ABSTRACT

Title of Document: IMPACT OF RECONSTRUCTED WETLANDS ON THE TREE SWALLOW (*TACHYCINETA BICOLOR)*

Karen Lynn Lohnes, M.S., 2006

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Tree swallow (*Tachycineta bicolor)* nestlings were used as a sentinel species to evaluate the bioavailability and effect of contaminants to terrestrial life at reconstructed wetlands, Kingman Lake and Kenilworth Marsh, on the Anacostia River. Nesting success, survival, growth and developmental parameters were recorded and compared to an established reference location. Metals, metalloids and organic contaminant concentrations in the eggs and nestlings were examined. In addition, biomarkers of exposure for some organic contaminants (cytochrome P450), some pesticides (cholinesterase activity), and lead (delta-aminolevulinic acid dehydratase) were measured. Only subtle differences between sites were observed for reproductive and growth parameters of the nestlings. Chemical analysis showed significant differences ($p<0.05$) between sites of relatively few contaminants. Of the biomarkers examined, only cytochrome P450 activity was found to be significantly different between sites ($p=0.04$). Contaminant concentrations and biomarker data

from the tree swallows suggest no serious impacts to insectivorous birds residing in these wetlands.

IMPACT OF RECONSTRUCTED WETLANDS ON THE TREE SWALLOW (*TACHYCINETA BICOLOR)*

By

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Dedication

Dedicated to my loving husband Edward, and my parents, Tom and Judy, without whom I could not have completed this study.

Acknowledgements

I am extremely grateful for the opportunity of being a part of this project. I am especially thankful for the financial support, patience and guidance of Dr. Mark J. Melancon from the USGS Patuxent Wildlife Research Center, Dr. Mary Ann Ottinger and Dr. Judd Nelson from the University of Maryland College Park, my graduate committee members.

The obstacles of attracting tree swallows to the study sites, getting them to use our nest boxes, and the collection of samples were some of the most daunting tasks I was faced with on this project. Without the generous assistance of Dr. Mark J. Melancon, Dr. Fred Pinkney, Pete McGowen, Peter Ossenton, Juliet Healy, Katie Schoen and volunteers from the US Fish and Wildlife Service, the installation, relocation and eventual removal of nest boxes would never have been possible. The monitoring of the nest boxes and collection of samples would never have been accomplished over the three years of field work for this study without the generous support of Dr. Mark Melancon, Kathy Dejong, Katie Schoen, Juliet Healy and Laura Senkowsky. I must also express my deepest gratitude towards those individuals for their help and diligence in the lab, especially during the many times that things did not work as planned. The advice and assistance with the biochemical assays of Dr. Mary Ann Ottinger, Nicola Thompson, Dr. Mark J. Melancon, and Dr. David J. Hoffman are particularly commendable. I must also thank Craig Hulse and the Patuxent Analytical Control Facility for their hard work in the chemical analysis of the samples. The

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Chapter 1: Introduction

A. Anacostia Wetlands

The Anacostia River is a channelized freshwater tidal tributary that flows from Montgomery and Prince George's Counties in Maryland to Washington, D.C., where it empties into the Potomac River and eventually the Chesapeake Bay (Figure 1). According to the Chesapeake Bay Program, the Anacostia River watershed encompasses 176 square miles and had an estimated population in 2000 of 867,408 individuals. This tributary is impacted by urban development and the input of untreated sewage from combined sewer outfalls. There is also a history of hazardous substance spills including moderate oil spills, and it continues to receive organic pollutants and heavy metals from urban non-point sources [1], [2]. In 1987, the Anacostia Watershed Restoration Agreement was signed by multiple government agencies. The Anacostia Watershed Restoration Committee (AWRC) was created with members from the District of Columbia, Montgomery and Prince George's counties in Maryland, the Metropolitan Washington Council of Governments, the interstate Commission on the Potomac River Basin, and the U.S. Army Corp of Engineers (USACOE) as the federal representative and liaison [3]. In 1991, the AWRC developed a six-point action plan for the restoration of the Anacostia River. The need to restore and/or create additional acreage of tidal wetland was determined as part of this action plan [4]. Under the direction of the USACOE, dredged sediments from the Anacostia River were placed on Park Service land at Kenilworth Marsh in early 1993 in an effort to restore 32 acres of tidal wetland that once existed there. Again, in the winter of 1999 – 2000, dredge spoil sediments from the

Anacostia and two marinas, the Washington Sailing Marina and Columbia Island Marina, were used to restore approximately 41 acres of tidal wetlands in Park Service land at Kingman Lake.

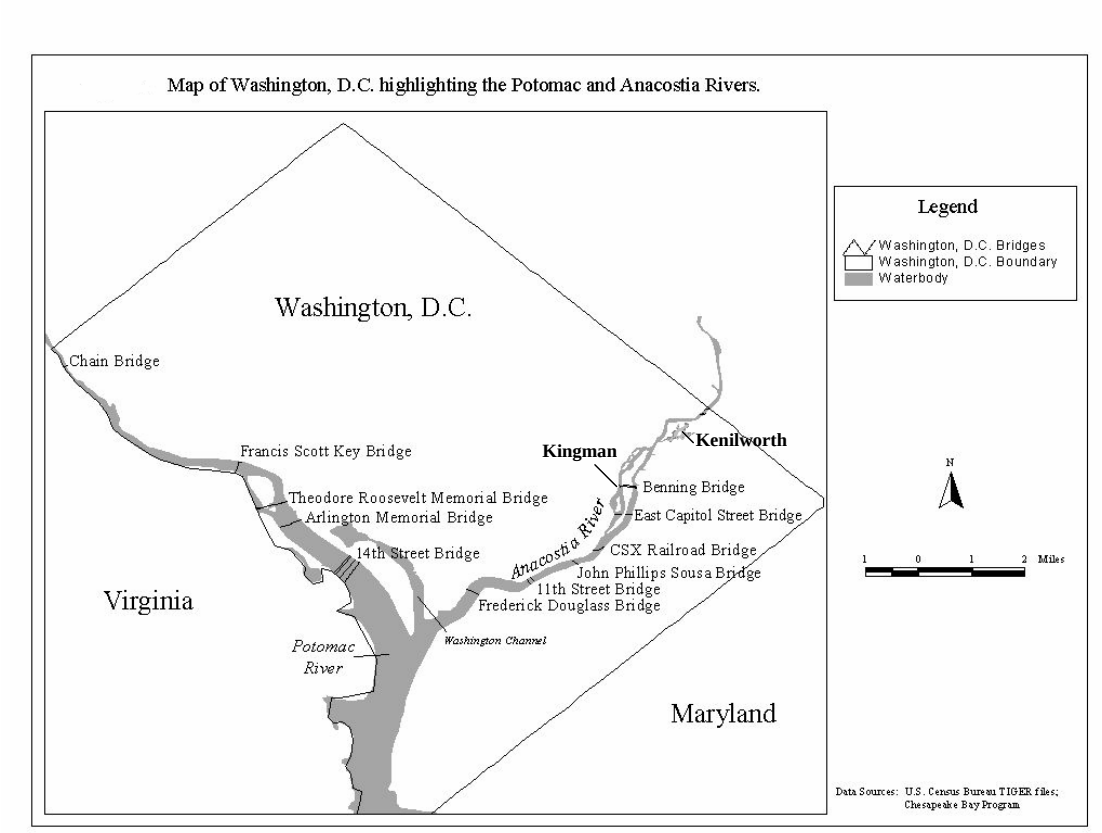


Figure 1: Map illustrating the location of the Anacostia River (picture courtesy of U.S.FWS).

A.1. Current status of the wetlands

Tests conducted by the U.S. Fish and Wildlife Service (USFWS) in the summer of 1993 on the sediments used to restore Kenilworth Marsh, showed that the new marsh sediments contained PCB's, chlordane, lead, nickel, chromium and zinc at levels above which biological impacts occur [2], [[5]. Sediments were collected again in 1996 and found to still contain chlordane, lead, nickel, and zinc at levels above that which toxic effects frequently occur [6], [7]. Prior to the reconstruction at

Kingman Lake, composites of sediments from the Anacostia River and the two marinas were analyzed and compared with similar analyses conducted on sediments from Kingman Lake. Toxicity tests showed that sediments from all sites inhibited the growth of aquatic organisms; however, there were no significant differences in toxicity between the locations [8]. It was determined that there was no substantial threat to organisms utilizing the marsh and reconstruction took place as planned. A monitoring plan prepared by a multi-agency group has taken into account the fact that dredging and placement of the dredge material may change the bioavailability of contaminants in the sediments [9]. As part of this plan, studies have been conducted to monitor the sediment contamination and the fate and effects of contaminants on wildlife residing in these marshes [6], [9].

Tests conducted on sediment toxicity and surveys on macroinvertebrates from the Anacostia River have indicated biological impairment [10]. Of particular significance has been the discovery of liver and skin tumor prevalence in Brown Bullheads from the Anacostia River. Pinkney et al. [11] reported in 2001 that liver tumors were found in 50% of animals collected in the Anacostia during the spring of 1996, and 60% of animals collected in the fall of 1996, significantly greater than any of the other sampled locations. Skin tumor frequency was also significantly different, with Anacostia Bullheads collected in the spring having the highest prevalence of these tumors at 37% [11]. In a follow-up study, Brown Bullheads were collected from the Anacostia in the fall of 2000 and the spring of 2001 [12]. This study found a 50 to 68% prevalence of liver tumors and 13 to 23% prevalence of skin tumors in large bullheads (3 years or older). Pinkney suggests that although the prevalence of

liver tumors was lower in animals one to two years of age (10 to 17%), a study of their liver DNA adduct concentrations and biliary polynuclear aromatic hydrocarbon (PAH) metabolites, indicates a probable increase of these tumors as these animals age [12]. Despite the breadth of studies on the Anacostia River in general and in the effects of these reconstructed wetlands on aquatic species, studies have not examined the impacts of contaminants on terrestrial wildlife in Kingman Lake and Kenilworth marsh. As a result of this deficit in the preliminary monitoring, there is a need to determine the impacts of these contaminated sediments on terrestrial wildlife in the wetlands.

A.1.a. How terrestrial species may be affected

Tidal freshwater wetlands such as those created at Kingman Lake and Kenilworth Marsh create an interdependent network between the species that live there. An abundance of amphibians and reptiles such as snakes, frogs and salamanders have been personally observed at these locations. Raccoons have also been personally observed at these sites, where they often feed on the eggs of nesting birds. Most migratory birds are dependent on the resources, such as wild rice, provided by a freshwater wetland for the energy required to complete their migration. A survey of the birds using these wetlands has revealed a total of 174 species from 2001 – 2003. Of these birds, 57 breeding birds were observed in and around Kingman marsh and 56 at Kenilworth in 2003 alone [13].

Given the species richness at these locations, the effect of contaminants in fish on piscivorous species is of great concern. Monitoring of osprey populations in the Anacostia region has been conducted [14]. In this study, sample eggs were collected

from three regions of concern, including the Anacostia and compared with reference locations. Osprey nests on the Anacostia River were grouped with other nests from the middle Potomac River for the purposes of this study partially due to the lack of accessible osprey nests within the Anacostia River. In addition to the collection of eggs for contaminant analysis, determination of egg shell thickness and a morphological examination of the embryo if a developing embryo within the egg is found, nests were also monitored for reproductive success and portions of food items at the nest sites analyzed for contaminants. Although the number of successful pairs was lower in the Anacostia and middle Potomac River, logistic regression analysis did not support a link between reproductive success and contaminant concentration. Due to the lack of ecotoxicological information on terrestrial vertebrates in the Chesapeake Bay tributaries including the Anacostia, the author concluded that a more thorough evaluation of contaminant exposure in ospreys may be necessary [14].

Terrestrial vertebrates which feed on aquatic insects are also of concern. These insects which laboratory testing has shown have biological impairments [10] as a result of exposure to the sediments from these sites, would be carrying toxins that they have accumulated in the larval stage of their growth. Any species which consumes these insects would be exposed to and potentially bioaccumulate the contaminants.

Chapter 2: Study Needs

It was the goal of the National Park Service to determine the direct impact of these reconstructed wetlands on terrestrial inhabitants. The addition of wetlands to a region of concern such as the Anacostia River would only help to improve the conditions there. Given cost limitations, the use of dredged sediments to reconstruct wetlands could provide the savings necessary to allow additional wetland acreage to be constructed. Given the concern of the impact of contaminated dredge material, finding a terrestrial species with a direct link to sediment bound contaminants was crucial to properly evaluate these wetlands. The species used as a sentinel species in this study should be well understood, easily attracted to researcher determined nesting sites, and feed primarily on a diet linked to the marsh sediments. It was also crucial to be able to compare these reconstructed wetlands to an established reference site for the sentinel species of choice. Given the goals of the National Park Service, it was determined that using an insectivorous species as a sentinel in this study would best confine the findings to the impact of the reconstructed wetland alone. The use of a piscivorous species could confound the results due to the fact that fish eaten by a sentinel species could originate from the Anacostia River proper or beyond and not necessarily the reconstructed wetlands themselves. Using a widely understood and studied species was also crucial given the fact that the impacts on this sentinel species would need to be extrapolated to their effects on other terrestrial vertebrates occupying the wetlands.

Chapter 3: Choice of the tree swallow

One insectivorous species that has been used often in toxicology studies of marshland is the tree swallow [15]. The tree swallow is a common breeding passerine throughout central and coastal North America. It is a species that prefers nesting near water, including areas such as marshes and swamps. Tree swallows are widely known as a hardy bird and are easily attracted to nest boxes making them ideal study subjects [16]. Tree swallows prefer to feed on emerging aquatic insects within close range to their nest site [16], [17]. The emergent insects that are the majority of the tree swallows diet are known to assimilate sediment-associated contaminants that are then accumulated by the tree swallows themselves [18]. This makes them an ideal species to evaluate the bioavailability of the contaminants present in the sediments at these sites. Recent studies have continued to illustrate the usefulness of the tree swallow in evaluating sites of sediment borne contamination. An additional reason to use tree swallows in a study is that they nest within close proximity to each other (approximately 20 meters apart, [15]), making the collection of many samples from a site possible [19]. Given the number of studies that have used tree swallows, a comparison between similar studies of the tree swallow can shed light on the magnitude of contamination between different locations.

Traditionally, tree swallow nestlings are collected in contaminant studies to avoid the effect of their migratory locations adding additional contamination that has not originated from the site of interest. To accomplish this, an egg is collected from the sampled nest, and the contaminants determined within that egg treated as a starting point and subtracted from the contamination burden found in the nestling.

With the use of the tree swallow as a sentinel species, a determination can be made if the levels of contaminants are a hazard to the tree swallows as well as other terrestrial species inhabiting these wetlands.

Chapter 4: Contaminants and Biomarkers investigated

A. Analytical Analysis

The trace contaminants found in the carcass, egg and feather of the animals collected serve a vital basis for comparison to the biomarker and biological data determined. The analytical method for determination of organochlorines in the carcass and egg contents of the animals including sample preparation, Soxhlet extraction and lipid removal are found in Cromartie *et al*., 1975 [20]. Traces of selected metals and metalloids in the feathers and egg shells were determined using ICP-MS. The gall bladders that were removed from the carcass at time of sacrifice were shipped out on dry ice in labeled chemically cleaned vials for PAH metabolite analysis. The analysis for PAH metabolites was conducted using HPLC.

B. Biomarkers for contaminants of concern

B.1. Lead

Delta aminolevulinic acid dehydratase (ALAD) is an enzyme involved in the second step of the biosynthesis of heme. ALAD catalyzes the reaction in which two molecules of delta-aminolevulinic acid are condensed to form porphobilinogen (Figure 2). Lead is a potent inhibitor of ALAD and its effects are long lasting (up to four months). The ALAD assay determines red-blood cell ALAD activity as a bioindicator of lead exposure. The procedure that was used for determining ALAD concentration in blood is based on the methods developed by Burch and Seigel [21]. The activity of ALAD has been determined, for most species, to be inversely

proportional to the log concentration of lead in the blood at detectable levels of exposure [21]. The assay for ALAD has been used in previous studies examining tree swallow exposure to lead [22].

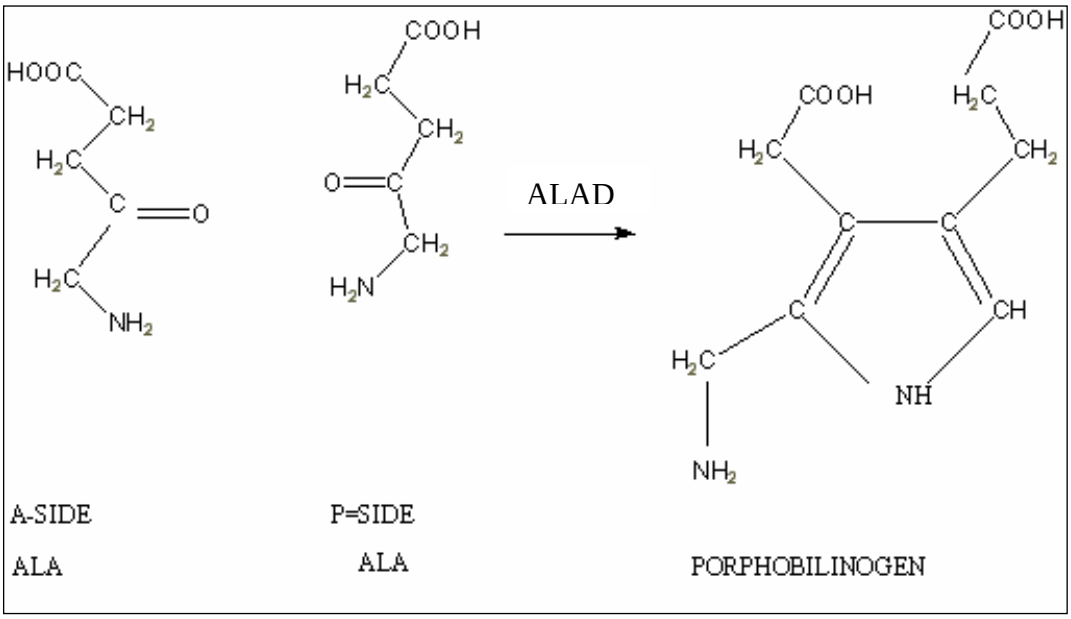


Figure 2: Reaction catalyzed by ALAD [23].

B.2. Organophosphate and carbamate pesticides

Acetylcholine is a neurotransmitter that affects several of the body's systems including cardiovascular, gastrointestinal, and respiratory systems. Organophosphorus and carbamate pesticides inhibit the ability of cholinesterase to hydrolyze acetylcholine by binding to acetlycholinesterase. Without the breakdown of acetylcholine, there is a build up at the nerve synapses which lead to tremors, motor dysfunction and eventually death. Although these pesticides are meant to target insects, their use often leads to exposure of birds and other insectivorous or herbivorous species. Brain cholinesterase activity is used to indicate exposure to

cholinesterase inhibiting pesticides. The procedure requires a comparison of cholinesterase activity in whole brain homogenate from the study specimens to a calculated normal cholinesterase level in normal specimens of the species; in the present study nestlings from Patuxent Research Refuge. The procedure was originally developed by Ellman *et al*. [24], and modified for the use of a microwell plate reader. Cholinesterase inhibition is a widely used technique and has proven successful in determining tree swallow exposure to pesticides in previous studies [25], [26].

B.3. Cytochromes P450

B.3.a. History of discovery and link to exposure to some polycyclic aromatic hydrocarbons (PAHs) and halogen substituted aromatic hydrocarbons

Cytochromes P-450 (P450s) are a large family of hemoproteins associated with cell membranes, particularly the endoplasmic reticulum membrane. They possess redox properties and are known as monooxygenases due to their ability to manipulate O_2 into reactive oxygen species (ROS), and then to place that oxygen into a wide variety of substrates [27] (Figure 3). P450s are also capable of catalyzing other reactions such as dealkylation, oxidative deamination, epoxidation, oxidative and reductive dehalogenation, and desulfuration reactions [28]. In addition to their role as catalysts in steroid hormone, fatty acid and cholesterol metabolism, they are also involved in xenobiotic metabolism [29]. Their function in this capacity has made them one of the most popular research topics in toxicological research since their discovery in the late 1950's. P450s are named according to gene family and

subfamily. The root of each name begins with CYP, an abbreviation of **cy**tochrome **P**450 and is followed by the numbers and letters to designate the particular family, subfamily and gene (e.g., CYP1A1 or CYP1A2) [29]. Two major types of cytochromes P450 are found in increasing amounts, or induced, when an animal is exposed to various types of organic contaminants in a laboratory setting. These two types of Cytochromes P450s are categorized as the Phenobarbital-inducible (PB) types and the 3-methylcholanthrene-inducible (3-MC) types [30].

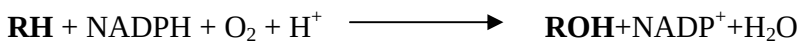


Figure 3: Common hydroxylation reaction catalyzed by Cytochromes P450 (from Newman, 2001).

Contaminants including polycyclic aromatic hydrocarbons (PAHs), coplanar or non-ortho-PCBs, crude petroleum, and halogenated dibenzofurans are 3-MC type inducers [31]. PB-type inducers include compounds such as barbiturates, DDT, and non-coplanar or ortho-PCBs [32]. The 3-MC inducible types are primarily associated with CYP1A while the PB inducible types are usually associated with CYP2B isozymes [33], [34].

B.3.b. Why use P450's?

P450 is classified as a biomarker of exposure to some contaminants, referring to the fact that they signify the exposure of an animal to a P450 inducer such as organohalides. This serves an advantage over a biomarker of effect such as ALAD, which is only detected after a measurable effect of exposure to lead has occurred.

The use of P450 is also beneficial due to the large number of contaminants that induce its activity such that it can serve the function of a broad biomarker of exposure. As with most biomarkers, the use of control animals is beneficial by providing comparison of levels of P450 in animals of the same age, etc during the same year. Studies have shown that P450 induction depends on many factors including the species being examined, the age of the animal, and the body weight of the animal [29]. By having control animals with which to compare study organisms, a baseline level of the induced P450 can be determined. It was thus necessary for the collection of control animals in each year of the study as a basis of comparison for P450 activity as well as the other biomarkers examined.

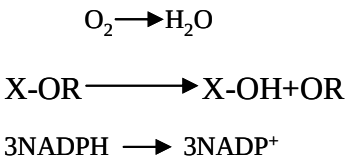
B.3.c. How do you detect P450?

P450s can be assessed by a number of methods that differ in sensitivity, specificity and convenience. The methods selected for this study rely on P450-associated monooxygenase activity and on a combination of size and antigenicity to specific antibodies.

B.3.c.1. Monooxygenase assay

One of the most common methods of measuring P450 induction is by direct fluorometric assay of monooxygenase activity. Dealkylation, a known CYP1 and CYP2 monooxygenase activity is measured in the fluorometric assay by the dealkylation of four synthetic substrates; ethoxyresorufin, benzyloxyresorufin, methoxyresorufin, and pentoxyresorufin (Figure 4). The product of these reactions, resorufin, is measured and quantified fluorometrically, providing the means to

compare specimens from the same location as well as comparing those animals with controls [27]. The particular family, subfamily and gene of the P450, determines which of these substrates are most suited to detect the activity of P450. For example, ethoxyresorufin-O-dealkylase (EROD) has been shown to be the substrate best suited to measure CYP1A in mammals [35]. This has also been shown to be generally true for birds as well. CYP1A is the type of P450 that is induced by coplanar PCBs, PCDFs and dioxin [36]. It should be noted that some O-dealkylases typically associated with CYP2B induction in mammals are associated with CYP1A in birds. This is likely due to the limited homology between the CYP1A produced in birds and that produced in mammals [37].



X = n-alkyl ether of phenoxazone with alkoxy chain, R
R = CH_3, methoxy-
C_2H_5, ethoxy-
C_5H_{11}, pentoxy-
$C_6H_5CH_2$, benzyloxy–

Figure 4: Dealkylation of resorufin ethers to resorufin [38], [39].

B.3.c.2. Western blotting – Mechanism and discovery

Western blotting combines the electrophoretic separation of proteins by size with the testing of the resulting protein bands for antigenicity to antibodies for

specific P450s; in this case CYP1A. The quantification of P450 using the immunological technique of Western Blotting employs the use of sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE). This technique utilizes a polyacrylamide gel in an electric field. The net charge on proteins would cause them to migrate toward the cathode or anode. The rate at which these proteins move toward the respective pole is dependent on the molecular weight of the protein, as the polyacrylamide gel creates a jungle gym like structure the proteins must navigate through [40]. Because the net charge determines in which direction the proteins migrate, SDS is employed to give a net negative charge to the proteins. SDS distributes itself on protein molecules in such a way as to give one SDS molecule for every 2 amino acid residues [41]. In this way, the negative charge is distributed evenly across all protein molecules [42]. With a uniform negative charge, all molecules will migrate toward the cathode, thus allowing efficient determination of molecular weights of all species in question. By the use of a known P450 containing sample, the protein band and thus the quantity of P450 can be determined from each sample. The proteins in gels produced from SDS-PAGE are transferred by electric current in buffer to the hydrophobic material, nitrocellulose [43]. Once bound to nitrocellulose, the process of western blotting, which includes blocking steps and binding of primary and secondary antibody to the P450 can be achieved. The dark bands produced by this procedure, can then be quantified using software programs and compared to a standard curve created with known P450 containing samples.

Since western blotting is an immunological technique, it takes advantage of the fact that it can detect antibody regardless of whether it is active or not. In this

sense, western blotting can be thought superior to the dealkylase assay, however immunoblotting has a more difficult time detecting low levels of P450 whereas the dealkylase assay is very sensitive tool for detecting activity in very small quantities of enzyme.

The antibody used in our laboratory was derived from rabbits in response to the products of mallard dosing with a known CYP1 inducer (Beta Napthoflavone) [44]. This antibody is a polyclonal antibody. An anti rabbit antibody produced in goat was used to bind to the primary antibody and produces a colored response where present.

Chapter 5: Materials and Methods

A. Field

A.1. Site selection

Nest boxes for tree swallows constructed of cedar wood were purchased and mounted on steel fence posts. Each nest box had a hinged front that allowed for easy access to the nest during breeding season. Three-foot lengths of six-inch PVC pipe were placed over each post to serve as a deterrent for predators. After losses to predation in the 2001 field season a 4“ piece of wood was installed over the entrance of the nest boxes to provide a longer entrance path to serve as an additional predator guard. Three foot pieces of galvanized metal were cut to fit around the base of the nest boxes and fitted around the metal posts in an inverted cone shape to prevent raccoons from climbing up the posts. Forty nest boxes were erected at each of the study locations (Kingman Lake, Kenilworth marsh, Patuxent Research Refuge). Nest boxes placed at the Patuxent Research Refuge were installed in the same location as tree swallow nest boxes used as a reference in a previous study [45]. The position of each nest box was recorded using a global positioning system (GPS) unit. In the winter, nest boxes were repaired and readied for the upcoming breeding season in the spring.

A.2. Sediment sampling

Sediment samples were collected from each study site with a PONAR sediment sampler. Six core samples were taken at different locations within a site

using a hand borer, and were divided into two subsets, to give 2 pooled samples per site. Each sample was placed in a chemically clean container and kept on ice until return to the laboratory where it was stored in the -20° C freezer until chemical analysis. Samples were analyzed for PAH's, organochlorines, metalloids and metals.

A.3. Nest monitoring and sample collection

In the Washington D.C. and Maryland area, tree swallows typically first enter the region in late March or early April (personal observation). As a result, nest boxes were checked from that time, biweekly at each study site for the arrival of the birds and nest building behavior. At the Anacostia study sites, field work was coordinated with high tide since nest boxes needed to be accessed with a canoe. Observations at each nest were recorded for each site and entered into a spreadsheet on the computer within the same week. At the first sign of egg laying, active nests were checked every other day until the last egg was laid. Eggs are usually laid each morning at dawn and clutches typically contain 4 to 6 eggs per nest [16]. One to two days after the complete clutch is laid, one egg was collected, placed in a chemically clean screw capped jar and kept on ice for transport to the laboratory where it was stored in the -20° C freezer until chemical analysis. An egg was collected only after the female laid a complete clutch to prevent abandonment of the nest. One egg was collected from a maximum of ten nests per study site in a field season.

Incubation of the eggs usually begins after the penultimate egg is laid [16]. Anticipated hatch dates can be determined after incubation begins, since tree swallow eggs hatch 11 to 19 days from the start of incubation [46]. Nests were monitored for hatching at day 11 and every other day after until the first nestling had hatched. The

day that the first nestling hatched was considered day one. Based on a technique developed by Orians [47], a food sample was collected from all of the nestlings in the nest when they were approximately 10 days old. A black cable tie was placed around the neck of each nestling to constrict its ability to swallow food items without restricting its ability to breathe. Any excess length of the cable tie was trimmed off with scissors. If any nestling showed signs of distress after the cable tie was applied, the initial cable tie was removed and a second attempt was made. If the second attempt was not successful, then that nestling was returned to the nest without further attempts. After all the nestlings were fitted with the cable ties and returned to the nest they were left undisturbed for one hour. Previous studies [45] have shown that the adults will return to feeding the nestlings no longer than 15 minutes after a disturbance. After an hour had passed, a pair of small rounded tweezers was used to remove the food bolus from the mouth of each nestling, if present. The food samples from each nest were placed in a labeled chemically cleaned screw capped jar and kept on ice until transferred to the -20° C laboratory freezer. The cable ties were removed from each nestling upon retrieval of the sample by cutting the cable ties with scissors and the nestlings replaced in the nest.

From each nest box where an egg was collected, one nestling was sacrificed by cervical dislocation on post-hatch day 14. A maximum of 10 nestlings were sacrificed per study site with the exception of Patuxent where 5 nestlings were collected each of the study years. All of the nestlings in each box were weighed and examined for gross physiological abnormalities. Tibia and radius-ulna measurements were taken for right and left sides to examine bilateral symmetry and culmen length

was also recorded. The nestling to be sacrificed was randomly chosen. All other nestlings were returned to the nest. These nests were left undisturbed for a one-week period to avoid premature fledging of remaining nestlings.

Blood was collected immediately after sacrifice in heparinized tubes for the ALAD assay. Two capillary tubes of blood were taken to measure the hematocrit, an indicator of general health and also a part of the ALAD assay. The remainder of each blood sample was placed on ice in the field until return to the laboratory where it was snap frozen and stored in the -80° C freezer. The gall bladder, if retrievable, was removed and placed in a small chemically cleaned vial for PAH metabolite analysis. These vials were kept on ice for transport to the laboratory where they were frozen at -20° C until analysis. Livers were removed, weighed, and placed in cryotubes that were snap-frozen in liquid nitrogen for transport to the lab. Upon arrival at the lab, liver containing cryotubes were transferred into the -80° C freezer until the cytochrome P450 analysis was run. The head was placed in a chemically clean screw cap jar and kept on ice for transport to the lab and later brain removal for the cholinesterase assay. The head was stored at -20° C until the cholinesterase assay was performed. All feathers of the sacrificed nestling were removed and placed in a chemically clean screw cap jar for metal and metalloid analysis. The remaining carcass was placed in a chemically clean screw cap jar on ice for transport to the lab for later organochlorine analysis. At the lab, the carcass was stored at -20° C.

After the remaining nestlings fledged, the nests were removed to allow a possible nesting attempt by other tree swallows. The nest boxes were cleaned of all

nesting material at the end of each field season and reproductive success of the birds inhabiting each study site was evaluated.

B. Laboratory

B.1. Biomarker assays

B.1.a. ALAD

Blood that was collected in heparinized tubes from the sacrificed nestling was used for the ALAD assay. The hematocrit for each nestling was determined from the average value of two capillary tubes of blood. The remaining blood was snap frozen and kept at -80° C until the assay was performed. The technical operating procedure (TOP) that was used is the Patuxent Wildlife Research Center (PWRC) procedure that is based on the methods developed by Burch and Seigel [21]. In short, two 100µl aliquots of each blood sample are placed in individual tubes, diluted with 1.4ml distilled water, and placed in a water bath at 38°C for 10 minutes. The completely hemolyzed blood sample is then incubated for one hour in the water bath in darkness with 1M 5-delta-aminolevulinic acid (SIGMA), the substrate of the ALAD enzyme, in 0.1M sodium phosphate buffer. After incubation, 1ml of 10% trichloroacetic acid is added to stop the reaction. The samples are then centrifuged at 2000rpm for 10 minutes. One ml of clear supernatant and 1ml of Ehrlich's reagent are placed in a cuvette and mixed. After 5 minutes, the sample absorbance is compared with a water blank at 555nm in a 1cm cell using a spectrophotometer. The ALAD activity (nmol ALA used/min/ml RBC) was calculated using the following formula: (Absorbance*3762)/hematocrit. This value was converted to the Burch and Siegel

value (the commonly reported value in most journals) by multiplying by 4.65. Activity of the samples from the study sites were then compared with reference samples collected at Patuxent Research Refuge.

B.1.b. Brain cholinesterase

The brain of the sacrificed nestling was removed from the head in the laboratory just prior to analysis for cholinesterase inhibition. The analytical procedure was originally developed by Ellman *et al*. [24] but has been modified for the use of a microwell plate reader. In brief, the "whole" brain was homogenized in 1ml 0.05M Tris buffer (pH 8.0) per 100mg sample weight. The homogenate was stored overnight in the refrigerator (4° C). The following day, photocells for each sample were prepared with 385 µl 0.25mM DTNB in 0.05M Tris buffered at pH 7.4, 2.56 µl aliquot of brain homogenate, and12.8 µl 0.156 M acetylthiocholine iodide. The sample was mixed by gently inverting parafilm covered photocells twice and then allowing it to stabilize for 1 minute. The samples were placed in the spectrophotometer and read at 30 second intervals for 2 to 3 minutes at 25º C. The average change in absorbance per minute was determined. The change in absorbance was multiplied by 130 to determine the mMol of acetylcholine iodide hydrolyzed/min/g of tissue.

B.1.c. Cytochromes P450

B.1.c.1. Monooxygenase assay

The liver of the nestling was stored in the cryotube in the ultra-cold freezer until assay for cytochrome P450. For the assay, livers were thawed and hepatic

microsomes prepared by differential centrifugation of homogenates following the protocol determined by Melancon in 2000 and based on Melancon [48]. In brief, liver samples were sectioned if necessary and weighed. Each sample was placed in a plastic test tube and suspended in a volume of homogenizing buffer (1.15% KCl in 0.01M Na/K Phosphate, pH 7.4) equivalent to 2 times the weight of the liver. A minimum of 1 ml homogenizing buffer was used even if the weight of the sample was <0.25g. These samples were homogenized using a Polytron (Model PT10/35, Brinkman, Westbury, NY; P-20 probe, setting 5, 20-sec pulse). The probe was rinsed with distilled water and wiped dry in between samples to avoid cross contamination. The homogenate was centrifuged at 4°C at 9,000g for 20 minutes (Fisher Scientific Marathon 21K/BR Centrifuge, AD8.9 rotor, 9200 RPM, Pittsburgh, PA). The pellet was discarded and the supernatant transferred to ultracentrifuge tubes and centrifuged at 4°C for 60 minutes at 100,000g (Beckman L5, fixed angle rotor type 50.4 at 32,000RPM, Beckman Instruments, Inc., Palo Alto, CA). The resulting pellet was transferred to a glass Potter-Elvehjem mortar and resuspended in resuspension buffer (0.05M Na/K phosphate containing 10^{-3}M EDTA, pH 7.6) equivalent to two times the original liver weight using a motorized PTFE pestle. The resulting microsomal suspensions were aliquoted into two labeled cryotubes and stored at -80° C until the monooxygenase assay was run.

The monooxygenase activity measured was the dealkylase ethoxyresorufin-O-dealkylase (EROD). The procedure followed that described by Melancon [48]. Tree swallows were one of the species used in the development of the procedure for avian species. In brief, the assays were run in 96 well plates at 37°C in a fluorometric

microwell plate scanner (Fluoroskan II, Titertek Instrument Co., Huntsville, AL). The incubation volumes contained microsomes from less than or equal to 4 mg of liver (pH 7.4), ethoxyresorufin (1.25 µM), NADPH (0.125mM) and 66mM Tris buffer (pH 7.4). Microsomes, ethoxyresorufin, and buffer were added to microwells in yellow light to avoid decomposition of the substrate from light exposure. All reactants except NADPH were preheated to 37°C for 15 minutes using a Jitterbug incubation/mixing chamber (Bookel Industries Inc., Philadelphia, PA). NADPH in 66mM Tris buffer solution was added and 8 readings were taken at 1.5 minute intervals. Each plate contained reference mallard duck liver microsomes to ensure the assay ran appropriately, reference microsomes prepared at the same time as tree swallow microsomes to ensure the microsomes were prepared properly and resorufin standards in triplicate in increasing concentration (0.02, 0.05, 0.1, 0.2, 0.3, and 0.6 µM resorufin). Each tree swallow sample was run in triplicate on the plate. Data from the fluoroscan microwell plate reader were entered in a computer file and manipulated using the protein levels determined for each sample to show the rate of product formation expressed as pmol product/min/mg microsomal protein [48]. Microsomal protein levels were determined by the bicinchoninic acid (BCA) method with a kit produced by Pierce^{tm} modified for use with a microwell plate. Bovine serum albumin was used as the protein standard. Each well contained either 30µl standard or diluted sample and 200µl of working assay reagent (25ml solution A and 0.5ml solution B from the Pierce^{tm} BCA Protein Assay Kit). The plates were incubated at 37°C for 30 minutes, cooled for 15 minutes and read using a Spectromax UV/VIS microwell plate scanner (Molecular Devices Corp., Sunnyvale, CA).

B.1.c.2. Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is a proven tool for detecting cytochrome P450 with the use of electrophoretic transfer and western blotting using polyclonal antibodies to avian cytochrome P4501A (CYP1A). SDS-PAGE and western blotting were conducted on liver microsomes and compared to monooxygenase activity. The protocol used for SDS-PAGE and western blotting was based on that written by Melancon (which followed modified methods of Burnette [43]) with minor adjustments for the tree swallow samples and updated materials. In brief, it was determined that the protein concentration of the tree swallow samples needed to be quite high in order to be detectable at a comparable level to reference microsomes from dosed black ducks. As a result, a minimum protein concentration of 6.35mg/ml was determined. With this protein level, 60µl of each sample was diluted as necessary with microsomal resuspension buffer (0.05M Na/K Phosphate containing 10^{-3}M EDTA). Samples were then diluted 1:1 with sample buffer (a solution containing 4ml 10% SDS, 2.5ml 0.5M Tris-HCl (pH 6.8), 2ml glycerol (to give the solution density), 0.125g dithiothreitol (DTT, Bio-Rad Laboratories, Richmond, CA), 1.5ml distilled water, and a small number of crystals of bromophenol blue (for visualization). Samples were pipetted into capped microcentrifuge tubes and denatured in boiling water for five to ten minutes. Samples were stored in a -20° C freezer in labeled containers until use.

Electrophoresis running buffer (pH 8.3) was diluted from a 5X stock solution (9g Tris base, 3g SDS, 43.2g glycine, and distilled water to a total volume of 600ml). Ready gel cassettes (7.5% Tris-HCl, Bio-Rad Laboratories, Richmond, CA) were

placed into the slots at the bottom of each side of the electrode notches of a Mini-PROTEAN 3 Electrophoresis Module Assembly. The inner chamber was filled with approximately 125ml of diluted running buffer. 2µl low range molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were loaded at the working ends (lane 2 and 14; it was discovered the 1st and 15th lanes would not run appropriately) of each gel. Samples were kept on ice, vortexed and loaded into the wells with a 10µl syringe. Optimal volumes of tree swallow test samples were found to be 8µl per lane. Each gel was always run with a standard curve of 1, 3, 5, and 10µl diluted reference black duck samples. Samples were also run concurrently with tree swallow samples collected from a highly contaminated site as a means of reference and consistency. The syringe was rinsed thoroughly with distilled water in between each sample to avoid cross-contamination. After sample loading, the Mini tank was filled with approximately 200ml of diluted running buffer. Color coded banana plugs and jacks were connected with the lid on the mini tank and the power supply. Samples were run for approximately 35 minutes at 200 volts, 15 watts and electrophoresis stopped when the blue sample fronts neared the bottom of the gels. When the run was completed, the gel sandwich was carefully opened using small forceps and distilled water, and the upper wells and bottom stacking gel were sliced off.

Pre-cut PVDF membrane (Bio-Rad Laboratories, Richmond, CA), filter paper and fiber pads were soaked in transfer buffer (6.1g Tris base, 28.8g glycine, and distilled water to a final volume of 2 L) and a cassette of the gel, membrane, filter paper and fiber pads was made according to manufacturer directions. The cassettes were placed into the Bio-Rad – Model 422 Electro-Eluter transfer apparatus along

with cooling ice molds for each cassette and the unit filled with transfer buffer. The lid was placed on the mini tank and color coded banana plugs and jacks are connected to the power supply. The protein bands were transferred to the PVDF membrane by wet electrophoretic transfer for 35 minutes at 50 constant volts.

At the end of the transfer, the membrane was removed from the cassette sandwich and placed in Ponceau Red solution (50ml acetic acid, 1g ponceau red in 950 ml distilled water) to confirm protein bands were properly transferred. Upon confirmation of protein transfer, the membranes were rinsed with distilled water and bathed for 1 hour on a shaker at low speed in 1% dry non-fat milk in Tris buffered saline with Tween 20 (TBST, Sigma, St Louis, MO) as a blocking step. After blocking, the membranes were rinsed (5 minutes/rinse) 3 times with 10ml TBST per blot. The blots were then incubated with 1:1000 anti-CYP primary antibody (20µl antibody: 20ml TBST) on a shaker at low speed for 1 hour. To produce the primary antibody, the major CYP induced by BNF administration to mallard duck was purified and injected into rabbits. The resulting polyclonal antibody was used [44]. The primary antibody was drained and the membrane again washed with TBST 3 times. The membrane was then incubated with 1:2000 (10µl antibody: 20 ml 1% milk in TBST) goat anti-rabbit IgG alkaline phosphatase conjugate secondary antibody (Sigma-Aldrich, Inc., St. Louis, MO) on the low speed shaker for 30 minutes and rinsed again 3 times with TBST. The membrane was then stained for 2 to 4 minutes (to develop desired band intensity) in 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT, Sigma, St. Louis, MO) substrate solution until desired intensity of protein bands. The protein band intensity was thus

caused by the reaction of the alkaline phosphatase conjugated secondary antibody with the BCIP/NBT substrate to produce a blue precipitate on the membrane. Once the desired intensity of the bands was reached, the solution was drained and the membranes washed with distilled water to stop the reaction. Membranes were covered and dried overnight. Membranes were photographed as TIFF files using an Olympus E-20 4.95 mega-pixel camera mounted on a fotodyne light box. Images were saved according to date and gel and developed bands quantified using UN-Scan-it gel^{tm} (Silk Scientific Inc., Orem, Utah).

B.2. Chemical analysis

B.2.a. Metal/metalloid analysis

The eggs collected from each of the active nests were frozen upon arrival to the laboratory. In preparation for analysis, they were thawed and the eggshells carefully separated from the egg contents. The shells were scored with a scalpel around the circumference of the egg and then separated from the contents using a small pair of scissors and tweezers. The shells were washed with deionized water and weighed after drying. They were then placed in labeled chemically clean screw capped jars and sent out for metal/metalloid analysis (a broad metal/metalloid scan). The feathers from the sacrificed nestling also underwent a metal/metalloid scan.

The scan included analysis for the following: Al, As, B, Ba, Cd, Cr, Cu, Fe, Hg, Mg, Ni, Pb, Sb, Se, Sr, V, and Z. The scan was conducted by a contract laboratory identified by the USFWS Patuxent Analytical Control Facility (USFWS PACF). According to the methods outlined by the Research Triangle Institute (RTI) that analyzed our samples, samples were first homogenized using a food processor.

The homogenized samples were then freeze dried for moisture content determination and ground to 100 mesh with a mill. Using a CEM microwave oven, 0.25 to 0.5g of the ground freeze dried sample was heated in a capped 120ml Teflon vessel with 5ml of Baker Instra-Analyzed nitric acid sequentially for three minutes at 120 watts, three minutes at 300 watts, and fifteen minutes at 450 watts. The residue was then diluted with laboratory pure water to a volume of 50ml. ICP measurements were made using either a Leeman Labs Plasma Spec I sequential or ES2000 simultaneous spectrometer (Leeman Labs, Lowell, MA). For the measurement of Hg, $SnCl_4$ was employed as the reducing agent and a Leeman PS200 Hg Analyzer was used (Leeman Labs, Lowell, MA).

B.2.b. Organochlorine analysis of animal tissue

The egg contents were weighed and placed in a labeled chemically clean screw capped jar and shipped on dry ice for organochlorine analysis. The nestling carcass was also weighed and placed in a labeled chemically clean screw capped jar and shipped on dry ice for organochlorine analysis. Analysis for organochlorines included DDT and its metabolites, PCB's, chlordane, etc.

Organochlorine analysis of egg contents and nestling carcasses were conducted for the 2002 and 2003 samples, however the 2004 samples were only analyzed for total PCBs. The methods used by the USFWS PACF for the 2002 and 2003 samples and by a USGS Chemist in 2004 followed methods described by Cromartie *et al*. [20]. In brief, samples were first homogenized using a Virtis homogenizer (Gardiner, NY). Each sample was placed in 150g Na_2SO_4 and blended with an Osterizer blender. Samples were then placed in glass extraction thimbles and

desiccated in an air-tight container with Drierite. After 24 hours of desiccation, the thimbles were placed in the bottom of the Soxhlet and extraction of lipids took place for 7 hours. The extracted lipids were then transferred to a beaker and allowed to dry under a fume hood. The samples were then dissolved in 10ml Hexane. Samples were then purified using a Florisil column. The samples were then condensed to 5ml using a hot water bath, transferred to a 10ml tube and diluted back up to 10ml with hexane. Contaminants in each sample were then separated using a Silica gel column. Each eluant fraction was then condensed to 5ml using a hot water bath and again diluted with hexane to a total of 10ml. The eluant fractions were then quantified with a 30m MEGABORE coated gas-liquid chromatograph (GLC) column with a 1.0 micron film of 7% cyanopropyl 7% phenyl polysiloxane and equipped with a 63Ni electron capture detector. Residues in 10% of the samples were then confirmed using a combined gas chromatograph-mass spectrometer (GC/MS) (Hewlett Packard 5890 Series II, Avondale, PA). The percent lipid content of each sample was determined. The nominal lower limit of detection based on a 10g (wet weight) sample was 0.01ppm for pesticides and 0.05ppm for PCBs.

B.2.c. Sediment analysis

Sediment samples were completely mixed by site and a 10g subsample was taken for each. Subsamples were heated in oven for 24 hours at 104°C and reweighed to determine moisture content. Samples were then diluted with a quantity of laboratory purified water equal to ten percent of the dry sample weight. The subsamples were placed in glass extraction thimbles with 10g of Florisil (U.S. Silica Company, Berkeley Springs, WV) and capped with 1.5 to 2.0 cm of sand for Soxhlet

extraction. A copper pad weighing five to ten grams was placed in the Soxhlet apparatus and samples were extracted for 8 hours with hexane:acetone:methanol (8:1:1). Extract was then mixed with 50 ml water in a seperatory funnel and allowed to separate over 15 minutes. The water layer was then discarded and the process repeated an additional two times. The resulting hexane layer was then concentrated and purified with a Florisil column (U.S. Silica Company, Berkeley Springs, WV). Contaminants were then separated using a Silica gel column (Mallinckrodt Baker, Inc., Phillipsburg, NJ). The Silica gel eluants were then quantified and confirmed using a combined gas chromatograph-mass spectrometer.

B.2.d. PAH metabolite analysis

The gall bladders were shipped on dry ice in labeled chemically cleaned vials for PAH metabolite analysis. Chemical analysis was conducted by the Geochemical Environmental Research Center (GERG) laboratory of Texas A&M identified as a USFWS PACF contract laboratory. A HPLC column was used for sample analysis by the injection of 5µl of each sample using an autosampler. Standards for benzo(a)pyrene, naphthalene and phenanthrene were dissolved in methanol in amber vials and stored at 20°C until use. Calibration standards were analyzed at the beginning of each sample run and required to agree within a relative standard deviation (RSD) of plus or minus 10%. Reference bile was also included for each sample run and had an RSD within plus or minus 15% of the previous value and an RSD that did not exceed plus/minus 25% for each batch. The column was run for 35 minutes and the fluorescence detector response was recorded with a HP-1000 computer at the excitation/emission wavelength pairs for naphthalene, phenanthrene,

and benzo(a)pyrene. The column conditions were set up so that no aromatic hydrocarbons could elute before 5 minutes and solvent contaminants for naphthalene, phenanthrene and benzo(a)pyrene metabolites elute after 28 minutes. These approximate elution times were verified for each system and were based on reports from the NOAA Technical Memorandum, NMFS F/NWC-102. The retention times for each contaminant were recorded and the areas of the reference standards were integrated such that the mean response factor (ng/integration unit) was used to calculate the sample analyte concentrations.

B.3. Data manipulation and statistical analysis

Data were recorded by hand in the field and entered regularly into an excel spreadsheet at the lab. Results of biomarker assays were directly stored electronically. All chemical analyses were entered into an electronic spreadsheet when data was received. Statistical analysis was conducted using SAS version 9.1 (SAS Inst., Inc., 2000, Cary, NC). Three types of data were generated: (1) contaminant concentrations in sediment, eggs and nestlings, (2) biomarker responses, and (3) reproductive success and growth. The assumption of normality was tested using the Shapiro-Wilkes statistic and the assumption of homogeneity of variance was determined by a plot of residuals for each variable.

Differences between study sites and among study years were evaluated using ANOVA (SAS Proc Mixed, SAS Inst., Inc., Cary, NC) to test for year, site and interaction (site X year) effects. If an interaction effect was determined, the effects were examined separately (sliced) by year and site to determine significance. When significant, a tukeys pairwise comparison was used to evaluate differences. Chemical

contaminants were only analyzed for those chemicals detected in greater than 50% of the samples. Half the detection limit was used for samples below the detection limit for the purposes of analysis. Linear relationships between contaminant levels and tree swallow biomarker responses were examined using regression analysis (SAS Proc Reg, SAS Inst. Inc., Cary, NC). Contaminant concentrations and biomarker responses were compared with values in previous literature.

Accumulation rates of total PCBs and p,p'-DDE, the most prevalent contaminants in the eggs and nestlings, were calculated. Accumulation rates were determined by taking the contaminant mass in the nestling minus the contaminant mass in the egg from the same nest divided by the age of the nestling. The contaminant mass was determined by multiplying the concentration of the contaminant in the sample by the mass of the sample. Accumulation rates were compared by ANOVA (SAS Proc Mixed, SAS Inst. Inc., Cary, NC) and when different, a means comparison using the Tukeys test.

Hatchability and nestling survival was determined by dividing the number of nestlings by the number of eggs and the number of fledglings by the number of nestlings, respectively. Nests that were lost to predation or flooding were not included in analyses. Hatchability and clutch size were compared between sites by ANOVA (SAS Proc Mixed, SAS Inst. Inc., Cary, NC) but since nestling survival was almost 100% at all sites it was not evaluated. In addition to the reproductive measures of hatchability and clutch size, similar examinations of the count of eggs and nestlings in nests at each site were examined and compared categorically (SAS Proc Catmod, SAS Inst. Inc., Cary, NC). Average bodyweight of nestlings in a nest

and physical measurements of the nestlings were also examined using ANOVA (SAS Proc Mixed, SAS Inst. Inc., Cary, NC) both as indicators of reproductive success as well as indicators of contaminant exposure.

Chapter 6: Results

A. In life data

A.1. Nest box use and predation

Attracting tree swallow pairs to the nest boxes at the Anacostia wetlands proved to be extremely difficult. Competition for nest-boxes with house wrens at the Anacostia sites may also have added to lower activity by tree swallows. Weather and predation related losses also largely contributed to problems. In the 2002 field season, what the USGS classified as moderate to severe drought conditions may have contributed to the death of nestlings in two nest boxes at Kenilworth Marsh. In that field season, another nest with 4 eggs was abandoned at that site. Overall, only one nest of 5 nestlings produced a nestling for sampling at Kenilworth Marsh. Kingman Lake did not fare much better, losing two nests to predation due to a black rat snake and a raccoon. Kingman Lake had two nests (of 5 and 4 nestlings respectively) that produced nestlings for sampling in 2002. Patuxent research refuge suffered from predation losses as well early in the field season in 2002, but still had a large amount of nesting or re-nesting taking place to provide samples. Five nests were sampled and an additional 5 nests fledged tree swallow offspring.

The second field season (2003) produced problems of a very different nature with greater than normal rainfall creating flooding of two nests at Kenilworth and abandonment of one nest with four eggs. Four nests were successfully sampled from Kenilworth. One of the sampled nests had two un-hatched eggs and another nest lost a nestling at day 3 post-hatch to unknown causes. Kingman was again the more

successful site with six sampled nests. A seventh nest was lost to predation by a black rat snake. Patuxent was a very productive site in 2003 with 5 nests utilized for sampling and an additional 8 nests producing fledglings. Three nests were lost to serpentine predation at Patuxent in 2003 but there was more than ample nesting at the site to sample from.

In the 2004 field season, Kenilworth became a more productive site than in previous years, providing the 5 nests needed for sampling. A sixth nest of 4 eggs was lost to predation by a black rat snake. One strange observation was made at Kenilworth that year. A nest which was never fully developed (containing only feathers which are behaviorally known to be supplied by the male in a breeding pair) had 5 tree swallow eggs. Unfortunately none of these eggs were ever incubated and tree swallows were rarely observed spending time at this nest box, but the laying and subsequent abandonment of 5 eggs in an incomplete nest was atypical. This may have been the activity of a first year pair, but I was never able to get close enough to the tree swallows that appeared to be using this box to determine the age of the birds. Kingman lost two nests to predation in 2004 but still produced seven successful nests, two of which were utilized to provide samples. In 2004, Patuxent again proved very successful with 16 nests including the five that were sampled to serve as a reference for that field season.

A.2. Clutch size

Tree swallows lay one egg per day until their clutch is complete with a typical clutch size of five to six eggs and a normal range from two to seven eggs [15]. In previous research, clutch size has been used as an indicator of the general condition

and energy level of the female in the pair [49]. Egg laying was monitored throughout the three field seasons and clutch size recorded, taking into account the egg collected for organochlorine analysis for sampled nests. Clutch sizes were then compared between sites and among years. Nests which were predated were not included in this analysis. There were minor non-significant differences in clutch size between sites but no significant differences (Table 1).

Table 1: Average clutch size at each site.

Site	Average Clutch Size	Standard Error	N
Patuxent	4.89	0.93	45
Kingman	4.50	1.15	20
Kenilworth	4.56	0.78	18

A.3. Hatchability and nestling survivability

Hatchability was calculated by dividing the number of nestlings by the number of eggs, and nestling survivability was calculated by dividing the number of fledglings by the number of nestlings. The sampled egg and nestling were not included in the tallies for these calculations. Also nests which were predated or flooded were not included in these calculations.

Despite 100% hatchability in 2002 at Kenilworth, the loss of two nests with nestlings (perhaps due to weather related issues) resulted in poor survivability of nestlings for Kenilworth in the first field season. There was one nest with an unhatched egg at Kingman in 2002 but nestling survivability was 100%. Patuxent had 4 nests where 1 or more eggs did not hatch but there was 100% survival of nestlings at Patuxent in 2002.

In 2003, Kenilworth had one nest with two unhatched eggs lowering the hatchability somewhat. One nestling was lost to unknown reasons however the other three nests with nestlings had 100% survivability. Three of the six nests sampled at Kingman this year contained unhatched eggs but the nestling survivability remained 100%. Patuxent had 6 of 13 nests with unhatched eggs but maintained 100% nestling survivability.

Kenilworth hatchability was less than perfect in 2004 with 2 of the 5 sampled nests having multiple un-hatched eggs. One of the sampled nests initially contained 5 eggs but only one successfully hatched. There was no loss of nestlings or fledglings in this field season at Kenilworth however. Kingman had two nests where not all the eggs hatched making the hatchability less than 100% but all hatchlings survived and fledged successfully. In 2004, Patuxent had 6 of 16 nests contained unhatched eggs but only one of 16 nests lost a nestling.

Overall, all of the sites had less than perfect hatchability in any of the three field seasons. Despite this fact, there were significant differences between sites in hatchability ($F = 3.87$, $df = 2, 60$, $p = 0.03$) as determined by the percentage of eggs that hatched (number eggs hatched divided by total number laid). A Tukeys test revealed that Kenilworth was significantly different (less successful) from Patuxent but not significantly different from Kingman. Kingman was not significantly different from Patuxent (Table 2).

Table 2: Average percent of eggs hatched by site over the 3 collection years.

Site	% Hatchability	Standard Error	N
Patuxent	0.89	0.04	42
Kingman	0.90	0.08	17
Kenilworth*	0.68	0.04	15

* Significantly lower than Patuxent ($p < 0.05$).

Nestling survivability was found to be almost identical at all sites despite the loss of some nestlings at Kenilworth and the loss of one nestling at Patuxent to undetermined causes. Perhaps had there been more nests available for sampling at Kenilworth, there would have been significant differences found between the sites but with the data collected over these three field seasons, no differences were found.

Some would argue that since the number of eggs and the number of nestlings in a nest are count data, differences between sites would be better evaluated as categorical data. A categorical analysis of the number of eggs in nests at each site revealed no significant differences between sites (Table 3). Likewise, the evaluation of the number of nestlings also revealed no significant differences (Table 4). Thus comparatively the ability to lay eggs and hatch nestlings by tree swallows inhabiting the Anacostia sites over the three study years was equivalent to that of tree swallows inhabiting Patuxent.

Table 3: Number of nests with each quantity of eggs at each site over three years.

Site	3 eggs	4 eggs	5 eggs	6 eggs	7 eggs
Kingman	0	5	7	2	1
Kenilworth	0	4	10	0	0
Patuxent	4	6	21	6	2

Table 4: Number of nests with each quantity of nestlings at each site over three years.

Site	0 nestlings	1 nestling	2 nestlings	3 nestlings	4 nestlings	5 nestlings	6 nestlings	7 nestlings
KL	0	0	1	1	7	5	0	1
KM	3	1	2	0	2	7	0	0
PRR	0	0	2	7	11	15	3	1

KL = Kingman Lake, KM = Kenilworth Marsh, PRR = Patuxent Research Refuge

A.4. Average body weight

Total body weight was recorded for all the nestlings in each sampled nest at the time of sacrifice. The weight of the nestling to be sacrificed was recorded just prior to euthanasia. Since statistical analysis was conducted at the nest level and not individual level, all weights for nestlings in a sampled nest were averaged at the time of statistical analysis. Average nest body weights for nestlings were compared between sites and among years. No significant differences between sites were found however there were significant differences between years (Table 5). Nestlings in the 2002 field season were significantly lower in weight than nestlings from the 2003 and 2004 seasons. This makes sense given the heat and drought that occurred during the 2002 field season that would have limited the food resources that year.

Table 5: Average weight of all nestlings (sacrificed and unsacrificed) at each site over the three study years.

Site	Average Weight	Standard Error	N
Kingman	21.56	0.57	10
Kenilworth	20.39	0.63	10
Patuxent	22.07	0.32	28

A.5. Tibiotarsus, radius-ulna and culmen measurements

Previous research by Watson and Thornhill [50], suggested that a deviation from the symmetry of bone length may indicate environmental or developmental disturbances. Measurements were taken for all nestlings in the nest (including the sacrificed nestling prior to euthanasia) of the right and left tibiotarsus and radius-ulna and the length of the culmen. Since this study was examining differences at the nest level, these values were averaged across all the nestlings in the nest prior to statistical analysis. Since the goal of these measurements were to examine symmetry the percent difference of the tibiotarsus and radius-ulna measurements were calculated by subtracting the minimum value from the maximum value, dividing by the maximum value and multiplying by 100. The percent differences of the measurements were compared across sites and over years. No significant difference between measurements over sites or years was discovered. This is to be expected given that gross physical abnormalities such as asymmetry of bone length would only be expected in areas of extreme contamination.

A.6. Ligatures

Ligature samples were collected from nestlings' at all three locations throughout the three years of the study however, only the 2002 field season ligature samples were identified and tallied according to their classification. Ligature samples were limited from all three sites and appeared to be biased towards larger food items that were unable to be swallowed. In many cases, food items were disgorged by the nestlings into the nest before collection. Despite careful examination of the nests for the disgorged items, some of these food items were fouled by excrement and trampled

making the collection difficult if not impossible. After careful consideration it was determined that these samples could not be effectively used to determine the food items of the tree swallows over the field season. Johnson *et al*. [51] documented the biases of the ligature method, supporting this decision.

B. Cytochromes P450

B.1. Monooxygenase activity

B.1.a. Site differences within each year

Thirty five nestlings were collected over the three field seasons of this study, twenty of which were collected from the Kingman (n = 10) and Kenilworth (n = 10). There were no significant differences between sites in the 2002 and 2003 collection year. A significant difference ($p = 0.04$) in monooxygenase activity was detected between sites in the 2004 field season. Only two samples were collected from the Kingman location that field season. Therefore, a comparison between combined Anacostia sites and Patuxent was conducted. There was a significant difference ($p = 0.01$) found between the Anacostia wetlands and the Patuxent Marsh with the Anacostia marshes having significantly higher monooxygenase activity.

B.1.b. Combined years monooxygenase activity

When the monooxygenase data was pooled by site and compared, a significant difference ($F = 3.61$, $df = 2, 26$, $p = 0.04$) was found (Table 6). Likewise, when the Anacostia sites were combined and compared with the Patuxent EROD results, there was a significant difference ($F = 5.80$, $df = 1, 26$, $p = 0.02$). When a pairwise comparison was conducted using Tukey's test, it was determined that Kingman Lake

was significantly different from Patuxent ($p < 0.05$) but not from Kenilworth marsh. Kenilworth marsh alone was not significantly different from Patuxent.

Table 6: Average EROD activity for pooled data reported as pmol product/min/mg microsomal protein.

Site	Average EROD activity	Standard Deviation	N
Kingman*	78.89	10.50	10
Kenilworth	63.13	11.71	10
Patuxent	44.80	7.53	15

* Significantly higher than Patuxent ($p < 0.05$).

B.1.c. Monooxygenase activity and it's relationship to residues

Regression analysis was conducted on the EROD activity (combined over all three years for each site) and the contaminants residues determined in the egg contents and carcass of the nestling. The only significant relationship between the EROD and a residue was found with the p,p'DDE levels detected in the carcass ($p=0.001$, $R^2=0.41$, df = 2, 21). The ANOVA conducted on the p,p'-DDE levels in the carcass did in fact reveal a significant site by year interaction, with Kingman having significantly higher concentrations than Patuxent in the 2003 collection year. As with the EROD activity, Kenilworth was found to not be significantly different from Patuxent. Despite the significant linear relationship between these contaminants, the likelihood of p,p'-DDE causing the difference between EROD activity at each site is highly unlikely given that p,p'-DDE does not induce CYP1A. This significant regression is likely just coincidental. The relationship between sediment contaminants and EROD was explored however no significant relationships were found.

B.2. Western Blotting

Western blots were performed on the 2003 and 2004 samples (n = 27) using antibodies specific for BNF-induced cytochromes P450 from mallard ducks [44]. A standard curve with which to compare and quantify the CYP1A in microsomal samples was prepared using microsomes from BNF-treated black ducks that were substantially diluted to 1µg protein/ 24µl sample. These black duck samples were pipetted on each gel in 1, 3, 5 and 10µl amounts. As mentioned in the methods, the Anacostia and Patuxent tree swallow samples were rather concentrated at 6.35µg protein/ 1µl sample. As another means of standardizing the study samples, tree swallow microsomes from a highly contaminated site in Rhode Island were also run on every gel (Figure 5).

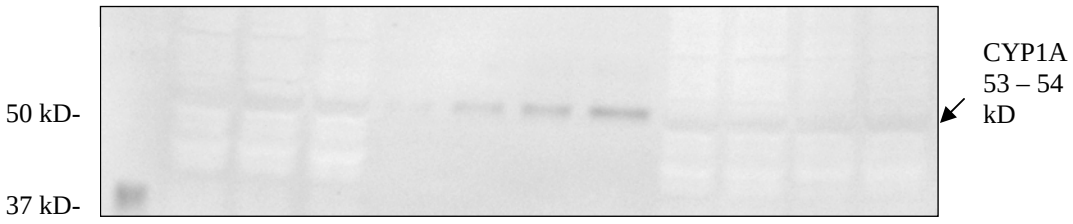


Figure 5: Western blot showing the CYP1A band in the tree swallow samples with the black duck samples used as a standard curve. Background coloration was automatically subtracted from the density calculations by the UN-Scan-it gel^{tm} software.

After the determination of the density of staining for each sample by the UN-Scan-it gel^{tm} (Silk Scientific Inc., Orem, Utah), readings were averaged across repeated samples. The density was then standardized to units of black duck (BD) using the values determined from the linear regression of the BD standard curve run with those samples. The BD units were then divided by the volume of sample used in

the gel to determine the BD units/µl of sample. A regression analysis of the western blot BD units with the EROD results for each sample revealed a significant relationship ($p = 0.001$, $R^2 = 0.3615$). This significant regression indicates that western blotting provided an accurate measure of CYP1A in tree swallows (Figure 6). When regression analysis included all of the highly induced tree swallow samples from Rhode Island, the linear relationship improved ($p = < 0.001$, $R^2 = 0.5648$) further supporting the accuracy of the western blot in determining P450 activity.

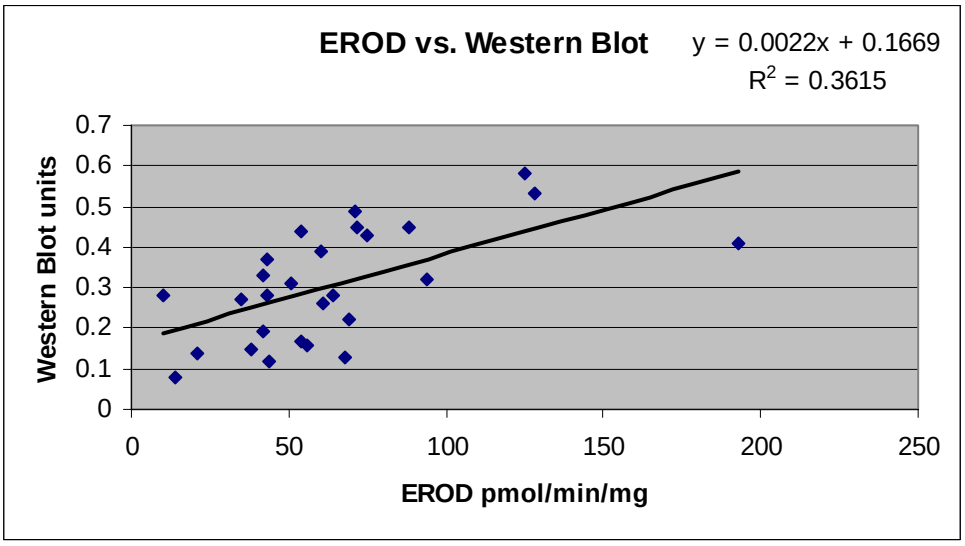


Figure 6: The linear relationship between EROD activity and corresponding western blot units (as determined by the standard curve).

C. Brain cholinesterase inhibition

Thirty five tree swallow brains were analyzed for cholinesterase inhibition. There were no significant differences found between sites within each year or when combined over the years (Table 7). Cholinesterase activity was found to be consistent with previous research in tree swallow nestlings [25]. Significant differences (F =

10.74, df = 2, 26, p = 0.0004) in cholinesterase activity were seen between the three years with the 2004 year having significantly higher activity than the 2002 or 2003 year. This difference was likely seen because all samples were analyzed after the 2004 field season likely leading to some loss of the enzyme activity in the older samples.

Table 7: Average Cholinesterase activity of 14 day-old nestlings pooled by site.

Site	Cholinesterase Activity	Standard Deviation	N
Kingman	7.15	1.02	10
Kenilworth	6.95	0.89	10
Patuxent	7.04	0.79	15

D. ALAD

Blood was collected and analyzed for ALAD activity from 33 tree swallow nestlings over the course of the three years of collection. ALAD activity was calculated in Burch & Siegel units of activity which is the increase in absorbance of 0.100ml at 555nm with a 1.0 cm light path/ml of erythrocytes/hr at 38°C [21]. No significant differences were found between sites within each year or when compared cumulatively by site (Table 8). Of note however is that one sample from Kingman in the 2002 year did have depressed ALAD activity (99.8) when compared to other nestlings from the same field season. This nestling likely was exposed to lead in an isolated food item. A significant difference among years was detected (F = 7.32, df = 2, 24, p = 0.003) with the 2003 samples having greater activity than either the 2002 or 2004 samples. All values fell within the expected normal range for nestling passerines [52]. Interestingly, the values for ALAD had a linear relationship with the

lead concentration from the egg ($p = 0.01$, $R^2 = 0.34$) but not the lead concentration in the nestling (Figure 7).

Table 8: Average ALAD activity (increase in absorbance of 0.100ml at 555nm with a 1.0cm light path/ml of erythrocytes/hr at 38°C) when samples were pooled by site.

Site	Average ALAD Activity	Standard Deviation	N
Kingman	362.54	136.17	9
Kenilworth	360.97	91.65	10
Patuxent	330.26	124.41	14

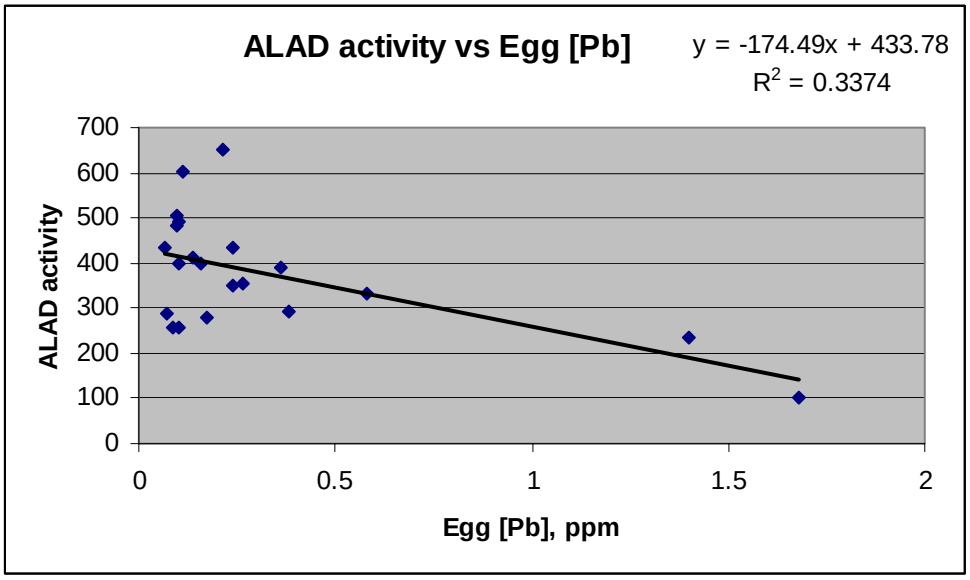


Figure 7: Linear relationship between ALAD activity and egg [Pb]. As egg [Pb] increases the activity of the ALAD enzyme decreases.

E. Chemical residues

E.1. Residues in egg and nestling carcass

All thirty-five nestling carcasses and egg contents from each of the sampled nests were analyzed for organochlorine residues. Concentrations are reported in parts per million wet weight. Total PCBs in the eggs had to be normalized for analysis by a square root transformation. For the total PCBs in the egg contents there were significant site by year interactions, making the teasing apart of a sole site or a year effect unfeasible. Less than 50% of the eggs in 2002 had detectable levels of total PCBs therefore analyses only examined samples from 2003 and 2004 (Table 9). When sites were compared within each year, 2003 was the only year where a significant difference between sites was detected ($F = 32.83$, $df = 2, 21$, $p = < 0.0001$). A Tukeys pairwise comparison revealed that both Kingman Lake and Kenilworth Marsh had significantly higher PCB in their egg contents than Patuxent. It should be noted that in 2003, Kenilworth had an egg that contained 31ppm PCB total, much higher than the next lowest contaminated egg with 19.1 ppm.

For the PCB residues detected in the carcass, there were significant differences between sites in all three years of collection (Table 10). In 2002, Kingman had significantly lower carcass [PCB] than Patuxent (but not Kenilworth). There were higher levels of PCBs in the carcass in 2003 while 2004 proved to have rather low levels of total PCBs all around. When samples were combined over the three years, a contrast demonstrated that the Anacostia marshes had significantly higher total PCBs than Patuxent. As previously mentioned, when nestlings were below detection, the samples were analyzed by using half the detection limit as long as more than 50% of

the samples in that year were above detection. A total of 8 of the 15 nestlings were below detection at Patuxent, so although Patuxent was included in this analysis for comparisons to Kingman and Kenilworth, the site itself had greater than 50% of nestlings below detection.

Table 9: Egg [PCB] values and pooled mean for the 2 study years where [PCB] was above detection in greater than 50% of samples. BDL = Below detection limit.

Site	Individual egg [PCB], ppm, 2003 & 2004	Mean, ppm	Std. Dev.	N
Kingman*	16, 8.54, 11.8, 11.7, 13, 19.4, 0.79, 0.16	10.17	6.79	8
Kenilworth*	8.67, 19.1, 6.67, 31, 0.47, BDL, 0.63, 2, 0.84	7.71	10.70	9
Patuxent	BDL, BDL, 4.3, BDL, BDL, 0.08, BDL, BDL, BDL, BDL	0.458	1.35	10

* Significantly higher than Patuxent ($p < 0.05$)

Table 10: Carcass [PCB] values and pooled means. BDL = Below detection level.

Site	Individual carcass [PCB], ppm	Mean, ppm	Std. Dev.	N
Kingman*	0.57, 0.36, 0.55, 0.6, 0.52, 0.58, 0.13, 0.11, 0.15, 0.93	0.54	0.11	10
Kenilworth*	0.42, 0.7, 0.4, 0.61, 1.72, 0.88, 0.64, 0.3, BDL, 1.25	0.58	0.09	10
Patuxent	BDL, 0.24, BDL, BDL, 0.1, 0.69, 1.04, 0.72, BDL, 1.17, BDL, 0.33, BDL, BDL, BDL	0.08	0.08	15

* Significantly higher than Patuxent ($p < 0.05$)

An examination of PCB accumulation rates in the nestlings showed a significant site by year interaction ($F = 2.91$, $df = 2,21$, $p = 0.03$). When teased apart it was discovered that a significant difference in sites was present in 2003 with the Anacostia sites being significantly lower in accumulation of PCBs than Patuxent. This difference is unexpected but attributable to the very high PCB concentrations that the Anacostia eggs contained making even moderate dietary intake of PCBs by

the nestlings negligible to the amount lost during growth (Table 11). The decrease in total PCBs in the nestlings at the Anacostia sites is likely due to the metabolism of PCBs by CYP1A. Unfortunately no linear relationship appears to exist between EROD activity and PCB uptake, but given the participation of CYP1A in the metabolism of other contaminants such as PAH's, any relationship is likely confounded by the metabolism of these other inducers.

For p,p'-DDE residues, the five eggs collected from Patuxent in 2002 contained both the highest and lowest levels that year (avg. = 0.1779). The four eggs collected from Kenilworth and Kingman each contained a similar range of p,p'-DDE residues with an average concentration of 0.2218 ppm and 0.189 ppm respectively. In 2003, 5 eggs collected from Patuxent ranged in p,p'-DDE residues from 0.767 to 1.20 ppm (avg. = 0.921) and were not significantly different from Kenilworth (0.996 – 1.42 , avg. = 1.179, n = 4) or Kingman (1.28 – 2.50, avg. = 1.585, n = 6). The p,p'-DDE residues found in the eggs over the two years this contaminant was examined were not significantly different between sites.

The p,p'-DDE concentration in the nestlings however revealed a significant interaction (site*year) effect ($F = 3.99$, $p = 0.04$, $df = 2, 17$). A test of effect slices (effect of year within each site and effect of site within each year) revealed that only in 2003 were there significant site differences ($F = 7.75$, $p = 0.004$, $df = 2, 17$). Further investigation with a Tukeys mean comparison revealed that in 2003, Kingman had significantly ($p < 0.05$) higher carcass [p,p'-DDE] than Patuxent. Patuxent and Kenilworth were not significantly different from one another nor were Kingman and Kenilworth (Table 12).

Accumulation rates of p,p'-DDE only showed differences between years at Kingman Lake but no differences in the uptake of this contaminant at the three sites (Table 11). This may indicate that exposure to p,p'-DDE occurred in the breeding pair prior to arrival at the nesting sites and thus became diluted enough in the nestlings over time through growth and metabolism of this contaminant.

Table 11: Sample calculations of PCB and p,p'-DDE accumulation rates at each site. Contaminant gained = (contam. nestling * nestling mass) – (contam. egg * egg mass). C. = carcass, BDL = below detection limit, PRR = Patuxent, KL = Kingman, and KM = Kenilworth. The nest box number and year corresponds to the particular box sampled in the given year. Notice how even in cases where both the egg and the carcass were below the detection limit, there was still calculated to be contaminant gained albeit very little. Also notice how an egg that started with 31 ppm at Kenilworth still had negative gain in total PCBs because of the dilution of PCBs with growth.

		Contam Carcass	Carcass X Mass	-	Contam. Egg	Egg X Mass	=	Contam gained
Contam	**Nest ID**	**Contam carcass (ppm)**	**C. (g)**	**Contam mass C.**	**Contam egg (ppm)**	**Egg (g)**	**Contam mass egg**	**Gain**
Total PCBs	PRR 14 2002	0.691	12.84	0.89	0.025 (BDL)	1.69	0.04	8.83
Total PCBs	PRR 33 2002	0.025 (BDL)	15.22	0.38	0.025 (BDL)	1.39	0.03	0.35
Total PCBs	PRR 11 2003	0.025 (BDL)	15.75	0.39	4.30	1.54	6.62	- 6.23
Total PCBs	KL 37 2003	0.584	14.31	8.36	19.4	1.44	27.94	- 19.58
Total PCBs	KM 35 2003	0.611	14.91	9.11	31	1.35	41.85	- 32.74
pp'-DDE	PRR 20 2002	0.039	12.97	0.51	0.114	1.40	0.16	0.34
pp'-DDE	KL 40 2003	0.039	14.51	0.57	1.48	1.39	2.06	- 1.50
pp'-DDE	KM 3 2003	0.024	13.89	0.33	1.42	1.24	1.76	- 1.43

Table 12: Individual carcass [p,p'-DDE] and averages by site within each year.

Site	Individual carcass [p,p'DDE], ppm	Mean, ppm	Std. Err.	N
Kingman 2002	0.023, 0.027	0.025	0.011	2
Kingman 2003*	0.055, 0.044, 0.103, 0.039, 0.045, 0.046	0.055*	0.006	6
Kenilworth 2002	0.053	0.053	0.016	1
Kenilworth 2003	0.024, 0.027, 0.014, 0.036	0.025	0.008	4
Patuxent 2002	0.018, 0.039, 0.017, BDL, 0.040	0.025	0.007	5
Patuxent 2003	0.010, 0.026, 0.026, 0.019, 0.025	0.021	0.007	5

*Kingman in 2003 was significantly higher than Patuxent ($p < 0.05$).

E.2. PAH metabolite analysis

Due to the small volume of bile in each gall bladder, gall bladders of nestlings from the same sites with similar monooxygenase activity were pooled to maximize the ability of the lab to analyze samples for PAH metabolites. Metabolites of three PAHs were examined; benzo(a)pyrene, naphthalene and phenanthrene. None of these metabolites exhibited significant differences between sites.

E.3. Metal/metalloid analysis

Feathers from one nestling and the shell from one egg collected from each of thirty-five nests were analyzed for select metals and metalloids. Concentrations are reported in parts per million dry weight. In cases where metals or metalloids could only be found in one or two of the study years, only the pertinent years were compared for site differences.

Arsenic, cadmium, chromium, magnesium, nickel and lead were found in feather and egg samples sporadically over the three collection years but did not vary significantly by site when found in greater than 50% of samples. Barium, copper, magnesium, and strontium were detected in feathers over all three years but no significant site differences were discovered. Beryllium and molybdenum were not detected in any samples.

Aluminum, although ubiquitous in animal tissues and water everywhere is a more prevalent compound in urban waters due to its use in water treatment and to the removal from water during such treatment organic compounds that would bind free aluminum [53]. No significant site differences were detected between aluminum in egg shells. Feather aluminum levels did not meet the assumptions of normality and thus were log transformed for the purposes of ANOVA. There were significant site differences in aluminum in the feathers ($F = 10.02$, $df = 2, 26$, $p = 0.0006$). Both Anacostia sites had significantly greater levels of feather Aluminum than Patuxent (Table 13).

Table 13: Average feather [Al], ppm pooled by site.

Site	Average feather [Al], ppm	Standard Deviation	N
Kingman*	16.14	2.27	10
Kenilworth*	15.28	2.53	10
Patuxent	7.72	1.63	15

* Significantly higher than Patuxent ($p < 0.05$).

Boron, ubiquitous in the environment, can reach elevated levels as a result of human activities such as drain water disposal and the use of certain laundry

detergents [54]. Boron did not show any significant site difference in egg shell but had a significant site by year interaction effect in feathers ($F = 5.07$, $p = 0.004$, $df = 4, 26$). An examination of site effects for each year and year effects for each site revealed significant site differences in 2003 ($F = 9.62$, $p = 0.001$, $df = 2, 26$) and 2004 ($F = 13.64$, $p = <0.0001$, $df = 2, 26$). Kenilworth was significantly higher than Patuxent in 2003 and 2004 while Kingman was only significantly higher than Patuxent in 2004 (Table 14).

Table 14: Average feather [B] at each site within each year.

Site	Average Feather [B], ppm	Standard Error	N
Kingman 2002	1.45	0.30	2
Kingman 2003	1.22	0.17	6
Kingman 2004*	3.36	0.30	2
Kenilworth 2002	1.49	0.42	1
Kenilworth 2003*	1.78	0.21	4
Kenilworth 2004*	2.58	0.19	5
Patuxent 2002	1.60	0.19	5
Patuxent 2003	0.56	0.19	5
Patuxent 2004	1.65	0.19	5

*Significantly greater than Patuxent feather [B] within the respective year ($p < 0.05$).

Iron is an essential dietary metal that can be toxic at elevated levels. Iron was detected at all sites over the three years of collection. There were no significant site differences in egg samples however there was a significant site effect in feather samples ($F = 4.39$, $p = 0.02$, $df = 2, 26$). The site by year interaction effect was nearly significant ($F = 2.74$, $p = 0.0503$, $df = 4, 26$). Due to this interaction effect, a closer examination of site differences by Tukeys mean comparison indicated that there were differences during the 2004 field season (Table 15).

Table 15: Average feather [Fe] by site within each year.

Site	Average feather [Fe], ppm	Standard Error	N
Kingman 2002	50.00	6.19	2
Kingman 2003	59.45	3.57	6
Kingman 2004*	75.90	6.19	2
Kenilworth 2002	52.10	8.75	1
Kenilworth 2003	54.08	4.37	4
Kenilworth 2004	66.62	3.91	5
Patuxent 2002	43.24	3.91	5
Patuxent 2003	58.96	3.91	5
Patuxent 2004	49.94	3.91	5

* Significantly higher feather [Fe] than Patuxent within the respective year ($p < 0.05$).

Mercury is a heavy metal that is carcinogenic, terratogenic, mutagenic, and causes death of embryos and cytochemical and histopathological effects. It is easily converted to the more toxic form, methylmercury, by biological and other means [55], [56]. Mercury levels, though detectable in many eggs, were below detection limits in greater than 50% of samples and were thus not statistically analyzed. Feather mercury levels were not normally distributed but with a log transformation was found to be significantly different by site ($F = 7.06$, $df = 2, 26$, $p = 0.004$) (Table 16). A Tukeys test revealed that Patuxent had significantly higher levels of mercury in the nestling feathers than Kingman but not Kenilworth.

Table 16: Average feather [Hg] pooled by site. Patuxent was significantly higher than Kingman.

Site	Average feather [Hg], ppm	Standard error	N
Kingman	0.700	0.12	10
Kenilworth	0.737	0.13	10
Patuxent	1.101	0.08	15

Selenium is an essential dietary metal that can be harmful in large amounts or in its deficiency [57]. Selenium was significantly different ($F = 4.73$, $df = 2, 22$, $p =$

0.02) in sites over the two years it was detected in egg shells (2002 and 2003) with mean comparison revealing that Kingman Lake had significantly lower levels of selenium than Patuxent (Table 17). A significant site by year interaction was found with the feather samples ($F = 6.15$, $df = 2, 26$, $p = 0.001$) with significant differences in sites being detected in 2002 and 2003 (Table 18). In 2002, feather samples from Kingman Lake were significantly lower in selenium than Patuxent and Kenilworth Marsh samples. In 2003, feather samples from Kenilworth Marsh had significantly lower selenium residues than samples from Kingman and Patuxent.

Table 17: Average egg [Se] pooled by site. Patuxent had significantly higher [Se] than Kingman.

Site	Average egg [Se], ppm	Standard Error	N
Kingman	0.274	0.113	8
Kenilworth	0.365	0.112	5
Patuxent	0.402	0.086	10

Table 18: Average feather [Se] at each site in each year of collection. Patuxent was significantly higher than Kingman in 2002 and higher than Kenilworth in 2003.

Site	Average feather [Se], ppm	Standard Error	N
Kingman 2002	0.985	0.276	2
Kingman 2003	2.535	0.159	6
Kingman 2004	3.395	0.276	2
Kenilworth 2002	2.460	0.390	1
Kenilworth 2003	1.883	0.195	4
Kenilworth 2004	3.162	0.174	5
Patuxent 2002	2.314	0.174	5
Patuxent 2003	2.722	0.174	5
Patuxent 2004	3.068	0.174	5

Vanadium is a ubiquitous metal found in many petroleum products. Although beneficial to plants in small amounts, in large amounts it is extremely toxic and

thought to be one of the fourteen most noxious heavy metals. The toxicity of vanadium is linked to its ability to inhibit many crucial enzyme systems [58]. Vanadium was only detected in feather samples from the 2003 collection year. Site differences ($F = 4.87$, $df = 2, 12$, $p = 0.03$) were determined with Kenilworth Marsh having significantly lower levels of vanadium than Patuxent (Table 19).

Table 19: Average feather [V] by site in 2003. Patuxent had significantly higher values than Kenilworth.

Site – 2003	Average feather [V], ppm	Standard Error	N
Kingman	0.147	0.010	6
Kenilworth	0.107	0.012	4
Patuxent	0.156	0.011	5

Zinc is an essential element present in the tissues of animals and plants under normal conditions. The interaction of zinc with other metals is key to its actions as a toxin in an organism, for example vanadium toxicity is intensified by high dietary zinc [59], [58]. Zinc is an important dietary element and can have adverse effects both in its deficiency and in its over-consumption [59]. Zinc levels in egg were found to have a significant site by year interaction ($F = 3.88$, $df = 2, 31$, $p = 0.01$) with a site effect in the 2004 collection year (Table 20). Due to the fact that only two eggs were collected from Kingman in that year, the means of Kingman and Kenilworth were compared collectively with Patuxent with Patuxent having significantly lower levels of zinc than those found at the Anacostia sites. No significant differences were observed between sites for feather zinc residues.

Table 20: Average egg [Z] in 2004 at each site.

Site – 2004	Average egg [Z], ppm	Standard Error	N
Kingman*	25.40	4.94	2
Kenilworth	9.88	3.13	5
Patuxent	8.84	3.13	5

Significantly higher than Patuxent ($p < 0.05$).

E.4. Residues in sediment

Sediments were analyzed at each site for select organic contaminants and metals. Significant site differences were detected for benzo(a)pyrene residues in sediment ($F = 5.73$, $df = 2, 6$, $p = 0.04$). Means comparison with Tukeys revealed that Patuxent had significantly lower levels of benzo(a)pyrene than both Kingman and Kenilworth. Total PCBs in the sediments also differed significantly between sites ($F = 7.92$, $df = 2, 6$, $p = 0.02$). As was the case with benzo(a)pyrene, total PCBs were significantly lower in sediment from Patuxent. Residues of phenanthrene in the sediment showed the same pattern with significant differences between sites ($F = 6.58$, $df = 2, 6$, $p = 0.03$) and Patuxent having a significantly lower concentration of phenanthrene than either of the Anacostia sites. Levels of naphthalene in the sediment showed no significant differences between sites (Table 21).

Metal and metalloid concentrations in the sediments were only found to be significantly different between sites in three metals/metalloids; selenium ($F= 15.75$, $df = 2, 6$, $p = 0.004$), vanadium ($F = 11.96$, $df = 2, 6$, $p = 0.008$), and zinc ($F = 9.75$, $df = 2, 6$, $p = 0.02$). For selenium, Patuxent had significantly higher concentrations of selenium in the sediments than either Anacostia site. In the case of Vanadium, Kingman Lake contained significantly lower concentrations of vanadium in it's sediments than Kenilworth but not Patuxent. Patuxent and Kenilworth Marsh were

not significantly different. Zinc concentrations were significantly higher at Kenilworth than Patuxent. Kingman Lake was not significantly different from either Kenilworth or Patuxent (Table 22). Detectable levels of aluminum, arsenic, boron, barium, chromium, copper, iron, mercury, magnesium, manganese, nickel, lead, and strontium were found at all study sites but did not differ significantly between sites.

Table 21: Average concentrations of sediment benzo(a)pyrene, total PCBs, phenanthrene and naphthalene by site.

Site	Average sediment [benzo(a)pyrene], ppm	Average sediment [Total PCBs], ppm	Average sediment [phenanthrene], ppm	Average sediment [naphthalene], ppm
Kingman (n = 5)	1.25 ± 0.19*	0.49 ± 0.07*	0.60 ± 0.08*	0.03 ± 0.01
Kenilworth (n = 2)	0.79 ± 0.30*	0.46 ± 0.11*	0.38 ± 0.13*	0.04 ± 0.01
Patuxent (n = 2)	0.06 ± 0.30	0.002 ± 0.11	0.04 ± 0.13	0.01 ± 0.01

Significantly higher than Patuxent ($p < 0.05$)

Table 22: Average sediment [Se], [V], and [Zn] for each site. [Se] was significantly higher ($p < 0.05$) at Patuxent than either Anacostia site. [V] was significantly higher ($p < 0.05$) at Patuxent than at Kingman. [Zn] was significantly higher ($p < 0.05$) at Kenilworth than Patuxent.

Site	Sediment [Se], ppm	Sediment [V], ppm	Sediment [Zn], ppm
Kingman (n = 5)	0.42 ± 0.05	50 ± 24	252 ± 21
Kenilworth (n = 2)	0.52 ± 0.08	266 ± 38	325 ± 33*
Patuxent (n = 2)	0.96 ± 0.08	149 ± 38	169 ± 33

Chapter 7: Discussion

A. In life data

The use of nest boxes at the Anacostia sites by the tree swallows were lower than originally anticipated and losses due to predation and flooding of nests led to collection over three field seasons to collect the necessary number of samples. Despite the low usage of nest boxes in the first collection year, the Anacostia sites did have a steady increase in nest box usage in consecutive years with the greatest number of successful nests being in the third year of collection. Average clutch size for the three sites (in individual years and over all three years) were all below average from tree swallow population data (5.40 ± 0.37) but did not differ significantly from one another [16]. It should be noted however, that the average clutch size improved at all sites over the three years of collection. Only minor differences were observed for reproductive and developmental parameters with hatchability being the only parameter with significant differences ($p < 0.05$) detected. Despite the significant differences in hatchability, the calculation of this variable was more stringent than other studies which count success as any nest where one egg in a clutch hatches. No differences in total body weight were found and average body weights were within the normal range determined in other studies [16], [19], [60]. No gross physical abnormalities were seen.

B. Cytochromes P450

Monooxygenase activities were not consistent over the three collection years with a gradual increase at both Patuxent and Kenilworth as the years progressed (Kingman had its lowest activity in the second year). When compared with the findings at Patuxent, the values at the Anacostia were only moderately higher, with Kingman being statistically different from Patuxent. In the 2003 collection year, the EROD activity was nearly identical between all sites. When compared with the findings of other tree swallow studies at known contaminated sites, the level of P-450 induction was moderate at worst [19], [18], [61] (Table 23). Even the nestling with the highest EROD activity (Kenilworth 2004) of 193 (pmol/min/mg) is extremely low compared to a nestling from a contaminated pond off of the Woonasquatucket River, RI which had EROD activity of 331.9 (pmol/min/mg) [19]. Western blots of the hepatic microsomes were shown to be significantly linearly related to the EROD activity. Although this supports the enzyme activities it did not provide any additional information that would be useful for understanding the cause of the P-450 induction.

C. Brain cholinesterase inhibition

No significant differences were seen in cholinesterase activity between the sites. When compared to another study that examined cholinesterase inhibition in tree swallow nestlings, the levels in this study were slightly lower for the age of the nestlings but not abnormal [25]. There was a significant year effect with 2004 having significantly higher activity than 2002 or 2003 but that is likely due to the fact that all

samples were analyzed together after the 2004 field season and the time earlier samples were stored likely led to the loss of some enzyme activity.

Table 23: Compilation of EROD activity in selected previous research and the current study. EROD activity expressed as pmol product/min/mg microsomal protein.

Study	Year(s)	Average EROD	Site
Wayland *et al*., 1998 [62]	1993	8.8	**Prince Albert, Saskatchewan, CA** - Upstream
		14.3	- Sewage
		9.6	- Downstream-near
		12.6	- Downstream-far
	1995	24	**Grand Prairie, Alberta, CA** - Upstream
		22	- Sewage
		17	- Downstream
Bishop *et al*., 1998 [63]	1994 – 1995	23.6	**Southern Ontario, CA** - Orchard 1
	1994/1995/1997	24.5	- Orchard 2
	1994	34.9	- Orchard 3
	1997	24.3	- Nonsprayed site 1
	1994	30.5	- Nonsprayed site 2
	1994 – 1995	10.7	- Nonsprayed site 3
Custer *et al*., 1998 [18]	1994 - 1995	109.23	**Fox River & Green Bay Area , WI** - Kidney Island
		89.21	- Arrowhead
		32.07	- High Cliff
		36.60	- Poygan
Custer *et al*., 2001 [61]	1998	16.7	**North Platte River, Casper, Wy** - Reference
		151.4	- Refinery
Yorks, 1999 [45]	1995	41.42	**PA, NY, IN, & MD*** - BCH, PA
	1995 – 1996	29.56	- BML, PA

	1995 – 1996	92.19	- FM, PA
	1995 – 1996	55.49	- JH, PA
	1995 – 1996	43.34	- VF, PA
	1997	37.51	- HR, NY
	1995 – 1996	28.36	- GP, IN
	1995 – 1996	60.91	- WT, IN
	1995	39.14	- Patuxent
	1996 - 1997	28.23	
Custer *et al*., 2005 [19]	2000	44.6	**Woonasquatucket River, RI** - Greystone
		66.9	- Allendale
		20.9	- E. Minnesota
		20.6	- W. Massachusetts
	2001	45.6	- Greystone
		210.3	- Allendale
		260.3	- Lyman
		49.4	- E. Minnesota
Thesis Research	2002 - 2004	78.89	Kingman
		63.13	Kenilworth
		44.80	Patuxent

D. ALAD

No significant differences were seen in ALAD activity between sites. Differences among years were detected but values were not outside the normal range expected for passerine nestlings [52]. A linear relationship between the ALAD activity and the egg lead concentrations were seen with 34% of the variation in the ALAD activity being explained by egg lead values. Thus this is not necessarily a causative relationship.

E. Chemical residues

Organic contaminants detected in the egg and nestling samples included Total PCBs, oxychlordane in one nestling (Kenilworth, 2002), p,p'-DDD in two nestlings (Kenilworth and Patuxent, 2002), p,p'-DDE (only analyzed in 2003 and 2003), and

trans-nonachlor in one nestling (Kenilworth, 2002). The finding of oxychlordane and trans-nonachlor in the same nestling at Kenilworth comes as no surprise given that oxychlordane is a metabolite of the chlordane isomer, trans-nonachlor. It is likely that this nestling consumed an insect or insects exposed to the pesticide. The concentrations for these contaminants (0.028 ppm w.w. oxychlordane and 0.034 ppm w.w. trans-nonachlor) were at background environmental levels seen in tree swallows in other studies [62], [63], [64]. The fact that the brain could not be included in the analysis leaves one to speculate how that might have contributed to levels of these nerve targeting contaminants. The presence of p,p'-DDD in two nestlings in 2002 may only indicate that this compound was a precursor to the p,p'-DDE metabolite found in all but one nestling from Patuxent in 2002 (in the two years this contaminant was analyzed).

Residues of p,p'-DDE in the eggs were low in 2002 (range 0.063-0.430) and significantly higher in 2003 (range 0.767-2.50). Despite the elevation of p,p'-DDE residues in eggs in the 2003 year, they are still comparatively much lower than levels seen in tree swallow eggs in contaminated areas of Central British Columbia, Canada [65]. Concentrations of p,p'-DDE in the nestlings (range 0.010-0.103 ppm) were at levels similar to those seen in Green Bay, Lake Michigan [18] and pool 15 of the Upper Mississippi River [64] and much lower than levels seen in some parts of Ontario, Canada [60] and in adult tree swallows from Montana, Colorado and New Mexico [66], [67]. The comparable concentrations from Green Bay and the Upper Mississippi were considered to be background levels [64] and significantly below the 3 to 5 ppm associated with reproductive effects in sensitive avian species [68]. It

should be noted that since the parent compound DDT was not detected in either the egg or nestling samples, the levels seen in these animals are likely due to exposure of the breeding pair in their wintering grounds in South America. To further support this idea, the accumulation rates of p,p'-DDE were not significantly different between sites and in most cases showed only a slight gain or overall loss of the contaminant in the nestlings at the time of sacrifice.

Total PCBs were below detection levels in more than half of the eggs collected in the 2002 year. In the 2003 and 2004 year, the majority of eggs contained detectable levels of total PCB residues. Four eggs in the 2003 year and 5 eggs in the 2004 year were below the detection level. The 2003 collection year was the only year where site differences in egg PCBs were observed; with the Anacostia sites having significantly higher residues of PCBs than Patuxent. In 2004, the levels of total PCBs in the eggs were much lower than in previous years. The level of total PCBs in the eggs in 2003 (Kingman avg. = 13.41ppm, Kenilworth avg. = 16.36ppm) were high when compared to other tree swallow studies [69], [64] but still much lower than those recorded from the Hudson River where total PCBs in tree swallow eggs have reached concentrations of 62 ppm [17]. When results were combined over the three years, total PCBs in the nestlings were found to be significantly higher at the Anacostia sites than at Patuxent. Despite that fact, levels of this contaminant were similar to background levels seen in other studies [63], [60], [18] and significantly lower than those seen in contaminated sites along the Hudson River [17]. Given that the levels in the nestlings were much lower than those seen in the eggs in 2003 and

that the accumulation rates were not significant at these sites, it appears that these PCBs were accumulated by the female elsewhere and deposited into the egg.

Gall bladders were pooled for the purposes of achieving a large enough bile sample size for PAH metabolite analysis. Metabolites of three PAHs, benzo(a)pyrene, naphthalene, and phenanthrene were analyzed for and detected. All of these contaminants were at or above the detection limit for these compounds. Statistical analyses revealed no significant site differences for any of these compounds. Concentrations of these contaminants in the bile were much lower than those found in mallard ducklings from oil contaminated wetlands in Alberta, Canada [70].

In the case of egg shell metal/metalloid concentrations, site differences were only detected with selenium and zinc. Given that metals and metalloids in the egg shells are considered to be accumulated predominantly outside of breeding locations, the observance of a site difference in zinc does not reflect badly on study sites. In fact the zinc concentration was significantly higher at Patuxent than either Anacostia site. With zinc being an essential dietary element, it comes as no surprise that it was detected at all sites. Another essential element, selenium, was found in concentrations in the egg shells that were also significantly higher at Patuxent than at the Kingman Lake location. Site differences in metal/metalloid concentrations of feathers were only observed with aluminum, boron, iron, mercury, selenium, and vanadium. Of these, aluminum, boron, and iron were the only ones found to be significantly higher at the Anacostia locations when compared with Patuxent. Levels

of these compounds were well below those documented to cause adverse effects in avian species [71], [72], [73].

Organic contaminant residues in the sediments of benzo(a)pyrene, total PCBs and phenanthrene were all significantly higher at the Anacostia sites than at Patuxent. Benzo(a)pyrene concentrations were slightly higher at the Kingman Lake location than at Kenilworth Marsh. Using the sediment quality guidelines outlined by MacDonald *et al*. [74] and discussed in relation to the Anacostia in Pinkney *et al*. [75] only Kingman was found to have levels above which adverse effects are expected to occur. Given that benzo(a)pyrene metabolite concentrations in bile were not different between sites however, these sediment concentrations did not appear to impact the tree swallows inhabiting Kingman Lake. Total PCBs and phenanthrene in the sediments although significantly different between sites were not at levels where adverse effects would be likely to occur [74].

Analysis of metal/metalloid concentrations in the sediments revealed only one instance where the concentration was higher than Patuxent at one of the Anacostia sites (Kenilworth) which in this case was zinc. Although these levels at Kenilworth were higher than those seen at Patuxent, a comparison to sediment quality guidelines reveals that zinc concentrations were well below those levels where adverse effects would be expected [74].

Chapter 8: Conclusions

Examination of the data presented in this paper for tree swallows at the reconstructed wetlands leads to the conclusion that the reconstructed wetlands are posing no substantial threat to tree swallow inhabitants. Given the benefit of additional wetland acreage to the Anacostia watershed, this study supports the continuation of wetland reconstruction.

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