FINAL REPORT TO THE HUDSON RIVER FOUNDATION

Project Title: An Analysis of Reproductive Ramifications of PCBs in Hudson River Snapping Turtles

Project # 009/03A

Term: 3/2003 to 3/2005, extended to 4/2007

Report Submitted: 7/2007

Investigators and Affiliations:
Christopher L. Rowe, Carys L. Mitchelmore, Joel E. Baker
University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, PO Box 38, Solomons, MD, 20688
Phone (Rowe): 410 326 7227
Rowe@cbl.umces.edu

Students Supported:

Shannon M. Kelly, M.S. 2007, University of Maryland School of Medicine Program in Toxicology.

Karen M. Eisenreich, Ph. D. in progress, University of Maryland Program in Marine, Estuarine, and Environmental Sciences.

Ref. No. [UMCES]CBL 07-096

Technical Report Series No. TS-533-07 of the University of Maryland Center for Environmental Science

CONTENTS

Executive Summary

This report summarizes results from Hudson River Foundation Project 009/03A, "An Analysis of Reproductive Ramifications of PCBs in Hudson River Snapping Turtles." The project was intended to assess potential impacts on adult and juvenile turtles of PCBs that remain entrained in environmental matrices in the Upper Hudson River despite cessation of active inputs to the River several decades past.

The report is divided among specific research areas associated with the project. An overview providing justification for the work is followed by results of field studies of traits of adults inhabiting regions contaminated with PCBs and relatively uncontaminated regions. The field studies revealed no discernable differences in sexual dimorphism that would indicate feminization of males due to PCBs. In 2004, average size of females captured in the contaminated region was lower than in the reference areas, yet there was no difference in 2005 and males did not differ in size during either year. Sizes of eggs collected in the contaminated region were reduced relative to the reference region with respect to wet weight (both years) and diameter (2005), whereas the diameter of eggs in 2004 was slightly larger in the reference region. When combined over both years, female size and egg size were significantly correlated in the contaminated area, but not in the reference area. Despite the subtle differences in some traits observed among the areas, we cannot conclude that the relationships are substantial enough to have overall negative impacts on the population residing in the contaminated area.

The report then discusses chemical analyses of blood from adults and egg contents from the 2004 and 2005 field studies. Total PCB concentrations in blood from

both males and females and in egg contents were significantly elevated in samples from the contaminated region relative to the reference region. In the contaminated region, whole blood concentrations of total PCBs correlated significantly with size of both males and females, suggesting that circulating concentrations were related to duration of accumulation and remobilization of some PCBs stored in lipids to the circulatory system. Blood borne PCBs in males from the contaminated area tended to be more enriched in octa-PCB congeners relative to females, whereas females were more enriched in hepta-congeners. Relative to reference areas, the congener profiles in blood from males and females were more dominated by hepta-congeners compared to the contaminated area. There was a strong correlation between female blood PCB concentration and egg concentration in animals from the contaminated site which was not observed in the reference area. High concentrations of PCBs in eggs due to maternal transfer may provide a mechanism through which reproductive effects would emerge. Hypotheses relating to this question were tested in ensuing laboratory studies (below).

Using blood collected during field studies we also attempted to develop and refine methodology for reliably quantifying vitellogenin (Vtg) concentrations in blood. Vtg is not typically expressed by males, therefore accurately detecting Vtg in blood would provide an indicator of feminization that may not be of a gross enough nature to be expressed as changes in gonadal development or secondary sexual traits. Despite intense efforts employing several techniques, we were unable to develop an assay that we consider reliable enough for application.

Finally, we conducted two years of experiments in which individuals hatched from field-collected eggs were raised in the presence or absence of PCBs in their food, to

compare effects of maternally transferred PCBs and environmental exposure to PCBs. A battery of endpoints were employed, ranging from molecular responses (DNA breakage) to respiration, growth, behavior, and survival. Sublethal endpoints revealed little or no consistent effects of PCBs from maternal or food sources. However, in the 2004 cohort, in which egg PCB concentrations were much higher than in 2005, a latent mortality effect emerged such that at approximately 10 months following hatching, survival rates of animals having received PCBs maternally declined precipitously. Mortality was correlated with concentration of PCBs in eggs, suggesting a dose-dependent response. Latent mortality was not observed in animals from the 2005 cohort in which PCB concentrations in eggs were, on average, about three-fold lower. Based upon the mortality rates observed in the 2004 cohort, we employed deterministic age/stage based matrix population models to assess the potential effects that sustained mortality could have on population dynamics. The models suggest that, if the effects observed in 2004 were representative over the entire population and expressed consistently over time, the population in the contaminated region would experience reduced population growth rates and increased generation turnover time. Iterated over a 10 year time span, the model projects a 15 % reduction in population size in the contaminated area relative to the reference area. Based upon only two years of field data, however, we cannot assess the generality of the effects observed and thus the likelihood that the model projections are valid for the population as a whole.

I. INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of 209 synthetic organic compounds consisting of a biphenyl group with a varying degree of chlorination. PCBs are characterized by non-flammability, electrical insulating properties, and extreme chemical and thermal stability, which made them desirable for use in industry and manufacturing. Commercially produced PCBs were synthesized in complex mixtures of congeners with an overall degree of chlorination suitable for specific applications. Approximately 700,000 metric tons of PCBs were manufactured in the United States from 1929-1977 for use in capacitors and transformers, and as plasticizers, hydraulic fluids and lubricants (CEC 1996). Until production was banned in the U.S. in 1977 due to increasing reports of the toxicity of these compounds to humans and wildlife, Monsanto Industrial Chemical Company was the major domestic producer of PCBs, with General Electric (GE) and Westinghouse Electric being their largest consumers. From the late 1940's until 1977, General Electric operated two capacitor plants on the upper Hudson River in New York, one in Glens Falls and the other in Fort Edward. Due to decades of PCB release from these now-closed plants, the 322-km stretch of the Hudson River downstream to New York's Harbor has been listed by the U.S. Environmental Protection Agency (USEPA) on the Federal Superfund National Priority List since 1984.

PCBs do not readily degrade by physical, chemical, thermal or metabolic pathways, and thus persist for decades or more in environmental matrices. As well, the highly non-polar, lipophilic nature of PCBs results in accumulation and magnification through the food web (James 2001). Thus PCBs are transported and cycled among air, water, soil, sediments and biota, resulting in ubiquitous contamination on a global scale.

PCBs have entered the environment through various pathways including: deliberate or accidental release directly into soil, water, or air; leaching from disposal sites; improper disposal or incineration; and volatilization or seepage from industrial sites. Prior to and in some cases following recognition of the toxicity of PCBs waste products rich in the chemical were dumped directly into rivers, landfills, and other environmental sites. This was the case in the Hudson River GE plants, which released 95-590 metric tons of PCBs directly into the river over the course of three decades (Bush *et al.* 1989). As a result, the Hudson River Superfund Site contains some of the most highly PCB-contaminated sediment in the United States. In the 2002 Record of Decision (ROD) for the site, the USEPA called for the selected dredging of the most severely contaminated sediments in the upper 40 miles of the Hudson River (USEPA 2002). In conjunction with federal and state agencies, GE has completed much of the sediment sampling and subsequent planning for cleanup and dredging is to begin in 2007. While remediation of the upper Hudson River may do much to protect future populations of humans and wildlife from PCB contamination, the effects of a legacy of exposure of humans and wildlife is an issue that scientists and regulators will continue to struggle to understand. Furthermore, the extremely long residence time of PCBs in biota and remaining undredged sediments suggest that PCBs will continue to cycle through the upper Hudson animal and plant communities for years to come.

Signs of toxicity related to PCB exposure began to emerge in 1936 as an occupational hazard during commercial production, when workers at Monsanto chemical manufacturing plants developed skin eruptions, fatigue and signs of liver disease (Drinker 1937). Over the ensuing decades, a tremendous body of science has emerged

documenting the diverse toxic properties of PCBs, which include adverse effects to the immune, reproductive, nervous and endocrine systems, as well as probable carcinogenicity (Longnecker 2001; Perskey 2001). Some PCBs can interfere with the thyroid (Zoeller 2001) and steroid hormone systems (Cooke *et al.* 2001). Of particular concern is the ability of certain PCB congeners and their hydroxylated metabolites to disrupt or mimic the actions of estrogen and androgen, with a potential cascade of reproductive effects. Numerous laboratory studies have shown that most PCB mixtures display net estrogenic activity (i.e. Bitman and Cecil 1970; Korach *et al.* 1988; Soontornchat *et al.* 1994), however at least seven PCB congeners and seven hydroxylated PCB metabolites display antiestrogenic activity (Jansen *et al.* 1993; Moore *et al.* 1997), while others appear to possess antiandrogenic activity (Hany *et al.* 1999; Vincent *et al.* 1992). The complex nature of these disparate effects is further compounded by the fact that environmental PCBs always exist as mixtures of congeners, and usually as part of an even more complex mixture of other contaminants, making it difficult to assign discrete cause and effects in ecological assessments. For these reasons, the extent of steroid hormone endocrine disruption by PCBs outside of the laboratory and under environmental conditions remains an area of active study.

The majority of research on PCB toxicity has focused on avian and mammalian models, leaving a paucity of knowledge of effects on other taxa such as reptiles. It is likely that the animals most susceptible to PCBs and other persistent, lipophilic contaminants are long-lived predators or omnivores which are exposed for decades to high levels of concentrations in their prey, such as turtles. Specifically, the common snapping turtle, *Chelydra serpentina*, has been identified as a particularly useful species

for studies of persistent contaminants such as PCBs (USEPA 1993). *Chelydra serpentina* is the second-largest fresh water turtle in North America, inhabiting freshwater ponds, lakes, and rivers throughout the much eastern half of continent, and extending into parts of Mexico and central America. Active only in the warm months, snapping turtles aestivate underwater in sediments, under logs or in muskrat burrows (Mitchell 1994). Snapping turtles spend the vast majority of their lives in the water and sediments, feeding primarily on smaller animals, such as fish, amphibians, waterfowl, and invertebrates, as well as some plant material (Mitchell 1994; Pell 1940; Punzo 1975). Snapping turtles may live for 60 years or more, and with relatively small home ranges (0.7-28.4 hectares, Bishop *et al.* 1994; Brown *et. al* 1994; Obbard and Brooks 1981), individuals can be expected to have accumulated body burdens of contaminants reflecting the condition of their immediate habitat. Numerous studies of snapping turtles inhabiting organochlorine-contaminated sites have demonstrated bioaccumulation in various tissues, including blood plasma (de Solla *et al.* 1998), skeletal muscle (Stone *et al.* 1980), liver, and adipose (Pagano *et al.* 1999, Stone *et al.* 1980). Accumulated contaminants in snapping turtles can subsequently be transferred to predators which feed on eggs, juveniles or adults.

Once reproductive maturity is reached at 10-18 years of age, reproduction usually occurs annually until death (Brooks *et al.* 1988; Congdon *et al.* 1994). In contaminated systems such as the upper Hudson River, female turtles are likely to accumulate high concentrations of contaminants over the decade or more before the first reproductive event. Female turtles produce sizable clutches (15-50) of large eggs, rich in lipids and proteins to sustain the developing embryo over the 2-3 month embryonic period. As lipids are shunted from maternal tissue to maturing oocytes, so too are the associated

lipophilic contaminants therein, representing both a significant depuration process for the female and chronic exposure throughout sensitive development stages for the embryo. Lipophilic contaminants rapidly distribute within both stored maternal somatic lipids (Rauschenberger *et al.* 2004) and absorbed dietary lipids (e.g. Russell *et al.* 1999), so that transfer of contaminants to embryos will occur regardless of the source of the lipids allocated to the eggs. More than two decades of studies of snapping turtles in the Great Lakes—St. Lawrence River basin has demonstrated that considerable maternal transfer of contaminants can occur in snapping turtles, making eggs an excellent source of contaminant information (Bishop *et al.* 1998; de Solla *et al.* 2001; Pagano *et al.* 1999).

Snapping turtles, like many other reptiles, exhibit a biological feature that makes them particularly well suited for use in studies of contaminants that may act as endocrine-disrupting contaminants (EDCs): temperature-dependent sex determination (TSD). Unlike vertebrates with genotypic sex determination (GSD), where sex of individuals is determined by genes on specific chromosomes, the sex of turtle embryos is determined after fertilization by incubation temperature during critical developmental stages (Ewert *et al.* 2004). However, research has shown that administration of both natural estrogens (i.e. 17β-estradiol, estrone) and synthetic estrogens (i.e. diethylstilbestrol) to eggs during the temperature-sensitive period can override the effects of temperature and produce female young at male-producing temperatures (Crews *et al.* 1991). In a similar manner, estrogenic contaminants, including some PCB congeners and metabolites, have produced sex reversal in TSD turtle embryos in laboratory experiments. Red-eared slider (*Trachemys scripta elegans*) eggs incubated at male-producing temperatures produced

phenotypic females after exposure to hydroxylated PCBs (Bergeron *et al*. 1994) and the PCB mixture Arochlor 1242 (Willingham and Crews 2000).

While endocrine-mediated sexual dysfunction in wild populations of animals has not been linked to environmental PCB contamination alone, such changes have been linked to mixtures of various organochlorines including PCBs. Lake Apopka, FL, a site historically contaminated with a mixture of contaminants (primarily toxaphene, dieldren, *p,p'*-DDE, *p,p'*-DDT, *trans*-non-achlor and PCBs), has been the location of some of the earliest and longest-term studies of effects of EDCs on reptiles. American alligators of both sexes inhabiting this lake have abnormal circulating hormone levels and aberrant gonadal histology, while juvenile males exhibit smaller penis sizes compared to cohorts in relatively pristine habitats (Guillette *et al.* 1996, 1999). Similarly, male snapping turtles inhabiting Canadian sites contaminated with PCBs, multiple organochlorine pesticides, and polychlorinated dibenzodioxins (PCDDs) indicate that males have altered external morphology suggesting feminization (de Solla *et al.* 1998).

Another biological trait of snapping turtles which may be highly in studies of environmental EDCs is the presence of the estrogen-dependent gene responsible for producing vitellogenin (Vtg). As with all oviparous animals, female snapping turtles respond to seasonal, pre-reproduction surges in estrogen by producing Vtg, a phospholipoglycoprotein which is broken down in developing oocytes to produce yolk components (Bergin and Wallace 1974). It appears that the Vtg gene is normally quiescent in males and reproductively immature individuals (Copeland *et al.* 1986), however, numerous studies have shown that the gene is inducible by natural or anthropogenic estrogens in any individual, regardless of sex or maturity (i.e. Colborn *et*

al. 1993; Hyllner *et al.* 1991). Researchers have taken advantage of this by developing assays for measuring Vtg in numerous oviparous species, including fish such as zebrafish (Fenske *et al.* 2001), fathead minnow (Panter *et al.* 2002), and rainbow trout (Thorpe *et al.* 2000), as well as reptile species such as green sea turtles (Herbst *et al.* 2003), red-eared sliders (Palmer and Palmer 1995), and American alligators (Gunderson *et al.* 2003). To date, these studies have been used primarily for screening contaminants and environmental samples (i.e. sewage effluent) for estrogenicity in a laboratory setting, and to demonstrate that these assays can be used in the field. However, there are very few published studies that quantify Vtg in wild populations of animals from contaminated sites, with the exception of fish. Thus, while the scientific technology is in place to screen contaminated populations of oviparous animals, it has yet to be widely applied but likely will be in the future.

Following a privately funded pilot study in 2003, we initiated two years of field studies (2004 – 2005) and laboratory studies (2004 – 2006) with financial support of the Hudson River Foundation. The overarching goal of these studies was to examine the relationship between PCB body burdens and fitness-related traits of individual snapping turtles of all life stages within the upper Hudson River Superfund Site. Until that time, limited data had existed documenting tissue concentrations of PCBs in snapping turtles in the vicinity (Bryan *et al.*1987; Stone 1980) and no assessments had been conducted to examine the health of resident turtles in relation to PCB bioaccumulation.

Throughout the nesting season (late May-late June) of 2004 – 2005 we captured adult and juvenile turtles throughout the contaminated and nearby reference areas for morphological measurements and blood sampling, followed by marking and release.

Measurement of anatomical parameters, including penis measurement in males, was used to ascertain whether the Hudson River PCBs are having a feminizing effect on males or any morphological effects on females. Blood samples were used for PCB analysis and for measurement of biochemical endpoints, including plasma vitellogenin. Egg clutches were also collected from both areas for PCB analysis and reproductive output metrics. following incubation of eggs in the laboratory, juveniles were raised for approximately 1 year post hatching during which a battery of molecular, physiological, and behavioral assays were performed periodically.

In the following sections, results are presented and discussed separately for the field-based measurements and laboratory studies.

II. FIELD STUDIES TO QUANTIFY MORPHOMETRIC AND REPRODUCTIVE TRAITS OF SNAPPING TURTLES IN THE UPPER HUDSON RIVER REGION

Study Areas

All collections and wildlife handling throughout the study duration were conducted under the terms of a License to Collect or Possess granted by the New York State Department of Environmental Conversation's (NYSDEC) Division of Fish and Wildlife and were also approved by the Institutional Animal Care and Use Committee of the University of Maryland Center for Environmental Science.

Study areas comprised two distinct geographical areas in eastern New York State, in the general vicinity of the city of Glens Falls (Figure 2.1). The 'contaminated' area consisted of the length of the Hudson River from just south of Fort Edward (location of the southernmost of the two GE plants) to the Northumberland Bridge. This 20 kilometer stretch of the Hudson encompasses some of the worst of the PCB-contaminated sediment 'hot spots,' defined by the EPA as areas of the river where sediment PCB concentrations exceed 50 parts per million (ppm) (Quantitative Environmental Analysis 1998). Twenty of the forty identified 'hot spots' are located in the Thompson Island Pool, the 13 km stretch of river between Fort Edward and the northern tip of Thompson Island, with another fifteen located in the stretch from Thompson Island to the Northumberland Bridge (see map, Figure 2.1). Ponds, marshes and abandoned canals within approximately 350 meters of that stretch of the river were also included**,** as snapping turtles may traverse neighboring waterways within their home ranges. Likewise, the perimeter of land extending ≈ 350 m around the contaminated waterways in this area was also included for surveying and egg collection purposes. Since most of the

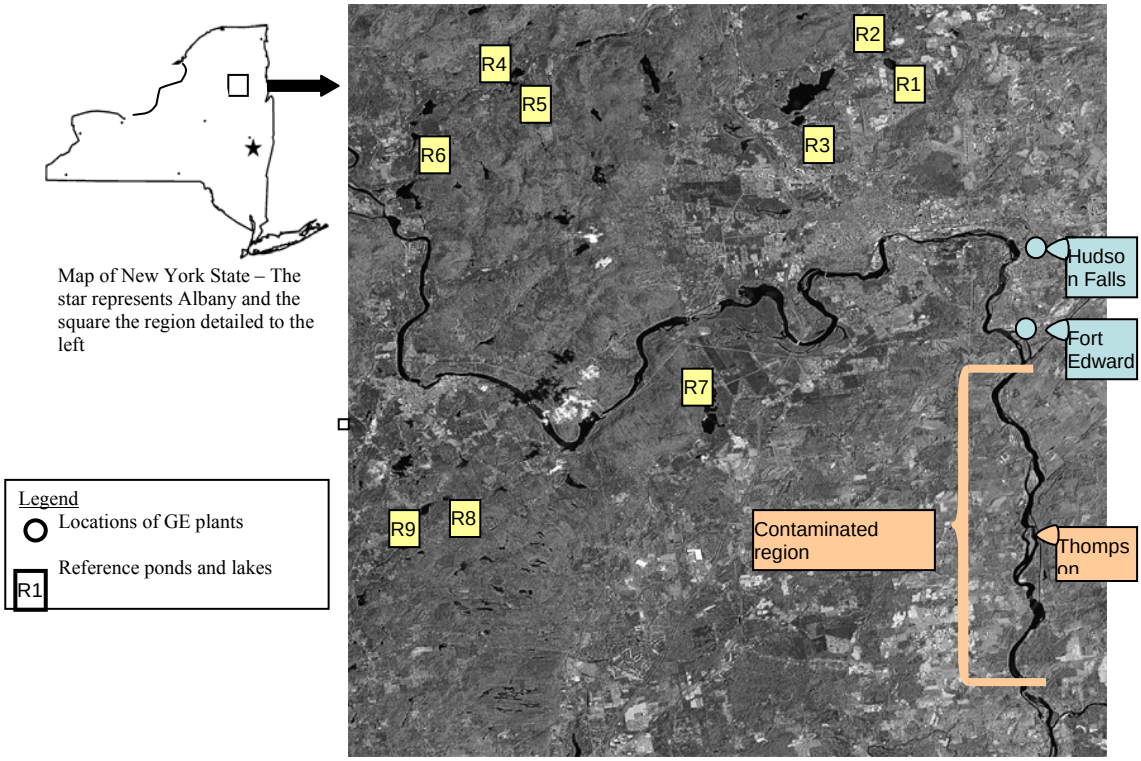

Figure 2.1 Locations of contaminated and reference study areas

land along this 16 km stretch of is residentially developed and privately owned, our specific trapping locations were determined not only by physical accessibility but also acquisition of explicit permission from landowners for access. Due to this restriction, very little trapping was available north of Griffin Island, such that the bulk of sampling in the contaminated area occurred between the northern end of Griffin Island and the Northumberland Bridge (Figure 2.1). The 'reference' area consisted of nine relatively pristine lakes and ponds located north and west of the northern tip of the Superfund site, as well as the associated ≈ 350 m perimeter of land around each. In both the contaminated and reference areas, specific trapping locations varied somewhat from year to year, depending on conditions, landowner permission, and prior experience in locating turtles and nests. For instance, initial attempts during the first field season to capture snapping turtles in the 10 km of the Hudson River upstream of the GE plants proved

ineffective, and thus I abandoned this area as a trapping site the following year. However, within the logistical constraints described above, reference sites were chosen to be as similar in habitat characteristics to the contaminated area as possible.

Snapping turtle densities, growth rates and reproductive parameters are known to vary with latitude, elevation, area of suitable habitat, and primary productivity (Brown *et al.* 1994; Galbraith *et al.* 1988; Iverson *et al.* 1997). Latitude was not an issue in this study, as both areas are in a very narrow range of latitude, from 43.13 to 43.39 North. Thus diel patterns in temperature, light intensity and precipitation are essentially uniform across all of the sampling sites. Furthermore, both areas are at the same elevation and encompass very similar habitats with regards to overall community composition. Measurement of other large-scale ecological parameters at each sampling site was beyond the scope and objectives of the study, which aimed not to elucidate the population dynamics at each area, but rather to gather contamination data and obtain a "snapshot" view of the condition of the populations and individuals in each area. In summary, for the purposes of our research, we have assumed that, PCB contamination aside, ecological conditions at all sampling sites are similar enough to support comparisons between the two areas in terms of all data collected.

Field Collection Materials and Methods

Adult and Juvenile Turtles

From late May through late June of both years, coincident with peak nesting season, we captured snapping turtles on land by daily surveys and in the waterways using baited hoop traps. All of the waterways in both areas were located close to

roadways and several animals were also captured along roadsides or crossing roads. Location of each capture was recorded using a handheld GPS unit.

Each turtle captured was brought to a temporary field station to be measured, weighed, marked and have a blood sample drawn. A unique pattern of marks was given to each turtle for immediate identification, as well as for identifying future recaptures, by drilling holes into the eight posterior marginal scutes (see Figure 2.2). This technique is a

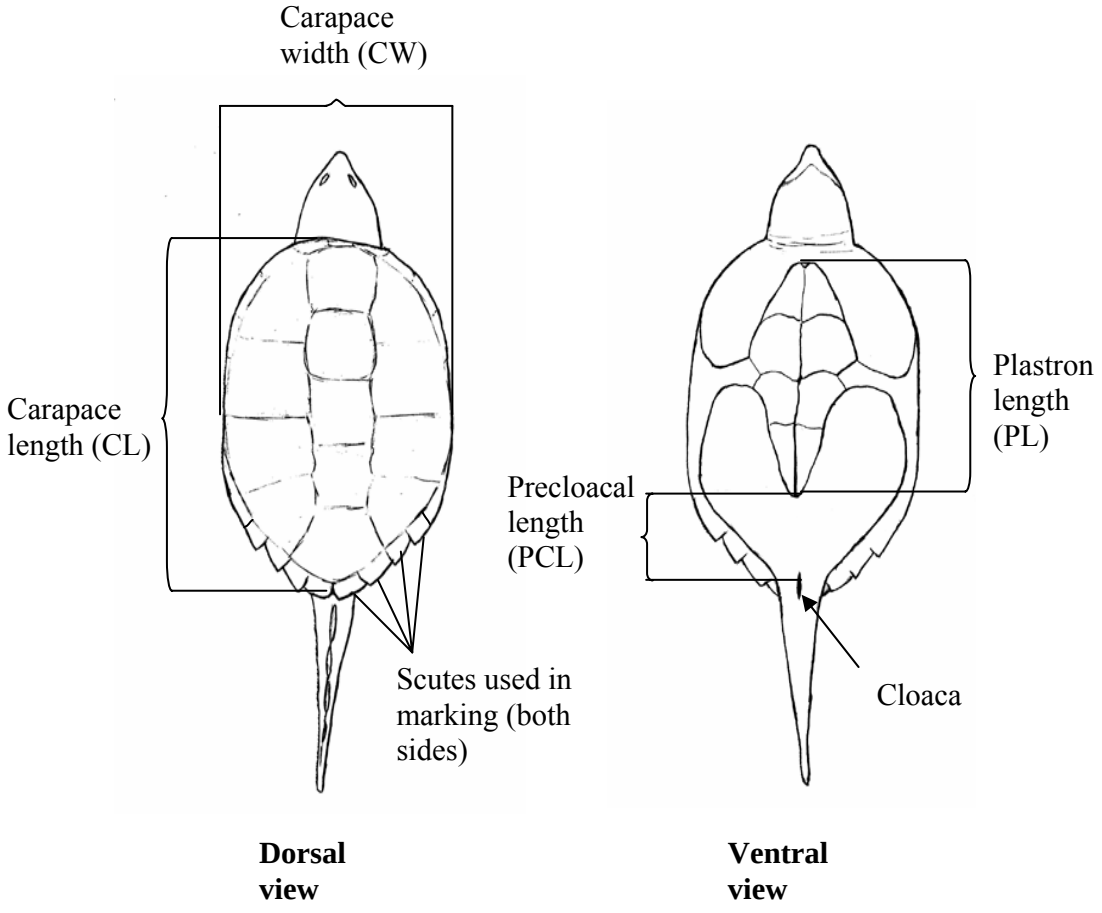

Figure 2.2 Schematic of snapping turtle shells, showing dorsal and ventral views and scutes to which marks were applied

standard method for mark/recapture identification of individual turtles, and beyond some superficial bleeding immediately after marking, does not harm the animals (Cagle 1939; Gibbons 1987). The data recorded for each individual included sex, mass, carapace length (CL), carapace width (CW), plastron length (PL), and precloacal length (PCL), the distance from the posterior edge of the plastron to the cloacal opening (Figure 2.2). The cloaca, the common urogenital opening, is located on the underside of the base of the tail, and PCL measurements were made with the turtle's tail oriented out in a straight, natural position. Mass was measured using a commercial dairy scale and linear measurements were taken using calipers.

While male snapping turtles grow to a larger size than females in most populations, this sexual dimorphism is only useful for gender identification in fully grown adults. Once males reach sexual maturity, the precloacal area, which overlays the penis, grows relatively faster than the carapace, while in females the two areas grow at the same rate (Gibbons and Lovich 1990; Mosimann and Bider 1960). This growth difference results in male turtles having a longer, thicker tail relative to body size, with the cloacal opening extending beyond the posterior edge of the carapace, unlike in females. The ratio of the PCL to carapace length or to the posterior lobe of the plastron has been used to identify the sex of snapping turtles, as well as a surrogate index for genitalia size (de Solla *et al.* 1998). The most definitive gender indicator in snapping turtles, however, is that when males are held in a vertical position, the penis distends from the cloaca. We also used this characteristic as an opportunity to measure male penis dimensions to determine if PCB contamination was contributing to feminization of males

in the form of reduced genital size, as seen in alligators inhabiting organochlorine-contaminated lakes in Florida (Guillette *et al.* 1996, 1999). We measured length as well as the maximum diameter at the base of the organ using calipers and calculated ratios of penis length or width to adult body mass or carapace length to determine whether site specific differences in secondary sexual traits existed.

For the purposes of analysis and comparisons between the two study areas, it is necessary to establish a morphological index for classifying individuals as reproductively mature. There are three traits that imply maturity in female turtles: initial enlargement of ovarian follicles, initiation of mating, and onset of nesting (Christiansen and Burken 1979; Galbraith *et al.* 1989). Due to the fact that the first two events cannot be easily assessed, size at first oviposition is the accepted benchmark for female turtle sexual maturity. The standard metric for snapping turtle size in relation to maturation is carapace length (CL), as mass measurements are variable depending on gut and bladder contents. There is significant geographical variation in size at maturity, with the general trend being that minimum reproductive size is negatively correlated with latitude, as lower latitudes have longer, warmer growing periods which allow individuals to grow at a faster rate (Iverson *et al.* 1997). Reported minimum CL for oviposition in female snapping turtles ranges from 18.5 cm in Tennessee (White and Murphy 1973) to 24.9 cm in Ontario (Galbraith *et al.* 1989) to 28.5 cm in Nebraska (Iverson *et al.* 1997). In the study conducted closest to our own research areas, Yntema (1970) reported first nesting at 20.0 cm CL for turtles in the vicinity of Syracuse, approximately 260 km east of Fort Edward and at a slightly lower latitude. Considering the number of studies addressing female sexual maturity, there is a striking paucity of published reports on male size or age

at maturity in snapping turtles. Mosimann and Bider (1960) dissected 28 females and 27 males near Lake Champlain in Quebec and concluded that both sexes of that population were mature at the same CL, while Christiansen and Burken (1979) found that male snapping turtles reach maturity 2-3 years earlier than females but at similar sizes due to a slightly faster growth rate. Based on their findings of maturity at equal sizes, the CL of the smallest nesting and/or gravid female from either study area was used as an estimate of the minimum size at maturity in both male and female turtles. Smaller individuals were classified as juveniles.

Blood samples were collected from the caudal vein, using a 21-gauge, 10-ml heparinized syringe. The needles and syringes were heparinized immediately prior to the blood draw with a 20 U/μl solution of sodium heparin to prevent blood clotting. The whole blood samples were immediately transferred to a 5 ml Vacutainer© tube containing 72 U sodium heparin and centrifuged at 3000 rpm (relative centrifugal force = 1100 xg) for 10 minutes. After centrifugation, the top layer of plasma was pipetted evenly into four microcentrifuge tubes, flash frozen in liquid nitrogen for transport and storage. Veterinarians have determined the total blood volume, TBV, of turtles to be equivalent to 5-8% of total body mass, and that 10% of the TBV may be collected without health effects (Mader 1996). Based on this index, we were able to draw 7-10 ml of whole blood from the majority of captured turtles, with ≈ 75% of this volume allocated to PCB analysis and the remainder separated for plasma for biochemical assays. In the case of juvenile turtles not large enough to sustain a blood draw greater than 4 ml, the blood was processed for biochemical assays only, since such small volumes would preclude analysis of PCB concentration.

After processing, turtles were released at their site of capture, usually within 24 hours, except for females held for several days for repeated oxytocin treatment (described below).

Eggs

During each field season, eggs were collected by three means: discovery of an actively-ovipositing female, excavation of previously oviposited nests, and capture of a gravid female followed by hormone-induced oviposition. The most ideal procurement of a clutch of eggs is finding a female mid-oviposition, as this allows collection of both maternal blood and the entire clutch of eggs. Capture of gravid females is less ideal, as hormone-induced oviposition tends to produce only partial clutches, precluding accurate estimates of clutch size. Previously oviposited clutches, which are often partially destroyed by predators, can yield data on egg contamination but not on clutch size or female traits.

Females found in the process of nesting were left undisturbed until they began covering the eggs and then the clutch and female were collected for processing. Previously oviposited nests, whether intact or partially destroyed, were carefully excavated to remove all eggs. The locations of all nests were recorded using a handheld GPS unit.

To determine whether a female was gravid, we palpated the oviductal region for shelled eggs. Gravid females were held for one to three days in individual plastic containers filled to a depth of approximately 10 cm with fresh water. Oxytocin, the peptide hormone responsible for contraction of the reproductive tract and passing of eggs through the cloaca, was obtained from Sigma Chemical in the form of 50 I.U./mg

lyophilized powder. The powder was reconstituted in ultra purified water and administered intraperitoneally near the rear limbs at a dosage of 20 I.U./kg (Ewert and Legler 1978). Females were injected in the evening hours and left undisturbed in the dark, except for occasional inspections for eggs. We have found that this method produces mixed results in that full or nearly-full clutches are obtained only when the eggs are fully developed, which is extremely difficult to determine by palpation. Gravid females found on land produced the largest induced clutches, as they were undoubtedly ready to oviposit and searching for a nesting location, while gravid females trapped from waterways often produced small, partial-clutches when induced and were thus less ready to oviposit naturally. In many cases, individual females were induced in two or three consecutive nights to maximize the number of eggs laid. Regardless of how many eggs were obtained, each female was released at the site of capture after a maximum of three successive attempts.

Following collection, each egg was cleaned with tap water, labeled with a pencil, weighed on a top loading balance, and measured using calipers. Three eggs were randomly selected from each clutch to be frozen at -20º C for PCB analysis, and the remainder stored in damp vermiculite at room temperature until transport to the Chesapeake Biological Laboratory for incubation. Eggs were incubated at 25 C, a temperature producing primarily males. Upon hatching, survival, size, and morphology were measured or scored, and hatchlings were used in ensuing experiments (Section III).

Statistical Analyses

All statistical analyses were performed using with MINITAB version 13 software (Minitab Inc., State College, PA), after examination of data for normality and equal

variance. A Type I error rate of $\alpha = 0.05$ was used for all statistical tests. One-way ANOVA was used for comparisons of morphology measurements of adult turtles and eggs between study areas. Regression analyses were used to examine relationships between metrics of female body size and clutch characteristics.

Results

Adults and Juvenile Turtles

In 2004, we captured 99 turtles, 48 animals from the reference area and 51 from the contaminated area. In 2005, 139 turtles were captured, 24.5 % across both study having been previously captured and marked in 2004 (26.4 % at the contaminated, 22.5 % at the reference). The smallest nesting and/or gravid female had a CL of 22.6 cm, setting the size limit below which we classified individuals as juveniles rather than adults.

As shown in Tables 2.1 and 2.2, we consistently captured more females than males across both years and both areas. This is likely due to the fact that the field season coincided with the nesting season, when females are much more active and conspicuous than males. It is also likely that gravid females were more prone to be caught in the traps that were set close to shorelines where females may be spending relatively more time than males in preparation for nesting. Due to those limitations, the data cannot be used to reliably estimate sex ratios of the populations studied.

There were no differences between collection areas in wet body mass or carapace length within sexes with the exception of 2004 females which were significantly larger in reference than contaminated areas (Tables 2.1 and 2.2). Because this pattern was not repeated in 2005 we cannot infer any overall relationships between site and size for the population.

Table 2.1. Sizes of adults captured in 2004 in contaminated and reference areas. CL = carapace length.

	Males			Females		
Site	**N**	**Mass (kg)**	**CL (cm)**	**N**	**Mass (kg)**	**CL (cm)**
Contaminated	20	7.1 ± 0.9	29.3 ± 1.6	21	3.4 ± 0.3	23.9 ± 0.6
Reference	15	7.6 ± 0.7	31.2 ± 1.2	29	4.1 ± 0.2	26.3 ± 0.4
		P = 0.688	P = 0.347		P = 0.009	P = 0.001

Table 2.2. Sizes of adults captured in 2005 in contaminated and reference areas. CL = carapace length.

	Males			Females		
Site	N	Mass (kg)	CL (cm)	N	Mass (kg)	CL (cm)
Contaminated	19	7.7 ± 0.9	29.5 ± 1.5	42	4.2 ± 0.2	25.8 ± 0.4
Reference	31	8.2 ± 0.6	30.4 ± 0.9	36	4.1 ± 0.2	25.8 ± 0.3
		P = 0.636	P = 0.582		P = 0.691	P = 0.952

The data on penis measurements are presented in Tables 2.3 and 2.4. There was no statistically significant difference in any of the three measurements between the two study areas during either year. Nor were there significant relationships between precloacal distance and total PCB in blood for males (Chapter III) in contaminated sites during either year (regression results P = 0.891 in 2004; P = 0.354 in 2005).

Table 2.3. Penis to body size ratios for males captured in contaminated and reference areas, 2004. CL = carapace length.

Site	**N**	**Penis length/body mass (cm/kg)**	**Penis length/CL (cm/cm)**	**Maximum diameter/body mass (cm/kg)**	**Maximum diameter/CL (cm/cm)**
Contaminated	13	0.65 ± 0.06	0.16 ± 0.01	0.50 ± 0.04	0.12 ± 0.01
Reference	17	0.84 ± 0.11	0.16 ± 0.01	0.59 ± 0.07	0.11 ± 0.01
		P = 0.187	P = 0.746	P = 0.287	P = 0.331

Table 2.4. Penis to body size ratios for males captured in contaminated and reference areas, 2005. CL = carapace length.

Site	N	Penis length/body mass (cm/kg)	Penis length/CL (cm/cm)	Maximum diameter/body mass (cm/kg)	Maximum diameter/CL (cm/cm)
Contaminated	22	0.84 ± 0.11	0.16 ± 0.01	0.54 ± 0.06	0.11 ± 0.01
Reference	29	0.77 ± 0.07	0.18 ± 0.01	0.49 ± 0.04	0.11 ± 0.01
		P = 0.588	P = 0.054	P = 0.400	P = 0.335

Eggs

Via induced oviposition and collection of natural nests we collected 556 and 1254 eggs in 2004 and 2005 respectively (Table 2.5). Mean clutch sizes (determined only for natural nests as induced oviposition often results in only a partial clutch being laid) ranged from 24 to 30 eggs per female and did not differ among sites during either year (Table 2.6).

Table 2.5. Total number of eggs of eggs collected from natural nests and via induced oviposition in females collected in contaminated and reference areas in 2004 and 2005. "N" is the total number of clutches from nests + induced females per year and site.

	2004		2005	
Site	N	Number of eggs	N	Number of eggs
Contaminated	18	299	49	686
Reference	16	257	36	568
	Total eggs	556	Total eggs	1254

Table 2.6. Number of eggs per clutch of eggs collected from natural nests in contaminated and reference areas in 2004 and 2005. Values are means ± 1 SE.

	2004		2005	
Site	N	Clutch size	N	Clutch size
Contaminated	6	29.7 ± 2.4	13	28.9 ± 2.1
Reference	5	30.0 ± 4.1	16	24.4 ± 2.0
		P = 0.946		P = 0.127

In both years, average egg wet mass was significantly greater in the reference area compared to the contaminated area, as was average egg diameter in 2005 (Tables 2.7 and 2.8). However in 2004, average diameter of eggs in the contaminated area were slightly (and significantly) larger than those from the reference area, despite the opposite pattern observed for average egg mass (Table 2.7). While the differences in egg sizes were statistically significant, their biological significance is questionable since the magnitude of the differences was quite small.

Table 2.7. Sizes of eggs collected from natural nests and induced females from contaminated and reference sites in 2004. Values are means ± 1 SE.

Site	**Egg diameter (mm)**	**Egg wet mass (g)**
Contaminated	26.5 ± 0.1	8.67 ± 0.10
Reference	25.8 ± 0.2	9.59 ± 0.19
	P = 0.006	P < 0.001

Table 2.8. Sizes of eggs collected from natural nests and induced females from contaminated and reference sites in 2005. Values are means ± 1 SE.

Site	**Egg diameter (mm)**	**Egg wet mass (g)**
Contaminated	26.5 ± 0.1	10.34 ± 0.04
Reference	27.0 ± 0.1	10.75 ± 0.10
	P < 0.001	P < 0.001

When analyzed over both years combined, there was a significant linear relationship between female size and egg size within the contaminated site, however this was not the case in the reference area (Figures 2.1 and 2.2).

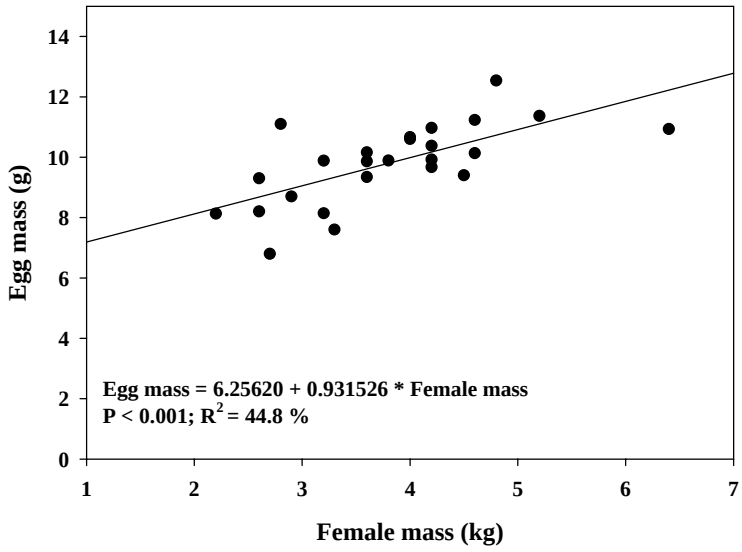

Figure 2.3. Relationships between female size and egg size in the Contaminated area, both years combined.

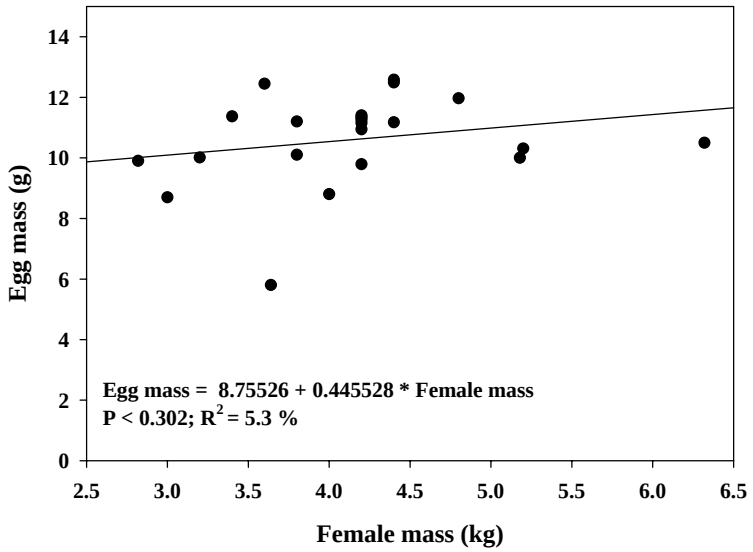

Figure 2.4. Relationships between female size and egg size in the Reference area, both years combined.

Discussion – 2004 and 2005 Adult Traits and Reproduction

Two years of data on adult and juvenile turtle morphometrics and egg and clutch characteristics did not reveal any obvious population differences between the snapping

turtles captured in the contaminated and reference areas. Both areas sustain turtles of diverse ages and sizes, with both sexes well represented. While there have been reports of populations in which strong sexual dimorphism in size does not exist (Galbraith *et al.* 1988; Lagler and Applegate 1943; Mosimann and Bider 1960), this was not the case at either study area, with males being significantly larger than females. Adult male turtles from the contaminated area did not differ from those from the reference area in body size or penis size. These data indicate that the PCB contamination in the upper Hudson River is not having a feminizing effect on the males that is expressed as adult body size or genital size. While de Solla *et al.* (1998) found reduced sexual dimorphism as expressed by relative precloacal length in male snapping turtles inhabiting contaminated Areas of Concern (AOCs) in southern Ontario, those populations were heavily exposed to pesticides as well as PCBs. Despite the possibility that this morphological aberration suggests feminization of males, the authors did not find any effects on testosterone or estrogen in either sex.

Numerous turtle life history studies have examined the relationship between female snapping turtle body size and clutch characteristics, finding that while both clutch size and egg size generally increase with female size, these correlations are moderate in strength and other maternal factors influence clutch characteristics (Galbraith *et al.* 1989; Iverson *et al.* 1997; Yntema 1970). The most comprehensive of these studies discuss the trade-off between egg and clutch size in terms of offspring fitness (i.e. larger individuals versus more individuals), as well as emphasize that individual variability in clutch size between reproductive events can be as great as the variability among individuals (Congdon and Gibbons 1990a; Gibbons and Greene 1990). Thus, despite the statistical

significance in overall egg sizes between years and study areas, it is difficult to assign a meaningful interpretation to these findings in terms of differences in reproductive biology parameters. For the females with full clutches in our study, the general trend at both study areas was an increase in egg size with increasing female body size (Figures 2.3 and 2.4), but the relationship was significant only in the contaminated area.

III. ANALYSIS OF SNAPPING TURTLE EGGS AND BLOOD FOR POLYCHLORINATED BIPHENYLS

Introduction

Polychlorinated biphenyls (PCBs) all have the general formula $C_{12}H_xCl_y$, where the 12 carbons form two conjugated phenyl rings (Figure 3.1) and x = 0-9 and y = 10-x (Alford-Stevens 1986). The ten positions on the biphenyl ring, labeled 2-6 and 2'-6', may be chlorinated in 209 unique possible structural arrangements, or congeners. Homologs or isomers are PCBs with different arrangements but the same number of chlorine atoms, such that there are ten homolog groups, mono- thru deca-chlorinated biphenyls. In terms of standard chemical nomenclature, different structures can be classified as meta-chlorinated (3, 3', 5, 5' positions), para-chlorinated (4 and 4' positions) and ortho-chlorinated (2, 2', 6, 6' positions). The numbers and positions of the chlorine atoms of PCB congeners impart different physicochemical characteristics that are important in such processes as partitioning, degradation, metabolism and bioaccumulation (Erickson 2001).

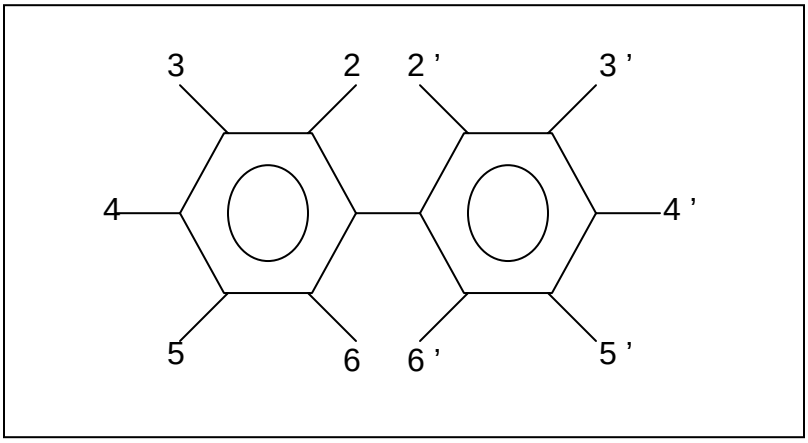

Figure 3.1 Schematic of a polychlorinated biphenyl (PCB) molecule.

PCBs are produced by heating biphenyl and anhydrous chlorine in the presence of ferric chloride, resulting in a liquid or resin with a high degree of chemical and thermal stability (Karcher *et al.* 2004). Large volumes of PCB mixtures were produced for use as dielectric fluids in capacitors and transformers, and as hydraulic fluids, solvents and plasticizers. Prior to the ban on production by the United States in 1977, the bulk of PCBs in the United States were produced commercially by Monsanto Chemical Company as complex mixtures of 60-90 congeners (Frame *et al.* 1996b). These PCB mixtures were designated with the Aroclor trademark, with each mixture assigned a four digit number (i.e. Aroclor 1260), with the last two digits signifying the percent weight of chlorine (with the exception of 1016 with 41% chlorine by weight) (Alford-Stevens 1986; Frame *et al.* 1996a). Extensive and widespread usage of Aroclor mixtures in industry and manufacturing is responsible for most of the environmental contamination of PCBs in the United States, resulting in the persistence of 140-150 congeners in environmental and biological matrices (Frame *et al.* 1996a). While PCBs are notably stable, they are susceptible to certain environmental processes that alter the relative congener distributions from the original contaminating mixtures over time. These dynamic processes include partitioning, adsorption, volatilization, atmospheric transport and deposition, microbial dechlorination, bioaccumulation, and metabolism (Connolly *et al.* 2000).

Upon entering the environment, PCB congeners differentially partition into environmental compartments (soil, water, air and sediment), depending on such physicochemical properties as vapor pressure, Henry's Law constant, and log octanol-water partition coefficient (log K_{ow}) (Erickson 2001). The higher chlorinated congeners

sorb tightly and persistently to organic material, soil and sediment, resisting degradation or metabolism (Cogliano 2001). Thus, although PCBs have low water solubility, high concentrations of dissolved organic carbon can dramatically increase PCB transport in the aqueous phase of a given system. Lower chlorinated congeners are more volatile and water-soluble, resulting in increased transport in air and water relative to heavier congeners. As the more volatile congeners evaporate out of a given system, an overall enrichment in higher chlorinated congeners results. Volatized PCBs are then subject to atmospheric transport and may be deposited into the same or other systems as dust or precipitation.

Other changes to the PCB congener profile at a given locale are mediated by biological endpoints. Microorganisms in aquatic sediments are capable of dechlorinating PCBs at variable rates, depending on PCB concentration, microbial diversity, temperature and other environmental conditions (Bedard 2001; Karcher 2004). The more heavily chlorinated congeners can be dechlorinated by anaerobic microbes, which preferentially target meta- and para-chlorines, producing less-chlorinated congeners (Rhee *et al,* 1993). Aerobic bacteria are capable only of dechlorinating the lower chlorinated congeners (mono-, di-, and tri-chlorinated biphenyls), but may completely degrade PCB molecules by breaking open the phenyl rings (Abramowicz *et al.* 1993; Bedard 2001). Numerous studies have demonstrated strong evidence for *in situ* microbial PCB dechlorination at various contaminated sites, including the Upper Hudson River (Brown *et al.* 1987a,b; Rhee *et al.* 1993). Animals inhabiting PCB contaminated sites can influence the environmental profile of PCB congeners through bioaccumulation, biomagnification and metabolism. In aquatic systems, movement of PCBs through the food chain begins with

passive (i.e. diffusion across biological membranes) and mechanical (i.e. ingestion of sediment) uptake of PCBs from the environment by benthic invertebrates and fish (DiPinto and Coull 1997; Lester and McIntosh 1994; Wirth *et al.* 1994). The major pathway for exposure in omnivorous and carnivorous animals is through ingestion of these contaminated food sources, resulting in increasing body burdens at each trophic level (Holoubek 2001). Differential bioaccumulation of PCB congeners in various species and individuals is influenced both by congener-specific factors (i.e. physicochemical properties, steric hindrance of large molecules, structure-based metabolism) and organismal factors (i.e. life history traits, fat content, enzyme characteristics and prey selection) (Cogliano 2001; Holoubek 2001). In general, higher-chlorinated congeners are more persistent in lipid-rich tissues than the more water-soluble lower-chlorinated congeners, while congeners with at least one ortho-chlorine are more resistant to metabolism than those without (James 2001).

Biotransformation of PCBs in animals can involve one or more enzyme systems, most notably cytochrome P450 (CYP) mixed-function oxidases and conjugating enzymes such as UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST). The most common PCB metabolites are mono- or dihydroxylated PCBs and methylsulfone PCBs, some of which are more readily excreted or more toxic than parent congeners (Erickson 2001; James 2001). Co-planar PCBs (the congeners with no more than one ortho-chlorine) can adopt a structure similar to highly toxic dioxins (specifically 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or TCDD) and behave similarly via a high affinity for the arylhydrocarbon receptor (AhR). In addition to mediating toxic responses such as hypothyroidism, carcinogenicity and neurotoxicity, the interaction of these congeners

with the AhR can induce CYP genes, potentially contributing to their biotransformation (James 2001; Van den Berg 1994). As animals in contaminated ecosystems differentially accumulate and metabolize PCB congeners from their environment, their body burdens may contribute to or detract from the system depending on such outcomes as death, migration, or capture for human consumption.

The cumulative effect of the above processes is that the congener profiles of abiotic and biotic compartments of a contaminated system become modified over time from that of the mixture originally introduced into the environment. Based on purchase records and statements from scientists from the two GE plants, the PCB input to the Upper Hudson River Superfund site consisted of approximately 80% Aroclor 1242, with the remainder comprised of Aroclors 1254, 1221, and 1016 (Bopp *et al.* 1981; USEPA 2000). Estimates for the amount of these PCBs that were released into the river from the plants prior to 1977 range from 95,000–603,000 kg (USEPA 2000). Even after commercial use of PCBs was restricted, transport into the river continued from several sources, including erosion of remnant deposits and seepage from bedrock fractures below the plants. The removal of an earthen dam in Fort Edward in 1973 and later spring flooding allowed a large volume of contaminated sediment to move downstream and settle into the Thompson Island Pool (TIP), which remains a major source of PCBs into the river system (USEPA 2000). PCB concentrations in the Hudson River generally decrease in with distance downriver from the GE plants (i.e. Steinbacher 2001), although significant heterogeneity exists even in the stretch of river closest to the source points, as evidenced by the forty 'hot spots,' 35 of which occur in the contaminated area of our study (USEPA 2000). In 1991, the maximum and average PCB concentrations in

surficial sediments were 2000 mg/kg and 42 mg/kg, respectively in the Thompson Island Pool and 4000 mg/kg and 26 mg/kg in the area between the TIP and Northumberland Bridge (USEPA 2000). The uppermost section of the Hudson River Superfund Site has largely retained a lower-chlorinated congener pattern, indicative of freshly released Aroclor 1242 from the bedrock below the Hudson Falls plant. Fish species studied in the same area, however have been characterized as having a pattern similar to Aroclor 1248 as a result of differential partitioning and accumulation (USEPA 2000).

Of the many studies conducted over the past several decades of vertebrate taxa inhabiting the PCB-contaminated upper Hudson River system, the majority are concerned with fish species (Baker *et al.* 2001; USEPA 2000). Scientists and regulatory agencies have closely monitored PCB levels in fish in various ecological niches in order to assess the scope of biological contamination, as well as for the purposes of setting advisories for human consumption in the Hudson River (see http://www.health.state.ny.us/environmental/outdoors/fish/fish.htm). Numerous bird species of the upper Hudson River ecosystem have also been relatively well characterized with regards to PCB contamination, including songbirds and waterfowl (Echols *et al.* 2004; Nichols *et al.* 2004; Hudson River Natural Resource Trustees 2005a). The relatively few studies of PCB exposure in mammals of the upper Hudson have focused primarily on mink, otters, and muskrats (Mayack and Loukmas, 2001). PCB accumulation by one of the most long-lived aquatic vertebrate residents of the upper Hudson, the snapping turtle (*Chelydra serpentina*) had only been briefly examined in two studies twenty years ago (Bryan *et al.* 1987; Stone 1980) until the 2005 release of a government-funded study on snapping turtle eggs conducted as part of an extensive

natural resources damage assessment of the area (Hudson River Natural Resource Trustees 2005b). In this study, I measured total PCBs and congener concentrations in the eggs and blood of snapping turtles in the most contaminated region of the Hudson River and compared them to those of turtles in nearby, relatively clean waterways, as well as to data from similar studies. Blood and/or plasma has become an increasingly favored endpoint in contaminant monitoring in wildlife as it allows researchers to assess body burdens in a non-destructive manner and to take repeated measures over time. The large, lipid-rich eggs of reptiles are easily obtained and have been shown to be highly indicative of maternal body burdens (i.e. Dabrowska *et al.* 2006; Pagano *et al.* 1999). Blood and eggs obtained from the same female provide information on total and congener-specific maternal PCB transfer to offspring.

Due to the lipophilic nature of PCBs, it is important to include corresponding lipid content data when reporting PCB concentrations in biological matrices. Lipid normalization of PCB data (i.e. concentration/lipid fraction) is essential in order to allow for meaningful comparisons between PCB loads in different tissues, life stages, and species, as well as across different studies. Throughout the current study, PCB concentrations are presented in both wet weight (ww) and lipid-normalized values to enable necessary comparisons both within and between studies. In deciding whether to use wet weight or lipid-normalized values for statistical comparisons, I will defer to the conclusion of Hebert and Keenleyside (1995) on the matter and use lipid-normalized data only if there is a significant relationship between PCB and lipid concentrations, or when comparing tissue types.

Extraction Materials and Methods

Eggs

The three eggs from each clutch that had been put aside for PCB analysis (Section II) were kept frozen at -20º C from collection in the field until processing in the laboratory at the Chesapeake Biological Laboratory (CBL) in Solomons Island, MD. In preparation for analyses, eggs were thawed, rinsed with distilled water and contents were removed. As previous research has found that eggs in a single snapping turtle clutch are not significantly variable with respect to contaminant levels (Bishop *et al.* 1994), the three eggs from each clutch were pooled for analysis. Egg contents were thoroughly homogenized in a clean, methanol-rinsed food processor and transferred to a pre-cleaned I-Chem® glass jar. In order to remove aqueous content, a sub-sample of approximately 5 g of the egg homogenate from each clutch was ground in a solvent-rinsed mortar and pestle with an excess of granular anhydrous Na_2SO_4 (previously pre-cleaned with a 1:1 v/v mixture of acetone:hexane, baked for 4 hours at 450º C and cooled to room temperature). This mixture was transferred to a pre-baked and cleaned Soxhlet extractor plugged with clean glass wool, and a 100 µl spike of a solution containing the following PCB surrogates was added: 3,5-dichlorobiphenyl (congener 14), 2,3,5,6-tetrachlorobiphenyl (congener 65) and 2,3,4,4',5,6-hexachlorobiphenyl (congener 166). These particular congeners, as well as those added later as internal standards, are not useful in this capacity as they are not typically found in the commercial PCB mixtures from which environmental contamination arise. All samples were then extracted for 24 hours with dichloromethane. Following extraction, samples were concentrated using rotary evaporation and exchanged them into hexane at a volume of ≈ 4 ml.

To measure the gravimetric lipid content of each sample, a 500 µl aliquot was removed and transferred it to a clean, pre-tared aluminum weigh boat, which was then placed in an oven at 60º C, dried overnight and weighed the next day. Gel permeation chromatography (GPC) was then used to remove the lipids from the remaining volume of each sample, using a 250-mm x 22.5-mm inner diameter Phenomenex Phenogel column (10 µm particles with a 100-Å pore size) with dichloromethane as the mobile phase at 5 ml/min and the lipid-free sample was concentrated using rotary evaporation while exchanging the solvent for hexane, resulting in a final volume of ≈ 1 ml.

The samples were further cleaned of interfering compounds and the PCB fraction separated from other organic components using Florisil (magnesium silicate) which was pre-cleaned Florisil with a 1:1 v/v mixture of acetone:hexane for 24 hours, allowed to thoroughly dry, and then activated with a four-hour baking period at 550 º C. The Florisil was then partially deactivated with the addition of 2.5% of its mass of deionized water and thoroughly mixed for 20 minutes. A chromatographic column was assembled with a clean glass wool plug at the bottom and then pre-rinsed with dichloromethane followed by petroleum ether. Eight grams of deactivated Florisil were poured into the column, covered by ≈1 g. of Na_2SO_4 and then pre-eluted once with 35 ml of 50:50 v/v petroleum ether/dichloromethane and then again with 35 ml petroleum ether. The sample and three small petroleum ether rinses of the sample vial were then added to the column and eluted with 35 ml of petroleum ether. The collected sample was then concentrated to a small volume (≈1.0 ml) and exchanged into hexane using a TurboVap® system with a warm water bath and nitrogen gas blow-down, followed by transfer to autosampler vials.

The exceedingly high PCB concentrations of some eggs collected from the contaminated area required that eggs from this area be prophylactically diluted at the beginning of the extraction procedure by using only 0.25 g of egg tissue, rather than the full 5 g typically extracted. However because of the wide range of egg concentrations, several of the diluted samples were subsequently found to be below detection limits, and thus were re-extracted at full strength (5 g of tissue) and re-analyzed.

Blood

The liquid-liquid extraction method for turtle whole blood in preparation for PCB analysis was adapted from a method for measuring organochlorines in sea turtle blood developed by Keller *et al.* (2004). In order to optimize the technique for our purposes we conducted numerous trial runs, resulting in several minor changes to Keller's method to achieve maximum surrogate recoveries and minimize background contamination. Blood samples, which had been frozen at -20º C since field collection (Section II), were thawed in their original I-Chem® glass vials and thoroughly mixed using solvent-rinsed metal spatulas. Each sample was transferred to a tared, clean and pre-baked glass test tube on a balance to measure the mass extracted and then spiked with 100 µl spike of a solution containing the following PCB surrogates: 3,5-dichlorobiphenyl (congener 14), 2,3,5,6-tetrachlorobiphenyl (congener 65) and 2,3,4,4',5,6-hexachlorobiphenyl (congener 166). Since PCBs bind to serum proteins (Gómez-Catalán *et al.* 1991; Norén 1999), samples received four ml of hexane-rinsed 98 % formic acid and were then sonicated for 15 minutes in order to denature the serum proteins and release bound PCBs. Eight ml of a 1:1 (v/v) mixture of methyl-tert-butyl-ether (MTBE):hexane was added to the test tubes followed by two minutes alternating inversion and vigorous vortexing. The test tubes

were centrifuged for 10 minutes at 2000 rpm, resulting in separation of the organic phase (containing partitioned lipophilic material including PCBs) from the denser aqueous phase. The organic upper layer was then transferred to another clean and pre-baked test tube. The liquid:liquid extraction of the remaining aqueous solution with 8 ml of 1:1 MTBE:hexane was repeated two more times, with the organic phases combined to achieve maximum extraction of hydrophobic components from the blood sample. In order to remove any residual water, the combined organic extracts were thoroughly mixed with 10 g. of anhydrous Na_2SO_4 and maintained at -20º C overnight. The following day, the sample was removed from the Na_2SO_4 and concentrated to ≈1.0 ml using a TurboVap® system with a warm water bath and nitrogen gas blow-down. Lipid content was determined gravimetrically by transferring a 100 µl aliquot of the sample to a clean, pre-tared and dried aluminum micro-weigh boat, which was then dried overnight at 60º C and re-weighed the next day on a microbalance.

Two chromatographic cleanup methods were used to removed lipids and other interfering biological matter: alumina (aluminum oxide) followed by Florisil. As with Florisil, alumina was pre-cleaned prior to use with a 1:1 v/v mixture of acetone:hexane for 24 hours, thoroughly dried, and then activated with a four-hour baking period at 550º C. The alumina was then partially deactivated with the addition of 6 % of its mass of deionized water and thoroughly mixed for 20 minutes. A chromatographic column was assembled with a clean glass wool plug at the bottom and then pre-rinsed with acetone, followed by hexane and petroleum ether. Four grams of the prepared alumina were poured into the column, covered by ≈1 g. of Na_2SO_4 and then pre-eluted with 25 ml petroleum ether. The sample and three small petroleum ether rinses of the sample vial

were then added to the column followed by 25 ml of petroleum ether, with the resultant elutant collected in a pre-baked and cleaned glass container. The sample was then concentrated to a small volume (≈1.0 ml) and exchanged into hexane using a TurboVap® system, followed by Florisil cleanup and transfer to autosampler vials as described earlier for egg sample extracts.

Instrumental Analysis of PCBs Materials and Methods

The final prepared extracts of all egg and blood samples were analyzed for PCBs in an identical manner. Each sample received 100 µl of a solution containing internal standards 2,3,6-trichlorobiphenyl (congener 30) and 2,2',3,4,4',5,6,6'-octachlorobiphenyl (congener 204). PCBs were quantified using a Hewlett Packard Model 5890 gas chromatograph equipped with a ^{63}Ni electron capture detector, with hydrogen and nitrogen used as carrier and make-up gases, respectively. A 60 m x 0.25mm 5% phenylmethyl silicon DB-5MS capillary column was used under the following conditions: inlet pressure was 100kPa and the temperature program was 100º C for two min, 100 to 170º C at 4º C/min, 170 to 280º C at 3º C/min, and 5 min at 280º C. The injector temperature was held at 225 º C and the detector at 285 º C. An autosampler (HP 7673) injected a 2 µl subsample from each sample vial in the split-less mode. Data were acquired and analyzed using Hewlett Packard Chemstation software.

Individual PCB congeners were identified based on chromatographic retention times in comparison to those of added internal standards and to those of a calibration standard prepared with commercial Aroclors mixtures. Quantification of PCB congeners was performed using the relative response factors generated from the calibration curve of the calibration standard (Mullin *et al.* 1984). Table 3.1 summarizes the congeners

quantified in all samples analyzed for PCBs; in cases of co-eluting congeners, combined values are reported. In order to classify congeners by homolog group, co-eluting congeners were separated based on the relative contribution in commercial Aroclor mixtures, based on the work of Frame *et al.* (1996a). Several congeners were excluded from all analyses (but are included in the full data sets in the appendices) due to either excessive analytical lability (congeners 1 and 3) or to high analytical interference from co-eluting compounds of unknown identity (congeners 49, 97, and 199).

Quality Assurance

In order to calculate detection limits for PCB congeners, as well as to monitor background contamination during sample processing, laboratory blanks were analyzed concomitantly with samples. For the egg samples, laboratory blanks consisted of 50 g of cleaned, baked sodium sulfate (from the same batch as that being used for samples) spiked with the PCB surrogate solution, extracted and processed alongside and in an identical manner as samples. Laboratory blanks for the whole blood samples were comprised of 4 ml of ultra purified water combined with 4 ml of formic acid, then spiked with surrogate solution. The detection limit for each peak (i.e. congener or group of

Table 3.1 PCB congeners and respective homolog groups for PCB analyses conducted on snapping turtle egg and blood tissues.

PCB congener	Homologue group (# of chlorine substituents)	PCB congener	Homologue group (# of chlorine substituents)
4,10	Di-/Di-	136	Hexa-
7,9	Di-/Di-	77,110	Tetra-/Penta-
6	Di-	82,151	Penta-/Hexa-
8,5	Di-/Di-	135,144	Hexa-/Hexa-
19	Tri-	107	Penta-
12,13	Di-/Di-	123,149	Penta-/Hexa-
18	Tri-	118	Penta-
17	Tri-	134	Hexa-
24	Tri-	146	Hexa-
16,32	Tri-/Tri-	132,153,105	Hexa-/Hexa-/Penta-
29	Tri-	141	Hexa-
26	Tri-	137,130,176	Hexa-/Hexa-/Hepta-
25	Tri-	163,138	Hexa-/Hexa
31,28	Tri-/Tri-	158	Hexa-
33,21,53	Tri-/Tri-/Tetra-	129,178	Hexa-/Hepta-
51	Tetra-	187,182	Hepta-/Hepta-
22	Tri-	183	Hepta-
45	Tetra-	128	Hexa-
46	Tetra-	185	Hepta-
52	Tetra-	174	Hepta-
47,48	Tetra-/Tetra-	177	Hepta-
44	Tetra-	202,171,156	Octa-/Hepta-/Hexa-
37,42	Tri-/Tetra-	157,200	Hexa-/Octa-
41,64,71	Tetra-/Tetra-/Tetra-	172	Hepta-
40	Tetra-	197	Octa-
100	Penta-	180	Hepta-
63	Tetra-	193	Hepta-
74	Tetra-	191	Hepta-
70,76	Tetra-/Tetra-	170,190	Hepta-/Hepta-
66,95	Tetra-/Penta-	198	Octa-
91	Penta-	201	Octa-
56,60,92,84	Tetra-/Tetra-/Penta-/Penta-	203,196	Octa-/Octa-
89	Penta-	189	Hepta-
101	Penta-	208,195	Nona-/Octa-
99	Penta-	207	Nona-
119	Penta-	194	Octa-
83	Penta-	205	Octa-
81,87	Tetra-/Penta-	206	Nona-
85	Penta-	209	Nona-

coeluting congeners) was determined by multiplying the mean mass measured in the blanks by a factor of three; sample masses at or below this value are reported as "ND," not detected. Total PCB detection limits ranged from 7.2-25.7 ng for the egg analyses from 8.4-22.9 ng for the blood analyses. The method detection limit was calculated for each congener in the samples on a concentration basis by dividing the detection limit mass by the mass of sample extracted. Total PCB (t-PCB) concentrations were calculated as the sum of the concentrations of the individual congeners.

The efficiency and accuracy of our sample processing was monitored by tracking the recovery of surrogate congeners, as well as by employing laboratory blanks spiked with PCB standard mixture and extracting standard reference materials. For the egg samples, the mean ± 1 SD of the recovery of surrogate congeners were 84.7 ±18.9 % for congener 14, 84.0 ± 9.0 % for congener 65 and 89.2 ± 10.6 % for congener 166. For the whole blood samples, surrogate recoveries were 46.8 ± 22.8% for congener 14, 62.7 ± 23.9 % for congener 65 and 76.5 ± 22.5 % for congener 166. Reported PCB concentration values for all samples were not corrected for surrogate recoveries. The mean ± 1 SD recoveries of PCB congeners spiked into laboratory blanks were 104.3 ± 62 % for those run with the egg samples and 80.7 ± 25 % for those run with the blood samples. The high standard deviation for the spiked blanks run with the egg samples can be attributed to elevated recovery levels (greater than 150%) for eight congeners; exclusion of these results in a mean recovery of 87.4 ± 20% of the spiked PCBs.

The standard reference material used for the snapping turtle egg analyses was "Lake Superior Fish Tissue" (1946), prepared by the National Institute of Standards and Technology (NIST). PCB concentrations measured in this material were 91.6 ± 22%.

The standard reference material used for the snapping turtle whole blood analyses was "PCBs, Pesticides, and Dioxins/Furans in Human Serum" (1589a), also prepared by the National Institute of Standards and Technology. Analysis of this material resulted in PCB concentrations averaging 109.8 ± 21.8% of the certified values.

Statistical analyses

Minitab version 13 software was used for all statistical tests. Examination of the total PCB concentration data for eggs and blood revealed marked non-normality and heteroscedasticity that was not fully controllable with $\log_{10}$ or other data transformations. Randomized resampling techniques (e.g. Manly 2007) were employed for one-way ANOVA analysis of total PCB concentration data, using 10,000 resampling iterations. Resampling regression analyses were used to examine the relationship between tPCB concentrations and such factors as lipid content and clutch characteristics. Because of the great difference in scale of tPCB concentrations between the factor levels (study area and/or gender depending on tissue type), it would be expected than any relationships would be more apparent within factor levels (i.e. contaminated eggs, reference female blood). For this reason, separate regression analyses were run on the data sets for each factor level, again using 10,000 resampling iterations. This analysis does not provide an r^2 value as part of the output, such that the only reported outcome of these analyses are the p-values related to the null hypothesis that the response variable is independent of the predictor. Comparisons of relative contributions of PCB homolog groups were made using parametric MANOVA (multivariate analysis of variance) with the associated univariate comparisons within each homolog group.

Data on maternal transfer by individual female turtles to their eggs was only gathered in 2004, since chemical analyses on blood samples were not conducted in 2005. Despite having large sample sizes at both study areas for adult females in 2004, there was an unfortunately low number of females from which blood and eggs could be collected, with four from the contaminated area and three from the reference area. Due to small sample size, data from these seven females were analyzed together rather than separately by study area. In addition to scrutiny of those paired data, examination of the overall homolog pattern differences in mean PCB burdens between female adults and eggs were also used to describe maternal transfer of PCBs at each study area. Comparison of PCB homolog group patterns between female turtles and eggs was conducted on the seven sample pairs, as well as on the data for all females and all eggs from each site. The low sample sizes for the paired data ($n = 4$ for contaminated area and $n = 3$ for the reference area) precludes formal statistical analyses, but MANOVA testing was conducted on the full data sets for females and eggs from both study sites with the data were analyzed separately for each study area in order to examine potential differences in maternal transfer. For all statistical analyses, significance was accepted at the $\alpha = 0.05$ level.

Results

Eggs

Table 3.2 contains the total PCB (tPCB) concentrations in snapping turtle eggs for both years. As regression analysis confirmed that egg lipid content was not a significant determinant of the variability in tPCB concentrations (Figure 3.1), concentrations based upon egg wet weights are presented and used for statistical analysis. In both years, tPCB in eggs from the contaminated area were significantly higher than in those from the reference area. In 2004 and 2005, average concentrations in the contaminated site were approximately 80 and 22 times greater respectively than in the reference area.

Table 3.2. Concentrations of total PCBs (ng/g ww) in eggs collected in 2004 and 2005. Values are Mean ± 1 SD (range).

	2004	**2005**
Reference	56.4 ± 26.6 (12.4, 86.5)	50.4 ± 23.8 (3.9, 81.3)
Contaminated	4578 ± 3672 (75, 10553)	1105 ± 1254 (56,3323)
	$P < 0.001$	$P = 0.041$

The relative percent contribution of all the measured congeners to the total PCB concentration, averaged across all egg samples for each study area, is shown in Figure 3.2. Of the 78 congeners or coeluting groups of congeners included in analysis of total PCBs in eggs, four were highly dominant, making up 45.5% of the total in the reference are eggs and 51.3% of the total in the contaminated area eggs. At both study areas, the coeluting congeners 132/153/105 were by far the highest, comprising 20.5% and 24.5% of the tPCB concentrations of eggs from the reference and contaminated areas, respectively. The next dominant congeners in eggs from both study areas were 163/138

(11.2% of total at both areas), 118 (6.8% of total in reference eggs and 10.0% of total in contaminated eggs), and 180 (5.6% of total in reference eggs and 7.0% of total in contaminated eggs).

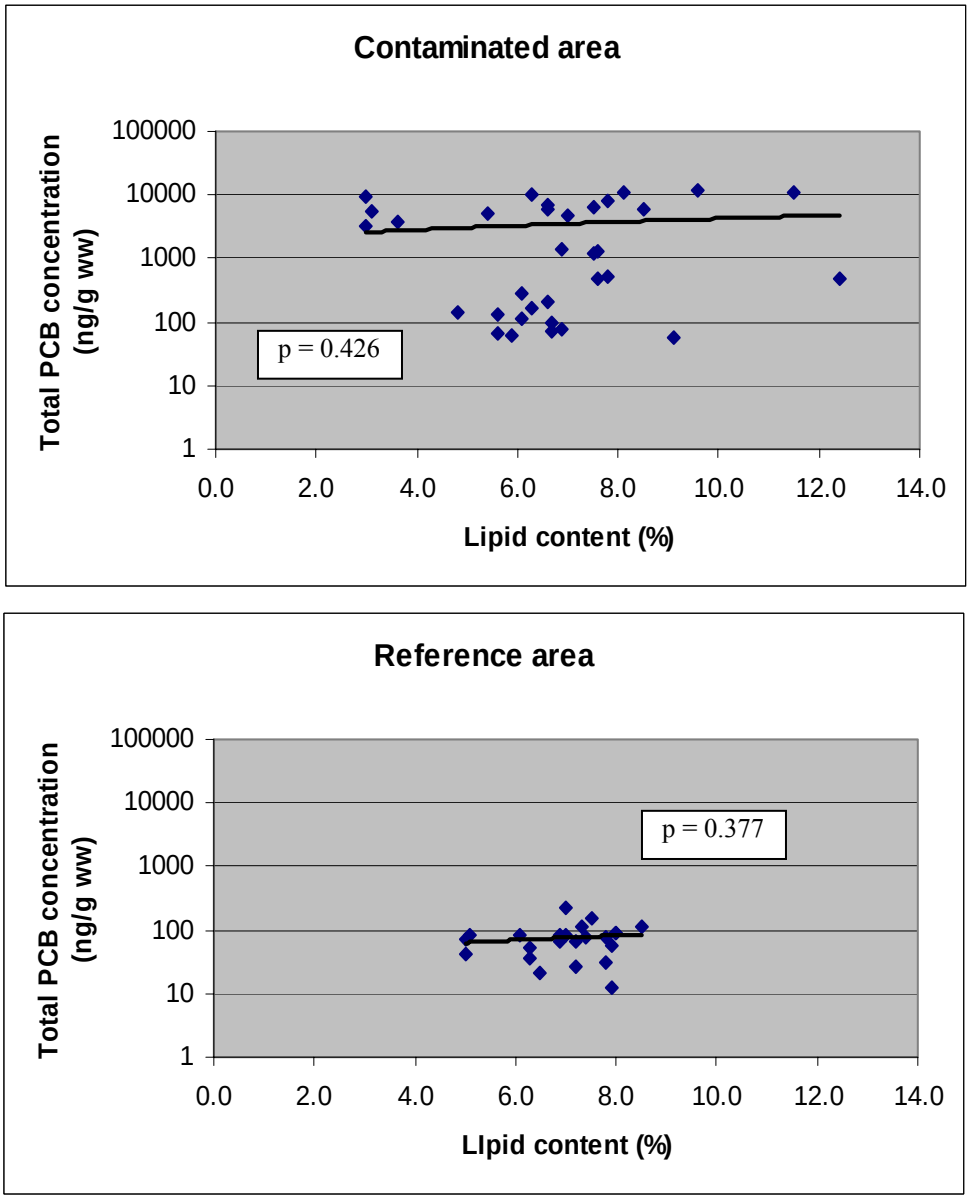

Figure 3.1 Total PCB concentrations in eggs vs. lipid for both study areas over 2 years combined.

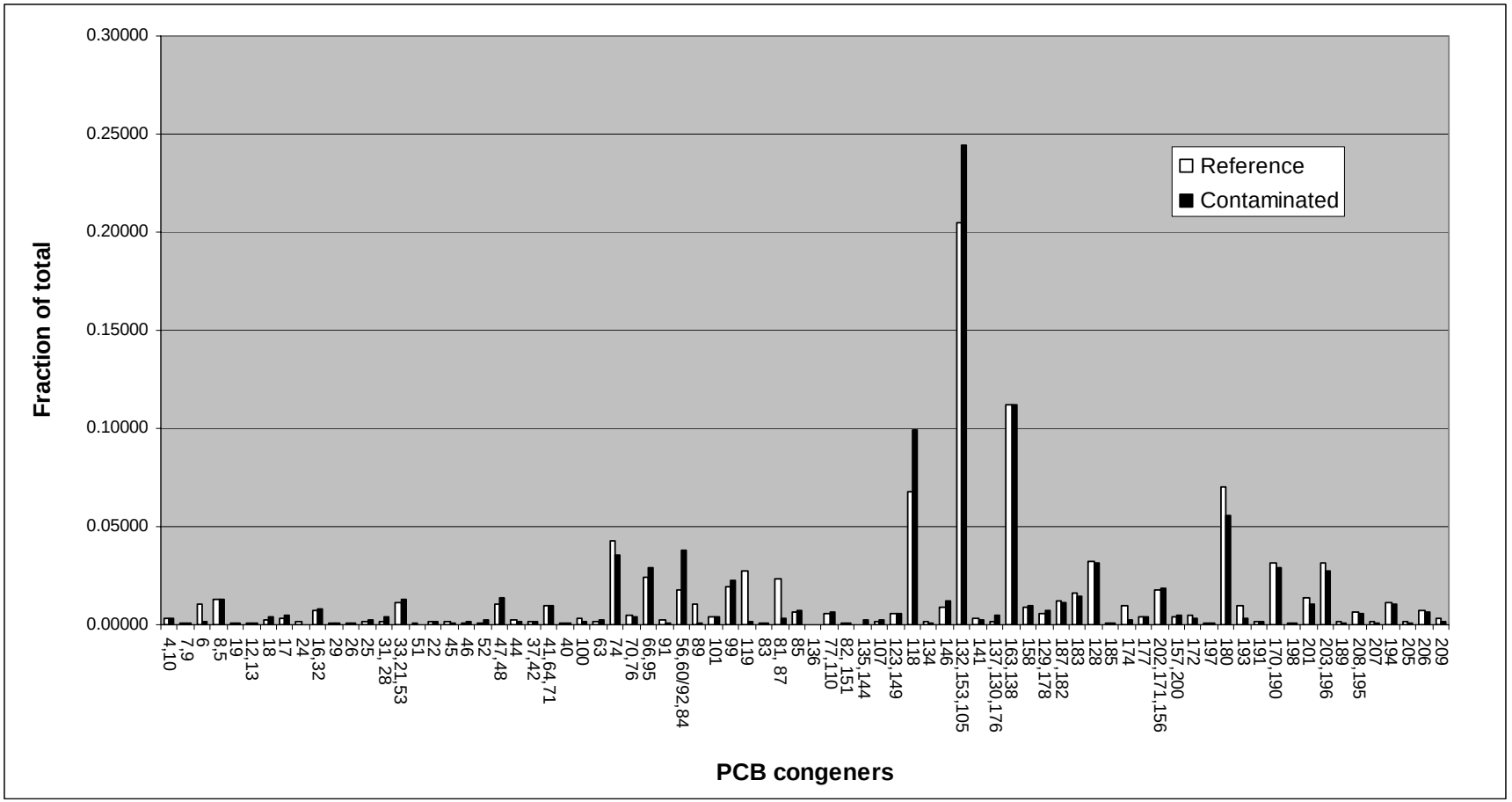

Figure 3.2 Mean relative contributions of individual congeners and co-eluting congeners for eggs from both study areas.

Blood

Table 3.3 contains the data for blood tPCB concentrations on a sex-specific basis, for all turtles sampled from the contaminated and reference study areas. Blood PCB concentrations were measured only in adults captured in 2004. Lipid content of whole blood ranged from 0.13 – 1.27% and was not significantly different between turtles from the two study areas, or between sexes. There was no significant relationship between whole blood lipid content and tPCB for either sex at either site (Fig. 3.3).

Table 3.3. Total PCBs in whole blood, 2004. Values are means $[PCB]_{Total}$ (ng/g ww) ± 1 SD.

	Females	**Males**
Contaminated	119.9 ± 31.9	450.3 ± 188.0
Reference	3.9 ± 0.5	60.7 ± 2.4
	$P < 0.001$	$P = 0.041$

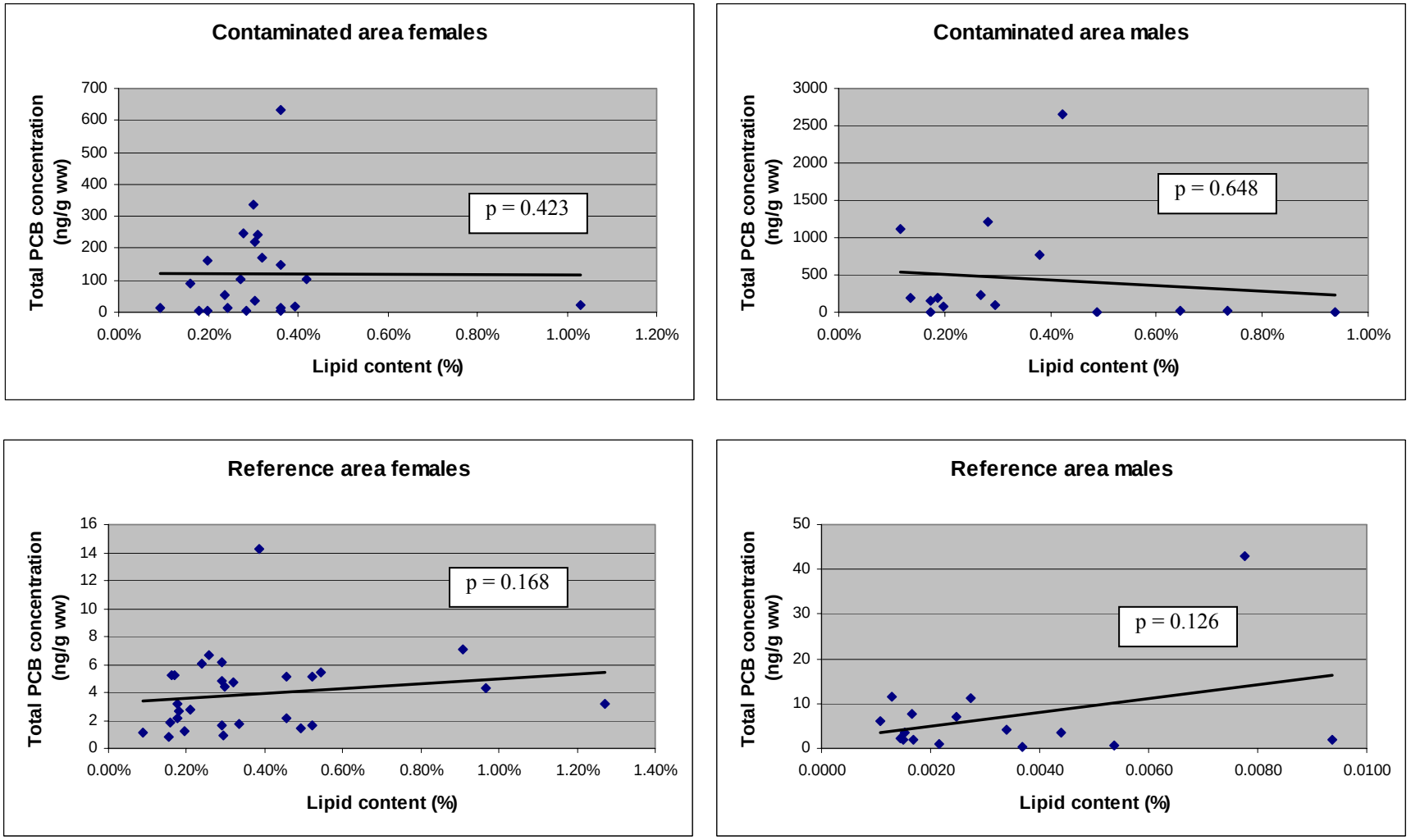

Figure 3.3 Total ww PCB concentrations in turtle blood vs. lipid content for each gender at both study areas.

At both study areas, male turtles had higher blood tPCB concentrations than females. At the reference site, the mean blood tPCB concentrations for males and females are 6.70 and 3.91 ng/g ww, respectively, a difference that is not statistically significant. The discrepancy is even higher in the contaminated area, where mean blood tPCB concentrations are 450.3 ng/g ww in males and 119.9 ng/g ww in females, a 3.8-fold difference that is statistically significant ($p = 0.020$). Total blood PCB concentrations from contaminated area turtles was significantly higher than from reference area turtles in both males ($p = 0.0001$) and females ($p = 0.0001$). As with the eggs, animals from the contaminated study area displayed a much greater degree of inter-individual variability in PCB body burdens, most notably in the males, with the most contaminated turtle having a tPCB concentration over 600-times greater that of the least contaminated turtle. There was no significant relationship between body size (carapace length, CL) and blood tPCB concentrations for either sex at the reference study area (Figure 3.4). In contrast, there was a significant positive relationship between body size and blood tPCB concentrations in both male turtles ($p = 0.005$) and female turtles ($p = 0.009$) at the contaminated study area. (Figure 3.4).

Figure 3.5 shows the relative percent contribution of all the measured congeners to the total PCB concentration, averaged across all blood samples for each gender at both

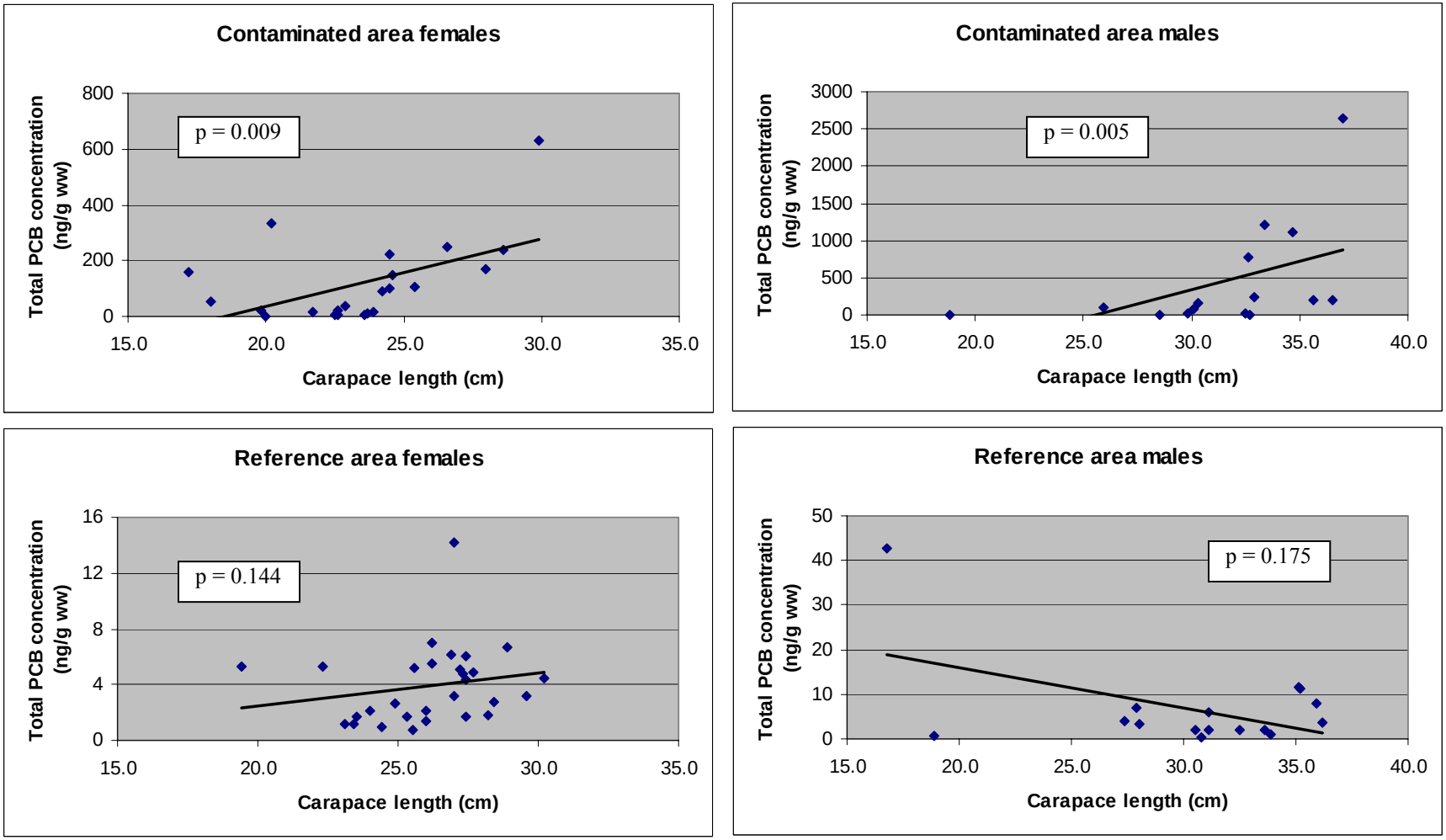

Figure 3.4 Blood total PCB concentrations vs. carapace length for female and male turtles at both study areas. P-values indicate the significance of the relationship between the two factors.

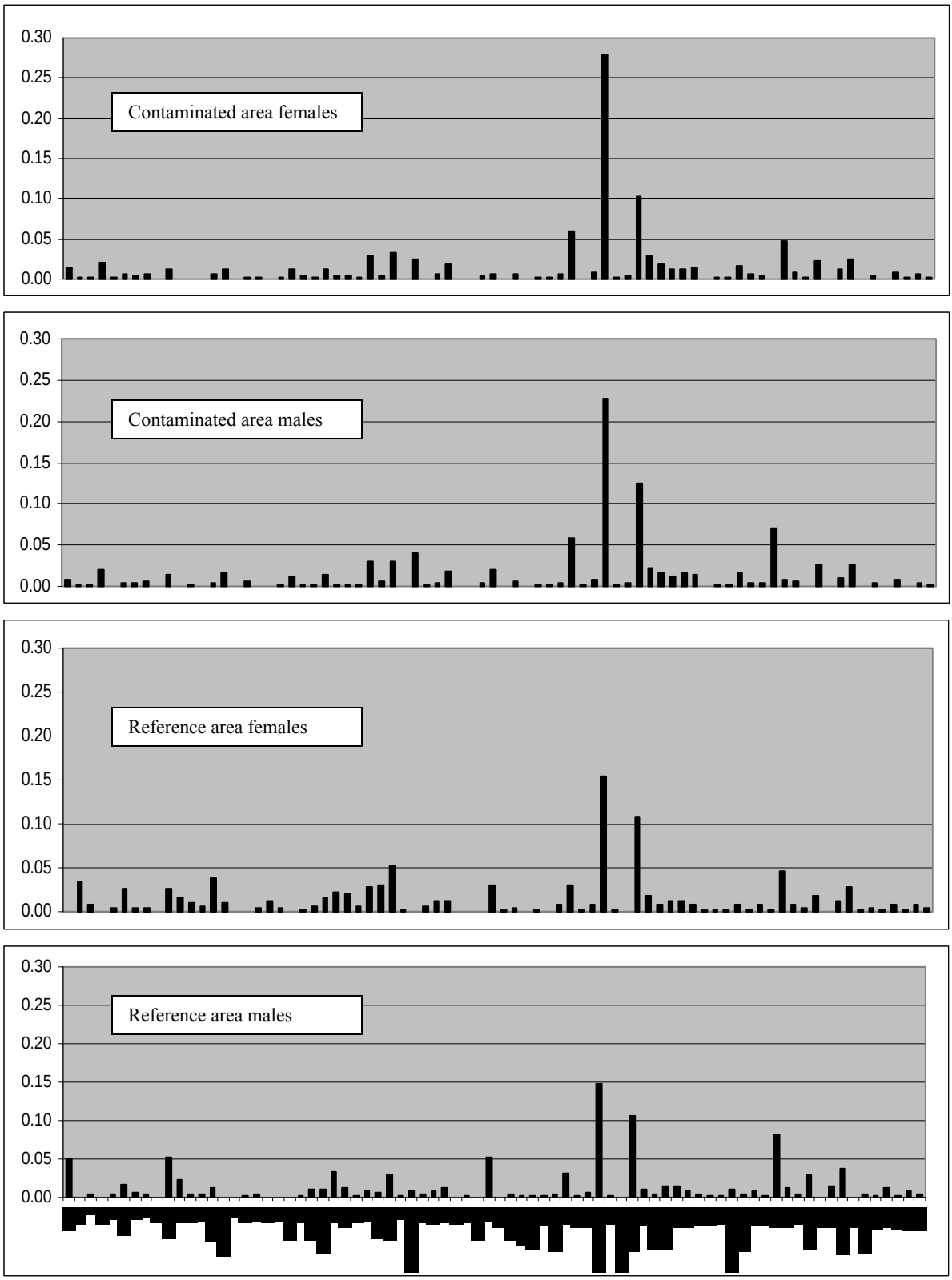

Figure 3.5 Relative contributions of individual congeners and co-eluting congeners for blood from turtles of each gender at both study areas.

study areas. The coeluting congeners 132/153/105 had the highest contribution, followed by 163/138, 118, and 180. At the reference area, the pattern is somewhat different, with congeners 132/153/105 dominating, followed by 163/138 and 180, but congener 118 is lower than several other congeners. In the reference area females, congeners 66/95 are the fourth most dominant contributor toward total mean concentrations, while in reference area males, congeners 4/10, 16/32, and 85 each comprise approximately 5% of the total.

Homolog group distributions for all turtle blood samples (regardless of sex) for each study area are presented in Figure 3.6. There is a significant difference between the two study areas with regards to the overall homolog patterns ($p<0.0001$), with turtle blood from the contaminated area strongly dominated by penta and hexa-homologs and samples from the reference area dominated by penta hexa, and hepta-homologs. The relative contributions of tetra and penta-chlorinated congeners were significantly higher in turtles from the contaminated area, while that of the tri, hepta, nona and deca homolog groups were significantly higher in the reference turtles. Figure 3.7 illustrates the same data separated by sex with intra-homolog group comparisons made between the sexes at each study area. For both male and female turtles inhabiting the contaminated site, the PCB homolog pattern is dominated by the penta and hexa homologs. The overall pattern of homolog distributions is significantly different between contaminated area males and females ($p = 0.002$), with hepta-chlorinated congeners significantly higher in females and octa-homologs significantly higher in males. At the reference study area, the homolog order of dominance in males is hepta, followed by hexa and penta homologs, while the

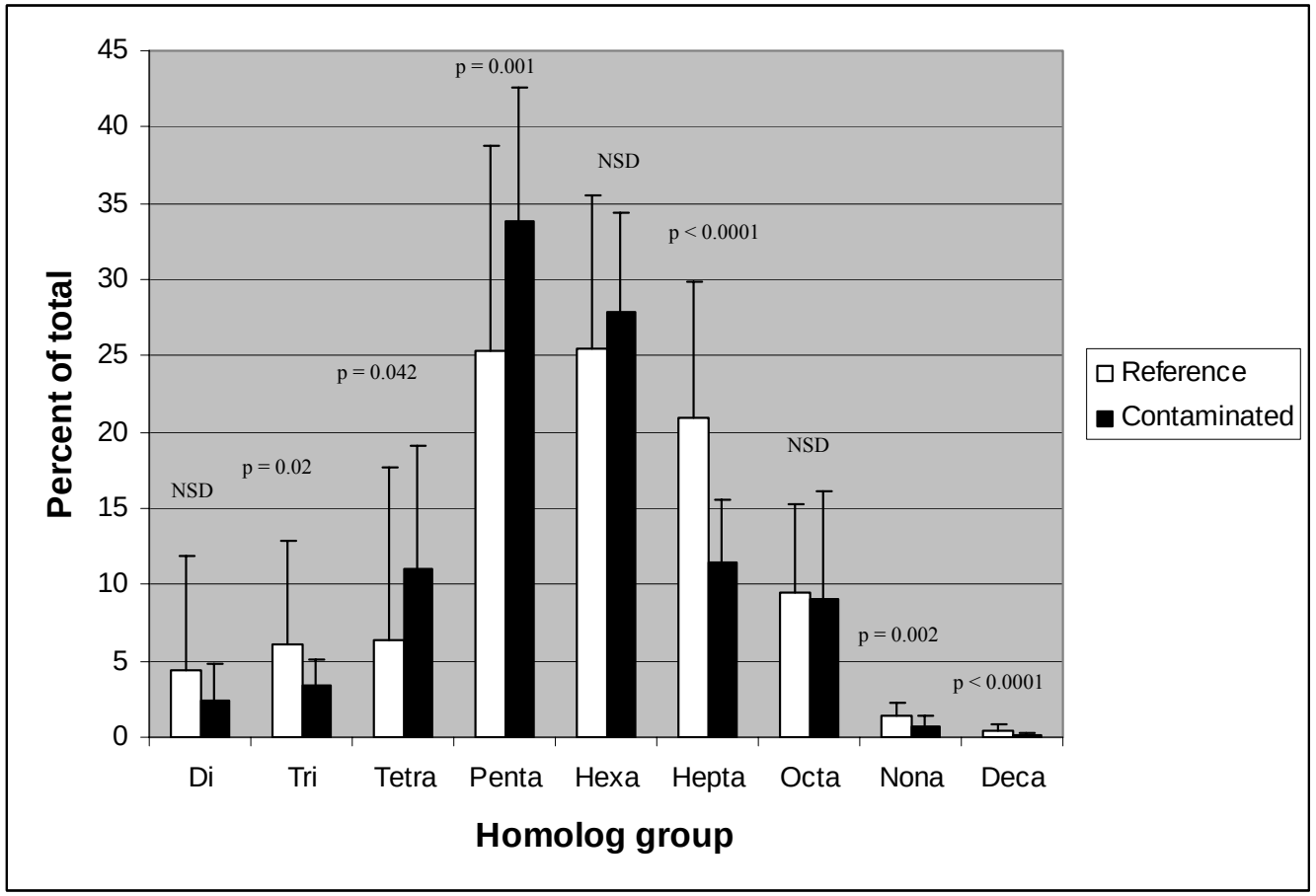

Figure 3.6 Homolog group profiles of whole blood of all turtles from contaminated and reference areas.
NSD = Not significantly different.

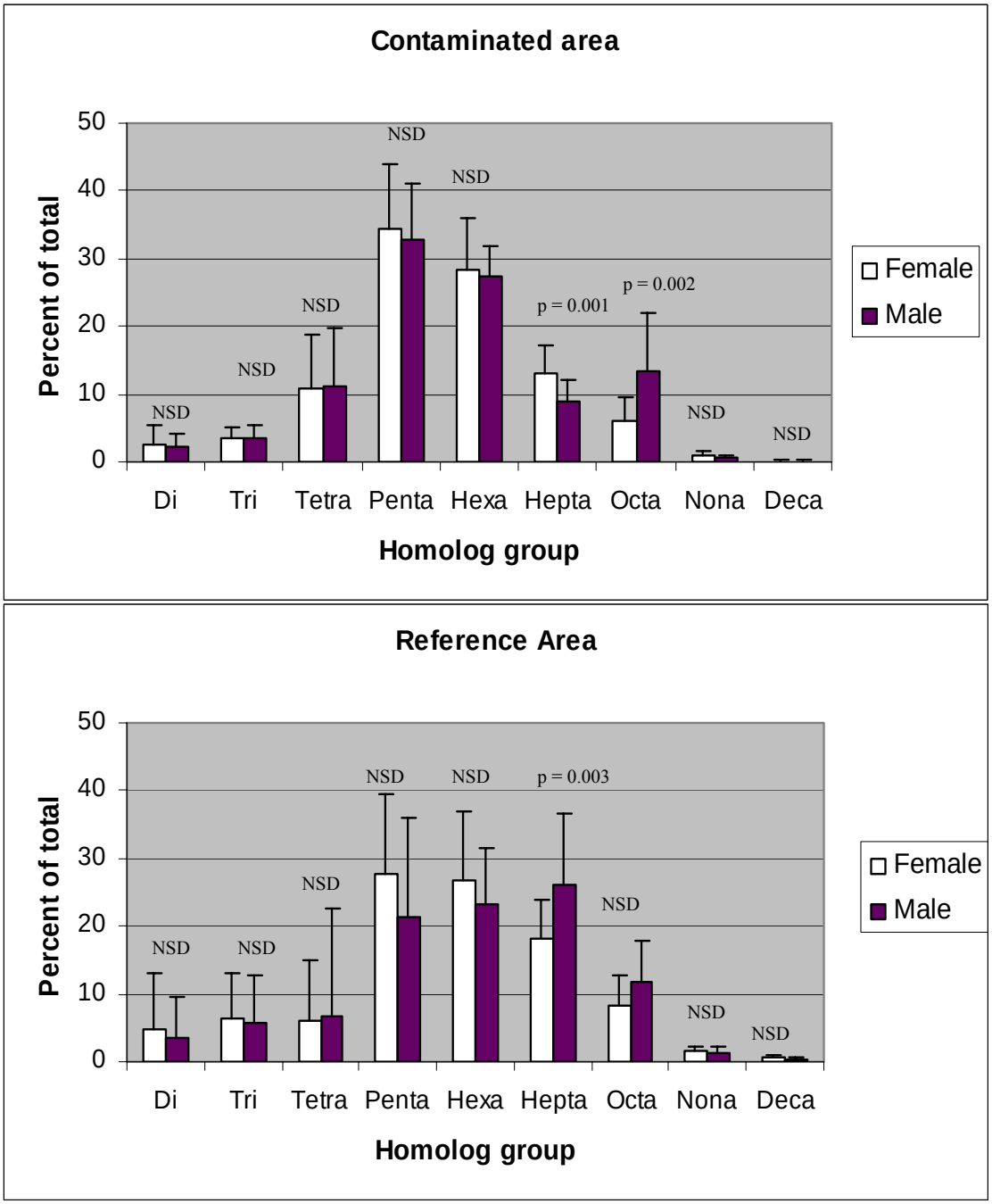

Figure 3.7 Comparison between genders at each study area with respect to PCB homolog group profiles in turtle whole blood. NSD = Not significantly different.

pattern in females is equally dominated by penta and hexa, followed by hepta homologs. There is a significant difference in the overall homolog group patterns between male and female turtles at the reference site ($p = 0.003$). Hepta-chlorinated homologs were significantly higher in reference males than females. The overall pattern of homolog group contribution was significantly different between the two study areas for each sex (Figure 3.8; $p<0.0001$ for both tests). In female turtles, hepta through deca homologs were significantly higher at the reference area and penta-chlorinated congeners were significantly higher at the contaminated area. Only two of the homolog groups were significantly different between the male turtles at each site: hepta homologs were significantly higher at the reference site while penta homologs were significantly higher at the contaminated site.

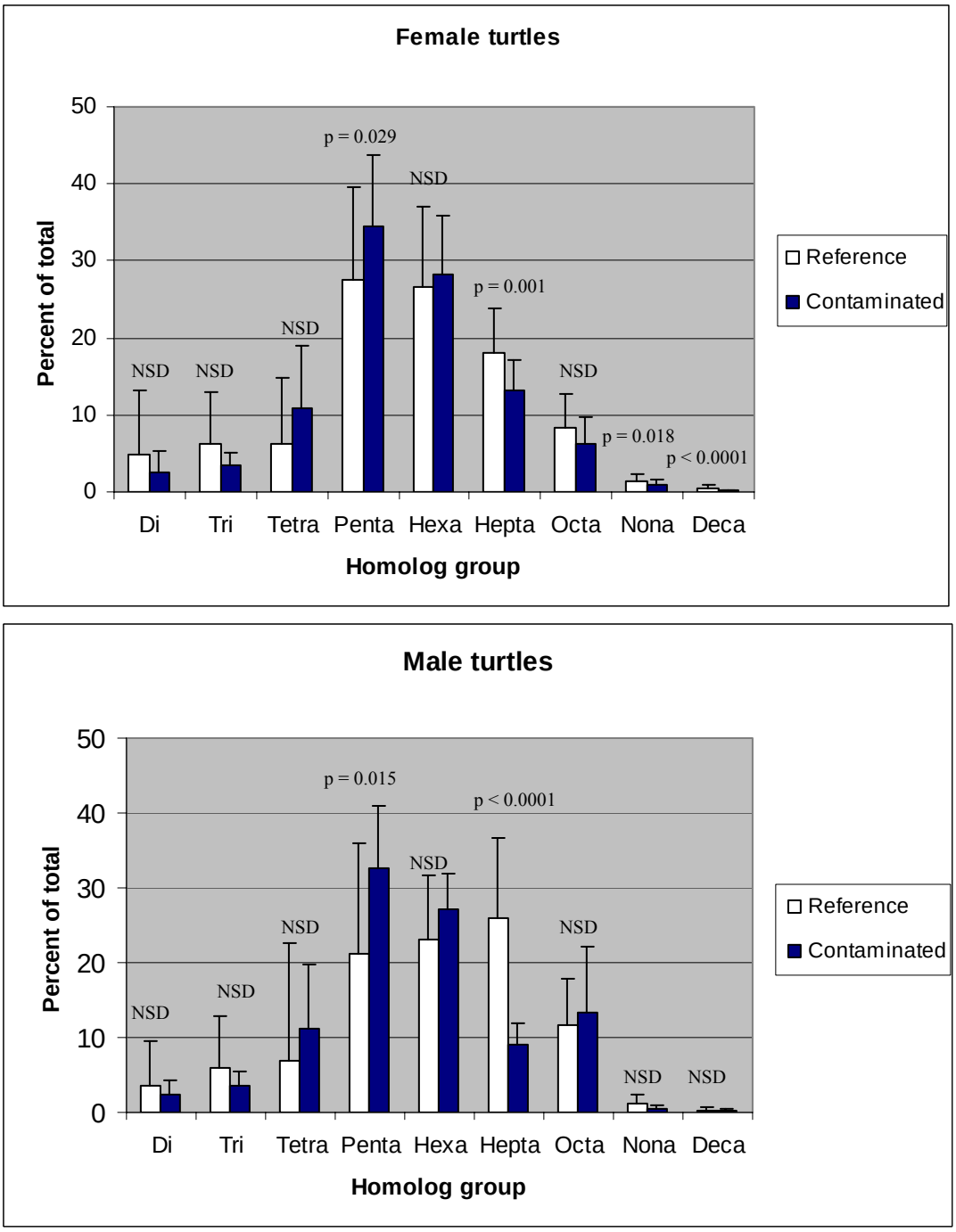

Figure 3.8 Comparison between study areas for each gender with respect to PCB homolog group profiles in turtle whole blood. NSD = Not significantly different.

Maternal Transfer

The relationships between lipid content and PCB levels in maternal blood and eggs in seven snapping turtles was examined using regression analyses. Although there is a trend of increasing lipid content in eggs with an increase in that of the mother's blood, the relationship is not significant (Figure 3.9). There was a significant positive relationship ($p = 0.002$), however, between lipid normalized tPCB concentrations in the whole blood and eggs of female turtles, shown in Figure 3.10. The ratios of tPCB (ng/g lipid) between eggs and whole blood ranged from 0.514-3.21 (mean = 1.33, SD = 0.958 and were not significantly different between the reference and contaminated areas ($p = 0.767$). Egg-blood ratios were also calculated for the lipid-normalized concentrations of the 14 congeners and/or coeluting congeners detected in all paired samples and are presented in Table 3.4. With the exception of coeluting congeners 12/13, there is a significant positive relationship between congener-specific concentrations in the two tissue types. The large standard deviations for the egg-blood ratios of the 14 congeners is primarily due to the influence of one of the sample pairs, which had the lowest recorded egg lipid content (3%), raising the lipid-normalized concentrations and thus the egg-blood ratio well above that of the other samples. Nonetheless, the general pattern for these congeners is an approximate 1:1 lipid-based concentration ratio between the mother turtle's blood and that of her eggs.

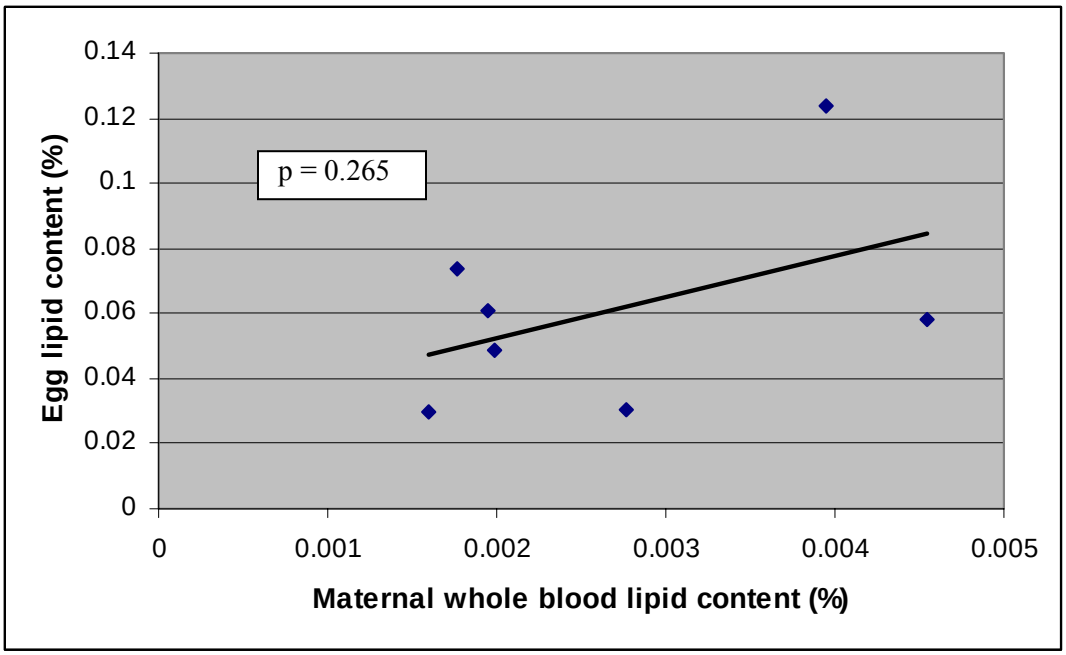

Figure 3.9 Maternal whole blood vs. egg lipid content for seven female turtles in 2004. P-value indicates the significance of the relationship between the two factors.

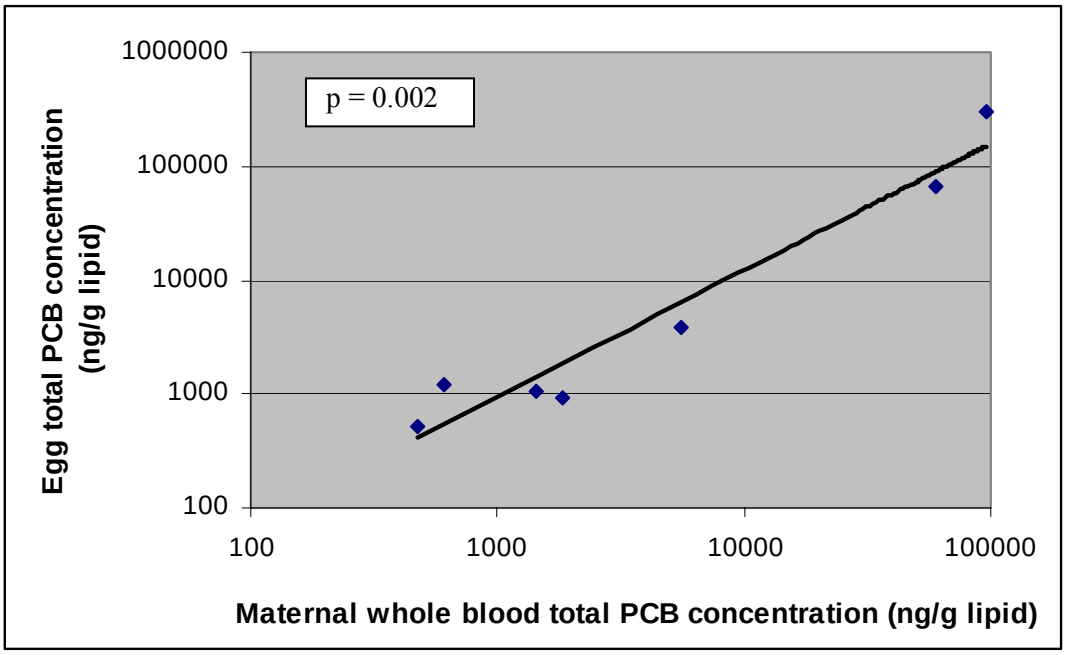

Figure 3.10 Maternal whole blood total PCB concentrations vs. egg total PCB concentrations for seven female turtles in 2004. P-value indicates the significance of the relationship between the two factors.

Table 3.4 Egg to blood ratios for the 14 congeners and coeluting congeners detected in the seven female turtles sampled for eggs and blood. Ratios are mean values ± SD and p-values indicate the significance of the relationship between the two factors based on regression analysis.

Congener	Egg-blood ratio	p-value
12/13	0.638 ± 1.04	0.081
18	0.248 ± 0.313	0.011
132/153/105	1.41 ± 1.42	0.006
163/138	0.945 ± 0.898	0.007
183	1.16 ± 0.809	< 0.0001
128	1.85 ± 1.48	0.001
202/171/153	1.08 ± 0.758	< 0.0001
180	0.951 ± 0.581	< 0.0001
170/190	1.02 ± 0.588	< 0.0001
201	0.844 ± 0.591	< 0.0001
203/196	0.847 ± 0.513	< 0.0001
208/195	0.810 ± 0.578	< 0.0001
194	0.839 ± 0.435	< 0.0001
206	0.659 ± 0.411	< 0.0001

The homolog group contribution patterns for the seven sample pairs and for all female blood and egg data at each study area are shown in Figure 3.11. At both study areas, the same pattern exists in homolog contribution patterns between eggs and maternal blood, but is more exaggerated for the paired samples. The only homolog group in which there was a significant difference at the contaminated study area was the di-chlorinated group, which was higher in maternal blood ($p = 0.002$). At the reference area, maternal blood is more enriched with tri ($p = 0.003$) and nona ($p = 0.025$) homologs than eggs, while eggs are higher in tetra ($p = 0.01$) and penta ($p = 0.025$). Despite the lack of significant differences in the other homolog groups, the overall pattern at both study area is that eggs are relatively enriched with tetra and penta homologs compared to the maternal blood, with all other homolog groups either equal in both tissues or higher in the females.

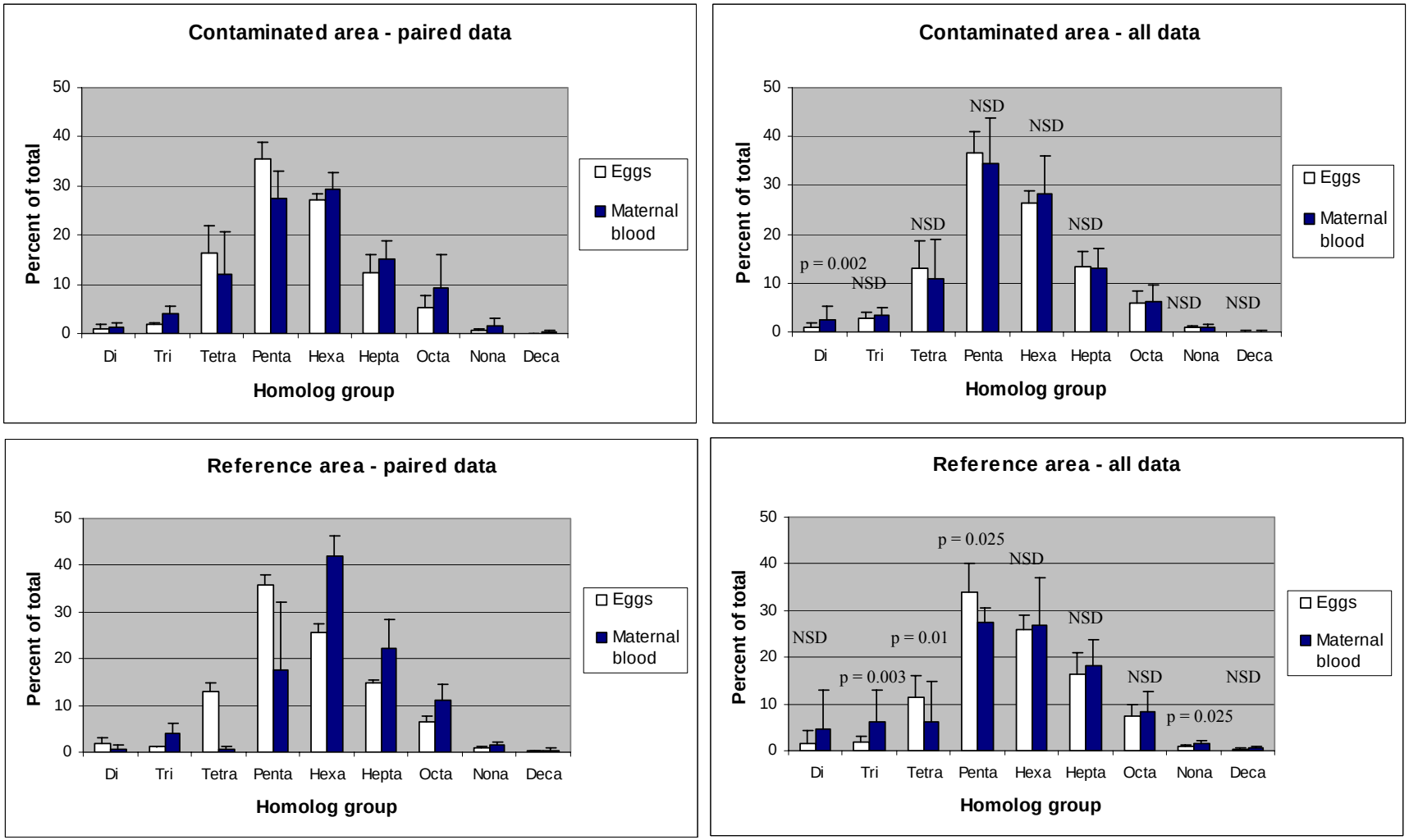

Figure 3.11 Homolog group contribution in maternal blood and eggs in paired samples and all data from both study areas.

Discussion

Total PCB Concentrations in Eggs and Blood

Based on the life history and longevity of the snapping turtle in conjunction with the long-term PCB contamination of the upper Hudson River, we expected that adult snapping turtles and eggs sampled from the area would have high concentrations of PCBs. This was certainly the case, as eggs and adult whole blood from turtles in the contaminated area had PCB concentrations exceeded those from the reference area by over an order of magnitude. Eleven snapping turtle nests sampled for PCBs by the Hudson River Natural Resource Trustees (HRNRT) in 2002 from an area of the Hudson River encompassing all but the southernmost few kilometers of our designated contaminated area were found to have average tPCB concentrations 2 to 3 times higher than the averages we measured. Six clutches analyzed from the upper Hudson River in 1978 (Stone *et al.* 1980) had average tPCB concentrations in nearly 10 times greater than the our average concentrations. The tremendous range of total PCB concentrations measured in snapping turtles eggs collected from the same limited area in these studies, however, indicates that individual turtles can accumulate very different concentrations of contaminants, and is likely only a matter of chance that we did not collect eggs with even higher tPCB levels. Nonetheless, the data from these three studies do seem to indicate a temporal decrease in tPCB concentrations in snapping turtle eggs in the contaminated area of the upper Hudson River, which is to be expected given that inputs into the river system have decreased since 1980. The relatively low tPCB concentrations that we measured in the eggs collected from the reference area display a much smaller degree of intra-site variation and reflect background contamination due primarily to atmospheric

transport and deposition into aquatic systems. The 2002 HRNRT study designated two reference regions: an "Upstream Reference Region" consisting of a 36 mile stretch of the Hudson River upstream from the GE plants and an "Other Reference Region" of various ponds and streams located in several surrounding counties. While none of these reference waterways coincides with any of the sampling locations in our designated reference area, all of the sites are removed from the contaminated sediments of the Hudson River as well as any known point sources of PCBs, and thus are comparable with respect to background contamination of the region. Total PCB concentrations measured in the HRNRT study ranged from 32-565 ng/g ww in the Upstream Reference Region and from 10-57 ng/g ww in the Other Reference Region, values which are in general agreement with our data. The highest of PCB measurements in these reference clutches may be the result of females foraging in highly localized areas of contamination within an otherwise clean aquatic environment due to improper disposal of PCB-containing materials. Local residents reported that it was not an uncommon practice to throw old electronic and industrial equipment into waterways.

The data that we collected on total concentrations of PCBs in whole blood of adult snapping turtles reinforces the utility of this nondestructive method for monitoring of contaminant exposure in freshwater systems. As with the turtle eggs, adult whole blood tPCB concentrations in the reference area were relatively low with a small degree of intra-site variability, indicative of background contamination due primarily to atmospheric deposition. Interestingly, the highest blood tPCB concentration measured at the reference area (42.8 ng/g ww) was that of a very small male collected from a lake which yielded several larger turtles with much lower concentrations. While it is

impossible to know this animal's history, it was possibly either transplanted from elsewhere by humans or encountered a highly localized point source within the lake. At the contaminated area, a relatively limited area of the uppermost region of the Hudson River Superfund Site, an even greater range of tPCB body burdens was detected in adult whole blood than in the population's eggs. Formal spatial analyses cannot be conducted on our findings as the study was not designed for such purposes, however a review of individual tPCB measurements from specific locations within the contaminated study area reveals some trends. Not unexpectedly, the lowest PCB concentrations measured in both eggs and whole blood samples were found in those collected in the waterways adjacent to but not directly connected to the river proper. However, one of these waterways, a eutrophic pond located approximately 40 meters from the river's edge, was the site of egg collections with PCB concentrations ranging from the lowest recorded in the contaminated study area (55.9 ng/g ww) to one of the highest (7885.2 ng/g ww). Turtles captured on Griffin Island, located in the Hudson River just north of the hotspots of the Thompson Island pool, had the highest maximum whole blood PCB concentrations in both males (2647.5 ng/g ww) and females (631.8 ng/g ww).

There are several ecological, physiological and environmental factors driving this pronounced intra-site variability, any one or more of which may affect a given individual. As omnivores, snapping turtles consume a diet that is approximately one-third plant material, one-third fish species, and one-third a wide array of other animals including birds, amphibians, snakes, turtles, insects, mollusks, crustaceans, annelids, and gastropods (Alexander 1943; Punzo 1975). This liberal diet, which encompasses a range of bioaccumulation risks, from very low (e.g. plants, short-lived insects) to very high

(e.g. other turtles and long-lived fish), in conjunction with individual variation in feeding locations and preferences, would be expected to produce such variability in snapping turtle PCB body burdens. The metabolic capacities of individual turtles may also differentially influence the PCB body burdens. Furthermore, the well-characterized heterogeneity of sediment contamination of the upper Hudson River will produce prey items of a large range of PCB levels, as well as vary direct PCB exposure from incidental sediment consumption. Given these numerous influential factors, it is not surprising that we observed such variability in PCB concentrations of snapping turtles in the contaminated area.

Although the difference was significant only at the contaminated area, male turtles at both study areas had higher tPCB levels in whole blood, likely a result of two phenomena. First, male turtles grow to a larger size than females and thus can be expected to accumulate higher concentrations of PCBs from the environment. As well, male turtles do not transfer significant portions of their body burdens to offspring as do females when producing large, lipid-rich egg clutches. While females experience an annual cycle of contaminant depuration via reproduction, the body burden of males continues to increase with age.

PCB concentrations in eggs and blood of snapping turtles inhabiting the upper Hudson River region are comparable to PCB concentrations in some of the other vertebrates at the top of the food chain of this riparian system and lower than others. Mammalian species in the area that have been monitored for PCB concentrations from 1989-2000 include omnivorous muskrats and carnivorous otters and mink (Mayack and Loukmas 2001). Liver PCB concentrations range from non-detectable to 2.18 μg/g lipid

in muskrats and from 0.154-139 µg/g lipid in mink. The largest of the three species, otters have diets very similar to snapping turtles (minus plant material) and were found to have liver PCB concentrations in the range of 18.5-431 µg/g lipid. Eggs of tree swallows, an insectivorous bird, sampled at two locations along the stretch of river comprising my contaminated study area were found to have mean PCB concentrations of 24 and 13 µg/g ww, while adult whole body PCB concentration at one of the sites was extremely high at 152 µg/g ww (Echols *et al.* 2004). In 2002, HRNRT collected eggs for PCB analysis from 11 avian species of diverse feeding regimes from the an area of the Hudson River encompassing all but the southernmost few kilometers of our designated contaminated area (HRNRT 2005a). The PCB concentration data for all species combined ranged from 65-56,200 ng/g ww, with a mean of 7,620 ng/g ww. The species with the highest egg PCB loads were the insectivorous spotted sandpiper (mean PCB concentration 15,200 ng/g ww) and the piscivorous belted kingfisher (mean concentration 13,900 ng/g ww). Similar to our data, within-species variability in PCB concentrations of this area of the Hudson River was very high, reflecting differential accumulation within a limited geographical location.

Due to their longevity, large size, and widespread occurrence, snapping turtles have been by far the most extensively studied of the turtle species with respect to contaminant exposure, while their distributions and life history also result in higher accumulations of organic contaminants than other turtles. For example, studies of PCBs body burdens in loggerhead sea turtles (*Caretta caretta*) have found concentrations comparable to those in our reference area samples: 89 ng/g ww in eggs collected in the Mediterranean (McKenzie *et al.* 1999), 1188 ng/g lipid in eggs collected on Florida's

Atlantic coast (Cobb and Wood 1997), and 8.9 ng/g ww in plasma from juvenile collected in the Outer Banks of North Carolina (Keller *et al.* 2004). Researchers have used an array of snapping turtle tissues for measuring PCB concentrations, including fat, eggs, testes, brain, liver, kidney, muscle, pancreas, lung, and blood or plasma (listed in decreasing order of lipid content) (Bishop *et al.* 1995; Bryan *et al.* 1987). Some of the highest body burdens ever recorded for wildlife have been in snapping turtle tissues, including those from the contaminated area of the upper Hudson River; most notably, Stone *et al*. (1980) reported PCB levels in adipose tissue of adult snapping turtles from upper Hudson River as high as 7990 μg/g lipid.

Canadian researchers associated with Environment Canada and University of Guelph in Ontario who have been studying contaminant burdens in snapping turtles inhabiting contaminated Areas of Concern (AOCs) in the Great Lakes-St. Lawrence River basin of Ontario since 1981 have reported two instances of extremely high PCB concentrations in egg tissues. Ashpole *et al.* (2004) detected 61.0 μg/g ww (1034 μg/g lipid) PCBs in a single clutch from Turtle Creek on the south side of the St. Lawrence River collected in 1999, while another solitary clutch sampled in the previous year by de Solla and (2001) was found to have an astonishingly high tPCB concentration of 737.7 μg/g ww (5083 μg/g lipid), almost 1% the mass of the egg. While these two findings are much greater than the highest value we measured in turtle eggs from the contaminated area (12.1 μg/g ww or 126.5 μg/g lipid), they are confined to one waterway with a very sparse snapping turtle population. These concentrations are also exceptional compared to the numerous samples collected by the same researchers in nearby Canadian Great Lakes-St. Lawrence River basin AOCs; mean total PCB concentrations in these studies

generally range from 0.34-11.0 µg/g ww (Ashpole *et al.* 2004; Bishop *et al.* 1996, 1998; Bonin *et al.* 1995; de Solla *et al.* 2001; Struger *et al*. 1993), similar to our results for contaminated-area eggs. The study design of several of these studies incorporates data on PCBs in snapping turtle eggs from comparative reference areas known to be relatively uncontaminated. The reported total PCB concentrations in eggs from reference areas ranged from 15.7-272 ng/g ww (Ashpole *et al.* 2004; Bishop *et al.* 1998; de Solla *et al.* 2001; Struger *et al*. 1993), also very similar to the concentrations that we measured in eggs collected from our reference study area.

Besides the aforementioned studies describing PCB accumulation in snapping turtle eggs in the Ontario area AOCs and the upper Hudson River, two other studies in the literature specifically report PCBs in snapping turtle eggs in other locales. Pagano *et al*. (1999) reported tPCB concentrations in snapping turtle eggs from six females collected from five diverse western and central New York sites ranging from 1.1-310.0 µg/g lipid, a range encompassing the combined lipid-normalized data from both of our study areas (see Table 3.2). In the most recent study reporting PCB concentrations in snapping turtle eggs, Dabrowska *et al*. (2006) found that specimens collected from four AOCs on the U.S. side of Lake Erie basin in Ohio had mean tPCBs concentrations ranging from 873-3683 ng/g ww, similar to our findings, although not reaching the highest concentrations that we observed. The reported mean tPCB concentrations at two reference sites in the Dabrowska *et al*. (2006) study were 183 and 352 ng/g ww.

In contrast to the numerous studies documenting PCB accumulation in snapping turtle eggs, relatively few studies of PCBs in snapping turtle blood exist in the literature. It should be noted that while these other studies measured PCBs in snapping turtle plasma

rather than whole blood, a study by Keller *et al.* (2004) concluded that, although 81-95% of PCBs partition into the plasma component of blood, whole blood analyses (which contain the plasma as well as serum) reflected the same concentrations. De Solla *et al.* (1998) examined PCB concentrations in plasma of male snapping turtles from the Ontario AOCs and found mean tPCB concentrations of 414.8 and 263.3 ng/g ww at contaminated sites and 17.8 and 18.2 at reference sites. These findings are similar to but not as extreme as our measurements in male turtle blood from the contaminated area (mean tPCB 450.3 ng/g ww with a high of 2647 ng/g ww). PCB concentrations in plasma from snapping turtle inhabiting Ohio AOCs measured by Dabrowska *et al*. (2006) were also lower than our observations.

Body size can strongly influence bioaccumulation of lipophilic contaminants in mammals (Honda *et al*. 1983; Letcher *et al.* 2000) and fish (Baumann and Whittle 1991; Borgmann and Whittle 1991). Researchers investigating the influence of body size on snapping turtle PCB body burdens have reported somewhat mixed results. Hebert *et al.* (1993) found relationships between snapping turtle body length and liver concentrations of 13 PCB congeners with Spearman rank correlation coefficients ranging from 0.19-0.90 (significance not given). The strength of the relationship increased with increasing hydrophobicity of congeners (K_{ow}), demonstrating the more persistent nature of the heavier congeners. Bishop *et al.* (1994) concluded that female snapping turtle body size and clutch characteristics are not significant predictors of organochlorine contaminants in eggs. Examination of the relationships between carapace length and plasma tPCB concentrations by de Solla *et al.* (1998) revealed a significant positive relationship between the factors at a contaminated site but not at a reference site. These results

suggest that when body burdens reflect only background contamination, body size is not a strong predictor of snapping turtle PCB concentrations, but at contaminated sites larger, older turtles are more likely to have accumulated higher body burdens despite the confounding effect of high intrasite variability. Our data support this trend, as whole blood PCB concentration was not significantly related to carapace length in reference area adults of either sex, but there was a significant positive relationship between the two factors in both males and females at the contaminated area.

Congener and Homolog Profiles in Blood

The observed patterns of PCB congeners and homolog groups provide information on exposure, metabolism and excretion in these animals. Certain emergent patterns were common to all samples. The coeluting congeners 132/153/105 were the highest of the measured congeners in all blood samples from both study areas, comprising 15-28% of the total concentrations. While PCB 105 would have been the highest of the three comprising this peak in the original Aroclor, it is relatively rapidly metabolized by cytochrome P450 enzymes (Auborg *et al.* 1994), such that the vast majority of this peak is PCB 153, considered a recalcitrant "steady state" congener nearly always detected in environmental samples (Hansen 2001). The other congeners considered to be in a steady state in environmental samples are PCBs 138, 180, and 118 (Hansen 2001), all of which are dominant congeners in all samples from both study areas.

Given that the contamination of the upper Hudson River sediments is characterized by a dominance of lighter congeners (Aroclor 1242), it was somewhat surprising that blood samples did not have proportions of di and tri homologs much greater than in those from the reference area. However, these lower chlorinated

congeners are the most easily metabolized and excreted by organisms, and being the most volatile and mobile of the congeners, may be unlikely to display patterns related to long term accumulation. The relative dominance of the tetra through hexa-chlorinated homologs (Figure 3.5) from the contaminated area, however, reflects the enrichment of the contaminated area sediments with Aroclor 1242. Higher proportions of hepta through deca-chlorinated congeners in the reference area blood are the result of a more general background contamination without the localized input of a particular mixture occurring in the contaminated area. The contributions of the heavier congeners whole blood at the contaminated site likely reflect a combination of background contamination, Aroclor 1242 (likely contributing only small amounts) and somewhat higher contributions from other Aroclor mixtures used at the GE plants. The higher proportions of heavier congeners at the reference site are also evident in homolog group profiles among study areas and genders. When homolog group profiles are compared between genders at each study areas the patterns are remarkably similar between the two areas.

Maternal Transfer

The usefulness of eggs as indicators of maternal contaminant burden and/or local contamination is contingent upon knowledge of the source of lipophilic contaminants found within freshly laid eggs. There has been considerable debate amongst toxicologists studying maternal transfer in turtles as to whether lipids and associated lipophilic contaminants in eggs originate from maternal reserves or recently consumed dietary sources. Over the course of the annual reproduction cycle of turtles, ovarian follicles undergo two periods of development (White and Murphy 1973). The majority of follicular enlargement occurs from mid-summer through late-fall when females enter

hibernation. Following emergence from hibernation the following spring, follicles undergo final development and enlargement, followed by shelling and ovulation over the course of several weeks. Congdon and Gibbons (1990b) report that in painted turtles (*Chrysemys picta*), the required energy for development of ovarian follicles over the 5-month pre-hibernation period is derived from recently harvested food sources, while energy input for post-hibernation derives from stored body lipids.

The basic physiology of lipid dynamics during reproduction in snapping turtles has not been thoroughly characterized, thus quantifying maternal transfer of lipophilic contaminants to eggs may provide useful information on the physiology of lipid storage and use. As noted previously, Bishop *et al.* (1994) found that larger/older female snapping turtles or those with larger clutches from a contaminated site in Ontario did not produce eggs having significantly higher in contaminant concentrations, and thus concluded that lipophilic egg contaminants are derived from dietary intake proximate to egg production. Although Bishop *et al.* (1994) did not measure maternal body burdens for any of the contaminants measured in eggs, this study formed the basis for the assumption that maternal somatic lipids and associated contaminants do not significantly contribute to those found in eggs. The Bishop *et al.* (1994) study has been cited numerous times in the snapping turtle contaminant literature (i.e. Bonin *et al.* 1995; de Solla *et al.* 1998, 2001) as the basis for using snapping turtle eggs solely as indicators of local contamination in studying geographical and temporal trends. Pagano *et al.* (1999) evaluated adipose, liver and egg tissues from six female snapping turtles exposed to a wide range of PCBs and other lipophilic contaminants and concluded that lower-chlorinated pattern in eggs compared to those of adipose and liver established recent food

sources as the major contributor to egg stores. The authors added, however, that since higher chlorinated maternal contamination levels resulted in higher egg chlorination levels, at least some portion of egg production is derived from maternal reserves and emphasized the need for additional research into the matter.

More recent studies of maternal PCB transfer in snapping turtles, including our data and that of Dabrowska *et al.* (2006), and well as that of organochlorine pesticides in another large, long-lived reptile, the American alligator (*Alligator mississippiensis*; Rauschenberger *et al.* 2004), suggest that maternal somatic stores may be a larger source of contaminants to eggs than was previously thought. All three of these studies found a significant relationship between total maternal body burdens and that of eggs, as well as egg-plasma ratios of a range of lipophilic contaminants close to 1:1. Dabrowska *et al.* (2006) found that while highly chlorinated congeners selectively partition into fat as K_{ow} decreases, the same was not true for partitioning between plasma and eggs, which was unaffected by $K_{ow.}$ These findings agree with our data for 14 congeners and/or coeluting congeners found in seven sample pairs of maternal whole blood and eggs, for which there was no trend of increasing egg-blood ratios with increasing chlorination (and thus decreasing K_{ow}). Thus while somatic lipids may contain higher proportions of heavier chlorinated congeners, light to moderate chlorinated congeners reach near steady state equilibrium between fat reserves, blood components and eggs regardless of lipid or contaminant content of recently harvested food items. This is further corroborated on a homolog group basis, in which we found eggs to be relatively enriched with tetra and penta-chlorinated homologs compared to maternal blood and Dabrowska *et al.* (2006) found eggs relatively enriched with penta and hexa homologs.

In the absence of accompanying data on contaminant concentrations in recently consumed food, it could be argued that blood component contaminant concentrations and subsequently egg concentrations have reached a steady state with recently consumed food, while adipose tissue reflects accumulation in the absence of egg production. This is not in agreement with our findings that blood PCB concentrations of turtles at the contaminated area, males and females alike, are significantly related to body size, indicating that blood concentrations reflect those of long-term accumulation in adipose. Unless larger individuals in the contaminated area consistently consume prey items of a higher PCB concentration which elevates blood concentrations rapidly before partitioning into eggs (an unlikely scenario given their indiscriminate feeding habits), egg PCB concentrations are most likely an accurate reflection of stored maternal reserves. Indeed, Russell *et al.* (1999), using a fugacity-based model and field verification of several oviparous organisms, including snapping turtles, concluded that dietary lipids rapidly adopt the same lipid-based contaminant concentrations as somatic lipids, such that eggs receive contaminant concentrations similar to maternal tissues rather than prey items. Ultimately, the exact nature of the dynamics of contaminant body burden transfer to offspring in snapping turtles can only be determined in controlled laboratory experiments, such as those conducted in birds species fed diets spiked with stable isotopes of PCBs to carefully monitor the source of egg contaminants (Drouillard and Norstrom 2001).

Conclusions

PCB concentrations in snapping turtle eggs and whole blood were greatly elevated at the contaminated study area of the upper Hudson River Superfund site compared to nearby reference sites. High variability in both tissues at the contaminated

study area reflects differential accumulation at the individual level as well as the pronounced spatial heterogeneity of PCBs in sediments. The significant trend of larger/older turtles having greater body burdens of PCBs, as well as the trends observed in maternal transfer dynamics, indicate that eggs and whole blood are both good indicators of long-term and accumulative sequestration of PCBs in snapping turtle adipose stores, rather than just recently consumed prey. Congener and homolog patterns in samples reflect different environmental sources of contaminants, as well as differential metabolism, excretion and accumulation. Temporal trends in PCB exposure in snapping turtle populations in the upper Hudson River are difficult to identify given the paucity of studies, but the limited data that exists suggests a decrease in body burdens since approximately 1980. Dredging of the selected Hudson River hotspots, scheduled to commence in the near future, is designed to remove the worst of the Aroclor 1242 contaminated sediments from the system. A gradual decrease in PCB body burdens of resident animals is a hopeful outcome of these remediation efforts.

IV. MEASUREMENT OF SNAPPING TURTLE PLASMA VITELLOGENIN

Introduction

In oviparous vertebrates, the glycolipophospoprotein vitellogenin (Vtg) is the egg yolk precursor manufactured in the liver of mature females for transport to developing oocytes. Structurally, Vtg is a high molecular weight (~ 200kDa) dimer with two protein subunits, lipovitellin and phosvitin which provide the biochemical and energetic basis for embryonic development (Brion *et al.* 2000; Herbst *et al.* 2003). Vitellogenesis is regulated by a complex interplay between the glands and hormones of the endocrine system. The entire process is under the control of the pituitary gland, which secretes gonadotropin-I (GtH-I) in varying levels to regulate the vitellogenic cycle of reproductively mature females, which is highest in late fall and early spring (see "Discussion: Maternal Transfer" section, Chapter 3) (Kime *et al.* 1999). GtH-I stimulates ovarian follicle cells to up-regulate the production of steroid hormone estradiol (Kime *et al.* 1999). Vtg production is specifically mediated in hepatocytes through binding of estradiol to estrogen receptors (ER), which in turn activate genetic estrogen response elements, initiating transcription and translation of Vtg (Krieg *et al.* 2004; Wheeler *et al.* 2005). Accumulated Vtg molecules are modified by the rough endoplasmic reticulum and Golgi apparatus prior to release into the blood plasma (Wheeler *et al.* 2005). Circulating Vtg passes into ovarian follicle cells through capillary networks to reach oocytes surfaces, where it is incorporated into oocytes by receptor-mediated endocytosis, under the influence of GtH-I (Korach and McLachlan 1995; Tyler *et al.* 1990).

In oviparous vertebrates, the same estrogen-inducible Vtg is found in males and immature individuals, but the protein is expressed at immeasurable or very low plasma

concentrations, since levels of circulating estrogen are low (Flouriot *et al.* 1993). The presence of this estrogen-activated gene in non-mature females has been of great interest to ecotoxicologists due to the presence of estrogen-mimicking chemicals in the environment (Denslow *et al.* 1999; Kime *et al.* 1999; Oberdörster and Cheek 2000) Numerous environmental contaminants have been shown to have estrogenic activity, including several pesticides, non-ionic surfactants, and some PCB mixtures, congeners and metabolites (Cooke *et al.* 2001; Jobling and Sumter 1993; Willingham and Crews 2000). Generally, the estrogenic PCBs are non-coplanar, lower chlorinated congeners, with the exceptions of some higher chlorinated congeners such as PCBs 153 and 184 (Cooke *et al.* 2001). PCB metabolites, particularly those with conformationally restricted structures, are also effective binding ligands for the ER (Korach *et al.* 1988). While most of the estrogenic potency of these congeners and metabolites appears to be mediated through direct binding and activation of the ER, some of the hydroxylated metabolites exert an ER-independent effect by increasing bioavailability of estradiol in target tissues (Kester *et al.* 2000). Confounding matters, the coplanar dioxin-like PCB congeners (see TEQ-related sections of Chapter 3) and their hydroxylated metabolites can function as estrogen antagonists, although the exact mechanism is unknown (Cooke *et al.* 2001; Geyer *et al.* 2000). However, estrogenic effects can be additive, such that several weakly-estrogenic PCBs in combination may elicit a strong effect capable of overpowering any antiestrogenic effects in the complex mixtures found in environmental matrices (Hany *et al.* 1999; Wheeler *et al.* 2005). This would explain the overall estrogenic effects of several Aroclor mixtures, including 1242, 1248, and 1254 (Cooke *et al.* 2001; Hany *et al.* 1999).

Environmental estrogens have been shown to induce Vtg production in males of numerous fish species (reviewed by Wheeler *et al.* 2005), amphibians (Selcer *et al.* 2001), crocodilians (Gunderson *et al.* 2003; Rey *et al.* 2006) and several turtles including red-eared slider (Palmer and Palmer 1995), painted turtle (Irwin *et al.* 2001), and green and black sea turtles (Herbst *et al.* 2003 and Sifuentes-Romero 2006, respectively). The direct physiological consequences of abnormal Vtg production by males and juveniles are not well established, however at very high concentrations correlated adverse effects have included decreased testicular growth, intersexuality, and reduced kidney function (Wheeler *et al.* 2005). In females, exposure to environmental estrogens could result in abnormally high or low circulating Vtg, with potential decreases in reproductive function (Kime *et al.* 1999).

Despite the number of laboratory-based studies establishing that vitellogenin induction can be used as a screening tool or as a potential biomarker for exposure to environmental estrogens, very few demonstrate the occurrence of xenoestrogen-induced Vtg induction in males of wild populations. The few studies that have successfully demonstrated this phenomenon in wild animals have been with fish species exposed to sewage treatment discharges (i.e. Purdom *et al.* 1994). In fact, juvenile alligators within populations from contaminated sites in Florida previously shown to have other endocrine-disruption effects such as reduced phallus size in males, perturbations to hepatic testosterone biotransformation, and altered thyroid and sex steroid concentrations did not have measurable levels of plasma Vtg. Nonetheless, given the unique contaminant profile of the upper Hudson River Superfund site, characterized by high levels of PCBs, and relatively low levels of other contaminants (USEPA 2000), we

sought to measure plasma Vtg in adult male and female snapping turtles inhabiting the area. we used Western blotting for qualitative identification of Vtg and developed a competitive, antibody-capture enzyme-linked immunosorbent assay (ELISA) for quantitative assessments.

Materials and Methods

Dr. Kyle Selcer (Duquesne University) generously donated purified polyclonal rabbit-anti-red-eared slider (*Trachemys scripta*) antibodies prepared by Palmer and Palmer (1995). Additionally, Dr. Selcer purified snapping turtle vitellogenin from a plasma sample pooled from nine reference area females using a protocol modified from that of Wiley (see Palmer and Palmer 1995). The lyophilized final protein product was reconstituted in ultra purified water and quantified using the bicinchoninic acid (BCA) assay with bovine serum albumen as the standard (Pierce, Rockford, IL). We used the rabbit-anti-red-eared slider as the primary (1º) antibody and the purified snapping turtle Vtg as the standard in both the Western blotting and ELISA.

Western blotting was employed to ensure cross-reactivity of the anti-red-eared slider Vtg antibodies with snapping turtle Vtg in samples and the reconstituted solution of purified protein. Several preliminary Western blots were conducted in order to optimize the dilutions of samples, the standard, and the 1º antibody. Turtle plasma was mixed with enough sample buffer (5% 2-mercaptoethanol in Laemmli solution) for a final dilution of 1:2 for male turtles (to maximize detection, if present) and 1:25 for females. Diluted samples were boiled for four minutes to facilitate protein denaturation and then loaded onto precast 7.5% Tris-HCl gels (Bio-Rad, Hercules, CA). Two identical gels were prepared so that one could proceed with Western Blotting and the other could be stained

with Coomassie protein stain to examine band patterns. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under a 100 v electrical field for 90 minutes, the proteins from one of the duplicate gels were transferred to polyvinylidene difluouride (PVDF) membranes for an additional 90 minutes at 100 v. Protein-embedded membranes were rinsed for 15 minutes with Tris-buffered saline (TBS; Bio-Rad, Hercules, CA), followed by a one hour incubation in blocking solution [5% (w/v) powdered milk in TBS] to block unoccupied binding sites. The membrane was then transferred to another 5% (w/v) powdered milk in TBS solution containing the 1º antibody at a 1:500 dilution and incubated at 4 ºC overnight. The following day, the membrane was rinsed in a 0.1% solution of Tween-20 (Bio-Rad, Hercules, CA) in TBS, followed by three rinses in TBS to remove all unbound 1º antibody. The secondary (2º) antibody, goat anti-rabbit horseradish peroxidase (GAR-HRP) was obtained as part of the Bio-Rad Opti-4CN colorimetric detection kit. Per the instruction manual, the membrane was incubated for 2 hours in a 1:3000 solution of GAR-HRP in blocking solution. The membrane was then rinsed twice for five minutes in 0.1% Tween-TBS and immersed in freshly prepared substrate solution (1:10 dilution of two proprietary solutions) for 30 minutes, resulting in colorimetric development of the HRP enzyme product.

Antibody-capture competitive ELISA begins with the adsorption of a known quantity of antigen (here, Vtg) to the wells of a microplate, a process allowed to proceed overnight (Specker and Anderson 1994). Concomitantly, individual dilutions of the antigen for each point of the standard curve, as well as each unknown (sample), are incubated with a set amount of 1º antibody in glass test tubes overnight. The following day, the microplate is rinsed to remove unbound antigen, and the standard curve and

sample incubations are added. In the incubation containing the zero point of the standard curve (i.e. no antigen), all of the antibodies will still be free to bind to the antigens sorbed to the microplate wells, representing the maximum binding, or B_{max}. Incubations of increasing concentrations of the standard curve will have decreasing levels of free antibody to bind to the sorbed antigens. Incubations of samples, when appropriately diluted, will fall somewhere between. After a set period of time, the plate is washed and the 2º enzyme-conjugated antibody is added. Finally, the enzyme substrate is added and a colorimetric response can be used to quantify the amount of antibody captured on the antigen, with substrate production being inversely proportional to levels of antigen in the incubations.

As there are no published protocols for snapping turtle Vtg ELISA, nor commercial kits available, we developed the competitive ELISA for snapping turtle vitellogenin using the "Starting from scratch" section of a book chapter entitled "Developing an ELISA for a model protein – vitellogenin" by Specker and Anderson (1994) using the general procedure described above. Dr. Kyle Selcer kindly shared his buffer recipes from his laboratory's competitive ELISA protocols. Carbonate buffer (0.1 M Na_2CO_3) was used for the vitellogenin coating buffer, phosphate buffered saline-Tween (PBST; 133 mM NaCl, 1.5 mM KH_2PO_4, 8.6 mM K_2HPO_4, and 0.05% Tween, pH 7.6) was used for plate washing steps and 5% (w/v) powdered milk in PBST was used for blocking and diluting antigens and/or antibodies. All solutions were made fresh daily and stored at 4º C. Nunc Immuno Maxisorp plates were used for all ELISA assays and all samples were run in triplicate. The overnight incubations were conducted at 4º C. Following each incubation step, plates were washed three times with PBST and tapped

gently over paper towels. Microplates were blocked for 1 hour prior to secondary antibody incubation for two hours, with both of these steps conducted at room temperature. The secondary antibody used, GAR-HRP, was part of a commercial detection kit using TMB Peroxidase substrate for colorimetric development (Bio-Rad, Hercules, CA). Following a 15 minute incubation with the substrate mix, the reaction was halted with addition of 2 N H_2SO_4 and absorbance was read at 450 nm.

Reduction of the standard curve absorbance data involves adjusting the response (absorbance) to the relative % antibody bound by normalizing all responses (designated as B in formulas) to that of B_{max}. The logit of the % bound is calculated as $\log[(B/B_{max})/(1-B/B_{max})]$. The logit is then plotted against the log(Vtg standard concentration) to obtain the working standard curve. Using this working standard curve, the logit of samples (unknowns) is fit to the curve to obtain log (sample Vtg concentration). Due to the inverse relationship between free antibody concentration and antigen in the solutions, lower response (absorbance) translates into higher Vtg concentration. It is important that samples are diluted in such a manner that they fall within the dynamic (linear) portion of the sigmoidal (prior to logit-adjustment) standard curve (Specker and Anderson 1994). The sensitivity of the assay is determined by running eight replicates of the zero standard (B_{max}) and the lowest four standards and then running a statistical analysis to determine which is different than zero. All statistical analyses were performed with Minitab version 13.0 using either ANOVA or regression randomized resampling analyses as appropriate. Significance was accepted at the $\alpha=0.05$ level.

Results and Discussion

Western blotting demonstrated the detection of snapping turtle Vtg by the rabbit anti-red-eared slider antibody in the standard and the females, but not in the males.

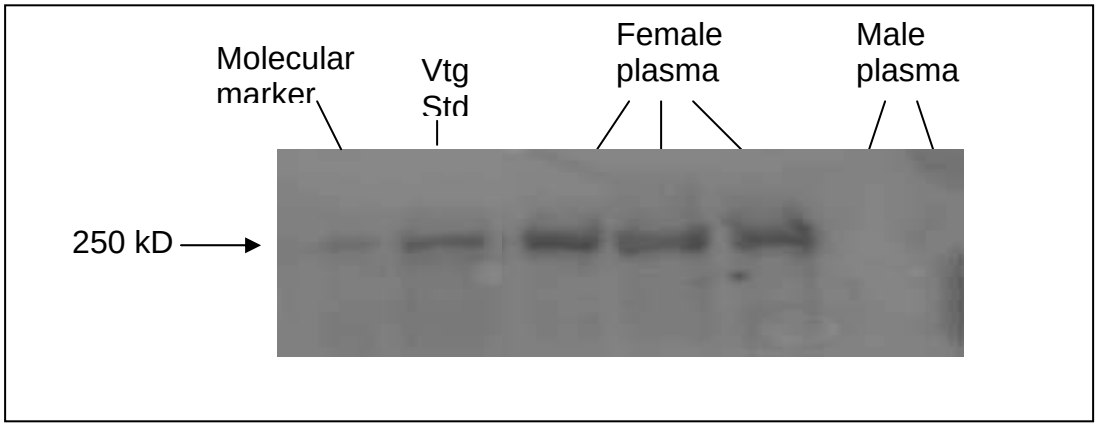

Figure 4.1 A representative Western blot membrane showing detection of Vtg in the purified standard and female plasma, but not male plasma (reference area).

Figure 4.1 shows a representative Western blot membrane of plasma from three female and two male turtles from the reference area. There is a distinct detection band corresponding to ~200 kD in the lanes with the Vtg standard and the female turtles, but not in those of male turtles. Poor resolution of the protein bands on the PVDF membrane precluded quantitative analyses of actual Vtg concentrations. Additional Western blotting utilizing plasma from contaminated area males did not show qualitative detection in any of the males from the contaminated area, even those with the highest blood PCB concentrations. However, while Western blotting has the advantage of providing a visual confirmation that a protein of the correct molecular weight is being detected, it also has the major disadvantage of having prohibitively high detection limits. Even if males are experiencing xenoestrogen-induced vitellogenesis, the concentrations may well still be much lower than that of mature females, and thus below the apparent detection limits of the Western blot technique. In order to lower detection limits substantially and allow for quantitative analysis, the ELISA assay was developed.

Considerable effort was expended on optimizing the protocol for appropriate antigen plate binding, as well as plasma and 1º antibody dilutions. In the first trials, we experimented with different amounts of Vtg for coating the microplate wells and several dilutions of the 1º antibody. The results of these experiments dictated the use of 400 ng of purified Vtg per well for the microplate coating step and a 1:3000 dilution of the 1º antibody. We used a standard curve ranging from 3.9-2200 ng/ml to maximize the dynamic range of the assay (i.e. the linear portion of the sigmoidal curve) (Specker and Anderson 1994), and determined the sensitivity to be 15.6 ng/ml. This value times the dilution factor then determines the practical detection limit for the assay (Nilsen *et al.* 2004).

In the next set of trial ELISAs, we experimented with different concentrations of plasma for both males and females, in order to find appropriate dilutions that would fall within the dynamic range of the standard curve. Given that male plasma Vtg concentrations would be expected to be much lower than females, even if xenoestrogen Vtg-induction is occurring, male turtle plasma would require less dilution. We ran several exploratory ELISAs with male plasma diluted to 1:50, 1:250 and 1:1000 and female plasma diluted to 1:1000, 1:10,000 and 1:50,000. Depending on the dilution, male plasma Vtg concentrations ranged from 7.1-75.1 μg/ml and female concentrations ranged from 1.1-8.4 mg/ml (note the different concentration units between genders). The strong lack of parallelism across different dilutions was of considerable concern: the higher the dilution, the greater the concentration of Vtg measured after correction for dilution factor. This phenomenon is attributed to a matrix effect in which other proteins or plasma components inhibit or reduce binding of the antigen and antibody (Nilsen *et al.*

2004; Sifuentes-Romero *et al.* 2006). The more dilute the plasma, the less interference is possible from other biomolecules, whether due to non-specific binding or physical/steric crowding. In order to try to elucidate the nature of this issue, we pooled serum from four reference area males and from four reference females to produce a representative matrix for each gender, removed from inter-individual variation. We then diluted the pooled male plasma to 1:50, 1:250 and 1:1000 and into each spiked the lower concentrations of the Vtg standard curve (0, 15.6, 62.5 and 250 ng/g Vtg). Pooled female plasma was diluted to 1:1000, 1:10,000 and 1:50,000 and spiked with the same low end Vtg standard curve (with the exception of 250 ng/g Vtg due to space constraints). The results of this matrix effect investigation for each sex are shown in Figure 4.2. The lack of parallelism is particularly pronounced in the male plasma, in which the three curves begin to converge at the highest Vtg concentration, however the trend in both sexes indicates that greater concentrations of Vtg will be detected in increasingly dilute plasma. Furthermore, it is impossible to measure plasma from both males and females at the same concentration (i.e. 1:1000) because at this dilution male plasma is at the far lower end of the dynamic range of the standard curve, while female plasma is at the high end. We

were not able to find any similar dilemma in any of the Vtg ELISA literature for any

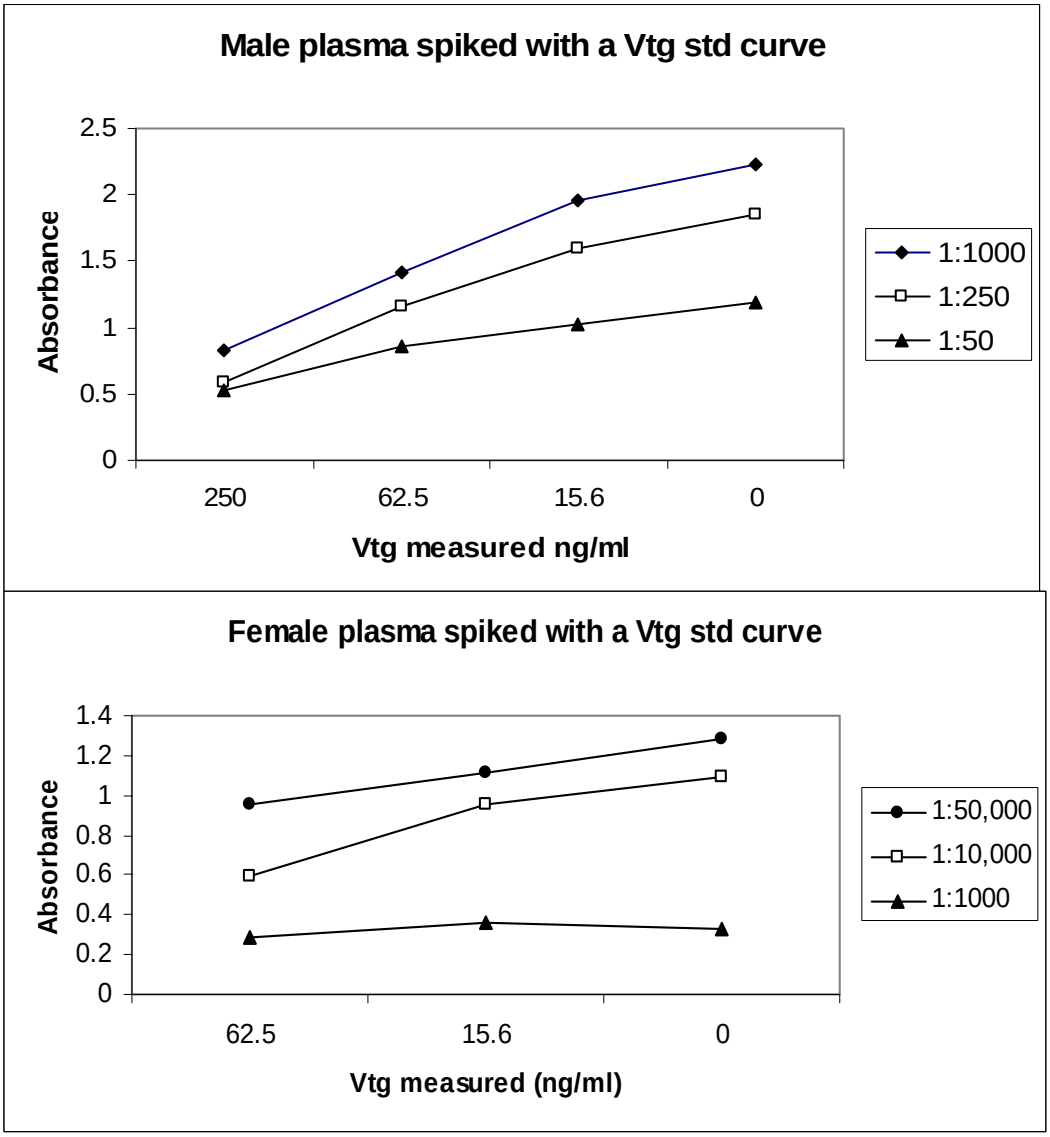

Figure 4.2 ELISA absorbance of a Vtg standard curve in male and female snapping turtle plasma at three dilutions.

years, the possibility of a matrix effect was rarely investigated, while the current studies that have encountered this problem have simply increased dilution until there is no indication of a matrix effect (i.e. Nilsen *et al.* 2004; Sifuentes-Romero *et al*. 2006). As this solution does not apply to the ELISA system we had developed, the ultimate problem

was that my measured concentrations in male and female snapping turtles are not necessarily comparable to each other, and may not reflect absolute values. In addition, the vast disparity between sexes regarding dilutions for this assay results in much higher detection limits for females than males.

After examining the ELISA results, we conducted one additional Western blot on six female turtles with a wide range of plasma Vtg concentrations ranging from non-detectable (ND, < 0.78 mg/ml) to 13.1 mg/g (Figure 4.3). There was no visible Vtg band for the females with ELISA-measured concentrations of ND and 1.5 mg/ml. The same results were obtained when these samples were analyzed by an outside laboratory using a novel, "universal" Vtg detection method based on phosphoprotein staining (van Veld, P.A. *et al.* 2005). However, neither the Western blot nor the phosphoprotein staining method were quantitative, or even have established detection limits. It is possible that: a) the detection limits of both are too high for the low levels of Vtg these samples may contain, or b) that the ELISA method is actually reporting non-specific or unknown specific binding when not preferentially bound to Vtg, or c) both.

Ultimately, however, we chose the most appropriate dilution for all Vtg

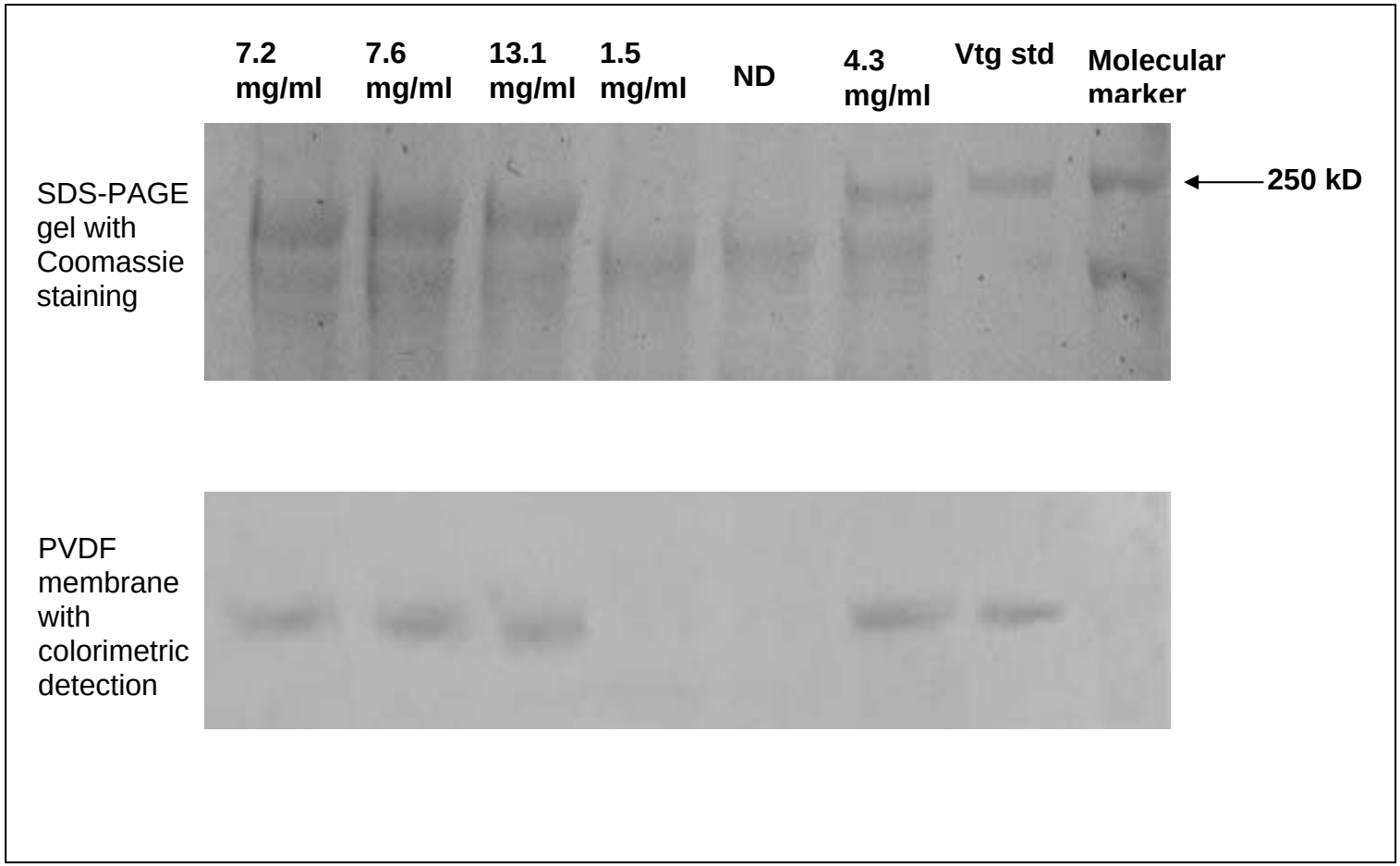

Figure 4.3 SDS-PAGE gel following Coomassie staining (upper panel) and PVDF membrane following Western Blotting for reference area female plasma. Values above each lane designate the measured concentration as determined by Western blot; ND = not detected.

measurements in each gender, and limited comparisons to intra-gender differences between study areas. Therefore, all male plasma was diluted to 1:250 for Vtg ELISAs (practical detection limit = 3.9 ng/ml) and female plasma was diluted to 1:50,000 (practical detection limit = 0.78 mg/ml). Unexpectedly, plasma Vtg was significantly higher ($p = 0.024$) at the reference site (mean 12.9 ± 17.6 μg/ml) than the contaminated site (mean 6.62 ± 2.5 μg/ml). One reference area individual, a small male (1.5 kg) with a low blood tPCB concentration (0.77 ng/g ww) had an usually elevated plasma Vtg concentration of 76.0 6 μg/ml. It is uncertain what, if any, underlying mechanisms, ecotoxicological or otherwise underlie these observations. There was no significant relationship between plasma Vtg concentration and either size or blood tPCB concentration at either study area. Similarly, in female turtles, plasma Vtg was significantly higher ($p = 0.012$) at the reference site for (mean 4.7 ± 1.8 mg/ml) than the contaminated site (3.1 ± 1.8 mg/ml). However, plasma Vtg was not significantly influenced by whole blood tPCB concentrations at either site, suggesting that the significant difference in Vtg between adults the two study areas is not related to PCB body burden. At both study areas, there was a significant positive relationship between female body size (CL) and plasma Vtg concentrations (reference area, $p = 0.013$; contaminated area, $p = 0.001$). This observation may be related to greater absolute yolk inputs made to eggs by larger females.

Conclusions

All things considered, there is considerable doubt that the competitive, antigen-capture ELISA that we developed is truly reflecting plasma Vtg concentrations at low levels, including the lower end of concentrations in females. Given that Vtg

sequences/structures are not extremely conserved between species and that multiple epitopes are found on Vtg molecules (Kime *et al.* 1999; Wheeler *et al.* 2005), the polyclonal antibody mixture in the anti-red-eared slider may very well have a low to moderate affinity for one or more other components of snapping turtle plasma. It is also not well known to what extent, if any, male and juvenile snapping turtles may express low levels of Vtg under normal conditions. In some fish species, researchers have reported "background" levels of plasma vitellogenin in males, ranging from <0.002 to 2.08 μg/ml, consistently several orders of magnitude lower than that of mature females (Wheeler *et al.* 2005). In studies of Vtg in turtle species, the few that measured concentrations in males reported no detection (Shelby and Mendonça 2001) or low levels in the range of 2-4 μg/ml (Herbst *et al.* 2003). It appears that, without snapping turtle specific antibodies (ideally monoclonal), or the application of other sensitive measurements of Vtg (i.e. mRNA measurement, RT-PCR; Wheeler *et al.* 2005), the normal background levels and potential contaminant-induced perturbations of snapping turtle vitellogenesis cannot be reliably quantified.

Snapping turtles of the upper Hudson River Superfund Site, like others of the species in other heavily contaminated waterways, can tolerate high body burdens of PCBs without overt adverse effects. As suggested by Lassiter and Hallum (1990), animals with large somatic stores of lipids may be afforded some protection by the sequestration of the more heavily chlorinated congeners into adipose. Indeed, as discussed in Chapter 3, studies of contaminant distribution in snapping turtles by other researchers suggest that this is the case in snapping turtles. Even still, PCB concentrations can be very high in eggs (and thus developing embryos) and adult blood

of upper Hudson River snapping turtles, which may be exerting sublethal effects not examined in this study.

V. EXPERIMENTAL ANALYSES OF EFFECTS OF PCBs ON JUVENILE TRAITS

Introduction

Through direct exposure or maternal transfer to offspring, PCBs have the potential to disrupt normal endocrinological functions similar to some other xenobiotics. For example, depending upon the species and conditions, organochlorine pesticides such as DDT, DDD, and DDE may act agonistically on estrogen and corticosterone, antagonistically on estrogen and androgens, or by modifying metabolism of testosterone, progesterone, and thyroxine (see Guillette et al., 2000). It has been demonstrated that PCBs tend to exert their effects in two primary ways. First, they behave estrogenically (as estrogen agonists), causing responses in organisms that would normally be associated with the presence of estrogen at higher than normal concentrations, or during periods of the life cycle when circulating estrogen concentrations are low (see Bergeron et al., 1994). Second, they can act to alter thyroxine metabolism and thyroid hormone transport and reception, potentially modifying developmental, metabolic, and reproductive processes that operate under thyroid control (see Morse et al., 1992, 1993; van Birgelen et al., 1992; Leatherland, 1999). While the activities of PCBs appear somewhat less diverse than the activities of some other xenobiotics, the pathways that are affected by PCBs are critical to normal developmental, growth, and reproductive patterns.

The biological actions of PCBs result largely from their entry into cells and binding to nuclear receptors, zinc finger proteins which act upon nuclear DNA when activated by specific ligands including steroid and thyroid hormones and retinoids. When the appropriate ligand binds to the receptor, a conformational change occurs which allows it to function as a transcription factor. The zinc finger portion of the activated

complex allows a close association with DNA, and the complex binds to response elements in the promoters of target genes, which are up- or down-regulated. It is through this signaling mechanism that PCBs have an endocrine disrupting effect; certain congeners can bind to hormone receptors and thus change the expression of estrogen receptor mediated genes (and possibly other hormones), through agonistic or antagonistic perturbations.

Some PCBs also have an affinity for the aryl hydrocarbon receptor (AhR), a nuclear receptor which is unique in that it has no known endogenous ligand. Certain contaminants, including some PCBs, bind to AhR and activate transcription of genes whose products mediate immune function, endocrine regulation, and metabolism, and also can contribute to teratogenesis and carcinogenesis. Previous research has established that the immunotoxic effects of PCBs on laboratory mammals are numerous, including changes in immune system tissues (i.e. spleen, lymph nodes and thymus) and suppression of humoral and cellular immunity (Tryphonas, 1995). Normal immune function is an integral aspect of reproductive fitness, as contaminant-induced compromises in the ability to combat pathogens and parasites can influence survival to reproduction.

PCBs can also affect organisms via disruption of thyroid hormone homeostasis. In addition to interference with the thyroid nuclear receptor, PCBs compete with thyroid hormones for binding sites on the plasma proteins which transport the hormones systemically, and stimulate induction of hepatic enzymes which metabolize thyroxine by glucurodination. The overall effect of these perturbations can be significant, particularly with regard to the developing embryo or juvenile, in which normal thyroid hormone levels are critical to normal neurological development. Several studies have shown an

association between PCB exposure and impairment of motor and mental function in laboratory mammals (Morse *et al.* 1993). However, much more research is required on wildlife species to determine the effects of EDCs acting through the thyroid system to affect neurological development and reproductive function (Colborn and Thayer, 2000). As well as regulating neurological development, thyroid homeostasis is required for proper metabolic function (consider the effects of hypo- and hyper-thyroidism in humans, for example). As metabolism regulates efficiency of the conversion of assimilated energy to somatic or reproductive tissues, alteration of metabolic processes can ultimately influence reproductive fitness of individuals (Rowe *et al*., 1998; Congdon *et al*., 2001).

Using experimental studies with juveniles hatched from eggs collected in the contaminated and reference study sites (see Chapter II) in 2004 and 2005, we quantified a suite of responses related directly or indirectly to potential endocrinological disruption. Our initial intent was to conduct these 1 year long studies only with animals derived from eggs collected in 2004, but lower than expected hatching rates (and thus sub optimal sample sizes) in 2004 required that we repeat the studies in 2005 through an extension of the project period. Using factorial experiments, we quantified these endpoints in response to maternally derived PCBs, food-borne PCBs, or their combination. Traits quantified in one or both years were: sexual differentiation, morphological development, metabolism/growth, survival, and behaviors related to survival or growth potential. We also assessed the potential effect of PCB exposures on DNA integrity by quantifying DNA single strand breakage. Due to unexpected results in 2004 in which mortality of juveniles from the contaminated areas was quite high, we also employed deterministic

stage structured population models to assess potential ramifications for population growth rates.

Materials and Methods

Incubation of field-collected eggs:

Following collection, marking, measurement, and transport of eggs from the field sites to the Chesapeake Biological Laboratory eggs were incubated in containers with damp vermiculite in a temperature controlled laboratory maintained at 25 °C, temperatures known to produce all male offspring via temperature-dependent sex determination (Yntema, 1976). Eggs were monitored at 2 day intervals and any eggs showing signs of arrested development or mortality were removed. When signs of pending hatching were apparent (exfoliation of eggshell mineral layers), we began inspecting eggs daily and removing individuals that had pipped (e.g. split the egg shell but had not yet emerged) to individual 4 L containers with a substrate of moist sphagnum moss. When animals completely emerged from the egg, they were measured (CL, CW, PL; Chapter II), weighed, and examined for gross abnormalities of the eyes, jaws, limbs, bridge, and scutes. Individuals were then assigned to groups for feeding studies conducted over the subsequent year following hatching (detailed below).

Studies of juvenile traits and performance:

These studies examined traits of juveniles during the first year of growth as they reflected maternal contribution of PCBs, accumulation of PCBs via feeding on contaminated food, and the interactive effects of these sources. In 2004 all hatchlings were used due to relatively small numbers available. For studies in 2005 we selected

subsets of individuals because of the large number of hatchlings (>1000) collected as eggs and logistics associated with long term husbandry of such large numbers of turtles. Turtles not selected for use were returned to the Hudson River and released in the vicinity in which the female was captured or the nest was located. In selecting turtles for the experiment we used 2 criteria when possible: 1) individuals were from large clutches so that several individuals per clutch would be represented in each treatment, and 2) clutches differed substantially in egg PCB concentrations (e.g. we attempted to test worst case versus best case scenario by using the most contaminated clutches from the contaminated site and the least contaminated ones from the reference site). The need to use all individuals in 2004 rather than select them by clutch as in 2005 resulted in use of hatchlings from eggs that varied considerably in egg total PCB concentration from this site (mean ± SE -- 3953 ± 1454 ppm ww) relative to those from the reference site (61 ± 8 ppm ww). Clutches used for the 2005 study were less variable but concentrations in samples from the contaminated area were lower on average than in 2004 (contaminated -- 1417 ± 404 ppm ww; reference 73 ± 9 ppm ww).

The design of these studies required that half of the hatchlings from each site be maintained on a food made from natural prey items collected from either the contaminated or reference sites (below) for the first year of the juvenile period. Thus there were four treatments consisting of the combination of the site of collection and food type provided (contaminated, contaminated; contaminated, reference; reference, contaminated; reference, reference; Figure 5.1).

Housing and feeding of juveniles: Hatchlings were held in a 20°C laboratory in individual 4 L containers with moist sphagnum moss and monitored for resorption of the remaining yolk sack prior to feeding. As a quantity of yolk remains within the abdominal cavity after the external sack is resorbed, we randomly selected individuals weekly and presented them with food to determine if they had reached feeding age. During both years, very few individuals displayed interest in food prior to overwintering (below) and thus feeding commenced the following spring. Because snapping turtles in northern areas undergo a period of dormancy at low

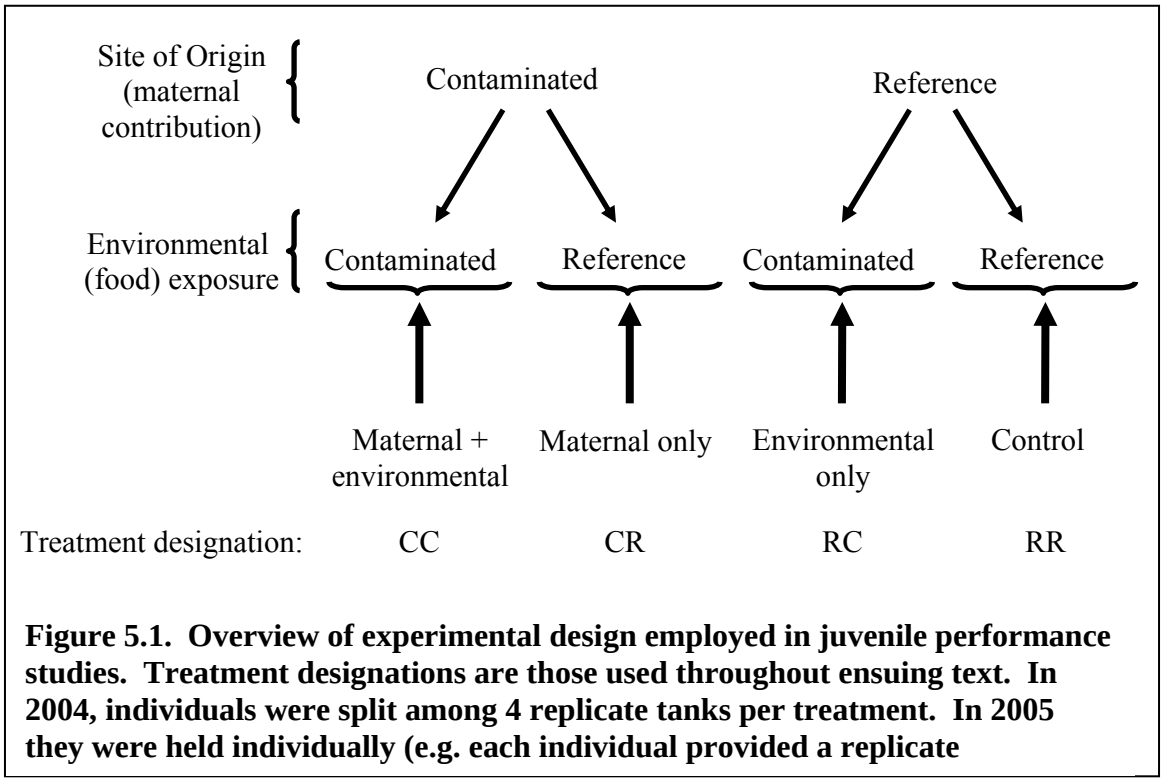

Figure 5.1. Overview of experimental design employed in juvenile performance studies. Treatment designations are those used throughout ensuing text. In 2004, individuals were split among 4 replicate tanks per treatment. In 2005 they were held individually (e.g. each individual provided a replicate

temperatures during winter, we simulated this pattern in the laboratory. In mid-October, hatchlings were transferred to a low-temperature room in which the temperature was lowered to 5° C gradually over a period of 6 wks. In late-February, temperature of the cold-room was gradually raised over 6 wks to 20° C, and transferred to

a 22-25° C laboratory for the remainder of the study. In 2004, each individual received a passive integrated transponder (PIT) tag inserted in the body cavity following methods subsequently published by Rowe and Kelly (2005). The PIT tags provided a unique identification for each individual which allowed us to hold them in groups in replicate tanks (below) for the remainder of the study while being able to keep records of each individual. In 2005 we did not tag individuals, but rather housed individuals in separate tanks (206 and 210 individual turtles from reference and contaminated sites respectively). Tanks were cleaned at 1 – 2 week intervals depending on need.

Food consisted of reconstituted aquacultural gelatin mixed with ground with fish and invertebrates which had been collected in the study sites at the time of turtle collection and frozen for subsequent use. Somewhat less food was obtained from the reference site than from the contaminated site, so reference food was supplemented with fish and shellfish purchased locally. Each food type was analyzed by bomb calorimetry to verify similarity in caloric content (average for both years: reference – 3908 ± 383 cal/g; contaminated 4652 ± 269 cal/g). The calorimetry values were also used in combination with measurements of standard metabolic rate (below) to select a ration that would exceed basal requirements (allowing for growth) but would not be in great excess such that holding tanks would quickly foul. Samples of food were also archived for subsequent analysis for contaminant concentrations (yet to be completed).

Growth, organosomatic indices, and metabolic measurements: Measurements of CL and wet mass were made monthly on all individuals. Wet mass at the end of the study were also used to calculate hepatosomatic and thyrosomatic indices (HSI or TSI) as (liver or

thyroid gland mass / body mass or CL) * 100 following dissection. Metabolic measurements were made bimonthly (2004 cohort) or monthly (2005) on a subset of four individuals from each clutch and feeding regime. The same individuals from each treatment combination were used for all metabolic measurements during the duration of the study. Resting metabolic rate was measured as oxygen consumption by resting, unfed animals. Oxygen consumption was measured using a computer-controlled micro-respirometry system attached to a 25 °C environmental chamber in which the juveniles were housed during measurement (methods detailed in Rowe, 1998; Rowe et al., 1998; 2001; Nagle et al., 2001). For animals collected in 2004, metabolic rates were measured bimonthly beginning in May, 2005 whereas those collected in 2005 were measured monthly beginning in June, 2006.

Behavioral assays: Behavioral assays were conducted one to three times during the experiment, depending upon the specific assay. As with metabolic rates, a subsample of individuals were used; individuals displaying gross morphological abnormalities were excluded. Individuals were subjected to three behavior/performance assays: 1) Responsiveness to a Visual Stimulus (2004, 2005), 2) Righting Response (2004), and 3) Foraging Efficiency (2004, 2005).

To examine the ability of juveniles to recognize and respond to a visual stimulus. adapted a technique recently employed by Winkelman (1996), in which a simulated avian predator was used to cast a shadow on or near the test animal. Individuals were placed alone in a water-filled aquarium lighted by a unidirectional light source. When the individual had relaxed and was oriented away from a tank wall, a heron-shaped silhouette

was passed through the light path (in a standardized fashion from behind a blind) to cast a shadow on the turtle. Specific behaviors were be recorded over three trials per individual and assigned categorical variables.

We also measured the time required for individual to right themselves from a ventrally-exposed position (e.g. Steyermark and Spotila, 2001). Righting response is critical to turtles when turned over by a predator, especially in species such as snapping turtles which have a very reduced plastron size. Using replicated tests on representative animals (as above), we placed individuals on their back on a packed-sand substrate and monitored times to righting. Measurements were made in a 25 °C laboratory under indirect light, and observations were made from behind a blind.

We assessed foraging success by individually placing four randomly selected juveniles from each clutch and treatment combination into replicated aquaria in the laboratory containing known numbers of food items (freshwater grass shrimp, tadpoles, minnows). Aquaria contained the same types and orientations of structural materials (plastic plants, shells) and were partially filled with well water and maintained on a natural seasonal light:dark cycle at 25 °C. After a 24 hr period, remaining food items were counted.

Assays of DNA Damage: We also assessed the potential effects of maternal and/or food borne PCB exposure on DNA integrity in blood and liver or juveniles using the Comet assay (detailed in Mitchelmore and Chipman, 1998) to quantify DNA single strand breakage. From 32 (8 per treatment) juvenile turtles sacrificed a the end of the studies for sex ratio determination and dissection, liver and blood samples were collected and

analyzed according to standard protocols. "Comet tail DNA" was compared among treatments as % DNA in tail correlates will amount of DNA breakage (detailed in Mitchelmore and Chipman, 1998). A positive control (hydrogen peroxide) was included in the assays.

Determination of sex ratios and histopathological examinations: At the conclusion of the studies above, final size measurements were made and individuals were sacrificed by inhalation of isoflorane followed by decapitation. Animals were dissected for inspection of the gonads for identification of sex (Yntema, 1976) and histological assessments for potential abnormalities in gonadal organization. Tissues were embedded in paraffin and sectioned by a commercial laboratory (American Histological Laboratory).

Population models: To assess potential population level ramifications of juvenile mortality in the 2004 cohort, we employed deterministic age/stage structured matrix population models (Caswell, 2001) to project population growth rates over time. Based upon demographic data and prior models of snapping turtle populations in Michigan and Ontario (Brooks et al., 1988; Congdon et al., 1994; Cunnington and Brooks, 1996), we incorporated vital rates for hatching and juvenile survival and projected population growth based upon the life cycle graph in Figure 5.2. For the purposes of this report, we refer the reader to Caswell (2001) for details of model parameters and construction.

Analysis of Organic Contaminant Concentrations: Chemical methods are detailed in Chapter III, as are results of analyses for concentrations of PCBs in eggs used in this study.

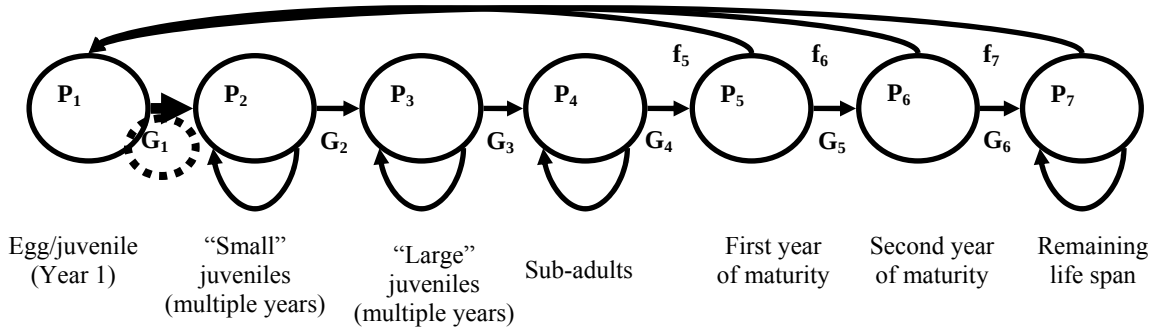

Figure 5.2. Life cycle graph forming the basis of the population models applied to results from the 2004 cohort. Pi = age/stage-specific survival probability (e.g. surviving and remaining in stage i); Gi = age/stage-specific transition probability (e.g. surviving and growing into the next stage); fi = age/stage-specific fertility. The model uses a 1 year time step. Only vital rate G_1 was modified from the base model to project effects of reduced juvenile survival as observed in the experiment.

Results:

Incubation studies

Hatching success: In 2004, hatching rates were extremely low, presumably due to stress incurred during transport from the field sites in NY to the laboratory in MD (average hatch regardless of site = 43 %). Hatching rate in 2005 were much more typical of laboratory incubations (reference 84.6 %; contaminated 86.6 %), resulting in 409 and 591 individuals hatching from reference and contaminated sites, respectively. Hatching rate did not differ statistically among sites.

Size at hatching and time to hatching: In both years, hatchlings from reference areas were significantly larger than those from contaminated areas, both in terms of wet mass and carapace length (Table 5.1). Time to hatching did not differ statistically in 2004. However in 2005 average time to hatching was significantly shorter for individuals from

the contaminated area compared to the reference areas, despite differing only by 3 days on average (Table 5.2).

Table 5.1. Wet mass and carapace length (CL) of hatchlings from contaminated and reference areas in 2004 and 2005. Values are means ± 1 SE.

	2004		2005	
Site	Wet mass (g)	CL (mm)	Wet mass (g)	CL (mm)
Contaminated	7.24 ± 0.36	27.0 ± 0.6	8.63 ± 0.04	27.7 ± 0.1
Reference	8.37 ± 0.42	29.3 ± 0.5	9.04 ± 0.06	28.0 ± 0.1
	$P < 0.001$	$P = 0.006$	$P < 0.001$	$P = 0.007$

Table 5.2. Time to hatching for eggs collected from contaminated and reference areas in 2004 and 2005. Values are means ± 1 SE.

	2004	2005
Site	Time (d)	Time (d)
Contaminated	85 ± 0.3	76 ± 0.1
Reference	84 ± 0.2	79 ± 0.2
	$P = 0.625$	$P < 0.001$

Deformities: Because of the low hatching rates in 2004 and concerns that eggs were compromised during transport or incubation, deformities were only analyzed for individuals from the 2005 collections. Frequency of deformities did not differ statistically between areas. 5.4 % of hatchlings from the contaminated area and 7.8 % from the reference areas displayed deformities. Deformities typically consisted of extra, missing, or abnormal carapacial scutes (19/41 from contaminated areas, 16/41 from

reference areas). Other deformities observed infrequently included spinal and tail curvatures, missing or abnormal eyes, and dented carapace.

Juvenile performance studies:

Survival: There was a large divergence in survival among treatments for the 2004 cohort which began to emerge approximately May, 2005 (Figure 5.3). Survival was affected only by site of egg collection ($P < 0.001$); feeding regime had no main effect nor was there an interaction between food and site of collection. This apparent effect of maternal contributions of PCB on subsequent survival of juveniles was not observed in 2005 in which 96% of juveniles from each site of origin survived but in which PCB concentrations in eggs/juveniles from contaminated areas were much lower than in 2004 (mean = 1417 and 3953 ppm ww respectively).

Juveniles that died during the 2004 study were derived from eggs having average concentrations of total PCBs of 3605 +/- 3438 ppm ww compared to 1404 +/- 2579 ppm ww in those that survived ($P < 0.001$; Figure 5.4). There was a positive relationship between PCB concentration in eggs and mortality (Table 5.3; Figure 5.5).

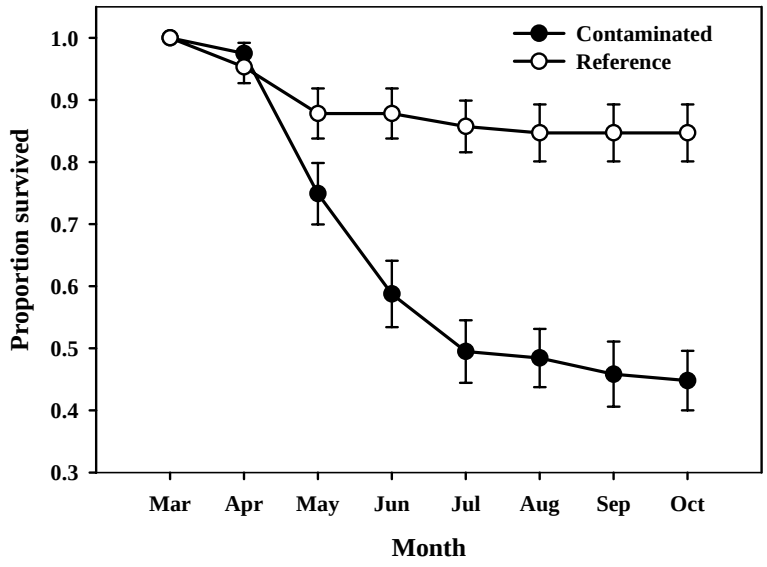

Figure 5.3. Survival of 2004 cohort over time in relation to site from which eggs were collected, regardless of type of food provided to juveniles. Points are means +/- 1 SD.

Figure 5.4. Total PCB concentrations in eggs versus survival of juveniles through the end of the study, 2004 cohort. Values are medians +/- 5th and 95th percentiles.

Table 5.3. Relationships between PCB concentrations in eggs from the contaminated site and mortality of juveniles from the 2004 cohort. With the exception of one very small clutch*, there appears to be a positive relationship between PCB concentration and mortality.

Clutch PCB (ppm ww)	N total	N died	Percent died
95.6	17	6	35.3
3291	16	9	56.3
5050	21	12	57.1
5987*	3	0	0
9220	15	10	66.7

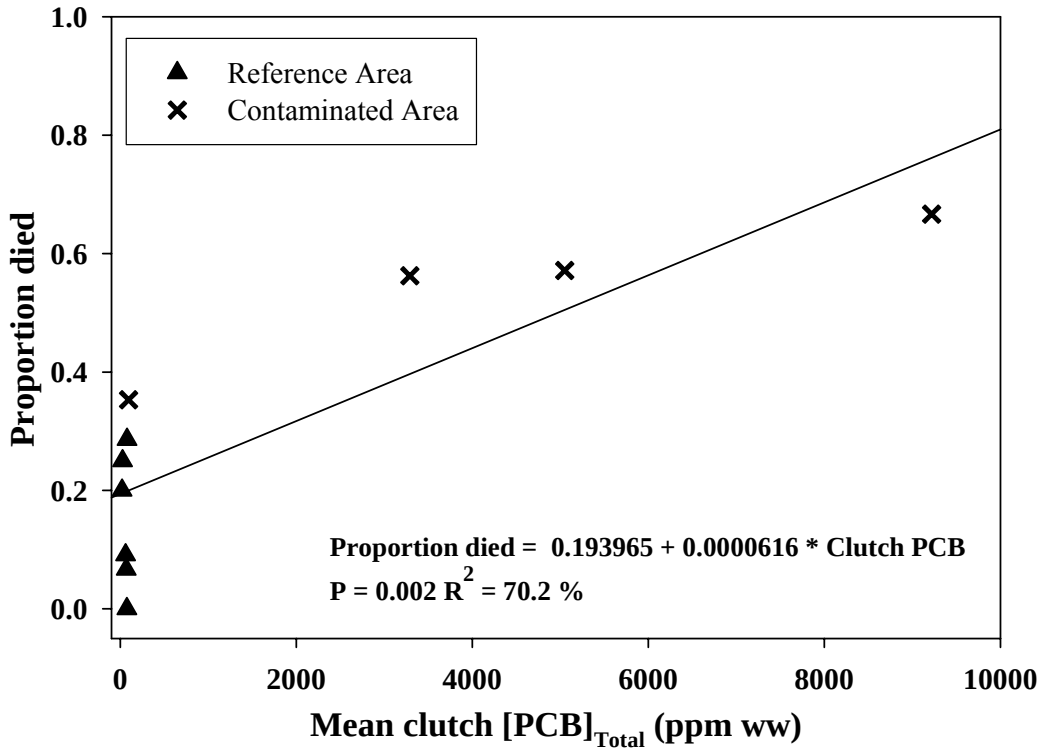

Figure 5.5. Relationship between PCB concentration in eggs from which juveniles were derived and mortality during the 2004 feeding study. Clutches from which fewer than 3 juveniles were used were removed from analysis.

Growth: Final sizes did not differ among treatments during either year of study (Figures 5.6 and 5.7) Final sizes were somewhat smaller in 2005 than in 2004 as the latter experiment lasted approximately 1 month longer than the former.

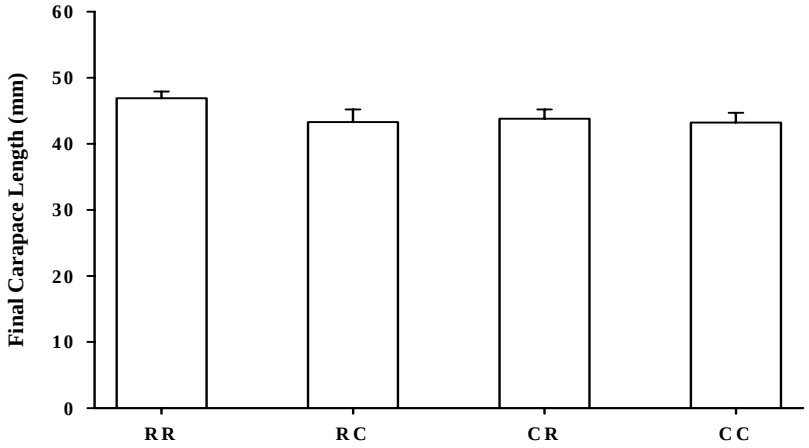

Figure 5.6. Final sizes of juveniles, 2004 study. Treatments did not differ statistically (P = 0.195). Treatment designations defined in Fig. 5.1.

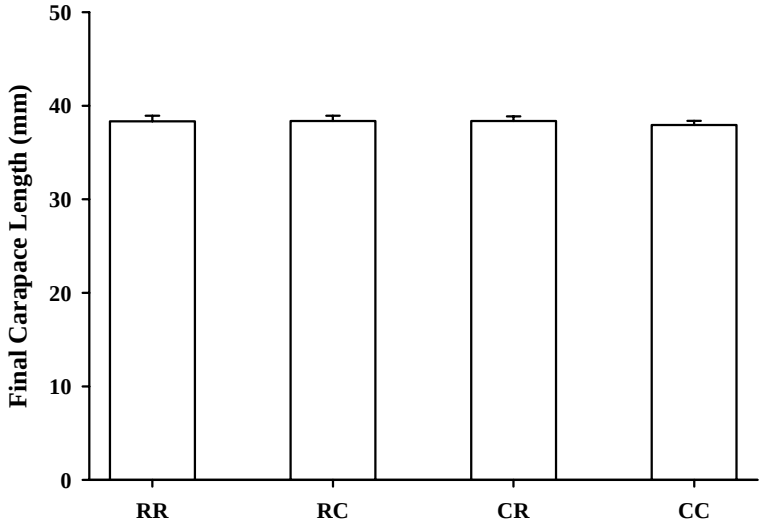

Figure 5.7. Final sizes of juveniles, 2005 study. Treatments did not differ statistically (P = 0.996). Final sizes are somewhat smaller than in 2004 as the 2005 study was 1 month shorter than 2004. Treatment designations defined in Fig. 5.1.

Organosomatic indices: For individuals collected in 2004, TSI did not differ among treatments (Figure 5.8), however HSI was greater in CC than CR (Figure 5.9) In 2005, TSI was significantly smaller for individuals in treatment RR compared to all other treatments (Figure 5.10) and HSI was greater in RR and CR compared to CC and RC (Figure 5.11).

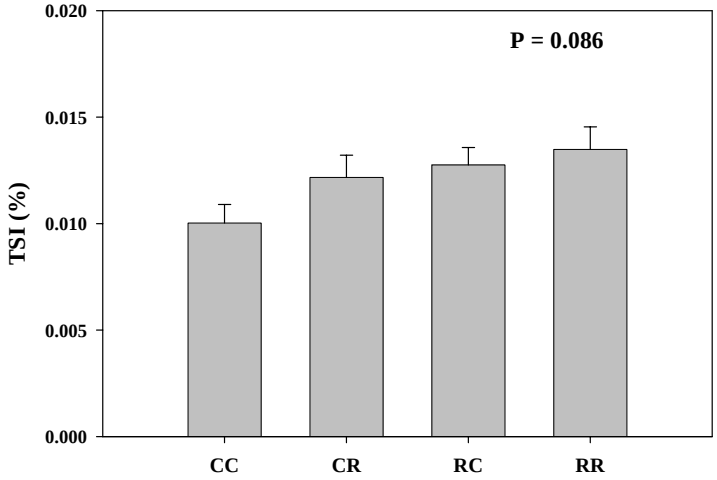

Figure 5.8. Thyrosomatic indices for juveniles, 2004 study.

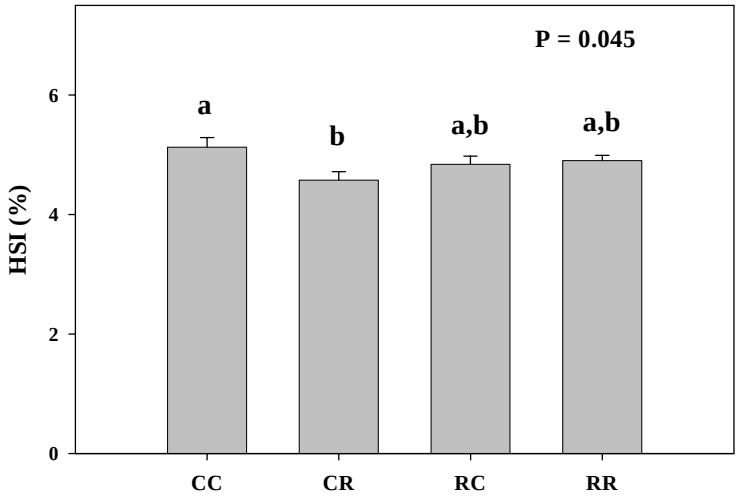

Figure 5.9. Hepatosomatic indices for juveniles, 2004 study. Different letters indicate treatments that differed following post-hoc pairwise comparisons.

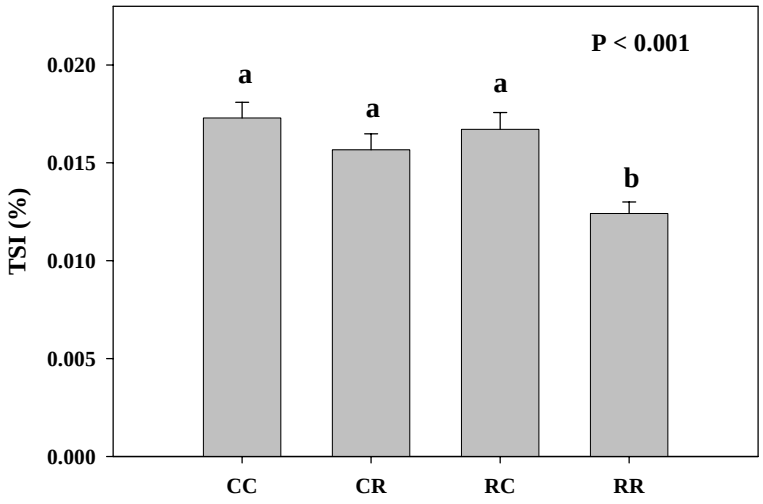

Figure 5.10. Thyrosomatic indices for juveniles, 2005 study. Different letters indicate treatments that differed following post-hoc pairwise comparisons.

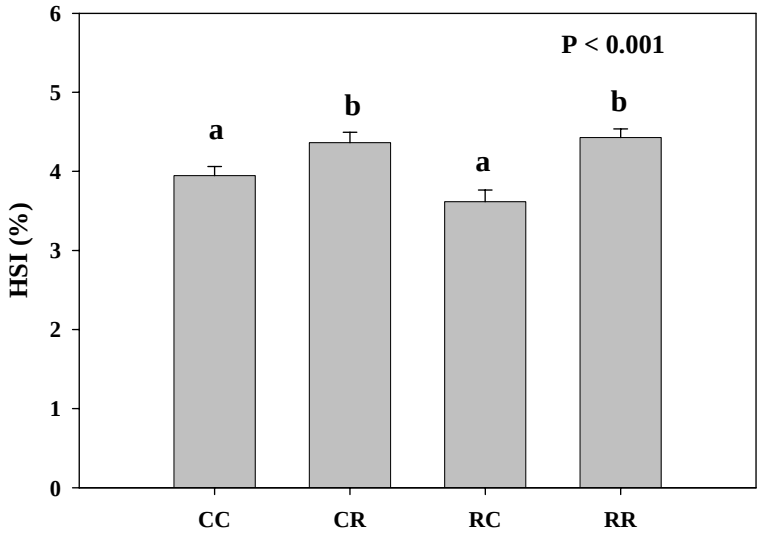

Figure 5.11. Hepatosomatic indices for juveniles, 2005 study. Different letters indicate treatments that differed following post-hoc pairwise comparisons.

Metabolic rate: Initial metabolic measurements on animals collected in 2004 (measured May and July, 2005) differed among treatments such that metabolic rates were elevated in treatments CR and RR relative to CC and RC. However by the final measurement differences were no longer detected. (Figure 5.12). Animals collected in 2005 did not differ statistically during any measurement interval (Figure 5.13).

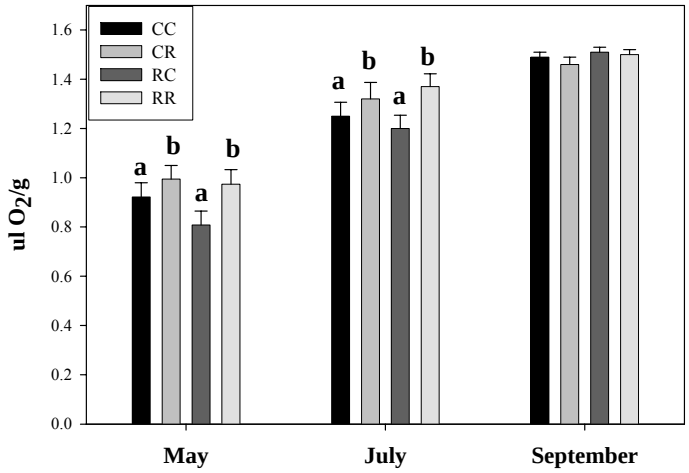

Figure 5.12. Standard metabolic rates, 2004 study. Different letters indicate treatments that differed following post-hoc pairwise comparisons (P < 0.05).

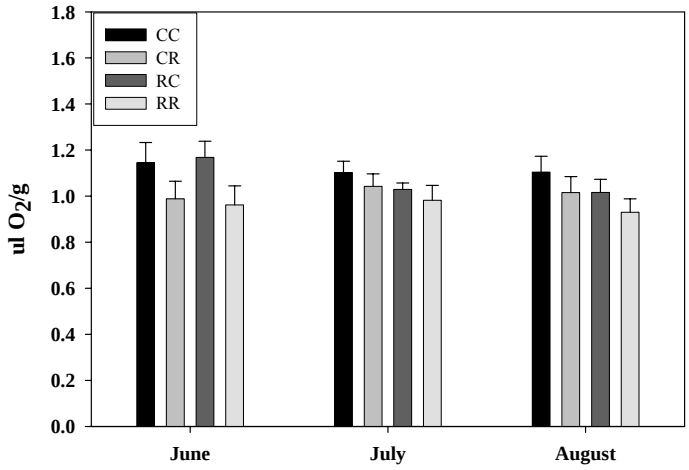

Figure 5.13. Standard metabolic rates, 2005 study.

Behavior: Assays for predator avoidance response for juveniles from the 2004 cohort suggested that individuals having been exposed to PCBs as embryos and via feeding exhibited slightly less activity than those from other treatments. However these results were not repeatable the following year. Other assays resulted in large variations among individuals and among assays for any given individuals. No patterns were discernable.

DNA damage: Assays for single strand DNA breakage on liver and blood from juveniles revealed no treatment-specific differences during either year (Figures 5.14 and 5.15).

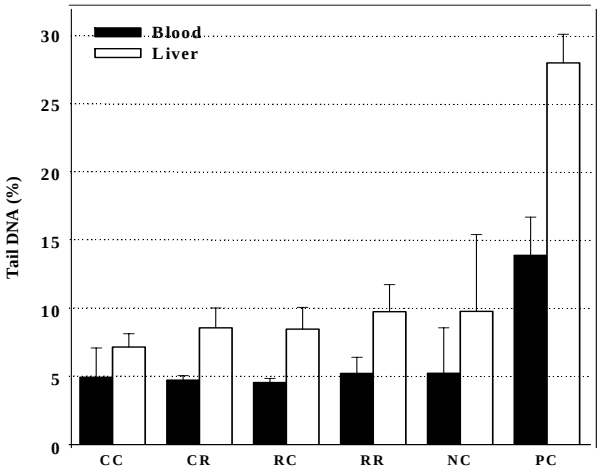

Figure 5.14. Results of DNA damage assays, 2004 animals. "NC = negative control (untreated turtle blood and liver), "PC" = positive control (hydrogen peroxide). Increased % tail DNA indicates increased DNA single strand breakage. There were no significant differences among treatments, excluding the positive control.

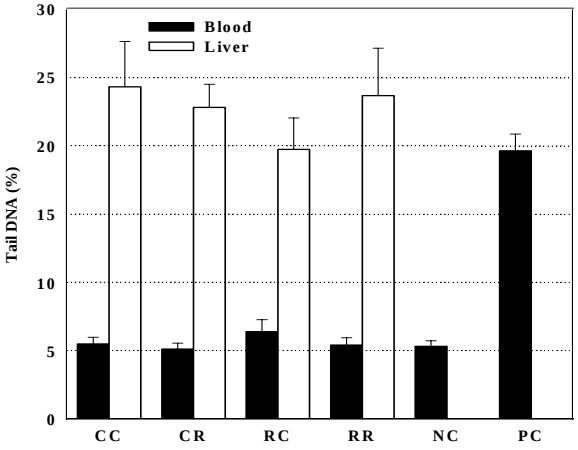

Figure 5.15. Results of DNA damage assays, 2004 animals. "NC = negative control (eastern painted turtle blood), "PC" = positive control (hydrogen peroxide). Increased % tail DNA indicates increased DNA single strand breakage. There were no significant differences among treatments, excluding the positive control.

Sexual differentiation: Eggs were incubated at a male producing temperature such that significant feminization would be apparent as an elevated frequency of morphologically-female individuals in contaminant exposed individuals rather than reference individuals.

Inspection of gonads following dissection revealed that feminization (presence of ovarian rather than testicular tissue) was exceptionally rare (Table 5.4).

Table 5.4. Percent of juveniles that were morphologically male (normal testes) versus site of collection, 2004 and 2005.

	2004	2005
Site	**% male**	**% male**
Contaminated	99.0	99.0
Reference	100	99.8

Population models: Matrix models performed well and provided the expected patterns in stable age distributions and reproductive values that are typical of long lived, iteroparous species such as turtles. Stable age/stage distributions are strongly biased toward early age classes, reflecting the high life time fecundity of turtles and low survival rates of these age classes (Figure 5.16). A slightly higher proportion of eggs and hatchlings in a stable population in the contaminated sites relative to reference sites reflects the elevated hatchling/juvenile mortality rates in the former. Reproductive value is strongly skewed toward older age classes regardless of site, reflecting high survivorship and increasing fecundity or reproductive fitness probability with age (5.17).

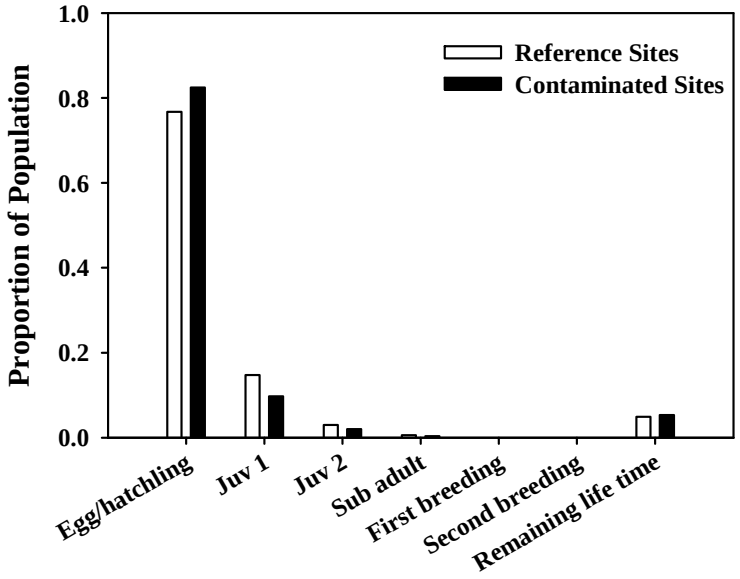

Figure 5.16. Stable age/stage distributions predicted from the matrix population models for the 2004 cohort.

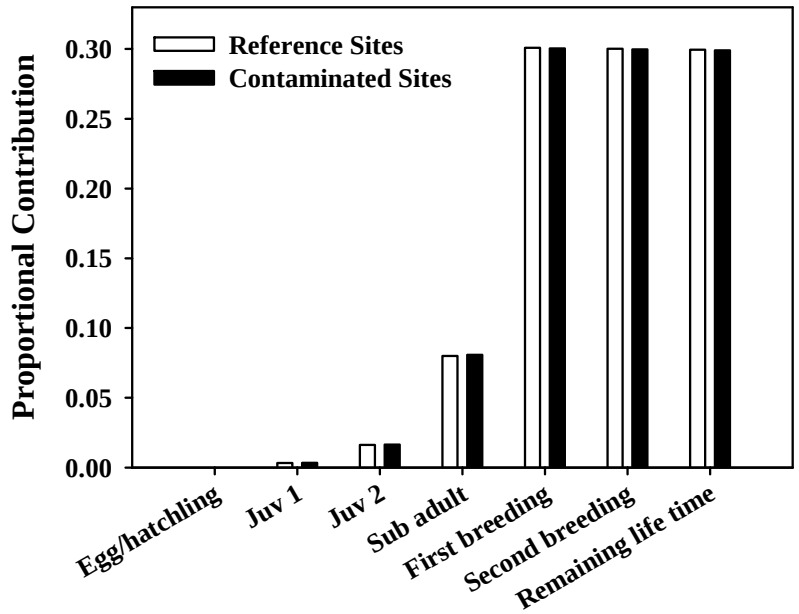

Figure 5.17. Relative reproductive values predicted from the matrix population models for the 2004 cohort.

Incorporating mortality rates observed in juveniles from the 2004 cohort and integrating these effects over a 10 year projection period projected a 9 % decrease in intrinsic rate of population growth and a 13 % increase in generation turnover time in the contaminated areas relative to reference areas (Figure 5.18). Number of replacements was projected to be 39 % lower in contaminated relative to reference areas. If juvenile mortality observed in the 2004 study for contaminated individuals were sustained over 10 years and representative of the population as a whole, a 15 % decline in population size was projected over that time span.

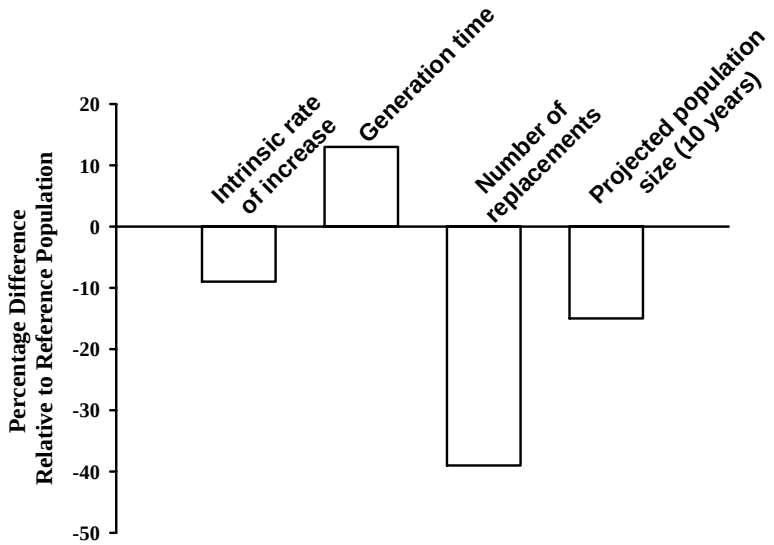

Figure 5.18. Summary of model outputs for population parameters for the population inhabiting the contaminated area relative to the reference area. Outputs reflect data from 2004 cohort.

Discussion

Responses that we hypothesized to be "early warning" signs of PCB toxicity typically did not differ among sites or feeding treatments or did not differ consistently

over time. For example, DNA single strand breakage is commonly quantified as a bioindicator of the onset of sublethal toxicity resulting from exposure to organic and inorganic contaminants (Mitchelmore and Chipman, 1998), yet there was no evidence of elevated DNA damage due to either maternal or food borne exposures. As well, malformations in gross morphology of hatchlings has been observed in snapping turtles in the Great Lakes (Bishop et al., 1991; 1998) where they were exposed to PCBs typically at somewhat lower concentrations than in our study where no site-specific differences in malformations were observed. However, the areas of the Great Lakes in which elevated frequencies of malformations were observed also were contaminated with high concentrations of other organic compounds, including organochlorine pesticides which may have been primarily responsible for the malformations (Bishop et al., 1991; 1998).

Metabolic rate has also been proposed as a sensitive indicator growth effects of contaminant effect (Calow and Sibly, 1990;Rowe et al., 2001) yet only varied in response to PCB exposure during one assay in 2004 and the pattern of response was not consistent over time. Significant reductions in metabolic rate observed in individuals from treatments in which they were fed contaminated food (CC and RC) may suggest a temporary narcotic effect of PCBs. Yet this effect was only observed in one of three assays in 2004 and in none of the assays in 2005. Other growth related metrics that we measured, hepatosomatic and thyrosomatic indices (HSI, TSI), also revealed inconsistent patterns from which we can draw no justified conclusions.

The lack of detection of early warning indicators of toxicity is particularly interesting in light of the larger, "whole animal" responses that we observed. Notably,

eggs collected from contaminated areas produced significantly smaller hatchlings during both years of the study. Whether these differences influence offspring fitness in natural habitats where predator avoidance, foraging rate, or other factors may be related to size is unknown. Size differences varied in magnitude depending upon the metric used (mass or CL) and year. In 2004, animals from the contaminated site were approximately 13% smaller in wet mass and 8 % smaller in CL compared to reference areas. In 2005 however, the sites differed only by 7 % (mass) and 1 % (CL). Identification of the latter in particular as statistically significant likely reflects a consistently small difference in combination with an very large number of eggs that were measured.

The most interesting and unexpected result of the study was the latent onset of mortality of juveniles from the 2004 cohort. Beginning approximately nine months after hatching, juveniles derived from clutches from the contaminated area (regardless of food treatment) began experiencing high rates of mortality in contrast to those from reference areas. Ultimately only approximately 40 % of contaminated individuals survived the entire study compared to approximately 90 % from the reference areas. For animals from the contaminated site, mortality rates correlated with maternal PCB contribution to eggs such that the highest mortality rates were observed in individuals from the most contaminated clutches. There was no correlation between mortality and PCB concentration for reference animals due to the small numbers of individuals that died and the narrow range in PCB concentrations in eggs from that area.

In the 2005 cohort, juvenile mortality rates were very low and thus did not correlate with egg PCB concentrations. This may reflect the differences in egg PCB concentrations in 2004 and 2005. Eggs from contaminated areas in 2004 were on

average 2.8 times higher in total PCBs than those used in 2005 (3953 vs. 1417 ppm ww), reflecting variation in PCBs among clutches collected from field sites. In both years eggs from reference areas were nearly the same (61 and 73 ppm ww).

Population models suggested that the mortality rates observed in juveniles from the 2004 cohort, if sustained over multiple years, could reduce total population sizes in the absence of immigration from uncontaminated areas. While turtle populations are typically most sensitive to mortality of late stage juveniles and adults, our models suggest that the cumulative impacts of reduced hatchling/early juvenile survival could reduce population growth rate by approximately 9 % and lead to a 15 % decline in population size if recurring over 10 years. However, the models assume not only that mortality rates would be unchanged over time, but also that the effects we observed are applicable to the entire population of turtles inhabiting the contaminated areas. The differences in PCB concentrations and effects observed over two years suggests that the proportion of the population captured and collected in a given year is unlikely representative of the population as a whole. In lieu of long term data sets on the demographics of the snapping turtle population in the upper Hudson River region, the population model results must be viewed cautiously as they reflect only a subset of individuals from the population. We conclude that reproductive fitness for some individuals occupying contaminated habitats in the region is likely compromised, yet the proportion of the population affected in this way remains unknown.

VI. REFERENCES

Ahlborg, U.G., Becking, G.C., Birnbaum, L.S., Brouwer, A., Derks, H.J.G.M., Feeley, M., Golor, G., Hanberg, A., Larsen, J.C., Liem, A.K.D., Safe, S.H., Schlatter, C., Wærn, F., Younes, M., and Yrjänheikki, E. Toxic equivalency factors for dioxin-like PCBs. *Chemosphere* **28**: 1049-1067.

Alexander, M.M. 1943. Food habits of the snapping turtle in Connecticut. *Journal of Wildlife Management* **7**: 278-282.

Alford-Stevens, A.L. 1986. Analyzing PCBs: basic information about PCBs and how they are identified and measured. *Environmental Science and Technology* **20**: 1194-1199.

Ashpole, S.L., Bishop, C.A., and Brooks, R.J. 2004. Contaminant residues in snapping turtle (*Chelydra serpentina*) eggs from the Great Lakes-St. Lawrence River Basin (1999-2000). *Archives of Environmental Contamination and Toxicology* **51**: 270-286.

Baker, J.E., Bohlen, W.F., Bopp, R., Brownawell, B., Collier, T.K., Farley, K.J., Geyer, W.R., and Nairn, R. 2001. PCBs in the Upper Hudson River: the science behind the dredging controversy. A white paper prepared for the Hudson River Foundation, New York, NY. Available online at http://www.seagrant.sunysb.edu/HEP/archive/hrfpcb102901.pdf.

Baumann, P.C. and Whittle, D.M. 1988. The status of selected organics in the Laurentian Great Lakes: an overview of DDT, PCBs, dioxins, furans, and aromatic hydrocarbons. *Aquatic Toxicology* **11:** 241-257.

Bedard, D.L. 2001. Microbial dechlorination of PCBs in aquatic sediments. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 27-33.

Bergeron, J.M., Crews, D. and McLachlan, J.A. 1994. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. *Environmental Health Perspectives* **102**: 780-781.

Bergink, E.W. and Wallace, R.A. 1974. Precursor-product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. *Journal of Biological Chemistry* **249**: 2897-2903.

Bishop, C.A., Brooks, R.J., Carey, J.H., Ng, P., Norstrom, R.J., and Lean, D.R.S. 1991. The case for a cause-effect linkage between environmental contamination and development in eggs of the common snapping turtle (*Chelydra s. serpentina*) from Ontario, Canada. *Journal of Toxicology and Environmental Health* **33**: 521-547.

Bishop, C.A., Brown, G.P., Brooks, R.J., Lean, D.R.S., and Carey, J.H. 1994. Organochlorine contaminant concentrations in eggs and their relationship to body size, and clutch characteristics of the female common snapping turtle (*Chelydra serpentina serpentina*). *Archives of Environmental Contamination and Toxicology* **27**: 82-87.

Bishop, C.A., Lean, D.R.S., Brooks, R.J., Carey, J.H., and Ng, P. 1995. Chlorinated hydrocarbons in early life stages of the common snapping turtle (*Chelydra serpentina serpentina*) from a coastal wetland on Lake Ontario, Canada. *Environmental Toxicology and Chemistry* **14**: 421-426.

Bishop, C.A., Ng, P., Norstrom, R.J., Brooks, R.J., and Pettit, K.E. 1996. Temporal and geographic variation of organochlorine residues in the eggs of the common snapping turtle (*Chelydra serpentina serpentina*) (1981-1991) and comparisons to trends in the herring gull (*Larus argentatus*) in the Great Lakes Basin in Ontario, Canada. *Archives of Environmental Contamination and Toxicology* **31**: 512-524.

Bishop, C.A., Ng, P., Pettit, K.E., Kennedy, S.W., Stegeman, J.J., Norstrom, R.J., and Brooks, R,J. 1998. Environmental contamination and developmental abnormalities in eggs and hatchlings of the common snapping turtle (*Chelydra serpentina serpentina*) from the Great Lakes-St Lawrence River basin (1989-1991). *Environmental Pollution* **101**: 143-156.

Bitman, J. and Cecil, H.C. 1970. Estrogenic activity of DDT analogs and polychlorinated biphenyls. *Journal of agricultural and food chemistry* **18**: 1108-1112.

Bonin, J., DesGranges, J.-L., Bishop, C.A., Rodrigue, J., Gendron, A., and Elliot, J.E. 1995. Comparative study of contaminants in the mudpuppy (*Amphibia*) and the common snapping turtle (*Reptilia*), St. Lawrence River, Canada. 1995. *Archives of Environmental Contamination and Toxicology* **51**: 270-286.

Brooks, R.J., Galbraith, D.A., Nancekivell, E.G., and Bishop, C.A. 1988. Developing management guidelines for snapping turtles. In: *Management of amphibians, reptiles, and small mammals in North America: Proceedings of a symposium, Flagstaff, Arizona, July 19-21, 1988.* U.S. Forest Service, pp. 173-179.

Bopp, R.F., Simpson, H.J., Olsen, C.R., and Kostyk, N. 1981. Polychlorinated biphenyls in sediments of the tidal Hudson River, New York. *Environmental Science and Technology* **15**: 210-216.

Borgmann, U. and Whittle, D.M. 1991. Contaminant concentration trends in Lake Ontario lake trout (*Salvelinus namaycush*): 1977 to 1988. *Journal of Great Lakes Research* **17**: 368-381.

Brion, F., Rodriguez, F., Noury, P., Migeon, B., Flammarion, P., Thybaud, E., and Porcher, J.M. 2000. Two-step purification method of vitellogenin from three teleost fish species: rainbow trout (*Oncorhynchus mykiss*), gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*). *Journal of Chromatography* **737**: 3-12.

Brown, G.P., Bishop, C.A., and Brooks, R.J. 1994. Growth rate, reproductive output, and temperature selection of snapping turtles in habitats of different productivities. *Journal of Herpetology* **28**: 405-410.

Brown, J.F., Jr., Bedard, D.L., Brennan, M.J., Carnahan, J.C., Feng, H., and Wagner, R.E. 1987a. Polychlorinated biphenyl dechlorination in aquatic sediments. *Science* **236:** 709-712.

Brown, J.F., Jr., Wagner, R.E., Feng, H., Bedard, D.L., Brennan, M.J., Carnahan, J.C., and May, R.J. 1987b. Environmental dechlorination of PCBs. *Environmental Toxicology and Chemistry* **6**: 579-593.

Bryan, A.M., Olafsson, P.G., and Stone, W.B. 1987. Disposition of low and high environmental concentrations of PCBs in snapping turtle tissues. *Bulletin of Environmental Contamination and Toxicology* **38**: 1000-1005.

Bush, B., Streeter, R.W., and Sloan, R.J. 1989. Polychlorinated biphenyl (PCB) congeners in striped bass (*Morone saxatilis*) from marine and estuarine waters of New York state determined by capillary gas chromatography. *Archives of Environmental Contamination and Toxicology* **19**: 49-61.

Cagle, F.R. 1939. A system of marking turtles for future identification. *Copeia* 1939: 170-173.

Calow P, Sibly RM. 1990. A physiological basis of population processes: ecotoxicological implications. *Functional Ecology* 4:283-288.

Caswell H. 2001. Matrix population models: construction, analysis, and interpretation. Second edition. Sunderland, MA: Sinauer Associates.

CEC. 1996. *Status of PCB management in North America.* Commission for Environmental Cooperation, Montreal, Canada.

Christiansen, J.L. and Burken, R.R. 1979. Growth and maturity of the snapping turtle (*Chelydra serpentina*) in Iowa. *Herpetologica* **35**: 261-266.

Colborn, T., von Saal, F.S., and Soto, A.M.. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* **101**: 378-384.

Cobb, G.P. and Wood, P.D. 1997. PCB Concentrations in eggs and chorioallantoic membranes of loggerhead sea turtles (*Caretta caretta*) from the Cape Romain National Wildlife Refuge. *Chemosphere* **34**: 539-549.

Cogliano, V.J. 2001. Considerations for setting reference values for environmental PCBs. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 429-435.

Congdon, J.D. and Gibbons, J.W. 1990a. The evolution of turtle life histories. In: Gibbons, J.W. (ed) *Life history and ecology of the slider turtle*. Smithsonian Institution Press, Washington, D.C., pp. 45-54.

Congdon, J.D. and Gibbons, J.W. 1990b. Turtle eggs: their ecology and evolution. In: Gibbons, J.W. (ed) *Life history and ecology of the slider turtle*. Smithsonian Institution Press, Washington, D.C., pp. 109-123.

Congdon, J.D., Dunham, A.E. and van Loben Sels, R.C. 1994. Demographics of common snapping turtles (*Chelydra serpentina*): Implications for conservation and management of long-lived organisms. *American Zoologist* **34**: 397-408.

Congdon JD, Dunham AE, Hopkins WA, Rowe CL, Hinton TG. 2001. Resource allocation based life history trait values: a conceptual basis for studies of environmental

toxicology. *Environmental Toxicology and Chemistry* 20:1698-1703.

Connolly, J.P., Zahakos, H.A., Benaman, J., Ziegler, C.K., Rhea, J.R., and Russell, K. 2000. A model of PCB fate in the Upper Hudson River. *Environmental Science and Technology* **34**: 4076-4087.

Cooke, P.S., Sato, T. and Buchanan, D.L. 2001. Disruption of steroid hormone signaling in PCBs. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 257-263.

Copeland, P.A., Sumpter, J.P., Walker, J.P., Croft, M. 1986. Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri* Richardson) at various stages of the reproductive cycle. *Comparative Biochemistry and Physiology* **83B**: 487-493.

Crews, D., Bull, J.J., and Wibbels, T. 1991. Estrogen and sex reversal in turtles: a dose-dependent phenomenon. *General and Comparative Endocrinology* **81**: 357-364.

Cunnington DC, Brooks RJ. 1996. Bet-hedging theory and eigenelasticity: A comparison of the life histories of loggerhead sea turtles (Caretta caretta) and snapping turtles (Chelydra serpentina). *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 74(2):291-296.

Dabrowska, H., Fisher, S.W., Estenik, J., Kidekhel, R., and Stromberg, P. 2006. Polychlorinated biphenyl concentrations, congener profiles, and ratios in the fat tissue, eggs and plasma of snapping turtle (*Chelydra s. serpentina*) from the Ohio Basin of Lake Erie, USA. *Archives of Environmental Contamination and Toxicology* **51**: 270-286.

Denslow, N.D., Chow, M.C., Kroll, K.J., and Green, L. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. *Ecotoxicology* **8**: 385-398.

de Solla, S.R., Bishop, C.A., Van Der Kraak, G., and Brooks, RJ. 1998. Impact of organochlorine contamination on levels of sex hormones and external morphology of common snapping turtles (*Chelydra serpentina serpentina*) in Ontario, Canada. *Environmental Health Perspectives* **106**: 253-260

de Solla, S.R., Bishop, C. A., Lickers, H., and Jock, K. 2001. Organochlorine pesticides, PCBs, dibenzodioxin, and furan concentrations in common snapping turtle eggs (*Chelydra serpentina serpentina*) in Akwesasne, Mohawk Territory, Ontario, Canada. *Archives of Environmental Contamination and Toxicology* **40**: 410-417.

de Solla, S.R. and Ferni, K.J. 2004. Characterization of contaminants in snapping turtles (*Chelydra serpentina*) from Canadian Lake Erie Areas of Concern: St. Clair River, Detroit River, and Wheatley Harbour. *Environmental Pollution* **132**: 101-112.

DiPinto, L.M. and Coull, B.C. 1997. Trophic transfer of sediment-associated polychlorinated biphenyls from meiobenthos to bottom-feeding fish. *Environmental Toxicology and Chemistry* **16**: 2568-2575.

Drinker, C.K., Warren, M.F., and Bennet, G.A. 1937. The problem of possible systemic effects from certain chlorinated hydrocarbons. *The Journal of Industrial Hygeine and Toxicology* **19:** 283- 311.

Drouillard, K.G. and Norstrom, R.J. 2001. Quantifying maternal and dietary sources of 2,2',4,4',5,5'-hexachlorobiphenyl deposited in eggs of the ring dove (*Streptopelia risoria*). *Environmental Toxicology and Chemistry* **20**: 561-567.

Echols, K.R., Tillitt, D.E., Nichols, J.W., Secord, A.L., and McCarty, J.P. 2004. Accumulation of PCB congeners in nestling tree swallows (*Tachycineta bicolor*) on the Hudson River, New York. *Environmental Science and Technology* **38:** 6240-6246.

Erickson, M.D. 2001. Introduction: PCB properties, uses, occurrence, and regulatory history. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. xi-xxx.

Ewert, M.A. and Legler, J.M. 1978. Hormonal induction of oviposition in turtles. *Herpetologica* **34**: 314-318.

Ewert, M.A., Etchberger, C.R., and Nelson, C.E. 2004. Turtle sex-determining modes and TSD patterns, and some TSD pattern correlates. In: Valenzuela, N. and Valentine, L. (eds) *Temperature-dependent sex determination in vertebrates*. Smithsonian Books, Washington, D.C., pp. 21-32.

Fenske, M., van Aerle, R., Brack, S., Tyler, C.R., and Segner, H. 2001. Development and validation of a homologous zebrafish (*Danio rerio* Hamilton-Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. *Comparative Biochemistry and Physiology* **129C:** 217-232.

Frame, G.M., Cochran, J.W., and Bowadt, S.S. 1996a. Complete PCB congener distributions for 17 Arochlor mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative, congener-specific analysis. *HRC—Journal of High Resolution Chromatography* **19**: 657-668.

Frame, G.M., Wagner, R.E., Carnahan, J.C., Brown, J.F., May, R.J., Smullen, L.A., and Bedard, D.L. 1996b. Comprehensive, quantitative, congener-specific analyses of eight Aroclors and complete PCB congener assignments on DB-1 capillary GC columns. *Chemosphere* **33**: 603-623.

Galbraith, D.A., Bishop, C.A., Brooks, R.J., Simser, W.L., and Lampman, K.P. 1988. Factors affecting the density of populations of common snapping turtles (*Chelydra serpentina serpentina*). *Canadian Journal of Zoology* **66:** 1233-1240.

Galbraith, D.A., Brooks, R.J., and Obbard, M.E. 1989. The influence of growth rate on age and body size at maturity in female snapping turtles (*Chelydra serpentina*). *Copeia* **1989**: 896-904.

Geyer, H.J., Rimkus, G.G., Scheunert, I., Kaune, A., Schramm, K.-W., Kettrup, A., Zeeman, M., Muir, D.C.G., Hansen, L.G., and Mackay, D. 2000. Bioaccumulation and occurrence of endocrine-disrupting chemicals (EDCs), persistent organic pollutants (POPs), and other organic compounds in fish and other organisms, including humans. In: Beek, B. (ed.) *The Handbook of Environmental Chemistry.* Springer-Verlag, Berlin-Heidelberg, pp. 112-118.

Gibbons, J.W. 1987. Why do turtles live so long? *Bioscience* **37**: 262-269.

Gibbons, J.W. and Greene, J.L. 1990. Reproduction in the slider and other species of turtles. In: Gibbons, J.W. (ed) *Life history and ecology of the slider turtle*. Smithsonian Institution Press, Washington, D.C., pp. 124-134.

Gibbons, J.W. and Lovich J.E. 1990. Sexual dimorphism in turtles with an emphasis on the slider turtle (*Trachemys scripta*). *Herpetological Monographs* **4**: 1-29.

Gómez-Catalán, J., To-Figueras, J., Rodamilans, M., and Corbella, J. 1991. Transport of organochlorine residues in the rat and human blood. *Archives of Environmental Contamination and Toxicology* **20**: 61-66.

Guillette Jr., L.J., Pickford, D.B., Crain, D.A., Rooney, A.A., and Percival, H.F. 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. *General and Comparative Endocrinology* **101**: 32-42.

Guillette Jr., L.J., Woodward, A.R., Crain, D.A., Pickford, D.B., Rooney, A.A., and Percival, H.F. 1999. Plasma steroid concentrations and male phallus size in juvenile alligators from seven Florida lakes. *General and Comparative Endocrinology* **116**: 356-372.

Gunderson, M.P., Breza, Jr., T.S., and Guillette, Jr., L.J. 2003. Lack of xenoestrogen-induced vitellogenin in wild juvenile American alligators (*Alligator mississippiensis*) from Lake Okeechobee and the Everglades. *Environmental Sciences* **10**: 193-204.

Hammer, D.A. 1969. Parameters of a marsh snapping turtle population: Lacreek Refuge, South Dakota. *Journal of Wildlife Management* **33**: 995-1005.

Hansen, L.G. 2001. Identification of steady state and episodic PCB congeners from multiple pathway exposures. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent*

advances in environmental toxicology and health effects. University Press of Kentucky, Lexington, KY, pp. 47-56.

Hany, J., Lilienthal, H., Sarasin, A., Roth-Harer, A., Fastabend, A., Dunemann, L., Lichtensteiger, W., and Winneke, G. 1999. Developmental exposure of rats to a reconstituted PCB mixture of Aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior. *Toxicology and Applied Pharmacology* **158**: 231-243.

Hebert, C.E., Glooschenko, V., Haffner, G.D., and Lazar, R. 1993. Organic contaminants in snapping turtle (*Chelydra serpentina*) populations from southern Ontario, Canada. *Archives of Environmental Contamination and Toxicology* **24:** 35-43.

Herbst, L.H., Siconolfi-Baez, L, Torelli, J.H., Klein, P.A., Kerben, M.J., and Schumacher, I.M. 2003. Induction of vitellogenin by estradiol-17β and development of enzyme-linked immunosorbent assays to quantify plasma vitellogenin levels in green turtles (*Chelonia mydas*). *Comparative Biochemistry and Physiology Part B* **135:** 551-563.

Holoubek, I. 2001. Polychlorinated biphenyl (PCB) contaminated sites worldwide. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 17-26.

Honda, K., Tatsukawa, R., Itano, K., Nobuyuki, M., and Fujiyama, T. 1983. Heavy metal concentrations in muscle, liver and kidney tissue of striped dolphin (*Stenella coeruleoalba*) and their variations with body length, weight, age and sex. *Agricultural and Biological Chemistry* **47**: 1219-1228.

Hong, C.S., Xiao, J., Bush, B., and Shaw, S.D. 1998. Environmental occurrence and potential toxicity of planar, mono-, and di-*ortho* polychlorinated biphenyls in the biota. *Chemosphere* **36**: 1637-1651.

Hudson River Natural Resource Trustees. 2005a. Data report for the collection of eggs from spotted sandpipers, American woodcock, belted kingfisher, American robin, red-winged blackbird, and Eastern phoebe associated with the Hudson River from Hudson Falls to Schodack Island, New York. U.S. Department of Commerce, Silver Spring, MD.

Hudson River Natural Resource Trustees. 2005b. Data report for the collection of eggs from the common snapping turtle (*Chelydra serpentina serpentina*) from the Hudson River, New York. U.S. Department of Commerce, Silver Spring, MD.

Hyllner, S.J., Oppen-Bernsten, D.O., Helvik, J.V., Walther, B.T., and Haux, C. 1991. Oestradiol 17ß induces major vitelline envelope proteins in both sexes in teleosts. *Journal of Endocrinology* **131**: 229-236.

Irwin, L.K., Gray, S., and Oberdörster, E. 2001. Vitellogenin induction in painted turtle, *Chrysemys picta*, as a biomarker of exposure to environmental levels of estradiol. *Aquatic Toxicology* **55**: 49-60.

Iverson, J.B., Higgins, H., Sirulnik, A., and Griffiths, C. 1997. Local and geographic variation in the reproductive biology of the snapping turtle (*Chelydra serpentina*). *Herpetologica* **53**: 96-117.

James, M.O. 2001. Polychlorinated biphenyls: metabolism and metabolites. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 35-46.

Jansen, H.T., Cooke, P.S., Porcelli, J., Liu, T-C., and Hansen, L.G. 1993. Estrogenic and anti-estrogenic actions of PCBs in the female rat. *Reproductive Toxicology* **7**:237-248.

Jobling, S. and Sumpter, J.P. 1993. Detergent components in sewage effluent are weakly estrogenic in fish—an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocyes. *Aquatic Toxicology* **27**: 361-372.

Karcher, S.C., Small, M.J., and VanBriesen, J.M. 2004. Statistical method to evaluate the occurrence of PCB transformations in river sediments with application to Hudson River data. *Environmental Science and Technology* **38**: 6760-6766.

Keller, J.M., Kucklick, J.R., and McClellan-Green, P.D. 2004. Organochlorine contaminants in loggerhead sea turtle blood: extraction techniques and distribution among plasma and red blood cells. *Archives of Environmental Contamination and Toxicology* **46**: 254-64.

Kester, M.H.A., Bulduk, S., Tibboel, D., Meinl, W., Glatt, H., Falany, C.N., Coughtrie, M.A.H., Bergman, A., Safe, S.H., Kuiper, G.G.J.M., Schuur, A.G., and Visser, T.J. 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology* **141**: 1897-1900.

Kime, D.E., Nash, J.P., and Scott, A.P. 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. *Aquaculture* **177**: 345-352.

Korach, K.S., Sarver, P., Chae, K, McLachlan, J.A. and McKinney, J.D. 1988. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. *Molecular Pharmacology* **33**: 120-126.

Korach, K.S. and McLachlan, J.A. 1995. Techniques for detection of estrogenicity. *Enviromental Health Perspectives Supplement* **103**: 5-8.

Korach, K.S., Sarver, P., Chae, K., McLachlan, J.A., and McKinney, J.D. 1988. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. *Molecular Pharmacology* **33**: 120-126.

Krieg, A. J., Krieg, S.A., Ahn, B.S., and Shapiro, D. J. 2004. Interplay between Estrogen Response Element Sequence and Ligands Controls *in Vivo* Binding of Estrogen Receptor to Regulated Genes. *Journal of Biological Chemistry* **279**: 5025 - 5034

Lagler, K.F. and Applegate, V.C. 1943. Relationship between the length and the weight in the snapping turtle *Chelydra serpentina* Linnaeus. *The American Naturalist* **77**: 476-478.

Lassiter, R.R. and Hallum, T.G. 1990. Survival of the fattest: implications for acute effects of lipophilic chemicals on aquatic populations. *Environmental Toxicology and Chemistry* **9**: 585-595.

Lester, D.C. and McIntosh, A. 1994. Accumulation of polychlorinated biphenyl congeners from Lake Champlain sediments in *Mysis relicta. Environmental Toxicology and Chemistry* **13**: 1825-1841.

Letcher, R.J., Norstrom, R.J., Muir, D.C.G., Sandau, C.D., Koczanski, K., Michaud, R., de Guise, S., and Béland, P. 2000. Methylsulfone polychlorinated biphenyl and 2,2-bis(chlorophenyl)-1,1-dichloroethylene metabolites in beluga whale (*Delphinapterus leucas*) from the St. Lawrence River estuary and western Hudson Bay, Canada. *Environmental Toxicology and Chemistry* **19**: 1378-1388.

Longnecker, M.P. 2001. Endocrine and other human health effects of environmental and dietary exposure to polychlorinated biphenyls (PCBs). In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 112-118.

Mader, D.R. 1996. *Reptile Medicine and Surgery.* W.B. Saunders: Philadelphia.

Manly, B.F.J. 2007. Randomization, bootstrap and Monte Carlo methods in biology, 3rd edition. Chapman and Hall, New York, NY.

Mayack, D.T. and Loukmas, J. 2001. Progress report on Hudson River mammals: polychlorinated biphenyl (PCB) levels in mink, otter, and muskrat and trapping results for mink, the upper Hudson River drainage, 1998-2000. Prepared for Hudson River Natural Resource Trustees, U.S. Department of Commerce, Silver Spring, MD.

McKenzie, C., Godley, B.J., Furness, R.W., and Wells, D.E. 1999. Concentrations and patterns of organochlorine contaminants in marine turtles from Mediterranean and Atlantic waters. *Marine Environmental Research* **47**: 117-135.

Mitchell, J.C. 1997. *Reptiles of Virginia*. Smithsonian Books: Washington, D.C.

Mitchelmore CL, Chipman JK. 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* 399(2):135-147.

Moore, M., Mustain, Ml, Daniel, K., Chen, I., Safe, S. Zacharewski, T., Gillesby, B., Joyeux, A. and Balaguer, P. 1997. Antiestrogenic activity of hydroxylated polychlorinated biphenyl congeners identified in human serum. *Toxicology and Applied Pharmacology* **142**: 160-168.

Mosimann, J.E. and Bider, J.R. 1960. Variation, sexual dimorphism, and maturity in a Quebec population of the common snapping turtle, *Chelydra serpentina*. *Canadian Journal of Zoology* **38**: 19-38.

Nichols, J.W., Echols, K.R., Tillitt, D.E., Secord, A.L., and McCarty, J.P. 2004. Bioenergetics based modeling of individual PCB congeners in nestling tree swallows from two contaminated sites on the upper Hudson River, New York. *Environmental Science and Technology* **38:** 6234-6239.

Nichols, J.W., Echols, K.R., Tillitt, D.E., Secord, A.L., and McCarty, J.P. 2004. Bioenergetics based modeling of individual PCB congeners in nestling tree swallows from two contaminated sites on the upper Hudson River, New York. *Environmental Science and Technology* **38:** 6234-6239.

Nilsen, B.M., Berg, K., Eidem, J.K., Kristiansen, S.-I., Brion, F., Porcher, J.-M., and Goksøyr, A. 2004. Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening.

Norén, K. Weistrand, C., and Karpe, F. 1999. Distribution of PCB congeners, DDE, hexachlorobenzene, and methylsulfonyl metabolites of PCB and DDE among various fractions of human blood plasma. *Archives of Environmental Contamination and Toxicology* **46**: 254-64**.**

Obbard, M.E. and Brooks, R.J. 1981. A radio-telemetry and mark-recapture study of activity in the common snapping turtle, *Chelydra serpentina*. *Copeia* **1987**: 630-637.

Oberdörster, E. and Cheek, A.O. 2000. Gender benders at the beach: endocrine disruption in marine and estuarine organisms. *Environmental Toxicology and Chemistry* **20**: 23-36

Pagano, J.J., Rosenbaum, P.A., Roberts, R.N., Sumner, G.M., and Williamson, L.V. 1999. Assessment of maternal contaminant burden by analysis of snapping turtle eggs. *Journal of Great Lakes Research* **25**: 950-961.

Palmer, B.D. and Palmer, S.K. 1995. Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. *Environmental Health Perspectives* **103**: 19-25.

Panter, G.H., Hutchinson, T.H., Lange, R., Lye, C.M., Sumpter, J.P., Zerulla, M., and Tyler, C. 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-) estrogenic substances. *Environmental Toxicology and Chemistry* **21**: 319-326.

Pell, S.M. 1940. Notes on the food habits of the common snapping turtle. *Copeia* **1940**: 131.

Punzo, F. 1975. Studies on the feeding behavior, diet, nesting habitats and temperature relationships of *Chelydra serpentina osceola* (Chelonia: Chelydridae). *Journal of Herpetology* **9**: 207-210.

Perskey, V.W. 2001. Endocrine and other human health effects of environmental and dietary exposure to polychlorinated biphenyls (PCBs). In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 99-102.

Quantitative Environmental Analysis. 1998. Final Report: Thompson Island Pool sediment PCB sources. Glens Falls, NY. (online at: http://www.ge.com/files/usa/en/commitment/ehs/hudson/tip1.pdf)

Rauschenberger, R.H., Sepúlveda, M.S., Wiebe, J.J., Szabo, N.J., and Gross, T.S. 2004. Predicting maternal body burdens of organochlorine pesticides from eggs and evidence of maternal transfer in *Alligator mississippiensis*. *Environmental Toxicology and Chemistry* **23**: 2906-2915.

Rey, F., Ramos, J.G., Stoker, C., Bussman, L.E., Luque, E.H., and Muñoz-de-Toro, M. 2006. Vitellogenin detection in *Caiman latirostris* (Crocodylia: Alligatoridae): a tool to assess environmental estrogen exposure in wildlife.

Rhee, G., Sokol, R.C., Bethony, C.M., and Bush, B. 1993. Dechlorination of polychlorinated biphenyls by Hudson River sediment organisms: specificity to the chlorination pattern of congeners. *Environmental Science and Technology* **27**: 1190-1192.

Rowe CL. 1998. Elevated standard metabolic rate in a freshwater shrimp (*Palaemonetes paludosus*) exposed to trace element-rich coal combustion waste. *Comparative Biochemistry and Physiology* 121 A:299-304.

Rowe CL, Kinney OM, Nagle RD, Congdon JD. 1998. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. *Physiological Zoology* 71:27-35.

Rowe CL, Hopkins WA, Zehnder C, Congdon JD. 2001. Metabolic costs incurred by crayfish (*Procambarus acutus*) in a trace element-polluted habitat: further evidence of a common response among diverse taxonomic groups. *Comparative Biochemistry and Physiology* 129C:275-283.

Rowe CL, Kelly SM. 2005. Marking hatchling turtles of via intraperotineal placement of pit tags: implications for long-term studies. *Herpetological Review*. 36:408-410.

Russell, R.W., Gobas, F.A.P.C., and Haffner, G.D. 1999. Maternal transfer and in ovo exposure of organochlorines in oviparous organisms: A model and field verification. *Environmental Science and Technology* **33**: 416-420.

Safe, S. 2001. PCBs as aryl hydrocarbon receptor agonists: implications for risk assessment. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 171-177.

Selcer, K.W., Nagaraja, S., Foret, P., Wagner, D., Williams, L. and Palmer, B.D. 2001. Vitellogenin as a biomarker for estrogenic chemicals. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 285-292.

Shelby, J.A. and Mendonça, M.T. 2001. Comparison of reproductive parameters in male yellow-botched map turtles (*Graptemys flavimaculata*) from a historically contaminated site and a reference site. *Comparative Biochemistry and Physiology Part C* **129:** 233-242.

Sifuentes-Romero, I., Vázquez-Boucard, C., Sierra-Beltrán, A.P., and Gardner, S.C. 2006. Vitellogenin in black turtle (*Chelonia mydas agassizii*): purification, partial

characterization, and validation of an enzyme-linked immunosorbent assay for its detection. *Environmental Toxicology and Chemistry* **25**: 477-485.

Soontornchat, S., Li, M., Cooke, P.S. and Hansen, L.G. 1994. Toxicokinetic and toxicodynamic influences on endocrine disruption by polychlorinated biphenyls. *Environmental Health Perspectives* **102**: 568-571.

Specker, J.L. and Anderson, T.R. 1994. Developing an ELISA for a model protein—vitellogenin. In: Hochachka, T. and Mommsen, F (eds) *Biochemistry and molecular biology of fishes*, Vol. 3. Elsevier Science, St. Louis, MO, pp. 567-578.

Steinbacher, J.C. 2001. The American eel, *Anguilla rostrata*, as a sentinel species of polychlorinated biphenyl contamination in the Hudson River. Master's Thesis, University of Maryland, College Park, Maryland

Stone, W.B., Kiviat, E., and Butkas, S.A. 1980. Toxicants in snapping turtles. *New York Fish and Game Journal* **27**: 39-50.

Struger, J., Elliott, J.E., Bishop, C.A., Obbard, M.E., Norstrom, R.J., Weseloh, D.V., Simon, M., and Ng, P. 1993. Environmental contaminants in eggs of the common snapping turtle (*Chelydra serpentina serpentina*) from the Great Lakes-St. Lawrence River Basin of Ontario, Canada (1981, 1984). *Journal of Great Lakes Research* **19**: 681-694.

Steyermark AC, Spotila JR. 2001. Body temperature and maternal identity affect snapping turtle (*Chelydra serpentina*) righting response. *Copeia* 2001:1050-1057.

Thorpe, K.L., Hutchinson, T.H., Hetheridge, M.J., Sumpter, J.P., and Tyler, C.R. 2000. Development of an *in vivo* screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* **19**: 2812-2820.

Tyler, C.R., Sumpter, J.P., and Handford, R.M. 1990. The dynamics of vitellogenin sequestration into vitellogenin ovarian follicles of the rainbow trout, *Salmo gaidneri. Fish Physiology and Biochemistry* **8**: 219-244.

United States Environmental Protection Agency (USEPA). 1993. Wildlife exposure factors handbook. EPA/600/R-93/187. U.S. Evironmental Protection Agency, Washington, DC.

USEPA. 2000. Phase 2 Report – Further Site Characterization and Analysis. Volume 2E – Revised Baseline Ecological Risk Assessment: Hudson River PCBs Reassessment. Region II, New York.

USEPA. 2002. Hudson River PCBs site, New York Record of Decision. U.S. Evironmental Protection Agency, Washington, DC. (online at: http://www.epa.gov/hudson/RecordofDecision-text.pdf).

Van den Berg, M., Craane, B.L.H.J., Sinnige, T., Van Mourik, S., Dirksen, S., Boudewijn, T., Van der Gaag, M., Lutke-Schipholt, I.J., Spenkelink, B., and Brouwer, A. 1994. Biochemical and toxic effects of polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in the cormorant (*Phalacrocorax carbo*) after in ovo exposure. *Environmental Toxicology and Chemistry* **13**: 803-816.

Van den Berg, M., Birnbaum, L., Bosveld, A.T.C., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Liem, A.K.D., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Wærn, F., and Zacharewski, T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environmental Health Perspectives* **106**: 775-792.

Wiley, H.S., Opresko, L., and Wallace, R.A. 1979. New methods for the purification of vertebrate vitellogenin. *Analytical Biochemistry* **97**: 145-152.

Willingham, E. and Crews, D. 2000. The red-eared slider turtle: an animal model for the study of low doses and mixtures. *American Zoologist* **40**: 421-428.

Wirth, E.F., Chandler, G.T., DiPinto, L.M., and Bidleman, T.F. 1994. Assay of polychlorinated biphenyl accumulation from sediments by marine benthic copepods using a novel microextraction technique. *Environmental Science and Technology* **28**: 1609-1614.

Van veld, P.A., Rutan, B.J., Sullivan, C.A., and Johnston, D. 2005. A universal assay for vitellogenin in fish mucus and plasma. *Environmental Toxicology and Chemistry* **24**: 3048-3052.

Vincent, D.R., Bradshaw, W.S., Booth, G.M., Seegmiller, R.E., and Allen, S.D. 1992. Effect of PCB and DES on rat monoamine oxidase, acetylcholinesterase, testosterone, and estradiol ontogeny. *Bulletin of Environmental Contamination and Toxicology* **48**: 884-893.

Weather Underground, Inc. 2003. History for Glens Falls, New York, May 2003. (online: http://www.wunderground.com/history/airport/KGFL/2003/5/30/MonthlyHistory.html).

Weather Underground, Inc. 2004. History for Glens Falls, New York, May 2004. (online: http://www.wunderground.com/history/airport/KGFL/2004/5/30/MonthlyHistory.html).

Wheeler, J.R., Gimeno, S., Crane, M., Lopez-Juez, E., and Morritt, D. 2005. Vitellogenin: a review of analytical methods to detect (anti) estrogenic activity in fish. *Toxicology Mechanisms and Methods* **15**: 293-306.

White, J.B. and Murphy, G.G. 1973. The reproductive cycle and sexual dimorphism of the common snapping turtle, *Chelydra serpentina serpentina. Herpetologica* **29**: 240-246.

Winkelman DL. 1996. Reproduction under predatory threat: trade-offs between nest guarding and predator avoidance in male dollar sunfish (*Lepomis marginatus*). *Copeia* 1996:845-851.

Yntema, C.L. 1970. Observations on females and eggs of the common snapping turtle, *Chelydra serpentina. American Midland Naturalist* **84**: 69-76.

Zoeller, R.T. 2001. Polychlorinated biphenyls as disruptors of thyroid hormone action. In: Robertson L.W. and Hansen, L.G. (eds) *PCBs: Recent advances in environmental toxicology and health effects.* University Press of Kentucky, Lexington, KY, pp. 265-271.