - 11*). While numerous contaminants have been shown to induce effects on offspring following maternal transfer, those most often implicated have been organochlorine pesticides, polychlorinated biphenyls (PCBs), and in some cases polybrominated diphenyl ethers (PBDEs; *4, 7 – 9, 11*).

As many aquatic habitats harbor complex mixtures of contaminants, it is often difficult to identify the particular compound(s) that may pose the greatest risk to resident species. For example, research suggests a correlation between exposure to PCBs and developmental anomalies in turtles, yet often the habitats studied have been contaminated by other compounds (*4, 8, 9 ,11*). However, there exist some systems in which contamination by PCBs is so severe that exposure to PCBs likely outweighs exposure to other, background contaminants. The upper Hudson River harbors some of the highest PCB concentrations in the U.S. (*12*). Contamination of the upper Hudson River is the result of historical discharge of PCBs from two electric capacitor plants operated by the General Electric Corporation, as well as from erosion of remnant deposits and seepage from bedrock fractures below the plants (*13*). Due to the extensive contamination of the upper Hudson River by PCBs, this system provides a rare opportunity to examine responses of wildlife to PCB exposure.

PCBs act as endocrine disrupting compounds (EDCs) that have several modes of action. PCBs and their metabolites can be estrogenic, antiestrogenic, or may exhibit both properties (*14 - 16*). PCBs can also alter the thyroid system, interfering with the transport, metabolism and receptor interactions of thyroid hormones (*17 - 19*). Alteration of thyroid hormone-modulated responses may result in abnormal metabolic function during the critical embryonic and juvenile stages of development. As a result, thyroid-disrupting compounds such as PCBs may alter energy assimilation efficiency, energy allocation to somatic and reproductive tissue growth, and immune response, potentially affecting survival and reproductive fitness (*20 - 23*).

While the effects of maternal transfer of PCBs on reptiles have been characterized, these assessments typically focused only on embryonic survival and development or traits of newly-hatched individuals (*1, 3, 4, 6, 24*). There remains little information regarding the potential for effects to emerge long after direct exposure of embryos via maternal transfer has ceased, or as a result of exposure of juveniles via consumption of contaminated food. Thus, the possible latent effects of embryonic exposure that may emerge during the juvenile period, alone and in combination with ongoing dietary exposure of juveniles, are unknown. If effects emerge beyond the scope in duration of most studies, estimates of environmental impacts based on those studies will likely be conservative. Subsequently, regulatory and management activities based upon results from relatively short term studies may not be sufficient to protect ecosystem health or to develop successful restoration/remediation strategies.

We assessed the long-term effects of maternally-transferred PCBs as well as proximate effects of exposure to food-borne PCBs in juvenile snapping turtles collected

from the upper Hudson River, NY. From early egg development through the ensuing 14 months of the juvenile period, we quantified development, metabolism/growth, and survival in individuals exposed or unexposed to maternally-derived PCBs, food-borne PCBs, and their combination.

Experimental Section:

Egg Collection and Incubation. Eggs were collected from areas designated as either "reference" or "contaminated" in northern New York, USA. The contaminated area was a section of the Hudson River just downstream of the Hudson Falls and Fort Edward General Electric facilities (43 ° 18 ´ N, 73 ° 35 ´ W and 43 ° 16 ´ N, 73 ° 35 ´ W respectively), including several areas known to be "hot spots" of sediment contamination (i.e., average PCB concentration > 40 mg/kg wet weight). The reference area consisted of lakes and ponds that were north and west of the contaminated area, distant from significant and direct sources of PCBs. Eggs from both areas were collected from nests or through induction of egg laying via intraperitoneal injection of females with oxytocin (*25*); clutches from oxytocin induced females were represented in both areas. Eggs were measured for diameter and wet weight. Three eggs per clutch were frozen at – 20 ° C for analysis of PCB concentrations. Detailed information on selection of study areas and collection methods is provided in Kelly et al. (*6*).

Eggs were incubated at 25 ° C laboratory, a temperature known to produce only males (*26*). Moisture and humidity were maintained by misting the eggs and nest substrate (vermiculite) with water at 2-3 day intervals. Eggs were periodically candled to determine viability and development. Hatchlings were measured for carapace length

("CL") and wet weight ("ww"), examined for gross abnormalities, and assigned to groups for subsequent 14-month studies of juveniles.

      Post-hatching Protocol. For 14 months post-hatching ("mph"), we assessed potential effects of maternal contribution of PCBs, food-borne exposure to PCBs, and the combination. We used a total of 144 hatchlings derived from 16 total egg clutches collected from females from the contaminated (n = 6) and reference areas (n = 10). Individuals from all clutches were used in assessing all endpoints with the exception of mortality, for which only those clutches having produced five or more hatchlings were used (contaminated n = 5; reference n = 6). Smaller clutches were excluded from mortality comparisons as they would have hindered analysis of these proportional data. Small clutch sizes resulted from limited numbers of viable eggs from natural nests due to predation as well as from induction of females via oxytocin, which often produces partial clutches. Mean (± 1 SE) total PCB concentrations were 3953 ± 1454 and 61 ± 8 ng/g ww (varying from 75 - 9220 and 21 - 86 ng/g ww) in the contaminated and reference area eggs, respectively. The eggs used for assessing mortality had concentrations of 3546 ± 1710 and 55 ± 10 ng/g ww in the contaminated and reference areas (varying from 75 - 9220 and 21 - 78 ng/g ww respectively). Hatchlings from each area were divided into two additional treatments, to be fed a diet of natural prey items collected from either area (see below). Thus there were four treatments for the remainder of the study, designated hereafter as the maternal (collection) area followed by food type: contaminated area, contaminated food = "CC"; contaminated area, reference food = "CR"; reference area, contaminated food = "RC"; reference area, reference food = "RR".

Hatchlings were held in a 20 ° C laboratory prior to a simulated over-wintering period. To mimic the winter-time period of dormancy that occurs in temperate climates, we over-wintered hatchlings beginning in mid-October by incrementally lowering temperature to a final temperature of 5 ° C, and incrementally increasing temperatures beginning in late-February. Turtles were maintained at 22 – 25 ° C for the remainder of the study. Each individual received a passive integrated transponder (PIT) tag following methods described by Rowe and Kelly (*27*), providing for identification of individuals, which were held in groups (below) per a common-garden design. Dietary exposures began following over-wintering when yolk stores were depleted and hatchlings began to display interest in food. Thus dietary exposures were approximately eight months in duration.

Food consisted of aquacultural gelatin (Aquatic Eco-systems, Inc., Apopka, FL) mixed with ground fish and invertebrates collected in the study areas and frozen for subsequent use. Food collected from the contaminated area and reference area contained mean total PCB concentrations of 5955 ± 427 ng/g and 83 ± 6 ng/g ww respectively. Food was provided *ad libitum* and was analyzed by bomb calorimetry to verify caloric content (reference: 4291 cal/g; contaminated: 3525 cal/g).

For each food treatment, we used eight replicate tanks (57 L) containing 5 L of water and nine to ten turtles (four to five from each area). As tanks were designated only by food treatment (not by collection area), each received animals from both areas (e.g., tanks in which animals were fed contaminated food contained only individuals of treatments CC and RC, and tanks designated to receive reference food contained only CR and RR).

Growth Rate, Size and Metabolic Rates. Instantaneous growth rate was calculated on ww and CL as [(ln(ww or CL final)-ln(ww or CL after over-winter))/199 days]. Sizes were measured monthly. Metabolic rates were measured bimonthly, beginning in May (9 mph), on a subset of 16 individuals from each treatment (representing all tanks; the same individuals were used during all assays). Resting metabolic rate (25 ° C) was measured as oxygen consumption by resting, fasted animals at 2 hr intervals over a period of 12 hr by micro-respirometry (Micro-Oxymax; Columbus Instruments, Columbus, OH) following a 24 hr acclimation period (*28*).

Sex Ratios, Histopathology, and Organosomatic Indices. At the conclusion of the experiment, ww and CL measurements were made and individuals were euthanized by inhalation of isoflorane followed by decapitation. Animals were dissected for inspection of the gonads for determination of sex (*26*) and abnormalities in gonadal organization. Those not defined as male were morphologically female (e.g., not inter-sex). We calculated hepatosomatic and thyrosomatic indices (HSI and TSI) as [(liver or thyroid gland weight / body weight)*100]. Individuals that died during the experiment were excluded since they may have remained in the tanks for up to approximately 12 hrs after death, possibly influencing organ and carcass wet weights.

Analysis of PCB Concentrations. Detailed PCB extraction and quantification methods were presented by Kelly et al. (*6*). Briefly, three eggs from each clutch were rinsed and the contents were composited and homogenized. Eggs were randomly selected from nests as research has shown little within-clutch variation in contaminant concentrations (*1*). A five gram sample of the homogenate from each clutch was dried with sodium sulfate, spiked with surrogate standards and Soxhlet extracted for 24 hours

using dichloromethane. Five gram samples of each food type were similarly processed.

Extracts were sub-sampled to gravimetrically measure lipid content. Gel permeation

chromatography was used to remove lipids followed by Florisil cleanup. Laboratory

blanks were processed with and in the same manner as all tissue samples by extracting 50

g of sodium sulfate spiked with surrogate standards.

Total PCB concentrations represent the sum of 110 individual congeners and/or

groups of co-eluting congeners (*6*). Samples were quantified using a Hewlett Packard

5890 gas chromatograph equipped with a ^{63}Ni electron capture detector, with hydrogen as

the carrier gas and nitrogen as the make-up gas. A 60 m x 0.25 mm 5 % phenyl-methyl

silicon DB-5MS capillary column was used with inlet pressure of 100 kPa. The oven

temperature program was: 100 ° C hold for two min, ramp to 170 ° C at 4 ° C/min, 170 to

280 ° C at 3 ° C/min, and a final 5 min hold at 280 ° C. The injector temperature was held

at 225 ° C and the detector at 285 ° C. Information on surrogates, internal standards and

recoveries see Kelly et al. (*6*).

Statistical Analyses. Mortality, growth rate, size by month, and PCB

concentrations were analyzed on tank means by analysis of variance (ANOVA) followed

by Tukey's pairwise comparisons. When assessing size by month, initial size was

defined as size following over-wintering. Mortality data were transformed by arcsine

square root. Bimonthly metabolic rates were analyzed on tank means by analysis of

covariance (ANCOVA) using ww as the covariate. Size and metabolic data collected

over the duration of the study were also analyzed using repeated measures ANOVA.

Relationships between PCB concentrations in eggs and juvenile mortality were assessed

by linear regression. Statistical significance was judged based upon a Type I error rate of

$\alpha = 0.05$. Prior to statistical analyses, data were tested to verify that assumptions of the models were met.

Results:

Eggs and Hatchlings. Eggs from the reference area were heavier and had higher lipid content than those from contaminated area, but were smaller in diameter (Table 1). Hatchlings from reference eggs were larger than those from the contaminated area, both in terms of ww and CL (approximately 13 % and 8 % larger, respectively; Table 1). For contaminated and reference areas respectively, neither time to hatching (84.7 ± 0.330 and 84.5 ± 0.240 days; $P = 0.625$) nor hatching success (73.2 and 66.8 %; $P = 0.472$) differed.

Juveniles. There was a large divergence in survival among treatments beginning 8 - 9 mph (2 months following the overwintering period; Figure 1). Survival was affected only by area of egg collection (e.g., maternal transfer only; $P < 0.001$); feeding regime had no main effect nor was there an interaction between feeding regime and area of collection. For juveniles from eggs from the contaminated area, those that died had received significantly higher maternal exposure than those that survived (3605 ± 481 versus 1404 ± 260 ng/g ww egg PCB, respectively; $P < 0.001$), but there was no such relationship for those from the reference area (58.1 ± 7.4 versus 63.1 ± 2.7 ng/g ww egg PCB, respectively; $P = 0.455$). There was a positive relationship between PCB concentration in eggs and juvenile mortality in the contaminated area ($R^2 = 0.864$; Figure 2). However, PCB concentration explained only 18 % of the variation in mortality in the reference area (proportion died = 0.254 - 0.00191 Clutch * PCB, $R^2 = 0.18$, $P = 0.0402$; not illustrated).

There were no differences in instantaneous growth rate, measured as CL, among treatments (P = 0.636). Nor were there statistical differences in monthly CL measurements among treatments when analyzed over the duration of the study by repeated measures (P = 0.059). However, CL comparisons within months revealed that individuals from CC, CR and RC were significantly smaller than those from RR in May (9 mph; P = 0.001, 0.026, and 0.026 respectively) while in June (10 mph) and July (11 mph) only individuals from CC and RC were smaller from those in RR (June P < 0.001 and P = 0.023; July P = 0.007 and 0.032 respectively; Figure 3). Within a treatment, individuals that died were smaller than those that survived although small sample sizes limited statistical power which may have precluded assigning statistical significance to the results. For those individuals that survived to the end of the study, there were no differences in size among any treatments (P = 0.195).

TSI did not significantly differ among treatments at P < 0.05, although TSI for individuals in CC appeared lower than TSIs in other treatments (P = 0.086; Table 2). HSI was significantly lower in CR than CC (P = 0.045; Table 2). Metabolic rates analyzed across the study by repeated measures ANOVA differed among treatments (P < 0.001). Metabolic rates in May (9 mph) and July (11 mph) were reduced in treatments CC and RC relative to CR and RR (P < 0.001). By the final measurement (13 mph) differences were no longer detected (Table 2). Dissections revealed that feminization (presence of ovarian rather than testicular tissue) was exceptionally rare. Ninety-nine and 100 % of juveniles from the contaminated and reference area were morphologically male.

Discussion:

The most striking and unexpected result of this study was the latent onset of mortality of juveniles having been exposed to PCBs via maternal transfer. Beginning eight to nine months after hatching, juveniles from clutches in the contaminated area (CC and CR) began experiencing high rates of mortality in contrast to those from the reference area. Ultimately, only approximately 40 % of individuals derived from the contaminated area survived the entire 14 month study versus 90 % from the reference area. Animals that died were smaller compared to survivors in the same treatment at the time of death (Figure 3). Reduced sizes in these individuals may reflect reduced feeding as a result of physiological stress; we observed no evidence of size-specific competitive hierarchies for food (e.g., heightened aggression of larger animals directed toward smaller animals), nor was food unavailable at any time. Animals that died had only slightly increased in size from their initial size following over-wintering, and in several cases the size at death was less than the initial size. Thus, the lack of a difference among treatments in size of turtles surviving to the end of the study most likely reflected the high mortality rate of small individuals from the contaminated area, such that only the largest survived the duration of the experiment.

Whereas studies with other species have reported correlations between embryonic mortality and egg PCB concentration (*29*), we observed little mortality of hatchlings prior to, during, and immediately following the over-wintering period. Rather, mortality rates increased only after the juveniles reached eight to nine months of age. Mortality rates reflected maternal PCB contribution to eggs such that the highest rates were observed in individuals from the most contaminated clutches. There was only a weak relationship

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between mortality and PCB concentration for reference animals, reflecting the small numbers of individuals that died and the narrow range in PCB concentrations (21 - 78 ng/g ww) in eggs from that area. While we cannot rule out the possibility that other factors may have played a role in inducing mortality, due to the strong relationship between mortality and egg PCB concentration in the contaminated area, it seems unlikely that other compounds may have played a substantial role in mortality.

Eggs collected from the contaminated area produced significantly smaller hatchlings than those from the reference area. During the juvenile growth period, animals from the contaminated area (CC and CR) remained significantly smaller than reference area animals fed reference food (RR). Even after taking into account the differences in sizes following overwintering (Table 1), animals from the reference area fed contaminated food (RC) remained consistently smaller relative to those fed reference food (RR) until late in the study when mortality led to variability in average sizes and numbers of survivors among treatments, limiting statistical power in making comparisons.

We observed reductions in metabolic rates in treatments in which animals were fed contaminated food (CC and RC) relative to reference food only in the first two assays (9 and 11 mph), prior to the onset of high mortality rates. The third assay, in which we observed no differences in MR among treatments, was conducted after the highest mortality rates occurred in treatments CC and RC (13 mph). Thus only the healthiest animals survived to the third assay, suggesting that these individuals may not have been metabolically compromised relative to those that had previously died.

Reductions in metabolic rates in treatments CC and RC were consistent with reductions in growth rates of juveniles in these treatments during the same period, suggesting a correlation between metabolic function and growth. Similarities in metabolic rate among treatments at the end of the study correspond with the similarities in final sizes at this time. This result further suggests a relationship between MR and growth in the least healthy individuals, such that loss of these individuals from the population reduced their influence on mean responses of survivors measured at the end of the study.

Hepatosomatic and thyrosomatic indices were inconsistent among treatments. Differences in TSI were expected as PCBs and their metabolites have been shown to cause thyroid hormone alterations that can lead to hypothyroidism and reduced glandular weights (*30, 31*). In addition, in other species exposure to PCBs causes a loss of body weight and mortality due to the degeneration of the liver (e.g., reduced HSI; *32*). Thus we expected to observe differences in HSI particularly in light of the limited weight gain or weight loss in some treatments. As with our measures of growth and MR, our inability to detect differences among treatments in organosomatic indices may reflect the time at which the measurements were made. Calculating these indices required dissection of the animals, therefore they represent measurements made at the end of the experiment, after mortality of juveniles (having been excluded from the calculations) exposed to the highest concentrations of PCBs had occurred.

Given the high concentrations of PCBs to which embryos were exposed, we expected that monitoring multiple endpoints over a relatively long period of time would reveal sublethal effects that would not be observed in shorter term studies (such as those

that span only the embryonic period). Aside from growth rate, metabolic rate was the only sublethal endpoint measured that we found to respond to PCB exposure, and it appeared to only be altered by proximate, dietary exposure rather than maternal exposure. Thus, under the conditions of this study, metabolic rate appears not to be an adequate indicator of maternal effects, thus would not be a sufficient metric for assessing organismal health in systems in which the primary route of exposure is via maternal transfer. Despite the numerous endpoints that we quantified, we are unable to suggest a putative mode of toxicity responsible for the latent onset of mortality of individuals exposed maternally to high concentrations of PCBs. Elucidating the underlying causes of the observed effects would require biochemical assessment of changes at the cell, tissue and organ levels that were beyond the scope of the study.

Maternally-derived PCBs alone have not previously been shown to induce mortality in juvenile snapping turtles, although in combination with co-occurring contaminants embryonic mortality has been observed (*9, 11*). The design of our study provided the opportunity to identify effects of PCBs that may emerge long after hatching, beyond the scope of most studies of maternal transfer, allowing us to quantify latent effects that otherwise would remain uncharacterized. Moreover, our results suggest that some indicators of sublethal effects may either be wholly insensitive to maternally-derived contaminants or reflect only effects of proximate (dietary) exposure, limiting their use as early warning signals of latent expression of maternal effects. Despite the logistical and financial challenges inherent to studies such as ours, the information they provide can be critical to developing cogent regulatory strategies in the light of dramatic responses to contaminants that may emerge long after maternal exposure has ceased.

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Acknowledgements:

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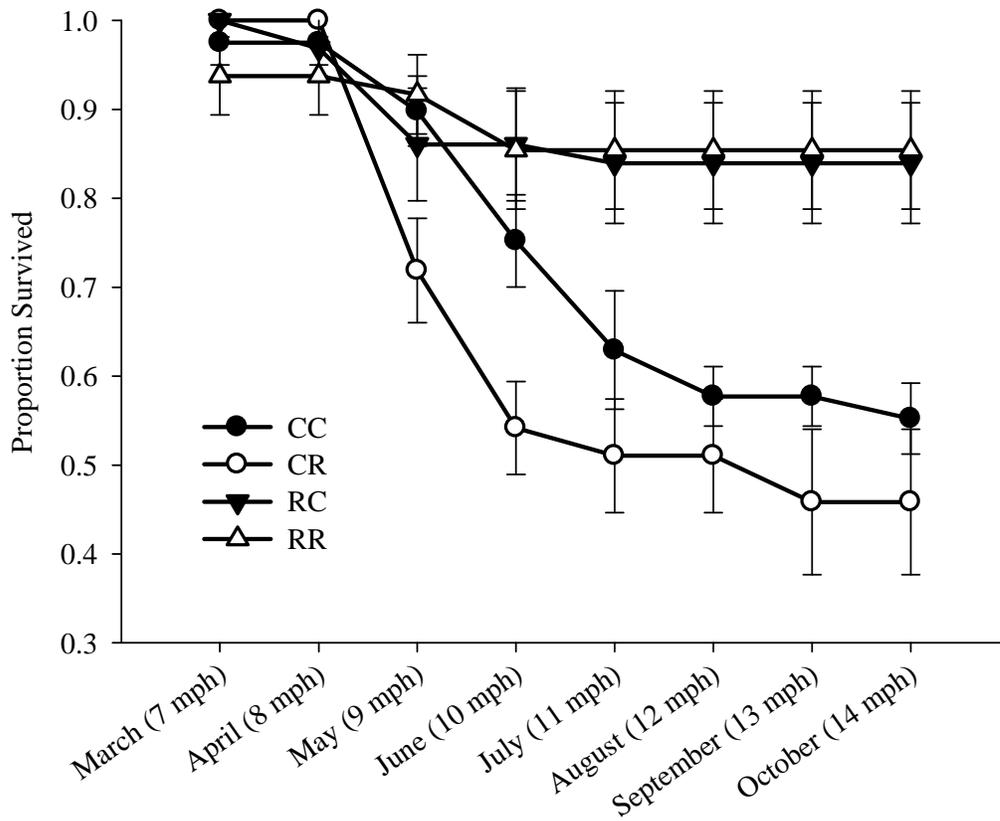
Figure 1. Temporal trends in survival. "mph" = months post-hatch. Symbols represent means ± 1 SE. CC = Contaminated area and contaminated food; CR = Contaminated area and reference food; RC = Reference area and contaminated food; RR = Reference area and reference food.
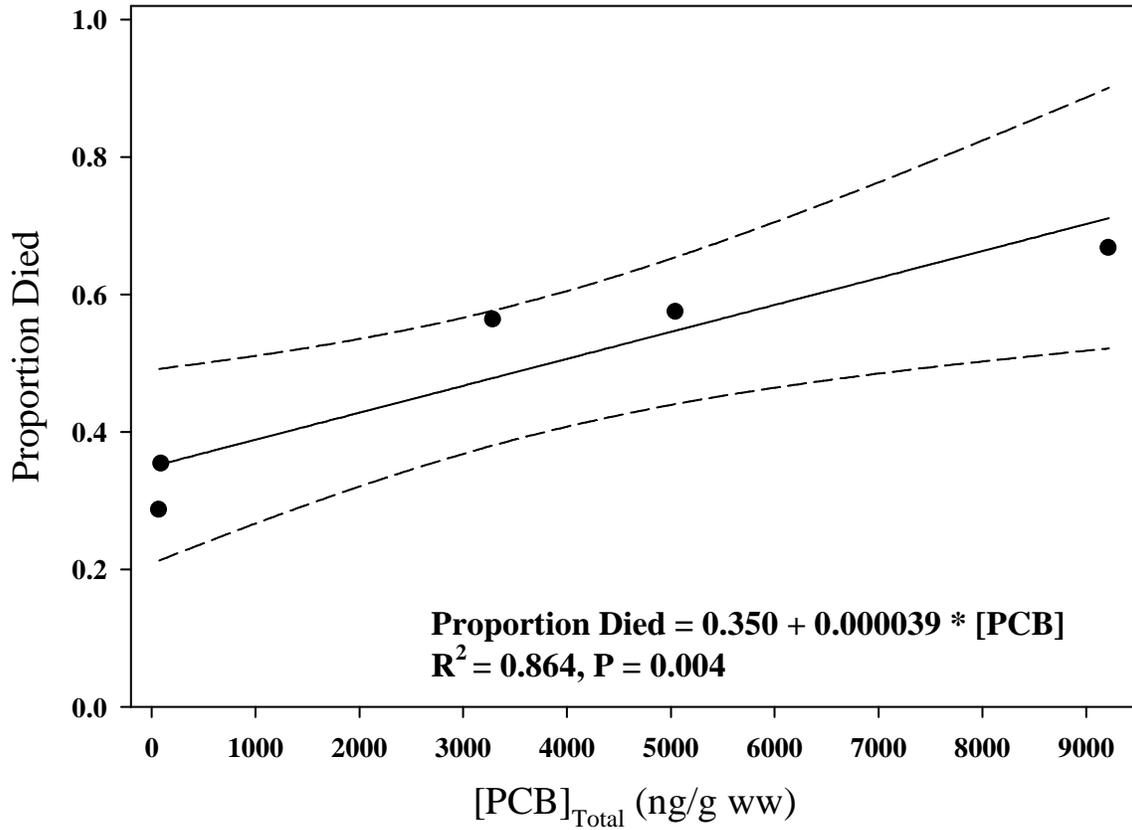
Figure 2. Relationship between PCB concentrations (ng/g ww) in eggs collected in the contaminated area and mortality of juveniles having hatched from them. Dotted lines indicate 95 % confidence interval.
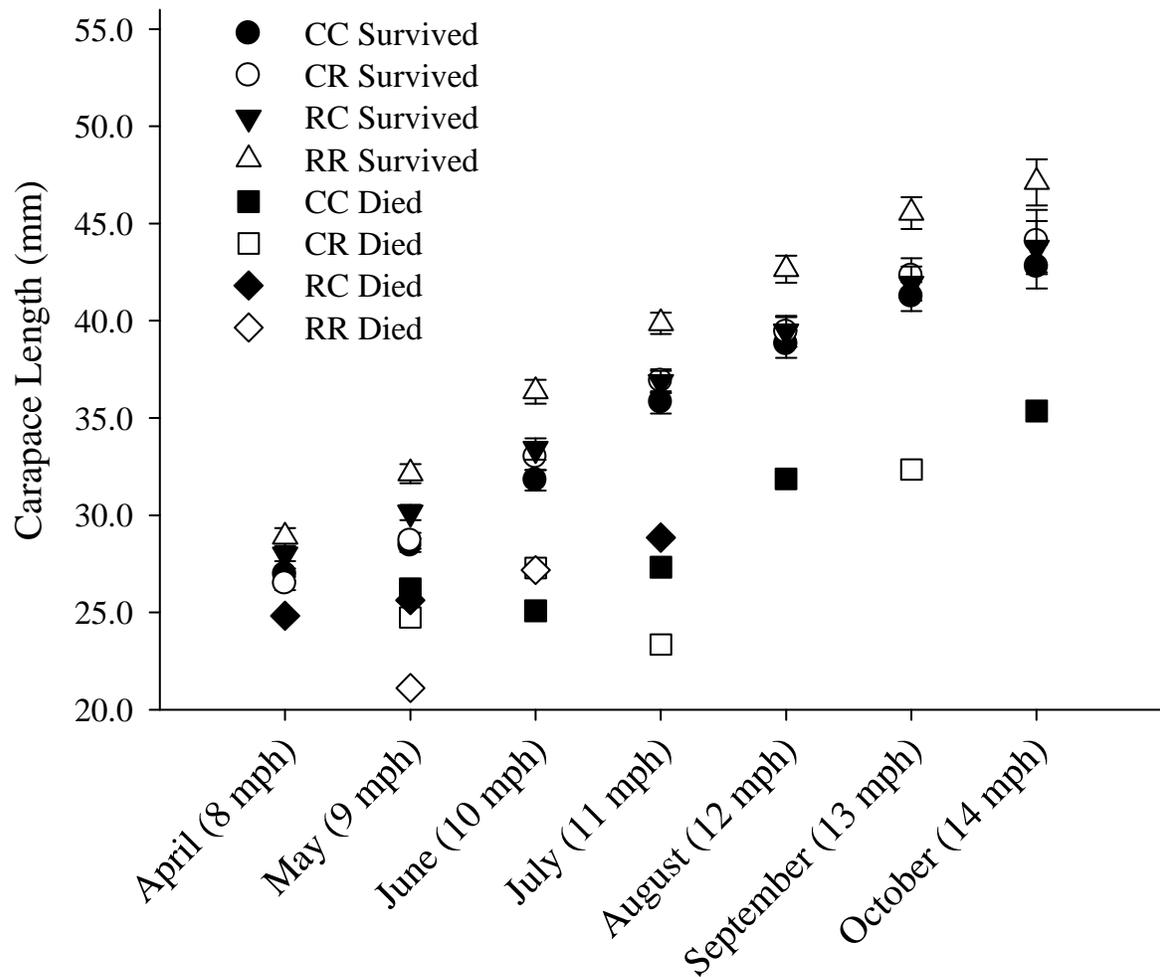
Figure 3. Mean monthly carapace length of juveniles that survived or died. "mph" = months post-hatch. Error bars represent 1 SE. CC = Contaminated area and contaminated food; CR = Contaminated area and reference food; RC = Reference area and contaminated food; RR = Reference area and reference food.

Table 1. Wet weight, diameter, and lipid content of eggs and wet weight and carapace length (CL) of hatchlings and post-overwintered juveniles from contaminated and reference areas. Values are means ± 1 SE.

| | Egg Size | | | Size at Hatching | | Size Post-overwintering | |
| | | | | | | (7 mph) | |
	Wet weight (g)	Diameter (mm)	Lipid (%)	Wet weight (g)	CL (mm)	Wet weight (g)	CL (mm)
Contaminated	8.67 ± 0.10	26.5 ± 0.1	4.93 ± 0.82	7.24 ± 0.36	27.0 ± 0.6	7.50 ± 0.12	26.7 ± 0.2
Reference	9.59 ± 0.19	25.8 ± 0.2	7.04 ± 0.23	8.37 ± 0.42	29.3 ± 0.5	8.41 ± 0.18	28.4 ± 0.3
	$P < 0.001$	$P = 0.006$	$P = 0.009$	$P < 0.001$	$P = 0.006$	$P < 0.001$	$P < 0.001$

Table 2. Standard metabolic rate measured on three dates and hepatosomatic (HSI) and thyrosomatic (TSI) indices of juveniles measured at the end of the study. Different letters within columns indicate treatments that differed following post-hoc pairwise comparisons. Values are mean ± 1 SE.   "mph" = months post-hatch. CC=Contaminated area and contaminated food.   CR=Contaminated area and reference food. RC=Reference area and contaminated food. RR=Reference area and reference

Treatment	Standard Metabolic Rate ($\mu l\ O_2/g*min$)			TSI (%)	HSI (%)
	May (9 mph)	July (11 mph)	September (13 mph)		
CC	$0.92 \pm 0.058\ ^A$	$1.25 \pm 0.057\ ^A$	1.49 ± 0.020	0.010 ± 0.0009	5.128 ± 0.161^A
CR	$1.00 \pm 0.055\ ^B$	$1.32 \pm 0.067\ ^B$	1.46 ± 0.030	0.012 ± 0.0010	4.577 ± 0.140^B
RC	$0.81 \pm 0.057\ ^A$	$1.20 \pm 0.054\ ^A$	1.51 ± 0.020	0.013 ± 0.0008	4.840 ± 0.138^{AB}
RR	$0.97 \pm 0.059\ ^B$	$1.37 \pm 0.052\ ^B$	1.50 ± 0.020	0.014 ± 0.0011	4.902 ± 0.090^{AB}
	P<0.001	P<0.001	P = 0.559	P = 0.086	P = 0.045

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