SUBLETHAL EFFECTS OF STRESSORS ON PHYSIOLOGICAL AND MORPHOLOGICAL PARAMETERS IN THE DIAMONDBACK TERRAPIN, *MALACLEMYS TERRAPIN*

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SUBLETHAL EFFECTS OF STRESSORS ON PHYSIOLOGICAL AND MORPHOLOGICAL PARAMETERS IN THE DIAMONDBACK TERRAPIN, *MALACLEMYS TERRAPIN*

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Sublethal effects of stressors on physiological and morphological parameters in the diamond back terrapin, *Malaclemys terrapin* (136 pp)

Director of Dissertation: Willem M. Roosenburg

Aquatic organisms inhabiting estuarine areas experience multiple natural (e.g. temperature and salinity) and anthropogenic physiological stressors. Stressed animals undergo many physiological adjustments, including the production of glucocorticoid stress hormones. Many stress responses are energetically expensive resulting in increased metabolic rate. If energy intake is finite, animals with higher metabolic rates may alter energy allocation. Such allocation adjustments occur at both the tissue level (e.g. decreased organ size) and the whole-organism level (e.g. decreased body size).

In this study, I examined the levels and effects of pollutant exposure in the obligate estuarine turtle, the Diamondback terrapin, *Malaclemys terrapin*. First, I determined PAH concentrations in turtle eggs and showed eggs from the eastern shore of the Patuxent River, Maryland had higher total hydrocarbons than eggs from the western shore. Thus analysis of terrapin eggs may provide insights into how contaminant impact geographically varies.

The remainder of this study examines the lethality and growth responses of three PCB 126 doses and the sublethal metabolic and growth responses of PCB 126 and salinity in combination. I exposed laboratory-reared, male terrapins to intraperitoneal injections of PCB 126. The terrapin ten-month LD50 was 29.8μg/g ±13.7, substantially higher than the LD50's reported for other vertebrates. PCB 126 exposure reduced growth

at all doses (4, 20, 40 and 80μg/g). Animals at 20μg/g PCB 126 exhibited impaired respiratory patterns and reductions in metabolic rate, hematocrit, and heart and gastrointestinal mass. There were no interactions between the two stressors, but salinity alone decreased growth and liver mass and altered respiratory patterns. There was no effect of PCB or salinity on corticosterone, however, plasma levels may have already returned to baseline at the time of the assay. These data suggest PCBs represent a more severe stressor, perhaps because terrapins are able to cope with salinity stress (e.g. salt glands).

This is the first study to provide experimental evidence for PCB 126 effects alone and in combination with another stressor in turtles. If we are to understand how stressors affect populations, we must provide causative, not merely correlative, evidence of the physiological responses to stress of all ecologically relevant stressors.

Approved: _____

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Chapter 1: Introduction

Dawn Ford

Stress and it's Energetic Costs

Most organisms inhabit fluctuating environments, yet must maintain physiological homeostasis within a range of biophysical conditions. For each biophysical factor (e.g. temperature, salinity) an organism has a defined zone of tolerance within which it can exist. Some tolerance zones are broad allowing individuals to exist in a wide range of environments, whereas other zones are narrow, physiologically restricting an organism's ability to occupy specific habitats. For example, estuarine species experience salinity fluctuations as the tides ebb and advance. The brackish water killifish, *Fundulus heteroclitus*, is euryhaline and tolerates a wide range of salinities along the eastern North American coast (Scott et al. 2004), but the intertidal Antarctic limpet, *Nacella concinna,* is stenohaline and relies on both physiological adaptations and behavioral responses to cope with minor salinity changes (Davenport 2001). Even though organisms can function within these zones of tolerance, deviations from their preferred or "optimal" level can be physiologically stressful to varying degrees. Within the zone of tolerance, physiological stress may be minimal, but beyond this zone, the same factors can be deleterious (Koehn and Bayne 1989).

Researchers have defined physiological stress in a variety of ways. Barton (2002) defines stress as any real or perceived threat to homeostasis. Beyers et al. (1999) and Grime (1989) consider stress to be the consequence of any factor that alters an organism's energy budget in terms of energy acquisition or allocation to maintenance and production. On the other hand, Sibly and Calow (1989) characterize stress as any environmental factor that when experienced reduces Darwinian fitness through the

currency of survivorship, fecundity or both. Historically, stress was viewed as being deleterious, but more recently it has been used as an adaptive framework within which to study population change (Greenberg et al. 2002). The ability of individuals to reallocate energy contingent on their physiological status enables an organism to survive under a number of environmental circumstances. For the purposes of this study, I define stress as the consequence of an environmental condition or external constraint that disrupts homeostasis and restricts resource acquisition or alters energy allocation in ways that may ultimately affect fitness through a reduction in survivorship or fecundity. This definition incorporates aspects from each of the definitions above and creates mechanistic hypotheses amenable to experimental testing so that responses may be understood at the population level.

Hans Seyle was one of the first researchers to formally define stress and the classical physiological stress response (Seyle 1976). Seyle divided the stress response into three distinct physiological stages (reviewed by Sapolsky 1992; Hoffman and Parsons 1991). Stage 1 is the alarm stage during which time the nervous system detects stressors. In stage 2, the resistance stage, organisms attempt to evade and minimize effects of a stress through a behavioral response (e.g. lizard changes basking posture to regulate body temperature, burrowing frog aestivates underground). If behavioral responses are inadequate, organisms up-regulate short-term, physiologically mechanisms to cope with the stress and reestablish homeostasis. If these mechanisms are unable to restore homeostasis and the organism is under chronic stress, exhaustion (stage 3) sets in,

and the organism may become more susceptible to disease or predation (sensu Seyle 1976).

Barton (2002) extended the characterization of the stress response by subdividing Seyle's resistance stage into primary, secondary and tertiary phases. Primary responses are neuroendocrine in nature and include the activation of the hypothalamus-pituitary adrenal/interrenal (HPA/I) axis. Secondary responses include the production of biochemicals (e.g., heat shock proteins), changes in cellular respiration, suppression of immune function, and mobilization of energy (Sapolsky 1992). Finally, tertiary responses occur at the whole animal level and include factors relating to feeding, growth, metabolism, dispersal behavior and performance (Greenburg et al. 2002).

When an organism perceives a stress, cells in the brain trigger a hormonal cascade. Stress activates the sympathetic nervous system causing the release of epinephrine and norepinephrine and triggers a number of pituitary secretions in efforts to prepare the organism for the impending danger (Sapolsky 2002). Energy and nutrients are reallocated immediately to essential body systems. For example, the pituitary secretes prolactin to shunt energy away from reproductive functions, vasopressin to conserve renal water and the pancreatic hormone glucagon to restrict carbohydrate usage (Sapolsky et al. 2000). Catecholamines and glucagons also play a role in energetics through the mobilization of energy stores (Sapolsky 2002).

However, the system of greatest interest, and the one studied in most detail in terms of the stress response, is the secretion of glucocorticoids. The major primary response of all vertebrates is an initiation of the HPA/I axis. In the presence of a stressor,

the paraventricular nucleus of the hypothalamus releases corticotropin releasing factor (CRF) which immediately stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) (Sapolsky 2002; Guillette et al. 1995). Within minutes, ACTH reaches the cortex of the adrenal gland and triggers the release of glucocorticoids (Guillette et al. 1995; Axelrod and Reisine 1984). Reptiles do not have a distinct adrenal cortex and instead ACTH triggers the interrenal cells (Gabe 1970) to secrete corticosterone (Greenburg and Wingfield 1987). In birds and mammals, this physiological cascade is termed the HPA (hypothalamus-pituitary-adrenal) axis, whereas in ectothermic vertebrates without a true and distinct adrenal cortex, this cascade is referred to as the HPI (hypothalamus-pituitary-interrenal) axis.

Sapolsky et al. (2000) provide an extensive review of the action of glucocorticoids, many of which are beyond the scope of this study. One focus of this project is the effect of corticosterone (CORT) on organismal energetics by inhibiting anabolic processes such as growth (Sapolsky 1992). Glucocorticoids are one of the underlying endocrinological mechanisms governing the reallocation of energy during the stress response. Organisms continually secrete low levels of CORT, but during a stress response the level of circulating CORT increases. Circulating levels of CORT are affected by factors such as season (Wingfield et al. 1992), sex (Valverde et al. 1999), experience (Simpkiss and Devine 2003), developmental stage (Barton 2002) and reproductive condition (Gregory et al. 1996). Typically, plasma CORT levels increase after a stress such as restraint or handling, and the rate of increase is an indication of the animal's sensitivity to stress (Cash et al. 1997).

Changes in glucocorticoids may signal the broader array of tertiary responses that impact fecundity, growth and performance. Physiological adjustments to stress that organisms must make to regain homeostasis (Calow 1989) typically involve energetic costs that may translate into energetic tradeoffs (Sibly and Calow 1989). Porter and Tracy (1983) developed a mass balance equation (Figure 1.1) describing the distribution of energy among growth, reproduction and storage, the effects of the biophysical environment and how maintenance is the central link between the energetics and the heat components. If energy intake is finite, an increase in the cost of any one component can result in a decrease in energy allocation to any of the other energetic components. My research focuses on the proximate tissue and whole-organism responses of an ecothermic reptile to stress. Maintenance accounts for 80% of an ectotherm's energy budget (Congdon et al. 1982) thus even a small effect of a stressor can translate into large scale changes in the energy allocation and acquisition (Congdon et al. 2001). For example, if increased maintenance costs result in a decrease in growth, stressed animals will remain smaller and more vulnerable to predation for longer period of time than unstressed individuals. Over time, a reduction in growth can lead to delayed maturity or a decline in reproductive output. Those individual that can restore homeostasis, will likely survive longer and have a higher fecundity than individuals that remain stressed. Resistant genotypes or those with an energetically less costly stress response are favored in specific environments and, in turn, organisms exhibit the ability, generation after generation, to tolerate specific stressors. For example, lab-reared, larval F_1 and F_2 generation killifish (*Fundulus heteroclitus*) from a superfund site were more resistant to t-butyl

hydroperoxide stress than killifish from a nearby reference (clean) site (Meyer et al. 2003). Additionally, many organisms possess specialized adaptations which allow them to cope with specific stressors (e.g. salt glands) which can lessen the effect and cost of the stress response.

Ecotoxicological Approaches

Ecotoxicology is the study of the interface between toxicology and ecological features either at the individual, population or community level. Researchers use the tools and applications provided by this field to better understand the fate and effects of anthropogenic toxicants on biological systems (Newman 1998). Thus, many ecotoxicological studies are stress biology studies where the research focuses on anthropogenic (e.g. chemical) stressors.

For many years predominant ecotoxicological methodologies involved a dose or concentration approach where the researcher determines the dose/concentration at which 50% of the exposed individuals succumb to the toxicant (lethal dose 50%, LD50; lethal concentration 50%, LC50). To calculate one of these lethality endpoints, one counts the number of organisms that perish at a few exposure levels, estimates the underlying distribution and uses a statistical analysis (e.g. PROBIT, SAS Institute 2001) to calculate the inflection point. If one is interested in the number that perish over a specific amount of time, a time series component is added and one can calculate the lethal endpoint using a Hazards analysis, failure time analysis or a Kaplan-Meier survival analysis (SAS Institute 2001). However, as Newman (1998) points out, the long-term ecological value of these estimates is difficult to measure. In this dissertation, I calculated the ten-month

LD50 for three doses of a common organic pollutant (PCB 126) and present a Kaplan-Meier survivorship plot.

Although death is a commonly measured endpoint, it does not provide any data that one can use to understand the long-term physiological response of the organism. Perkins (1979) suggested that examining sublethal effects of toxicants on reproductive potential (e.g. fecundity, metamorphosis) across various life stages and on productivity (e.g. growth, performance, physiology) of the individual, population, and community is more informative. Widdows and Donkin (1991) summarized the biological responses of *Mytilus edulis* to various chemical contaminants and argued that significant energetic responses are evident at lower xenobiotic exposure than levels that are lethal, thus sublethal responses may be more useful for management practices. For ecotoxicological studies, it has become increasingly important to develop a cause-effect framework because lethality studies may underestimate the ecological impacts of stressors. This entails identification of the amount of biologically available environmental contaminant, estimation of how much of these xenobiotics end up in animal tissues, and finally, evaluation of their biological effects (Widdows and Donkin 1991). Exposure to chemical contaminants is likely to be energetically costly either through the additive expenses of ostensibly small costs such as increased activity to relocate to a less stressful area (Sibly and Calow 1989) and induction of detoxifying factors such as CYP1A (e.g., Yawetz et al. 1997), or through larger scale energetic trade-offs such as changes in whole organism metabolic rates (Hopkins et al. 1999) or decreases in production (e.g. growth, Albers et al. 1986).

By definition, an animal that experiences an environmental stressor will incur an energetic cost. The energy used to regain homeostasis is no longer available for other physiological or behavioral processes. This cost may be manifested as an increase in metabolic rate, which commonly causes a decrease in growth or reallocation of energy from storage or reproduction (Fair and Ricklefs 2002). For example, water snakes from coal ash polluted sites had higher standard metabolic rates than those from nearby reference sites (Hopkins et al. 1999) and cichlids exposed to high concentrations of cadmium exhibited slower growth rates than those exposed to low doses (Pratap 1999). As in stress physiology studies, toxin exposure carries a number of physiological costs at the individual level that can affect individual fitness in turn, affecting population production and biomass (Calow 1991). In this dissertation I also examine the sublethal effects of stressors on growth and metabolic rate for an ectothermic, estuarine reptile, the diamondback terrapin, *Malaclemys terrapin*.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are organic structures comprised of two to five benzene rings (ATSDR 1995) and these compounds can act as physiological stressors. PAHs can either be of petrogenic (e.g. non-naturally occurring oil spills) or pyrogenic (e.g. natural volcanic activity) origin. PAHs typically occur as complex mixtures in the environment and the largest portion of environmental PAHs are byproducts of human activities (Rand et al. 1995). PAHs result from the incomplete combustion of coal, oil and wood and they are present in some pesticides, plastics, asphalt, and road oil and are common ingredients of crude oil, creosote, automobile

exhaust and roofing tar. The health effects of all PAHs are diverse, some are mutagenic, carcinogenic or tetratogenic, whereas others have no known biological activity (Rand et al. 1995). For example, benzo(a)pyrene, a high molecular weight PAH, has effects ranging from craniofacial and musculoskeletal developmental abnormalities to effects on oogenesis and sperm morphology (Lewis 1991).

On April 7, 2000, approximately 140,000 gallons of #6 crude oil and a small amount of #2 fuel oil leaked from an underground pipeline into Swanson's Creek, a tributary of the Patuxent River, Maryland. Due to shifting winds, the oil slick escaped the creek and contaminated approximately 17 miles of the Patuxent River (Collins et al. 2003). The oil impacted a few beaches along the eastern shore and several creeks and beaches along the western shore of the Patuxent River (Collins et al. 2003) many of which have been the focus of a 17-year demographic study of the diamondback terrapin by my dissertation advisor, Willem Roosenburg. In this dissertation, I present gas chromatographic analyses for the presence and concentrations of PAHs found in terrapin eggs collected along the shores of the Patuxent River one year after the oil spill.

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are chlorinated organic chemicals. Chlorine substitution can occur at up to 10 different locations on the biphenyl molecule resulting in up to 209 different congeners with widely varying toxicity. Coplanar PCBs (e.g. PCB 126) are among the most toxic congeners (McFarland and Clarke 1989) and are structurally similar to that of the most potent organochlorine known, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). PCBs commonly occur in the

environment as mixtures called Aroclors. Each Aroclor is designated with a number that corresponds to the percentage of chlorine present by mass, including Aroclor 1254 (54% chlorine) and Aroclor 1260 (60% chlorine) produced by the Monsanto Chemical Company of St. Louis, Missouri (Safe 1994). PCBs function in diverse applications ranging from pesticide extenders to dielectric fluids in transformers to adhesives and sealants (for review see Safe 1994). PCBs are persistent organic pollutants that are found in a variety of matrices (soil, water, ice, biological tissue) on all continents including Antarctica (Montone et al. 2003 and references therein). Organisms vary in their level of contamination based on age, lipid content and history of exposure (Huestis et al. 1996). Although their manufacture in the United States was banned in 1977 (USEPA 2002), their ubiquitous presence in our environment continues to pose a serious toxicological threat. Today, PCBs still enter the environment via certain combustion practices, seepage from waste disposal sites and improper industrial disposal (Safe 1990).

Polychlorinated biphenyls are persistent, lipophilic contaminants that tend to bioaccumulate and biomagnify in carnivorous organisms (for review, see Safe 1994). PCBs occur throughout many North American estuaries, including the Chesapeake Bay (Ko and Baker 2004). Estuaries and other freshwater aquatic waterways are habitats for numerous species of turtles (Ernst et al. 1994). Several studies have reported PCB concentrations in tissues (e.g. Albers et al. 1986; Bishop et al. 1998; Corsolini et al. 2000) and eggs (e.g. Bishop et al. 1995; Alam and Brim 2000) of wild-caught turtles. However, few studies have directly measured contaminant effects in turtles through experimental exposure (but see Willingham and Crews 1999, 2000; Yawetz et al. 1997, 1998). PCB

exposure has estrogenic effects (*Trachemys scripta*, Bergeron et al. 1994) and induces mechanisms involved in the depuration of contaminants (e.g., CYP1A in *Chrysemys picta*, Yawetz et al. 1997). There are suggestions that PCB exposure may also lead to changes in growth (Albers et al. 1986), development (Bishop et al. 1998) and susceptibility to disease (Aguirre et al. 1994), but these correlative studies have either shown patterns contrary to expectations or been unable conclusively to attribute the effects to PCB exposure. In this dissertation, I will provide experimental evidence documenting the sublethal tissue and whole-organism level effects of one of the most toxic, coplanar PCB congeners, PCB 126 in the diamondback terrapin.

The Importance of Multiple Stressors

Natural ecosystems are replete with numerous abiotic and biotic factors that elicit a stress response. These stressors can be acute, sequential, episodic, chronically intermittent, or sustained (Sapolsky et al. 2000). Because of the complex nature of environments, multiple stressors are likely simultaneously experienced. Multiple stressors can interact in four ways: 1) the effects can equal the sum of all single stressors (additive), 2) the effects can be greater than the combined sum of the single effects (synergistic), 3) the effects can be less than the combined sum of the single effects (antagonistic), or 4) no interactive effects. For example, chemical contaminants such as flouranthene (Hatch and Burton 1998) and carbamate (Zaga et al. 1998) in the presence of UV light have produced synergistic deleterious effects in anuran larval physiology and development. Synergism of multiple contaminants may be due to the toxic effects of one contaminant interfering with the effects of another stressor, or the presence of one

stressor leading to an overall decrease in the tolerance of the organism to stress (Hatch and Blaustein 2000). Numerous studies have examined the effects of multiple stressors and how they relate to amphibian declines (e.g. Hatch and Blaustein 2000; Kiesecker and Blaustein 1995; Long et al. 1995; Zaga et al. 1998), yet there is still much to be done regarding the effects of multiple stressors on other ectothermic organisms. The importance of multiple stressors is becoming increasingly apparent if we are to understand their effects and how to affectively manage populations in their presence (Briston and Threlkeld 2000).

Chapter summaries

The research presented here uses a stress paradigm to examine the ecotoxicological consequences of environmental contaminants on turtle ecophysiology. I conducted these studies using the diamondback terrapin, *Malaclemys terrapin*, because this species inhabits estuaries along the eastern U.S. coast from Cape Cod, Massachusetts to Galveston, Texas (Ernst et al., 1994). According to a recent report by the Environmental Protection Agency (USEPA, 2005), coastal areas, and thus terrapin habitat, range from fairly clean sites in the southeast, to poor environmental conditions in the northeast. In this dissertation, I present PAH levels in field collected diamondback terrapin eggs (Chapter 2). Then, the remaining four chapters examine dose effects of PCB 126 on growth and survival (Chapter 3), one concentration of PCB 126 in combination with varying salinity stress on whole-organism effects (Chapter 4), tissue levels effects (Chapter 5) and the production of corticosterone (Chapter 6).

First, I describe PAH levels in diamondback terrapin eggs collected from the shores of the Patuxent River, Maryland (Chapter 2). I sampled nesting locations on the eastern and western shores of the river in 2001, one year after a large crude oil spill. The recently laid eggs were homogenized and analyzed for PAHs using a Gas Chromatograph with a Flame Ionization Detector (GC-FID). I also calculated a fossil fuel pollution index (FFPI) to determine the type of PAHs (petrogenic versus pyrogenic). Eggs from the western shore contained fewer PAHs and had FFPIs characteristic of pyrogenic PAHs, whereas eggs from eastern shores had significantly higher PAH levels and contained a larger proportion of petrogenic PAHs. Comparison of terrapin eggs from sites along the river may elucidate geographic differences in hydrocarbon exposure level. The most likely route of PAH exposure was transfer from the female parent to the egg via the yolk, and future studies should further investigate the maternal transfer of contaminants. However, without a "fingerprint" of the exact crude oil spilled in 2001, it is impossible to determine if the PAHs detected in the eggs were from the spill or from other sources.

Because of some extenuating difficulties with measuring the physiological effects of PAHs in turtles due to our inability to obtain a sample of the oil spilled in 2000, the remaining four chapters employ the environmental toxicant PCB 126. Chapter 3 is a dose ranging study that examines how variation in exposure to PCBs can disrupt physiological processes and in some situation can cause death. Direct causal evidence of sublethal and lethal effects of PCB exposure in reptiles is generally lacking. I exposed diamondback terrapins to three concentrations of PCB 126 or two control solutions and measured growth and survivorship over a period of ten months. At three months after

exposure, all PCB levels inhibited growth. I also determined that the LD50 for PCB 126 in terrapins was 29.8μg/g ±13.7, but was not calculable until ten months after exposure. My data suggests terrapins may be a useful indicator species for the study of the sublethal physiological effects of estuarine contaminants.

The dose ranging chapter enabled me to identify a PCB level that would elicit only sublethal effects. Based on these data, in Chapter 4, I chose to examine additional sublethal physiological traits in turtles exposed to a PCB 126 dose of 20μg/g. I collected eggs, incubated them at male-producing temperatures, and reared hatchlings for eight months prior to PCB exposure. I exposed all animals (PCB-exposed and unexposed) to a second stress, four ecologically relevant salinity levels. I measured growth and standard metabolic rate at the end of monthly salinity cycles for six months. All PCB exposed animals exhibited reduced growth and lower standard metabolic rates with an altered respiratory pattern. It is possible that PCB stressed terrapins lowered their metabolic rate and altered their respiratory pattern as an energy conservation mechanism. Salinity stress caused reduced growth, but had no affect on metabolic rate. Contrary to expectations, there were no interactive effects of the two stressors. These data suggest that either PCBs are a more severe stressor or terrapins have a better physiological capacity to cope with salinity stress because they are estuarine organisms.

Chapter 5 presents additional physiological data obtained from the same individuals studied in chapter 4. At six months after exposure (14 months of age), a subset of animals were euthanized, the organs removed, and blood was collected for hematocrit determinations. Salinity level only affected liver mass, but PCB exposure

reduced heart and gastrointestinal mass as well as hematocrit. This pattern is consistent with energy conservation mechanisms where stressed animals shunt energy away from immediately non-essential organs with high metabolic activity which are energetically expensive to maintain (e.g. heart and GI tract) whereas other organs involved in depuration of toxins (e.g. liver) are essential and thus maintained despite increased energy demands. Animals with reduced hematocrit will have a decreased ability to transport carbon dioxide, but the data suggest that stressed individuals have a smaller heart and lower carbon dioxide production. Future studies should investigate the mechanisms that led to decreased organ size and determine if these effects are a method of energy conservation and determine the significance of reduced hematocrit.

The sixth and final chapter presents corticosterone (CORT) data. Individuals that have recently experienced stress, typically exhibit elevated plasma levels of the steroid hormone corticosterone. In this study, I used controlled laboratory exposure to salinity, a natural stressor and 3,3',4,4'5-pentachlorobiphenyl (PCB 126), an anthropogenic stressor, to assess effects of single and multiple stressors on plasma corticosterone (CORT) levels in the diamondback terrapin. I collected plasma from recently euthanized juvenile terrapins and used an enzyme immunoassay to determine corticosterone concentrations. I compared CORT concentrations between PCB-exposed and control turtles and at each of four salinities. Terrapin CORT levels were some of the lowest levels reported for any turtle species. In addition, I found no affect of either PCB or salinity stress on CORT. However, because I assayed CORT six months after the PCB stress exposure, it is possible that PCBs initially increased CORT, but over the long-term,

plasma levels had decreased back to baseline. A single measurement of CORT may not reflect the current level of stress due to other mitigating factors and should be included in a larger study using additional variables and a time series to more fully quantify the overall sensitivity and effects of stressors on terrapin physiology.

Overall Conclusions

This research is one of the first studies to provide data on the causal effects of PCB 126 in reptiles. Through laboratory exposure of diamondback terrapins to PCB 126, I have determined a ten month LD50 of 29.8 ±13.7 μg/g. Additionally, I documented numerous sublethal effects. PCB 126 at all tested doses (4, 20, 40, and 80 μg/g) caused significantly decreased growth. Additionally, individuals exposed to PCB 126 (20 μg/g) exhibited depressed metabolic rates, altered respiratory patterns, reduced heart and GI tract dry masses, and decreased hematocrit. Although there were no interactive effects between salinity and PCB 126, individuals at some salinity levels exhibited reduced growth and smaller livers. Finally, CORT did not differ among any of the treatments. Yet, initially the stressors may have caused CORT to increase, but by the time of the assay, CORT had returned to baseline levels. All of these data support the hypothesis that PCB 126 and salinity can act as physiological stressors and alter organismal physiology and morphology in the diamondback terrapin. I found no evidence of any interactive effects of the stressors experienced multiply and conclude that this is either due to the overwhelming stress produced by exposure to PCB 126 or the very limited stress caused by salinity due to the presence of morphological adaptations (e.g. salt glands). Future terrapin ecotoxicological studies should investigate the transfer

of maternal contaminants via lipid-rich eggs, the affects of stressors on energy acquisition and digestive efficiency, and incorporate all of these data into a model to predict population level effects.

Chapter 2: Spatial Variation in Polycyclic Aromatic Hydrocarbon Concentrations in *Malaclemys terrapin* Eggs from the Patuxent River, Maryland

Dawn Ford, Adria Elskus, and Willem Roosenburg

Introduction

Organisms inhabiting industrialized aquatic environments experience a number of environmental contaminants including heavy metals, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAH). The contamination of aquatic habitats by these compounds frequently results in developmental anomalies and other teratogenic effects (Portelli and Bishop 2000).

Polycyclic aromatic hydrocarbons are organic structures comprised of two to five benzene rings (ATSDR 1995) and these compounds can act as physiological stressors. PAHs can either be of petrogenic (e.g. non-naturally occurring oil spills) or pyrogenic (e.g. natural volcanic activity) origin. PAHs typically occur as complex mixtures in the environment and the largest portion of environmental PAHs are byproducts of human activities (Rand et al. 1995). PAHs result from the incomplete combustion of coal, oil and wood and they are present in some pesticides, plastics, asphalt, and road oil and are common ingredients of crude oil, creosote, automobile exhaust and roofing tar. The health effects of all PAHs are diverse, some are mutagenic, carcinogenic or tetratogenic, whereas others have no known biological activity (Rand et al. 1995). For example, benzo(a)pyrene, a high molecular weight PAH, has effects ranging from craniofacial and musculoskeletal developmental abnormalities to effects on oogenesis and sperm morphology (Lewis 1991).

The route of toxicant uptake in aquatic ecosystems varies depending on the species, developmental stage, the contaminant, and a number of other ecophysiological and biochemical factors. For example, PCBs can be absorbed through amphibian skin

(Johnson et al. 1999) and heavy metals are ingested when water snakes consume contaminated prey (e.g. Hopkins et al. 1999). Although studies have documented contaminant tissue burdens (e.g. Albers et al. 1986) and adverse effects of exposure in adult turtles (e.g. Lutcavage et al. 1995), but younger developmental stages are often more susceptible to toxicant effects (Bishop et al. 1991). Furthermore, many environmental contaminants are lipophilic and thus, can bind to the lipoprotein vitellogenin (Monteverdi and Di Guilio 2000). Therefore, eggs can be exposed to contaminants via maternal transfer when the female allocates vitellogenin to developing embryos (Sakai et al. 1995). Maternal transfer of several contaminants have been reported for a number of species, including heavy metals maternally transferred in common grackles (Bryan et al. 2003) and PCBs in leghorn chickens (Bargar et al. 2001). Russell et al. (1999) reported that maternal transfer of contaminants in fish results in embryo contaminant concentrations roughly equivalent to those in the mother, yet this pattern was not as consistent in snapping turtles.

For many vertebrate species, such as turtles, eggs can be useful indicators of contaminant exposure. Previous research (Bishop et al. 1994; Bishop et al. 1996; Bonin et al. 1995) found that organochlorine levels in snapping turtle eggs reflect the spatial variation of these contaminants within the turtle's habitat. Therefore, diamondback terrapin (*Malaclemys terrapin*) eggs may also be useful in elucidating spatial contamination patterns and levels of organismal exposure. Diamondback terrapins are estuarine turtles found throughout the eastern seaboard and the gulf coast of the United States. Terrapins in the Patuxent River are locally abundant and show strong site fidelity

throughout their life time (Roosenburg unpublished). Therefore, analysis of terrapin eggs is a convenient way to assess toxin exposure of particular subpopulations.

On April 7, 2000, approximately 140,000 gallons of #6 crude oil and a small amount of #2 fuel oil leaked from an underground pipeline into Swanson's Creek, a tributary of the Patuxent River, Maryland. Due to shifting winds, the oil slick escaped the creek and contaminated approximately 17 miles of the Patuxent River (Collins et al. 2003). The oil impacted a few beaches along the eastern shore and several creeks and beaches along the western shore of the Patuxent River (Collins et al. 2003) many of which have been the focus of a 17-year demographic study of the diamondback terrapin by one of the authors (WMR). Here I present a chemical analysis of Patuxent River terrapin eggs to show small scale geographic variation in hydrocarbon exposure, possibly from the Swanson's Creek oil spill. I hypothesized that the greatest total amount and variety of PAHs would be detected in eggs collected from sites closest to the spill and eggs from areas of heavy shoreline oiling would also have high total PAHs.

Methods and Materials

One year after the Swanson's Creek oil spill, I located terrapin nests on beaches directly affected by the 2000 spill along the western (from Golden Beach south to Cremona) and eastern shores (Sheridan point and Craney Creek) of the Patuxent River (Figure 2.1A and Appendix). I located a total of twelve recently constructed nests (<24 hours old) as evidenced by fresh tracks and pink eggs that had not yet begun to chalk (turn white). For PAH analysis, I removed three eggs from each nest, measured (length and width to the nearest mm) and weighed (to the nearest 0.1 g) each egg. Eggs were

frozen at -10C in solvent-washed (HCl and petroleum ether) glass jars to avoid any contamination of the eggs from the freezing containers.

Frozen samples were brought to the University of Kentucky for hydrocarbon analysis. Eggs were thawed, unshelled, pooled by clutch and homogenized with a polytron. A subsample of homogenate was retained for dry weight determination. I combined the remaining homogenate with 4g sodium sulfate (drying agent) and ground the sample with a mortar and pestle. I spiked each sample with 50µl of the surrogate standard p-terphenyl and placed the spiked samples in pre-extracted cellulose thimbles. I extracted samples in a soxlet refluxing 1:1 acetone:hexane for twelve hours. For every five samples, I ran one procedural blank. I removed extract from the soxlet and concentrated it to 1ml with a Labconco® rotoevaporator. To remove any lipids that could interfere with the gas chromatographic signal, samples were pre-processed in chromatography columns containing 5g of 5% deactivated silica and 2.4g of 5% deactivated alumina. Samples were eluated with hexane and methylene chloride. I collected and concentrated the eluate to 0.5ml in the rotoevaporator. I spiked the concentrate with 50µl of the internal standard m-terphenyl and brought the volume up to 1ml with heptane. One microliter of each sample was injected into a Shimadzu GC-FID with a DB5 (J& W Scientific) chromatography column with the dimensions of 30m x 0.25mm ID and a 0.25 micron coating. Hydrogen was used as a carrier gas. The temperature was incrementally increased from 80°C to 120°C at 30 degrees/minute, from 120°C to 150°C at two degrees per minute, from 150°C to 275°C at 5 degrees per minute and held at 275°C for 15 minutes, maintaining both the injection port and the detector

temperatures at 275°C. Each sample had a run time of 59 minutes. I identified compounds detected in eggs by comparing sample retention times with the retention times of PAH standards obtained from Ultra Scientific with a retention time error of 2.5%. Peak areas were calculated using the Normalize option. I calculated percent recoveries for each sample using the amount recovered from both the surrogate and internal standards. Any sample with a recovery less than 70% was not used in statistical analyses. This rejection criterion resulted in a sample size of 4 western nests and 2 eastern nests.

I used a t-test to compare total PAH concentrations from eggs collected on the western shore to those collected on the eastern shore. In order to determine the relative contributions of PAHs from petrogenic origins (e.g. from oil spills) versus pyrogenic origins (e.g. combustion), I calculated the fossil fuel pollution index (Boehm and Farrington 1984; Wenchuan et al. 2003) using the following formula:

FFPI = [naphthalene+phenanthrene+0.5*(phenanthrene+2-methylphenanthrene+ 1-methylphenanthrene) + dibenzothiophene]/ total PAH

Boehm and Farrington (1994) states that an FFPI of 26% or greater is indicative of PAHs from petrogenic origins and lower values are indicative of pyrogenic hydrocarbons.

Results

Eggs collected from the eastern shore had significantly higher concentrations of total PAHs than eggs collected from the western shore ($t = 5.49$, $df = 5$, $p = 0.02$). One western shore egg sample had no detectable hydrocarbons, two exhibited only one PAH (either perylene or benzo(a)pyrene), and in the two remaining samples I detected small amounts of 3,6 dimethylphenanthrene (Table 2.1). I detected a larger diversity and

greater quantity of hydrocarbons, ranging from naphthalene to 3,6 dimethylphenanthrene and other heavier PAHs in eggs collected from the eastern shore. Fossil fuel pollution indices of western shore eggs ranged from 0-16.3% with a mean of 4.8%, indicative of PAHs from pyrogenic origin. In comparison, pollution indices calculated for the two eastern shore samples were 20.6% and 30.4% (mean = 25.5%), more indicative of PAHs from petrogenic origins.

Discussion

The total concentrations of polycyclic aromatic hydrocarbons (tPAH) in terrapin eggs collected from the Patuxent River indicated a spatial difference in the level of contamination. Eggs collected on the eastern side of the river had higher concentrations of PAHs then eggs from the western shore of the Patuxent (Figure 2.1B). The April 2000 oil pipeline rupture released oil into Swanson's Creek and subsequently contaminated seventeen miles of the Patuxent River. The beaches on the eastern shore were closest to the spill site (e.g. Craney Creek) and most affected by the oil spill (Figure 2.1A). The single clutch analyzed from this heavily impacted nesting area contained almost five times more tPAHs than the highest tPAHs found in eggs from the western shore. The other eastern shore sample was from Sheridan Point, a lightly contaminated area approximately 3 nautical miles (5.5km) down river from Craney Creek. The Sheridan point eggs contained the greatest quantity of PAHs, almost two times the tPAH concentration found in the eggs from Craney Creek, and the greatest diversity of PAH of any eggs sampled (Table 2.1). Terrapins regularly move along the shores (Roosenburg, unpublished) and thus the PAHs detected in these eggs could have originated from a

number of points along the eastern shore, including more highly impacted sites, such as Craney Creek. The oil spill also affected nesting areas on the river's western shore (Figure 2.1A). Eggs from these sites contained very few PAHs, a finding somewhat surprising given the extent of the beach oiling.

One potential route of egg PAH exposure is via incubation in contaminated beach sands. Some reports document deleterious physiological effects in eggs laid in areas with persistent heavy metals and organochlorines (Nagle et al. 2001; Bishop et al. 1991, 1998). However, PAH exposure from contaminated beach sand is an unlikely route of exposure for these terrapin eggs because PAHs are not as persistent as heavy metals and organochlorines. Fritts and McGehee (1981) provided experimental evidence demonstrating that oil spilled on beaches, even just a few weeks prior to nesting, showed no significant effects on sea turtle egg mortality. The eggs collected in this study were deposited 14 months after the oil spill and thus PAHs were likely no longer present in the surface and subsurface sands or not biologically available because the hydrocarbons would need to dissociate from the sand and traverse across the egg shell. I collected eggs within 24 hours of oviposition, thus even if PAHs could transverse the egg membranes they would need to have done so within hours in order to explain these data.

The more likely origin of PAHs in the eggs of this study is maternal transfer. During egg development, the female liver produces vitellogenin, a lipid-rich protein, which then enters the blood stream and is incorporated into developing follicles. Hydrocarbons are lipophilic and can transfer to the developing oocytes in the lipid- rich vitellogenin. Sakai et al. (1995) have shown that eggs in the oviduct of adult female sea

turtles exhibited high levels of heavy metals deposited during the yolking of follicles. Because terrapins have strong site fidelity (Roosenburg et al. 1999), turtles that lay eggs on the eastern shore most likely foraged in heavily oiled areas and thus would have accumulated greater PAH concentrations to pass onto their developing follicles. Yet there is no physical or ecological barrier separating eastern from western shores and individuals marked on the western shore have been captured on the eastern shore albeit at a lower frequency (pers. obs). Future studies must include toxicological assays of livers, fat bodies and developing follicles from adult female turtles in order to conclusively demonstrate that PAHs detected in eggs are due to maternal transfer.

A number of studies have used contaminants detected in turtle eggs to document geographic variations in pollution (de Solla et al. 2001; Alam and Brim 2000). However, the inability of this study to discriminate among areas with heavy to light shoreline contamination was unexpected. I expected to find much higher tPAH levels in eggs along the western shore, due to the levels of shoreline oiling. However, from this study it appears that only animals on the eastern shore that may have moved along the coastline and foraged in contaminated areas had any toxicological level of tPAHs. However, because each crude oil is slightly different in chemical composition, in order to link tissue contaminant levels with a specific oil spill event, researchers must analyze a sample of the spilled oil and compare its PAH "fingerprint" to that of the tissue. Without knowing the exact PAH "fingerprint" of the crude oil #6 that spilled in Swanson's Creek in 2000, it is impossible to trace the origin of the PAHs detected in the terrapin eggs. With the increased industrialization of the Patuxent River, it remains a possibility that the

petrogenic PAHs detected in eastern shore eggs were from other, less obvious, fuel oil leaks. Additionally, the total load of PAHs to which organisms are exposed is increased via PAH exposure from a variety of sources including boating activities and leaching from creosote pilings. The fossil fuel pollution indices suggest the PAHs detected in eastern shore eggs were of petrogenic origin, whereas those on the western shore were more likely pyrogenic from run off or the combustion of fossil fuels. Clearly, further work is needed to understand the mechanism of PAH uptake in turtles and the resolution with regard to understanding geographic variation. Nonetheless, this study suggests that the terrapin may be a good organism in which to study and evaluate small scale geographic variation in contaminant exposure.

Chapter 3: Lethal and Sublethal Dose Effects of PCB 126 in the Diamondback Terrapin, *Malaclemys terrapin*.

Dawn Ford, Willem Roosenburg, and Adria Elskus

Introduction

Polychlorinated biphenyls (PCBs) are synthetic organochlorines, first manufactured in the United States in 1929. North American industries used PCBs in electrical transformers, pesticide extenders, and asphalt sealant and for fifty years, released these toxicants into the environment. Although the United States banned production of these chemicals in 1977 (USEPA 2002), their ubiquitous presence in our environment continues to pose a serious threat. Today, PCBs still can enter the environment via certain combustion practices, seepage from waste disposal sites and improper industrial disposal (Safe 1990). PCBs are persistent organic pollutants that have been found in a variety of matrices (soil, water, ice, biological tissue), on all continents (Montone et al. 2003 and references therein). They have multiple biological effects including immune suppression, wasting syndrome and other carcinogenic and estrogenic effects (reviewed in Safe 1994).

PCBs are highly stable, lipophilic chemicals and because of these properties tend to bioaccumulate in higher trophic level consumers (Safe 1994). A large body of literature has focused on the occurrence of PCBs in mammals and birds, but comparatively few studies have analyzed tissue contaminant levels in wild-caught reptiles (Portelli and Bishop 2000). PCB levels reported in turtle eggs (Table 3.1) ranged from low (0.3 μg total PCB/g wet weight, Bishop et al. 1996) to the highest concentrations ever reported in tissues of wild-caught individuals (737 μg total PCB/g w.w. with 0.015 μg PCB 126/g w.w., de Solla et al. 2001). While it is useful to quantify contamination patterns in the field, such studies cannot directly determine if PCBs are causing any

adverse biological effects. Field studies can identify correlations between PCB levels and various biological parameters, but cannot directly address causation. Furthermore, the coplanar congeners (PCB 77, 126, and 169) are the most biologically reactive and potent PCBs (McFarland and Clarke 1989), yet most previous turtle studies reported only total PCBs and not concentrations of the specific congeners (Portelli and Bishop 2000). Although animals experience PCBs as mixtures (e.g. Aroclors) in the environment, typically the co-planar congeners are responsible for harmful biological effects (Safe 1990).

Commonly, researchers measure both lethal and sublethal effects to evaluate the relative potency of chemical toxicants. Lethal dose experiments determine the chemical concentration that kills a certain percentage of the study species within a specified period of time (e.g. LD50, lethal dose killing 50% of the exposed individuals). The LD50 has become a useful tool for comparing the acute toxicity of one substance to that of another. However, the mammalian LD50 may be very different from that for a reptile because ecothermic reptiles have very different metabolic rates than mammals and turtles have been shown to have a reduced ability to detoxify contaminants based on lower levels of CYP1A activity (Rie et al. 2000). While LD50 values are useful in some applications, they can be less effective for studying long-term effects of contaminants in long-lived species. Chemical exposure of field individuals may not cause immediate death, but cause more subtle effects on their physiology and life history. Therefore, exposure levels that elicit quantifiable sublethal effects in these biological variables may still cause substantial changes in population dynamics. For example, sublethal effects on growth can

lead to population decline through decreased juvenile survivorship or delayed time to reproductive maturity. Therefore, in long-lived species with unknown chemical sensitivity, it is beneficial to have both LD50 values and data documenting sublehtal physiological and morphological effects of contaminant exposure.

The goals of this study were two-fold. First, I wanted to identify the LD50 for turtles exposed to PCB 126, a toxic, coplanar congener found in most ecosystems, thought to be largely responsible for many of the effects of PCB mixtures, and similar in bioreactivity to dioxin (Safe 1990). Another coplanar congener (PCB 77) has been shown to cause decreased in growth rate in brook trout (Ndayibagira et al. 1994). I chose *Malaclemys terrapin* as the study species because this turtle inhabits highly industrialized estuarine areas in the United States. To date, all published reports have been unable to identify PCBs as a direct cause of death in turtles despite turtles possessing some of the highest PCB loads ever recorded in any vertebrate (de Solla et al. 2001). Here, I provide direct causative evidence of a specific level of PCB 126 exposure causing death. Second, I wanted to identify doses that elicit sublethal effects in stage-specific growth. In turtles, growth positively correlates with survivorship and fecundity (Congdon et al. 1982). Thus, slower growth can decrease survivorship and the number of offspring produced, resulting in long-term decreases in population numbers.

Methods and Materials

In May 2002, I collected five clutches of recently laid *Malaclemys terrapin* eggs from the shores of the Patuxent River, Chesapeake Bay, Maryland. I transported the eggs to Ohio University and incubated them at male-producing temperatures (28°C, Jeyasuria

et al. 1994). Turtles were measured (carapace length $\bar{x}$ = 30.2 mm ± 1.1; mass $\bar{x}$ = 7.7g ± 0.57) after yolk plugs were nearly resorbed at approximately 4 days old. Hatchlings were assigned to one of five treatments, three PCB exposure groups and two controls. PCB 126 neat was obtained from Ultra Scientific (Rhode Island, USA), dissolved in dimethylsulfoxide (DMSO)-corn oil and serially diluted into three concentrations (1ug/ul, 10ug/ul and 20ug/ul), maintaining a 7% level of DMSO. Over the course of four days, individuals in the three PCB treated groups received four intraperitoneal injections (tuberculin syringe with a 27 gauge needle) of one of the three PCB 126 concentrations to achieve total body burdens of 4ug/g (n = 6), 40ug/g (n= 5) and 80ug/g (n = 6). Control turtles either received four injections of DMSO in corn oil ("control", n = 4) or four sham injections ("sham", n = 7). Turtles were housed individually at 28°C (±1C) in 5ppt salt water (Coralife®) in 12.5 cm x 17.5 cm x 6 cm plastic containers. Twice a week I fed turtles a known quantity of brine shrimp and 24 hours later changed their water completely. I measured growth (carapace length and mass) every 30 days for eight months after treatment.

To relate growth differences to changes in feeding, I measured apparent food intake during feeding trials for all treatments at five months after exposure. I continued to feed the turtles equal amounts of brine shrimp in their individual containers and monitored the amount of food remaining (orts). Turtles ate for twelve hours after which I collected orts, vacuum filtered the water using 25um fluted filters, air-dried the filters and weighed them (0.001g). I determined ort mass as the weight of the dry filter plus orts

minus original filter weight. Apparent food intake was calculated as food provided (dry mass) minus orts remaining (dry mass).

Statistics

I used a repeated measures ANOVA (Proc mixed, unstructured covariance matrix; SAS Inc., 2001) to test for growth differences among the groups and orthogonal contrasts to test for differences between the two control groups and the three PCB treatment groups. I analyzed growth data from the first 180 days because after this time increased mortality at higher PCB levels reduced sample sizes and precluded statistical analysis.

I used an ANCOVA (Proc mixed; SAS Inc. 2001) with body mass as a covariate to test for food intake differences between the two groups. For statistical comparisons, I compared pooled PCB treatment levels with pooled control groups.

I observed turtles for ten months after exposure to calculate an LD50 using a probit analysis (Hintz 2004) and constructed Kaplan-Meier survivorship curves for each treatment. I tested homogeneity in survival among the treatments using the logrank statistic (Hintz 2004) which equally weights all time intervals and calculates a Chi-square value.

Results

Initially, size and mass among the groups were similar (mass: $F_{4,23} = 0.02$, $p = 0.99$; CL: $F_{4,23} = 0.07$, $p = 0.99$), as were growth trajectories for the first 60 days (Figures 3.1A and 3.1B). After 60 days, slight growth differences developed between control and all PCB treated groups. At four months after PCB exposure, large size and mass differences between PCB-treated and control groups were evident (Figures 3.2A and

3.2B). After 120 days, growth patterns continued to diverge resulting in smaller turtles (carapace length: $F_{4,23} = 5.89$, $p = 0.002$, Figure 3.1A; mass: $F_{4,23}=9.09$, $p = 0.001$, Figure 3.1B) in the PCB exposed treatments. PCB treated turtles at all levels of exposure were significantly smaller in mass (orthogonal contrasts: $T_{24} = 5.73$, $p<0.001$) and in carapace length (orthogonal contrasts: $T_{24} = 4.67$, $p<0.001$) than turtles in both control groups. There were no dose-related differences, growth was suppressed to the same levels regardless of PCB-concentration (contrasts $T_{23} = 0.73$, $p = 0.47$). Although PCB exposed turtles were smaller, this result could not be explained by a difference in apparent food intake (Figure 3.2; $F_{1,25} = 1.7$, $p = 0.21$).

There was no mortality due to PCB exposure for the first six months after exposure. However, by ten months after exposure, 50% (n = 3) of the 40ug/g turtles had died and 83% (n = 5) of the 80ug/g turtles had died. I found no mortality in the uncontaminated and low dose (4μg/g) groups. Survival probability curves (Figure 3.3) showed differences in survival (logrank $\chi^2 = 19.14$, df = 3, $p = 0.0003$) among the treatments. The calculated 10 month LD50 for PCB 126 in terrapins was 29.8 μg/g ± 13.7 (Probit; Hintz 2004).

Discussion

Juvenile male hatchling terrapins exposed to PCB 126 grew more slowly as compared to control individuals (Figure 3.1). Terrapins in all treatments initially increased in body size (day 0 to day 30, Figure 3.1) followed by no growth during the next 30 days (day 30-day 60). The initial increase in hatchling mass most likely resulted from water gain when I moved turtles from semi-dry incubation boxes to an aquatic

environment. The initial increase in body size may be attributable to actual somatic growth or to the further development, restructuring and mineralization of the carapace. After the initial change in body size, growth arrested for the next 60 days and resumed at ninety days but only in the control animals (Figure 3.1). This growth arrest may be the result of an endogenous metabolic or growth component (W. H. Karasov pers. comm.) responsible for shunting energy from growth to storage for overwintering survival. Female turtles provision eggs with yolk for both embryogenesis and post-hatching survivorship, thus in many species including terrapins, there is a large amount of residual yolk remaining at hatching (Congdon 1989; Morafka et al. 2000). Although I housed turtles under conditions to maximize growth, hatchlings may have still been experiencing maternal yolk-derived hormone-regulation of their initial activity and growth. If there was an endogenous growth component governing energy allocation and metabolism, it is possible that PCB 126 interfered with the animal's transition to a new metabolic state and thus treated animals were not able to break the established pattern of slowed growth and continued to grow slowly for the remainder of the experiment.

Within 120 days of exposure, PCB exposed turtles at all doses had decreased growth relative to control groups. Even though slight growth differences within the first few months were not statistically significant, small differences at this stage can still be biologically significant. Chelonian mortality is moderate to high in early size classes (Iverson 1991; Parker 1996; Frazer et al. 1991). Contaminated turtles with delayed or slower growth will remain at smaller size classes with lower survivorship for longer periods. Furthermore, if the slower growth pattern observed in this study continues

through later size classes, then it may result in delayed maturity or reduced fecundity for PCB exposed turtles.

Rats exposed to dioxin, a compound similar in toxicity to PCB 126, exhibited reduced body mass and a reduction in food intake (Seefeld et al. 1984; Seefeld and Peterson 1984). PCB 126 exposure can lead to a growth reduction via a decrease in energy acquisition, damage to the digestive system, or an alteration of energy allocation patterns. However, in this study, I found no difference in food intake. Therefore, the observed growth effect was not a function of a disparity in energy ingested. Although we did not see a refusal of food, a symptom typical of wasting syndrome, PCB-exposed turtles may exhibit reduced digestive processing ability or lowered assimilation efficiency. Wasting syndrome and reduced growth in dioxin-exposed fishes is a function of a reduced ability to absorb and mobilize nutrients due to a decrease in blood flow from edema and swelling restricting flow through blood vessels (Spitsbergen et al. 1991). If edema inhibits gut motility affecting throughput time or intestinal uptake, then digestive efficiency is subsequently affected (Sibly 1981). Furthermore, rats exposed to PCB 126 had lowered intestinal levels of cholecystokinin (Lee et al. 2000), a hormone regulating gall bladder and pancreatic function (Randall et al. 2002). PCB 126 exposed turtles may have reduced growth due to constricted or damaged intestinal vasculature, increased gut throughput time, or altered digestive hormones. An organism displaying a combination of any of the above symptoms would be exhibiting wasting syndrome, a toxicological effects common to PCB and dioxin exposure.

Alternatively, the growth difference may be due to an energetic trade-off with another physiological parameter, such as metabolic rate. Up-regulating mechanisms for detoxification and depuration are energetically expensive (Calow 1991), thus contaminated organisms may need to reallocate energy from somatic production (i.e. growth) to maintenance (i.e. metabolic rate). Hopkins et al. (1999) showed water snakes exposed to coal ash increase oxygen consumption compared to unexposed snakes. Maintenance accounts for more than 80% of an ectotherm's energy budget (Congdon et al. 1982). Even a small change in the amount of energy required for daily physiological maintenance impacts other energy allocations, such as growth.

All PCB doses tested produced a similar pattern of reduced growth. Because the 4μg/g dose had similar effects to the higher doses, we could not identify the lowest tolerable level of PCB 126 contamination for terrapins. However, terrapins did not experience an increase in mortality at 4μg/g compared to the 40 and 80μg/g at ten months (see below). Future studies should investigate whether lower doses of PCB 126 have similar effects on aquatic turtle life history parameters in order to establish an ED50. Factors that affect survivorship and fecundity (e.g. growth) can have long lasting implications at the population level. Although an LD50 might be useful to understand the short-term effects of exposure levels, an ED50 can help elucidate long-term sublethal and cryptic effects.

Other studies have used injections with adult turtles to examine CYP1A production (Yawetz et al. 1997, 1998) or topically introduced contaminants to eggs to examine estrogenic effects (Willingham and Crews 1999, 2000). However, this is the

first study to use intraperitoneal injections in a laboratory setting to understand the sublethal whole-organism physiological effects of PCB 126 in hatchling turtles. In this study, I saw no mortality at even the highest (80µg/g) in the first 5 months after exposure. However, by ten months, fifty percent of the individuals exposed to 40µg/g died and 80% died at 80µg/g. At ten months after exposure, the approximate LD50 for PCB 126 in terrapins was 29.8µg/g. Concentrations lower than 4µg/g might have lower effects and are worth investigating. This LD50 is substantially higher than the LD50s reported from intraperitoneal injections in birds eggs (terns: 0.104µg/g, kestrels: 0.0004µg/g, Hoffman et al. 1998; cormorants: 0.158µg/g, Powell et al. 1997). However, it is more informative to compare LD50s at equivalent developmental stages than it is across stages (Thomas and Colborn 1992). The most important outcome is that an LD50 could not be determined until 9-10 months after exposure (Figure 3.3). Ten months is considerably longer than most LD50 studies and further exemplifies the need for long-term studies documenting lethal and sublethal effects.

Direct evidence of the physiological effects of PCBs in reptiles is generally lacking (Portelli and Bishop 2000). In this study, I have shown that specific PCB levels decreased survival and suppressed growth. This study also provides evidence that turtles can tolerate relatively high acute exposure levels to PCB 126 without dying. Our doses were slightly larger than the 0.025µg/g PCB 126 concentrations in eggs reported by Bishop et al.(1996) , but given that total PCBs can range from 5.9-737µg/g (de Solla et al. 2001) in eggs and Albers et al. (1986) reported a mean of 291.1µg/g in adult lipid stores, we believe they have ecological relevance.

Malaclemys terrapin inhabits estuarine areas along the eastern coast and gulf coast of the United States that include industrialized regions. Meyers-Schone and Walton (1994) note that some turtle species are valuable indicators of environmental contamination. Terrapins are a good candidate for a turtle indicator species because of the following reasons: **1)** Chemical contaminants from both point- and non-point sources impacted estuaries that provide terrapin habitat. Thus, terrapin are exposed to chemicals ranging from heavy metals to organochlorines. **2)** Terrapins exhibit strong site fidelity (Roosenburg et al. 1999) and therefore contaminant tissue loads will better reflect the local toxic burden relative than tissues of a more vagile species. **3)** The terrapins' diet consists of clams, mussels, blue crabs (Tucker et al. 1995) and other filter feeders or opportunistic omnivores with little ability to breakdown PCBs. Thus, terrapins will bioaccumulate toxins from contaminated prey (Kennish and Ruppel 1998). **4)** Large, older, reproductive females can have a high contaminant burden that can transfer to their eggs. Thus, hatchlings from contaminated sites experience exposure to contaminants *in ovo* during critical times of development, such as embryogenesis and sex determination (Willingham and Crews 1999; Bergeron et al. 1994). **5)** Terrapins and other turtles are typically less sensitive to contamination than fish (Hall 1980), thus ecologically relevant levels of contaminants can be monitored over the long-term. **6)** Turtles exhibit low levels of specific enzymes that detoxify contaminants (e.g. CYP1A1) and thus have a limited ability to metabolize toxins (Rie et al. 2000). **7)** Because of decreased sensitivity and a longer life span (up to 40 years), terrapins may be a more relevant indicator of sublethal stressors than certain fish.

Chapter 4: Sublethal Physiological Effects of Multiple Stressor Exposure in Juvenile Diamondback Terrapin, *Malaclemys terrapin*.

Dawn Ford

Introduction

The vast majority of reptilian ecotoxicology studies has examined tissue contamination levels and survivorship, but do not provide evidence of causation even though the need and importance for these studies has been emphasized in the literature (Perkins 1979; Portelli and Bishop 2000). Furthermore, many studies tend to focus on short-term toxicological effects and not long-term sublethal consequences. Furthermore, laboratory studies often examine a single stressor at a time and extrapolate the observed effects to wild populations. This design can generate misleading conclusions because organisms in the field are likely to encounter more than one stressor at any given time. Organisms in industrialized aquatic areas may experience both natural stressors and anthropogenic stressors. Researchers have begun to look at the effects of multiple stressors in efforts to increase ecological realism, applicability of their results, and efficacy of explaining trends at the population level.

The diamondback terrapin *(Malaclemys terrapin)* is the only obligate North American brackish water turtle. Terrapins are long-lived and locally abundant in the Chesapeake Bay, Maryland. These turtles live in industrialized estuaries and experience a number of natural and anthropogenic physiological stressors. Therefore, terrapins are good model organisms for studying long-term sublethal effects of multiple stressors.

One potential physiological stress is salinity. Because of their unique natural history, terrapins experience a range of salinities depending upon their specific habitat and during different life stages. Exposure to high salinity (34ppt) caused mature male terrapins held without access to freshwater to decrease their food intake (Davenport and

Ward 1993). Exposure to high salinities can also cause changes in energetic variables, such as growth and maintenance. Dunson (1985) exposed hatchling terrapins to 5 salinity levels ranging from 0ppt to 35ppt for 33 days and showed high salinities caused a reduction in mass gain with the largest gain in mass at approximately 9ppt. Additionally, adult terrapins kept at 33ppt increased their oxygen consumption (Bentley et al. 1967). Despite osmoregulatory organs for the removal of salt (e.g., salt glands; Schmidt-Nielsen and Fange 1958), salinity stress causes short-term changes in growth and metabolic rate.

Terrapins also encounter a wide variety of man-made environmental contaminants. Polychlorinated biphenyls (PCBs) are persistent, lipophilic toxicants which tend to bioaccumulate and biomagnify in carnivorous organisms (for review, see Safe 1994). PCBs occur throughout much of the terrapin's habitat, including the Chesapeake Bay (Ko and Baker 2004; USEPA 1999). PCB concentrations in wild-caught turtles include 29ppm total PCBs in New Jersey snapping turtle fat tissues (Albers et al. 1986), 334ppb in Adriatic Sea loggerhead sea turtle fat tissues (Corsolini et al. 2000), 2.9ppb of PCB #126 in eggs of some snapping turtle populations in the St. Lawrence River (Bishop et al. 1998), 28,574ppb total PCBs in the lipid fraction of snapping turtle eggs collected in Lake Ontario, Canada (Bishop et al. 1995), and 1494ppb of PCB #126 in Florida loggerhead sea turtle eggs (Alam and Brim 2000). However, a few studies have directly measured PCB effects in turtles through experimental exposure. For example, Willingham and Crews (1999; 2000) experimentally exposed red-eared slider turtle eggs to PCB mixtures and documented effects on sexual development. Additionally, Yawetz et al. (1997, 1998) exposed two species of freshwater turtles to

intraperitoneal injections of PCB 77 and measured the up-regulation of CYP1A, a gene responsible for the biotransformation of toxins. Many studies document PCB exposure and correlate tissue loads with difference in growth (Albers et al., 1986), development (Bishop et al. 1998) and disease (Aguirre et al. 1994), but these correlative studies are unable to attribute the effects directly to PCB exposure because they cannot make the cause and effect link and rule out other ecological variables in these wild-caught animals. In order to develop a cause and effect link and directly attribute the effects to PCB exposure, scientists must experimentally expose laboratory-reared animals to the toxins, keep as many other possible sources of variation (e.g. temperature, diet) constant, and measure the resultant physiological, morphological or biochemical effects of interest.

Separately, salinity and PCBs affect growth, metabolic rate and numerous other physiological variables. However, multiple stressors encountered simultaneously can interact in four ways: 1) the effects can equal the sum of all single stressors (additive), 2) the effects can be greater than the combined sum of the single effects (synergistic), 3) the effects can be less than the combined sum of the single effects (antagonistic), or 4) there are no interactive effects. Multiple stressor effects have not been well documented in reptiles and amphibians, however Hatch and Burton (1998) and Zaga et al. (1998) exposed tadpoles to flouranthene and carbamate respectively in the presence of UV light and documented synergistic deleterious effects in anuran larval physiology and development.

In this study I documented how two stressors (polychlorinated biphenyl-PCB #126 and salinity) affected growth and respiration over a period of six months in the

diamondback terrapin, *Malaclemys terrapin*. Because stressors can cause long-term changes in physiology and ultimately affect life histories, I chose to analyze ecophysiological variables that affect organismal energetics (Dunham et al. 1989; Congdon et al. 2001). If up-regulation of physiological processes necessary to cope with stress is energetically expensive, then stressed terrapins should exhibit higher metabolic rates than unstressed individuals, as reported for other reptiles (Hopkins et al. 1999). If stressed turtles are unable to increase their energy intake to meet these increased physiological demands, then individuals with higher maintenance costs would exhibit decreased growth due to reallocation of energy from growth to maintenance (Hoffman and Parsons 1991; Fair and Ricklefs 2002). Overall, I expected PCB 126 to be a more severe stressor than salinity and cause substantial affects on the ecophysiological parameters of growth and respiration. Furthermore, I expected to observe additive or synergistic deleterious effects when stressors were experienced in combination.

Methods and Materials

Experimental Exposure

In June 2002, I collected twelve clutches of recently laid diamondback terrapin eggs from the shores of the Patuxent River, Chesapeake Bay, Maryland. I transported eggs to Ohio University and incubated them at male-producing temperatures (28°C, Jeyasuria et al. 1994). I individually reared animals for eight months and those exhibiting abnormal growth (i.e. those growing much faster or more slowly than the average turtle) were excluded from study. One hundred four male juvenile turtles were randomized and assigned to one of 8 treatments. The treatments were: 20μg/g PCB 126 and 0μg/g control

with the sub-treatments 30, 20, 10, and 0ppt salt water (Coralife®). There were 13 individuals per treatment based on a power analysis from the previous dose-ranging experiment (Ford, chapter 3). PCB 126 neat was obtained from Ultra Scientific (Rhode Island, USA). Turtles in the PCB treatments received one intraperitoneal (i.p.) injection (27 gauge tuberculin syringe) of 20µg/g PCB 126 dissolved in 1% dimethylsulfoxide (DMSO) delivered with a corn oil vehicle (7% final DMSO concentration). Turtles in the uncontaminated (0µg/g) treatment received a sham injection (no corn oil or DMSO). Based on previous research suggesting terrapins cannot survive long periods in full strength sea water (Dunson 1985), I cycled turtles through the treatment salinities. All turtles spent the first 10 days of a thirty day cycle at an acclimation salinity (10ppt) and then were switched to ecologically relevant test salinities (0, 10, 20 or 30ppt) for the remaining 20 days. I repeated this salinity cycle every thirty days for six months.

For measuring metabolic rate, I divided the turtles into eight random groups with representatives from each of the treatments and staggered the initial start day of the first experimental month. In other words, the group 2 turtles began their first 30 day cycle 2 days after the group 1 turtles, and the group 3 turtles began two days after that, and so on for each of the eight groups. Therefore, all measurements (growth and metabolic rate) and all feedings occurred on specific days of the 30-day cycle and not on specific dates. Turtles were housed individually at 28°C (±2C) in 12.5 cm x 17.5 cm x 6 cm plastic containers in a 12:12 light cycle. Twice a week I fed turtles 2 tablespoons of Kordon® frozen brine shrimp and 24 hours later changed their water completely.

Growth

All one hundred four turtles were measured every 30th day for six months. I measured straight line carapace length with dial calipers to the nearest 0.1mm and determined mass to the nearest 0.1g. I used two repeated measures ANOVAs (SAS Inc. 2001) with treatments as factors and carapace length and mass as the dependent variables to determine differences among the treatment groups. I used a Bonferroni adjustment to decrease the experiment wise error rate (Sokal and Rohlf 1995) and a Tukey-Kramer post hoc test for multiple comparisons.

Standard Metabolic Rate (SMR)

I measured carbon dioxide production in air using a Sable System flow-through respirometer. Metabolic studies of aquatic reptiles are typically conducted in air (e.g. Hopkins et al. 1999; O'Steen and Janzen 1999; Peterson and Stone 2000). Although this procedure may be slightly desiccating, all turtles in this experiment lost less than 5% of their body mass during metabolic determinations regardless of PCB or salinity treatment. Therefore, this procedure did not appear to introduce additional desiccation stress unevenly among the treatments.

Juvenile terrapins were held at 28°C (±2C) and fasted for 7 days prior to SMR determination to ensure metabolic rates did not include metabolic increases associated with digestion, such as specific dynamic action (e.g. Peterson and Stone 2000). All metabolic determinations occurred at the end of the 30 day salinity cycle (i.e. after 20 days of exposure to the test salinity). I patted dry and weighed each turtle and placed it in 100ml glass respirometry chamber in an incubator set to 26°C. Turtles were acclimated to the chamber and allowed to recover from any handling stress for 4 hours prior to SMR

measurement. I only measured carbon dioxide during the quiescent period of scotophase. The respirometry chamber received dry, carbon dioxide-free ambient air and Datacan (Sable Sysytems, Inc.) sampled every chamber individually for fifteen minutes, determining a baseline before and after each sample reading. The flow rate was set to 110ml/minute with a Sierra Sidetrak® flow meter, was continuously monitored by Datacan, and was periodically confirmed using a Gilmont® calibrated flowmeter. I determined total carbon dioxide production over the 15 minute measurement period by integrating carbon dioxide level and adjusting for flow rate. I measured SMRs twice for each individual on days 0, 30, 90, 150 and 180 to calculate mean CO_2 production. A repeated measures ANCOVA (SAS Inc. 2001) with treatments as factors, mean CO_2 production as the dependent variable and body mass as a covariate was used to determine differences among the treatment groups. In all figures, I plotted the least squared means of carbon dioxide which are mathematically adjusted for body size.

In addition to total carbon dioxide production, I measured the number of breaths taken by each individual during the SMR determinations. I analyzed each Datacan file for the number of distinct carbon dioxide spikes for each individual during each measurement period. A spike was defined as one that possessed a well defined peak bounded on each side by two well defined valleys. I eliminated any file containing an indistinct peak from analysis, thus sample sizes were 86 at 0 days, 97 at 30 days, 89 at 90 days, 91 at 150 days, and 92 at 180 days. I defined each peak to equal one release of carbon dioxide. Many turtles are capable of cutaneous gas exchange through the skin, buccopharynx and cloaca (Wood and Lenfant 1976) and thus a carbon dioxide peak may

also represent an extrapulmonary bout. However, because Datacan cannot distinguish between a breath and an extrapulmonary release of carbon dioxide, they were considered together. Thus, some peaks represent carbon dioxide released during a breath whereas other peaks represent extrapulmonary releases. Data were analyzed using a repeated measures ANCOVA with treatments as factors, number of CO_2 peaks as the dependent variable, and body mass as a covariate to determine differences among the treatment groups. I used a Tukey-Kramer post hoc test to evaluate multiple comparisons.

Instead of using a full ANOVA model which considers all possible interactions, I analyzed growth and metabolic data using a reduced model to increase my power and degrees of freedom. The reduced model included only the specific interaction terms in which I was interested. Here, I present the terms of the reduced model and their associated p-values and degrees of freedom (Table 4.1).

Results

Growth

At the onset of the experiment, turtles in all treatment groups were similar in mass (ANOVA $F_{7,88} = 0.22$, $p = 0.98$; some values were missing) and carapace length (ANOVA $F_{7,103} = 0.36$, $p = 0.92$). At the end of six months, all turtles exposed to PCB 126 were smaller in length and mass than uncontaminated individuals. Turtles that remained at the acclimation (and source population) salinity (10ppt) were the largest in mass and length, followed by those cycled at 20ppt and 0ppt. Individuals at 30ppt exhibited further reduced mass and length (Table 4.1). Interestingly, turtles in the uncontaminated treatments exhibited a greater variance in body size among the salinities

than turtles in the PCB 126 treatments. Furthermore, PCB turtles at all salinities other than 10ppt were significantly different in mass and length than uncontaminated turtles at all salinities except 30ppt (Tukey-Kramer $q = 4.38$ $p < 0.05$). Over the course of the experiment, there was a PCB 126 by salinity by time interaction, but there was no significant interaction between PCB 126 and salinity (Table 4.1). These data suggest that although experiencing cycles of near full strength sea water (30ppt) is stressful, PCB 126 is a more severe stressor than the ecologically relevant levels tested in this study (Figure 4.1 and 4.2).

Standard Metabolic Rate

Salinity had no effect on mass-adjusted standard metabolic rate (Table 4.1). However, exposure to PCB 126 caused a significant reduction in the amount of carbon dioxide produced (Table 4.1), with differences becoming evident at 90 days after exposure (Figure 4.3 and 4.4). The decrease in metabolic rate was associated with a change in breathing behavior. At day zero, all individuals took equal number of breaths during the 15 minute measurement period, as shown by the equal number of carbon dioxide exhalations (Figures 4.5 and 4.6). Turtles in higher salinities increased the frequency of their peaks and turtles held at 0ppt produced the fewest number of peaks (Table 4.1, Figure 4.5) compared with those at 20 and 30ppt (Tukey-Kramer $q = 3.69$, $p < 0.05$). PCB treated turtles also took more breaths than uncontaminated turtles (Figure 4.6), but there was no interaction between PCB 126 and salinity (Table 4.1).

Discussion

In this study, I exposed estuarine diamondback terrapins from the Chesapeake Bay to ecologically relevant levels of stress from PCB 126 and salinity. Exposure to PCB 126 decreased growth, decreased metabolic rate (carbon dioxide production), and increased the number of breaths as determined by carbon dioxide peaks (Figures 4.1-4.6). High and low salinity also acted as stressors, but only caused modest growth lags. Interactive stressor effects have been shown in amphibians (e.g. Long et al. 1995; Hatch and Blaustein 2000; Zaga et al. 1998), however, I observed no interactive effects of PCB 126 and salinity in terrapins. Data suggest that PCB 126 is a more severe stressor than salinity.

Growth

PCBs inhibit growth in many vertebrates including sea gulls (Gilbertson 1983), chickens (Gould et al. 1997) and mink (Wren et al. 1987). Some field evidence suggests that PCBs may inhibit growth in snapping turtles (Bishop et al. 1994). However, in the latter study, investigators were unable to determine if PCB exposure caused the observed decline in body size or if the observation was merely correlative and caused by other ecological variables. In this study, individuals exposed to PCB 126 in the lab exhibited significantly reduced growth compared to sham injected turtles from the same population, of the same age and held under equivalent conditions (Figure 4.1 and 4.2). To our knowledge, this is the strongest evidence to date to show that PCB 126 exposure significantly reduced growth in reptiles. Furthermore, I did not detect significant growth effects until 60-90 days after PCB exposure. Typical short-term laboratory trials which follow animals for a period of a few weeks would end well before the effects became

apparent. Thus, sublethal studies of turtles and other reptiles must increase the duration of experiments or risk grossly underestimating the impacts of contaminants on important life history and population parameters.

Salinity level also caused a decrease in growth. Dunson (1985) reported lethality in terrapins continuously exposed to full strength sea water, but the cycling of experimental treatments in this study was more ecologically relevant. Cycling from 10ppt to the treatment test salinities increased ecological realism because terrapins inhabit coastal areas that are subject to fluctuations in salinity corresponding to the tidal cycle and weather patterns. At each experimental measurement period, turtles that remained at 10ppt (the acclimation salinity) attained the largest size. The similar response of turtles at 0ppt or 20ppt suggested that these animals have a near equal ability to compensate for a 10ppt increase or decrease from 10ppt (Figure 4.1 and 4.2). Turtles exposed to 30ppt exhibited lower growth but by 120 days these high salinity turtles began to exhibit a growth rate (slope, Figure 4.1) similar to the uncontaminated turtles. Differences in growth due to salinity exposure were not evident until at least 60 days after exposure. Thus, short-term laboratory studies would underestimate the effects of high and low salinities on turtle growth. Therefore, irrespective of the stressor, researchers must increase toe duration of short-term experiments in order to completely understand the extent of sublethal effects of stress. Yet, individuals with an initially slower growth rate may not be able to reach a body size equivalent to those which exhibited faster, sustained growth rates. Smaller animals may achieve comparable adult body sizes if they sustain

growth for longer periods of time at the expense of other activities (i.e. reproduction) or by increase growth rate at later time intervals (Congdon et al. 1982; Wieser 1994).

Standard metabolic rate

Physiological mechanisms to cope with stressors may be energetically expensive and can lead to a measurable increase in metabolic rate. Hopkins et al. (1999) have shown that contaminant-exposed reptiles exhibited higher metabolic rates (oxygen consumption) than those from reference populations. Similarly, I hypothesized salinity and/or PCB 126 exposure would be physiologically stressful and thus increase terrapin metabolic rate. Early in the experiment, all individuals exhibited similar SMRs. By 90 days after exposure, there were clear differences in carbon dioxide production in uncontaminated turtles versus the PCB-exposed individuals (Figure 4.4). The observed metabolic pattern suggests PCB 126 exposure causes a reduction in mass-specific metabolic rate, not an increase as hypothesized. In this study, I measured carbon dioxide production during scotophase during which time metabolic rates tend to be lower, in some cases by as much as 50% (Bennett and Dawson 1976), thus a further decrease in metabolic rate during scotophase can have measurable effects on energetics. On the one hand, a depressed metabolic rate during scotophase would enable PCB-stressed turtles to conserve energy for future activities whereas uncontaminated individuals have lower overall energy demands and can thus maintain higher metabolic rates during this time period. On the other hand, a depressed metabolic rate could be a symptom of overall illness, thus not an adaptive response. This design of this study does not enable me to distinguish the mechanism by which turtles are decreasing their metabolic rates, but perhaps the latter

hypothesis of illness is suggested due to mortality of turtles exposed to other PCB 126 doses (Ford, chapter 3).

Even though salinity had a significant effect on growth, I saw no effect of salinity on metabolic rate. This pattern implies that there is not a direct link between the energy used for growth and the energy used for maintenance. If an organism increased its metabolic rate to combat toxins, energy resources may not need to be reallocated from growth. Therefore, perhaps metabolic rates are conserved and not as flexible as other energetic parameters (i.e. growth) or more perhaps it is more likely that increases in metabolic costs are transient and do not always substantially impact growth.

Although measures of both oxygen consumption and carbon dioxide production are used as surrogates of metabolic rate, carbon dioxide production may not be as accurate an indicator as oxygen consumption (McDonald 1976). Turtles, and most other vertebrates, store carbon dioxide in body tissues at much higher concentrations than they store oxygen, in the form of bicarbonate ions in the extracellular fluid. Furthermore, aquatic turtles often periodically hold their breath whether in or out of water (pers. obs; Seymour 1982). In aquatic species with long breath-holding (apneic) capabilities, carbon dioxide accumulates in blood (Jackson et al. 1976). Carbon dioxide is eliminated by diffusion back into the lung or via extrapulmonary pathways, such as through the integument or cloaca. The more aquatic the species, the more CO_2 is lost extrapulmonarily, with an average 21.2% cutaneous loss (Jackson et al. 1976). Thus over a short period of time, the elimination of CO_2 can be a less reliable indicator of metabolic rate than oxygen consumption (Randall et al. 2002). However, over longer time measurements, carbon

dioxide measurements are more accurate because the signal to noise ratio is much smaller than it is for oxygen. In flow-through respirometry the incurrent air is scrubbed of carbon dioxide, thus the study animal produced all the carbon dioxide detected in the metabolic chamber and even very small fluctuations in carbon dioxide production are detectable. If metabolic rate is determined by oxygen consumption, researchers measure the amount of oxygen depleted from the container based on how much oxygen was present at the start of the experiment. Because of the physiological difference between oxygen consumption and carbon dioxide production, the rate of oxygen exchange may not parallel the rate of CO_2 production making comparisons among studies using these varied metabolic measures increasingly complex. Although, measurements of SMR in fasted individuals during scotophase are not ecologically as realistic as measuring activity metabolic rates in fed reptiles (Niewiarowski and Waldschmidt 1992).

Not only did PCB affect carbon dioxide production, but I also observed significant differences in ventilation frequency (carbon dioxide peaks). Yet, it is unclear whether ventilation was via alveoli or extrapulmonary elimination, it was higher in PCB-stressed individuals that had lower standard metabolic rates. Thus, although ventilation frequency increased with PCB exposure, the amount of carbon dioxide released with each ventilation must have been smaller. Faster, shallower breathing is typical of stressed organisms, including reptiles that are attempting to maximize respiratory intake while minimizing energy expenditure (Perry 1998). Although salinity did not affect SMR, treatments did affect the ventilatory pattern. Turtles held at 0ppt ventilated at a lower

frequency than those at higher salinities. Thus, fewer carbon dioxide releases produced the same overall amount of carbon dioxide eliminated over a given period of time.

At the end of the 6 month experiment, I dissected all PCB turtles and a fraction of the uncontaminated individuals to confirm sex and collect tissue samples for additional analyses. During dissection, the PCB turtles seemed to have decreased muscle tone with muscle fibers appearing gray in color and separating easily from bony elements. It is possible the decreased muscle tone played a role in altering respiratory patterns and turtles may have been physically unable to increase their tidal volume.

Further considerations

Turtles exposed to PCB 126 decreased growth and metabolic rate. PCB 126 exposure may lead to a growth reduction via a decrease in energy acquisition, damage to the digestive system, or an alteration of energy allocation patterns. Rats exposed to dioxin exhibited reduced body mass and a reduction in food intake (Seefeld et al. 1984; Seefeld and Peterson 1984). In previous studies I have shown turtles exposed to PCB 126 consume the same amount of food as control animals (Ford, chapter 3). Therefore, it is unlikely that the observed growth effect is a function of a disparity in energy ingested. Although we did not see a refusal or decrease in food ingested, PCB-exposed turtles may exhibit reduced digestive processing ability, lowered assimilation efficiency or other gastrointestinal abnormalities. Wasting syndrome and reduced growth in TCDD-exposed fishes may be a function of a reduced ability to absorb and mobilize nutrients due to edema compromising blood flow (Spitsbergen et al. 1991). If edema affects throughput time or intestinal uptake, digestive efficiency may be affected (Sibly 1981).

Furthermore, exposure to PCB 126 decreases intestinal cholecystokinin (Lee et al. 2000), a hormone regulating gall bladder and pancreatic function (Randall et al. 2002). PCB 126 exposed turtles may have reduced growth due to constricted or damaged intestinal vasculature, increased gut throughput time, or altered digestive efficiency due to decreased hormone levels. The presence of any of these symptoms could lead to the common effect of reduced growth. To better understand the energetics of this stress response, it would be useful to compare the assimilation efficiency of PCB exposed turtles to uncontaminated individuals.

Another consequence of PCB exposure is vitamin A deficiency. In herring gulls and terns, as PCB levels increased, the plasma levels of retinol decreased (Grasman et al. 1996). High organochlorine levels in box turtles (*Terrapene carolina*) have been correlated with low plasma and liver vitamin A (Holladay et al. 2001). Vitamin A is essential for healthy skin, thus a decrease in vitamin A could lead to skin sloughing. Approximately three months after PCB 126 exposure, contaminated turtles began to slough large amounts of skin, a condition also seen in an earlier dose-ranging experiment (Ford, chapter 3) and in oiled sea turtles (Lutcavage et al. 1995). This response is interesting for a number of reasons. First, PCBs can accumulate in many tissues, including skin (Huang and Karasov 2000). Edema caused by PCB exposure can compromise vasculature (Spitsbergen et al. 1991). If interstitial fluid collects in tissues, peripheral blood flow may become restricted causing skin cells to die and slough off (Karasov pers. com).

Finally, it is well documented that endocrine disrupting chemicals, such as PCBs, disrupt thyroid gland function (for review see Leatherland 2002). Thyroid hormones are involved in regulating metabolism, activity, growth, temperament, skin production and shedding (Randall et al. 2002). Froglets orally dosed with PCBs tended to produce less thyroxine (Gutleb et al. 2000), whereas thyroxine levels in alligators from contaminated areas were either normal or elevated based on location (Hewitt et al. 2002). Increased thyroxine levels generally increase metabolic rate (John-Alder 1990). However, in this study, PCB exposure actually decreased metabolic rate. Because of numerous physiological effects of thyroid hormones, future energetics studies of PCB stress should include measures of thyroxine and thyroid function.

Conclusions

With decreases in habitat availability and large scale ecological changes (e.g. global warming), species may be forced to colonize areas once normally avoided. Additionally, further industrialization, continuing manufacture of PCBs in other countries, and aging disposal sites will lead to an increase in the ubiquitous and persistent presence of organochlorines in our environment. Estuarine turtles that inhabit these impacted areas will physiologically have to overcome the additional stressors that these changes are likely to impose. Here, I have shown that exposure to salinities other than the acclimation salinity led to a decrease in growth and change in respiratory pattern, but not a change in metabolic rate. However, effects of PCBs were more severe, decreasing growth and metabolic rate and causing turtles to employ a respiratory pattern

characteristic of severe stress (Perry 1998). The effects of PCB 126 and salinity in the slow-growing diamondback terrapin only became apparent at 60 to 90 days after exposure. A study of shorter duration would have completely missed any effects of either stressor. To better understand the effects of pollutants on species, we must carry out laboratory experiments for sufficient duration to see important sublethal effects. In addition, experiments must provide causative, not simply correlative, evidence of the disruption of the ecophysiological mechanisms that govern important life history strategies and affect population structure.

Chapter 5: Environmental Stressors Adversely Affect Organ Size and Other Physiological Traits

Dawn Ford and Willem Roosenburg

Introduction

Traditionally, ecotoxicological studies document the effects of single contaminant stressors and extrapolate the observed effects to wild populations. However, field populations are likely to encounter multiple stressors at any one time. Organisms that inhabit industrialized estuaries and aquatic environments encounter multiple natural stressors (e.g. pH, salinity) in addition to anthropogenic stressors (e.g. thermal, and chemical pollution). Researchers must look at the effects of simultaneous multiple stressors to increase ecological realism and field applicability of ecotoxicological studies. It is crutial for studies to integrate natural stressors into models of anthropogenic pollutant effects.

The diamondback terrapin *(Malaclemys terrapin)*, a North American turtle found exclusively in brackish water, inhabits many industrialized areas along the eastern United States. Many terrapin habitats are tidal creeks that experience large fluctuations in salinity with the ebb and advance of tides. Although terrapins possess osmoregulatory organs for the removal of salt (e.g., lacrimal salt glands; Schmidt-Nielsen and Fange 1958), salinity can still be a stressor. Terrapins exposed to high salinities grew slowly (Ford chapters 3 and 4; Dunson 1985), have decreased appetites (Davenport and Ward 1993) and altered metabolic patterns (increased oxygen consumption, Bentley et al. 1967; decreased carbon dioxide production, Ford chapter 4) when compared to those held at lower salinities.

Many industrialized estuaries contain polychlorinated biphenyls (PCBs) and other persistent synthetic organochlorines (e.g. Ashley et al. 2003) in sediments and prey items.

PCBs occur on all continents in soil, water, ice, and biological tissues (Montone et al. 2003 and references therein). These stable, lipophilic contaminants tend to bioaccumulate in higher trophic level consumers (Safe 1994) including turtles (Portelli and Bishop 2000). PCBs occur throughout much of the terrapin's habitat, including the Chesapeake Bay (Ko and Baker 2004; USEPA 1999). PCB concentrations in wild-caught turtles include 29ppm total PCBs in New Jersey snapping turtle fat tissues (Albers et al. 1986), 334ppb in Adriatic Sea loggerhead sea turtle fat tissues (Corsolini et al. 2000), 2.9ppb of PCB #126 in eggs of some snapping turtle populations in the St. Lawrence River (Bishop et al. 1998), 28,574ppb total PCBs in the lipid fraction of snapping turtle eggs collected in Lake Ontario, Canada (Bishop et al. 1995), and 1494ppb of PCB #126 in Florida loggerhead sea turtle eggs (Alam and Brim 2000). However, a few studies have directly measured PCB effects in turtles through experimental exposure. For example, Willingham and Crews (1999; 2000) experimentally exposed red-eared slider turtle eggs to PCB mixtures and documented effects on sexual development. Additionally, Yawetz et al. (1997, 1998) exposed two species of freshwater turtles to intraperitoneal injections of PCB 77 and measured the up-regulation of CYP1A, a gene responsible for the biotransformation of toxins. Many studies document PCB exposure and correlate tissue loads with difference in growth (Albers et al., 1986), development (Bishop et al. 1998) and disease (Aguirre et al. 1994), but these correlative studies are unable to attribute the effects directly to PCB exposure because they cannot make the cause and effect link and rule out other ecological variables in these wild-caught animals. In order to develop a cause and effect link and directly attribute the effects to PCB

exposure, scientists must experimentally expose laboratory-reared animals to the toxins, keep as many other possible sources of variation (e.g. temperature, diet) constant, and measure the resultant physiological, morphological or biochemical effects of interest.

Animals exposed to chronic stressors can behaviorally avoid, tolerate or make physiological adjustments in efforts to respond to stress (Calow 1991). Individuals unable to evade or tolerate a specific stressor may attempt to regain homeostasis by shunting energy from tissues of low demand to those of higher demand (Sapolsky 1992). With these changes in energy allocation, we may see changes in physiological and morphological properties. Rats exposed to chronic stress had increased blood volume (Elliott et al. 2003) thus increasing the ability or nutrient and waste products to move to and from sites of high demand. Additionally, rats exposed to PCB 126 altered organ sizes (e.g. Chu et al. 1994) possibly as a mechanism of energy reallocation.

Here, I hypothesize multiply stressed terrapins will exhibit a size reduction in those organs not essential to the stress response, while those essential to detoxification processes will undergo hypertrophy. Additionally, Sapolsky (2002) notes that during the stress response, vasopressin causes increased blood volume through blood water retention, thus I expected stressed terrapins to have lower hematocrit. In this study, I present data documenting how two stressors (PCB 126 and salinity) affect these physiological and morphological parameters in the diamondback terrapin.

Methods

Experimental Exposure

In June 2002, I collected twelve clutches of recently laid diamondback terrapin eggs from the shores of the Patuxent River, Chesapeake Bay, Maryland. I transported eggs to Ohio University and incubated them at male-producing temperatures (28C, Jeyasuria et al. 1994). I individually reared animals for eight months and those exhibiting abnormal growth (i.e. those growing much faster or more slowly than the average turtle) were excluded from study. One hundred four male juvenile turtles were randomized and assigned to one of 8 treatments. The treatments were: 20µg/g PCB 126 and 0µg/g control with the sub-treatments 30, 20, 10, and 0ppt salt water (Coralife®). There were 13 individuals per treatment based on a power analysis from the previous dose-ranging experiment (Ford, chapter 3). PCB 126 neat was obtained from Ultra Scientific (Rhode Island, USA). Turtles in the PCB treatments received one intraperitoneal (i.p.) injection (27 gauge tuberculin syringe) of 20µg/g PCB 126 dissolved in 1% dimethylsulfoxide (DMSO) delivered with a corn oil vehicle (7% final DMSO concentration). Turtles in the uncontaminated (0µg/g) treatment received a sham injection (no corn oil or DMSO). Based on previous research suggesting terrapins cannot survive long periods in full strength sea water (Dunson 1985), I cycled turtles through the treatment salinities. All turtles spent the first 10 days of a thirty day cycle at an acclimation salinity (10ppt) and then were switched to ecologically relevant test salinities (0, 10, 20 or 30ppt) for the remaining 20 days. I repeated this salinity cycle every thirty days for six months. Turtles were housed individually at 28°C (±2C) in 12.5 cm x 17.5 cm x 6 cm plastic containers in a 12:12 light cycle. Twice a week I fed turtles 2 tablespoons of Kordon® frozen brine shrimp and 24 hours later changed their water completely.

At six months after exposure, I euthanized (by decapitation) a subset of individuals (n = 39) representing each of the eight treatment groups. I dissected turtles to confirm the presence of testes (based on egg incubation at male-producing temperatures) and removed the liver, heart, testes and kidneys (urogenital), and gastrointestinal tract (GI, esophagus to rectum). Hearts, GIs and the carcasses were oven-dried at 55°C to constant mass. Livers and urogenital dissections were not oven dried due to the need to use these organs in future analyses, thus I recorded wet masses instead. I analyzed GI dry mass using an ANCOVA with carcass dry mass as a covariate. Because there was no significant effect of body size (heart: $F_{1,38} = 1.66$, $p = 0.21$; liver: $F_{1,38} = 0.09$, $p = 0.76$; urogenital: $F_{1,38} = 1.62$, $p = 0.21$) on the other morphological parameters, I analyzed heart dry mass and liver and urogenital wet mass data using an ANOVA. To determine differences in liver mass among the groups, I used a Tukey-Kramer post-hoc test. I used NCSS for all statistical analyses (Hintz 2004).

Immediately after decapitation of each turtle, I collected a small blood sample in a heparinized capillary tube for hematocrit determination. Capillary tubes were spun in a microhematocrit Readacrit™ centrifuge at 8,000rpm for five minutes. Hematocrit was determined from a direct reading scale. I used an ANOVA to determine if there were differences in hematocrit among the treatment groups (Hintz 2004).

Results

Generally, individuals exposed to high salinities had smaller livers (Table 5.1). Turtles that remained at 10ppt (acclimation salinity) had greater liver masses than those cycled at 30ppt (Tukey-Kramer $q = 3.79$, $p < 0.05$). In terms of contaminant exposure,

PCB turtles at 0 and 10ppt had larger livers than uncontaminated turtles at the same salinities. However, PCB turtles at 20 and 30ppt had reduced liver mass as compared to uncontaminated individuals at the same salinity (Figure 5.1). Thus, overall there was no effect of PCB on liver mass and the interaction between PCB and salinity was marginally insignificant (Table 5.1). Neither PCB exposure nor salinity affected urogenital mass (Table 5.1).

In contrast, PCB exposure decreased GI mass and heart mass, but there was no effect of salinity (Figure 5.2 and 5.3). Similarly, carcass dry mass was lower in PCB exposed animals, but was not affected by salinity and there was no interaction between the two stressors (Table 5.2). Hematocrit was lower in PCB exposed animals, but was not affected by salinity (Table 5.2, Figure 5.4). There were no significant interactions between PCB and salinity for any measured variable.

Discussion

Physiological responses of exposure to stress may incur an energetic cost and thus stressed individuals may need to make physiological and morphological adjustments to maintain energy balance. If maintenance costs are elevated because of energy demands associated with toxin depuration, then stressed animals may not be able to maintain overall body size. Here, animals exposed to PCBs were smaller than control individuals in both wet mass (Ford chapter 4) and dry mass. Similarly, stressed animals might not be able to maintain all organ tissues. Therefore, decreased organ mass might be a toxic response or an adaptive response to decrease the size of organs with high metabolic activities that are energetically expensive to maintain. Turtles exposed to PCB 126

underwent a number of physiological changes including reduced growth and carbon dioxide production (Ford chapter 4) as well as reduced GI, heart and carcass mass. Decreased GI mass leads to changes in energy and nutrient assimilation efficiency via differences in throughput time or digestive mixing patterns (Stevens and Hume 1995). However, large snakes decrease GI size but quickly up-regulate the gut in the presence of a meal (Secor and Diamond 1995) so it is possible that decreased gut function does not lead to overall changes in energy assimilation if the gut is quickly up-regulated when the stress diminishes. Although, terrapins, like many other aquatic turtles, may not have the ability to efficiently up-regulate the gut after a stress (Secor and Diamond 1999) and thus a smaller gut size may not be a strategy for energy conservation, but rather a necessity due to energy imbalance or simply a toxicological response to PCB exposure.

Organs involved in detoxification and waste removal will likely be maintained or under some circumstances undergo hypertrophy. Chu et al. (1994, 1998) have shown that PCB exposure increased liver and kidney dry mass in rats. In turtles, neither PCB nor salinity stress affected urogenital mass and only salinity affected liver mass. The reduced liver mass in salinity stressed individuals may be a function of water content as opposed to a decrease in other tissue components. Comparisons of liver and urogenital dry masses are more informative because both salinity stress and PCB exposure (e.g. Rosenshield et al. 1999) can alter cellular water levels. Interestingly, PCB exposure alone did not affect liver mass, but there may be an interactive affect of the multiple stressors. Although the effect observed here is not statistically significant (Table 5.1, $p = 0.06$), low salinity, PCB turtles tended to have larger livers, but high salinity, PCB turtles had smaller livers than

uncontaminated turtles. It is unclear as to what mechanism might explain this pattern and what affect larger samples sizes would have on the results.

The observed pattern of decreased mass in these large, metabolically active tissues is consistent with the decrease in standard metabolic rate reported elsewhere (Ford chapter 4) because metabolic rate is often highly correlated with liver and kidney masses (Daan et al. 1990) and during stress these organs may be important in energy conservation (Lopez-Calleja and Bozinovic 2003).

Contrary to previously published research on rats (Lind et al. 2004; Chu et al. 1994), PCB stressed turtles had smaller hearts than controls (Figure 5.2). A decrease in heart mass has far reaching physiological consequences including affecting rates of nutrients and waste product transport.

Previous research has shown that turtles exposed to high salinities exhibited higher hematocrit, although not during the summer months (Gilles-Baillien 1973). In this study, I found no affect of salinity on hematocrit, a pattern consistent with Gilles-Baillien (1973) because these laboratory animals experienced near-summer conditions. PCB exposure also reduced hematocrit, consistent with previous research on the effects of crude oil on loggerhead sea turtles (Lutcavage et al. 1995). Consequently, PCB stressed individuals with lower hematocrit may be unable to transport high concentrations of metabolic waste products. However, animals with decreased hematocrit also exhibited decreased carbon dioxide production (Ford chapter 4). Even though stressed individuals may have a more limited ability to transport carbon dioxide, they are also producing lower quantities of this metabolic waste.

Stressed animals mobilize all forms of stored energy, including that stored as protein (Sapolsky et al. 2002). The primary site of protein storage is muscle tissue. Thus, the continuous mobilization of stored protein coupled with the effect of glucocorticoids on muscle fibers can lead to myopathy, general muscle fatigue and weakness (Oshima et al. 2004; Sapolsky 1992). During dissections, I noticed that in a majority of the PCB exposed turtles the skeletal elements of the limb girdles easily dissociated from the overlying muscle tissue. Although I did not quantify muscle tone, PCB exposed animals appeared to be experiencing myopathy.

While energetic constraint is a possible explanation for the reduction in organ size, there are also other mechanisms which may account for a similar pattern. Polychlorinated biphenyls may act directly on tissues. For example, Hennig et al. (1999) showed that exposure to co-planar PCB 77 caused dysfunction of vascular endothelial cells and cellular oxidative stress. PCB 126 is also a coplanar PCB congener and thus may elicit similar effects at the cellular level in both vascular tissues and perhaps in all endothelial tissues. Additionally, PCB 126 may directly cause cell death, thus reducing the organ size through necrosis. However, PCB-induced cellular death is not attributable to exposure to coplanar PCBs, such as PCB 126, but rather to other ortho-substituted congeners (Tan et al. 2004a, 2004b). PCB exposure can also reduce organ size through other indirect means not mediated by energetic tradeoffs. For example, wasting syndrome and reduced body size in TCDD-exposed fishes may be a function of a reduced ability to absorb and mobilize nutrients due to edema compromising blood flow (Spitsbergen et al. 1991). If edema effects blood flow, specific tissues may not be

receiving adequate blood supply which could lead to cellular death and thus reduced organ size. Future studies should investigate the specific mechanisms responsible for reduced organ size in PCB-exposed animals.

Terrapins and other organisms inhabiting industrialized estuarine areas experience stress from exposure to multiple biotic and abiotic factors. Some stressors may interact to exacerbate effects, however in this study, terrapins were under extreme PCB 126 stress and the additional salinity stress had little or no overall effect. Previous studies have shown PCB exposure decreases growth and standard metabolic rate and can lead to increased skin sloughing (Ford chapter 4). Here, I have added to list of PCB effects and shown PCB exposure to reduce hematocrit and affect organ mass. Future studies of PCB 126 stress should investigate whether the observed patterns are mechanisms to manage stress-induced energy imbalance. Furthermore, if PCB-stressed, juvenile turtles are generally smaller, myopathic and exhibit other energetic-associated changes, they may also exhibit reduced activity and running performance which are important parameters associated with fitness (Janzen 1993). Thus, PCB stress may ultimately reduce hatchling fitness in many ways.

Chapter 6: Plasma Corticosterone Response to Variation in Salinity and Exposure to PCB 126 in Diamondback Terrapins, *Malaclemys terrapin*

Dawn Ford, Mathew Milnes and Willem Roosenburg

Introduction

Polychlorinated biphenyls and other persistent environmental pollutants can disrupt an organism's homeostasis and are just a few of many chemical, thermal and physical anthropogenic stressors (Beyers, et al. 1999) that trigger a broad scale physiological response. In many situations, this stress response can be adaptive (e.g. organisms inhabiting fluctuating environments; Hoffman and Parsons 1991) and has the ultimate goal of restoring homeostasis.

There are numerous physiological responses to stress including changes in metabolic rate and respiratory pattern (e.g. Ford chapter 4; Hopkins et al. 1999), growth (Ford chapters 3 and 4), immune function (e.g. Svesson et al. 1998; Guillette et al. 1995) and hormone production (Mahmoud et al. 1989). One of the major endocrinological features of the stress response is the hypothalamus-pituitary-interrenal (HPI) axis cascade. Briefly, in the presence of a stressor, the hypothalamus stimulates the pituitary to release adrenocorticotropic hormone (ACTH) which triggers the interrenal cells of the adrenal to release a glucocorticoid stress hormone (Sapolsky 2002; Guillette et al. 1995; Axelrod and Reisine 1984). Corticosterone (CORT), the stress hormone released in reptiles (Greenburg and Wingfield 1987), is a common physiological index of stress (Dunlap and Wingfield 1995) that increases in response to a variety of natural (e.g. density of conspecifics; Comendant et al. 2003) and anthropogenic stressors (e.g. environmental contaminants; Gunderson et al. 2003).

The majority of chelonian stress studies have focused on measuring stress sensitivity through increases in plasma CORT induced by handling or captivity in sea

turtles (e.g. Gregory et al. 1996; Gregory and Schmid 2001) or red-eared sliders (Cash et al. 1997; Cash and Holberton 1999). Perhaps only a single study has measured chelonian CORT levels under natural stress, such as during times of mass nesting (Valverde et al. 1999). However, many turtle species regularly confront stress during reproductive bouts (Valverde et al. 1999) and through their residence in potentially stressful habitats such as cold North American winters (e.g. *Chrysemys picta*, Costanzo et al. 2004) or industrialized estuaries (e.g. *Malaclemys terrapin*, Kannan et al. 1998).

In this study, I investigate the effects of the natural stressor, salinity, and an anthropogenic stressor, 3,3',4,4'5-pentachlorobiphenyl (PCB 126), on plasma corticosterone levels in the diamondback terrapin, *Malaclemys terrapin*. I expected that controlled laboratory exposure of terrapins to ecologically relevant levels of one of the most potent PCBs would trigger the HPI axis and elicit an increase in plasma CORT. Similarly, I expect large changes in salinity levels to induce a stress response. These data will provide the first measurements of corticosterone in the terrapin as well as provide insight into how turtles might cope with multiple stressors including persistent organic pollutants.

Methods and Materials

In June 2002, I collected fifteen clutches of recently laid *Malaclemys terrapin* eggs from the shores of the Patuxent River, Chesapeake Bay, Maryland. Eggs were brought to Ohio University and incubated at male-producing temperatures in Percival BLL 30 incubators (28°C, Jeyasuria et al. 1994). I reared hatchlings for eight months prior to experimentation. PCB 126 neat was obtained from Ultra Scientific (Rhode Island, USA).

Turtles in the PCB treatment received one intraperitoneal (i.p.) injection (27 gauge tuberculin syringe) of 20µg/g PCB 126 dissolved in 1% dimethylsulfoxide (DMSO) delivered with a corn oil vehicle (7% final DMSO concentration). Turtles in the uncontaminated (0µg/g) treatment received a sham injection (no corn oil or DMSO). Based on previous research suggesting terrapins cannot survive long periods in full strength sea water (Dunson 1985), I cycled turtles through the treatment salinities. All turtles spent the first 10 days of a thirty day cycle at an acclimation salinity (10ppt) and then were switched to ecologically relevant test salinities (0, 10, 20 or 30ppt Coralife®) for the remaining 20 days. I repeated this salinity cycle every thirty days for six months. Turtles were housed individually at 28°C (±2C) in 12.5 cm x 17.5 cm x 6 cm plastic containers in a 12:12 light cycle. Twice a week I fed turtles 2 tablespoons of Kordon® frozen brine shrimp and 24 hours later changed their water completely.

At six months after PCB exposure (14 months old and the end of the sixth salinity cycle), I euthanized thirty-six individuals (18 PCB and 18 unexposed). In efforts to minimize the effect of handling stress on glucocorticoid production, the same individual (WMR) decapitated each turtle in less than ten seconds. Immediately following decapitation, I collected 400-600µl of blood from each turtle, centrifuged samples at 6000 rpm for 10 minutes, collected the plasma, and stored each sample at -10C until assayed for corticosterone concentration in the Guillette lab at the University of Florida.

I analyzed terrapin plasma samples for CORT using a standard enzyme immunoassay kit (900-097, Assay Designs, Inc.). I extracted plasma samples twice with 5ml diethyl ether, flash froze the lower aqueous phase in dry ice, super-cooled methanol,

and poured off the upper organic phase. Each sample was dried down with air, resuspended in 250µl of buffer #15, and assayed in duplicate. I used 200µl of each sample per assay and simultaneously ran 5 serially diluted standards, two extraction efficiencies, other blanks and controls (e.g. total activity, non-specific binding) in duplicate on a single 96 well plate. I incubated and lightly shook (500rpm) the plate for two hours then washed the wells in triplicate with 400µl of wash solution and aspirated. I added 200µl of substrate to each well and incubated the plate at room temperature for one hour. After the reaction was stopped, I used a microplate reader (optical density set to 405nm) to determine absorbance. I used a four parameter logistic curve fit in calculating the standard curve ($Y = -14.249\text{Ln}(X) + 155.99$, $R^2 = 0.98$). The minimum detectable concentration was 48pg/ml. To determine if PCB or salinity stress caused an increase in CORT, I statistically analyzed $\log_{10}$ transformed mean CORT concentrations (pg/ml) using an ANCOVA to test for a covariate relationship between body size and CORT. Because there was no significant effect of body size ($F_{1,35} = 1.16$, $p = 0.29$), I reanalyzed all data using an ANOVA (SAS Inc. 2001) with PCB exposure and salinity level as the dependent variables.

Results

Plasma CORT values ranged from 85.14-1246.54 pg/ml in unexposed turtles and 78.0-1349.25 pg/ml in turtles exposed to PCB 126. PCB exposure was not associated with an increase in terrapin CORT ($F_{1,35} = 0.07$, $p = 0.79$). Similarly, there were no differences in CORT among the four salinity levels ($F_{3,35} = 1.38$, $p = 0.27$) and no interaction between PCB exposure and salinity ($F_{3,35} = 0.20$, $p = 0.89$). Interestingly, all

turtles held at 20ppt, irrespective of PCB exposure status, tended to have slightly lower CORT levels than all other groups (Figure 6.1) including those that experienced no fluctuations in salinity (i.e. they remained at 10ppt for the entire experimental period).

Discussion

I did not detect differences in corticosterone attributable to either PCB exposure or salinity. Typically, during a stress response to an acute stressor, CORT increases upon perception of the stress, continues to increase for some period of time, peaks or plateaus and eventually declines back to baseline after removal of the stress. Plasma corticosterone levels typically return to baseline within hours after the acute stress has been removed (Paris et al. 1987). If PCB causes physiologically acute stress at the time of exposure, rather than as a chronic stress due to tissue burden, then CORT levels six months later, when I tested, likely had sufficient time to return to baseline. Thus, the lack of CORT differences among treatments may be a consequence of the time frame of this study and not due to the lack of a response. It is also possible, that PCB exposure never elicited an increase in plasma CORT in terrapins. Love et al. (2003) have shown that in American kestrels, exposure to PCB mixtures actually reduces CORT perhaps through a PCB-induced disruption of the HPI axis. The corticosterone stress response is an adaptive mechanism to restore homeostatis and its possible suppression by PCB exposure is an extremely important environmental concern that warrants further investigation (Love et al. 2003). However, without a time series of plasma CORT levels, the effect of PCBs on terrapin CORT profiles will remain unclear.

In addition to stressors, many other non-stress-related factors affect CORT. Other variables shown to affect CORT have included size (Gregory et al. 1996) and sex (Valverde et al. 1999). Gregory et al. (1996) hypothesized the size-dependent difference in *Lepidochelys kempi* CORT levels likely were due to differences in reproductive condition, but Gregory and Schmid (2001) suggested metabolic rate may also be a contributing factor. In this study, I controlled for sex (all males), age (reproductive condition), and ambient temperature, thus removing the influence of any of these variables on CORT levels. Despite the finding that PCB-exposed animals exhibited reduced metabolic rates and smaller body sizes (Ford chapters 3 and 4), I did not observe any difference in CORT suggesting metabolic rate and body size do not affect CORT in terrapins.

Similarly, salinity had no effect on terrapin CORT. Previously, I determined salt levels both above and below the acclimation salinity initially reduced growth, but over time terrapins acclimated to the salinity changes and within five months animals at all salinity levels exhibited equivalent growth rates (chapter 4). Decreased growth is one physiological response to stress (Sapolsky 1992), therefore it is possible CORT initially increased. However, after 30 days, normal growth resumed suggesting that the turtles acclimated to the salinity stress and that, CORT would have returned to baseline levels. Because the turtles resumed normal growth prior to these CORT assays, it is difficult to determine how CORT responded to the salinity stress. Although early mammalian studies refuted the possibility of stress acclimation (Seyle 1976), there is evidence supporting acclimation during critical life stages (see Sapolsky 2002 and references

therein), evidence for general habituation to stressors (Piersma and Ramenofsky 1998), and a growing body of literature supporting evolutionary adaptation to stressors (Meyer et al. 2003).

The terrapin CORT levels reported here are among the lowest reported in any reptile. Initial or baseline levels of CORT in turtles range from as low as 0.55ng/ml (Gregory et al. 1996) to 6.16ng/ml (Gregory and Schmid 2001). In this study, terrapin CORT levels were far lower, ranging from 0.078 to1.35ng/ml. Assayed terrapin plasma levels were more similar to sea turtle baseline levels (e.g. Gregory et al. 1996) than to the more closely related red-eared slider (Cash et al. 1997). Contrary to what I predicted, turtles cycled at 20ppt salinity tended to have lower mean CORT than those that had no change in salinity throughout the experiment (Figure 6.1). I expected the terrapins that remained at 10ppt, which was both the experimental acclimation salinity and natural salinity level for this population, to show the lowest CORT levels. It is unclear what physiological process might explain this trend for lower CORT at 20ppt. However, I did correctly predict that the largest difference in mean CORT would occur between uncontaminated and PCB exposed terrapins cycled at 30ppt (Figure 6.1). A large difference in this experimental group might be indicative of an interactive effect between the salinity stress and the stress caused by PCB exposure. Interactive stressor effects occur in numerous physiological variables, including CORT (Laugero and Moberg 2000). Because stressors can interact in multiple ways (e.g. additively, synergistically), it is important for studies to increase ecological realism by studying the physiological effects of stressors both singly and in combination.

Although there are numerous morphological and physiological signs of stress, determination of plasma CORT levels can provide a quick measure of stress and survival (Romero and Wikelski 2001) in wild caught individuals. However, CORT levels in many organisms fluctuate based on food intake and tidal cycles as seen in marine iguanas (Woodley et al. 2003). Far more data are available for the role of glucocorticoids in the stress responses of birds and mammals than they are for reptiles. Future terrapin studies of contamination affects on CORT need to include a time series component in order to determine a CORT profile and the overall role of CORT in this species sensitivity to stress.

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Table 2.1. PAH residues detected in each clutch from western eggs (Golden, Cremona 1 and 2, Trent Hall and Washington) and eastern eggs (Craney and Sheridan). Values are presented as ng/g concentrations calculated on both a wet weight (ww) and dry weight (dw) basis. A dash (-) denotes values were below detection limits. See appendix for site GIS coordinates.

	Golden		Cremona 1		Trent Hall		Washington		Cremona 2		Craney		Sheridan	
PAH	ww	dw	ww	dw	ww	dw	ww	dw	ww	dw	ww	dw	ww	dw
naphthalene	-	-	-	-	-	-	-	-	-	-	-	-	28.0	106.6
acenaphthylene	-	-	-	-	-	-	-	-	-	-	-	-	12.4	46.9
acenaphthene	-	-	-	-	-	-	-	-	-	-	-	-	13.8	52.4
fluorene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
dibenzthiophene	-	-	-	-	-	-	-	-	-	-	-	-	12.9	49.1
phenanthrene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
anthracene	-	-	-	-	-	-	-	-	-	-	21.2	86.9	17.9	67.9
2 methylphenanthrene	-	-	-	-	-	-	-	-	-	-	11.7	47.9	28.0	106.2
1 methylphenanthrene	-	-	-	-	-	-	-	-	-	-	16.5	67.8	23.8	90.6
3,6 dimethylphenanthrene	3.8	11.8	-	-	-	-	-	-	6.7	24.0	76.2	312.5	64.2	244.0
fluoranthene	-	-	-	-	-	-	-	-	-	-	54.5	223.5	114.0	433.3
pyrene	-	-	-	-	-	-	-	-	-	-	26.4	108.3	23.2	88.1
benz(a)anthracene	-	-	-	-	-	-	-	-	2.8	10.0	-	-	15.9	60.6
chrysene	-	-	-	-	-	-	-	-	-	-	1.2	5.0	-	-
benzo(b)fluoranthene	-	-	-	-	-	-	-	-	16.2	58.5	2.2	9.1	-	-
benzo(k)fluoranthene	-	-	-	-	-	-	-	-	-	-	86.8	355.8	21.6	82.1
benzo(a)pyrene	37.6	116.6	20.4	85.7	-	-	-	-	15.3	54.9	-	-	25.1	95.5
perlyene	8.5	26.5	-	-	4.9	18.1	-	-	-	-	-	-	29.8	113.2
indeno(1,2,3-cd)pyrene	-	-	-	-	-	-	-	-	-	-	-	-	105.8	402.0
dibenz(ah)anthracene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
benzo(ghi)perylene	-	-	-	-	-	-	-	-	-	-	-	-	36.9	140.3
total PAH	**49.9**	**154.8**	**20.4**	**85.7**	**4.9**	**18.1**	**0.0**	**0.0**	**41.0**	**147.4**	**296.7**	**1216.6**	**573.4**	**2178.9**

Table 3.1. PCB concentrations reported in the literature for aquatic turtles. All concentrations are total PCB levels detected in tissues with the exception of Yawetz et al. which was a study using experimental exposure of turtles using intraperitoneal (i.p.) injections of PCB congener 77.

Species	**Levels (ug/g)**	**Tissue**	**Citation**
C. serpentina	0.2	fat	Albers et al. 1986
D. coriacea	1.2	fat	Eckert and Eckert 1990
C. serpentina	0.6-10	eggs	Bonin et al. 1995
C. serpentina	0.3-25.75	eggs	Bishop et al. 1996
C. serpentina	2-737	eggs	de Solla et al. 2001
C. picta	125	i.p. of 77	Yawetz et al. 1997

Table 4.1. Results from repeated measures ANOVAs examining the effects of PCB, salinity and their interaction on mass, carapace length (CL), carbon dioxide production (CO_2) and number of carbon dioxide exhalation peaks (Peaks).

	Mass				CL				CO_2				Peaks		
Variable	df	F	p		df	F	p		df	F	p		df	F	p
PCB	1,96	62.27	<0.0001		1,96	70.8	<0.0001		1,147	26.56	<0.0001		1,179	22.74	<0.0001
Salinity	3,96	11.53	<0.0001		3,96	9.02	<0.0001		3,147	1.15	0.33		3,179	11.64	<0.0001
PCB*salinity	3,96	1.17	0.33		3,96	1.37	0.26		3,147	2.61	0.31		3,179	0.56	0.64
PCB*salinity*time	21,95	2.19	0.005		21,95	1.53	0.09		12,138	1.46	0.15		12,146	1.04	0.41

Table 5.1. Summary of analysis of variance (ANOVA) statistics for the effects of PCB, salinity, and an interaction between the two stressors on Diamondback terrapin urogenital and liver wet mass.

	Urogenital			Liver		
Factor	df	F	p	df	F	p
PCB	1,47	2.80	0.10	1,47	1.29	0.26
salinity	3,47	0.88	0.45	3,47	5.18	0.004
PCB*salinity	3,47	0.29	0.83	3,47	2.62	0.06

Table 5.2. Summary of analysis of covariance (ANCOVA) for the effects of PCB, salinity, and an interaction between the two stressors on Diamondback terrapin gastrointestinal dry mass with dry carcass mass as a covariate, and analysis of variance (ANOVA) for heart dry mass, hematocrit and dry carcass mass.

	GI			Heart			Hematocrit			Carcass		
Factor	df	F	p	df	F	p	df	F	p	df	F	p
PCB	1,39	14.89	<0.001	1,39	59.44	<0.001	1,39	33.71	<0.001	1,39	17.53	<0.001
salinity	3,39	0.18	<0.001	3,39	0.25	0.86	3,39	0.57	0.64	3,39	2.42	0.08
PCB*salinity	3,39	0.18	0.9	3,39	1.22	0.31	3,39	0.19	0.9	3,39	0.54	0.65

Figure 1.1. Mass-balance and heat balance coupled equations describing the distribution of energy among growth, reproduction and storage, the effects of the biophysical environment and how maintenance is the central link between the energetics and the heat components. Figure adapted from Porter and Tracy (1983).

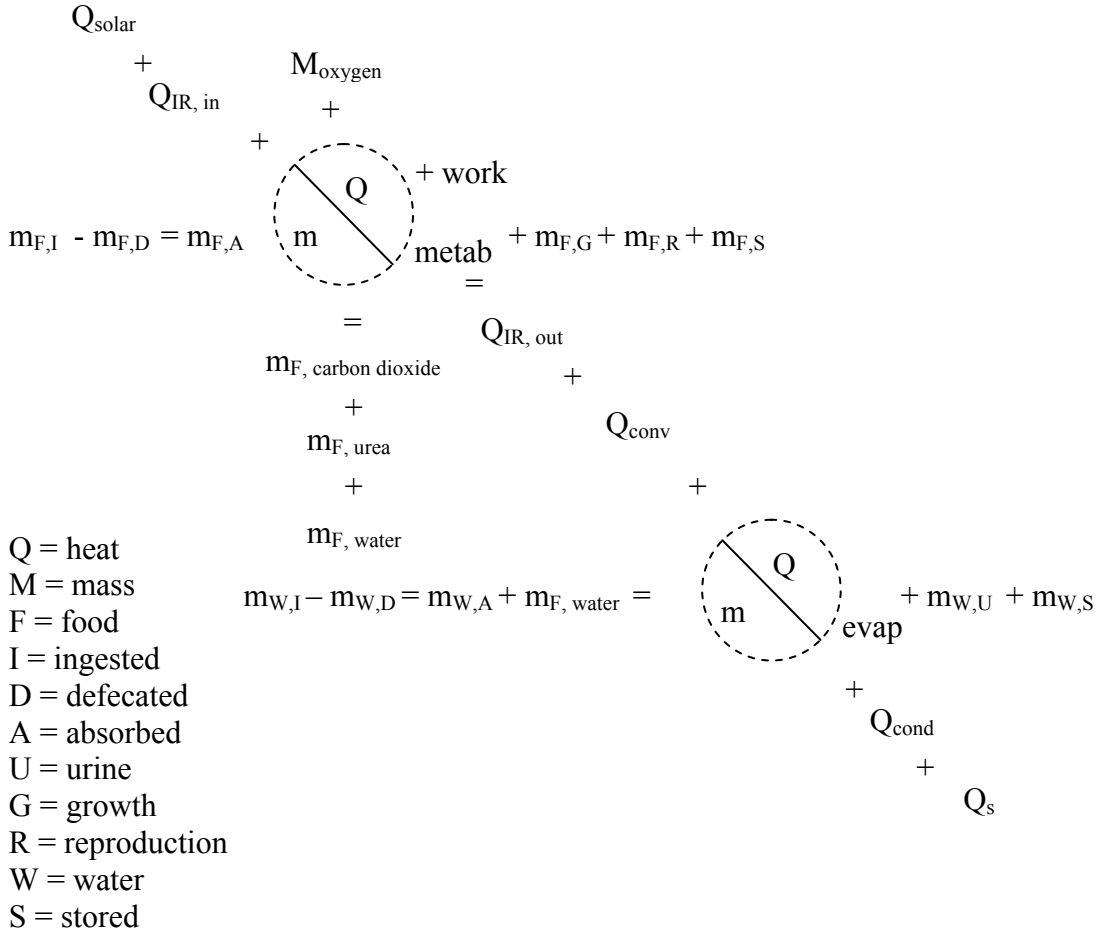
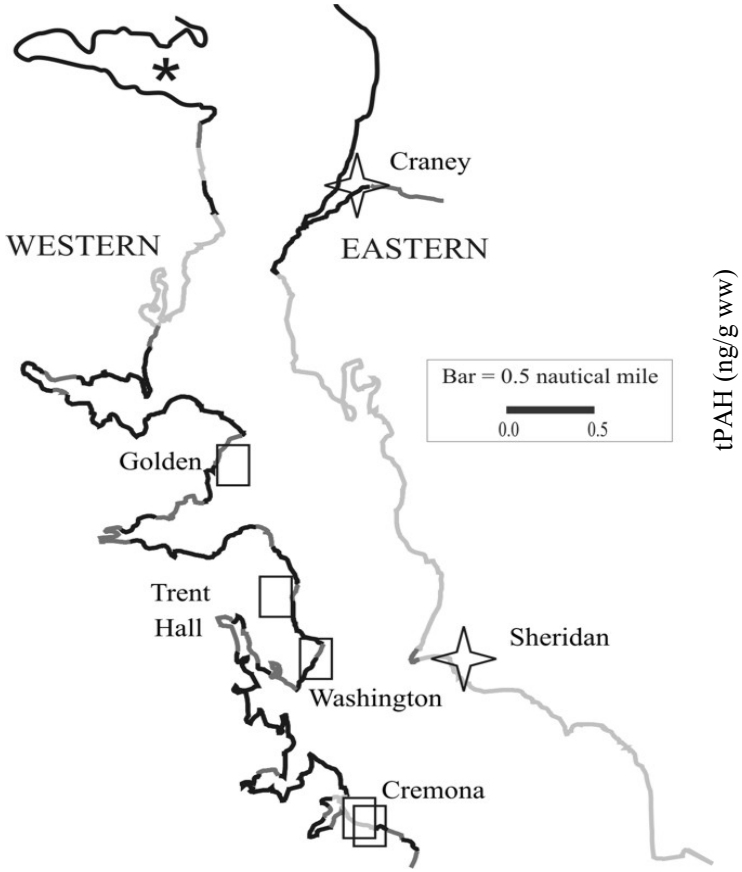


Figure 2.1A. Map of Diamondback terrapin egg collection sites along the Patuxent River, Maryland. Squares indicate low PAH eggs collected from western shore sites and stars indicate high PAH eggs collected from sites along the eastern shore. The gray scale value of the shoreline represents the level of shoreline oiling (black = moderate oil, medium gray = light oil, light gray = no oil detected). The asterisk (*) denotes the site of the 2000 oil spill in Swanson's Creek.

Figure 2.1B. Diamondback terrapin eggs collected on the eastern shores (n = 2 clutches) had significantly greater total PAHs ($p = 0.02$) than those collected on the western shores (n = 5 clutches) of the Patuxent River, Maryland. Gray bars represent contaminated eggs from eastern shore sites and white bars represent eggs from western shore sites.

2.1A.


Craney
WESTERN
EASTERN
Bar = 0.5 nautical mile
0.0
0.5
Golden
Trent
Hall
Sheridan
Washington
Cremona

2.1B.

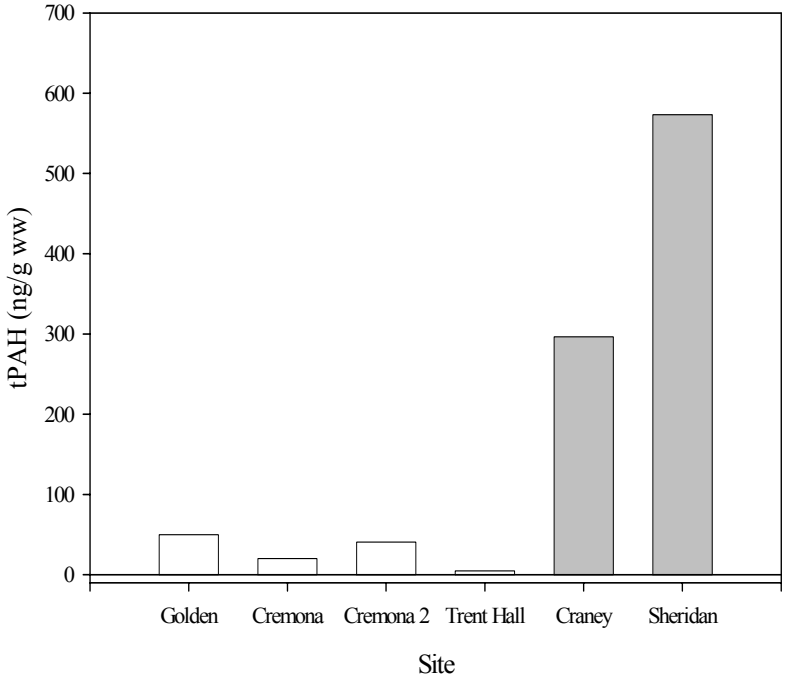
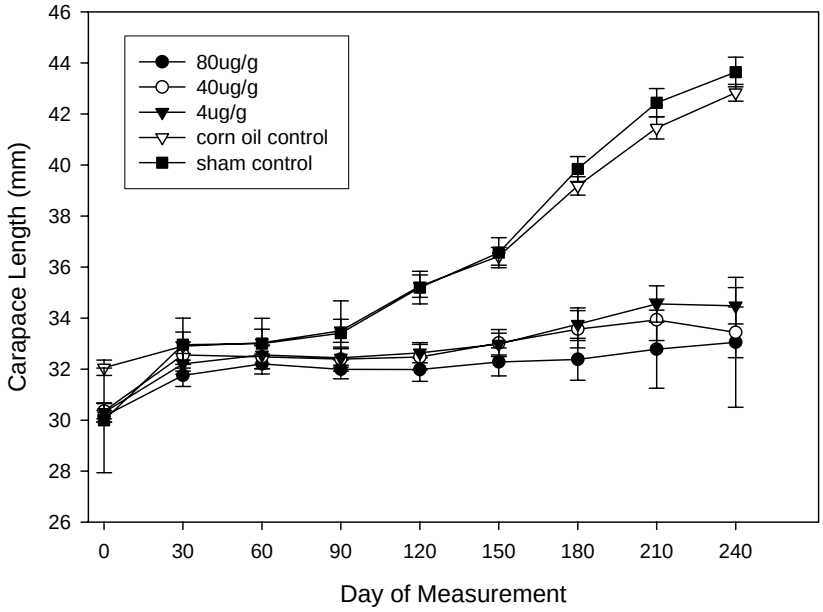

700
600
500
400
300
200
100
0
tPAH (ng/g ww)
Golden
Cremona
Cremona 2
Trent Hall
Craney
Sheridan
Site

Figure 3.1A (top). PCB126 treated turtles were significantly smaller in body size (repeated measures ANOVA: $F_{4,24} = 6.21$, $p = 0.001$), but there were no differences among the dose levels (orthogonal contrasts: $T_{24} = 4.67$, $p<0.001$). Values are means ±1SE (n = 4-6).
Figure 3.1B (bottom). PCB 126 treated turtles were significantly smaller in mass (RANOVA: $F_{4,24} = 8.99$, $p = 0.001$), but there were no differences among the dose levels (orthogonal contrasts: $T_{24} = 5.73$, $p<0.001$). Values are means ±1SE (n = 4-6).

A.



B.

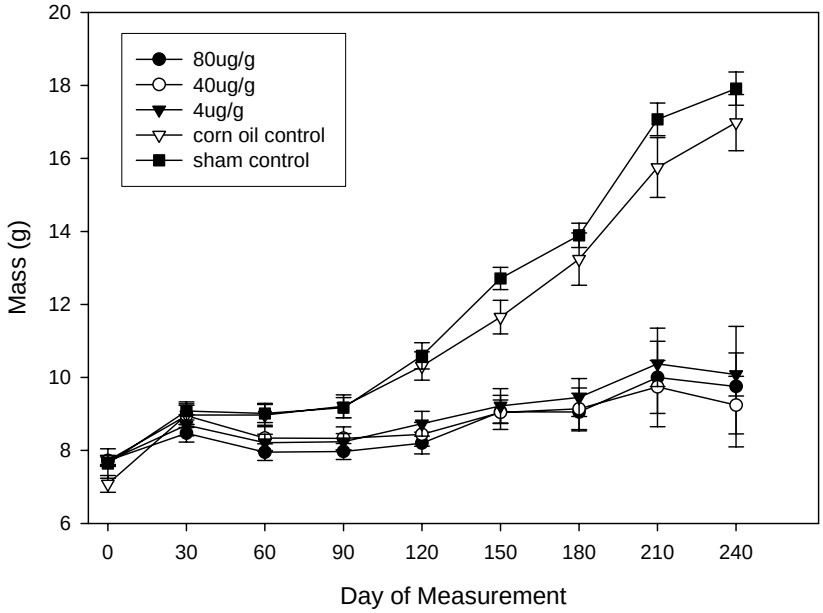


Figure 3.2. PCB 126 exposed animals (gray bars)did not differ significantly in the amount of food eaten as compared to food eaten by uncontaminated turtles (white bars). Values plotted are least squared means after adjusting for body size using body mass as a covariate in an ANCOVA. Bars are means +1SE.

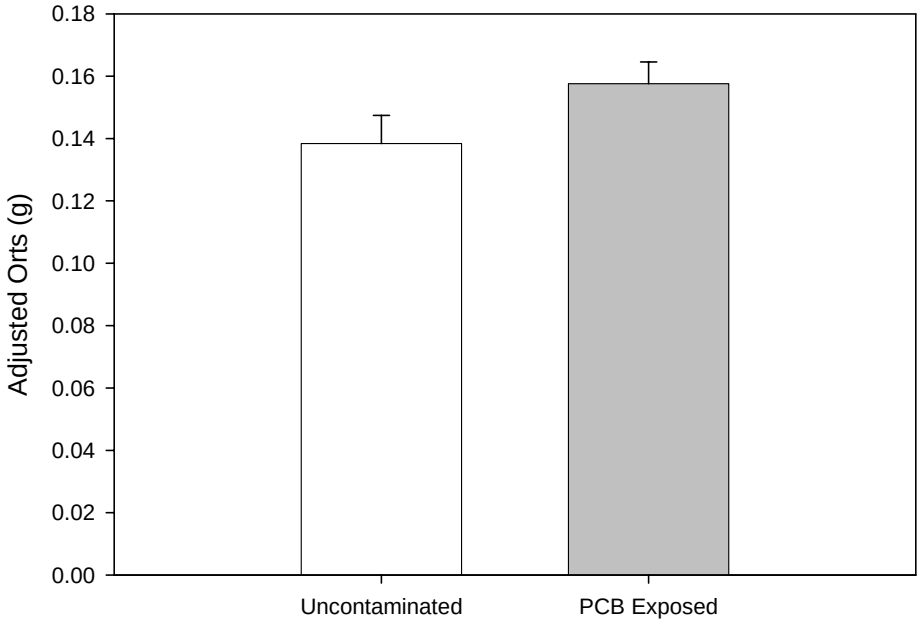


Figure 3.3. Kaplan-Meier survivorship curves for uncontaminated turtles (vehicle control and sham injection) and the three PCB 126 exposure levels. There were significant differences in survivorship among the treatments (logrank $\chi^2 = 19.14$, df = 3, p = 0.0003). The calculated LD50 was 29.8 ug/g (± 13.7).

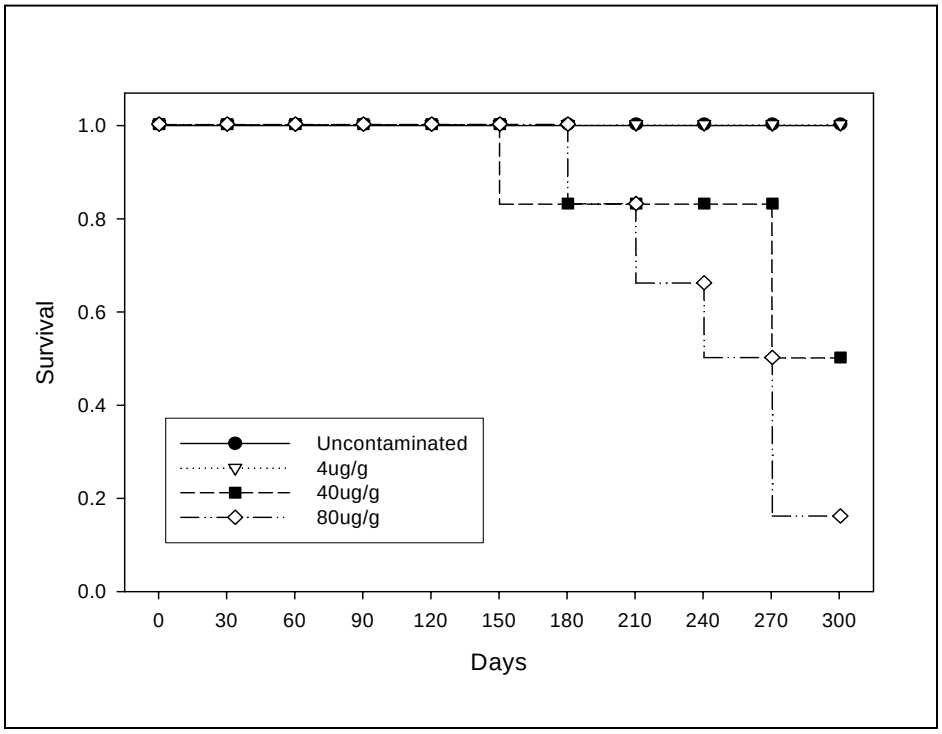


Figure 4.1 Reduction in carapace length after exposure to PCB 126 (repeated measures ANOVA: $F_{1,96} = 70.8$, $p < 0.001$) and salinity ($F_{3,96} = 9.02$, $p < 0.001$). Differences only became evident at 60 days after the initial exposure. Bars are means ± 1SE.

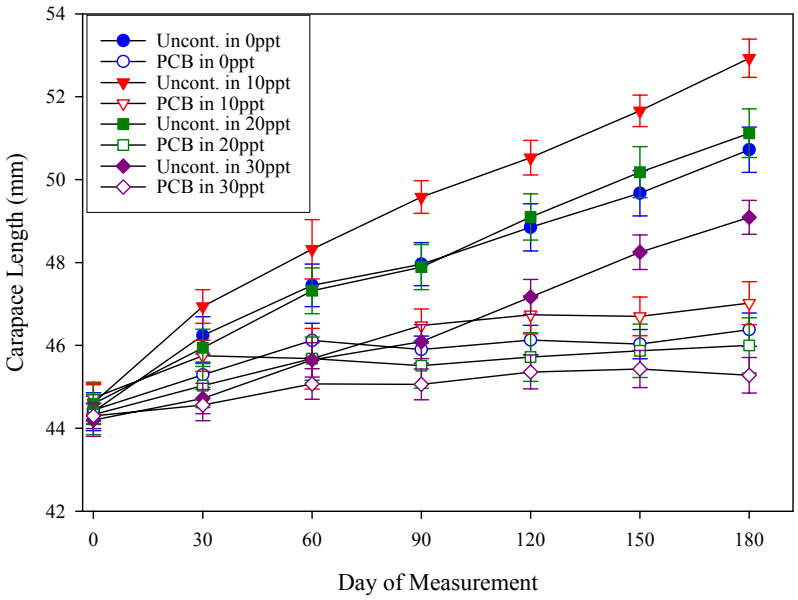


Figure 4.2 Reduction in body wet mass after exposure to PCB 126 (repeated measures ANOVA: $F_{1,96} = 62.27$, $p < 0.001$) and salinity ($F_{3,96} = 11.53$, $p < 0.001$). Difference only became evident at 60 days after the initial exposure. Bars are means ± 1SE.

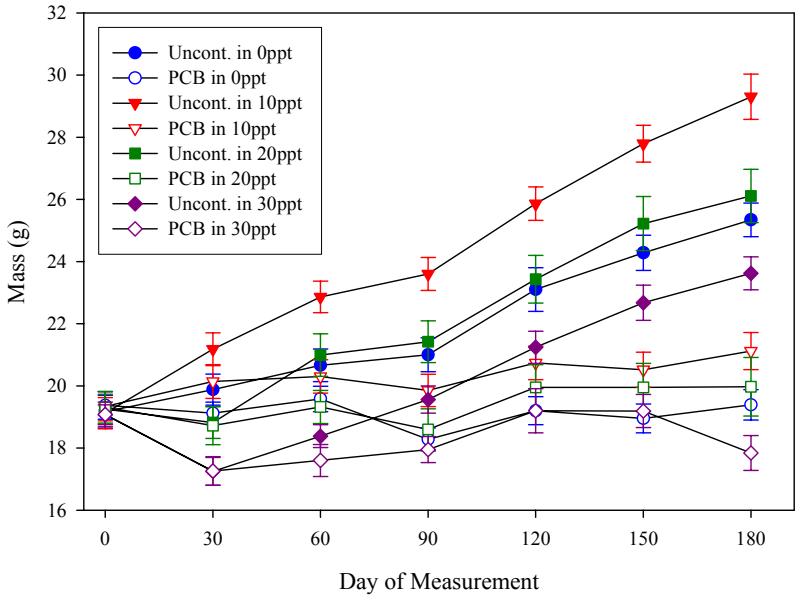


Figure 4.3 Carbon dioxide production after exposure to various salinities ($F_{3,147} = 1.15$, $p = 0.33$). Values plotted are least squared means after adjusting for body size using body mass as a covariate in an ANCOVA. Bars are means ± 1SE.

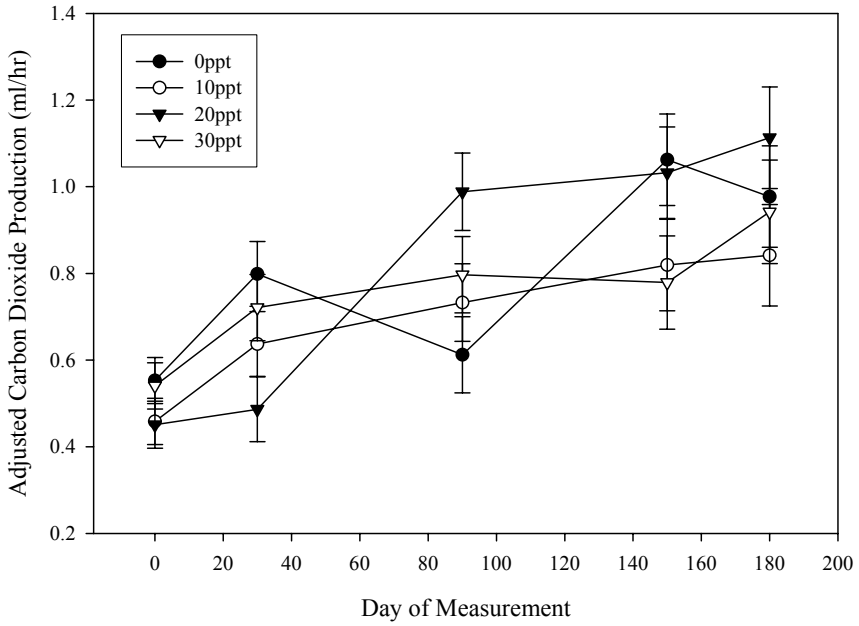


Figure 4.4 Reduction in carbon dioxide produced after exposure to PCB 126 (repeated measures ANOVA: $F_{1,147} = 26.56$, $p < 0.001$). Differences only became evident at 90 days after the initial exposure. Values plotted are least squared means after adjusting for body size using body mass as a covariate in an ANCOVA. Bars are means ± 1SE.

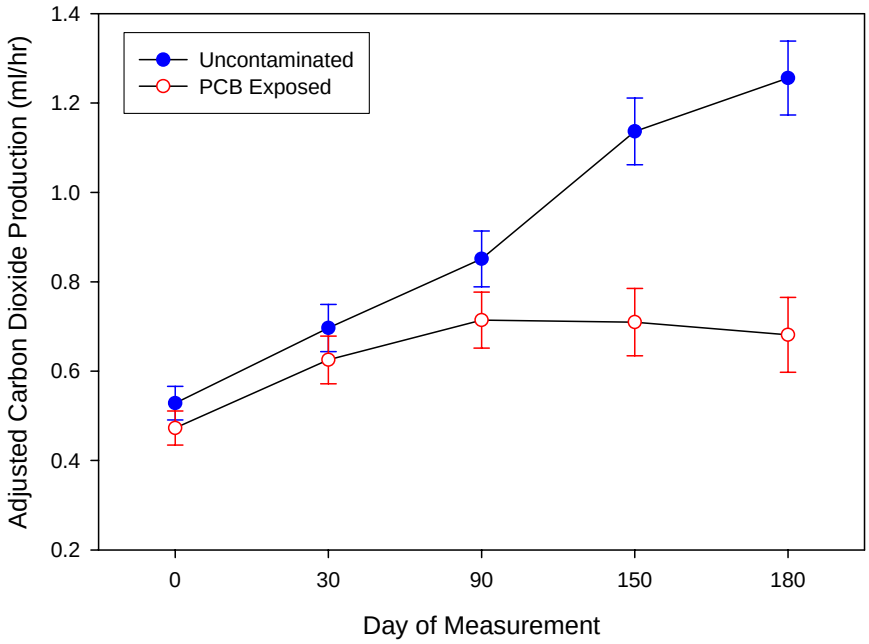


Figure 4.5 Respiratory pattern after exposure to various salinities as seen by a difference in the number of carbon dioxide peaks ($F_{3,179} = 11.64$, $p < 0.001$). This difference appears to be largely driven by the reduction in peak number of individuals held in 0ppt. Bars are means ± 1SE

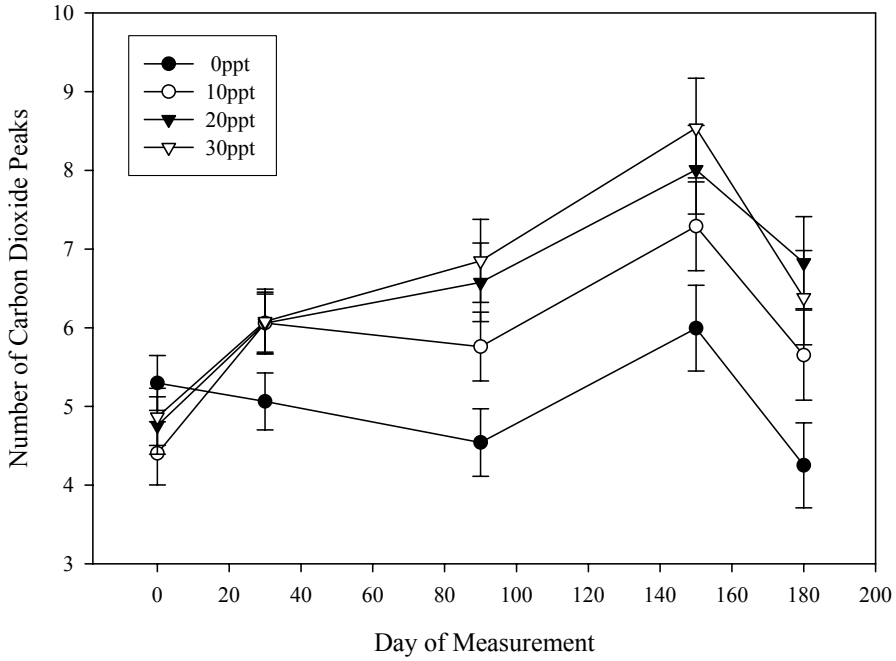


Figure 4.6 Respiratory pattern after exposure to PCB 126 ($F_{1,180} = 22.74$, $p < 0.001$), suggestive of hyperventilation. Bars are means ± 1SE.

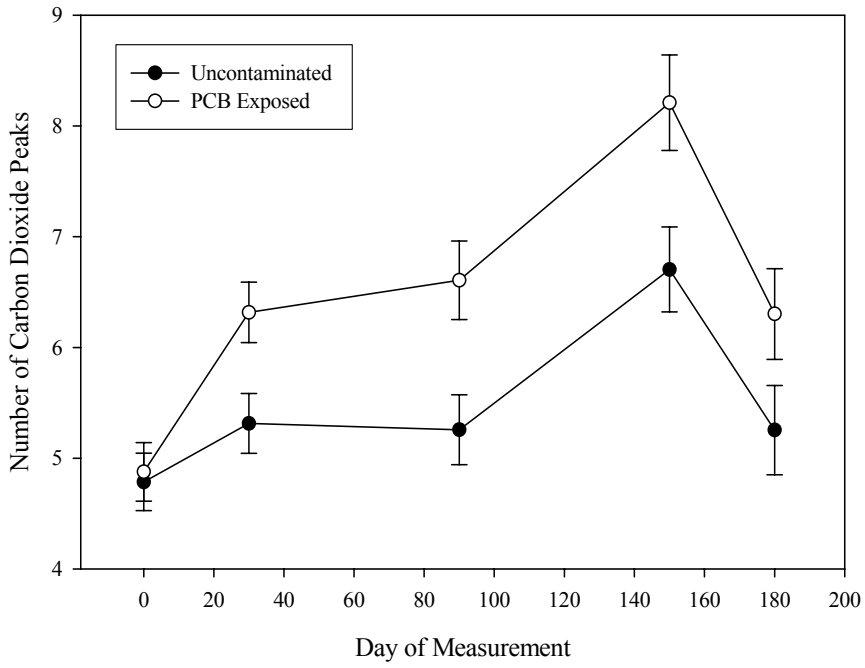


Figure 5.1. Salinity significantly affected liver wet mass. Turtles held at salinities above 10ppt exhibited reduced liver size. PCB 126 treatment did not affect liver mass and the interaction between PCB and salinity was marginally insignificant. The horizontal represents the mean and the box represents the 10^{th} and 90^{th} percentiles (n = 48).

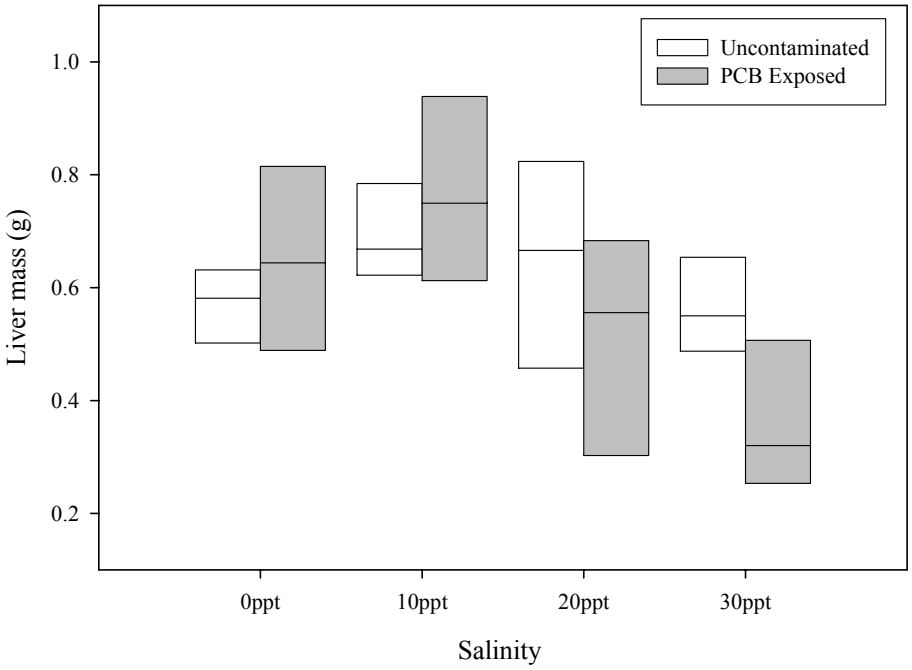


Figure 5.2. Mean heart dry mass (g) of turtles exposed to varying stressors (salinity, PCB or both). PCB 126 exposed individuals (gray bars) had significantly smaller heart dry masses compared to uncontaminated individuals (white bars), but there was no effect of salinity and no interaction. Bars represent mean dry mass and numbers are sample sizes for each treatment.

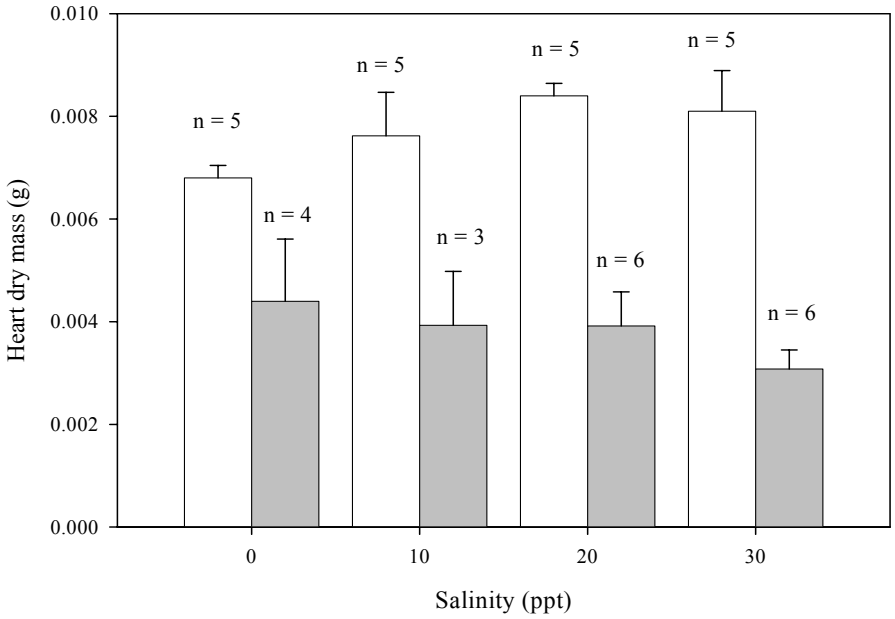


Figure 5.3. Least square means (+1SE) of gastrointestinal dry mass of turtles exposed to PCB 126 and varying salinity. PCB 126 exposed turtles (gray bars) had smaller GI dry masses compared to uncontaminated turtles (white bars). There was no effect of salinity and no interaction between PCB and salinity.

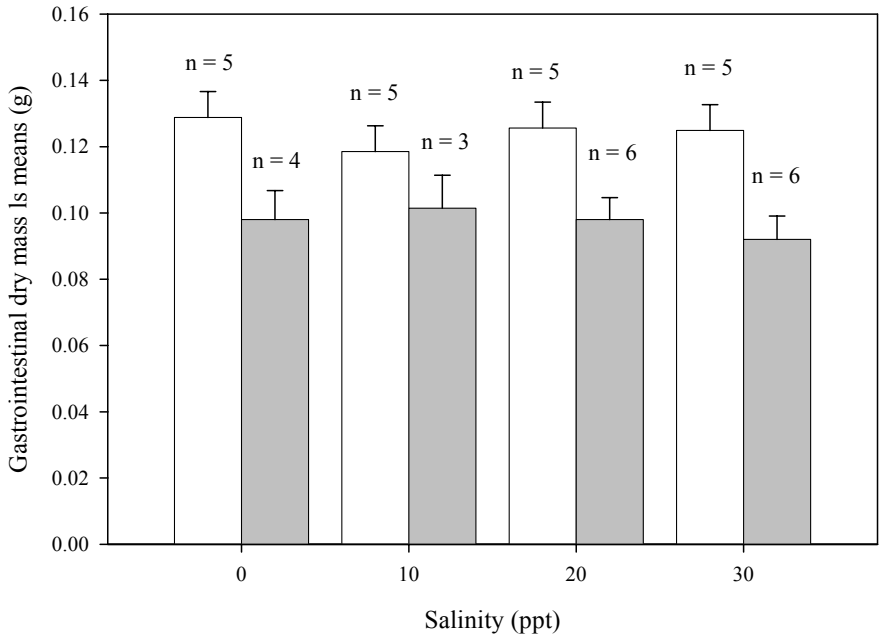


Figure 5.4. Mean hematocrit (+1SE) was reduced in turtles exposed to PCB 126 (gray bars) compared to uncontaminated individuals (white bars). There was no effect of salinity and no interaction between PCB126 and salinity. Numbers above each bar indicate sample size.

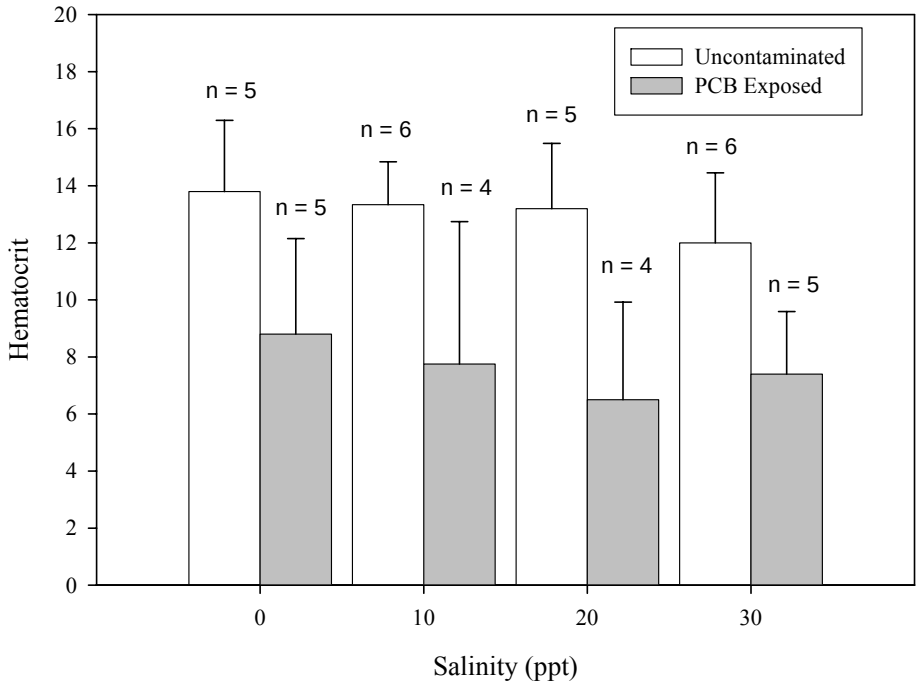
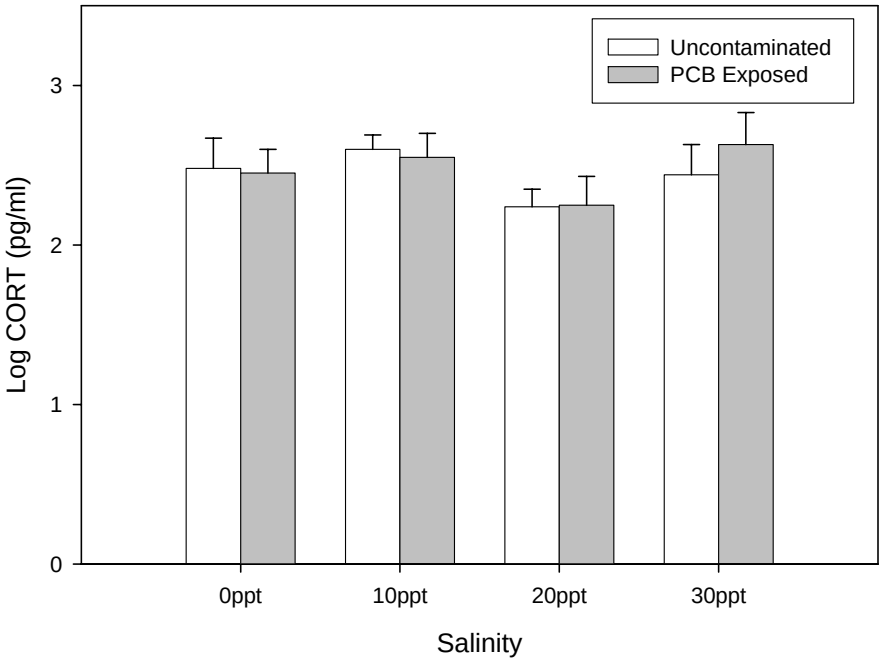


Figure 6.1. PCB exposure and varying levels of salinity showed no significant effect (PCB p = 0.79; salinity p = 0.27) on plasma corticosterone levels in the diamondback terrapin. Bars represent means ±1SE.



Appendix

GIS Coordinates of nest locations of eggs used in Chapter 2 PAH analyses

Golden Beach (38.29.389 N, 76.40.439 W)
Trent Hall Creek (38.28.469 N 76.39.804 W)
Washington Creek (38.28.090 N, 76.39.804 W)
Cremona Site 1 (38.27.076 N, 76.39.332 W)
Cremona Site 2 (38.27.041 N, 76.39.276 W)
Craney Creek (38.31.146 N, 76.39.235 W)
Sheridan Beach (38.27.904 N, 76.38193 W)