ABSTRACT

Title of Dissertation: THE EFFECTS OF POLYCHLORINATED BIPHEYNLS (PCBs) ON AVIAN CARDIAC DEVELOPMENT

Tiffany Carro, Doctor of Philosophy, 2012

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Polychlorinated biphenyls (PCBs) are a class of synthetic organochlorines that are thermally stable, resistant to degradation, and persistent in the environment as a result of bioaccumulation and intermittent redistribution through trophic levels. These compounds were sold commercially as mixtures in the twentieth century and later banned due to their biological toxicity. There are 209 known PCB congeners, each with different toxicities and physical properties that cause a variety of adverse health effects. Moreover, the effects of PCB mixtures vary with exposure concentrations, PCB congener toxicity, and species sensitivity. However, limited information is available about the impact of PCBs on the development of the embryonic cardiovascular system. There is a major site of contamination along the upper Hudson River in New York; wildlife in that region have shown evidence of exposure to PCBs. The purpose of this research was to determine the impact of embryonic exposure to a PCB mixture and a single congener, both found in the upper Hudson River on the developing avian cardiovascular system. In study 1, tree swallow eggs (*Tachycineta bicolor*) were dosed with PCB 77 and incubated to hatch. Similarly, domestic chicken eggs (*Gallus domesticus*) were dosed with the PCB mixture at embryonic day zero and incubated to hatch in study 2. Eggs were monitored through

incubation; other measures were taken at hatch along with tissue collection. Results showed that embryonic exposure to PCBs resulted in an absence of the ventricular wall compact layer and hypertrabeculation in tree swallow hatchlings in spite of no effect on survival. Embryonic exposure to a PCB mixture in domestic chickens resulted in compact layer absence as well as additional cardiomyopathies, including absence of the ventricular wall trabeculated layer, ventricular chamber dilation, abnormal heart wall and septal formations, and arrhythmias during embryonic development. In study 3, embryonic exposure to a PCB mixture was studied at Hamburger Hamilton stages 10, 16, and 20. Embryonic exposure to a PCB mixture resulted in abnormal proliferation of cardiomyocytes early in heart development. Dose-dependent mortality occurred in chicken embryos exposed to the PCB mixture. These results support other findings demonstrating PCB effects on the cardiovascular system. Further, these data showed dramatic adverse effects of the PCB mixture as well as a single congener found in the region of the upper Hudson River on the developing avian cardiac system.

THE EFFECTS OF POLYCHLORINATED BIPHENYLS (PCBs) ON AVIAN CARDIAC DEVELOPMENT

by

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Dedicated to my Mother and Father

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LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASD	atrial septal defect
bHLH	basic-helix-loop-helix
CAVSD	complete atrioventricular septal defect
CCVM	congenital cardiovascular malformation
CERCLA	Comprehensive Environmental Response and Liability Act
CL	compact layer
CYP	cytochrome P450
ED	embryonic day
EDC	endocrine disrupting chemical
EPA	Environmental Protective Agency
EtOH	ethanol
GE	General Electric
HH	Hamburger Hamilton chick embryonic stage
HLHS	hypoplastic left heart syndrome
HPA	hypothalamus-pituitary-adrenal
HPT	hypothalamus-pituitary-thyroid
HR	heart rate
hsp90	heat shock protein 90
KCl	potassium chloride
LD_{50}	lethal dose for 50% of the population
LHR	lower Hudson River
LV	left ventricle
PCB	Polychlorinated biphenyl
PCB mix	58 congener PCB mixture from egg contaminant analysis profile
PFA	paraformaldehyde
pHH3	phospho-histone-H3
PRR	Patuxent research refuge
ROD	record of decision
RV	right ventricle
TA	truncus arteriosis
TAPVC	total anomalous pulmonary venus connection
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	toxic equivalency factor
TEQ	toxic equivalency
TGA	transposition of great arteries
TOF	Teratology of Fallot
TSCA	Toxic Substance Control Act
UHR	upper Hudson River
VMHC	ventricular myosin heavy chain
VSD	ventricular septal defect
XAP2	X-associated protein 2
XRE	xenobiotic response element

CHAPTER 1

INTRODUCTION

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are synthetic organochlorines consisting of one biphenyl molecule with one to ten chlorine substitutions. There are 209 known PCB congeners, which are in a variety of PCB mixtures, such as Aroclors used in industrial applications in the United States. PCBs were introduced in 1929 and manufactured through 1976, with a total global production estimate of 13 billion pounds (Durfee *et al*., 1976). PCBs were manufactured for a variety of purposes, including use in transformers, capacitors, regulators and switches, motor oil, hydraulic systems oil, fluorescent light ballasts, cable insulation, adhesives and tapes, caulking, oil based paints, carbonless copy paper, plastics, and floor finish (Durfee *et al*., 1976). These compounds are thermally stable and are resistant to degradation, making them appealing for industrial use (Blectchy, 1983). In the United States, PCBs were used industrially from 1929 until their ban in 1979 as Aroclors, produced solely by Monsanto, and production totaled 1.25 billion pounds (Blectchy, 1983). Aroclors were named based on their percent chlorination by weight: for example, Aroclor 1254 has 54% chlorination by weight. Further, individual lots of the same Aroclors can contain varying congener distributions, thereby altering their environmental impact (Frame *et al*., 1996; Frame, 1997; Rushneck *et al*., 2004). While this makes assessing the biological impact of PCB mixtures difficult, it does make each mixture and lot unique, affording the ability to trace environmental exposures to specific sites of release (Brown *et al*., 1984).

In 1979, PCBs were officially banned from manufacture, processing, and distribution in the United States: three years after the Toxic Substances Control Act (TSCA) authorized the Environmental Protective Agency (EPA) to control any chemicals

posing potentially hazardous risks to human populations and the environment (TSCA, 1976). Until then, PCBs were released and distributed into the environment primarily through manufacture for industrial applications, some having "open system" procedures, which often released PCBs into waterways (USEPA, 1992a; 1992b). Since their ban, PCBs have been intermittently released from deteriorating hazardous waste sites, leaks from industrial transformers containing PCBs, disposal of consumer products containing PCBs, and burning of contaminated waste in industrial incinerators (USEPA, 1992a; 1992b). Ironically, the compound's resilience which made them popular in the twentieth century is the characteristic that makes PCBs environmentally persistent today in a variety of locations, including surface waters and throughout natural cycling processes (Hoffman *et al*., 1996).

The difficulty in determining the environmental impact of PCBs is related to structural differences between congeners and PCB compositional differences in the mixtures at contaminated release or exposure sites (Frame *et al*., 1996; Frame, 1997; Rushneck *et al.*, 2004). Generally, congeners substituted at the *ortho* positions (in relation to the biphenyl bond) cause a rotation in the molecule making the phenyl rings non-planar and as a rule, less biologically active. Congeners substituted at the *meta* or *para* positions generally have coplanar or planar orientations, with the phenyl rings in a single plane. In most instances these compounds are more biologically active, due to their proposed mechanism of action (Kennedy *et al*., 1996; Schuur *et al*., 1998). There are species-specific biological impacts of PCBs. PCBs bioaccumulate in fish and invertebrates with little metabolic alteration of ingested congeners (Norstrom, 1988). Conversely, reptiles, birds, and mammals do metabolize some PCB congeners, with the

exception of species with blubber stores, thereby altering the patterns of congener exposure and ultimately biological impact (Norstrom, 1988; Carey, 1994). Therefore, assessing the potential environmental impact of PCB mixtures between sites of contamination and across trophic levels is challenging. It is important to study specific sites of exposure and individual animal species to assess the complete impact of a PCB exposure or contamination site.

The Hudson River Superfund Site

The Hudson River in New York, USA is a site of PCB contamination, designated by the EPA as a Superfund site in need of remediation and/or restoration (USEPA, 1992a; 1992b). The Hudson River contamination project encompasses approximately 200 miles of waterway, from Hudson Falls to the Battery in New York City. The major area of contamination is in the Upper Hudson River (UHR) from Hudson Falls to Federal Dam, Troy, NY and is approximately 40 miles long. The remaining 160 miles, from Federal Dam, Troy to the Battery in Manhattan is identified as the Lower Hudson River (LHR). Environmental contamination along the Hudson River impacts sixteen counties in New York and New Jersey and has been associated with effects on resident wildlife (USEPA, 1992a; 1992b; Echols *et al*., 1996; Custer *et al*., 2010a; b; c; d).

In 1947, General Electric (GE) first began manufacturing capacitors at the Fort Edward plant on the UHR, and a second manufacturing plant was producing PCBs at Hudson Falls, NY within five years. For the next few decades PCBs entered the waterway at these two sites, with contamination contained at the region below the Fort Edward Dam, which blocked high concentrations of PCBs from moving downriver. However, when the Federal Dam at Troy was removed from the UHR in 1973 a bolus

release of PCBs occurred, contaminating downriver regions. The removal of the dam altered the contaminated area from a few miles, to approximately 200 miles of exposed waterway (USEPA, 1992a; 1992b). By 1976, studies revealing the adverse health effects of PCB exposure prompted the Department of Environmental Conservation to ban fishing along the Hudson River, which remains today. During that same year, natural flooding resulted in further spread of PCBs downstream, prompting national attention and leading to close both manufacturing plants (Clearwater, 2011). Unfortunately, an estimated 1.3 million pounds of PCBs entered the Hudson River from Fort Edward and Hudson Falls between 1947 and 1976 (TAMS *et al*., 1991).

Three mixtures were released from these GE plants along the Hudson River in the twentieth century. From 1952 until 1955, Aroclor 1254 (54% chlorine by weight) was released, accounting for approximately 4% of the total PCB contamination in the Hudson River. From 1955 until 1971, Aroclor 1242 (42% chlorine by weight) was released and determined to be the source of 90% of total detected PCB contamination. Finally, from 1971 until 1977, Aroclor 1016 (42% chlorine by weight) was released, accounting for approximately 1% of the total PCB contamination (TAMS *et al*., 1991). As previously mentioned, Aroclor mixtures are made up of different congeners and named by percent chlorine by weight. Individual lots of the same mixtures can have variable congener compositions, making each Aroclor lot a traceable fingerprint to the source site of contamination (Frame *et al*., 1996; Frame, 1997). Based on soil analysis of downstream PCB congener profiles, concentrations, and distributions, PCB contamination of the Hudson River has been linked solely to the GE manufacturing plants (TAMS *et al*., 1991; ATSDR, 1997).

The link between PCB contamination and GE is a milestone for environmental stewardship under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), established in 1980 by the EPA. The purpose of CERCLA was to investigate claims for natural resource damage relative to injury, destruction, or loss of natural resources as a result of release of hazardous substances. In essence, CERCLA can retroactively identify responsible parties of hazardous substance release (CERCLA, 1980). In the case of the UHR, CERCLA findings resulted in a Record of Decision (ROD) by the EPA in 2002 that required General Electric to remediate affected areas of the Hudson River. Currently, the ROD calls for six miles (2.65 million cubic yards) of dredging to remove PCB-contaminated sediment from the riverbed in the UHR region of the Hudson River (USEPA ROD, 2002). The legality of this remediation and restoration project is very challenging to the environmental cause, cleanup is now forty years beyond the halt in manufacture and PCB exposure continues during periods of high river flow and drought. The continuous, intermittent release of PCBs in the environment presents a constant problem for the ecosystem along the Hudson River and is a current issue of concern to both scientists and the community.

PCB mechanism of action

Polychlorinated biphenyls act as agonists that induce the aryl hydrocarbon receptor pathway to varying degrees to upregulate downstream genes in this pathway. The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) transcription factor family (Denison and Nagy, 2003). The functional domains of the AhR include a DNA binding domain (bHLH region), two ligand-binding domains (PAS-A and PAS-B), and a glutamine rich domain, which

functions as a co-activator (Fukunaga *et al*., 1995). This protein is inactive in cellular cytoplasm, prior to ligand-binding transduction. The inactive form of AhR is part of a complex with a dimer of heat shock protein 90 (hsp90), Ptges3, X-associated protein 2 (XAP2), and p23 co-chaperone. Hsp90 and co-chaperone p23 protects the complex against proteolysis, with p23 also regulating AhR ligand responsiveness (Kazlauskas *et al*., 1999), and XAP2 is a transcriptional enhancer while preventing AhR transfer into the nucleus prior to ligand-binding (Meyer *et al*., 1998).

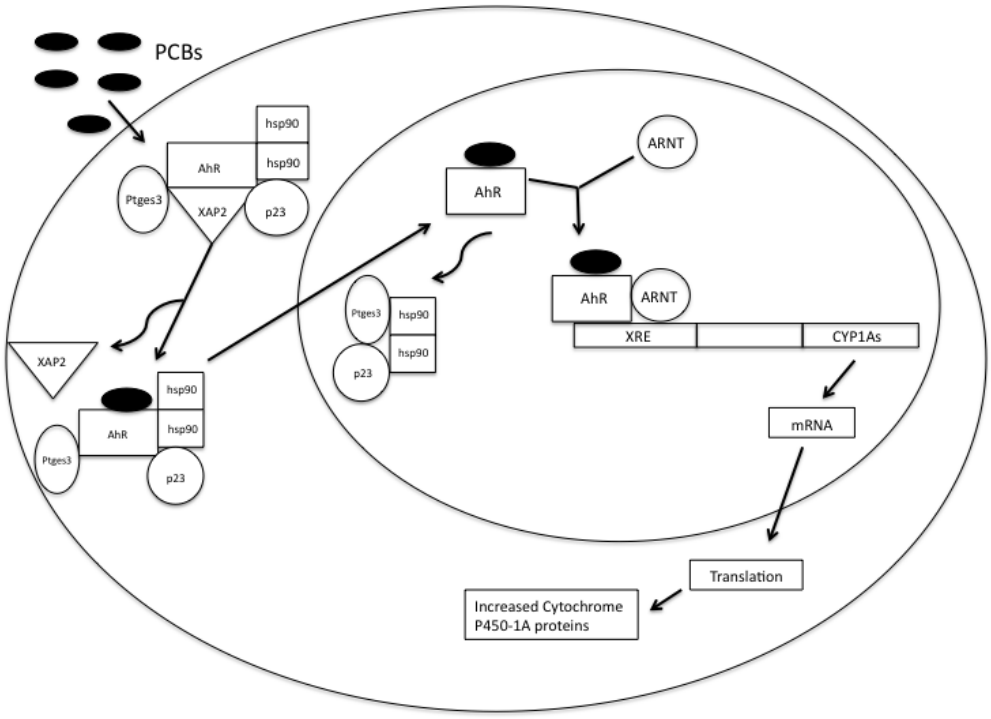
Coplanar (planar) PCBs act as ligand agonists on this cytoplasmic complex. The ligand binds to the PAS-B domain of the AhR, releasing XAP2 and exposes the bHLH region of the AhR protein, activating a nuclear localization signal and shuttling the complex into the nucleus, where the hsp90 dimer, co-activator p23, and Ptges3 dissociate (Hord and Perdew, 1994; Pollenz *et al*., 1994). The dissociation exposes the PAS-A binding domain, which is the binding site for the aryl hydrocarbon receptor nuclear translocator (ARNT) (Lindebro *et al*., 1995). Bound ARNT converts AhR into its active form, with a high affinity for a specific DNA recognition site, xenobiotic response elements (XRE) (Shen and Whitlock, 1992). The ligand bound AhR-ARNT complex binds to XRE, causing an activation cascade stimulating gene transcription downstream (Hankison, 1995; Schmidt *et al*., 1996; Denison *et al*., 1998). As such, the XRE genes serve a regulatory role in xenobiotic metabolism in response to AhR pathway activation (Shen and Whitlock, 1992; Whitlock, 1999; Denison *et al*., 1988).

AhR protein expression has been detected in multiple cell types, including neural ganglia, smooth muscle, cardiac muscle, skeletal muscle, and epithelial cells in the developing chick embryo, with mRNA expression occurring in all cells except

myocardium (Walker *et al*., 1997). Downstream targets of cytochrome P450 (CYP), subfamily 1A, genes are upregulated at varying rates in different tissues following activation by ligand agonists, with the induction of AhR-dependent avian CYP1A4 and CYP1A5 shown in a subset of these protein-rich cells. CYP1A4 was induced in the blood vessels, small intestine, liver, kidney, and outflow myocardium in response to dioxin (TCDD) exposure, a chemical that activates the AhR similar to PCBs (Walker and Catron, 2000; Head *et al*., 2008). CYP1A5 is also induced, but to a lesser degree than CYP1A4, in response to this potent AhR stimulant (Jones and Kennedy, 2009). In birds, the activation of the AhR pathway by dioxin-like PCBs induces CYP responses of both CYP1A4 and CYP1A5.

The importance of the CYP-1A gene family in response to AhR activation resides in the regulatory function of the resulting proteins. The CYP1A subfamily is responsible for detoxification and oxidative metabolism of organic or exogenous compounds. Unfortunately, the detoxification process may also result in the bioactivation of some PCBs, due to the strong affinity of the CYP1A subfamily to aromatic compounds (Goldstone and Segeman, 2006). The detoxification effectiveness of CYP1As that lead to pollutant bioactivation is a major factor contributing to the susceptibility of different organisms (Gooch *et al*., 1989; Arzuaga and Elskus, 2002).

Figure 1: Schematic of the activation of the Aryl Hydrocarbon Receptor pathway in the cell following PCB exposure.



Adapted from Denison and Nagy, 2003

Conservation of the AhR protein and its function across species has been well established. Comparisons of AhR protein sequences from chick embryos to mammalian, amphibian, and piscine species revealed that the bHLH domain of the AhR had a conservation identify of 87-100% amino acids (mammalian and amphibian) and 69-74% amino acids (piscine). The PAS region is 97% conserved between avian species, 81-86% conserved in amphibians and mammals, and 64-69% conserved in piscine species. However, the carboxyl terminus is less than 53% conserved between the domestic chick and these other species (Walker and Catron, 2000). Because the bHLH domain and the

PAS regions are highly conserved across species, these regions are thought to play a major role in AhR pathway activation (Jones and Kennedy, 2009; Hankinson, 1995; Whitcock, 1999; Schmidt and Bradfield, 1996).

The efficacy of a PCB to activate the AhR pathway has been linked to congener toxicity. Using this toxicological characteristic, the World Health Organization developed a toxic equivalency (TEQ) paradigm with tetrachlorodibenzo-p-dioxin (TCDD) serving as the standard for activation of the AhR pathway for both mammalian and avian species (van den Berg *et al*., 1998; 2005). The TEQ schematic was developed as a cumulative yardstick of biological toxicity of PCB coplanar (planar) congeners compared to TCDD activation in the AhR pathway. To summarize, each "dioxin-like" PCB congener is assigned a toxic equivalency factor (TEF), which represents a toxicity relative to TCDD. The TEQ for PCB mixtures is calculated by multiplying the TEF by the concentration of each congener and then summing all values (van den Berg *et al*., 1998; 2006). Due to the high variability within PCB mixtures and lots, the TEQ assessment becomes increasingly important to establish safe levels of PCB exposure (van den Berg *et al*., 1996; 1998; 2005). Moreover, it is critical to evaluate wildlife from individual sites of contamination to gauge the impact of mixtures with site-specific variations in partitioning, chemical transformations, and bioaccumulation (TAMS, 1991). While TEQs do not account for variability in responsiveness between species for specific congeners, the TEQ provides valuable information in the initial assessment of potential toxicity and risk (USEPA, 1996; van den Berg *et al*., 2006).

Adverse health effects of PCBs

PCBs have been linked to a variety of adverse health effects, with species-specific differences in sensitivity (Hoffman *et al*., 1996). PCBs are regarded as carcinogenic, based on occupational studies that showed increased mortality of exposed workers from liver, gall bladder, and skin cancers following exposures to Aroclors 1254, 1242, 1016 in the United States and around the world (Bertazzi *et al*., 1987; Brown, 1987; Silberhorn *et al*., 1990; Sinks *et al*., 1992; USEPA, 1996). Similarly, rodent studies confirmed that ingested PCB mixtures induced liver tumors (Kimbrough *et al*., 1975; Silberhorn *et al*., 1990; Mayes *et al*., 1998). In addition, maxillary and mandibular tumors occurred with two months of dietary exposure to a dioxin-like PCB congener in mink (Render *et al*., 2000). Interestingly, nonplanar PCB congeners are associated with increased risk of cancer, suggesting a mechanism of action unrelated to the AhR pathway. These PCB congeners are general cancer promoters that generate reactive oxygen species, with prolonged exposure leading to oxidatitve DNA damage (Tharappel *et al*., 2002). Additonally, PCB metabolites are thought to be responsible for chromosomal abnormalities seen in animal exposures (Carpenter, 2006). Animals with higher bioaccumulative PCB loads such as marine mammals with blubber have increased cancer rates similar to those found in exposed human populations (Guise *et al*., 1995; Ross *et al*., 2000; Martineau *et al*., 2002; Yilato *et al*., 2005).

PCBs also adversely affect the immune system of humans with exposed individuals at increased risk for infections, lower white blood cell and immunoglobulin levels, decreased antibody production rates, and increased incidences of respiratory diseases (Weisglas-Kuperus *et al*., 1995; 2000; 2004; Dewailly *et al*., 2000; Kudyakov *et*

al., 2004). These data are further supported by the finding of depressed immunoglobulin levels in non-human primates that were exposed to PCBs (Tryphonas *et al*., 1988). Fish, birds, and rodents have impaired immune system function following PCB exposure (Dunier 1994; Tryphonas, 1995). Moreover, immune responsiveness decreased in blubber marine mammals, inferring decreased fitness associated with secondary immune challenges (Lahvis *et al*., 1995; Ross *et al*., 1996). Similarly, fish exposed to a dioxin-like PCB congener showed depressed antibody production for up to two weeks following an acute exposure (Duffy *et al*., 2002). In addition, gulls and tree swallows have also shown decreased immune responsiveness with dietary PCB congener exposure (Smits *et al*., 2000; Grasman, 2002; Bustnes *et al*., 2004). PCB exposure altered immune system organ morphological effects, including reduced thymus gland, decreased germinal proliferative capacity, and altered immunological cells in the peripheral blood (Carpenter, 2006). Finally, immunologically impaired individuals are at greater risk for PCB induced immunological dysfunctions (Weisglas-Kuperus *et al*., 1995; 2002). The effects of PCBs on the immune system have been categorized, with estrogen-like PCB congeners being key compounds impacting dysfunction. Estrogen receptors located in lymphocytes and non-lymphoid cells are susceptible to estrogen-like PCB congeners. Research suggests that these contaminants can upregulate the expression of molecular messangers such as cytokines, impair developmental immune organs including the thymus and bone marrow, and alter patterns of T and B cell apoptosis (Ahmed, 2000), suggesting various mechanisms of action. In summary, data from human health, domestic species, and wildlife studies implicate PCBs as responsible for immune system impairment.

PCBs also impact nervous system function. *In utero* or dietary PCB exposure through breast milk resulted in reduced performance of children on intelligence quotient, diminished overall intelligence, and poorer performance on behavioral tests (Yu *et al*., 1996; Jacobson and Jacobson, 1996; Chen *et al*., 1992). Additionally, exposed children also had neurobehavioral deficits including abnormal reflex to stimuli, lower degree of muscle tone, and amplified responses to stressors (Lonky *et al*., 1996; Rogen *et al*., 1986). While neurological and behavioral effects are prevalent in children due to their developmental stages, adults exposed to PCBs also had decreased memory associated with increased concentrations of PCB loads (Schantz *et al*., 2001). These data are supported by non-human primate studies, in which exposure to Aroclors impaired spatial learning and memory in cognitive tests (Schantz *et al*., 1991). Other mammalian studies showed PCB treatment induced spontaneous behavioral patterns, decreased cognitive function in neonatal mice, and altered concentrations of neurotransmitters in the brain in mink kits (Fischer *et al*., 2008; Aulerich *et al*., 1985). Studies in birds have confirmed the presence of measureable PCB levels in the brain, making the central nervous system a potential target of PCB action (Naert *et al*., 2007). Delayed developmental milestones were also observed in the higher trophic level American kestrel following PCB exposure (Fernie *et al*., 2003). Neurological effects following PCB exposures have been associated with the perturbation of calcium homeostatis during development. In animals exposed to PCBs, decreased calcium transfer enzyme concentrations and reduced enzymatic function were identified. Many developmental processes are calcium dependent and perturbations in homeostasis can affect neurological growth as well as cause cellular injury (Tilson *et al*., 1998). As such, the evidence implicates PCBs in

impacts on neurological systems, especially with exposure during critical developmental stages.

PCBs are an established endocrine disrupting chemical (EDC), with adverse effects on endocrine function. In humans, dietary PCB exposure through maternal breast milk has been correlated with decreased levels of thyroid hormones T3 and T4 and increased levels of thyroid stimulating hormone and thyrotropin in infants (Koopman-Essenboom *et al*., 1994; Osius *et al*., 1999; Schell *et al*., 2002). Similarly, the hypothalamus-pituitary-thyroid (HPT) axis appears to be a target of PCBs, with decreased thyroid function, thyroid gland hypertrophy, and follicle cell hyperplasia in neonatal rats, harbor seals, and kestrel hatchlings (Crofton *et al*., 2000; Tabuchi *et al*., 2006; Hoffman *et al*., 1996). Increased turnover of thyroid horomones as a result of increased hormone excretion leads to decreased circulating levels of thyroid hormones. This causes the HPT axis to overcompensate, eventually leading to hypothyroidism in chronic cases. Exposures to PCBs have been shown to disrupt thyroid status through different mechanisms of action. PCBs can disrupt biotransformation enzymes of the liver such as transferaces, which are essential in proper thyroid function. PCB mixtures and select congeners have also been shown to interfere with thyroid hormone transport by plasma proteins and individual congeners can also interfere directly with thyroid gland function (McNabb and Fox, 2002). In short, multiple congeners can be linked to thyroid dysfunction through a myriad of mechanisms.

In addition to thyroid dysfunction, PCBs have been linked to functional abnormalities in the hypothalamus-pituitary-adrenal (HPA) axis in animals. Increased PCB loads in polar bears and fish have been linked to decreased cortisol levels in

peripheral blood as well as atrophy of the pituitary corticotropes (Oskam *et al*., 2004; Hontela *et al*., 1992). Similarly, American kestrels and tree swallows exposed to environmental mixtures had an inverted-U dose response in corticosterone and repressed adrenal responsiveness (Love *et al*., 2003; Franceschini *et al*., 2008). While the underlying mechanism of reduced cortisol responsiveness following PCB exposure is not clearly understood, there is evidence implicating a direct effect on the adrenal tissue (Franceschini *et al*., 2008). It is clear that PCBs can have significant implications for organisms relative to normal growth and development, as well as stress responses necessary for survival.

PCB exposures have been associated with reproductive abnormalities, likely associated with endocrine disruption. In humans, males exposed to PCB mixtures had decreased concentrations of free testosterone in peripheral blood, decreased sperm motility, and increased sperm abnormalities (Ritchthoff *et al*., 2003; Hsu *et al*., 2003). Laboratory studies in male rats confirmed these effects: exposure inhibited testosterone production, impacted Leydig cell proliferation, and showed competitive inhibition of the testosterone binding receptor (Kovacevic *et al*., 1995; Portigal *et al*., 2002). PCBs also affect the female reproductive system. In humans, PCB exposure was linked to early onset of the menstrual cycle in adolescents, increased duration of menstruation, and higher rates of endometriosis in adults (Denham *et al*., 2005; Cooper e*t al*., 2005; Pauwels *et al*., 2001). Similar effects of PCBs were observed in studies on the rhesus macaques (Rier *et al*., 2001). In addition, human infants born to PCB exposed mothers have decreased birth weights and shorter periods of gestation, compared to non-exposed mothers (Taylor *et al*., 1984; Baibergenova *et al*., 2003). Reproductive failure, impaired

reproduction, and reduced litter size was also correlated to PCB concentrations in marine mammals, mink, and rats (Addison, 1989; Heaton *et al*., 1995; Kimbrough, 1985). In birds, embryonic mortality, reduced reproductive fitness, and declines in brooding behavior followed Aroclor exposure (Peakall and Peakall, 1973; Custer and Heinz, 1980; Carro *et al*., unpublished). The underlying mechanism of action of PCBs on the reproductive system is not well understood. PCB congeners have been shown to be estrogenic or anti-androgenic, and the sensitivity of reproductive function varies across species. Further work is needed to elucidate the mechanistic cause for decreased reproductive fitness following PCB exposure (Schwacke *et al*., 2002). In summary, PCB exposure was associated with decreased overall reproductive fitness.

PCB exposure has also been linked to a variety of other effects including increased rates of diabetes in women, increased risk of asthma, elevated incidence of joint inflammation, and arthritis in humans (Longnecker *et al*., 2001; van den Heuvel *et al*., 2002; Kuratsune *et al*., 1980; Guo *et al*., 1990). In birds, exposure to a PCB mixture have inhibited proper heme production, reduced growth rates, altered oxidative responsiveness, and increased liver necrosis (Elliot *et al*., 1990; 1991; McKinney *et al*., 1976; Hoffman, 1995). PCBs have adverse health effects on multiple physiological systems across a range of organisms. PCBs are carcinogenic and adversely affect the immune, neurological, endocrine, and reproductive systems in humans and other species.

Specific effects of PCBs on the cardiovascular system

Exposure to PCBs has been linked to a variety of cardiovascular defects in developing embryos and adults. In humans, dietary and dermal PCB exposures in adults correlated with increased plasma triglyceride levels and total serum cholesterol, elevated

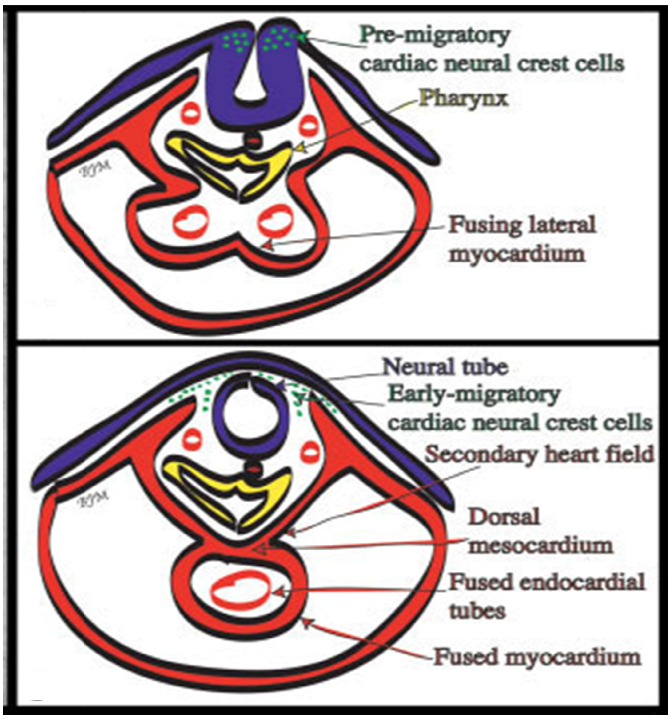
blood pressure, and increased hypertension (Baker *et al*., 1980; Morgan *et al*., 1980; Wilsgaard *et al*., 1981; Kreiss *et al*., 1981; Chase *et al*., 1982; Moysich *et al*., 2002). Adults residing in contaminated areas along the upper Hudson River had higher diagnostic rates for coronary heart disease and myocardial infarction at 35.8% and 39.1%, respectively compared to national averages (Sergeev and Carpenter, 2005). Similar findings of increased coronary heart disease were obtained in rat and non-human primate studies in which animals were exposed to PCB mixtures and dioxin-like congeners (Mochizuki *et al*., 1998; 2000; Bell *et al*., 1994; Lind *et al*., 2004). PCB exposure was correlated with increased heart weight in adult rats and mink (Lind *et al*., 2004; Aulerich and Ringer, 1977; Restum *et al*., 1998). In fish, dioxin-like PCB exposure was linked to decreased blood flow to the periphery, decreased heart size, depressed rates of contraction, and altered heart looping during embryonic exposure (Kopf and Walker, 2009). In birds, exposure to PCB mixtures or dioxin-like congeners resulted in increased heart weight in chick embryos and passerine nestlings (Powell *et al*., 1996; Walker *et al*., 1997; Walker and Catron, 2000; DeWitt *et al*., 2006). Passerine nestlings in areas contaminated by PCB mixtures had thinning of the ventricular wall, microsurface roughness, and overall heart deformities (DeWitt *et al*., 2006). Chicks exposed to dioxin-like PCB congeners also had abnormal chamber dilation, ventricular wall thinning, depressed heart rate stimuli responsiveness, and reduced rates of cardiomyocyte proliferation when examined at embryonic day 10 of development (Walker and Catron, 2000; Kopf and Walker, 2009). Our research suggests similar effects of PCB embryonic exposure on the developing heart in birds.

Stages of chick heart development vulnerable to PCB exposure

The cardiovascular system begins to function very early in embryonic development, with the heart being the first functional organ in vertebrates. At Hamburger Hamilton (HH) stage 5, the early progenitors of the heart field have moved through the primitive streak and are located in the anterior lateral plate mesoderm (Yang *et al*., 2002). Within the splanchic layer, these progenitors form a bilateral pair of cardiogenic field, or a two-dimensional plane of heart precursors (Rosenquist, 1970; Antin *et al*., 1994). By HH9 these bilateral, separate fields begin to indent, forming a trough-like structure before folding towards the ventral midline as the ventral foregut closes. These cardiogenic fields constitute the myocardium that forms the primordial cells of the trabeculated layer of the ventricles (de la Cruz *et al*., 1989).

These two newly three-dimensional heart fields fuse at the ventral midline along its cranial border, forming the outer curvature of the future heart tube (de la Cruz *et al*., 1989; Meilhac *et al*., 2003). Following cranial fusion, the myocardium begins to fuse along the dorsal midline, which forms the inner curvature of the future heart, and the simple heart tube by HH10 (Linask, 2003). This heart tube is made up of three distinct layers; myocardium along the outer surface, cardiac jelly secreted by the myocardium in the middle, and endocardium lining the inner surface (Linask, 2003). At HH10, the heart is oriented dorsal to the foregut and pulsations or initial heartbeats begin as sodium-calcium exchange pumps appear in the myocardial membrane (de la Cruz, 1977; Wakimoto *et al*., 2000; Linask *et al*., 2001). These pulsations eventually become a rhythmic heartbeat controlled by the sinus venosus, allowing muscle contractions to control blood flow before the completion of heart valve formation (Linask *et al*., 2001).

Figure 2: Representation of the formation of the heart tube at HH10 in the chick.

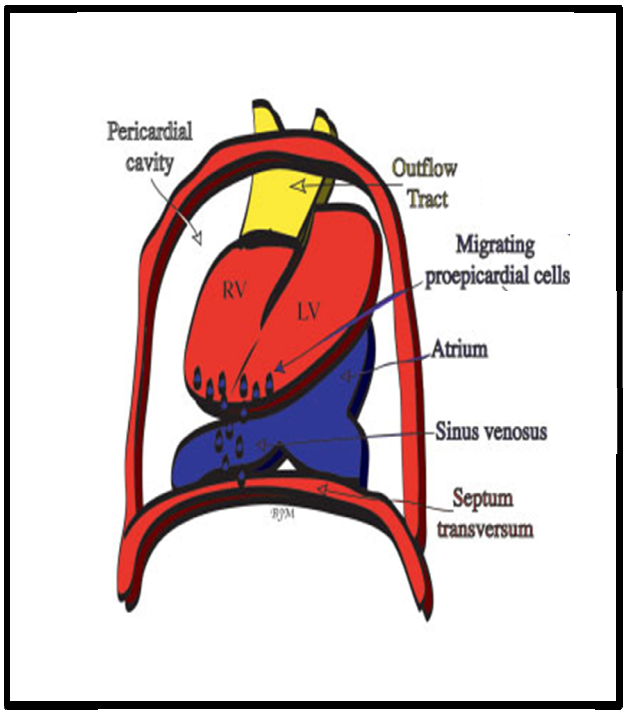


(From Martinsen, 2005)

The looping of the heart is the first visible sign of right-left asymmetry in the developing embryo. In the chick, the complete looping process occurs from embryonic day 2-8, with critical stages occurring between HH14 and HH16 (Manner, 2000). At this point, the anterior and posterior regions of the heart have different polarities, with the anterior region the beginning of the outflow tract and the posterior region the beginning of the inflow tract (van den Hoff *et al*., 2001; Waldo *et al*., 2001). Addition of cells to the myocardial regions of the outflow and inflow tracts elongates the simple heart tube. The looping process requires the heart tube to bend, rotate and twist around the foregut to converge into a C-shaped loop directed towards the right side of the body (DeHaan, 1965; Icardo, 1996). When the outflow and inflow regions of the heart converge, a S-shaped loop is formed with the outflow tract cranially oriented and the inflow tract

oriented caudally (Manner, 2000). During this portion of looping, the right ventricle and a portion of the left ventricle are present in the tube (de la Cruz *et al*., 1989). As myocardium is added to the inflow and outflow ends of the tube, the atria and outflow tract begins to get incorporated into the heart (Cai *et al*., 2003). By the end of looping, the developing heart contains all of the regions that will give rise to the adult four-chamber heart.

Figure 3: Representation of proper heart looping at HH16 in the developing chick. Developing heart forms an "S-shape" at this stage.



(From Martinsen, 2005)

Chamber development occurs from HH16 to HH29, with major events occurring at HH17, 21, and 25. While the heart tube is looping, the myocardium signals for detachment of the endocardial cells. These cells migrate to the cardiac jelly forming a endocardial cushion which divides, as the atrioventricular septum elongates, the looping tube into right and left atrioventricular channels (Potts *et al*., 1991; Larsen 1993). These

divided channels give rise to the mitral and tricuspid valves in the mature heart (Icardo, 1996). As this occurs the enlarged primitive atrium developing along the inflow tract is undergoing partitioning by septal formation (Larsen, 1993). Interestingly, atria are derived from left and right cardiogenic fields and retain this left-right axis specification in the adult heart (Manner, 2000). As the atrioventricular and primitive atrium undergo septation, ventricles are also septating. The part of the S-shaped cardiac loop that will join the developing inflow and outflow tracts, known as the bulboventricular fold, will ultimately divide the right and left ventricles. As the outer curvature of the cardiac loop grows and expands, it gives rise to the trabeculated region of the ventricular wall. The ventricular septum also grows from the bulboventricular fold, ultimately dividing the right and left ventricles (Cristofells *et al*., 2000; Sedmera *et al*., 2004). Finally, the conus eventually becomes the area connecting the right ventricle with the pulmonary trunk and the left ventricle with the aorta and the truncus becomes the semilunar valves in both ventricles, both undergo septation (Cai *et al*., 2003; Waldo *et al*., 2001; 2005). The aortic sac, a sinus in the outflow tract region of the developing heart, initially branches into the pulmonary trunk and aorta by a preliminary septation (de la Cruz *et al*., 1977; Thompson *et al*., 1987). Remodeling, further septation, and apoptosis of this region then connect the right ventricle to the pulmonary artery and the left ventricle to the aorta (Cai *et al*., 2003; Schaefer *et al*., 2004). In summary, septation of the primordial atria, atrioventricular canal, ventricles, and conotruncus gives rise the four chambered, valved, vascularized heart.

Another important component to heart development is the formation of the trabecular and compact zones or layers of the heart wall. After looping and as early as

HH16, trabeculations can be seen in the ventricles (Challice and Viragh, 1973; Icardo and Fernandez-Teran, 1987). These trabeculations orient themselves as sheets, with protrusions that increase surface area (Van Mierop and Kutsche, 1984). The increased surface area enables the myocardium to increase in size in the absence of complete circulation. As the heart undergoes septation, trabeculations in the left ventricle become thicker than the right ventricle and intertrabecular spaces increase in size (Rychter and Rychterova, 1981). Trabeculations enhance contractility and coordinate intraventricular conduction in the heart (Challice and Viragh, 1973; Hogers *et al*., 1995). The trabeculated layer initially starts developing before septation in birds. The compact layer or outer myocardial layer of the heart wall is not developed until long after the presence of the trabeculated layer. At very early stages of embryonic development, the compact myocardial layer is only one or two cell layers thick and much of the myocardial mass at these stages can be attributed to the trabeculated layer (Rychterova, 1971; Blausen *et al*., 1990). The early embryonic compact layer is the source for proliferating cells, although differentiation rates are lower than those found in the trabeculated layer (Jeter and Cameron, 1971; Challice and Viragh, 1973). Compaction of the myocardial wall, which occurs concurrently with cardiac vascularization, is the driving force behind increases in wall thickness (Rytchterova, 1971). During this process, proliferation of cardiomyocytes in the compact layer contributes significantly to heart wall thickness compared to the trabeculated layer (Blausen *et al*., 1990). Compaction occurs at greater rate in the left ventricle, contributing to the substantial growth of the left versus right ventricular wall, with growth and compaction still occurring after embryonic development (Hirokawa, 1972). In birds, thickness of the compact layer in the left ventricle is approximately five

times greater than the right ventricle in adults, but only three times as thick in hatchlings (King and MacLelland, 1986). Notably, adult mammals have a left to right ventricular wall thickness ratio of three to one (Komarek *et al*., 1982). The difference in ratio suggests an adaptation for flight in birds (King and MacLelland, 1986).

Congenital cardiovascular malformations

Congenital cardiovascular malformations (CCVMs) are one of the most common forms of birth defects in human infants, occurring in approximately 1.0 % of live infant births (Hoffman, 1995). Prenatal death associated with congenital heart disease is greater and estimated to account for approximately 7.3% of all fetal deaths (Hoffman, 1995). Of the live infant births with identified CCVMs, 15% will die in the first year of life. Four percent of the surviving cases of CCVMs will die before 16 years of age (Knowles *et al*., 2005). Additionally, 30% of postnatal infant deaths attributed to other birth defects also have CCVM, as these malformations are genetically linked to other high mortality birth defects (Jenkins *et al*., 2007).

The severity of CCVMs, their diagnosis and treatments, and the incidence in the human populations varies greatly. Many postnatal CCVMs are associated with improper septation during development. Atrial septal defects (ASDs) are one of the most common malformations of the heart. They occur in 50-100 per 100,000 live births and are usually isolated malformations, meaning they usually do not occur with other birth defects (Hoffman, 1987). Notably, fetal environment impacts the prevalence of ASD, for example neonates born with fetal-alcohol syndrome have increased incidences of ASD (Hoffman, 1987). Another septal malformation is Complete Atrioventricular Septal Defect (CAVSD), a major malformation of the lower part of the atrial septum, upper

portion of the ventricular septum, and atrioventricular valves. The incidence of CAVSD is 30 per 100,000 live births and is generally linked to other genetic defects including Down Syndrome (Wilson *et al*., 1993). Ventricular septal defect (VSD) is one of the most common CCVMs in live births, occurring in an estimated 300 per 100,000 cases. Only an estimated 60 per 100,000 live births require surgical correction. In many instances, infants with VSD can live normal lifestyles without being diagnosed with this CCVM (Hoffman, 1987). Teratology of Fallot (TOF) is a septal defect with aortal displacement, usually existing with right ventricular obstruction and decreased outflow (Hoffman, 1987). This defect is severe and life threatening, usually occurring as an isolated malformation in 30 per 100,000 live births. Finally, Hypoplastic Left Heart Syndrome (HLHS) occurs in 20 per 100,000 live births, and is a combination of underdeveloped left ventricle, underdeveloped valve formation and aortic arch and aorta. This leads to poor circulation to the periphery and lungs (Wren *et al*., 2008).

In addition to malformations during septation of the various regions of the heart, improper vascular formation is another cause of CCVMs. Total anomalous pulmonary venus connection (TAPVC) occurs if the pulmonary veins do not properly connect to the left atrium, instead connecting to the systemic venous system. This malformation is severe and infants with TAPVC are very ill. TAPVC usually occurs with an additional ASD (Bleyl *et al*., 1995; Hancock-Friesen *et al*., 2005). Pulmonary atresia is defined by the absence of a proper connection between the heart and lungs, usually results from malformations of the septum, valves and vessels, and occurs in 20 per 100,000 live births (Leonard *et al*., 2000). Pulmonary atresia is a severe malformation, with infant mortality rates greater than 50% within the first year of life (Leonard *et al*., 2000). Transposition

of the Great Arteries (TGA) is a severe CCVM if untreated but can successfully be corrected in newborns. TGA is identified by incorrect orientation of the pulmonary artery and aorta, with the pulmonary artery attaching from the left ventricle and the aorta attaching from the right ventricle. TGA occurs in 30 per 100,000 births (Wren *et al.*, 2003). Finally, truncus arteriosis (TA) occurs in 10 per 100,000 live births and is one of the most major malformations. Characterized by failed septation of the ventricular outflow tract, TA affects the pulmonary arteries and the aortic arch (Hoffman, 1987).

Cardiomyopathies involving the ventricular wall also inhibit proper cardiovascular function in infants. The heart wall thickness forms from myocardium. As the compact layer forms, compaction of the trabeculated layer is essential. Noncompaction, a myocardial disorder in which the trabeculated layer does not get compacted due to a failure of differentiation by the myocardium, is a rare development that is categorized by hypertrabeculation of the ventricular wall, and deep intertrabecular recesses. This disorder prevents proper proliferation of the ventricular wall and can affect one or both ventricles (Chin *et al*., 1990; Oechslin *et al*., 2000; Oda *et al*. 2005). Proliferation is essential to normal ventricular wall development, and decreased rates of proliferating cardiomyocytes during development can be linked to defects in ventricular wall thickness in association with environmental exposures to contaminants (Henshel *et al*., 1993).

In summary, embryonic exposure to environmental contaminants during critical developmental stages increases the risk of a child born with a CCVM. The incidence of CCVMs has increased in the last decade, suggesting a link between environmental exposures and adverse heart effects (Pierpont *et al*., 2007). Exposures to environmental contaminants such as pesticides and solvents during embryogenesis account for 2.8-4.7

fold increases in the TGA and 3 fold increases in HLHS based on risk assessment modeling (Ferencz *et al*. 1997). The estimated incidence of CCVM attributed to environmental contaminants is approximately 30% (Wilson *et al*., 1998). Embryos exposed to dioxins, specifically TCDD, have shown increases in heart weight, ventricular cavity size, and myosin protein during development (Kopf and Walker, 2009). These hearts exhibited cardiac stress and cellular hypertrophy (Walker and Catron, 2000). Dioxins work mechanistically through the AhR, similar to dioxin-like PCBs, although the AhR mediated process is not the exclusive target pathway for PCB exposures (Carpenter, 2006). As such, there is an indication for human health and wildlife that embryonic exposure to PCBs can be linked to increases in CCVMs and cardiomyopathies seen in developing embryonic hearts.

Summary and purpose of this research

PCB mixtures are still environmentally persistent today, with some congeners more biologically toxic based on their chemical structure and mechanism of action through the aryl hydrocarbon receptor. PCBs are resistant to degradation and bioaccumulative, with higher trophic animals at greater risk for biological effects. PCBs have been linked to health effects including carcinogenicity, and adversely impacting the nervous, endocrine, reproductive, and immune systems. Additionally, PCBs have been thought to impact the cardiovascular system, although PCB effects on avian embryonic cardiac development to unique PCB mixtures are not well categorized. One major site of PCB contamination is along the UHR in New York, where PCB mixtures have been intermittently released into the ecosystem since the 1920's. The goal of this dissertation is to assess the effects of

PCB mixtures and dioxin-like congeners that are environmentally relevant at the UHR on the avian embryo, with an emphasis on impact to cardiac development.

References

Addison RF. 1989. Organochlorines and marine mammal reproduction. Can J of Fish and Aquati Sci 46:360-368

Ahmed SA. 2000. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology 150:191-206.

Andric SA, Kostic TS, Dragisic SM, Andric N, Stojikovic SS, Kovacevic RZ. 2000. Acute effects of polychlorinated biphenyl containing and free transformer fluids on rat testicular steroidogenesis. Environ Health Perspect 108:955-959.

Antin PB, Taylor RG, Yatskievych T. 1994. Precardiac mesoderm is specified during gastrulation in quail. Dev Dynam 200:144-154.

Arzuaga X, Elskus A. 2002. Evidence for resistance to benzo[a]pyrene and 3,4,3',4'-tetrachlorobiphenyl in chronically polluted Fundulus heteroclitus population. Mar Environ Res 54:247-251.

Aulerich RJ, Ringer RK. 1977. Current status of PCB toxicity to mink and effect on their reproduction. Environ Contam and Toxicol 6:279-292.

Aulerich RJ, Bursian SJ, Breslin WJ, Olson BA, Ringer RK. 1985. Toxicological manifestations of 2,4,5,2',4',5', 2,3,6,2',3',6', and 3,4,5,3',4',5'-hexachlorobiphenyl and Aroclor 1254 in mink. J Toxicol and Environ Health 15:63-79.

Baibergenova A, Kudyakov R, Zdeb M, Carpenter DO. 2003. Low birth weight and residential proximity to PCB contaminated waste sites. Environ Health Perspect 111:1352-1357.

Baker EL, Landrigan PJ, Glueck CJ, Zack MM, Liddle JA, Burse VW. 1980. Metabolic consequences of exposure to polychlorinated biphenyls (PCB) in sewage sludge. Am J Epidemiol 112: 553-563.

Bell FP, Iverson F, Arnold D, Vidmar TJ. 1994. Long- term effects of Aroclor 1254 (PCBs) on plasma lipid and carnitine concentrations in rhesus monkey. Toxicology 89: 139–153.

Bertazzi PA, Riboldi L, Pesatori A, Radice L, Zoochetti C. 1987. Cancer mortality of capacitor manufacturing workers. Am J Ind Med 11:165-176.

Blausen BE, Johannes RS, Hutchins GM. 1990. Computer-based reconstructions of the cardiac ventricles of human embryos. Am J Cardiovasc Pathol 3:37–43.

Blectchy JD. 1983 Polychlorinated biphenyls: production, current use, and possible rates of future disposal in OECD member countries. Proceedings, PCB Seminar, Netherlands Ministry of the Environment. Hague, Netherlands, 343-365.

Bleyl S, Nelson L, Odelberg SJ. 1995. A gene for familial total anomalous pulmonary venous return maps to chromosome 4p13-q12. Amer J Hum Genet 56:408-415.

Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, Bergman A, Visser TJ. 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. Toxicol and Ind Health 14:59-84.

Brown JF, Wagner RE, Bedard DL, Brennan MJ, Carnahan JC, May RJ, Tofflemire TJ. 1984. PCB transformations in upper Hudson River sediments. Northeastern Environ Sci 3:167-179.

Brown JE, Bedard DL, Brennan MJ, Carnahan JC, Feng H, Wagner RE. 1987. Olychlorinated biphenyl dechlorination in aquatic sediments. Science. 236:709-712.
Brown DP. 1987. Mortality of workers exposed to polychlorinated biphenyls- an update. Environ Health 42:333-339.

Bustnes JO, Hanssen SA, Folstad I, Erikstad KE, Hasselquist D, Skaare JU. 2004: Immune Function and Organochlorine pollutants in Arctic breeding Glaucous Gulls. Environ Contam and Toxicol 47:530-541.

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. 2003. Isl1 identified a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 5:877-889.

Carey JH. 1994. Transformation processes of contaminants in rivers. Hydrological, chemical, and biological processes of transformation and transport of contaminants in aquatic environments. International Association of Hydrological Sciences 219:41-50.

Carpenter DO. 2006. Polychlorinated biphenyls (PCBs): Routes of exposure and effects on human health. Rev Environ Health 21:1-22.

Challice CE, Viragh S. 1973. The architectural development of the early mammalian heart. Tissue Cell 6:447–462.

Chase KH, Wong O, Thomas D, Berney BW, Simon RK. 1982. Clinical and metabolic abnormalities associated with occupational exposure to poly- chlorinated biphenyls. J Occup Med 24: 109–114.

Chen YCJ, Guo YL, Hsu CC, Rogan WJ. 1992. Cognitive development of Yu-Cheng (Oil Disease) children prenatally exposed to heat-degraded PCBs. JAMA 268:3213-3218.

Chin TK, Perloff JK, Williams RG, Jue K, Mohrmann. 1990. Isolated noncompaction of left ventricular myocardium. A study of eight cases. Circulation 82:507-513.

Christofells VM, Gabets PE, Franco D, Campione M, de Jong F, Lamers WH, Bao ZZ, Palmer S, Biben C, Harvey RP, Moorman AF. 2000. Chamber formation and morphogenesis in the developing mammalian heart. Dev Biol 223:266-278.

Clearwater. 2011. Phase I and II monitoring report and revised updates. Clearwater, Beacon, NY.

Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). 1980. EPA 540-R-98-024. EPA, Washington, DC.

Cooper GS, Klebanoff MA, Promislow J, Brock JW, Longnecker MP. 2005. Polychlorinated biphenyls and menstrual cycle characteristics. Epidemiol 16:191-200.

Crofton KM, Kodavanti PRS, Derr-Yellin EC, Casey AC, Kehn LS. 2000. PCBs, thyroid hormones and toxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. Toxicol Sci 57:131-140.

Custer TW, Heinz GH. 1980. Reproductive success and nest attentiveness of mallard ducks fed Aroclor 1254. Environ Pollut 21: 313-318.

Custer TW, Gray BR, Custer CM. 2010a. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicol 19:391-404.

Custer TW, Custer CM, and Gray BR. 2010b. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in belted kingfisher eggs from Upper Hudson River Basin, New York, USA. Environ Toxicol Chem 29:99-110.

Custer CM, Gray R, Custer TW. 2010c. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallow (Tachycineta bicolor). Environ Toxicol Chem 29:909-921.

Custer CM, Custer TW, Dummer PM. 2010d. Patterns of organic contaminants in eggs of an insectivorous, an omnivorous, and a piscivorous bird nesting on the Upper Hudson River, New York, USA. Environ Toxicol Chem 29: (epublished).

de la Cruz MV, Gomez CS, Arteaga MM, Arguello C. 1977. Experimental study of the development of the truncus and the conus in the chick embryo. J Anatomy 123:661-686.

de la Cruz MV, Gomez CS, Palomino. 1989. The primitive cardiac regions in the straight tube heart (stage 9) and their anatomical expression in the mature heart: an experimental study in the chick embryo. J Anat 165:121-131.

DeHaan RL. 1965. Morphogenesis of the vertebrate heart. Organogenesis. Eds: DeHaan RL and Ursprung H. Holt, Rinehart, and Winston. New York, New York.

Denham M, Schell LM, Dean G, Gallo MV, Ravenscroft J, DeCaprio AP. 2005. Relationship of lead, mercury, mirex, dichlorodiphenyldichloroethylene, hexachlorobenzene, and polychlorinated biphenyls to timing of menarche among Akwesasne Mohawk girls. Pediatrics 115:127-134.

Denison MS, Fisher JM, Whitlock JP. 1988. The DNA recognition site for the dioxin-Ah receptor complex: nucleotide sequence and functional analysis. J Biol Chem 263:17721-17724.

Denison MS, Elferink CF, Phelan D. 1998. The Ah receptor signal transduction pathway. Toxicant-Receptor Interactions in the Modulation of Signal Tranduction and Gene expression. MS Denison, WG Helferich. Taylor and Francis, PA, USA, pp 3-33.

Denison MS, Nagy SR. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Ann Rev Pharm Tox 43:309-334.

Dewailly E, Ayotte P, Bruneau S, Gingras S, Belles-Isles M, Roy R. 2000. Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. Environ Health Perspect 108:205-211.

DeWitt JC, Millsap DS, Yeager RL, Heise SS, Sparks DW, Henshel DS. 2006. External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol and Chem 25: 541–551.

Dhont M, Brouwer A. 2001. The risk of endometriosis and exposure to dioxin and polychlorinated biphenyls: a case control study of infertile women. Human Repro 16:2050-2055.

Duffy JE, Carlson E, Li Y, Prophete C, Zelikoff JT. 2002. Impact of polychlorinated biphenyls (PCBs) on the immune function of fish: age as a variable in determining adverse outcome. Marine Environ Res 54:559-563.

Dunier M. 1994. Modulators of fish immune response models for environmental toxicology-biomarkers immunostimulators. Stolen JS, Fletcher TC, eds. Modulators of fish immune responses Fair Haven NY, USA, pp 123-139.

Durfee RL, Contos G, Witmore FC, Barden JD, Hackman EE, Westin RA. 1976. PCBs in the United States – Industrial Use and Environmental Distribution Report. EPA 56016-16-005. Versar Inc., Springfield, VA.

Echols KR, Meadows, JC, Tillitt DE, Secord A. 1996. Congener-specific analysis of polychlorinated biphenyl residues in tree swallow chicks, eggs, and other biota from the Hudson River, W.U. 30096. USGS Final Laboratory Report FY97-30-01.

Elliott JE, Kennedy SW, Peakall DB, Won H. 1990. Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds. I. Japanese quail. Comp Biochem Physiol 96:205-210.

Elliot SE, Kennedy SW, Jeffry D, Shutt L. 1991. Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds. II. American Kestrel. Comp Biochem Physiol 99:141-145.

Emmett EA, Maroni M, Jefferys J, Schmith J, Levin BK, Alvares A. 1988. Studies of transformer repair workers exposed to PCBs: II. Results of clinical laboratory investigations. Amer J of Ind Med 14:47-62.

Ferencz C, Loffredo CA, Correa-Villasenor A, Wilson PD. 1997. Genetic and environmental risk factors of major cardiovascular malformations. Futura Publishing Company, Armonk, NY, USA.

Fernie K, Bortolotti G, Drouillard K, Smits J, Marchant T. 2003. Developmental toxicity of in ovo exposure to polychlorinated biphenyls. II. Effects of maternal or paternal exposure on second-generation nestling American kestrel. Environ Toxicol and Chem 22:2688-2694.

Fischer C, Fredriksson A, Eriksson P. 2008. Neonatal co-exposure to low doses of ortho PCB 153 and methyl mercury exacerbate defective developmental neurobehavior in mice. J Toxicol and Enviorn Health 244:157-165.

Frame GM, Cochran JW, Bowad SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, qualitative, congener-specific analysis. J High Res Chromat 19:657-668.

Frame GM. 1997. Congener specific PCB analysis. Analyt Chem 69:468-478.

Franceschini MD, Custer CM, Custer TW, Reed JM, Romero LM. 2008. Corticosterone stress response in tree swallows nesting near polychlorinated biphenyl and dioxin-contaminated rivers. Environ Toxicol and Chem 27:2326-2331.

Fukunaga BN, Probst MR, Reisz-Porszasz S, Hankinson O. 1995. Identification of functional domains of aryl hydrocarbon receptor. J Bio Chem 270:29270-29278.

Goldstone HM, Stegeman JJ. 2006. A Revised evolutionary history of the CYP1A Subfamily: Gene duplication, gene conversion, and positive selection. J of Molec Evol 62:708-717.

Gooch JW, Elskus AA, Kloepper-Sams PJ, Hahn ME,Stegeman JJ. 1989. Effects of ortho and non ortho substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (Stenotomus chrysops). Toxicol Appl Pharm 98:422-433.

Grasman KA. 2002. Assessing immunological function in toxicological studies of avian wildlife. Integ Comp Biol 42:34-42.

Guo YL, Yu ML, Hsu CC, Rogan WJ. 1999. Chloracne, goiter, arthritis, and anemia after polychlorinated biphenyl poisoning: 14-year follow-up of the Taiwan Yucheng cohort. Environ Health Perspect 107:715–719.

Hankinson O. 1995. The aryl hydrocarbon receptor complex. Ann Rev Pharm Toxicol 35:307-340.

Hancock-Friesen CL, Zurakowski D, Thiagarajan RR. 2005. Total anomalous pulmonary venous connection: an analysis of current management strategies in a single institution. Ann Thorac Surg 79:596-606.

Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environ Sci Technol 42:7535-7541.

Heaton SN, Bursian SJ, Giesy JP, Tillitt DE, Render JA, Jones PD, Verbrugge DA, Kubiak TJ, and Aulerich RJ. 1995. Dietary exposure of mink to carp from Saginaw Bay, Michigan. Effects on reproduction and survival, and the potential risks to wild mink populations. Environ Contam and Toxicol 28:334-343.

Henshel DS, Hehn B, Vo MT, Steeves JD. 1993. A short-term test for dioxin teratogenicity using chicken embryos, Environmental Toxicology and Risk Assessment, Vol 2. STP 1216. American Society for Testing and Materials, Philadelphia, PA, USA pp 159–174.

Hirokawa K. 1972. A quantitative study on pre and postnatal growth of human heart. Acta Path Jpn 22:613–624.

Hoffman JIE. 1995. Incidence of congenital heart disease: II. Prenatal incidence. Pediat Cardiol 16:155-165.

Hoffman DJ, Melancon MJ, Klein PN, Rice CP, Eisemann JD, Hines RK, Spann JW, Pendleton GW. 1996. Developmental toxicity of PCB 126(3, 3', 4, 4', 5-pentachlorobiphenyl in nestling American kestrels (Falco sparverius). Fundam Appl Toxicol 34:188-200.

Hogers B, de Ruiter MC, Baasten AMJ, Gittenberger-de Groot AC, Poelmann RE. 1995. Intracardiac flow patterns related to the yolk sac circulation of the chick embryo. Circ Res 76:871–877.

Hontela A, Rasmussen JB, Audet C, Chevalier G. 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Environ Contam and Toxicol 22:278-283.

Hord NG, Perdew GH. 1994. Physiochemical and immunochemical analysis of aryl hydrocarbon receptor nuclear translocator characterization of two monoclonal antibodies of the aryl hydrocarbon receptor nuclear translocator. Molec Pharm 46:18-24.

Hsu PC, Huang W, Yao WJ, Guo YL, Lambert GH. 2003. Sperm changes in men exposed to polychlorinated biphenyls and dibenzofurans. JAMA 289:2943-2944.

Icardo JM, Fernandez-Teran A. 1987. Morphologic study of ventricular trabeculation in the embryonic chick heart. Act Anat 130:264 –274.

Icardo JM. 1996. Developmental biology of the vertebrate heart. J Exp Zool 275:144-161.

Jacobson JL, Jacobson SW. 1996. Intellectual impairment in children exposed to polychlorinated biphenyls in utero. N Engl J Med 335:783-789.

Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, Elixson M, Warnes CA, Webb CL. 2007. Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American heart association council on cardiovascular disease in young: endorsed by the academy of pediatrics. Circulation 115:2995-3014.

Jeter JR, Cameron II. 1971. Cell proliferation patterns during cyto-differentiation in embryonic chick tissues: liver, heart and erythrocytes. J Embryol Exp Morphol 25:405–422.

Jones SP, Kennedy SW. 2009. Chicken embryo cardiomyocyte cultures: a new approach for studying effects of halogenated aromatic hydrocarbons in the avian heart. Toxicol Sci 109:66-74.

Kazlauskas A, Poellinger L, Pongratsz I. 1999. Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon receptor). J Biol Chem 274:13519-13524.

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol Appl Pharm 141:214-230.

Kimbrough RD, Squire RA, Linder RE, Strandberg JD, Montali RJ, Burse VW. 1975. Induction of liver tumors in Sherman strain female rats by polychlorinated biphenyl Aroclor 1260. J Nat Cancer Inst 55:1453-1459.

Kimbrough RD. 1985. Laboratory and human studies on polychlorinated biphenyls (PCBs) and related compounds. Environ Health Perspect 59:99-106.

King AS, McLelland J. 1986. Birds: their structure and function. Baillere Tindal, London, UK.

Knowles R, Griebsch I, Dezateuz C, Brown J, Bull C, Wren C. 2005. Newborn screening for congenital heart defects: a systematic review and cost-effectiveness analysis. Health Tech Assess 9:1-152.

Komarek V, Malinovsky L, Lemez L, eds. 1982. Anatomia avium domesticarum et embryologia galli. Bratislava: Piroda.

Koopman-Essenboom C, Morse DC, Weisglas-Kuperus N, Lutkeschipholt IJ, Van der Paauw CG, Tuinstra LG. 1994. Effects of dioxins and polychlorinated biphenyls on thyroid hormone status of pregnant women and their infants. Pediat Res 36:468-473.

Kopf PG, Walker MK. 2009. Overview of developmental heart defects by dioxins, PCBs, and pesticides. J Environ Sci Health 27(4): 276-285.

Kovacevic R, Vojinovic-Miloradov M, Teodorovic I, Andric S. 1995. Effect of PCBs on androgen production by suspension of adult rat Leydig cells in vitro. J Steroid Biochem Molec Biol 52:595-597.

Kreiss K, Zack MM, Kimbrough RD, Needham LL, Smrek AL, Jones BT. 1981. Association of blood pressure and polychlorinated biphenyl levels. JAMA 245: 25050–2509.

Kudyakov R, Baibergenova A, Zdeb M, Carpenter DO. 2004. Respiratory disease in relation to patient residence near to hazardous waste sites. Pediatrics 18:249-257.

Kuratsune M. Yusho. In: Kimbrough RD. 1980. Halogenated Biphenyls, Terphenyls, Napththalenes, Dibenzodioxins and Related Products. Amsterdam, Elsevier North Holland. 287–292.

Lahvis GP, Wells RS, Kuehl DW, Stewart JL, Rhinehart HL, Via CS. 1995. Decreasing lymphocyte responses in free ranging bottlenose dolphins (Tursiops truncates) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ Health Perspect 103:67-72.

Larsen WJ. 1993. Human embryology. Churchill-Livingstone, New York, NY, USA.

Leonard H, Derrick G, O'Sullivan J, Wrenc C. 2000. Natural and unnatural history of pulmonary atresia. Heart 84:499-503.

Linask KK, Han MD, Artman M, Ludwig CA. 2001. Sodium-calcium exchanger (NCX-1) and calcium modulation: NCX protein expression patterns and regulation of early heart development. Dev Dynam 221:249-264.

Linask KK. 2003. Regulation of heart morphology: current molecular and cellular perspectives on coordinated emergence of cardiac form and function. Birth Defects Res 69:14-24.

Lind PM, Orberg J, Edlund UB, Sjoblom L, Lind L. 2004. The dioxin like pollutant PCB 126 (3,3',4,4',5-pentachlorobiphenyl) affects risk factors for cardiovascular disease in female rats. Toxicol lett 150:293-299.

Lindebro MC, Poellinger L, Whitelaw ML. 1995. Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. J Emb 14:3528-3539.

Longnecker MP, Klebanoff MA, Brock JW, Zhou H. 2001. Polychlorinated biphenyl serum levels in pregnant subjects with diabetes. Diabetes Care 24:1099–1101.

Lonky E, Reihman J, Darvill T, Mather J, Daly H. 1996. Neonatal behavioral assessment scale performance in humans influenced by maternal consumption of environmentally contaminated Lake Ontario fish. J Great Lakes Res 22:198-212.

Love OP, Shutt LJ, Silfies JS, Bortolotti GR, Smits JEG, Bird DM. 2003. Effects of dietary PCB exposure on adrenocortical function in captive American kestrels (Falco sparverius). Ecotoxicology 12:199-208.

Manner J. 2000. Cardiac looping in chick embryo: a morphological review with specific reference to terminological and biomechanical aspects of the looping process. Anat Rec 259:248-262.

Martineau D, Lemberger K, Dallaire A, Labelle P, Lipscomb TP, Michel P, Mikaelian I. 2002. Cancer in wildlife, a case study: beluga from the St. Laurence estuary, Quebec, Canada. Environ Health Perspect 110:285-292.

Martinsen BJ. 2005. Reference Guide to Stages of Chick Heart Embryology. Dev Dyn 233:1217-1237.

Mayes BA, McConnel EE, Neal BH, Brunner MJ, Hamilton SB, Sullivan TM. 1998. Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclors 1016, 1242, 1254, 1260. Toxicol Sci 41:62-76.

McKinney JD, Chae K, Gupta BN, Moore JA, Goldstein JA. 1976. Toxicological assessment of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. Toxicol Appl Pharm 36:65-80.

McNabb A, Fox G. 2003. Avian thyroid development in chemically contained environments: Is there evidence of alterations in thyroid function and development? Evol and Dev 5:76-82.

Meilhac SM, Kelly RG, Rocancourt D, Eloy-Trinquet S, Nicolas JF, Buckingham ME. 2003. A retrospective clonal analysis of myocardium reveals two phases of clonal growth in the developing mouse heart. Development 130:3877-3889.

Meyer BK, Pray-Grant MG, Heuvel JP, Perdew GH. 1998. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Molec Cell Biol 18: 978-988.

Mochizuki H, Oda H, Yokogoshi H. 1998. Amplified effect of taurine on PCB-induced hypercholesterolemia in rats. Adv Exp Med Biol 1998. 442: 285– 290.

Mochizuki H, Oda H, Yokogoshi H. 2000. Dietary taurine alters ascorbic acid metabolism in rats fed diets containing polychlorinated biphenyls. J Nutr 130: 873–876.

Morgan DP, Lin LI, Saikaly HH. 1980. Morbidity and mortality in workers ccupationally exposed to pesticides. Environ Contam Toxicol 9:349–382.

Moysich KB, Ambrosone CB, Mendola P, Kostyniak PJ, Greizerstein HB, Vena JE. 2002. Exposures associated with serum organochlorine levels among postmenopausal women from western New York State. Am J Ind Med 41: 102–110.

Naert C, Van Peteghem C, Kupper J, Jenni L, Naegeli H. 2007. Distribution of polychlorinated biphenyls and polybrominated diphenyl ethers in birds of prey from Switzerland. Chemosphere 68:977-987.

Norstrom RJ. 1988. Patterns and trends of PCB contaminations in Canadian wildlife. Crine JP, ed. Hazards, decontamination and replacement of PCB. Plenum, New York, NY, USA pp 85-100.

Oda YS, Tsunoda S, Doue T, Iida Y, Matsubara H. 2005. Clinical characteristics and morphological features of isolated noncompaction of the ventricular myocardium: electrocardiogram-gated magnetic resonance imaging can elucidate more accurate biventricular structures. Heart Vessels 20:126-127.

Oechslin EN, Attenhofer-Jost CH, Rojas JR, Kaufmann PA, Jenni R. 2000. Long-term follow-up of 34 adults with isolated left ventricular noncompaction: a distinct cardiomyopathy with poor prognosis. J Am Coll Cardiol 36:493-500.

Osius N, Karmaus W, Kruse H, Witten J. 1999. Exposure to polychlorinated biphenyls and levels of thyroid hormones in children. Environ Health Perspect 107:843-849.

Rogan WJ, Galden BC, McKinney JD, Carreras N, Hardy P, Thullen J. 1986. Neonatal effects of transplacental exposure to PCBs and DDE. J Pediat 109:335-341.

Oskam I, Ropstad E, Lie E, Derocher A, Wilg O, Dahl E, Larsen S, Skaare JU. 2004. Organochlorines affect the steroid hormone cortisol in free-ranging polar bears (Ursus maritimus) at Svalbard, Norway. J Toxicol and Environ Health 67:959-977.

Peakall DB, Peakall ML. 1973. Effects of polychlorinated biphenyl on reproduction of artificially and naturally incubated dove eggs. J Appl Ecol 10:863-868.

Pierpont ME, Basson CT, Benson DW, Gelb BD, Giglia TM, Goldmuntz E, McGee G, Sable CA, Srivastava D, Webb CL. 2007. Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American heart association congenital cardiac defects committee, council on cardiovascular disease in the young: endorsed by the American academy of pediatrics. Circulation 115:3015-3038.

Pollenz RS, Sattler CA, Poland A. 1994. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharm 45:428-438.

Portigal CL, Cowello SP, Feboruk MN, Butler CM, Rennie PS, Nelson CC. 2002. Polychlorinated biphenyls interfere with androgen induced transcriptional activation and hormone binding. Toxicol Appl Pharmacol 179:185-194.

Potts JD, Dagle JM, Walder JA, Weeks DL, Runyon RB. 1991. Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor B3. Proc Natl Acad Sci 88:1516-1520.

Powell DC, Aulerich RJ, Meadows JC, Tillitt DE, Giesy JP, Stromborg KL, Bursian SL. 1996. Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) injected into the yolks of chicken (Gallus domesticus) eggs prior to incubation. Environ Contam and Toxicol 31:404-409.

Reijnders PJH. 1986. Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature 324:456.

Render JA, Aulerich RJ, Bursian SJ, Nachreiner RF. 2000. Proliferation of maxillary and mandibular periodontal squamous cells in mink fed 3,3',4,4' 5-pentachlorobiphenyl (PCB126). J Vet Diagn Inves 12:477-479.

Restum, JC, Giesy JP, Render JA, Shipp EB, Verbrugge DA, Aulerich RJ. 1998. Multigenerational study of the effects of consumption of PCB-contaminated carpt from Saginaw Bay, Lake Huron, on Mink. 1. Effects on mink reproduction, kit growth and survival, and selected biological parameters. J Toxicol and Environ Health 54:345-375.

Rier SE, Turner WE, Martin DC, Morris R, Lucier GW, Clark GC. 2001. Serum levels of TCDD and dioxin like chemicals in rhesus monkeys chronically exposed to dioxin: correlation of increased serum PCB levels with endometriosis. Toxicol Sci 59:147-159.

Ritchthoff J, Rylander L, Jonsson BA, Akesson H, Hagmar L, Nilsson-Ehle P. 2003. Serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CP-153) in relation to markers of reproductive function in young males from the general Swedish population Environ Health Perspect 111:409-413.

Rosenquist GC. 1970. Location and movements of cardiogenic cells in the chick embryo: the heart-forming portion of the primitive streak. Dev Biol 22:461-475.

Ross P, DeSwart R, Addison R, Loveren HV, Vos J, Osterhaus A. 1996. Contaminant-induced immunotoxicity in harbor seals: Wildlife at risk? Toxicol 112:157-169.

Ross PS, Ellis GM, Ikonomou MG, Barett-Lennard LG, Addison RF. 2000. High PCB concentrations in free-ranging Pacific killer whales, orcinus orca: effects of age, sex, and dietary preference. Marine Poll Bull 40:504-515.

Rushneck DR, Beliveau A, Fowler B, Hamilton C, Hoover D, Kaye K, Berge M, Smith T, Telliard WA, Roman H, Ruder E, Ryan L. 2004. Concentrations of dioxin-like PCBs in unweather Aroclors by HRGC/HRMS using EPA method 1668A. Chemosphere 54:79-87.

Rychter Z, Rychterova V. 1981. Angio- and myoarchitecture of the heart wall under normal and experimentally changed conditions. In: Pexieder T, ed. Perspectives in cardiovascular research, Vol. 5, mechanisms of cardiac morphogenesis and teratogenesis. Raven, New York, New York, USA pp 431–452.

Rychterova V. 1971. Principle of growth in thickness of the heart ventricular wall in the chick embryo. Folia Morphol Praha 19:262– 272.

Schaefer KS, Doughman YQ, Fisher SA, Watanabe M. 2004. Dynamic patterns of apoptosis in the developing chicken heart. Dev Dyn 229:489-499.

Schantz, SL, Levin ED, Bowman RE. 1991. Long-term neurobehavioral effects of perinatal polychlorinated biphenyl (PCB) exposure in monkeys. Environ Toxicol and Chem 10:747-756.

Schantz SL, Gasior DM, Polverejan E, McCaffrey RJ, Sweeny AM, Humphrey HE. 2001. Impairments of memory and learning in older adults exposed to polychlorinated biphenyls via consumption of Great Lakes fish. Environ Health Perspect 109:605-611.

Schell LM, DeCaprio A, Gallo M, Hubicki L. 2002. Polychlorinated biphenyls and thyroid function in adolescents of the Mohawk Nation at Akwesansne. In Gill G, Schell LM, Benson L, eds. Human Growth from Conception to Maturity, Chapter 30. Smith-Gordon, London, UK pp 289-296.

Schmidt JV, Bradfield CA. 1996. Ah receptor signaling pathways. Ann Rev Cell Dev Biol 12:55-89.

Schuur AG, Brouwer A, Bergman A, Coughtrie MW, Visser TJ. 1998. Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polychlorinated biphenyls. Chem Biol Inter 109:293-297.

Schwacke LH, Voit EO, Hansen LJ, Wells RS, Mitchum GB, Hohn AA, Fair PA. 2002. Probabilistic risk assessment of reproductive effects of polychlorinated biphenyls on bottlenose dolphins (Tursiops truncatus) from the southest United States coast. Environ Toxicol and Chem 21:2752-2764.

Sedmera DM, Reckova M, Bigelow MR, Dealmeida A, Stanley CP, Mikawa T, Gourdie RG, Thompson RP. 2004. Developmental transitions in electrical activation patterns in chick embryonic heart. Anat Rec 280: 1001-1009.

Sergeev A V , Carpenter DO. 2005. Hospitalization rates for coronary heart disease in relation to residence near areas contaminated with persistent organic pollutants and other pollutants. Environ Health Perspect 113:756-761.

Shen ES, Whitlock JP. 1992. Protein-DNA interactions at a dioxin-responsive enhancer. Mutational analysis of the DNA-binding site for the liganded Ah receptor. J Bio Chem 267:6815-6819.

Silberhorn EM, Glauert HP, Robertson LW. 1990. Carcinogenicity of Polyhalogenated biphenyls: PCBs and PBBs. Critical Reviews in Toxicol 20:440-496.

Sinks T, Steele G, Smith AB, Watkins K, Shults RA. 1992. Mortality among workers exposed to polychlorinated biphenyls. Am J Epidem 136:389-398.

Smits JE, Wayland ME, Miller MJ, Liber K, Trudeau S. 2000. Reproductive, immune, and physiological end points in tree swallows on reclaimed oil sands mine sites. Environ Toxicol and Chem 19:2951-2960.

Tabuchi M, Veldhoen N, Dangerfield N, Jeffries S, Helbing CC, Ross PS. 2006. PCB-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging harbor seals (Phoca vitulina). Environ Health Perspect 114:10924-1031.

Taylor PR, Lawrence CE, Hwang HL, Laulson AS. 1984. Polychlorinated biphenyls: influence on birth weight and gestation. Amer J Public Health 74: 1153-1154.

TAMS/Gradient. 1991. Phase 1 Report, Review Copy, Interim Characterization and Evaluation, Hudson River PCB Reassessment RI/FS. USEPA Work Assignment No. 013-2N84. Prepared for USEPA by TAMS Consultants, Inc. and Gradient Corporation.

Tharappel JC, Lee EY, Robertson LW, Spear BT, Glauert HP. 2002. Regulation of cell proliferation, apoptosis, and transcription factor activities during prootion of liver carcinogenesis by polychlorinated biphenyls. Toxicol Appl Pharmacol 179:172-184.

Thompson RP, Abercrombie V, Wong M. 1987. Morphogenesis of the truncus arteriosus of the chick embryo heart: movements of autographic tattoos during septation. Anat Rec 218:434-440.

Tilson HA, Kodavanti PS, Mundy WR, Bushnell PJ. 1998. Neurotoxicity of environmental chemicals and their mechanism of action. Toxicol Lett 102:631-635.

Toxic Substances Control Act. (TSCA).1976. Public Law 94-469.

Tryphonas H. 1995. Great Lakes health effects: immunotoxicity of PCBs (Aroclors). Environ Health Perspect 103:33-46.

U.S. Environmental Protection Agency. 1992a. Estimating exposure to dioxin-like compounds. EPA 600-6-88-005B. Office of Research and Development, Washington, DC.

U.S. Environmetnal Protection Agency. 1992b. Framework for ecological risk assessment. EPA 630-R-92-001. Risk assessment forum, Washington, DC.

U.S. EPA. 1996. PCBs: Cancer Dose-Response Assessment and Application to Environmental Mixtures. EPA 600-P-96-001F. Office of Research and Development, National Center for Environmental Assessment, Washington, DC.

U.S. Environmental Protective Agency. 2002. Hudson River PCBs Site New York: Record of Decision (USEPA ROD). Washington, DC.

Van den Berg M, Birbaum LS, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson PRE, Poellinger L, Safe S, Schrenk D, Tillitt DE, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg M, Birnbaum LS, Denison M, DeVito, M, Farland W, Feeley W, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. 2005. The 2005 world health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93:223-241.

Van den Heuvel RL, Koppen G, Staessen JA, Hond ED, Verheyen G, Nawrot TS. 2002. Immunologic biomarkers in relation to exposure markers of PCBs and dioxins in Flemish adolescents (Belgium). Environ Health Perspect 110:595-600.

Van den Heuvel RL, Koppen G, Staessen JA, Hond ED, Verheyen G, Nawrot TS. 2002. Immunologic biomarkers in relation to exposure markers of PCBs and dioxins in Flemishadolescents (Belgium). Environ Health Perspect 110:595–600.

Van den Hoff MJB, Kruithof BP, Moorman AF, Markwald RR, Wessels A. 2001. Formation of myocardium after the initial development of the linear heart tube. Dev Biol 240:61-76.

Van Mierop LHS, Kutsche LM. 1984. Comparative anatomy and embryology of the ventricles and arterial pole of the vertebrate heart. In: Nora JJ, Takao A, eds. Congenital heart disease: causes and processes. Futura, New York, NY, USA pp 459–479.

Wakimoto K, Kobayashi K, Kuroo M, Yao A, Iwamoto T, Yanaka N, Kita S, Nishida A, Azuma S, Toyoda Y, Omori K, Imahie H, Oka T, Kudoh S, Kohmoto O, Yazaki Y, Shigekawa M, Imai Y, Nabeshima Y, Komuro I. 2000. Targeted disruption of Na+/Ca2+ exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. J Biol Chem 275:36991-36998.

Waldo KL, Kumiski DH, Wallis KT, Stadt HA, Hutson MR, Platt DH, Kirby ML. 2001. Conotruncal myocardium arises from secondary heart field. Development 128:3179-3188.

Waldo KL, Hutson MR, Ward CC, Zdanowicz M, Stadt HA, Kumiski D, Abu-Issa R, Kirby ML. 2005. Secondary heart field contributes myocardium and smooth muscle to the arterial pole of the developing heart. Dev Biol 281:78-90.

Walker MK, Pollenz RS, Smith SM. 1997. Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with tetrachlorodibenzo-p-dioxin induced heart defects. Toxicol and Appl Pharmacol 107:1075-1083.

Walker MK, Catron TF. 2000. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early embryo development. Toxicol and Appl Pharmacol 167:210-221.

Weisglas-Kuperus N, Sas TC, Koopman EBC, van der Zwan CW, DeRidder MA, Beisbuizen A. 1995. Immunologic effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. Pediat Res 38:404-410.

Weisglas-Kuperus N, Patandin S, Berbers GA, Sas TC, Mulder PG, Sauer PJ. 2000. Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. Environ Health Perspect 108:1203-1207.

Weisglas-Kuperus N, Vreugdenhil HJ, Mulder PG. 2004. Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. Toxicol Lett 149:281-285.

Whitlock JP. 1999. Induction of cytochrome P450A1. Ann Rev Pharmacol Toxicol 39:103-125.

Wilsgaard T, Jacobsen BK, Schirmer H, Thune I, Lochen ML, Njolstad I. 1981. Tracking of cardiovascular risk factors. Am J Epidemiol 154: 418–426.

Wilson L, Curtis A, Korenberg JR, Schipper RD, Allan L, Chenevix-Trench G, Stephenson A, Goodship J, Burn J. 1993. A large, dominant pedigree of atrioventricular septal defect (AVSD): exclusion from the down syndrome critical region on chromosome 21. Amer J Hum Gen 53:1262-1268.

Wilson PD, Loffredo CA, Correa-Villasenor A, Ferencz C. 1998. Attributable fraction for cardiac malformations. Am J Epidemiol 148:414-423.

Wren C, Birrell G, Hawthorne G. 1993. Cardiovascular malformations in infants of diabetic mothers. Heart 89:1217-1220.

Wren C, Reinhardt Z, Khawaja K. 2008. Twenty year tends in diagnosis of life threatening neonatal cardiovascular malformations. Arch Dis Child Fetal Neonat Ed 93:F33-F35.

Yang QH, Khoury MJ, Mannino D. 1997. Trends and patterns of mortality associated with birth defects and genetic diseases in the United States, 1972-1992: an analysis of multiple-cause mortality data. Genet Epidem 14:493-505.

Yang X, Dormann D, Munstergberg A, Weijer CJ. 2002. Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8. Dev Cell 3:425-437.

Yitalo GM, Stein JE, Hom T, Johnson LL, Tilbury KL, Hall AJ, Rowles, T, Greig D, Lowenstine LJ, Gulland FMD. 2005. The role of organochlorines in cancer-associated mortality in California sea lions (Zalophus californianus). Marine Poll Bull 50:30-39.

Yu ML, Hsu CC, Guo YL, Lai TJ, Chen SJ, Luo JM. 1994. Disordered behavior in early-born Taiwan Yucheng children. Chemosphere 29:2413-2422.

CHAPTER 2

THE EFFECTS OF *IN OVO* EXPOSURE TO 3, 3', 4, 4'-TETRACHLOROBIPHENYL (PCB 77) ON THE DEVELOPMENT OF HEART MORPHOLOGY IN TREE SWALLOW (*TACHYCINETA BICOLOR*) HATCHLINGS

Abstract

Polychlorinated biphenyls (PCBs) are environmentally ubiquitous, synthetic compounds that are resilient to degradation, lipophilic, and bioaccumulative in the environment. The purpose of this study was to determine embryonic effect of PCB 77, administered at two doses on wild tree swallows and with respect to impacts on the developing cardiac system. Tree swallow (*Tachycineta bicolor*) eggs from two uncontaminated sites, Patuxent Research Refuge (PRR), MD and Cobleskill Reservoir, NY were dosed with a single dioxin-like PCB congener, PCB 77. To ensure embryonic viability, treatments were administered into the air cell at embryonic day (ED) 2.5 of development and treated as follows: untreated, vehicle, 100 and 1,000 ng/g egg wt. Eggs were dosed, returned to the nest for incubation, and then collected at ED13, hatched, and necropsied. PCB 77 treated hatchlings were compared to environmentally exposed hatchlings collected from a PCB contaminated site along the upper Hudson River (UHR). Results showed no effects of PCB 77 on hatching success or hatchling mortality, heart index (heart wt/body wt %), or morphological measures of four distinct heart layers (heart width, length, septal thickness, total area, ventricular cavity area) compared to controls. However, hatchlings dosed with 1,000 ng/g PCB 77 had increased incidence of a specific cardiomyopathy, the absence of the compact layer of the ventricular heart wall (Chi sq; $p<0.001$). The compact layer is essential for proper heart growth and overall heart function because of its role in ventricular cardiomyocyte proliferation and normal heart contraction. The finding that *in ovo* exposure to PCB 77 resulted in a distinct cardiomyopathy (absence of the compact layer) in tree swallow hatchlings has implications for the fitness of affected individuals. Furthermore, these

data point to the importance of considering impacts of non-lethal PCB effects on lifetime fitness for exposed individuals.

Introduction

Polychlorinated biphenyls (PCBs) are synthetic compounds composed of a biphenyl molecule with one to ten chlorine substitutions, with 209 known congeners. PCBs were used extensively in manufacturing from their introduction in 1929 until their ban in the late 1970s. PCBs were released from the General Electric plants at Fort Edwards and Hudson Falls during this time as mixtures or Aroclors, synthesized according to percent chlorine by weight. Each lot mixture utilized different concentrations of congeners, altering toxicity effects between mixtures and lots, making source sites easily traceable (Brown *et al*., 1987; Frame *et al*., 1996; Frame, 1997; Rushneck *et al*., 2004). Avian species are variable in their sensitivity to PCB congeners, but it has been well established that coplanar "dioxin-like" congeners, such as PCB 126, 81, and 77, induce toxicity responses at higher rates than non-dioxin-like congeners (McKinney and Waller, 1994; Kennedy *et al*., 1996; Elliot *et al*., 1997; Head *et al*., 2008; Hervé *et al*., 2010). Recent contaminant analysis showed concentrations of PCB 77, 81, and 126 account for approximately 86%, 9%, and 3% respectively, of the total PCB-TEQ in tree swallows eggs collected along the Upper Hudson River (UHR) (Custer *et al*., 2010c). These data, along with contaminant analysis performed in the 1990s, suggest that PCB 77 is a primary dioxin-like congener that causes adverse health effects in exposed birds along the UHR (Echols *et al*., 1996; Secord *et al*., 1999).

Environmental exposure to PCBs has been linked to cancer (Safe, 1994; Silberhorn *et al*., 1990), immune system dysfunction (Thomas and Hindsdill, 1978;

McNabb, 2005; Lavoie and Grasman, 2007), decreased reproductive fitness (Barsotti *et al*., 1976; Aulerich and Ringer, 1977; Golub *et al*., 1991), endocrine disruption (van de Berg, 1990; Gould *et al*., 1999), and neurological and cognitive dysfunction (Jacobson *et al*., 1990). Additionally, the effects of environmental exposure to PCBs was linked to improper cardiovascular development in wild birds (DeWitt *et al*., 2006). Previous research also demonstrated that exposure to dioxins and PCB 126 resulted in increased ventricular dilation, thinned ventricular wall, elongated apical heart region, increased overall heart weight, and decreased chronotrophic responsiveness (Walker *et al.*, 1997; Walker and Catron, 2000; Kopf and Walker, 2009).

The effects of environmental exposure to PCBs on tree swallow nestlings and embryos have been well documented at the UHR site and other locations across the country (Bishop *et al*., 1995; Echols *et al*., 2004; Custer *et al*., 2002; 2003; 2010c, d). Moreover, contaminant analysis of bird eggs and nestlings collected from the UHR showed that PCBs were detectable, including elevated levels of PCB 77 (Secord *et al*., 1999; Custer 2010 a, b, c, d). PCB 77 has dioxin-like characteristics and a relatively high toxicity equivalency factor (TEF) of 0.05 in birds, which suggests that PCB 77 may be the principle congener linked to adverse health effects (Kennedy *et al*., 1996; Karchner *et al*., 2006).

Tree swallow eggs were collected from two uncontaminated sites, PRR and Cobleskill and one contaminated site on UHR. Eggs from the UHR were not dosed and regarded as "environmentally exposed" embryos. Eggs collected from PRR and Cobleskill were dosed with one of two concentrations of PCB 77 and necropsied at hatch. The purpose of this experiment was to determine if PCB 77 increased mortality in tree

swallows eggs and to assess any other impacts of this congener, including heart defects following *in ovo* embryonic exposure.

Materials and Methods

Egg injection

All fieldwork and experimental procedures were conducted under an approved Institutional Animal Care and Use Committee protocol and with appropriate permits from the Fish and Wildlife Service. Tree swallow (*Tachycineta bicolor*) eggs were collected from two reference field sites; PRR and Cobleskill, and one contaminated field site; UHR. The UHR eggs were identified as "environmentally exposed" embryos and were not dosed with PCB 77 treatments. Viable eggs were collected from the Upper Hudson River at ED13 and transported to the University of Maryland for in incubation and hatch. PCB 77 was solubilized in a filter sterilized fatty acid mixture (10% palmitic acid, 30% oleic acid, 60% linoleic acid) at two concentrations: 100 and 1,000 ng/g egg wt (1 and 10 µg/µl, respectively). Nests were checked daily and egg laying was monitored. Eggs were candled and at approximately 18% embryonic development or ED 2.5, individual eggs were randomly assigned the following treatments: untreated, 0, 100, and 1,000 ng/g egg wt. Treatments were administered in the field. A small hole was drilled (Dremel, WI) into the blunt side of the egg and a total injection volume of 0.2 µl per egg (based on egg weight) was deposited into the air cell using a reverse displacement pipettor (Rainin Instrument LLC, Oakland, CA). Eggs were returned to nests for incubation and at ED13, eggs from all treatment groups were collected, transported to the University of Maryland, and placed in hatching trays until hatch at 37°C and 60% humidity.

Heart collection

Hatchlings were euthanized by cervical dislocation within 12 hrs of hatch; the heart was dissected while beating and placed in ice-cold 25 mM KCl until beating ceased (about 30 sec). The heart was then rinsed thoroughly in 1x PBS solution, blotted dry, weighed, and immersed in ice-cold 10% neutral-buffered formalin for storage at 4°C for 48 hr. Following fixation, hearts were removed from fixative and immersed in 1x PBS solution for 30 minutes (2x) at 4°C and transferred to a 5% sucrose solution overnight at 4°C. The heart was dehydrated by a stepwise immersion into ethanol at the following increments: 50% EtOH, 70% EtOH, 80% EtOH, 95% EtOH, 100% EtOH (twice) for 60 minutes each. The heart was immersed in 1:1 Hemo-De:EtOH mixture for 30 minutes at room temperature, transferred to 100% Hemo-De for 15 minutes at 58°C, followed by 1:1 Hemo-De:paraffin for 60 minutes at 58°C and then immersed in 100% paraffin for 30 minutes at 58°C (repeated 3 times). Following the last immersion, the heart was transferred to 100% paraffin filled mold, oriented uniformly, and allowed to solidify for 24 hours.

Heart sectioning and staining

Hearts were sectioned transversely, from apex to cranial region at 10 µm, and wet mounted onto glass slides. Once dried, sections were taken through a standard hematoxylin and eosin staining procedure. Briefly, slides were dewaxed in Hemo-De for 3 min (3X) and rehydrated using reverse stepwise increments of EtOH: 100% EtOH, 95% EtOH, 70% EtOH, and 1x PBS for 3 min each; nuclear staining using Gill's hematoxylin (< 5 sec) and cytoplasmic staining using 0.5% eosin solution (6 min).

Tissue was dehydrated again for 30 sec at 70% EtOH and 100% EtOH, immersed in Hemo-De for 3 min, coverslipped, and dried for 24 hr before storage.

Analysis of heart indices and morphological measurements

Heart weight and indices including heart weight/ body weight percent were recorded and calculated for each individual. Detailed morphological analysis was conducted on a subset of samples to examine more specific morphology of the heart. A total of 25 tree swallow hearts were analyzed, representing controls, 100, and 1,000 ng/g treatment groups. This subset of hearts analyzed was selected randomly and all subsequent imaging and measurements were conducted blind to experiment. Hearts were checked for septal defects and any additional gross anatomical defects.

Images of sections were captured using the 4x Achroplan Zeiss objective, Zeiss microscope (Model 451888,Carl Zeiss, Inc., New York), and Photometrics Coolsnap *fx* camera (Photometrics, Tuscan, AZ). Four layers were evaluated as defined by morphometric structures as follows. Layer 1- most caudal section, identified by the left ventricular (LV) wall and cavity (~95% of the section), and the very beginning of the right ventricular opening; Layer 2- contains both left and right ventricles (RV), with both clearly prevalent, with no evidence of papillary muscles or valves; Layer 3- identified by the upper portion of both ventricles with emerging aorta present in the center of the section, left ventricle papillary muscles emerging, and evidence of the right ventricle papillary muscle; Layer 4- identified by the emergence of the pulmonary artery without the emergence of the aortic valve, and was the most cranial section analyzed. Images were captured using IP Lab 3.6 software (Biovision Technologies, Inc.) and analyzed using ImageJ software (NIH resources, Bethesda, MD). Treated groups were compared

to controls with all measures taken in millimeters (mm). Measurements taken for Layer 1 included width, depth, LV compact layer, LV ventral compact layer, total area, and LV cavity area. Layer 2 measurements were width, depth, LV dorsal compact layer, LV ventral compact layer, and total area. Layer 3 measurements were width, depth, LV dorsal compact layer, RV ventral compact layer, RV ventral compact layer, septal thickness, and total area. Layer 4 measurements included width, depth, and total area.

Compact layer analysis

Because the measurements taken indicated that PCB 77 exposure impacted the development of the compact layer (CL) in hatchlings, additional hearts were sectioned and analyzed at Layers 1 and 2. These hearts, as well as hearts used for morphological measurements were scored to determine whether the CL was present or absent in Layers 1 and 2, along the dorsal and ventral walls of the sectioned samples. Differentiation between the CL and the trabeculated heart layer were clearly identified and visualized using the 4x objective. Sample sizes varied due to hatch success rates as follows: untreated (n=15), vehicle (n=11), 100 (n=14), 1,000 ng/g (n=15), and environmentally exposed (n=8). Histology and imaging were completed as described previously and performed blind.

Statistical analysis

Individual measurements were used to test for treatment effects by one-way ANOVA at significance criteria of $p<0.05$ for each heart layer. CL analysis was conducted on the differences across treatments for either the presence or absence of the CL, using the chi square statistic. All statistics were analyzed using JMP 8 software (SAS, Cary, NC).

Results

Heart Weights & Indices

Tree swallow hatchling hearts from the three collection sites showed no difference in wet weight (Table 1) or heart index (heart weight/body weight %; based on wet weight; Table 1). Two samples from the vehicle group at PRR were eliminated due to improper collection, which made it impossible to section and conduct histological analaysis. PCB 77 treatment did not affect embryo hatch success rate or survivability to hatch (data not shown). PCB 77 did not affect morphological measurement analysis of any layer (Table 2). CL deformities significantly increased with PCB 77 exposure (Table 3).

Table 1: Heart weights and heart indices for PCB 77 treated eggs from uncontaminated sites: Patuxent Research Refuge (PRR), MD and Cobleskill Reservoir, NY and a contaminated site, Upper Hudson River (UPR), NY exposed to environmental PCBs.

Treatment	PRR	Cobleskill	UHR
Heart weight (mg)			
100 ng/g egg	12.65 ± 0.57 (15)	11.25 ± 0.77 (12)	
1000 ng/g egg	10.66 ± 0.47 (17)	11.08 ± 0.64 (17)	
Untreated	12.19 ± 0.74 (12)	12.45 ± 0.67 (17)	
Vehicle	13.88 ± 1.66 (12)	12.15 ± 1.12 (12)	
Environmentally exposed			11.95 ± 0.42 (33)
Heart index (heart weight/body weight %)			
100 ng/g egg	1.05 ± 0.04 (15)	0.98 ± 0.03 (12)	
1000 ng/g egg	0.91 ± 0.04 (17)	0.93 ± 0.05	
Untreated	0.99 ± 0.05 (12)	1.02 ± 0.05	
Vehicle	1.24 ± 0.14 (12)	1.04 ± 0.10 (12)	
Environmentally exposed			0.96 ± 0.03 (33)

Mean ± SE (n) of heart weights (mg) and indices (heart weight/body weight %) for 100ng/g egg, 1000ng/g egg, untreated, and vehicle PCB 77 treated tree swallow hatchlings from PRR and Cobleskill. Eggs from UHR are environmentally exposed to PCB levels and were not dosed with PCB 77. Heart indices were compared between uncontaminated, injected eggs, and UHR (environmentally exposed) eggs.

Heart morphological measurements

Exposure to 100 and 1,000 ng/g egg wt concentrations of PCB 77 did not affect heart width, depth, length of dorsal and ventral compact layers (when present), total area, septal thickness, or ventricular cavity area when compared to controls or environmentally exposed hearts (Table 2). Heart morphological structures, with the exception of ventricular compact layer (Figure 1; Layers 1, 2, 3) did not differ between PCB 77-treated and control animals. There was a significant ($p<0.05$) absence of the CL in Layers 1, 2, and 3 in the high dose treatment (1,000 ng/g) compared to the control group (Table 3). Similarly, there was a significant absence ($p=0.03$) in Layer 1 in the 1,000 ng/g PCB 77 treated hatchlings of the dorsal and ventral CL. Layers 2 and 3 ventral right ventricular CL were significantly absent ($p=0.01$ and $p=0.04$, respectively, Figure 1) in the 1,000 ng/g PCB 77 treated hatchlings. There was no difference in the thickness of the CL from other PCB-treated groups when the compact layer was intact (Table 2) and the trabeculated ventricular layers was present in all of the animals sampled. No significant difference was observed between control samples and environmentally exposed hearts from the UHR. Atrial formation was not affected by PCB 77 treatments (data not shown).

Additional hearts analyzed from PRR and Cobleskill at Layers 1 and 2 for CL analysis showed an increase in compact layer deformities with PCB 77 exposure. Analysis determined that PCB 77-treated embryos at both 100 and 1,000 ng/g dose concentrations had CL deformities in layers 1 and 2 (Chi sq $p<0.001$; Table 3). There was no difference in these layers for control hearts and environmentally exposed hearts from UHR.

Table 2: Average spatial measurements of morphological heart structures at Layers 1, 2, 3, and 4 in tree swallow hatchlings dosed with PCB 77 during early embryonic development, control groups, and environmentally exposed (UHR embryos).

Layer 1

Treatment	Width (mm)		Depth (mm)		D LV CL (mm)		V LV CL (mm)		Total Area (mm^2)		LV area (mm^2)	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
PRR untreated	1.68	1.56-1.76	1.20	1.03-1.72	0.14	0.11-0.18	0.15	0.11-0.23	2.2	1.55-2.76	0.31	0.25-0.42
PRR 100 ng/g	1.59	1.57-1.61	1.15	1.01-1.30	0.11	0.11-0.12	0.13	0.12-0.13	2.27	2.13-2.4	0.30	0.20-0.39
PRR 1,000 ng/g	1.67	1.57-1.79	1.32	1.07-1.49	0.16	0.0*-0.2	0.16	0.0*-0.19	2.02	1.5-2.54	0.31	0.16-0.45
UHR e.e.	1.68	1.48-1.90	1.25	0.92-1.54	0.13	0.09-0.20	0.17	0.09-0.25	1.7	1.24-2.33	0.34	0.13-0.48

Layer 2

Treatment	Width (mm)		Depth (mm)		D LV CL (mm)		V RV CL (mm)		Total Area (mm^2)		LV area (mm^2)		Septum (mm)	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
PRR untreated	1.68	1.20-2.17	1.65	1.20-1.88	0.14	0.0-0.19	0.11	0.0-0.17	1.73	1.60-1.88	0.17	0.14-0.25	0.16	0.13-0.18
PRR 100 ng/g	1.83	1.81-1.85	1.31	1.12-1.5	0.10	0.09-0.11	0.12	0.10-0.13	2.15	1.79-2.52	0.27	0.25-0.30	0.13	0.10-0.21
PRR 1,000 ng/g	1.61	1.22-2.14	1.57	1.17-1.81	0.14	0.0-0.2	0.09	0.0*-0.12	2.29	1.85-2.71	0.26	0.13-0.41	0.14	0.10-0.18
UHR e.e.	1.91	1.72-2.11	1.43	1.27-1.61	0.15	0.11-0.17	0.14	0.11-0.17	1.73	1.64-2.50	0.17	0.13-0.35	0.16	0.10-0.21

Layer 3

Treatment	Width (mm)		Depth (mm)		D LV CL (mm)		V RV CL (mm)		Total Area (mm^2)		Septum (mm)	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
PRR untreated	2.25	1.86-2.70	1.73	1.20-1.91	0.16	0.13-0.18	0.45	0.12-1.84	2.33	1.67-2.70	0.17	0.11-0.21
PRR 100 ng/g	2.22	1.9-2.60	1.73	1.59-1.82	0.14	0.13-0.16	0.13	0.10-0.14	2.42	2.17-2.57	0.13	0.11-0.15
PRR 1,000 ng/g	2.37	1.92-2.66	1.78	0.99-2.45	0.16	0.0-0.18	0.14	0.0*-0.17	2.22	1.83-2.63	0.19	0.13-0.25
UHR e.e.	2.2	1.82-2.80	1.58	1.46-1.71	0.18	0.15-0.24	0.14	0.09-0.19	2.28	1.80-2.64	0.22	0.15-0.28

Layer 4

Treatment	Width (mm)		Depth (mm)		Total Area (mm^2)	
	Average	Range	Average	Range	Average	Range
PRR untreated	0/93	0.15-1.90	1.03	0.15-1.90	2.47	1.92-2.88
PRR 100 ng/g	2.01	1.84-2.18	1.67	1.65-1.68	2.88	2.79-2.98
PRR 1,000 ng/g	1.42	1.13-1.9	1.72	1.32-1.91	2.38	1.85-2.87
UHR e.e.	2.02	1.80-2.21	1.66	1.61-1.73	2.45	1.92-2.75

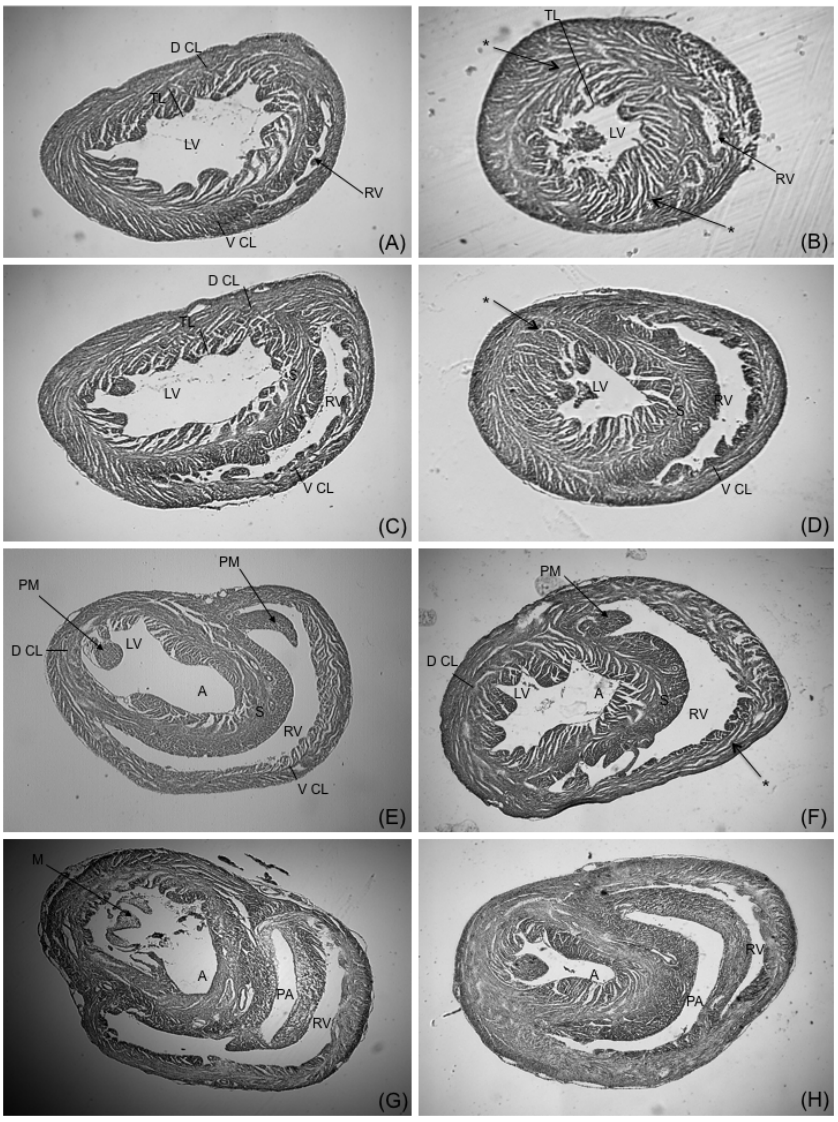
Untreated, 100 ng/g, and 1,000 ng/g PCB 77 treated hearts were randomly selected from PRR uncontaminated site. UHR environmentally exposed (e.e.) hearts were randomly selected for a contaminated site reference. Layer identification was determined using morphological landmarks, described in the methods section. There was no difference across treatments in morphology measurements, including CL depth when CL was present (D=dorsal; LV=left ventricle; RV= right ventricle; CL=compact layer; PRR= Patuxent Research Refuge; UHR=upper Hudson River; e.e.= environmentally exposed). Averages and ranges are expressed in millimeters or mm^2.

Table 3: Percent of tree swallow hatchling hearts with Compact Layer absences following exposure *in ovo* to PCB 77.

Treatment	(n)	# hearts w/ intact CL	% hearts with CL deformities
Untreated	15	13	13.33
Vehicle	11	10	9.09
100 ng/g*	14	8	42.86
1000 ng/g*	15	4	73.33
UHR e. e.	8	8	0.00

Compact layer (CL) analysis (presence or absence of this morphological structure) was determined on the dorsal and ventral ventricular wall regions in Layers 1 and 2. Hearts with compact layer absence in any location were identified as "CL deformed hearts." Chi square analysis determined significant differences between PCB 77 treated hearts and control samples ($p<0.001$), indicated by an asterisk (*). Environmentally exposed group, collected from Upper Hudson River (UHR e.e.), was not significantly different from control groups. Untreated, vehicle, 100 ng/g and 1,000ng/g hatchling hearts were randomly sampled from Patuxent Research Refuge (PRR), MD and Cobleskill, NY treatment groups.

Figure 1: A comparison of tree swallow hatchling hearts treated *in ovo* with PCB 77 [1,000 ng/g] with untreated hearts, analyzed at Layer 1, 2, 3, and 4 for morphological heart defects.



Tree swallow hatchling heart samples shown are representative of untreated and 1,000 ng/g PCB 77 treated embryos collected from Patuxent Research Refuge (PRR). There was no significant difference for all morphological measurements taken across treatments. PCB 77 dosed hatchlings (100 ng/g and 1,000 ng/g concentrations) showed a significant increase in compact layer absence when compared to the control. (LV=left ventricle; RV=right ventricle; D CL=dorsal compact layer; V CL=ventral compact layer; TL=trabeculated layer; S=septum; A=aorta; PM=pulmonary muscles; M=mitrial valve; PA=pulmonary artery; (*)= CL absence). Layer 1: (A) control, (B) PCB 77 treated; Layer 2: (C) control, (D) PCB 77 treated; Layer 3: (E) control, (F) PCB 77 treated; Layer 4: (G) control, (H) PCB 77 treated. Layer identification described in Materials and Methods.

Discussion

Exposure to PCB 77 did not affect survivability

It has been well established that environmental contaminants are maternally deposited into eggs where they can cause adverse effects on embryonic heart development (Hill and Hoffman, 1984; Rufer *et al*., 2009). PCBs have been detected in mammalian cardiac tissue at elevated concentrations (Wang *et al*., 2010). PCB congener toxicity associated with activation of the aryl hydrocarbon receptor (AhR) revealed tree swallows have greater resistance to PCB exposure compared to other avian species (Kennedy *et al*., 1996; Carney *et al*., 2006; Head *et al*., 2008; Hervé *et al*., 2010). Dioxin-like PCBs with similar chemical structures to dioxins such as TCDD are categorized as more toxic based on their toxic equivalency factors (TEF) (Van den Berg, *et al*., 1998; 2005). PCB 77, a dioxin-like PCB congener, TEF of 0.0004 in kestrels, 0.005 in turkeys, and 0.05 in chickens (Brunstrom, 1988; Elliot *et al.*, 1996; Lavoie and Grasman, 2007). Previous dosing studies with PCB 77 have shown no effects on herring gulls and mallards at concentrations of 1,000 to 5,000 ng/g. Moreover, there was no effect on animal survivability (Brunstom, 1988), which is consistent with the data we collected in tree swallows. Based on the TEF of PCB 77, however, the concentrations we used in this study had an elevated toxic equivalency (TEQ), which should have resulted in embryo mortality (Van den berg *et al*., 1998). Therefore, these data suggest a limitation of TEQs for estimating risk to wild bird populations, especially if the mechanism of action does not involve AhR mediated effects (Dean *et al.*, unpublished).

PCB 77 affects heart development in tree swallow hatchlings

Hearts from nestlings exposed to environmental mixtures of PCBs had heart defects including abnormal heart indices (heart weight/body weight %), deformities, microsurface roughness, apical and ventricular deformities, and thinning of the ventricular wall (DeWitt *et al*., 2006). In laboratory studies, domestic chick embryos exposed to dioxin-like PCB congeners and dioxins in chick embryos had ventricular dilation, thinning of the ventricular wall, and reduced chronotropic responsiveness (Canga *et al*., 1993; Henshel *et al*., 1993; Walker *et al*., 1997; Walker and Catron, 2000; Heid *et al*., 2001; Kopf and Walker, 2009). We also observed a cardiomyopathy of the ventricular wall, specifically an absence of the CL. The CL is a portion of the myocardial wall formed by proliferation of the cardiomyocytes. The myocardial wall is comprised of a highly proliferative region, the CL, and a highly trabeculated inner zone (Jeter and Cameron, 1971; Tomanek *et al*., 1999). The CL is absolutely necessary for myocardial growth and proper overall heart growth, resulting from cardiomyocyte proliferation, and proliferates at high rates through hatch in avian species, with the major increase in ventricular wall thickness occurring between ED 8-14 (Rychterova, 1978). The high volume of cardiomyocytes in the CL is essential for normal muscle contraction and cell turnover in the organ (Kirby, 2007). Absence of such a vital portion of the ventricular wall in PCB 77-treated tree swallow hearts would lead to decreased fitness in these animals, especially during periods of increased stress. Further, absence of the CL is associated with compromised heart contraction and reduced myocardial proliferation throughout the life of the organism, another contributing factor to decreased overall fitness (Sedmera and McQuinn, 2008). In some species, even minor localized absences

of compact muscle layer can result in cardiomyopathies, heart failure, and even cardiac death (Waller *et al*., 1980; Jenni *et al*., 2001). In tree swallows, there are extensive cardiac requirements as they feed and protect their nests; any compromise of the heart morphology presents a great disadvantage to the individual (Custer *et al*., 1980; 2002; 2003, 2010a,b,c,d). In humans, exposure to environmental contaminants *in utero* increases the possibility of an underdeveloped LV (Ferencz *et al*., 1997), a similar phenotype to the thinning ventricular walls seen in PCB exposed birds. It has been estimated that 30% of congenital cardiovascular malformations in infants could be linked to environmental risk factors (Wilson *et al*., 1998). The link between environmental contaminants and congenital cardiovascular malformations in humans is reminiscent of some observations in wild birds. Our data confirms that PCB 77 adversely affects the proper formation of the CL, which suggests that although PCB 77 is not lethal, it critically affects tree swallow cardiac fitness.

While our study demonstrated increased CL defects with PCB 77 exposure, we did not observe some of the adverse heart effects seen in previous avian studies. One possible explanation is that treatments were administered at approximately 18% of incubation. As such, we were able to minimize embryonic mortality by injecting after development had begun (Heinz *et al*., 2006). While this approach minimized mortality and verified the presence of a developing embryo, the timing of injection occurred after the initiation of heart development. In chickens, embryonic heart development begins at approximately Hamburger Hamilton stage (HH) 7, with the emergence of the cardiogenic mesoderm at approximately 25 hrs of incubation (Hamburger and Hamilton, 1951). In tree swallows, which have an 18-day incubation period, heart formation based on a

relative HH staging would occur at 21 hrs of incubation, approximately 40 hrs earlier than the time of PCB exposure in this experiment. Therefore, the embryos in this study were treated *in ovo* to PCB 77 well after the initiation of heart development (Bellairs and Osmond, 2005). Studies that have linked PCB exposure to altered heart weight and dilated cavities have exposed embryos prior to incubation, which might account for the differences relative to heart size and weight (Walker *et al*., 1997; Walker and Catron, 2000; DeWitt *et al*., 2006; Kopf and Walker, 2009). This suggests that we would have observed an even greater effect, including impact on weight, additional morphological abnormalities, and size, if *in ovo* dose treatments had been administered earlier.

Future work

Our lab will test an environmentally relevant, 58 congener PCB mixture, derived from contaminant analysis of eggs collected along the upper Hudson River, on heart development in an avian lab model with high sensitivity to PCBs, the chicken embryo. Due to the availability of samples, this study will dose eggs prior to the initiation of heart development, using a larger dose-curve, in an effort to identify a dose-dependent lethality curve, as well as a complete spectrum of cardiomyopathies seen in other PCB studies. Additionally, future work will endeavor to link environmentally relevant PCB mixtures to adverse cardiovascular effects and determine embryonic stages most sensitive to these exposures.

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References

Aulerich RJ, Ringer RK. 1977. Current status of PCB toxicity to mink, and effect on their reproduction. Arch Environ Contam Toxicol 6:279-292.

Barsotti DA, Marlar RJ, Allen JR. 1976. Reproductive dysfunction in rhesus monkeys exposed to low levels of polychlorinated biphenyls (Aroclor 1248). Food and Cosmet Toxicol 14:99-103.

Bellairs R, Osmond M. 2005. The Atlas of Chick Development 2nd Edition. Elsevier Academic Press, San Diego, CA, USA.

Bishop CA, Koster MD, Chek AA, Hussell DJ, Jock K. 1995. Chlorinated hydrocarbons and mercury in sediments, red winged blackbirds (Agelaius phoeniceus) and tree swallows (Tachycineta bicolor) from wetlands in Great Lakes, St. Lawrence River basin. Environ Toxicol and Chem 14:491-510.

Brown JF, Wagner RE, Feng H, Bedard DL, Brennan MJ, Carnahan JC, May RJ. 1987. Environmental dechlorination of Polychlorinated biphenyls. Environ Toxicol and Chem 6: 579-593.

Brunstrom B. 1988. Sensitivity of embryos from duck, goose, herring gull, and various chicken breeds to 3,3', 4,4'-tetrachlorobiphenyl. Poult Sci 67:52-57.

Canga L, Paroli L, Blanck TJ, Silver RB, Rifkind AB. 1993. 2,3,7,8-tetrachlorodibenzo-p-dioxin increases cardiac myocyte intracellular calcium and progressively impairs ventricular contractile responses to isoproterenol and to calcium in chick embryo hearts. Molec Pharmacol 44:1142-1151.

Carney SA, Jing Chen C, Burns G, Xiong KM, Peterson RE, Heideman W. 2006. Aryl hydrocarbon receptor activation produces heart specific transcriptional and toxic responses in developing zebrafish. Molec Pharmacol 70:549-561.

Custer TW and Heinz GH. 1980. Reproductive success and nest attentiveness of mallard ducks fed Aroclor 1254. Environ Pollut 21:313.

Custer TW, Custer CM, Hines RK. 2002. Dioxins and congener specific polychlorinated biphenyls in three avian species from Wisconsin River, Wisconsin. Environ Pollut 119:323-332.

Custer CM, Custer TW, Dummer PM, Munney KL. 2003. Exposure and effects of chemical contaminants on tree swallows nesting along the Housatonic River, Berkshire County, Massachusetts, USA, 1998-2000. Environ Toxicol Chem 22:1605-1621.

Custer TW, Gray BR, Custer CM. 2010a. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicol 19:391-404.

Custer TW, Custer CM, and Gray BR. 2010b. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in belted kingfisher eggs from Upper Hudson River Basin, New York, USA. Environ Toxicol Chem 29:99-110.

Custer CM, Gray R, Custer TW. 2010c. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallow (Tachycineta bicolor). Environ Toxicol Chem 29:909-921.

Custer CM, Custer TW, Dummer PM. 2010d. Patterns of organic contaminants in eggs of an insectivorous, an omnivorous, and a piscivorous bird nesting on the Upper Hudson River, New York, USA. Environ Toxicol Chem 29: (epublished).

DeWitt JC, Millsap DS, Yeager RL, Heise SS, Sparks DW, Henshel DS. 2006. External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol and Chem 25: 541–551.

Echols KR, Meadows, JC, Tillitt DE, Secord A. 1996. Congener-specific analysis of polychlorinated biphenyl residues in tree swallow chicks, eggs, and other biota from the Hudson River, W.U. 30096. USGS Final Laboratory Report FY97-30-01.

Echols KR, Tillitt DE, Nichols JW, Secord AL, McCarty JP. 2004. Accumulation of PCB congeners in nestling tree swallows (Tachycineta bicolor) on the Hudson River, New York. Environ Sci Tech 38:6240-6246.

Elliott JE, Wilson LK, Langelier KW, Norstrom RJ. 1996. Bald eagle mortality and chlorinated hydrocarbon contaminants in livers from British Columbia, Canada, 1989-1994. Environ Pollut 94: 9-18.

Ferencz C, Loffredo CA, Correa-Villasenor A, Wilson PD. 1997. Genetic and environmental risk factors of major cardiovascular malformations. Futura Publishing Co, Armonk, NY, USA.

Frame GM, Cochran JW, Bowad SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, qualitative, congener-specific analysis. J High Res Chromat 19:657-668.

Frame GM. 1997. Congener specific PCB analysis. Analyt Chem 69:468-478.

Golub MS, Donald JM, Reyes JA. 1991. Reproductive toxicity of commercial PCB mixtures: LOAELs and NOAELs from animal studies. Environ Health Perspect 94:245-253.

Gould JC, Cooper KR, Scanes CG. 1999. Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeiodinase in the chick embryo. Ecotoxicol Environ Saf 43:195-203.

Hamburger V, Hamilton HIL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49-92.

Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environ Sci Technol 42:7535-7541.

Heid SE, Walker MK, Swanson HI. 2001. Correlation of cardiotoxicity mediated by halogenated aromatic hydrocarbons to aryl hydrocarbon receptor activation. Toxicol Sci 61:187-196.

Heinz GH, Hoffman SL, Kondrad SL, Erwin CA. 2006. Factors affecting the toxicity of methylmercury injected into eggs. Environ Contam Toxicol 50:264-279.

Henshel DS, Hehn B, Vo MT, Steeves JD. 1993. A short-term test for dioxin teratogenicity using chicken embryos, Environmental Toxicology and Risk Assessment, Vol 2. STP 1216. American Society for Testing and Materials, Philadelphia, PA, USA, pp 159–174.

Hervé JC, Crump D, Jones SP, Mundy LJ, Giesy JP, Zwernick MJ, Bursian SJ, Jones PD, Wiseman SB, Wan Y, Kennedy SW. 2010. Cytochrome P450A induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. Toxicol Sci 113:380-391.

Hill EF, Hoffman DJ. 1984. Avian models for toxicity testing. J Am Coll Toxicol 3:357.

Jacobson JL, Jacobson SW, Humphrey HEB. 1990. Effects of in utero exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. J Pediat 116;38-45.

Jenni, R, Oechslin, E, Schneider, J. 2001. Echocardiographic and pathoanatomical characteristics of isolated left ventricular non-compactions: a step towards classification as a distinct cardiomyopathy. Heart 86: 666.

Jeter JR, Cameron IL. 1971. Cell proliferation patterns during cytodifferentiation in embryo chick tissues: liver, heart and erythrocytes. J Embryol Exp Morphol 23:403-422.

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol and Appl Pharmacol 141:214-230.

Kirby ML. 2007. Cardiac Development. Oxford University Press, New York, NY, USA.

Kopf PG, Walker MK. 2009. Overview of developmental heart defects by dioxins, PCBs, and pesticides. J Environ Sci Health 27: 276-285.

Lavoie ET, Grasman KA. 2007. Effects of in ovo exposure to PCBs 126 and 77 on mortality, deformities, and post-hatch immune function in chickens. J Toxicol Environ Health, Part A 70:547-558.

McKinney JD and Waller CL. 1994. Polychlorinated biphenyls as hormonally active structural analogues. Environ Health Perspect 290-298.

McNabb FM. 2005. Biomarkers for the assessment of avian thyroid disruption by chemical contaminants. Avian Poult Biol Rev 16:3-10.

Rufer ES, Hacker TA, Flentke GR, Drake,VJ, Brody MJ, Lough J, Smith SM. 2009. Altered cardiac function and ventricular septal defect in avian embryos exposed to low-dose trichloroethylene. Toxicol Sci 113:444-452.

Rushneck DR, Beliveau A, Fowler B, Hamilton C, Hoover D, Kaye K, Berge M, Smith T, Telliard WA, Roman H, Ruder E, Ryan L. 2004. Concentrations of dioxin-like PCBs in unweather Aroclors by HRGC/HRMS using EPA method 1668A. Chemosphere 54:79-87.

Rychterova V. 1978. Development of proliferation structure of ventricular heart wall in the chick embryo between 6^{th} and 14^{th} day of embryogenesis. Folia Morphol 26:131-143.

Safe SH. 1994. Polychlorinated biphenyls (PCBs). Environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87-149.

Secord AL, McCarty JP, Echols KR, Meadows JC, Gale RW, Tillitt DE. 1999. Polychlorinated bipheyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from Upper Hudson River, New York State, USA. Environ Toxicol Chem 18:2519-2525.

Sedmera D and McQuinn T. 2008. Embryogenesis of heart muscle. Heart Fail Clin 4: 235-245.

Silberhorn EM, Glauert HP, Robertson LW. 1990. Carcinogenicity of Polyhalogenated biphenyls: PCBs and PBBs. Crit Rev Toxicol 20:440-496.

Thomas PT, Hinsdill RD. 1978. Effects of polychlorinated biphenyls on the immune responses of rhesus monkeys and mice. Toxicol Appl Pharmacol 44:41-51.

Tomanek RJ, Hu N, Phan B, Clark EB. 1999. Rate of coronary vascularization during embryonic chicken development is influences by rate of myocardial growth. Cardiol Res 41:663-671.

Van den Berg KJ. 1990. Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin, and thyroid binding globulin. Chem Biol Interact 76:63-75.

Van den Berg M, Birbaum LS, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson PRE, Poellinger L, Safe S, Schrenk D, Tillitt DE, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106:775-792.

Van den Berg M, Birnbaum LS, Denison M, DeWitto, M, William F, Feeley M, Fidler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. 2005. The 2005 World Health Organization re-evaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93:223-241.

Walker MK, Pollenz RS, Smith SM. 1997. Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with tetrachlorodibenzo-p-dioxin induced heart defects. Toxicol and Appl Pharmacol107:1075-1083.

Walker MK, Catron TF. 2000. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early embryo development Toxicol and Appl Pharmacol 167:210-221.

Waller BF, Smith ER, Blackbourn BD. 1980. Congenital hypoplasia of portions of both right and left ventricular myocardial walls. Clinical necropsy observations in two patients with parchment heart syndrome. Am J Cardiol 46:885.

Wilson PD, Loffredo CA, Correa-Villasenor A, Ferencz C. 1998. Attributable fraction for cardiac malformations. Am J Epidem 148:414-423.

CHAPTER 3

EFFECTS OF AN ENVIRONMENTALLY RELEVANT POLYCHLORINATED BIPHENYL (PCB) MIXTURE ON CARDIAC DEVELOPMENT IN THE DOMESTIC CHICK HATCHLING

Abstract

A 58-congener polychlorinated biphenyl (PCB) mixture based on a contaminant analysis of eggs collected along the Upper Hudson River, New York was used to study *in ovo* PCB effects on cardiac development in the domestic chicken. Fertile eggs were injected prior to incubation with a dose range of the PCB mixture, including: untreated, sham, 0.0, 0.03, 0.08, 0.30, 0.50, 0.70, and 2.06 µg/g egg wt. Embryonic development was monitored throughout incubation; chicks were necropsied at hatch. Survivability data at hatch followed a dose-dependent curve with significant ($p<0.05$) mortality above the 0.50 µg/g egg wt treatment concentrations compared to controls. The LD_{50} of this PCB mixture in chicks was estimated as 0.40 µg/g egg wt at hatch based on the lethality curve. Cardiac arrhythmia was observed at embryonic day (ED) 14 of development at treatment concentrations 0.50 µg/g egg wt and above. Histological analysis was utilized to characterize any cardiac abnormalities. Cardiomyopathies increased across treatments in a dose-dependent manner when compared to the control groups. Identified abnormalities included absence of the trabeculated layer of the ventricular wall, ventricular dilation, thinning of the ventricular walls, malformation of the septal wall, and most commonly, absence of the compact layer of the ventricular wall. Chick heart width, depth, total area, compact layer depth, septal width, chamber area, and ventricular wall dimensions did not differ across treatments. With the exception of heart defects, additional analyses of organ indices and neurological behavior did not differ with treatment. This study supports prior reports of adverse developmental effects of PCBs on cardiovascular systems in birds. Although the birds hatched, the measured cardiomyopathies suggest potential deleterious impacts on individual health and fitness.

Introduction

Polychlorinated biphenyls are a class of synthetic compounds used extensively in manufacturing in the United States until 1979, at which time PCBs were banned from manufacture, processing, and distribution. These compounds are made up of one biphenyl molecule with one to ten chlorine substitutions along the rings, for a total of over two hundred known PCB congeners. PCBs are lipophilic, resistant to degradation, and bioaccumulative, making them a continuous source of exposure for animals living near contaminated sites long after their ban in the late 1970's (Johnson *et al*., 2000). Two contaminated sites identified along the upper Hudson River (UHR) in New York are Fort Edwards and Hudson Falls. PCBs were used in manufacturing plants at these two locations from the late 1940s until the 1970s. Many PCBs occur as mixtures and were marketed by percent chlorination under various names. For example, three mixtures, or Aroclors, have been identified as the most common mixtures released from Fort Edward and Hudson Falls; Aroclor 1254, Aroclor 1242, and Aroclor 1016, with 54%, 42%, and 42% chlorine by weight, respectively (TAMS *et al*., 1991). While Aroclors are identified by % chlorine by weight, individual lots of the same Aroclors can contain varying congener distributions, thereby altering their toxicity to vertebrates (Frame *et al*., 1996; Frame, 1997; Rushneck *et al.*, 2004). Avian species are exposed to PCB congeners primarily through dietary consumption, metabolize PCBs to varying degrees, and deposit these lipophilic compounds into their eggs (Barron *et al.*, 1995; Custer *et al*., 2002; 2003; 2010a; 2010b; 2010c; 2010d). The wide variability between Aroclor site exposure, ingested PCB congeners, and ultimately maternal deposition of congeners into eggs makes identification of specific effects difficult to interpret between sites. Moreover,

movements of birds between sites through migration make it even more difficult to determine effects and assess risk (Secord *et al*., 1999). In recent years, analysis of the PCB profile in spotted sandpiper eggs collected from the UHR, New York has aided in estimating potential uptake and exposures in developing birds. A 58-congener mixture (PCB mix) was developed based on relative concentrations of congeners found in the sandpiper eggs (Echols *et al*., 1996; Custer *et al*., 2010d). This PCB mix has been formulated for use in laboratory and field studies to assess effects in avian species.

Environmental exposure to PCBs has been linked to carcinogenicity (Safe, 1994; Silberhorn *et al*., 1990), immune system dysfunction (Thomas and Hindsdill, 1978; McNabb, 2005; Lavoie and Grasman, 2007), reproductive defects (Barsotti *et al*., 1976; Aulerich and Ringer, 1977; Golub *et al*., 1991), neurological effects (Tilson *et al*., 1990; Jacobson *et al*., 1990) and endocrine disruption in mammalian, avian, and fish studies (van de Berg, 1990; Gould *et al*., 1999). Although some exposures are potentially lethal, many infer a decreased fitness in surviving animals (Meeker and Hauser, 2010; Jacobson *et al.*, 1990; Nebeker *et al*., 1974). The effects of individual PCBs on embryonic heart development have been documented; however, a complex PCB mixture such as that found at the UHR has not been studied.

Proper heart development is essential for animal survivability, long-term health, and overall fitness. In fact, congenital cardiovascular malformations account for upwards of 20% of all human infant death related to non-genetic birth defects (Jenkins *et al*., 2007). Previous studies have shown a link between PCB exposure and adverse health effects at different contaminant sites for several bird species. Nestlings at a PCB contaminated site in Indiana exhibited cardiac malformations including decreased heart

indices (heart weight as a percent of body mass), thinning of the ventricular walls, and abnormal heart apexes (DeWitt *et al*., 2006). Domestic chick embryos exposed to dioxin-like PCBs had increased ventricular-septal defects, ventricular dilation, and higher overall heart weight by ED10 (Walker *et al*., 1997; Walker and Catron, 2000). Tree swallow hatchlings that were exposed to a dioxin-like PCB congener 77 (PCB 77) showed an absence of the compact layer of the ventricular wall of the heart at high doses but did not induce embryonic death based on toxic equivalencies (TEQ) (Carro *et al*., unpublished; van den Berg *et al*., 1998). Interestingly, PCB 77 was introduced at approximately 18% of embryonic development, which is well after the initiation of heart development. This suggests that the PCBs exerted effects after documented developmental events, including looping of the heart tube, differentiation of the atrial and ventricular cavities, and initiation of proliferating cardiomyocytes, but before compact layer formation is completed (Kirby, 2007; Bellairs and Osmond, 2005). The absence of the compact layer in this tree swallow study potentially links wild bird nestling studies (DeWitt *et al*., 2006) to embryonic studies (Walker *et al*., 1997; Walker and Catron, 2000; Kopf and Walker, 2009) through a common cardiomyopathy of ventricular wall thinning with the suggested origin of that cardiomyopathy being compact layer malformation for birds exposed to PCBs.

The purpose of this study was to further investigate the link between *in ovo* PCB exposure and congenital cardiac malformations in an avian lab model. An environmental mixture, the PCB mix, was formulated based on PCBs found in wild egg samples (Tillitt *et al*., 2011). Previous work has shown the domestic chicken is a highly responsive avian model. This is due to the mechanism of action, which acts through the aryl hydrocarbon

receptor (AhR) (Kennedy *et al*., 1996; Head *et al*., 2008). As such, the chick embryo may respond to the PCB mix below concentrations found in the environment (Kennedy *et al.*, 1996; Jones and Kennedy, 1999; Custer *et al*., 2010a; b; d). Interestingly, non-dioxin like congeners that do not act through the AhR pathway are at greater concentrations in our PCB mix than dioxin-like congeners (Tillitt *et al*., 2011). In another lab study utilizing Japanese quail, our lab determined that this PCB mix was twice as toxic as another environmental mixture containing 66 congeners, based on TEQs, which was unexpected (TEQs predicted the 66 congener mixture to be more toxic) (Dean *et al*., unpublished; van den Berg *et al*., 1998). These data suggest that the previously accepted mechanism of action for PCBs, specifically action primarily through the AhR, may not be the sole mechanism of action in avian species (Ahlborg *et al*., 1992; Kennedy *et al*., 1996; Head *et al*., 2008). The aims of this study were to determine the 50% lethal dose (LD_{50}) of the PCB mix in the domestic chicken model and determine if cardiac malformations result from *in ovo* exposure.

Materials and Methods

Egg injections and solution preparation

Fertile broiler chicken (*Gallus domesticus*) eggs were purchased from Allen's hatchery (Seaford, DE). The PCB mix, made of primary PCB congeners in relative concentrations found in sandpiper eggs collected at the UHR, was prepared by the USGS Biochemistry and Physiology Branch (Tillitt *et al*., 2011). The PCB mix was solubilized in activated charcoal-stripped corn oil (Dean *et al*., unpublished) at the following concentrations: 0.03, 0.08, 0.30, 0.50, 0.70, and 2.06 µg/g egg wt. A vehicle treatment of

0.0 µg/g egg wt, sham eggs (shell punctured but no vehicle administered), and untreated eggs were used as control treatments.

Eggs were weighed at ED0 and grouped randomly to have egg weights evenly distributed across treatments (n=20 eggs/treatment). Treatments were administered as follows: the egg was swabbed with alcohol and a small hole was drilled into the side of the egg using a sterile Dremel® bit (Dremel Co., Racine, WI). Injection volume was calculated for each egg and a total of 0.9-1.11 µl (based on egg wt) was administered into the hole using a reverse displacement pipettor (Rainin Instrument LLC, Oakland, CA). Eggs were sealed with melted paraffin wax and incubated (air cell up) at 37ºC and 65% humidity and randomly distributed through the incubator. At ED20, eggs were transferred to individual, compartmentalized hatching trays and allowed to hatch.

Incubation period and hatchling collections

Eggs were candled on ED0, ED2, ED4, ED6, ED10, ED14, ED18, and ED20. Infertile eggs were removed from treatments and not incorporated into statistical analyses. Early embryonic death was recorded as mortality prior to ED6; embryos that died later were necropsied. At ED10, ED14, and ED18, heart rate was determined using a digital egg monitor (Avian Buddy International, Tallahassee, FL) at 37±0.5°C. All heart rate monitoring was randomized and performed blind.

Hatchlings were tested for neurological deficits using behavior assessments, sacrificed, and necropsied. Body weight, organ weights and gender were recorded; liver, thyroid, feces, blood, yolk, lung, brain, and gastrointestinal tracts were collected and frozen at -80°C for future analysis. The protocol followed for collecting the hearts were as follows: beating hearts were dissected immediately following euthanization and placed

in ice-cold 25 mM KCl until beating ceased. Hearts were rinsed thoroughly in 1xPBS solution, blotted dry, weighed, immersed in ice-cold 10% neutral buffered formalin, and stored at 4°C for 48 hrs. Next, hearts were washed in 1xPBS solution for 30 min (2x) at 4°C, stepwise dehydrated to 100% ethanol (Pharmco-Aaper, Shelbyville, KY) in 60 min increments: 50%, 70%, 80%, 95%, 100% (2x) ethanol (EtOH) and transitioned in 1:1 mixture of the clearing agent Hemo-De® and EtOH at room temperature for 30 min. Hearts were transferred to 100% Hemo-De for 30 min at 58°C, moved to 1:1 Hemo-De:paraffin for 60 min at 58°C and then immersed in 100% paraffin (Triangle Biomedical Sciences, Inc., Durham, NC) for 120 min at 58°C (replacing paraffin every 30 min). The heart was then blocked in paraffin filled mold, oriented uniformly, and allowed to solidify for 24 hrs prior to sectioning.

Heart sectioning and histology

Hearts were sectioned transversely at 10 µm from the apex to the cranial region, wet mounted on glass slides, dried, and stained with standard hemotoxylin and eosin procedure. Briefly, slides were dewaxed in Hemo-De for 3 min (3x) and rehydrated using reverse stepwise increments of EtOH: 100%, 95%, 70% EtOH, and 1xPBS for 3 min each. Samples were nuclear stained using Gill's hematoxylin (Fisher Scientific, Fair Lawn, NJ), followed by a 2 min water rinse, and cytoplasmic stained using 0.5% eosin Y (Fisher Scientific, Fair Lawn, NJ) solution, followed by a 6 min water rinse. Tissue was dehydrated again in 70% and 100% EtOH for 30 sec each, immersed in HemoDe for 3 min, coverslipped with Cytoseal^{TM}, and dried overnight before storage. Ten control hearts were analyzed for baseline morphology; five hearts were analyzed from each

treatment, with the exception of 0.70 and 2.06 µg/g egg wt treatments (only 2 and 0 embryos survived to hatch, respectively).

Four layers were evaluated in each heart as defined by the following morphometric features: Layer 1- most caudal section analyzed, identified by the left ventricular wall and cavity (~95% of the section) and the early right ventricular opening; Layer 2- both left and right ventricles visible, with no evidence of papillary muscles or valves; Layer 3- the upper portion of both ventricles visible with emerging aorta present in the central region, left ventricle papillary muscles emerging, and evidence of the right ventricle papillary muscle; Layer 4- emergence of the pulmonary artery without the emergence of the aortic valve.

Images were captured using a 1.25 and 2.5x Zeiss Acroplan objective under a Zeiss Axioplan microscope with a Coolsnap Fx™ photometric camera attachment (Carl Zeiss, Inc., New York, NY). Images were captured using IP Lab36 software (Biovision Technologies, Inc., Exton, PA) and analyzed using ImageJ software (NIH resources, Bethesda, MD). A number of comparisons were made between treatments at each layer, as described below. Measurements for width, depth, left ventricle (LV) dorsal and ventral compact layers, total area, and LV cavity area was analyzed for Layer 1. Width, depth, LV dorsal and ventral compact layers, RV wall, and total area were measured in Layer 2. Width, depth, LV dorsal and ventral compact layer, RV wall, septal thickness, and total area was measured in Layer 3. Finally, width, depth, and total area were measured in Layer 4. Each measurement was taken three times and the average score was used for analysis. Hearts were also scored for the presence of the compact layer in

Layers 1-3 and additional cardiomyopathies were identified in all hearts analyzed. All analysis was performed blind to prevent bias.

Statistical analyses

Herein survivability was taken to be viability at hatch with treatment and was analyzed by one-way ANOVA and Tukey's post-hoc tests (JMP9, SAS Institute Inc., Cary, NC). Infertile eggs were removed from the analysis, as determined via egg necropsies of non-viable eggs. Samples sizes ranged from 16 to 20 eggs per treatment associated with infertility. Embryonic heart rates were analyzed by one-way ANOVA and Tukey's post-hoc test at ED 10, 14, and 18. Cardiomyopathies were analyzed in hearts as described above and subjected to the Chi Square Test to determine the presence or absence of a cardiomyopathy. Finally, compact layer testing was performed at 6 regions in the heart (across 3 layers) in the subgroup of heart samples, with a maximum score of 6 and a minimum score of 0 and analyzed by one-way ANOVA followed by a Tukey's post-hoc test.

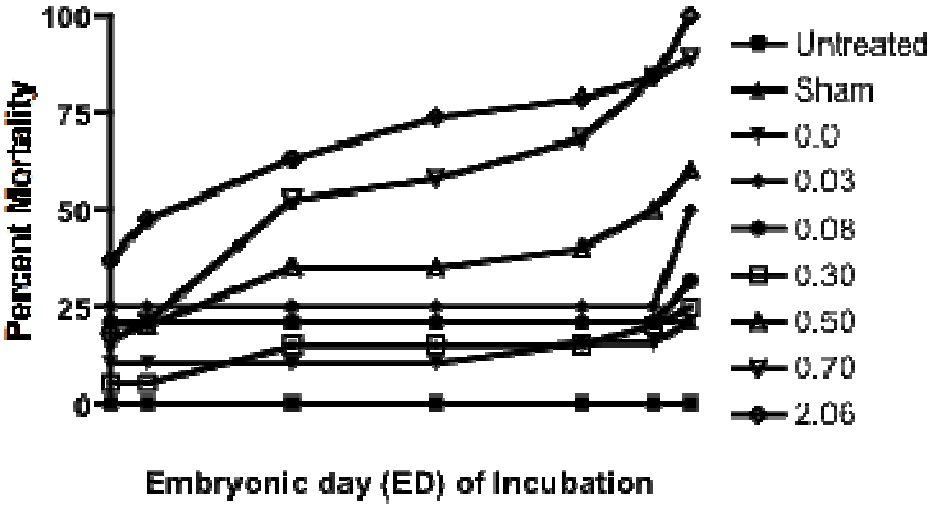
Results

PCB mix exposure and embryo survival

Exposure *in ovo* to this PCB mix resulted in a dose-dependent increase in mortality across PCB treatments (Figure 1). Mortality during incubation of low doses and controls occurred early (before ED6) and late (after ED18), with the exception of the three highest PCB treatments (0.50, 0.70, 2.06 µg/g egg wt) in which mortality increased across all incubation time points. The calculated LD_{50} established in hatchlings was

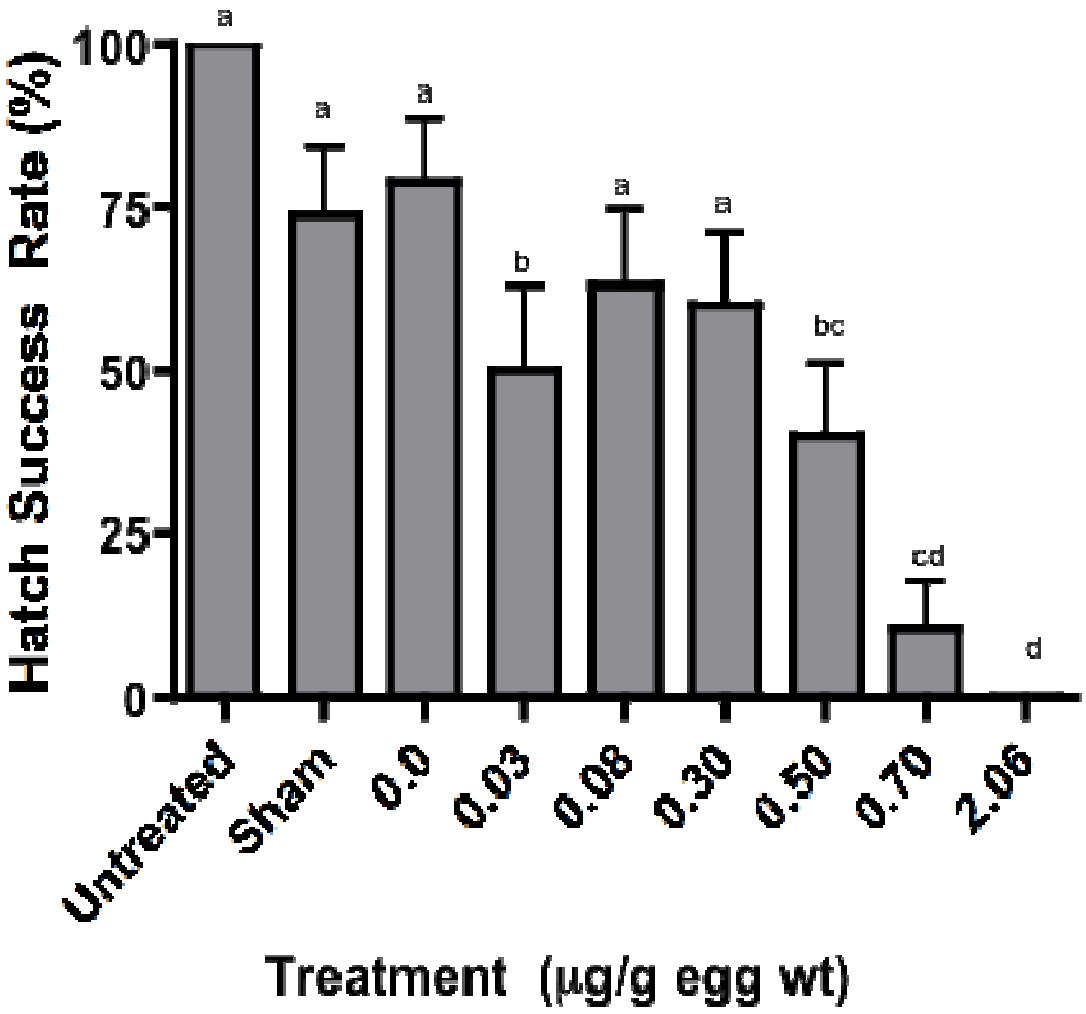
0.40 µg/g egg wt, based on this lethality curve. Hatch success rates significantly (p<0.05) decreased compared to controls in PCB treated embryos (Figure 2).

Figure 1: Percent mortality of chick embryos exposed *in ovo* to a 58-congener PCB mixture and dosed at ED0.



Percent mortality for all treatments throughout incubation. Sample size (n=20) across treatments at ED0. Infertile eggs were removed from subsequent analysis.

Figure 2: Percent of chick embryos per treatment that successfully hatched following exposure *in ovo* to a 58-congener PCB mixture.

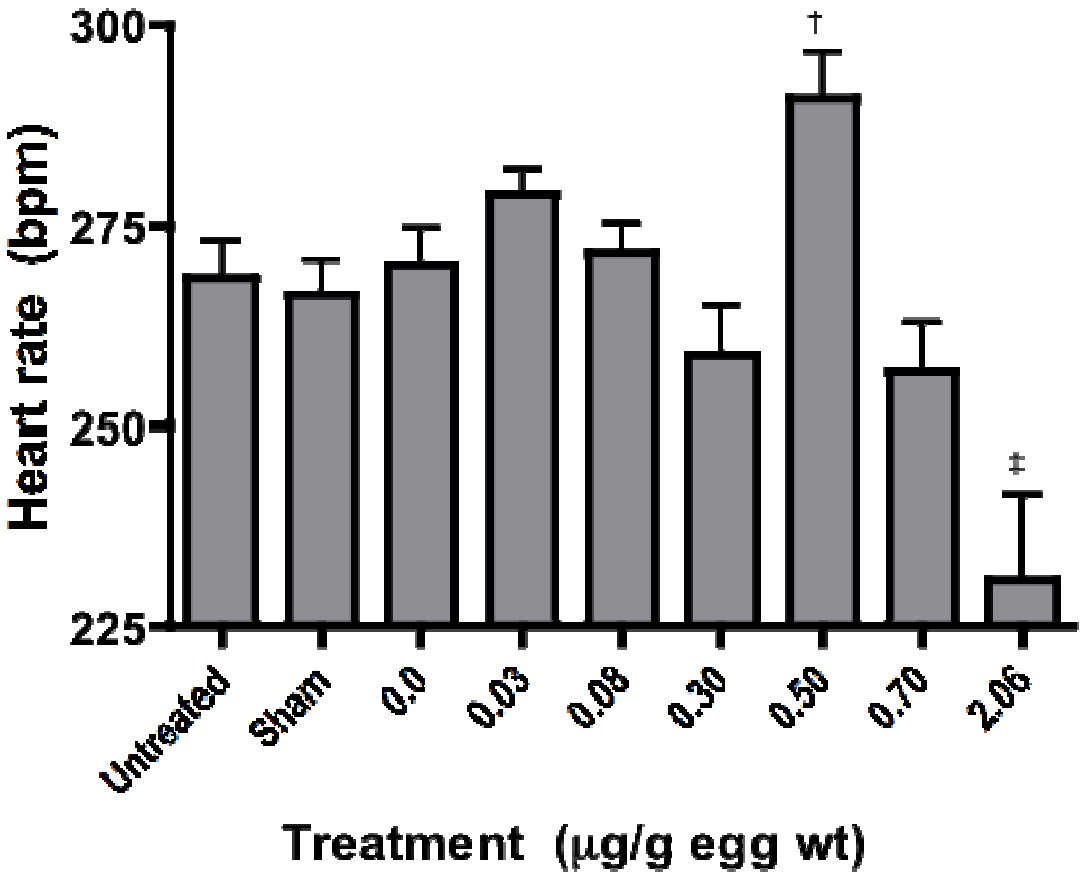


One way ANOVA showed significant effects across treatments ($p<0.0001$). Tukey's post-hoc test determined dose-dependent differences in survivability at hatch across treatments. Levels not connected by the same letter are significantly different.

PCB mix affects on heart rate during embryonic development

Heart rate (HR) in beats per minute, measured at ED10, ED14, and ED18 showed no significant differences across treatments at ED10 or ED18 (data not shown). A significant ($p<0.05$) difference was detected at ED 14 at 0.50 µg/g egg wt and 2.06 µg/g egg compared to control groups (Figure 3).

Figure 3: Embryonic heart rate (beats per minute) at ED14 of chick development following exposure *in ovo* to a 58-congener PCB mixture.



One way ANOVA showed significant effects across treatments ($p<0.05$). Tukey's post-hoc test determined a heart rate difference at embryonic day (ED) 14 of development. Treatment 0.5 µg/g egg wt was significantly higher than controls (†) and treatment 2.06 µg/g egg wt was significantly lower than controls (‡).

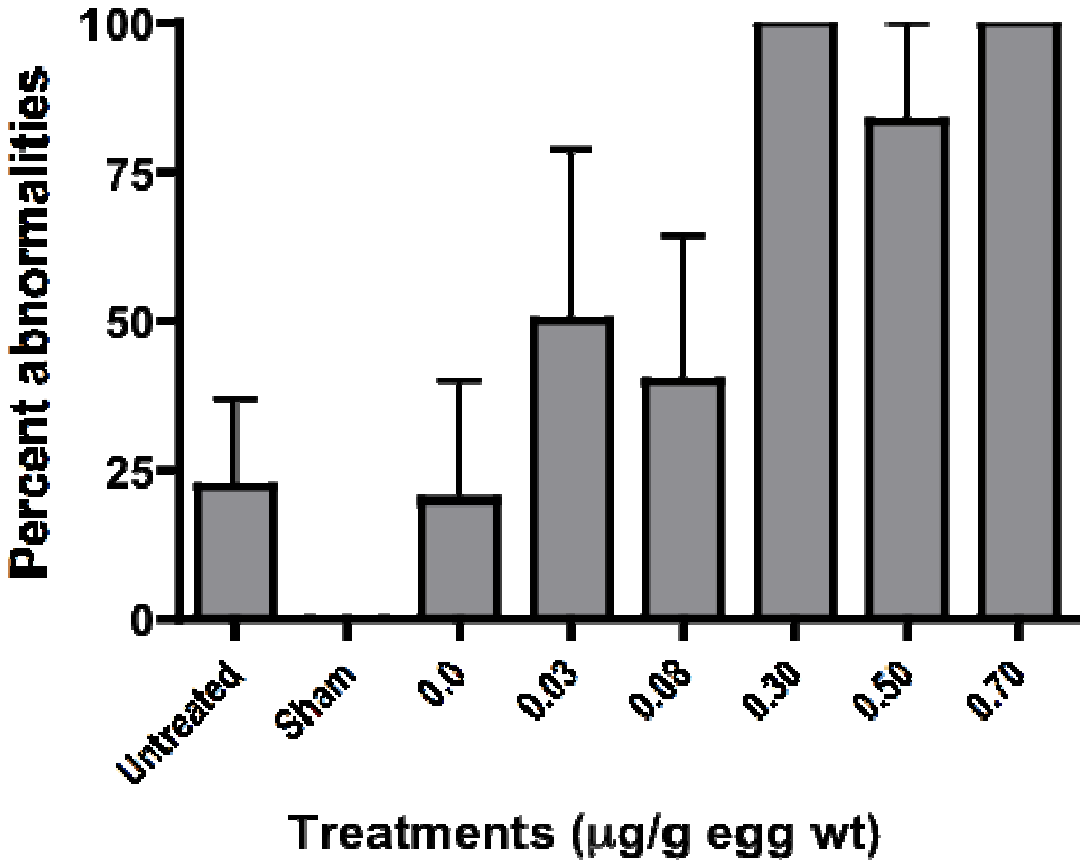
Cardiomyopathies in hatchlings exposed in ovo to the PCB mix

The percent cardiomyopathies showed a dose-dependent increase across PCB treatments when compared to the control groups (Figure 4; $p<0.05$). Hearts with multiple deformities were not scored for severity of cardiomyopathy; rather they were identified as abnormal hearts. The most prevalent heart abnormality identified in this analysis was an absence of the compact layer of the ventricular heart wall (Figure 5). Additional heart abnormalities noted were an absence of the trabeculated layer of the heart wall, enlargement of the ventricular chambers, thinning of the ventricular walls, and malformation of the septal wall (Figure 5).

Effects of the PCB mix on the compact layer of the ventricular heart wall

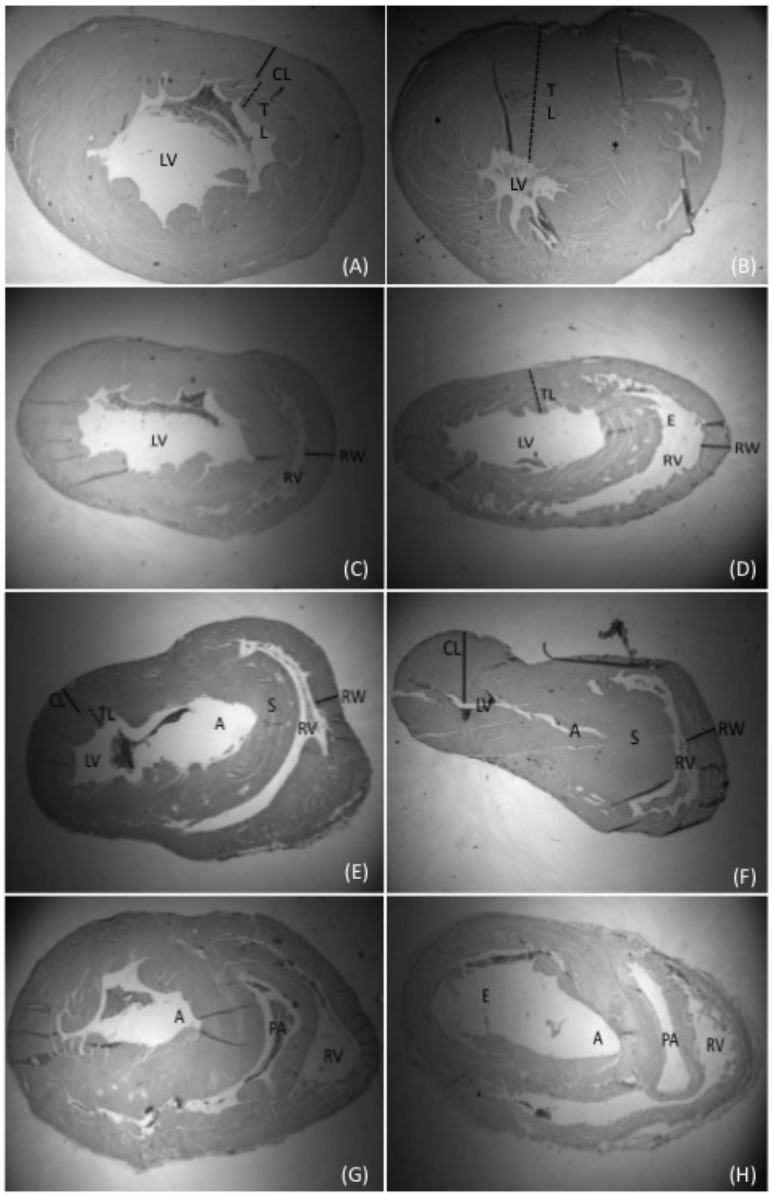
Chick hearts were analyzed at the four distinct layers for heart width, depth, area, compact layer depth, septal width, chamber area, and ventricular wall dimensions revealed no differences across treatments (data not shown). Analysis of the compact layer in hatchling hearts from treatments 0.30, 0.50, and 0.70 µg/g egg wt, however, showed an absence of compact layer in the ventricular wall (Figure 6; $p<0.05$).

Figure 4: Percent of hatchling hearts with cardiomyopathies in surviving chicks following exposure *in ovo* to a 58-congener PCB mixture.



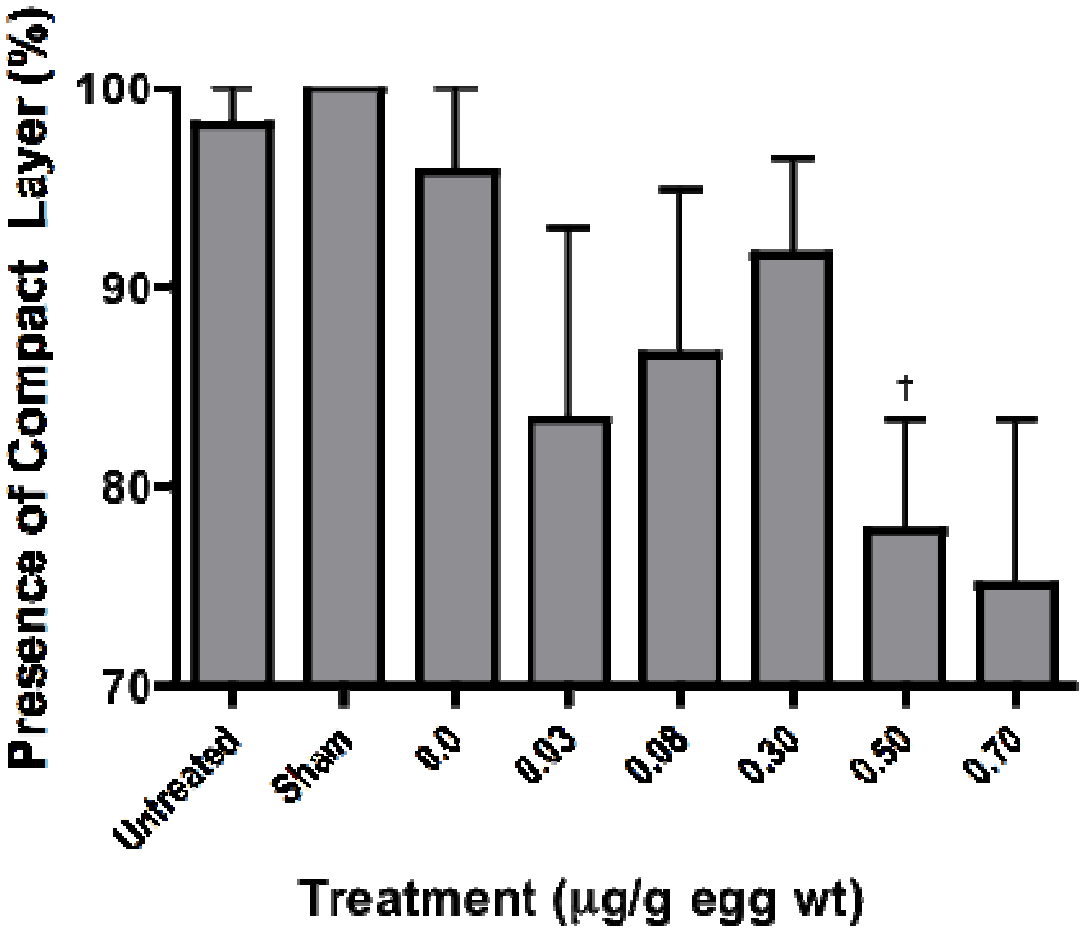
Chi square test showed a significant effect across treatments at hatch ($p<0.05$). The trend seen here is that the percent of hearts with abnormalities increased as dose concentration increased.

Figure 5: Histological representation of hatchling hearts compared to controls at four morphological transversely sectioned layers in surviving chicks following exposure *in ovo* to a 58-congener PCB mixture.



*Description of layer identification can be found in material and methods. Layer 1 compares normal control heart (A) to enlarged trabeculated layer (TB) of the ventricular wall (*) and absence of compact layer (CL) in PCB treated heart (B). Layer 2 compared normal control (C) to enlarged right ventricular chamber (E) of PCB treated heart (D). Layer 3 compares normal control heart (E) to a PCB treated heart (F) with no TL. Layer 4 compares a normal control heart (G) to a PCB treated heart (H) with an enlarged aorta. (CL-compact layer; TL-trabeculated layer; LV-left ventricle; RV-right ventricle; RW-right ventricular wall; S-septum; A-aorta; PA-pulmonary artery; E-enlarged chamber/cavity; *-abnormal heart wall morphology).*

Figure 6: Average ventricular wall compact layer scores by treatment in chicken hatchling hearts following exposure *in ovo* to a 58-congener PCB mixture.



Scores based on presence or absence of compact layer in six zones of the chick hatchling heart (two zones in Layers 1, 2, 3: layers described in materials and methods section). Maximum score per heart was six, lowest score recorded was three, hearts averaged by treatment in this graph. One way ANOVA followed by Tukey's post-hoc test showed significant difference in 0.50µg/g egg wt when compared to control ($p<0.05$). No hearts were analyzed at 2.06 µg/g egg wt because no embryos survived to hatch.

Discussion

Exposure to a PCB mix decreased embryo survival

Exposure *in ovo* to the PCB mix resulted in a dose-dependent decrease in survivability at hatch with a calculated LD_{50} of 0.40 µg/g egg wt (Figure 2). There was a gradual increase in mortality throughout development at PCB mix doses of 0.50 µg/g egg wt and higher. Treatment groups at lower doses showed a trend of increased mortality before ED06 and after ED18, with limited mortality between ED7 and ED17 (Figure 1). Our data suggest chickens are more sensitive to concentrations of PCB mixtures that would occur in the environment when compared to other avian lab models such as Japanese quail (*Coturnix japonica*), which had increased mortality above baseline 7.5 µg/g egg wt (threshold 3.11µg/g egg wt) using the same congener mixture (Dean *et al*., unpublished). The differences in sensitivity to this PCB mixture among our chick and Japanese quail models is further supported by previous work showing differences in sensitivity to PCB congeners between bird species (Head *et al*., 2008).

Increased cardiomyopathies in hatchlings dosed with PCB mix

The PCB mix utilized in this study significantly increased hatchling cardiomyopathies in a dose-dependent manner. Our results concur with observations in a wild bird nestling study, which identified cardiomyopathies in passerine nestlings, including the house wren (*Troglodytes aedon*), tree swallow (*Tachycineta bicolor*), and the Carolina chickadee (*Parus carolinesis*), at a PCB contaminated Superfund site. In this study, hearts from nestlings showed thinning of the ventricular wall, microsurface roughness, abnormal heart indices, and changes in overall heart deformities (DeWitt *et al*., 2006). Separate studies in embryonic chicks exposed to dioxin-like PCBs showed

abnormal dilation of the chambers, ventricular wall thinning, and reduced heart rate responsiveness to stimuli at ED10 (Kopf and Walker, 2009; Walker and Catron, 2000; Heid *et al*., 2001). Primary cardiomyopathies identified in our study were abnormal ventricular walls, most commonly an absence of the compact layer. Moreover, we observed an absence of the trabeculated layer, in addition to ventricular chamber dilation, thinning of the ventricular walls, and septal malformation. This is a notable finding because it begins to isolate specific effects of the PCB mix on the developing heart. While previous studies have conclusively shown heart effects in nestlings or at ED10 of development, the lethality effect of exposure *in ovo* in hatchlings was not assessed (DeWitt *et al*., 2006; Kopf and Walker, 2009; Walker and Catron, 2000; Heid *et al*., 2001). Additionally, our study confirms the effects of an environmentally relevant PCB mixture, containing both dioxin-like and non-dioxin PCB congeners, identified in egg contaminant analysis along the Hudson River (Echols *et al*., 2004; Custer *et al*., 2010a; Tillitt *et al*., 2011). Our study also suggests PCB effects on heart rate, especially relatively late in incubation at ED14. In previous chick embryo studies, dioxin-like PCB congeners were linked to a reduced heart rate response to stimuli at ED10 (Kopf and Walker, 2009). While we did not find this effect at ED10, we did confirm cardiac arrhythmia later in development. Dioxin exposed embryos showed decreased chronotropic responses to stimuli following *in ovo* exposure (Sommer *et al*., 2005; Walker and Catron, 2000), suggesting similar effects with PCBs.

Compact layer absence with PCB mix treatment

Exposure to dioxin-like PCBs has been linked to heart abnormalities in previous studies. The most common heart abnormality identified in both nestling populations and

ED10 embryos was a thinning of the ventricular wall (DeWitt *et al*., 2006; Walker and Catron, 2000; Kopf and Walker, 2009). We found an absence of the compact layer to be the most common ventricular wall cardiomyopathy. The importance of cardiomyocyte proliferation as it pertains to compact layer formation has been well documented between ED07-15 (Jeter and Cameron, 1971; Kirby, 2006). Studies categorizing the effects of contaminants that act similar to PCBs, such as dioxins, also suggest a decline in proliferation rates by ED15 (Walker and Catron, 2000; Kopf and Walker, 2009). The consequence of this developmental defect due to a lack of proliferation during critical stages of embryonic heart development could cause inhibited myocardial growth, leading to myocardial developmental disorders, such as thinning of the ventricular wall and absence of the compact layer, or hypertrabeculation. This occurs in humans, where non-compaction of the ventricular wall and hypertrabeculation is indicative of ventricular dysfunction (Chin *et al*., 1990). When chick embryos are exposed to dioxins, reduced proliferation rates are observed, further supporting a potential link between PCB exposures, reduced cardiomyocyte proliferation, and ventricular wall thinning (Henshel *et al*., 1993; Kopf and Walker, 2009).

PCB mix concentrations and environmentally relevant concentrations

Our study utilized a 58 congener PCB mix developed from contaminant analysis of sandpiper eggs collected along the Hudson River (Echols *et al*., 1996; 2004; Tillitt *et al*., 2011). Contaminant analysis from eggs collected from the UHR showed wild bird populations to have concentrations ranging from 5.9 to 29.5 µg/g egg wt, with variability occurring from year to year (McCarty and Secord, 1999; Secord *et al*., 1999; and Custer *et al*., 2010c). The PCB mix observed in these populations was obtained from tree

swallow eggs that contained 66 primary PCB congeners (Echols *et al*., 2004; Tillitt *et al*., 2011; Dean *et al*., unpublished). While the concentrations utilized in our study are 10-100 fold lower than wild bird populations, it has been well established that chickens are more sensitive to PCB exposure through AhR mediated responsiveness and CYPIA induction (Head *et al*., 2008; van den Berg, *et al.*, 1998; Kennedy *et al*., 1996; Elliot *et al*., 1997). By using a highly sensitive model for our experiment, we were able to use dose concentrations well below environmental ranges found in wild eggs. Moreover, this dose was sufficient to result in heart effects related to both lethal and non-lethal dose concentrations. This becomes very important in identifying how relevant PCB mixtures affect heart development in surviving and non-surviving bird populations.

Future direction

Through this study, our lab conclusively determined that exposure to an environmentally relevant PCB mixture causes dose-dependent lethality. In addition, embryos taken to hatch showed severe cardiomyopathies, including thinning of the ventricular wall, hypertrabeculation, ventricular dilation, arrhythmias, and most commonly, absences of the compact layer of the ventricular wall. Future work will identify (1) early embryonic stages of cardiac development affected by PCB exposure, and determine if these effects are related to (2) proliferation and apoptosis of cardiomyocytes, or (3) proteins critical in ventricular wall morphology. The PCB mix affects heart development in chickens, implicating a decreased overall fitness in these animals following *in ovo* exposure. Future studies should be aimed at determining the impact of PCBs, heart effects, and overall avian fitness.

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References

Ahlborg UG, Brouwer A, Fingerhut MA, Jacobson JL, Jacobson SW, Kennedy SW, Kettrup AA, Koeman JH, Poiger H, Rappe C. 1992. Impact of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls on human and environmental health, with special emphasis on application of the toxic equivalency factor concept. Eur J Pharmacol 288:179-199.

Aulerich RJ, Ringer RK. 1977. Current status of PCB toxicity to mink, and effect on their reproduction. Arch Environ Contam Toxicol 6:279.

Barron MG, Galbraith H, Beltman D. 1995. Comparative biochemistry and physiology Part C: Pharmacology, toxicology, and endocrinology 112:1-14.

Barsotti DA, Marlar RJ, Allen JR. 1976. Reproductive dysfunction in rhesus monkeys exposed to low levels of polychlorinated biphenyls (Aroclor 1248). Food Cosmet Toxicol 14:99-103.

Bellairs R, Osmond M. 2005. The Atlas of Chick Development 2nd Edition. Elsevier Academic Press, San Diego, CA, USA.

Chin TK, Perloff JK, Williams RG, Jue K, Mohrmann R. 1990. Isolated noncompaction of left ventricular myocardium. A study of eight cases. Circulation 82: 507-513.

Custer TW, Custer CM, Hines RK. 2002. Dioxins and congener specific polychlorinated biphenyls in three avian species from Wisconsin River, Wisconsin. Environ Pollut 119:323-332.

Custer CM, Custer TW, Dummer PM, Munney KL. 2003. Exposure and effects of chemical contaminants on tree swallows nesting along the Housatonic River, Berkshire County, Massachusetts, USA, 1998-2000. Environ Toxicol Chem 22:1605-1621.

Custer TW, Gray BR, Custer CM. 2010a. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicol 19:391-404.

Custer TW, Custer CM, and Gray BR. 2010b. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in belted kingfisher eggs from Upper Hudson River Basin, New York, USA. Environ Toxicol Chem 29:99-110.

Custer CM, Gray R, Custer TW. 2010c. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallow (Tachycineta bicolor). Environ Toxicol Chem 29:909-921.

Custer CM, Custer TW, Dummer PM. 2010d. Patterns of organic contaminants in eggs of an insectivorous, an omnivorous, and a piscivorous bird nesting on the Upper Hudson River, New York, USA. Environ Toxicol Chem. 29: (epublished).

DeWitt JC, Millsap DS, Yeager RL, Heise SS, Sparks DW, Henshel DS. 2006. External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol Chem 25: 541–551.

Echols KR, Meadows, JC, Tillitt DE, Secord A. 1996. Congener-specific analysis of polychlorinated biphenyl residues in tree swallow chicks, eggs, and other biota from the Hudson River, W.U. 30096. USGS Final Laboratory Report FY97-30-01.

Echols KR, Tillitt DE, Nichols JW, Secord AL, McCarty JP. 2004. Accumulation of PCB congeners in nestling tree swallows (Tachycineta bicolor) on the Hudson River, New York. Environ Sci Tech 38:6240-6246.

Eisler R. 1986. Polychlirinated biphenyl hazards in fish, wildlife, and invertebrates: a synoptic review. Biol Rep 85:1.7, USFWS, Washington DC.

Elliott JE, Wilson LK, Langelier KW, Norstrom RJ. 1996. Bald eagle mortality and chlorinated hydrocarbon contaminants in livers from British Columbia, Canada, 1989-1994. Environ Pollut 94: 9-18.

Frame GM. 1997. Congener specific PCB analysis. Analyt Chem 69:468-478.

Frame GM, Cochran JW, Bowad SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, qualitative, congener-specific analysis. J High Res Chromat 19:657-668.

Golub MS, Donald JM, Reyes JA. 1991. Reproductive toxicity of commercial PCB mixtures: LOAELs and NOAELs from animal studies. Environ Health Perspect 94:245-253.

Gould JC, Cooper KR, Scanes CG. 1999. Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeidinase in the chick embryo. Ecotox and Environ Saf 43:195-203.

Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environ Sci Technol 42:7535-7541.

Heid SE, Walker MK, Swanson HI. 2001. Correlation of cardiotoxicity mediated by halogenated aromatic hydrocarbons to aryl hydrocarbon receptor activation. Toxicol Sci 61:187-196.

Henshel DS, Hehn B, Vo MT, Steeves JD. 1993. A short-term test for dioxin teratogenicity using chicken embryos, *Environmental Toxicology and Risk Assessment,* Vol 2. STP 1216. American Society for Testing and Materials, Philadelphia, PA, pp 159–174.

Jacobson JL, Jacobson SW, Humphrey HEB. 1990. Effects of in utero exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. J Pediat 116:38-45.

Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, Elixson M, Warnes CA, Webb CL. 2007. Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation 115:2995-3014.

Jeter JR, Cameron IL. 1971. Cell proliferation patterns during cytodifferentiation in embryo chick tissues: liver, heart and erythrocytes. J Embryol Exp Morphol 23:403-422.

Johnson BL, Hicks HE, Cibulas W, Faroon O, Ashizawa AE, DeRosa CT, Cogliano J, Clark M. 2000. Public health implications of exposure to polychlorinated biphenyls (PCBs). Agency for Toxic Substances and Disease Registry, Div of Toxicol, Atlanta, GA, USA.

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol and Appl Pharmacol 141:214-230.

Kirby ML. 2007. Cardiac Development. Oxford University Press, New York, New York, USA.

Kopf PG, Walker MK. 2009. Overview of developmental heart defects by dioxins, PCBs, and pesticides. Environ Sci Health 27:276-285.

Lavoie ET, Grasman KA. 2007. Effects of *in ovo* exposure to PCBs 126 and 77 on mortality, deformities, and post-hatch immune function in chickens. Toxicol Environ Health, Part A 70:547-558.

McCarty JP, Secord AL. 1999. Reproductive ecology of tree swallows (Tachycineta bicolor) with high levels of polychlorinated biphenyl contamination Environ Tox Chem 18:1433-1439.

McNabb FM. 2005. Biomarkers for the assessment of avian thyroid disruption by chemical contaminants. Avian Poult Biol Rev 16:3-10.

Meeker JD, Hauser R. 2010. Exposure to polychlorinated biphenyls (PCBs) and male reproduction. Sys Biol Repro Med 56:122-131.

Nebeker AV, Puglisi FA, DeFoe DL. 1974. Effect of polychlorinated biphenyl compounds on survival and reproduction of the fathead minnow and flagfish. Trans Am Fish Soc 103:562.

Rushneck DR, Beliveau A, Fowler B, Hamilton C, Hoover D, Kaye K, Berge M, Smith T, Telliard WA, Roman H, Ruder E, Ryan L. 2004. Concentrations of dioxin-like PCBs in unweather Aroclors by HRGC/HRMS using EPA method 1668A. Chemosphere 54:79-87.

Safe SH. 1994. Polychlorinated biphenyls (PCBs). Environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87-149.

Secord AL, McCarty JP, Echols KR, Meadows JC, Gale RW, Tillitt DE. 1999. Polychlorinated bipheyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from Upper Hudson River, New York State, USA. Environ Toxicol Chem 18:2519-2525.

Silberhorn EM, Glauert HP, Robertson LW. 1990. Carcinogenicity of Polyhalogenated biphenyls: PCBs and PBBs. Crit Rev Toxicol 20:440-496.

Sommer RJ, Hume AJ, Ciak JM, Vannostrand JJ, Figgens M, Walker MK. 2005. Early developmental 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure decrease chick embryo heart chronotropic response to isoproterenol but not to agents affecting signaling downstream of beta-adrenergic receptor. Toxicol Sci 83:363-371.

TAMS/Gradient. 1991. *Phase 1 Report, Review Copy, Interim Characterization and Evaluation, Hudson River PCB Reassessment RI/FS.* USEPA Work Assignment No. 013-2N84. Prepared for USEPA by TAMS Consultants, Inc. and Gradient Corporation.

Thomas PT, Hinsdill RD. 1978. Effects of polychlorinated biphenyls on the immune responses of rhesus monkeys and mice. Toxicol and Appl Pharmicol 44:41-51.

Tillitt DE, Zajicek JL, Nicks DK. 2011. Preparation of individual and custom PCB mixture dosing solutions for avian egg injection studies associated with injury determinations under Hudson River NRDA. USCG Biochemistry and Physiology Branch. Final Report. Hudson River Natural Resource Trustees, New York.

Tilson HA, Jacobson JL, Rogan WJ. 1990. Polychlorinated biphenyls and the developing nervous system: Cross-species comparisons. Neurotox Teratol 12:239-248.

Van den Berg M, Birbaum LS, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson PRE, Poellinger L, Safe S, Schrenk D, Tillitt DE, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg KJ. 1990. Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin, and thyroid binding globulin. Chem Biol Interact 76:63-75.

Walker MK, Pollenz RS, Smith SM. 1997. Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with tetrachlorodibenzo-p-dioxin induced heart defects. Toxicol and Appl Pharmacol107:1075-1083.

Walker MK, Catron TF. 2000. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early embryo development. Toxicol and Appl Pharmacol 167:210-221.

CHAPTER 4

THE EFFECTS OF AN ENVIRONMENTALLY RELEVANT 58 CONGENER POLYCHLORINATED BIPHENYL (PCB) MIXTURE ON CARDIAC DEVELOPMENT AT THREE CRITICAL STAGES OF EMBRYONIC DEVELOPMENT IN THE DOMESTIC CHICK

Abstract

Embryonic exposure *in ovo* to a 58 congener PCB mixture containing relative proportions of primary congeners measured in sandpiper eggs collected along the upper Hudson River, NY, resulted in observed teratogenic heart defects in chick embryos at critical heart developmental stages (Hamburger Hamilton (HH) 10, HH16, and HH20.) Embryos were treated with 0.08 and 0.50 µg/g egg wt at embryonic day 0, prior to incubation. Mortality of exposed embryos was increased at all developmental stages, with a marked rise in cardiomyopathies at HH16 and HH20 ($p<0.05$). Heart abnormalities occurred across all treatments, including abnormal elongation and expansion of the heart tube at HH10, improper looping and orientation, indentations in the emerging ventricular wall (HH16, HH20), and irregularities in overall heart shape (HH10, HH16, HH20). Histology was conducted on two cardiac proteins critical to embryonic heart development, ventricular myosin heavy chain (VMHC) and titin, to investigate potential mechanistic effects of PCBs on heart development, but no difference was observed in spatio-temporal expression. Similarly, cellular apoptosis in the developing heart was not affected by exposure to this PCB mixture. Conversely, cardiomyocyte proliferation rates dramatically declined ($p<0.01$) at HH16 and HH20, correlating with increased PCB exposure concentrations. Early embryonic cardiomyocyte proliferation contributes to proper formation of the morphology and overall thickness of the ventricular wall. Therefore, *in ovo* exposure to a PCB mixture at critical stages adversely impacts embryonic heart development.

Introduction

Polychlorinated biphenyls (PCBs) are synthetic compounds made up of a biphenyl molecule with one to ten chlorine substitutions. PCBs were used extensively in manufacturing until their ban in 1979. Because PCBs are lipophilic, bioaccumulative, and resistant to degradation, they have become ubiquitous in the environment, representing a continuous source of exposure for animals residing in contaminated areas (Johnson *et al*., 2000). PCB mixtures found in the environment have been identified as probable carcinogens in animals (Silberhorn *et al*., 1990; Safe, 1994), linked to serious effects of the immune system in non-human primates (Thomas and Hinsdill, 1978), reproductive defects in non-human primates and other animals (Barsotti *et al.*, 1976; Golub *et al*., 1991), and endocrine disruption in mammalian, avian, and fish studies (van de Berg 1990; Gould *et al*., 1999). Furthermore, studies of human populations exposed to PCB mixtures indicate neurological effects, including learning deficits, decreases in visual recognition, and lower aptitudes on standardized cognitive tests (Jacobson *et al.*, 1990).

While some exposure effects are potentially lethal, sub-lethal exposures still may confer decreased fitness of surviving animals. For example, effects of PCBs on embryonic heart development and implications of overall health and fitness have recently received attention. Adult passerine birds exposed to multiple environmental contaminants, including PCBs, had offspring with cardiac malformations including decreased heart index and abnormalities in heart morphology such as ventricular wall thinning and abnormal apical shape (DeWitt *et al*., 2006). Additional studies in domestic chick embryos have shown dioxins and dioxin-like PCB congeners increase ventricular

septal defects, overall heart weight, and dilate ventricular cavities when examined on embryonic day (ED) 15 of development (Walker *et al*., 1997; Walker and Catron, 2000). Similarly, wild bird (tree swallow) embryos exposed to a single dioxin-like congener (PCB77) did not properly form the compact layer of the ventricular heart wall at hatch (Carro *et al*., unpublished). To elucidate the etiology of the heart defects observed in wild birds, we tested an environmentally relevant PCB mixture in the chick embryo to determine a lethality curve and investigate potential cardiomyopathies in hatchlings (Carro *et al*., unpublished). This mixture contained 58 congeners in relative proportions of primary congeners measured in sandpiper eggs collected along the upper Hudson River (UHR), NY (Tillitt *et al*., 2011). Based on the results of our chick study, two doses of the 58-congener PCB mixture (PCB mix) were selected for low dose (0.08 µg/g egg wt) and high dose (0.50 µg/g egg wt) treatments.

An important limitation of previous studies on PCB heart effects is confounding factors operating in tandem in wild bird exposures. While many wildlife studies have been comprehensive and elegant, they have usually occurred at Superfund sites with different Aroclor mixture exposures, varying concentrations of maternal deposition into eggs, and fluctuating annual PCB exposure rates (Ankley *et al*., 1993, DeWitt *et al*., 2006, Custer *et al*., 1998; 2000; 2002; 2003). The variation of environmental conditions makes the determination of specific effects of local PCB mixtures difficult in wild bird populations. Extensive measurement of PCB congener concentrations in eggs, embryos that did not survive, and nestlings have been sampled from the UHR; however, cardiac morphology has not been examined (Secord *et al*., 1999; Custer *et al*., 2010 a,b,c, and d). We propose that non-surviving embryos impacted by PCB exposure effects, ultimately

leading to mortality, may be the result of developmental impairment of the cardiovascular system. The chicken embryo provides an ideal model for studying effects of environmental contaminants on embryonic heart development. The chicken egg is a closed system model, the developing heart is very similar to mammalian species, PCB mechanism of action in birds has been identified (Kennedy *et al*., 1996) and chicken embryonic development has been well categorized in recent years (Bellairs and Osmond, 2005). Therefore, the purpose of this study was to investigate developmental periods in which heart development is most sensitive to PCB exposure while limiting environmental variation in a lab model, the chick embryo.

Our previous study documented cardiomyopathies in chick hatchlings following exposure to the PCB mix (Carro *et al*., unpublished). The most prevalent abnormality was an absence of the compact layer of the ventricular heart wall. PCBs also affected heart wall morphology overall, particularly in the ventricles. Ventricular myosin heavy chain (VMHC) protein was identified as a potential target protein for PCB exposure because it is essential for developing the structural integrity of the heart wall during differentiation (Bourke *et al*., 1991; Bisaha and Bader 1991; Bandman and Rosser, 2000; Somi *et al.*, 2006). A second protein, titin (connexin in mammals), was identified as a potential PCB target because of its role in the development of proper myofibril orientation and its expression during early embryonic heart formation (Tokuyasu and Maher, 1987). Additionally, phospho-histone-H3 (pHH3) was the protein identified to study cardiomyocyte proliferation, which is essential to proper compact layer formation, the most prevalent heart defect in the chick hatchling study (Jeter and Cameron, 1971;

Tapia *et al*., 2006). Finally, apoptosis was examined using a TUNEL assay to detect cell death in the heart at critical stages of embryonic development (Schaefer *et al*., 2004).

Although previous work has shown a correlation between PCB exposure and heart defects in hatchlings, the stages of embryonic development in which effects first manifested have not been identified. Taking into account a low and high concentration PCB dose and potential target proteins, we now show that PCBs affect embryonic heart development at very early stages, Hamburger Hamilton stage (HH) 10, HH16, and HH20 (Hamburger and Hamilton, 1951). Additionally, we now report decrease in proliferation of the heart field following PCB exposure in chicken embryos.

Materials and Methods

Egg injections

Fertile broiler chicken (*Gallus domesticus*) eggs were purchased from Allen's hatchery (Seaford, DE). The PCB mix was made up of primary PCB congeners prepared in the relative concentrations found in sandpiper eggs collected at the UHR. The mixture was prepared by the USCG Biochemistry and Physiology Branch (Tillitt *et al*., 2011). The PCB mix was solubilized in activated charcoal-stripped corn oil (Dean *et al*., unpublished) at two concentrations: 0.08 and 0.50 µg/g egg wt. Both a charcoal-stripped corn oil vehicle treatment and untreated eggs served as controls.

Eggs were weighed at ED0 and grouped randomly to have egg weights evenly distributed across treatments (n=20 eggs/treatment). Treatments were administered as follows: the egg was swabbed with alcohol and a small hole was drilled into the side of the egg using a sterile Dremel bit (Dremel Co, Racine, WI). Injection volume was calculated for each egg and a total of 0.9-1.11 µl (based on egg wt) was administered into

the hole using a reverse displacement pipettor (Rainin Instrument LLC, Oakland, CA). Eggs were sealed with melted paraffin wax and incubated (air cell up) at 37ºC and 65% humidity.

Embryo collections

Embryos were staged based on HH staging criteria; number of paired somites and visible morphological differences between stages (Hamburger and Hamilton, 1951). Embryos were collected at HH10, HH16, and HH20. Unfertile eggs were not included and fertile eggs with dead embryos at the time of embryonic harvest were recorded only for mortality data. Embryos were stained using India ink and visualized under a stage microscope (Wild M3Z Type S, Heerbrugg, Switzerland). Embryos were then harvested off of the yolk, transferred to Ringer's solution and trimmed. For HH16 and HH20 embryos, the thoracic region and extraneous membranes were removed to facilitate penetration of antibodies.

Embryo fixation and immunohistochemistry

Fresh embryos were rinsed in 1x phosphate-buffered saline (PBS) and transferred to 4% buffered paraformaldehyde (PFA) solution overnight. Embryos were then washed in 1xPBS and prepared for immunohistochemistry immediately to detect VMHC and titin in whole mount. Additional detection of VHMC, titin, and pHH3 was completed on serial transverse sections of the heart region to determine spatio-temporal protein expression and cardiomyocyte proliferation in the heart field. Transverse sections were obtained serially at a thickness of 14 µm (Wu *et al*., 2011). The spatial distribution of VMHC and titin were assessed using anti-VMHC and anti-titin primary antibody supernatant (Developmental Studies Hybridoma Bank, Iowa University).

VMHC Immunohistochemistry: Following fixation in 4% PFA, embryos were washed with 1xPBS. Embryos were blocked in 10% heat-treated sheep serum (HT ss) in PBS containing 0.1% Tween-20 (PTW). Anti-VMHC antibody (1:100 in 5% HT ss in PTW) was added and the embryos were incubated at 4ºC overnight. Primary antibody was washed off with PTW and Alexa-Fluor-488 secondary antibody (goat anti-mouse IgG_1-488; Molecular probes, 1:200) was diluted in 5% HT ss in PTW and incubated at 4ºC overnight. The embryo was washed in PTW and imaged on an inverted microscope (Zeiss AxioObserver.Z1, Carl Zeiss, Inc., Thornwood, NY), with images captured using the AxioCam HRc and Axiovision software (Carl Zeiss, Inc., Thornwood, NY).

Titin immunohistochemistry: Following fixation and wash in 4% PFA and 1xPBS respectively, embryos were blocked in 0.1% Triton X-100 in 1x PBS-A (0.02% sodium azide, 0.01M glycine, and 0.002% Triton X-100 solubilized in 1xPBS) overnight at 4ºC. An enzymatic procedure ensued for 30 min at room temperature (2,000 U/ml Hyaluronidase in PBS-A). Anti-titin antibody (1:50 in 5% HT ss in PBS-A) was added and embryos were incubated overnight at 4ºC. Primary antibody was washed off in PBS-A and AlexaFluor 594 secondary antibody (goat anti mouse-IgM-594, Molecular Probes; 1:200) was diluted in PBS-A and incubated overnight at 4ºC. The embryo was washed in PBS-A and imaged on an inverted microscope (protocol adapted from Tokuyasu and Maher, 1987) as described above.

pHH3 immunohistochemistry: Phospho-histone H3 immunochemistry was performed on sections according to the protocol of Wu *et al*. (2011). Briefly, mounted sections were incubated in a primary antibody concentration of 1:500 in PTW (Millipore Ser10, Billerica, MA), sections were washed, then incubated with a secondary antibody used

was AlexaFluor 594 (goat anti rabbit IgG-594) at a concentration of 1:200 in 5% HT ss in PTW. Sections were rinsed, cover-slipped with Fluoromount G (Fisher Scientific, Pittsburg, PA), and imaged on an inverted microscope as described above.

TUNEL assay

The TUNEL procedure was used to detect apoptotic cells (*in situ* cell death detection kit *fluorescein*, Roche Applied Sci, IN). Sections were degelatinized and post-fixed in 4% PFA. Following a wash in 1xPBS, sections were permeabilized, reacted, and incubated according to instructions (Roche Diagnostics, Mannheim, Germany). All sections were DAPI (4',6-diamidine-2-phenylidole-dichloride) stained to identify cell nuclei, rinsed in PTW, and mounted using Fluoromount G (Fisher Scientific, Pittsburg, PA). Four embryos per stage for each treatment were analyzed (n=4).

Identification of cardiomyopathies, cell counts, and heart field area

Cardiomyopathies were identified and recorded during whole mount imaging and confirmed by analysis of cross sections. Serial images were captured from at least 7 embryos per treatment and the average numbers of pHH3 positive cells (nucleus identified by DAPI staining) in the heart field was recorded (heart field identified by VMHC staining). The mean number of proliferating cells per treatment (averaged across serial sections) and standard error was calculated, and significant differences between treatments were analyzed using a one-way ANOVA and Tukey's post-hoc test. The area of the heart field was measured across serial sections and captured from embryos for each treatment. The average area across sections was used to calculate mean area per

treatment and standard error. Measurements were taken using ImageJ 1.43 U software (NIH, Bethesda, MD).

Statistical analysis

Significant differences in mortality and cardiomyopathies across treatments were analyzed by a one-way ANOVA and a Tukey's post-hoc test. Proliferative, apoptotic, and heart area differences were determined using a one-way ANOVA and a Tukey's post-hoc test.

Results

Embryo survival and cardiomyopathies

Significant embryonic mortality was observed in PCB mixture-exposed embryos (Figure 1, HH16 and HH20). Cardiomyopathies were observed in whole mount and confirmed in sections across all treatments, with significantly more abnormalities at the high dose of HH16 and the low dose of HH20 (Figure 2). Heart abnormalities included elongation (Figure 3B) and shortening of the heart tube (Figure 8B,C) (HH10, 16, 20), improper looping (Figure 3H: Figure 8) and orientation (Figure 3E) of the heart (HH16, 20), and irregularities in heart wall morphology (Figure 3C, I: Figure 8E,F,I) (HH10, 16, 20), with abnormalities identified across all treatments.

VMHC protein immunohistochemistry

Whole mount immunohistochemistry, followed by transverse sectioning, showed VMHC expression throughout the developing chick heart at HH10 (Figures 3, 4). At HH16 and 20, VMHC distribution was prevalent in the differentiating ventricular chamber and expressed to a lesser degree in the differentiating atrial chamber (Figures 3,

5, 6). This expression pattern was expected, as described previously (Bourke *et al*., 1991). There was no difference in spatio-temporal expression of VMHC following PCB treatment.

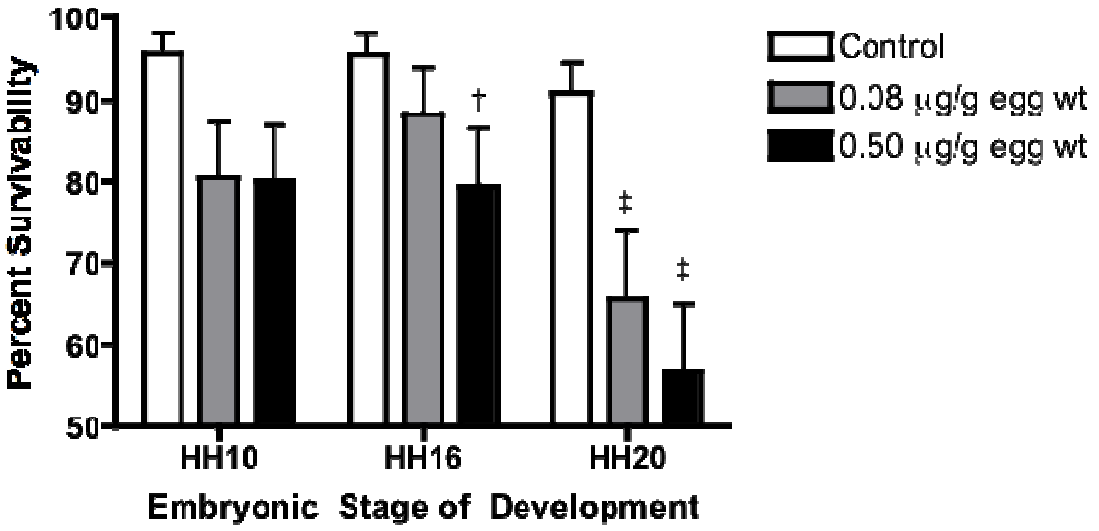
Titin protein and immunohistochemistry

Whole mount immunohistochemistry, followed by transverse sectioning (Figure 8, transverse sections not shown) showed titin expression at HH10 throughout the developing chicken heart. At HH16 and 20, titin was expressed throughout the developing heart, in both differentiating chambers, and was distributed as striations in the developing somites (micrographs not shown). This expression was expected as described previously (Greaser, *et al.*, 1985).

Measurements of cellular proliferation and apoptosis

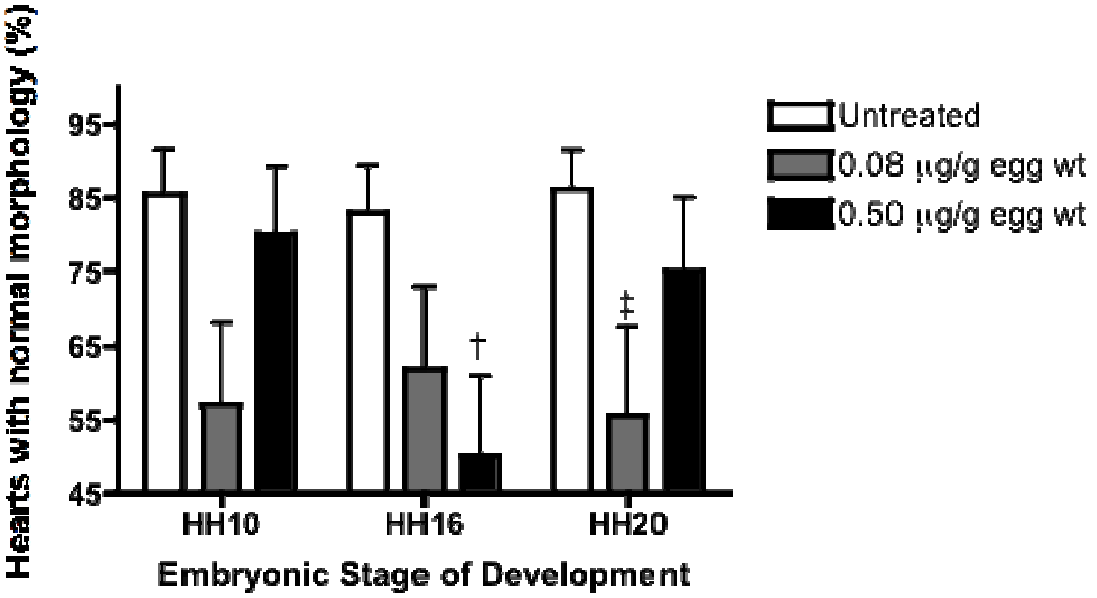
Average proliferation rates of cells (Figure 7) within the heart field by pHH3 immunostaining in transverse sections revealed no difference in proliferation rates in the heart at HH10 (Figure 4; *white arrowheads indicate proliferating cells in the heart field*) but a significant decrease at HH16 and HH20 (Figures 5, 6; *white arrowheads indicate proliferating cells in the heart field*) in PCB treatment groups. Apoptosis rates within the heart field at HH10, HH16, and HH20 were not affected (data not shown).

Figure 1: Percent survival of domestic chicken embryos at HH10, HH16, and HH20 following PCB exposure.



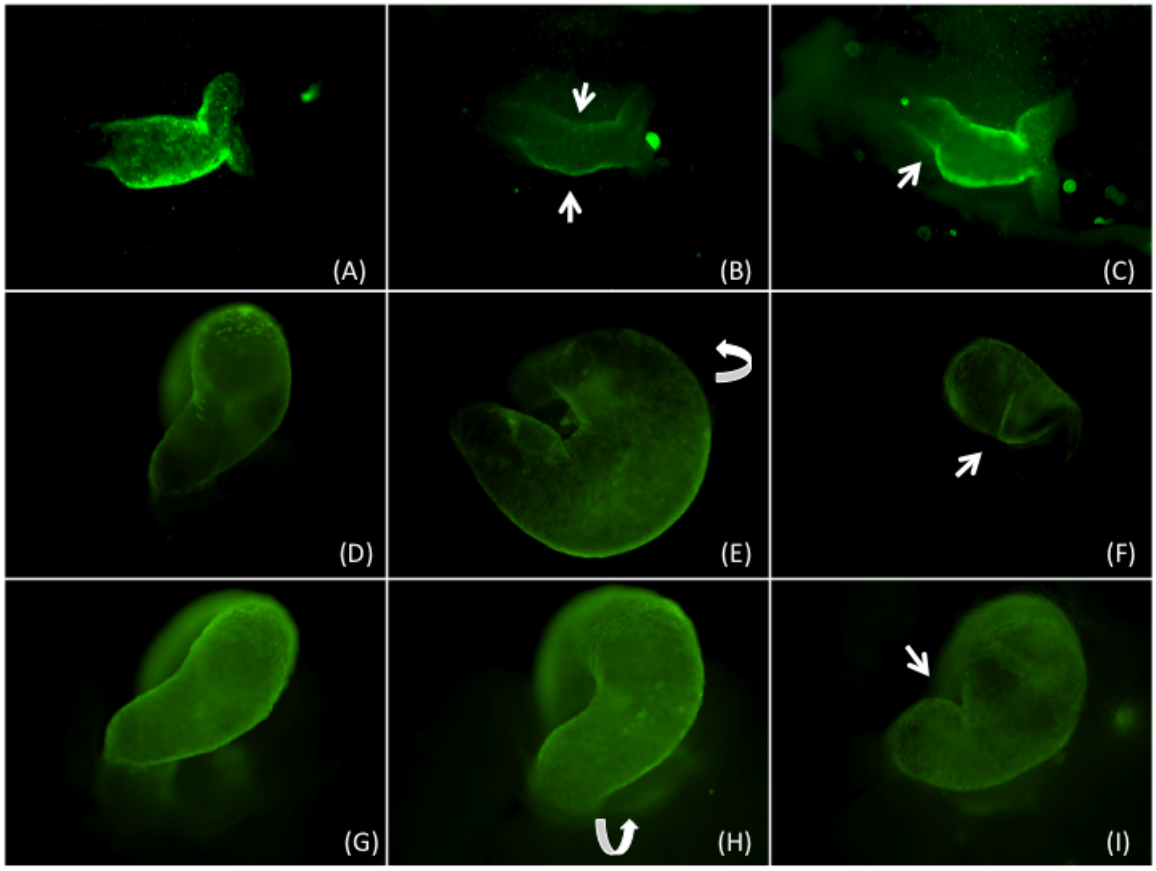
One-way ANOVA showed no differences across treatments at HH10. There was a significant effect of PCB mixture exposure in chick embryos on mortality at HH16 (†) at the high dose and at HH20 (‡) in both PCB treatments (Tukey's $p<0.05$, $p<0.01$, respectively).

Figure 2: Percentage of hearts with normal morphology in surviving chicken embryos at HH10, HH16, and HH20 following PCB exposure.



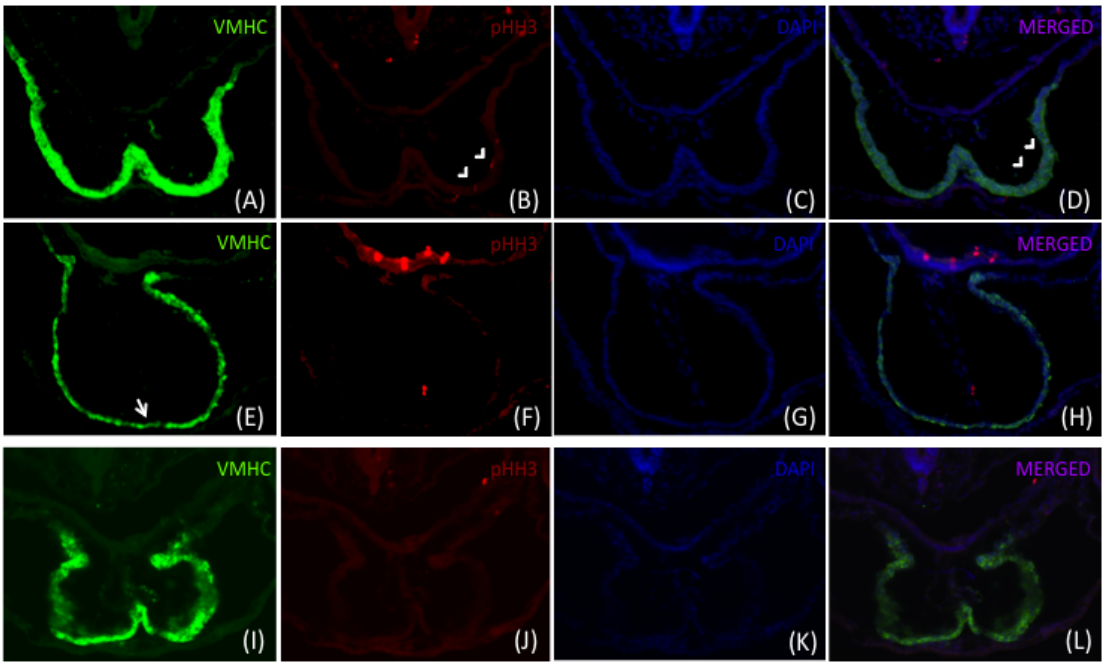
One-way ANOVA did not reveal a difference across treatments at HH10 following exposure to the PCB mixture. There was a significant increase in cardiomyopathies when compared to the control group in the high dose at HH16 and in the low dose at HH20 (Tukey's; $p<0.05$). Note: untreated and vehicle groups were combined in this data set as a control group.

Figure 3: Whole mount immunohistochemistry of ventricular myosin heavy chain in the developing heart in surviving chicken embryos at HH10, HH16, and HH20 following PCB exposure.



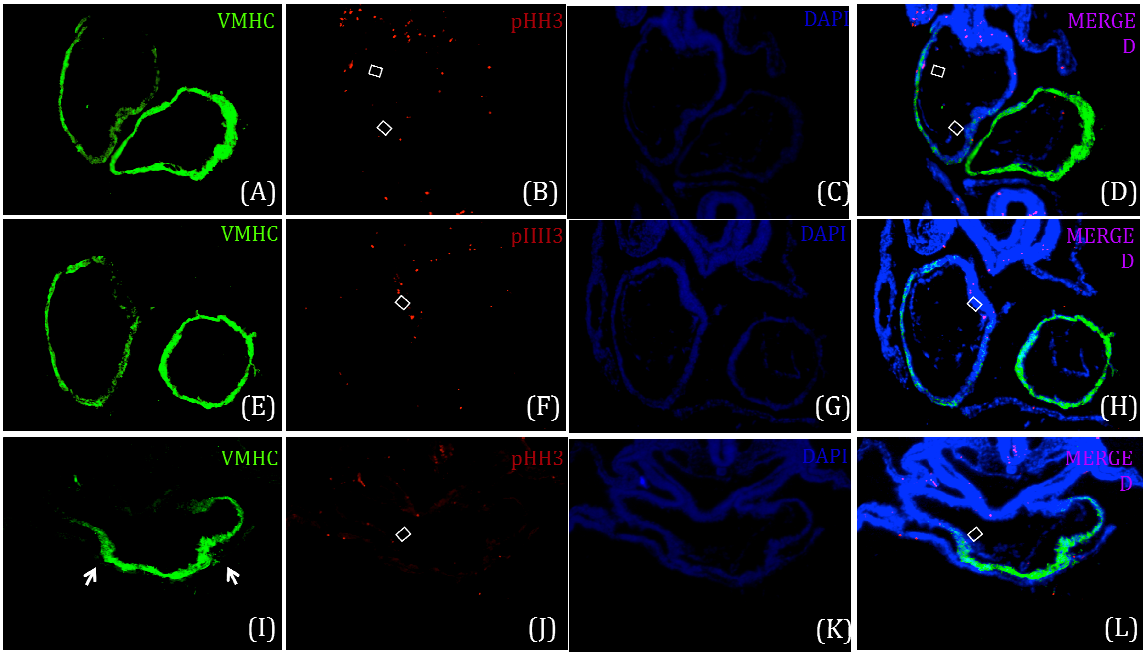
VMHC protein distribution across treatments at each stage showed no difference in spatio-temporal expression (images captured using 5x objective). HH10 embryos (A), (B), and (C) are control, low dose, and high dose treatments, respectively. HH16 embryos (D), (E), (F) are control, low dose, and high dose treatments, respectively. HH20 embryos (G), (H), (I) are control, low dose, and high dose, respectively. Single arrowheads in (B), (C), (F), and (I) indicate cardiomyopathies. Looped arrowhead in (E) and (H) indicate improper looping pattern and orientation. Abnormalities and incorrect looping pattern were not exclusive to individual treatments.

Figure 4: Ventricular myosin heavy chain and phospho-histone-H3 protein distributions at HH10 in the heart of the developing chicken embryo.



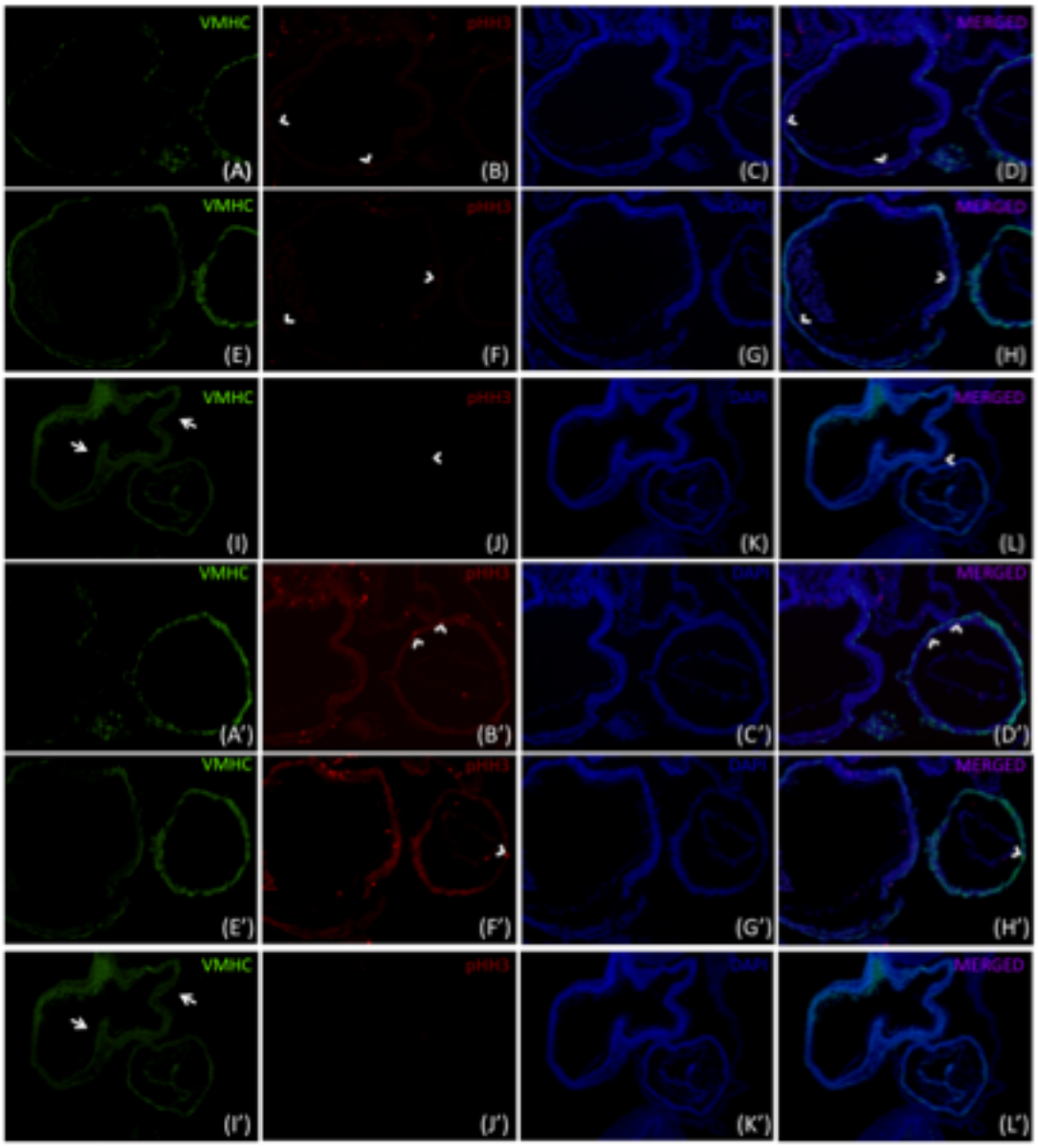
Transverse sections taken through the chick embryo at HH10 following whole-mount immunohistochemistry for VMHC protein; (A), (E), (I). PHH3 staining of (A,E,I) to mark proliferating cells (B), (F), (J). DAPI (40, 6-diamidine-2-phenylidole-dihydrochloride) staining (blue) of (A,E,I) to mark cell nuclei; (C), (G), (K). Merge images; (D), (H), (L). White arrows indicate cardiomyopathies (heart abnormalities) while white arrowheads point to proliferating cells in the heart. Images were captured using a 20x objective.

Figure 5: Ventricular myosin heavy chain and phospho-histone-H3 protein distributions at HH16 in the heart of the developing chicken embryo.



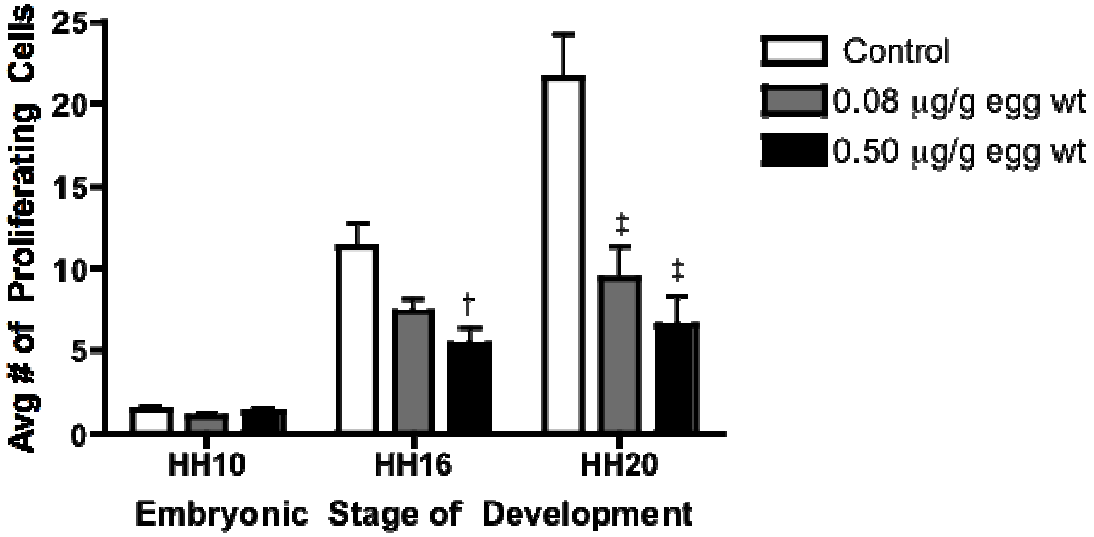
Transverse sections taken through the chick embryo at HH10 following whole-mount immunohistochemistry for VMHC protein; (A), (E), (I). PHH3 staining of (A,E,I) to mark proliferating cells (B), (F), (J). DAPI (40, 6-diamidine-2-phenylidole-dihydrochloride) staining (blue) of (A,E,I) to mark cell nuclei; (C), (G), (K). Merged images; (D), (H), (L). White arrows indicate cardiomyopathies (heart abnormalities) while white arrowheads denote proliferating (PHH3-positive) cells in the heart. Images were captured using a 10x objective.

Figure 6: Ventricular myosin heavy chain and phospho-histone-H3 protein distributions at HH20 in the heart of the developing chicken embryo.



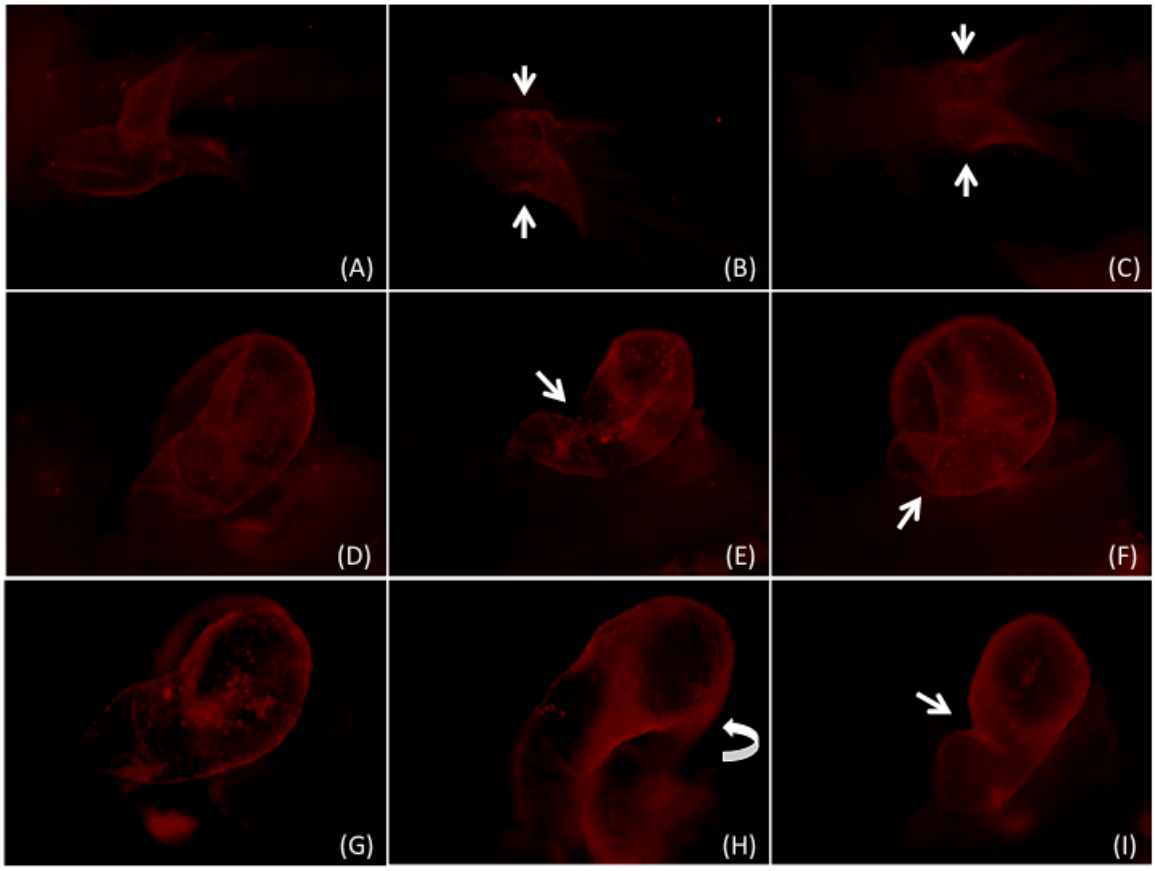
Transverse sections taken through the chick embryo at HH10 following whole-mount immunohistochemistry for VMHC protein; (A), (E), (I). PHH3 staining of (A,E,I) to mark proliferating cells (B), (F), (J). DAPI (40, 6-diamidine-2-phenylidole-dihydrochloride) staining (blue) of (A,E,I) to mark cell nuclei; (C), (G), (K). Merged images; (D), (H), (L). Prime letters (') are identical sections taken of the left chamber of the developing heart. White arrows indicate cardiomyopathies (heart abnormalities) while white arrowheads are examples of proliferating cells in the heart. Images were captured using a 10x objective.

Figure 7: Average number of proliferating cells within the heart field in surviving chicken embryos at HH10, HH16, and HH20 following exposure to PCB mixture.



One-way ANOVA showed no significant effects across treatments at HH10. There was a significant effect in the average number of proliferating cells in the heart field at HH16 and HH20. Tukey's post-hoc test showed a significant decrease (†) at the high dose when compared to control ($p<0.05$) at HH16 and at HH20 between control:low dose and control:high dose (‡) ($p>0.001$).

Figure 8: Whole mount immunohistochemistry of Titin in the developing heart in surviving chicken embryos at HH10, HH16, and HH20 following PCB exposure.



Titin protein distribution across treatments at each stage showed no difference in spatio-temporal expression (images captured using 5x objective). HH10 embryos (A), (B), and (C) are control, low dose, and high dose treatments, respectively. HH16 embryos (D), (E), (F) are control, low dose, and high dose treatments, respectively. HH20 embryos (G), (H), (I) are control, low dose, and high dose, respectively. Single arrowheads in (B), (C), (E), (F), and (I) indicate cardiomyopathies. Looped arrowhead (H) indicates improper looping pattern and orientation. Abnormalities and incorrect looping pattern was not exclusive to individual treatments.

Discussion

PCB mixture affects survivability

Decreased survival occurred with PCB mixture exposure in chick embryos at HH16 (0.50 µg/g egg wt) and HH20 (0.08 and 0.50 µg/g egg wt) (Tukey post hoc, $p<0.05$). Our previous hatchling study showed a similar percent survival at the low dose (Carro *et al*., unpublished). Although hatchling survival was not significantly lower in the previous study, there were fewer individuals than in this current study, attributing to this statistical difference. The range of survivability of controls was similar to those observed in other chick embryos studies (Brunstom and Danerud, 1983; Brunstrom and Orberg, 1982, Henshel 1993). Furthermore, the decrease in survival following PCB mixture exposure in this study is consistent with other avian studies using different mixtures (Gilbertson *et al.*, 1991; Peakall *et al*., 1973; Anne *et al.*, 1980; Barron *et al*., 1995).

PCB mixture impacts on heart development

Exposure to a PCB congener mixture affected normal heart development as early as HH10, with significant cardiomyopathies by HH16 and HH20. These data support previous studies using dioxin-like PCBs in which the chicken embryo was shown to have thinner ventricular walls and cardiac dilation at later stages of development (Kopf and Walker, 2009). The authors also compared piscine and murine embryos, which displayed teratogenic effects of the developing heart following exposure (Kopf and Walker, 2009). Additionally, studies conducted on wild bird nestlings at a PCB-contaminated site in Indiana further support our observations. In that study, environmentally exposed

passerine nestlings had evidence of thinning ventricular wall, microsurface roughness, abnormal heart indices, and changes in overall heart shape. Additionally, up to 30% of the surviving nestling populations had visible heart deformities (DeWitt *et al*., 2006). Exposure to PCBs in songbirds also affected heart development (Henshel and Sparks, 2006). Our data suggests that PCBs can affect heart development as early as HH10. Interestingly, it has been shown that TCDD, a dioxin with a similar chemical structure to PCBs, affected heart development as early as HH16 (Ahlborg *et al*., 1993; Henshel *et al*., 1993; 1994; Van den Berg *et al*., 1998; Elliot *et al*., 1996; Head *et al*., 2008).

Importantly, our data reveals that PCBs show a consistent, dose-related response at HH10 and HH16 associated with heart abnormalities. Higher concentrations of toxins lead to more detrimental effects on heart development (Stickel *et al*., 1997). Conversely, by HH20 our data reveals a higher percent of cardiomyopathies in our low dose (0.08µg/g egg wt) over our high dose (0.50 µg/g egg wt). This could be due to the fact that there was lower survival in high dose embryos at HH20. It is important to relate mortality or lethal dose with sub-lethal effects. The survivability of embryos at HH20 was 56% in the high dose and 65% in the low dose (a difference of 11%), while the percent cardiomyopathies in the high and low doses were 38% and 50%, respectively (a difference of 12%). Therefore, there are a greater number of low dose (0.08 µg/g egg wt)-surviving embryos may account for the higher detection of cardiomyopathies compared to the high dose treatment (0.50 µg/g egg wt) at HH20. This has been observed in other studies in which a lower percentage of abnormalities in a high dose may be attributed to the increase in mortality of the high dose following treatment (Calabrese and Baldwin, 2002). In risk assessments, the lethal effect of exposure does

not account for sub-lethal teratogenic effects, as suggested by this apparent disparity in the cardiomyopathy data (Davis and Svendsgaard, 1990). Therefore, the higher percent cardiomyopathies identified in the low dose of the HH20 embryos, although not consistent with dose concentration effects, can be interpreted as a sub-lethal teratogenic effect.

Spatio-temporal expression of proteins of interest

Exposure to the PCB mixture did not affect expression of VMHC or titin proteins. VMHC protein was expressed in the heart tube at HH10 and in the looped heart tube at HH16, with a diminished expression pattern in the atria at HH16. By HH20, when atrial-ventricular chamber differentiation was complete (Hamburger Hamilton, 1951), expression was still prevalent in the differentiated ventricle, but greatly diminished in the atrial chamber. This expression pattern is similar to observations in previous studies (Gonzalez-Sanchez and Bader, 1984, 1985; Sanders *et al*., 1986; Evans *et al*., 1988, Bourke *et al*., 1991). Titin protein was expressed throughout the heart at all stages examined; HH10, 16, and 20. In addition, titin was visible in striations within the somites at these stages (data not shown). The distribution pattern in the heart and somites was similar to previous observations (Wang and Greaser, 1985; Wang *et al*., 1988; Trombitas *et al*., 1997). VMHC and titin did not show abnormal patterns of expression at HH10, HH16, and HH20; however, the identification of heart abnormalities and decreased cardiomyocyte proliferation were present at these stages. This suggests VMHC and titin were not potential target proteins for PCB exposure at these stages of embryonic heart development (Somi *et al*., 2006; Kirby, 2006).

PCBs mixtures impacts rates of cardiomyocyte proliferation, but not cardiac apoptosis

Our study showed reduced proliferation of cardiomyocytes at HH16 and 20, but no change in rates of apoptosis at any stage studied. Cellular proliferation is essential to proper heart formation. During middle stages of embryonic development (HH31-HH41), the cardiomyocytes orient themselves into a compact layer that is highly proliferative and a trabeculated layer that has a much lower rate of proliferating cells (Jeter and Cameron, 1971; Kirby 2006). Myocardial cells, predominantly in the compact layer, proliferate until hatch in chicks (Kirby, 2006), with ventricular wall rates of proliferation peaking during these middle stages of development (Rychterova, 1971, 1976). When proliferation does not occur, myocardial growth is inhibited, usually leading to myocardial developmental disorders. In humans, noncompaction of the ventricular wall was identified as a disorder that could be linked to further ventricular dysfunction (Chin *et al*., 1990). The morphological thinning of the ventricular wall in nestlings and avian embryos could be explained by the reduced rates of proliferation seen in this study (DeWitt *et al*., 2006; Kopf and Walker, 2009). This idea is further supported by observations of reduce proliferation rates in chicken embryos exposed to dioxins (Henshel *et al.*, 1993).

Future direction

Through this study, we have identified three critical stages of embryonic heart development that are affected by PCB exposure. The cardiomyopathies identified at these early stages are possible precursors for abnormalities in heart morphology seen in previous studies at later stages of development, in nestlings and in adult birds. The identification of myocardial proliferation as a target for PCB exposure makes it possible

to focus on the understanding of the molecular mechanisms affected by PCB exposure such as (i) those underlying myocardial growth and proliferation, (ii) factors linked to ventricular wall abnormalities such as non-compaction and hypertrabeculation, and (iii) the gene and protein expression of these factors in the heart field at early embryonic stages of heart development.

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References

Ahlborg UG, Brouwer A, Fingerhut MA, Jacobson JL, Jacobson SW, Kennedy SW, Kettrup AA, Koeman JH, Poiger H, Rappe C. 1992. Impact of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls on human and environmental health, with special emphasis on application of the toxic equivalency factor concept. Eur J Pharmacol 288:179-199.

Ankley GT, Niemi GJ, Lodge KB, Harris HJ, Beaver DL, Tillitt DE, Schwartz TR, Giesy JP, Jones PD, Hagley C. 1993. Uptakes of planar polychlorinated biphenyls and 2,3,7,8-substituted polychlorinated dibenzofurans and dibenzo-p-dioxins by birds nesting in the lower Fox River and Green Bay, Wisconsin, USA. Environ Contam Toxicol 24:332-344.

Anne M, McLane R, Hughes DL. 1980. Reproductive success of screech owls fed Aroclor 1248. Environ Contam and Toxicol 9:661-665.

Bandman E, Rosser BW. 2000. Evolutionary significance of myosin heavy chain heterogeneity in birds. Miscrosc Res Tech 50: 473-491.

Barron MG, Galbraith H, Beltman D. 1995. Comparative reproductive and developmental toxicology of PCBs in birds. Comp Biochem and Physiol Part C 112:1-14.

Barsotti DA, Marlar RJ, Allen JR. 1976. Reproductive dysfunction in rhesus monkeys exposed to low levels of polychlorinated biphenyls (Aroclor 1248). Food Cosmet Toxicol 14:99-103.

Bellairs R, Osmond M. 2005. The Atlas of Chick Development 2nd Edition. Elsevier Academic Press, San Diego, CA, USA.

Bisaha JG, Bader D. 1991. Identification and characterization of a ventricular-specific avian myosin heavy chain, VMHC1: expression in differentiation cardiac and skeletal muscle. Dev Biol 148:355-364.

Bourke DL, Wylie SR, Wick M, Bandman E. 1991. Differentiating skeletal muscle cells initially express a ventricular myosin heavy chain. BAM 1:13-21.

Brunstrom B, Orberg J. 1982. A method for studying embryotoxicity of lipophilic substances and experimentally introduced into hens' eggs. Ambio 11:209-211.

Brunstrom B, Danerud PO. 1983. Toxicity and distribution in chick embryos of 3,3', 4, 4'-tetrachlorobiphenyl and in concentration/affinity of the hepatic receptor for 2,3,7,8-tetra-chlorodibenzo-p-dioxin. Comp Biochem Physiol 91:507-512.

Calabrese EJ, Baldwin LA. 2002. Hormesis: the dose-response revolution. Pharmacol Toxicol 43:175-197.

Chin TK, Perloff JK, Williams RG, Jue K, Mohrmann R. 1990. Isolated noncompaction of left ventricular myocardium. A study of eight cases. Circulation 82: 507-513.

Custer CM, Custer TW, Allen PD, Stromborg KL, Melancon M. 1998. Organochlorine contaminants and tree swallows nesting along the Fox River and in Green Bay, Wisconsin. Environ Toxicol Chem 17:1786-1798.

Custer CM, Custer TW, Coffey M. 2000. Organochlorine chemicals in tree swallows nesting in pool 15 of the upper Mississippi River. Bull Environ Contam Toxicol 64:341-346.

Custer TW, Custer CM, Hines RK. 2002. Dioxins and congener specific polychlorinated biphenyls in three avian species from the Wisconsin River, Wisconsin. Environ Pollut 119:323-332.

Custer CM, Custer TW, Dummer PM, Munney KL. 2003. Exposure and effects of chemical contaminants on tree swallows nesting along the Housatonic River, Berkshire County, MA, USA, 1998-2000. Environ Toxicol Chem 22:1605-1621.

Custer TW, Gray BR, Custer CM. 2010a. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicol 19:391-404.

Custer TW, Custer CM, and Gray BR. 2010b. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in belted kingfisher eggs from Upper Hudson River Basin, New York, USA. Environ Toxicol Chem 29:99-110.

Custer CM, Gray R, Custer TW. 2010c. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallow (Tachycineta bicolor). Environ Toxicol Chem 29:909-921.

Custer CM, Custer TW, Dummer PM. 2010d. Patterns of organic contaminants in eggs of an insectivorous, an omnivorous, and a piscivorous bird nesting on the Upper Hudson River, New York, USA. Environ Toxicol Chem. 29: (epublished).

Davis JM, Svendsgaard DJ. 1990. U-shaped dose-response curves: their occurrence and implications for risk assessment. J Toxicol Environ Health 30:71-83.

DeWitt JC, Millsap DS, Yeager RL, Heise SS, Sparks DW, Henshel DS. 2006. External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol Chem 25: 541–551.

Elliott JE, Wilson LK, Langelier KW, Norstrom RJ. 1996. Bald eagle mortality and chlorinated hydrocarbon contaminants in livers from British Columbia, Canada, 1989-1994. Environ Pollut 94: 9-18.

Evans D, Miller JB, Stockdale FE. 1988. Developmental patterns of expression and coexpression of myosin heavy chains in atria and ventricles of the avian heart. Dev Biol127:376–383.

Gilbertson M, Kubiak T, Ludwig J, Fox G. 1991. Great lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. J Toxicol Environ Health 33:455-520.

Gonzalez-Sanchez A, Bader D. 1985. Characterization of myosin heavy chain in myocardial cells of the conductive system of the chicken heart. J Cell Biol 100:270-275.

Gonzalez-Sanchez A, Bader D. 1984. Immunochemical analysis of myosin heavy chains in the developing chicken heart. Dev Biol 103:151-158.

Golub MS, Donald JM, Reyes JA. 1991. Reproductive toxicity of commercial PCB mixtures: LOAELs and NOAELs from animal studies. Environ Health Perspect 94:245-253.

Gould JC, Cooper KR, Scanes CG. 1999. Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeidinase in the chick embryo. Ecotox and Environ Saf 43:195-203.

Greaser ML, Handel SE, Wang SM, Schultz E, Bulinski JC, Lin JJ, Lessard JL. 1989. Assembly of titin, myosin, actin, and tropomyosin into myofibrils in cultured chick cardiomyocytes. In Stockdale F, Kedes L, eds, Cellular and Molecular Biology of Muscle Development, 93. New York, NY, USA, pp247-257.

Hamburger V, Hamilton HIL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49-92.

Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environ Sci Technol 42:7535-7541.

Henshel DS, Hehn B, Vo MT, Steeves JD. 1993. A short-term test for dioxin teratogenicity using chicken embryos, *Environmental Toxicology and Risk Assessment,* Vol 2. STP 1216. American Society for Testing and Materials, Philadelphia, PA, pp 159–174.

Henshel DS. 1994. Early embryonic teratogenic changes induced by *in ovo* exposure to TCDD. The Toxicologist 14:383.

Henshel DS, Sparks DW. 2006. Site Specific PCB-correlated interspecies differences in organ somatic indices. Eocotoxicol 15:9-18.

Jacobson JL, Jacobson SW, Humphrey HEB. 1990. Effects of *in utero* exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. J Pediat 116;38-45.

Jeter JR, Cameron IL. 1971. Cell proliferation patterns during cytodifferentiation in embryo chick tissues: liver, heart and erythrocytes. J Embryol Exp Morphol 23:403-422.

Johnson BL, Hicks HE, Cibulas W, Faroon O, Ashizawa AE, DeRosa CT, Cogliano J, Clark M. 2000. Public health implications of exposure to polychlorinated biphenyls (PCBs). Agency for Toxic Substances and Disease Registry, Div of Toxicol, Atlanta, GA, USA.

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 141:214-230.

Kirby ML. 2007. Cardiac Development. Oxford University Press, New York, New York, USA.

Kopf PG, Walker MK. 2009. Overview of developmental heart defects by dioxins, PCBs, and pesticides. J Environ Sci Health 27: 276-285.

Peakall DB, Peakall ML. 1973. Effect of polychlorinated biphenyl on the reproduction of artificially and naturally incubated dove eggs. J Appl Ecol 10:863-868.

Rychterova V. 1971. Principle of growth in thickness of the heart ventricular wall in the chick embryo. Folia Morphol 19:262-272.

Rychterova V. 1978. Development of proliferation structure of the ventricular heart wall in the chick embryo between the 6^{th} and 14^{th} day of embryogenesis. Folia Morphol 26:131-143.

Safe SH. 1994. Polychlorinated biphenyls (PCBs). Environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87-149.

Sanders E, de Groot IJ, Geerts WJ, de Jong F, van Horssem AA, Los JA, Moorman AF. 1986. The local expression of adult chicken heart myosins during develop- ment. II. Ventricular conducting tissue. Anat Embryol (Berl) 174:187–193.

Schaefer KS, Doughman YQ, Fisher SA, Watanabe M. 2004. Dynamic patterns of apoptosis in the developing chicken heart. Dev Dyn 229:489-499.

Secord AL, McCarty JP, Echols KR, Meadows JC, Gale RW, Tillitt DE. 1999. Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from Upper Hudson River, New York State, USA. Environ Toxicol Chem 18:2519-2525.

Silberhorn EM, Glauert HP, Robertson LW. 1990. Carcinogenicity of Polyhalogenated biphenyls: PCBs and PBBs. Crit Rev Toxicol 20:440-496.

Somi S, Klein AT, Houweling AC, Ruijter JM, Buffing AAM, Moorman AFM, van den Hoff MJB. 2006. Atrial and ventricular myosin heavy-chain expression in the developing chicken heart: strengths and limitations of non-radioactive *in situ* hybridization. J Histochem Cytochem 54:649-664.

Stickel WH, Stickel LF, Dyrland RA, Hughes DL. 1997. Aroclor 1254 residues in birds: lethal levels and loss rates. Environ Contam Toxicol 13:7-13.

Tapia C, Kutzner H, Mentzel T, Savic S, Baumhoer D, Glatz K. 2006. Two mitosis-specific antibodies, MPM-2 and phopho-histone H3 (Ser28), allow rapid and precise determination of mitotic activity. Amer J Surgical Path 30:83-89.

Thomas PT, Hinsdill RD. 1978. Effects of polychlorinated biphenyls on the immune responses of rhesus monkeys and mice. Toxicol Appl Pharmacol 44:41-51.

Tillitt DE, Zajicek JL, Nicks DK. 2011. Preparation of individual and custom PCB mixture dosing solutions for avian egg injection studies associated with injury determinations under Hudson River NRDA. USCG Biochemistry and Physiology Branch. Final Report. Hudson River Natural Resource Trustees, New York.

Tokuyasu KT, Maher PA. 1987. Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. II. Generation of alpha-actinin dots within titin spots at the time of the first myofibril formation. JCB 105:2795-2801.

Trombitas K, Greaser ML, Pollack GH. 1997. Interaction between titin and thin filaments in intact cardiac muscle. J of Muscle Reactivity and Cell Motility 18:345-351.
Van den Berg M, Birnbaum L, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson PRE, Poellinger L, Safe S, Schrenk D, Tillitt DE, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg KJ. 1990. Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin, and thyroid binding globulin. Chem Biol Interact 76:63-75.

Walker MK, Pollenz RS, Smith SM. 1997. Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with tetrachlorodibenzo-p-dioxin induced heart defects. Toxicol Appl Pharmacol107:1075-1083.

Walker MK, Catron TF. 2000. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early embryo development. Toxicol Appl Pharmacol 167:210-221.

Wang SM, Greaser ML. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. J Muscle Res Cell Motil 6:293-312.

Wang SM, Greaser ML, Schultz E, Bulinski JC, Lin JJ, and Lessard JL. 1988. Studies on cardiac myofibrillogenesis with antibodies to titin, actin, tropomyosin, and myosin. JCB 107:1075-1083.

Wu CY, Jhingory S, Taneyhill LA. 2011. The tight junction scaffolding protein cingulin regulates neural crest cell migration. Dev Dyn 240:2309-2323.

CHAPTER 5

DISCUSSION

PCB mixtures are ubiquitous in the environment, with some congeners having high biological toxicity in certain avian species. These compounds are linked to a variety of adverse health effects and have been thought to impact cardiovascular development. Because of their persistence in the environment and bioaccumulative properties, these compounds still pose a great exposure risk for developing embryos. The purpose of this research was to determine the effects of *in ovo* exposure to a PCB mix and PCB 77, both present (ubiquitous) at a Superfund site along the upper Hudson River, on avian cardiovascular development.

Summary of results

The data collected in this dissertation demonstrated that exposure *in ovo* to polychlorinated biphenyls (PCBs) adversely affect cardiovascular development in two avian species, tree swallows (*Tachycineta bicolor*) and chickens (*Gallus domesticus*). As such, there is a clear correlation between PCB exposure and cardiomyopathies demonstrated for both species.

The tree swallow experiment (Chapter 2) dosed wild bird embryos from two reference sites at 18% of embryonic development (ED2.5) with a single dioxin-like congener, PCB 77, at two doses; 100 and 1,000 ng/g egg wt. The treated groups were compared to controls, and also to environmentally exposed embryos collected from a contaminated site along the upper Hudson River. PCB 77 did not affect hatchling viability and survival at any dose compared to controls. Hatchling hearts showed no difference in heart wet weight, heart index, or morphological measures. However, this study revealed that in chicks exposed to PCB 77, there was a significant absence

(p<0.001) of the dorsal and ventral compact layers in the 100 and 1,000 ng/g egg wt PCB 77 dose along the heart ventricular wall compared to the controls.

In the chick hatchling experiment (Chapter 3), fertile eggs were dosed at embryonic day 0 (prior to incubation) with a 58 congener PCB mix at the following doses: 0.0, 0.03, 0.08, 0.30, 0.50, 0.70, and 2.06 µg/g egg wt. Hatching success decreased with PCB treatment dose-dependently (p<0.05) and the calculated LD_{50} for hatchlings was 0.40 µg/g egg wt. Chick embryo heart rates were significantly different at 0.50 and 2.06 µg/g egg compared to control groups (p<0.05) and percent cardiomyopathies in hatchlings showed a dose-dependent increase across PCB treatments when compared to controls (p<0.05). The most common heart abnormality observed was an absence of the compact layer of the ventricular wall.

The objective of the early embryonic chick experiment (Chapter 4) was to examine very early developmental effects of the PCB mix, particularly relative to impacts on heart development at Hamburger Hamilton (HH) stages HH10, HH16, and HH20. We observed decreased survival of treated embryos and detected cardiomyopathies at all stages across all treatments, with significant differences at HH16 and HH20 compared to controls. There was no difference in spatio-temporal expression of ventricular myosin heavy chain and titin proteins across treatments. Average rates of proliferation in the heart decreased significantly at HH16 and HH20 in PCB treatments but rates of apoptosis were not affected at any HH stage.

Implications of heart defects

The PCB mix and the single congener, PCB 77, both significantly increased cardiomyopathies in chicks and tree swallows, respectively. While the PCB mix showed

a broad spectrum of morphological abnormalities in chicks, both species experienced deleterious effects on the ventricular wall compact layer. Compact layer absence in the ventricular wall corroborates previous reports from avian exposure studies to dioxin-like compounds, which identified abnormal thinning of the ventricular wall as a common heart abnormality (DeWitt *et al*., 2006; Walker and Catron, 2000; Kopf and Walker, 2009). As the heart develops, cardiomyocytes orient themselves as trabeculated layer in what will give rise to the ventricles and during the middle stages of embryonic development the compact layer forms and becomes highly proliferative, adding thickness to the ventricular wall of the heart (Jeter and Cameron, 1971; Rychterova, 1971; Kirby, 2006). Our study showed a reduction in early embryonic heart proliferation, suggesting exposure to PCBs could impact normal ventricular wall formation as early as HH16. Interestingly, there may be a parallel absence of the compact layer that occurs in humans, which results from noncompaction and hypertrabeculation of the ventricular wall in one or both ventricles (Chin *et al*., 1990). This cardiomyopathy, a myocardial developmental disorder, carries a risk of progressive cardiac dysfunction including arrhythmias, systemic embolism, and myocardial infarctions, all of which can be lethal (Kirby, 2006). In wild birds, noncompaction or compact layer absence can reduce individual overall fitness, as cardiovascular requirements in avian species intensify during periods of extended flight such as foraging, behavioral reproductive displays, and migration (Butler *et al*., 1977; 2001). In short, *in ovo* exposure to PCBs resulted in abnormal compact layer formation in tree swallows and chicken hatchlings, which is likely to lead to a progressive reduction in the overall fitness of individuals.

Interestingly, reduced proliferation during early embryonic heart development appears to be the first abnormality in heart development associated with the morphological differences in compaction of the ventricular wall identified in our study of hatchlings with the ventricular wall thinning seen in adult wild birds (DeWitt *et al.*, 2006). Our findings support previous studies that showed reduced proliferation in developing embryos exposed to dioxins, which have similar chemical structures to coplanar PCBs (Kopf and Walker, 2009). PCBs have been shown to reduce proliferation in other tissues besides the heart, including human breast tumor cells, through 17-*beta* estradiol stimulated inhibition of cellular proliferation (Oenga *et al*., 2004). Further, the reduced proliferative effects through estrogen-like inhibition can be further exacerbated by perturbation of insulin-like growth factors (Dhar *et al*., 2007). Furthermore, there is a link between inhibition of insulin-like growth factors and reduced cardiomyocyte proliferation (McDevitt *et al*., 2005). This suggests that PCB effects on cardiomyocyte proliferation may be linked to inhibition of essential factors, such as insulin-like growth factors. Future work in this area may identify the cause of reduced cardiomyocyte proliferation following PCB exposure.

Beyond noncompaction of the ventricular heart wall, additional cardiomyopathies were identified in PCB exposed chick hatchlings including thinning of the ventricular wall, obliteration of the trabeculated layer, hypertrabeculation, left and right ventricular hypertrophy, septal malformation, and cardiac arrhythmias. These abnormalities appear similar to PCB effects observed in passerine nestlings and chick embryos, which included thinning of the ventricular wall, chamber dilation, reduced chronotropic responses, microsurface roughness, abnormal heart indices, and overall gross heart deformities

(Walker and Catron, 2000; Heid *et al*., 2001; DeWitt *et al*., 2006; Kopf and Walker, 2009). Congenital cardiovascular malformations (CCVMs), including the cardiac abnormalities affected by PCB exposure, occur in 1% of live human births. It has been estimated that 15% of infants with identified CCVMs will die in the first year of life (Jenkins *et al.*, 2007). Environmental contaminant estimations suggest that 13-30% of all CCVMs might have been avoided by elimination of known environmental risk factors, such as embryonic exposure to organochlorides (PCBs) (Wilson *et al*., 1998). Development of the avian heart is similar to that in humans (Kirby, 2006). As such, exposures to environmental contaminants such as PCBs have been shown to contribute to cardiomyopathies at hatch. The effects of PCB exposure on the cardiovascular system determined cardiomyopathies that carry a risk for progressive cardiac dysfunction, ultimately suggesting decreased overall fitness and increased risk of early mortality in avian neonates (Chin *et al*., 1990).

Limitations in avian risk assessments

Environmental risk assessments are performed to determine the probability of a harmful effect on individuals or populations, through evaluations of quantitative and qualitative hazards on biology and ecology, following exposures to particular environmental contaminants. Risk assessments are determined through hazard identification, dose-response analysis, and exposure assessments. In the United States, the Environmental Protection Agency (EPA) requires dose-response analyses to be performed by determining the relationship between dose and the probably incidence of an effect in specified animal species. These data are then extrapolated from experimental animals to humans and wildlife at risk for exposures. In developing these extrapolations,

the EPA integrates estimations for variability between individuals, populations, species, and susceptible populations. While this approach is currently the best way to perform risk assessments, the variability among avian species following PCB exposure makes risk assessment predictions very challenging.

PCBs are found in the environment as mixtures of congeners, with mixtures varying in concentrations of congener compounds between species and exposure sites. To predict biological impact, the World Health Organization established toxic equivalency factors (TEFs) for twelve dioxin-like PCB congeners that are biologically toxic (van den Berg *et al*., 1998). These TEFs are then used to calculate toxic equivalency (TEQ), which is a standard measurement that enables comparisons between mixtures based on dioxin activation of the aryl hydrocarbon receptor (AhR). In mammalian species, TEQs are used effectively to predict PCB toxicity, however these measurements may not be as useful in avian species (van den Berg *et al*., 2005).

Contaminant analysis of tree swallow eggs and nestlings from the upper Hudson River (UHR) in the 1990's had mean PCB concentrations ranging from 721-62,200 ng/g, with a TEQ range of 0.41-25.4 ng/g (Secord *et al*., 1999). In 2008, analysis of tree swallow eggs from the UHR had concentrations ranging from 2,400-12,000 ng/g egg wt, with a TEQ range of 0.76-3.8 ng/g egg wt. Notably, the variability of mean PCB concentrations and TEQs in a single species speaks to the difficulty of risk assessments for wild bird populations. Measurements of PCB congeners in wild bird eggs suggested that the highly toxic congener, PCB 77 could be responsible for decreased survival in wild birds (Echols *et al*., 1996; Custer *et al*., 2010d). In our tree swallow study, we tested two PCB 77 doses: 100 and 1,000 ng/g egg wt with TEQs of 5 and 50 ng/g respectively,

to determine if PCB77 decreased survival to hatch. Based on the established TEQs, we hypothesized significant increases in mortality at the 1,000 ng/g egg wt PCB 77 treatment (van den Berg *et al*., 1998). However, we did not observe an effect of PCB 77 on survival at any dose. These data suggest a limitation in risk assessment predictions based on TEQs alone (Table 1).

Table 1: PCB concentrations and TEQ values in tree swallow eggs and nestling studies

PCB Exposure	[PCB] (ng/g)	TEQ (ng/g)
UHR mix[a]	721-62,200	0.41-25.4
UHR mix[b]	2,400-12,000	0.76-3.8
PCB 77[c]	100	5
PCB 77[c]	1,000	50*

TEQs were determined using World Health Organization calculations and TEFs for avian species (van den Berg et al., 1998). PCB contaminant analysis in tree swallow eggs and nestlings along the upper Hudson River determined PCB exposures from (a): Secord et al. 1999 and (b): 2008 contaminant egg analysis, unpublished. (c): PCB 77 concentrations selected for tree swallow injection study. (*): PCB high dose (1,000 ng/g) was outside the range of environmentally relevant TEQs, dose hypothesized to affect survival.

Further support for the limitations of TEQs in assessing risk for endocrine disrupting chemicals was obtained in a study in which we dosed exposed chicks to a 58 congener PCB mix at concentrations ranging from 30-2,060 ng/g egg wt, with TEQs ranging from 0.004-0.266 ng/g (van den Berg *et al.*, 1998). The LD_{50} for this PCB mixture in chick hatchlings was calculated to be 400 ng/g egg wt with a TEQ of 0.052 ng/g (see appendix). Previous studies using cell cultures following exposures to dioxin and PCB 126 determined the LD_{50} TEQ in chicks was 0.18 and 1.1 ng/g, respectively (Kennedy *et al*., 1996; Head and Kennedy, 2008). Based on these data, the LD_{50} of the 58 congener PCB mix should be between treatments 700 and 2,060 ng/g egg wt, using

TEQ calculations of 0.90 and 0.266 ng/g, respectively (Table 2). However, this is not what we observed in our study, suggesting that the TEQ does not fully consider critical deleterious biological activities of non-AhR active compounds. The lack of predictability of the TEQ calculation for this PCB mix in chicks is further support of the limitations of TEQs and TEFs in avian populations.

Table 2: TEQs at 50% population lethal dose in chickens calculated for various contaminants and mixtures.

Avian Species	Contaminant	LD_{50} TEQ dose (ng/g)
Chicken[a]	PCB 126	1.1
Chicken[a]	Dioxin (TCDD)	0.18
Chicken[b]	58 congener mix	0.052

TEQs were determined using World Health Organization calculations and TEFs for avian species (van den Berg et al., 1998). Lethal dose for 50% of test populations (LD50) was calculated on a TEQ basis for comparison. Data presented are from (a): Kennedy et al., 1996 and (b): 58-congener PCB mix presented in this dissertation (see appendix for composition). TEQs established for all three contaminants are not consistent using World Health calculations.

To further complicate risk assessments in birds, there are several critical sources of uncertainty in determining avian effects that make extrapolating research data difficult. The main source of uncertainty regarding PCB effects in birds is interspecific variability of sensitivity or variability in effects between species. There is a broad spectrum of biological toxicity following PCB exposure in birds, leading classifications of species based on sensitivity testing through the activation of the AhR pathway (Kennedy *et al.*, 1996; Head and Kennedy, 2008; Jones and Kennedy, 2009). These differences in species sensitivity to PCB exposure make effect extrapolations very difficult, potentially contributing to difficulties in avian risk assessments.

Our domestic chick heart assay provides a novel approach for addressing the

limitations of the TEQ approach during PCB assessments in avian species. In fact, there are several advantages for utilizing the domestic chick assay. First, the domestic chicken is sensitive to PCB exposure compared to other bird species based on AhR activation (Kennedy *et al*., 1996). By utilizing a sensitve avian model during critical life stages (embryonic development), this assay provides the most conservative assessment available. Second, a major limitation of previous PCB assessments is that coplanar or "dioxin-like" PCB congeners were tested separately (Walker and Catron, 2000; Kopf and Walker, 2009). However, it has been established that environmental PCB exposure occurs through mixtures of these PCB congeners. Furthermore, mixtures from one site of contamination can differ dramatically from another site of contamination. Our novel assay allows for testing of multiple environmental mixtures in a single study, which can aid damage assessors in accurately categorizing PCB damage in birds and make comparisons between exposed environmental sites. Finally, the cardiovascular system is one of the first systems to develop in the chick embryo and normal morphology and function is essential for survival in avian species (Kirby, 2007). Through our novel heart assay, we have developed a way to identify abnormalities in cardiac development during very early embryonic stages (Hamburger and Hamilton, 1951). Our assay provides a cost-effective, fast technique for determining PCB effects on critical systems in the developing embryo. In summary, the development of this chick heart assay addresses key limitations of previous PCB assessments by utilizing a conservative model, allowing for mixture assessments and site-to-site comparisons, and incorporating novel scientific approaches that focus on cardiac abnormalities that could lead to decreased animal fitness.

Additional sources of uncertainty in avian risk assessments of PCBs include route of exposure and variability between individuals. In birds, most PCB exposures are dietary (Custer *et al*., 2010 a, b, c, and d). Due to the bioaccumulative properties of PCBs, concentrations of PCBs in the diet vary considerably, with birds at higher trophic levels exposed to elevated concentrations of PCBs and higher proportions of biologically toxic congeners (Hoffman *et al*., 1995). Moreover, variability between exposed individuals within a population also contributes to uncertainty. Developing embryos exposed through maternal deposition of PCBs are exposed to unpredictable concentrations of PCBs and as discussed previously, concentration analyses determined mean concentrations to range from 721-62,200 ng/g in a single species of wild birds along the UHR (Secord *et al*., 1999). Finally, PCB mixtures vary across exposure sites, making extrapolation between one environmentally contaminated location to another difficult (Frame *et al*., 1996; Rushneck *et al*., 2004). The combination of the poor predictability of TEQs, uncertainty factors including variability of species sensitivity, differences in dietary concentrations, and inconsistencies of PCB mixtures between all contaminated sites make PCB risk assessments in avian species exceptionally challenging. Our studies suggest a re-evaluation of current risk assessment methods regarding PCB exposures in avian species, as the limitations of current methods make risk assessment predictions that may be inaccurate or certainly inadequate for a thorough assessment.

Adverse health effects and the Hudson River

The purpose of this dissertation was to assess potential effects of PCBs on cardiovascular development. For each study, treatments with environmental relevance to

exposure concentrations and TEQs in wild birds along the upper Hudson River were selected. The adverse cardiovascular effects and lethality shown through our studies suggests additional remediation may be necessary to protect wild bird overall fitness. The UHR is already a Superfund site, as a result of PCB contamination from 1947 to 1977 (TAMS *et al*., 1991). The contamination spans the entire river, 200 miles of waterway, with the major concentration of PCBs located along 40 miles of the UHR, from Hudson Falls, to Federal Dam Troy in New York. In 2002, the EPA issued a Record of Decision (ROD) requiring riverbed dredging of 40 miles along the Hudson River. This remediation plan targets 2.65 million cubic yards of riverbed sediment to be removed. The ROD created a phase approach to this remediation plan. In 2009, Phase 1 began with General Electric (GE) removing 283,000 cubic yards of sediment through dredging along six miles of the upper Hudson River near the Ford Edward plant in New York. In 2011, Phase 2 began with a remediation plan to remove 2.4 million cubic yards of sediment from the remaining 40 miles of the UHR. Phase 2 of this remediation plan was determined by a compilation of research data from avian, mammalian, aquatic, and amphibian studies. These data, including the experiments conducted for this dissertation, were used to assist in the planning of the Hudson River Phase 2 remediation plan.

Future directions

These studies conclusively demonstrated that exposure to an environmentally relevant PCB mixture resulted in a dose-dependent lethality in the domestic chick. Additionally, our data demonstrate that the estimated impact calculated using the TEQ does not accurately predict observed effects in avian species and therefore is insufficient to completely assess potential damage for compounds having endocrine disruption on

non-AhR effects. PCBs convincingly affected the developing cardiovascular system in tree swallows and the domestic chick and reduced cardiomyocyte proliferation as early as embryonic stage HH16 (Hamburger and Hamilton, 1951).

Based on these data, our lab suggests that an alternative method for predicting adverse effects in avian species may be needed. While TEQs have successfully been used for mammalian species, perhaps a reassessment of their usefulness is necessary based on the data collected in our lab. PCBs are known carcinogens, and also inhibit normal function of the immune, endocrine, nervous, reproductive, and cardiovascular systems. These data support the development of a new method for risk assessment predictions that incorporate previous knowledge of TEQs with assessment assays of affected systems, while accounting for known uncertainty factors in avian assessments.

Another avenue worthy of investigation is examining how PCB exposure affected the early domestic chick embryo. Cardiomyocyte proliferation was severely reduced by PCB exposure. Future studies may investigate if the reduced proliferation is affected by 1) PCB action on essential proteins necessary for cellular proliferation, 2) indirect reduction of proliferation following PCB action on related systems during these critical stages of development or 3) upstream gene expression critical to cardiomyocyte proliferation is altered. Future studies identifying likely targets that directly or indirectly inhibit proliferation of the heart wall would potentially show an additional mechanism of action for these compounds. Additional target pathways may lend themselves to creating a more robust risk assessment for avian species.

References

Butler PJ, West NH, Jones DR. 1977. Respiratory and cardiovascular responses of the pigeon to sustained, level flight in a wind tunnel. J Exp Biol 71:7-26.

Butler PJ. 2001. Diving Beyond Limits. News Physiol Sci 16:222-227.

Chin TK, Perloff JK, Williams RG, Jue K, Mohrmann R. 1990. Isolated noncompaction of left ventricular myocardium. A study of eight cases. Circulation 82: 507-513.

Custer TW, Gray BR, Custer CM. 2010a. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicol 19:391-404.

Custer TW, Custer CM, and Gray BR. 2010b. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in belted kingfisher eggs from Upper Hudson River Basin, New York, USA. Environ Toxicol Chem 29:99-110.

Custer CM, Gray R, Custer TW. 2010c. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallow (*Tachycineta bicolor*). Environ Toxicol Chem 29:909-921.

Custer CM, Custer TW, Dummer PM. 2010d. Patterns of organic contaminants in eggs of an insectivorous, an omnivorous, and a piscivorous bird nesting on the Upper Hudson River, New York, USA. Environ Toxicol Chem 29: (epublished).

DeWitt JC, Millsap DS, Yeager RL, Heise SS, Sparks DW, Henshel DS. 2006. External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol Chem 25: 541–551.

Dhar K, Banerjee S, Dhar G. 2007. Insulin-like Growth factor-1 (IGF-1) induces WISP-2/CCN5 via multiple molecular cross-talks and is essential for mitogenic switch by IGF-1 axis in estrogen receptor positive breast tumor cells. Cancer Res. 67:1520-1526.

Echols KR, Meadows, JC, Tillitt DE, Secord A. 1996. Congener-specific analysis of polychlorinated biphenyl residues in tree swallow chicks, eggs, and other biota from the Hudson River, W.U. 30096. USGS Final Laboratory Report FY97-30-01.

Frame GM, Cochran JW, Bowad SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, qualitative, congener-specific analysis. J High Res Chromat 19:657-668.

Frame GM. 1997. Congener specific PCB analysis. Analyt Chem 69:468-478.

Hamburger V, Hamilton HIL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49-92.

Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environ Sci Technol 42:7535-7541.

Heid SE, Walker MK, Swanson HI. 2001. Correlation of cardiotoxicity mediated by halogenated aromatic hydrocarbons to aryl hydrocarbon receptor activation. Toxicol Sci 61:187-196.

Hoffman DJ, Rattner BA, Burton GA, Cairns J. 1995. Handbook of Ecotoxicology. Lewis Publishers, Boca Raton, Fl. 424-456.

Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, Elixson M, Warnes CA, Webb CL. 2007. Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart association council on cardiovascular disease in the young: endorse by the American Academy of Pediatrics. Circulation 115:2995-3014.

Jeter JR, Cameron II. 1971. Cell proliferation patterns during cytodifferentiation in embryonic chick tissues: liver, heart and erythrocytes. J Embryol Exp Morphol 25:405–422.

Jones SP, Kennedy SW. 2009. Chicken embryo cardiomyocyte cultures: a new approach for studying effects of halogenated aromatic hydrocarbons in the avian heart. Toxicol Sci 109:66-74.

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 141:214-230.

Kirby ML. 2007. Cardiac Development Oxford University Press. New York, New York

Kopf PG, Walker MK. 2009. Overview of developmental heart defects by dioxins, PCBs, and pesticides. J Environ Sci Health 27: 276-285.

McDevitt TC, Laflamme MA, Murry CE. 2005. Proliferation of cardiomyocytes derived from human embryonic stem cells is mediated via the IGF/PI 3-kinase/Akt signaling pathway. J Molec Cell Cardiol 39:865-873.

Oenga GN, Spink DC, Carpenter DO. 2004. TCDD and PCBs inhibit breast cancer cell proliferation in vitro. Toxicol in vitro 18:811-819.

Rushneck DR, Beliveau A, Fowler B, Hamilton C, Hoover D, Kaye K, Berge M, Smith T, Telliard WA, Roman H, Ruder E, Ryan L. 2004. Concentrations of dioxin-like PCBs in unweather Aroclors by HRGC/HRMS using EPA method 1668A. Chemosphere 54:79-87.

Rychterova V. 1971. Principle of growth in thickness of the heart ventricular wall in the chick embryo. Folia Morphol Praha 19:262– 272.

Secord AL, McCarty JP, Echols KR, Meadows JC, Gale RW, Tillitt DE. 1999. Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from Upper Hudson River, New York State, USA. Environ Toxicol Chem 18:2519-2525.

TAMS/Gradient. 1991. *Phase 1 Report, Review Copy, Interim Characterization and Evaluation, Hudson River PCB Reassessment RI/FS.* USEPA Work Assignment No. 013-2N84. Prepared for USEPA by TAMS Consultants, Inc. and Gradient Corporation.

Van den Berg M, Birnbaum LS, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson PRE, Poellinger L, Safe S, Schrenk D, Tillitt DE, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg M, Birnbaum LS, Denison M, DeVito, M, Farland W, Feeley W, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. 2005. The 2005 world health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93:223-241.

Walker MK, Catron TF. 2000. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early embryo development. Toxicol Appl Pharmacol 167:210-221.

Wilson PD, Loffredo CA, Correa-Villasenor A, Ferencz C. 1998. Attributable fraction for cardiac malformations. Am J Epidem 148:414-423.

APPENDIX

Fixation, Dehydration and Embedding of Large Heart Specimens
(procedure modified from M.K. Walker, University of New Mexico)

1. Dissect the heart and place in ice-cold 25 mM KCl until it stops beating. Rinse thoroughly with 1x PBS.

2. Immerse the specimen in a large (10x more than amount of tissue) volume of ice-cold 10% neutral-buffered formalin.

3. Allow tissue to fix overnight @ 4°C. The specimen should not remain in fixative for longer than 24 hrs.

4. Pour off fixative. Add a similar volume of 1x PBS for 30 min @ 4°C, 2x. This is a wash step.

5. Immerse the specimen in 5% sucrose overnight @ 4°C. Ideally, this is an overnight procedure. Do not exceed three days. Enhancement step: aids in penetration of embedding wax.

6. Remove specimen and place in a labeled cassette immersed in 50% ETOH to be transferred to the dehydrator. Dehydration step: 50%, 70%, 80% 95%, 100%, 100% for 1 hr each.

7. Immerse tissue in 1:1 HemoDe: EtOH mixture for 1x 30min at room temperature (HemoDe replaces xylene).

8. Immerse tissue in 100% HemoDe for 1x15min at 58°C (in oven).

9. Immerse tissue in 1:1 HemoDe: paraplast for 1hr at 58 °C (in oven).

10. Immerse tissue in paraplast for 3x 30min at 58°C (in oven).

11. Orient tissue in mold using a probe that has been heated w/ the Bunsen burner after filling mold with paraffin wax. Mold can be placed in water bath on hotplate to prevent paraffin from solidifying. Upon properly orienting specimens in molds, water bath can be lifted and dried at room temperature to prevent bubbles, joints, and cracks. Let paraffin solidify for 12 hrs prior to sectioning.

Note: Steps 7-9 should not be lengthened as prolonged exposure of tissues to HemoDe or xylene will make them brittle.

Recipes for Fixation, Dehydration, Embedding Solutions

10x PBS

1. Mix the following in about 800 ml ddH_2O:

    80 g NaCl
    2 g KCl anhydrous
    2.4 g KH_2PO_4
    26.8 g $Na_2HPO_4@7H_2O$

2. pH to 7.4
3. q.s. to 1 L
4. autoclave and dilute to 1x as needed

10% Neutral-buffered Formalin

1. For 1 L, mix the following:
    800 ml of ddH_2O
    100 ml of 37% formaldehyde
    100 ml of 10X PBS
2. Chill before use to 4°C

Paraplast:Xylene mixture

1. Melt solid wax in 250 ml beaker
2. Place 125 ml xylene in a second beaker and place in 58°C oven
3. When the wax is melted, add the appropriate amount of hot xylene to make the 1:1 mixture.

Hematoxylin and Eosin Staining for Morphology
(procedure modified from M.K. Walker, University of New Mexico)

1. Dewax slides in Hemo-De 3x3min, pour used solution back into bottle for reuse (~10x reuse).

2. Rehydrate slides in 100%, 95%, 70% EtOH for 3min each, pour solution back into bottle for reuse (~10x reuse).

3. Rehydrate in 1xPBS solution for 3min.

4. Stain in Gill's Hematoxylin (Fisher CS400-ID) for 2-5 seconds, pour used stain back into bottle for reuse.

5. Rinse for 3 min in running tap water.

6. Stain in 0.5% Eosin for 6min (Fisher E-511 Eosin Y; 2.5g in 500 ml 1xPBS. Autoclave. Store in refrigerator 4°C).

7. Rinse for 30 seconds in running tap water.

8. Dehydrate in 70%, 100% EtOH for 30 seconds each, pour solution back into bottle for reuse.

9. Place in Hemo-De solution for 3min; slides should be removed for coverslipping one at a time, as to prevent drying out.

10. Coverslip w/ Cytoseal, removing slides one at a time. No air bubbles should be present under slide.

Notes: After using Hemo-De and EtOH ~10x, dispose of it and use fresh. Hematoxylin stains nuclei, Eosin stains cytoplasm.

WHOLE MOUNT Ventricular Myosin Heavy Chain Immuno Protocol
(adapted from L.A. Taneyhill general procedure, University of Maryland)

1. Embryo collection: Place eggs on side, reinforce shell will packing tape. Withdraw 5-10 ml albumen using a needle and syringe. Inject embryos with India ink to stage them under a microscope. Embryos at the correct stage of development can be cut off of the yolk, scooped out of the egg and collected in Ringer's solution on ice. Using needles, trim embryos. For HH16 and HH20 hearts, dissect thoracic region under the microscope. HH10 embryos can be fixed without dissection. Rinse in 1x PBS prior to fixation on ice.

2. Fixation: Place embryos (no more than 5 per blacktop glass vial) in 2ml 4% FRESH PFA overnight at 4°C on shaker.

3. Wash Step: Wash with PTW (1xPBS + 0.1% Tween) for 10 min @ room temperature on shaker (Repeat 3x).

4. Blocking Step: 10% heat treated sheep serum in PTW. (1ml per vial total: 100 µl HT sheep serum into 900 µl PTW) overnight at 4°C on shaker.

5. Primary antibody Step: Use 1:100 dilution of 1°ab into 5% heat-treated sheep serum in PTW solution. (400µl per vial total: 20µl sheep serum into 360 µl PTW. Add 4µl 1°ab). Place embryos overnight @ 4°C on shaker.

6. Wash Step: Rinse 4x 30 min in PTW at room temperature on shaker.

7. Secondary antibody Step: Use 1:200 dilution of 2°ab. 5µl goat-anti-mouse IgG_1-488 into 1ml 5% heat-treated sheep serum in PTW. Place overnight at 4°C on shaker.

8. Wash Step: Rinse 4x 30 min in PTW at RT on shaker.

9. Mount using premade whole mount slides with hearts oriented in the same direction for imaging. Coverslip, image on inverted microscope.

10. Heart abnormalities identified in whole mount should be noted prior to embedding and confirmed during sectioning.

WHOLE MOUNT Titin Immuno Protocol
(procedure modified from Tokuyasu & Maher, 1987)

1. Embryo collection: Place eggs on side, reinforce shell will packing tape. Withdraw 5-10 ml albumen using a needle and syringe. Inject embryos with India ink to stage them under a microscope. Embryos at the correct stage of development can be cut off of the yolk, scooped out of the egg and collected in Ringer's solution on ice. Using needles, trim embryos. For HH16 and HH20 hearts, dissect thoracic region under the microscope. HH10 embryos can be fixed without dissection. Rinse in 1x PBS prior to fixation on ice.

2. Fixation: Place embryos (no more than 5 per blacktop glass vial) in 2ml 4% FRESH PFA overnight at 4°C on shaker.

3. Wash Step: Wash with 1x PBS for 15 min @ room temperature on shaker (Repeat 2x).

4. Blocking Step: Place embryos in 2 ml of 0.1% Triton in PBS-A (see recipe at the end of the protocol) overnight at 4°C on shaker.

5. Enzyme Step: make 2,000 U/ml Hyaluronidase in PBS-A. (6.67 mg hyaluronidase per ml of PBS-A. Hyaluronidase used was 3,000 U/mg). Place in solution for 30 min @ room temperature on shaker (time is CRITICAL).

6. Wash Step: Wash 3x in PBS-A for 10 min each.

7. Primary antibody Step: Use 1:50 dilution of 1°ab. 20μl titin in 980 μl PBS-A. Place embryos in it overnight @ 4°C on shaker.

8. Wash Step: Rinse 4x 30 min in PBS-A at RT on shaker.

9. Secondary antibody Step: Use 1:200 dilution of 2°ab. 5μl goat-anti-mouse IgM-594 into 1ml PBS-A. Place overnight at 4°C on shaker.

10. Wash Step: Rinse 4x 30 min in PBS-A at RT on shaker.

RECIPE: PBS-A (1xPBS, 0.02% azide, 0.01M glycine, 0.002% TritonX-100)
To make 100 ml volume
75mg glycine, 2.0 mg NaN_3, 200 μl Triton X-100, 10 ml 10xPBS, 90 ml diH_20

58 Congener PCB Mixture: Proportion of Individual Congeners for Chicken Studies as concentrations and percentages. (Page 1 of 2).

PCB congener	58 Congener Mix [µg/ml]	58 Congener Mix [µg/g egg]	58 Congener Mix (%)
28	817.600	0.0818	10.463
31	236.000	0.0236	3.020
41	39.960	0.0040	0.511
42	15.900	0.0016	0.203
43	36.320	0.0036	0.465
47	288.400	0.0288	3.691
48	188.100	0.0188	2.407
49	316.400	0.0316	4.049
52	402.000	0.0402	5.144
56	115.600	0.0116	1.479
59	3.950	0.0004	0.051
60	115.200	0.0115	1.474
64	135.600	0.0136	1.735
66	733.200	0.0733	9.383
70	290.300	0.0290	3.715
71	32.280	0.0032	0.413
74	536.800	0.0537	6.869
75	16.040	0.0016	0.205
77	8.000	0.0008	0.102
81	4.008	0.0004	0.051
85	138.800	0.0139	1.776
87	96.360	0.0096	1.233
89	12.000	0.0012	0.154
92	68.270	0.0068	0.874
95	47.960	0.0048	0.614
97	51.680	0.0052	0.661
99	318.700	0.0319	4.078
101	399.600	0.0400	5.114

58 Congener PCB Mixture: Proportion of Individual Congeners for Chicken Studies as concentrations and percentages. (Page 2 of 2).

PCB congener	58 Congener Mix [µg/ml]	58 Congener Mix [µg/g egg]	58 Congener Mix (%)
105	245.600	0.0246	3.143
109	24.120	0.0024	0.309
110	144.400	0.0144	1.848
114	20.160	0.0020	0.258
115	7.968	0.0008	0.102
117	39.840	0.0040	0.510
118	510.600	0.0511	6.534
123	11.980	0.0012	0.153
126	1.687	0.0002	0.022
128	80.520	0.0081	1.030
130	27.800	0.0028	0.356
137	23.970	0.0024	0.307
138	340.000	0.0340	4.351
139	1.360	0.0001	0.017
141	35.760	0.0036	0.458
146	51.670	0.0052	0.661
149	67.600	0.0068	0.865
153	324.300	0.0324	4.150
156	48.240	0.0048	0.617
157	11.880	0.0012	0.152
158	56.440	0.0056	0.722
163	79.600	0.0080	1.019
164	32.140	0.0032	0.411
167	15.920	0.0016	0.204
169	0.014	0.0000	0.000
170	36.280	0.0036	0.464
180	56.040	0.0056	0.717
187	43.930	0.0044	0.562
189	1.404	0.0001	0.018
190	8.016	0.0008	0.103
Total	**7814.267**	**0.7814**	**100.000**

58 Congener PCB Mixture: Calculated Toxic Equivalency (TEQ) based on World Health Organization (WHO) Avian Toxic Equivalency Factors (TEFs).

										Calculated LD_{50}
				0.03	0.08	0.30	0.50	0.70	2.06	0.40
PCB congener	WHO TEF	Congener Mix	58 Congener Mix	[µg/g]						
	(avian)	[µg/g egg]	Proportion of TEQ	TEQ [ng/g]	TEQ [ng/g]	TEQ [ng/g]	TEQ [ng/g]	TEQ [ng/g]	TEQ [ng/g]	TEQ [ng/g]
77	0.05000	0.000800	0.000051	0.002	0.004	0.015	0.026	0.036	0.105	0.020
81	0.10000	0.000401	0.000051	0.002	0.004	0.015	0.026	0.036	0.106	0.021
105	0.00010	0.024560	0.000003	0.000	0.000	0.001	0.002	0.002	0.006	0.001
114	0.00010	0.002016	0.000000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
118	0.00001	0.051060	0.000001	0.000	0.000	0.000	0.000	0.000	0.001	0.000
123	0.00001	0.001198	0.000000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
126	0.10000	0.000169	0.000022	0.001	0.002	0.006	0.011	0.015	0.044	0.009
156	0.00010	0.004824	0.000001	0.000	0.000	0.000	0.000	0.000	0.001	0.000
157	0.00010	0.001188	0.000000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
167	0.00001	0.001592	0.000000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
169	0.00100	0.000001	0.000000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
189	0.00001	0.000140	0.000000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Total:	0.781427	PCB Mix TEQ [ng/g]:	0.004	0.010	0.039	0.064	0.090	0.266	0.052
			PCB Mix [ng/g]:	30	80	300	500	700	2060	400

COMPILED REFERENCES

Addison RF. 1989. Organochlorines and marine mammal reproduction. Can J Fish Aquat Sci 46:360-368

Agency for Toxic Substances and Disease Registry (ATSDR). 1997. Public health implications of exposure to polychlorinated biphenyls (PCBs). Atlanta, GA.

Ahlborg UG, Brouwer A, Fingerhut MA, Jacobson JL, Jacobson SW, Kennedy SW, Kettrup AA, Koeman JH, Poiger H, Rappe C. 1992. Impact of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls on human and environmental health, with special emphasis on application of the toxic equivalency factor concept. Eur J Pharmacol 288:179-199.

Ahmed SA. 2000. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicol 150:191-206.

Andric SA, Kostic TS, Dragisic SM, Andric N, Stojikovic SS, Kovacevic RZ. 2000. Acute effects of polychlorinated biphenyl containing and free transformer fluids on rat testicular steroidogenesis. Environ Health Perspect 108:955-959.

Ankley GT, Niemi GJ, Lodge KB, Harris HJ, Beaver DL, Tillitt DE, Schwartz TR, Giesy JP, Jones PD, Hagley C. 1993. Uptakes of planar polychlorinated biphenyls and 2,3,7,8-substituted polychlorinated dibenzofurans and dibenzo-p-dioxins by birds nesting in the lower Fox River and Green Bay, Wisconsin, USA. Environ Contam Toxicol 24:332-344.

Anne M, McLane R, Hughes DL. 1980. Reproductive success of screech owls fed Aroclor 1248. Environ Contam Toxicol 9:661-665.

Antin PB, Taylor RG, Yatskievych T. 1994. Precardiac mesoderm is specified during gastrulation in quail. Dev Dyn 200:144-154.

Arzuaga X, Elskus A. 2002. Evidence for resistance to benzo[a]pyrene and 3,4,3',4'-tetrachlorobiphenyl in chronically polluted Fundulus heteroclitus population. Mar Environ Res 54:247-251.

Aulerich RJ, Ringer RK. 1977. Current status of PCB toxicity to mink and effect on their reproduction. Environ Contam Toxicol 6:279-292.

Aulerich RJ, Bursian SJ, Breslin WJ, Olson BA, Ringer RK. 1985. Toxicological manifestations of 2,4,5,2',4',5', 2,3,6,2',3',6', and 3,4,5,3',4',5'-hexachlorobiphenyl and Aroclor 1254 in mink. Toxicol Environ Health 15:63-79.

Baibergenova A, Kudyakov R, Zdeb M, Carpenter DO. 2003. Low birth weight and residential proximity to PCB contaminated waste sites. Environ Health Perspect 111:1352-1357.

Baker EL, Landrigan PJ, Glueck CJ, Zack MM, Liddle JA, Burse VW. 1980. Metabolic consequences of exposure to polychlorinated biphenyls (PCB) in sewage sludge. Am J Epidemiol 112: 553–563.

Bandman E, Rosser BW. 2000. Evolutionary significance of myosin heavy chain heterogeneity in birds. Microsc Res Tech 50: 473-491.

Barron MG, Galbraith H, Beltman D. 1995. Comparative biochemistry and physiology Part C: Pharmacology, toxicology, and endocrinology 112:1-14.

Barsotti DA, Marlar RJ, Allen JR. 1976. Reproductive dysfunction in rhesus monkeys exposed to low levels of polychlorinated biphenyls (Aroclor 1248). Food Cosmet Toxicol 14:99-103.

Bell FP, Iverson F, Arnold D, Vidmar TJ. 1994. Long- term effects of Aroclor 1254 (PCBs) on plasma lipid and carnitine concentrations in rhesus monkey. Toxicol 89: 139–153.

Bellairs R, Osmond M. 2005. The Atlas of Chick Development 2nd Edition. Elsevier Academic Press, San Diego, CA, USA.

Bertazzi PA, Riboldi L, Pesatori A, Radice L, Zoochetti C. 1987. Cancer mortality of capacitor manufacturing workers. Am J Ind Med 11:165-176.

Bisaha JG, Bader D. 1991. Identification and characterization of a ventricular-specific avian myosin heavy chain, VMHC1: expression in differentiation cardiac and skeletal muscle. Dev Biol 148:355-364.

Bishop CA, Koster MD, Chek AA, Hussell DJ, Jock K. 1995. Chlorinated hydrocarbons and mercury in sediments, red winged blackbirds (Agelaius phoeniceus) and tree swallows (Tachycineta bicolor) from wetlands in Great Lakes, St. Lawrence River basin. Environ Toxicol Chem 14:491-510.

Blausen BE, Johannes RS, Hutchins GM. 1990. Computer-based reconstructions of the cardiac ventricles of human embryos. Am J Cardiovasc Pathol 3:37–43.

Blectchy JD. 1983. Polychlorinated biphenyls: production, current use, and possible rates of future disposal in OECD member countries. Proceedings, PCB Seminar, Netherlands Ministry of the Environment. Hague, Netherlands, 343-365.

Bleyl S, Nelson L, Odelberg SJ. 1995. A gene for familial total anomalous pulmonary venous return maps to chromosome 4p13-q12. Amer J Hum Gen 56:408-415.

Bourke DL, Wylie SR, Wick M, Bandman E. 1991. Differentiating skeletal muscle cells initially express a ventricular myosin heavy chain. BAM 1:13-21.

Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, Bergman A, Visser TJ. 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. Toxicol Ind Health 14:59-84.

Brown JF, Wagner RE, Bedard DL, Brennan MJ, Carnahan JC, May RJ, Tofflemire TJ. 1984. PCB transformations in upper Hudson River sediments. Northeastern Environ Sci 3:167-179.

Brown JE, Bedard DL, Brennan MJ, Carnahan JC, Feng H, Wagner RE. 1987. Polychlorinated biphenyl dechlorination in aquatic sediments. Science 236:709-712.

Brown DP. 1987. Mortality of workers exposed to polychlorinated biphenyls- an update. Environ Health 42:333-339.

Brunstrom B, Orberg J. 1982. A method for studying embryo toxicity of lipophilic substances and experimentally introduced into hens' eggs. Ambio 11:209-211.

Brunstrom B, Danerud PO. 1983. Toxicity and distribution in chick embryos of 3,3', 4, 4'-tetrachlorobiphenyl and in concentration/affinity of the hepatic receptor for 2,3,7,8-tetra-chlorodibenzo-p-dioxin. Comp Biochem Physiol 91:507-512.

Brunstrom B. 1988. Sensitivity of embryos from duck, goose, herring gull, and various chicken breeds to 3,3', 4,4'-tetrachlorobiphenyl. Poult Sci 67:52-57.

Bustnes JO, Hanssen SA, Folstad I, Erikstad KE, Hasselquist D, Skaare JU. 2004. Immune Function and Organochlorine pollutants in Arctic breeding Glaucous Gulls. Environ Contam Toxicol 47:530-541.

Butler PJ, West NH, Jones DR. 1977. Respiratory and cardiovascular responses of the pigeon to sustained, level flight in a wind tunnel. Exp Biol 71:7-26.

Butler PJ. 2001. Diving Beyond Limits. News Physiol Sci 16:222-227.

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. 2003. Isl1 identified a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 5:877-889.

Calabrese EJ, Baldwin LA. 2002. Hormesis: the dose-response revolution. Pharmacol Toxicol 43:175-197.

Canga L, Paroli L, Blanck TJ, Silver RB, Rifkind AB. 1993. 2,3,7,8-tetrachlorodibenzo-p-dioxin increases cardiac myocyte intracellular calcium and progressively impairs ventricular contractile responses to isoproterenol and to calcium in chick embryo hearts. Molec Pharmacol 44:1142-1151.

Carey JH. 1994. Transformation processes of contaminants in rivers. Hydrological, chemical, and biological processes of transformation and transport of contaminants in aquatic environments. International Association of Hydrological Sciences 219:41-50.

Carney SA, Jing Chen C, Burns G, Xiong KM, Peterson RE, Heideman W. 2006. Aryl hydrocarbon receptor activation produces heart specific transcriptional and toxic responses in developing zebrafish. Mol Pharmacol 70:549-561.

Carpenter DO. 2006. Polychlorinated biphenyls (PCBs): Routes of exposure and effects on human health. Rev Environ Health 21:1-22.

Challice CE, Viragh S. 1973. The architectural development of the early mammalian heart. Tissue Cell 6:447–462.

Chase KH, Wong O, Thomas D, Berney BW, Simon RK. 1982. Clinical and metabolic abnormalities associated with occupational exposure to polychlorinated biphenyls. J Occup Med 24: 109–114.

Chen YCJ, Guo YL, Hsu CC, Rogan WJ. 1992. Cognitive development of Yu-Cheng (Oil Disease) children prenatally exposed to heat-degraded PCBs. JAMA 268:3213-3218.

Chin TK, Perloff JK, Williams RG, Jue K, Mohrmann. 1990. Isolated noncompaction of left ventricular myocardium. A study of eight cases. Circulation 82:507-513.

Christofells VM, Gabets PE, Franco D, Campione M, de Jong F, Lamers WH, Bao ZZ, Palmer S, Biben C, Harvey RP, Moorman AF. 2000. Chamber formation and morphogenesis in the developing mammalian heart. Dev Biol 223:266-278.

Clearwater. 2011. Phase I and II monitoring report and revised updates. Clearwater, Beacon, NY.

Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). 1980. EPA 540-R-98-024. EPA, Washington, DC.

Cooper GS, Klebanoff MA, Promislow J, Brock JW, Longnecker MP. 2005. Polychlorinated biphenyls and menstrual cycle characteristics. Epidemiology 16:191-200.

Crofton KM, Kodavanti PRS, Derr-Yellin EC, Casey AC, Kehn LS. 2000. PCBs, thyroid hormones and toxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. Toxicol Sci 57:131-140.

Custer TW, Heinz GH. 1980. Reproductive success and nest attentiveness of mallard ducks fed Aroclor 1254. Environ Pollut 21: 313-318.

Custer CM, Custer TW, Allen PD, Stromborg KL, Melancon M. 1998. Organochlorine contaminants and tree swallows nesting along the Fox River and in Green Bay, Wisconsin. Environ Toxicol Chem 17:1786-1798.

Custer CM, Custer TW, Coffey M. 2000. Organochlorine chemicals in tree swallows nesting in pool 15 of the upper Mississippi River. Bull Environ Contam Toxicol 64:341-346.

Custer TW, Custer CM, Hines RK. 2002. Dioxins and congener specific polychlorinated biphenyls in three avian species from Wisconsin River, Wisconsin. Environ Pollut 119:323-332.

Custer CM, Custer TW, Dummer PM, Munney KL. 2003. Exposure and effects of chemical contaminants on tree swallows nesting along the Housatonic River, Berkshire County, Massachusetts, USA, 1998-2000. Environ Toxicol Chem 22:1605-1621.

Custer TW, Gray BR, Custer CM. 2010a. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicol 19:391-404.

Custer TW, Custer CM, and Gray BR. 2010b. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in belted kingfisher eggs from Upper Hudson River Basin, New York, USA. Environ Toxicol Chem 29:99-110.

Custer CM, Gray R, Custer TW. 2010c. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallow (Tachycineta bicolor). Environ Toxicol Chem 29:909-921.

Custer CM, Custer TW, Dummer PM. 2010d. Patterns of organic contaminants in eggs of an insectivorous, an omnivorous, and a piscivorous bird nesting on the Upper Hudson River, New York, USA. Environ Toxicol Chem 29: (epublished).

Davis JM, Svendsgaard DJ. 1990. U-shaped dose-response curves: their occurrence and implications for risk assessment. Toxicol Environ Health 30:71-83.

de la Cruz MV, Gomez CS, Arteaga MM, Arguello C. 1977. Experimental study of the development of the truncus and the conus in the chick embryo. J Anatomy 123:661-686.

de la Cruz MV, Gomez CS, Palomino. 1989. The primitive cardiac regions in the straight tube heart (stage 9) and their anatomical expression in the mature heart: an experimental study in the chick embryo. J Anatomy 165:121-131.

DeHaan RL. 1965. Morphogenesis of the vertebrate heart. Organogenesis. Eds: DeHaan RL and Ursprung H. Holt, Rinehart, and Winston. New York, New York, USA.

Denham M, Schell LM, Dean G, Gallo MV, Ravenscroft J, DeCaprio AP. 2005. Relationship of lead, mercury, mirex, dichlorodiphenyldichloroethylene, hexachlorobenzene, and polychlorinated biphenyls to timing of menarche among Akwesasne Mohawk girls. Pediat 115:127-134.

Denison MS, Fisher JM, Whitlock JP. 1988. The DNA recognition site for the dioxin-Ah receptor complex: nucleotide sequence and functional analysis. J Biol Chem 263:17721-17724.

Denison MS, Elferink CF, Phelan D. 1998. The Ah receptor signal transduction pathway. Toxicant-Receptor Interactions in the Modulation of Signal Transduction and Gene expression. MS Denison, WG Helferich. Taylor and Francis, PA, USA, pp 3-33.

Denison MS, Nagy SR. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Ann Rev Pharmicol Toxicol 43:309-334.

Dewailly E, Ayotte P, Bruneau S, Gingras S, Belles-Isles M, Roy R. 2000. Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. Environ Health Perspect 108:205-211.

DeWitt JC, Millsap DS, Yeager RL, Heise SS, Sparks DW, Henshel DS. 2006. External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol and Chem 25: 541–551.

Dhar K, Banerjee S, Dhar G. 2007. Insulin-like Growth factor-1 (IGF-1) induces WISP-2/CCN5 via multiple molecular cross-talks and is essential for mitogenic switch by IGF-1 axis in estrogen receptor positive breast tumor cells. Cancer Res 67:1520-1526.

Dhont M, Brouwer A. 2001. The risk of endometriosis and exposure to dioxin and polychlorinated biphenyls: a case control study of infertile women. Human Repro 16:2050-2055.

Duffy JE, Carlson E, Li Y, Prophete C, Zelikoff JT. 2002. Impact of polychlorinated biphenyls (PCBs) on the immune function of fish: age as a variable in determining adverse outcome. Marine Environ Res 54:559-563.

Dunier M. 1994. Modulators of fish immune response models for environmental toxicology-biomarkers immunostimulators. Stolen JS, Fletcher TC, eds. Modulators of fish immune responses. Fair Haven NY, USA, pp 123-139.

Durfee RL, Contos G, Witmore FC, Barden JD, Hackman EE, Westin RA. 1976. PCBs in the United States – Industrial Use and Environmental Distribution Report. EPA 56016-16-005. Versar Inc., Springfield, VA, USA.

Echols KR, Meadows, JC, Tillitt DE, Secord A. 1996. Congener-specific analysis of polychlorinated biphenyl residues in tree swallow chicks, eggs, and other biota from the Hudson River, W.U. 30096. USGS Final Laboratory Report FY97-30-01.

Echols KR, Tillitt DE, Nichols JW, Secord AL, McCarty JP. 2004. Accumulation of PCB congeners in nestling tree swallows (Tachycineta bicolor) on the Hudson River, New York. Environ Sci Tech 38:6240-6246.

Eisler R. 1986. Polychlorinated biphenyl hazards in fish, wildlife, and invertebrates: a synoptic review. Biol Rep 85:1.7, USFWS, Washington DC, USA.

Elliott JE, Kennedy SW, Peakall DB, Won H. 1990. Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds. I. Japanese quail. Comp Biochem Physiol 96:205-210.

Elliot SE, Kennedy SW, Jeffry D, Shutt L. 1991. Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds. II. American Kestrel. Comp Biochem Physiol 99:141-145.

Elliott JE, Wilson LK, Langelier KW, Norstrom RJ. 1996. Bald eagle mortality and chlorinated hydrocarbon contaminants in livers from British Columbia, Canada, 1989-1994. Environ Pollut 94: 9-18.

Emmett EA, Maroni M, Jefferys J, Schmidt J, Levin BK, Alvares A. 1988. Studies of transformer repair workers exposed to PCBs: II. Results of clinical laboratory investigations. Amer J of Ind Med 14:47-62.

Evans D, Miller JB, Stockdale FE. 1988. Developmental patterns of expression and coexpression of myosin heavy chains in atria and ventricles of the avian heart. Dev Biol 127:376–383.

Ferencz C, Loffredo CA, Correa-Villasenor A, Wilson PD. 1997. Genetic and environmental risk factors of major cardiovascular malformations. Futura Publishing Company, Armonk, NY, USA.

Fernie K, Bortolotti G, Drouillard K, Smits J, Marchant T. 2003. Developmental toxicity of in ovo exposure to polychlorinated biphenyls. II. Effects of maternal or paternal exposure on second-generation nestling American kestrel. Environ Toxicol Chem 22:2688-2694.

Fischer C, Fredriksson A, Eriksson P. 2008. Neonatal co-exposure to low doses of ortho PCB 153 and methyl mercury exacerbate defective developmental neurobehavior in mice. Toxicol Environ Health 244:157-165.

Frame GM, Cochran JW, Bowad SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, qualitative, congener-specific analysis. J High Res Chromat 19:657-668.

Frame GM. 1997. Congener specific PCB analysis. Analyt Chem 69:468-478.

Franceschini MD, Custer CM, Custer TW, Reed JM, Romero LM. 2008. Corticosterone stress response in tree swallows nesting near polychlorinated biphenyl and dioxin-contaminated rivers. Environ Toxicol Chem 27:2326-2331.

Fukunaga BN, Probst MR, Reisz-Porszasz S, Hankinson O. 1995. Identification of functional domains of aryl hydrocarbon receptor. J Bio Chem 270:29270-29278.

Gilbertson M, Kubiak T, Ludwig J, Fox G. 1991. Great lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. Toxicol Environ Health 33:455-520.

Goldstone HM, Stegeman JJ. 2006. A Revised evolutionary history of the CYP1A Subfamily: Gene duplication, gene conversion, and positive selection. J Mol Evol 62:708-717.

Golub MS, Donald JM, Reyes JA. 1991. Reproductive toxicity of commercial PCB mixtures: LOAELs and NOAELs from animal studies. Environ Health Perspect 94:245-253.

Gonzalez-Sanchez A, Bader D. 1985. Characterization of myosin heavy chain in myocardial cells of the conductive system of the chicken heart. Cell Biol 100:270-275.

Gonzalez-Sanchez A, Bader D. 1984. Immunochemical analysis of myosin heavy chains in the developing chicken heart. Dev Biol 103:151-158.

Gooch JW, Elskus AA, Kloepper-Sams PJ, Hahn ME,Stegeman JJ. 1989. Effects of ortho and non ortho substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (Stenotomus chrysops). Toxicol Appl Pharmacol 98:422-433.

Gould JC, Cooper KR, Scanes CG. 1999. Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeiodinase in the chick embryo. Ecotox and Environ Saf 43:195-203.

Grasman KA. 2002. Assessing immunological function in toxicological studies of avian wildlife. Integ Comp Biol 42:34-42.

Greaser ML, Handel SE, Wang SM, Schultz E, Bulinski JC, Lin JJ, Lessard JL. 1989. Assembly of titin, myosin, actin, and tropomyosin into myofibrils in cultured chick cardiomyocytes. In Stockdale F, Kedes L, eds, Cellular and Molecular Biology of Muscle Development, 93. New York, NY, USA, pp247-257.

Guo YL, Yu ML, Hsu CC, Rogan WJ. 1999. Chloracne, goiter, arthritis, and anemia after polychlorinated biphenyl poisoning: 14-year follow-up of the Taiwan Yucheng cohort. Environ Health Perspect 107:715–719.

Hamburger V, Hamilton HIL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49-92.

Hankinson O. 1995. The aryl hydrocarbon receptor complex. Ann Rev Pharmacol Toxicol 35:307-340.

Hancock-Friesen CL, Zurakowski D, Thiagarajan RR. 2005. Total anomalous pulmonary venous connection: an analysis of current management strategies in a single institution. Ann Thorac Surg 79:596-606.

Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environ Sci Tech 42:7535-7541.

Heaton SN, Bursian SJ, Giesy JP, Tillitt DE, Render JA, Jones PD, Verbrugge DA, Kubiak TJ, and Aulerich RJ. 1995. Dietary exposure of mink to carp from Saginaw Bay, Michigan. Effects on reproduction and survival, and the potential risks to wild mink populations. Environ Contam Toxicol 28:334-343.

Heid SE, Walker MK, Swanson HI. 2001. Correlation of cardiotoxicity mediated by halogenated aromatic hydrocarbons to aryl hydrocarbon receptor activation. Toxicol Sci 61:187-196.

Heinz GH, Hoffman SL, Kondrad SL, Erwin CA. 2006. Factors affecting the toxicity of methylmercury injected into eggs. Environ Contam Toxicol 50:264-279.

Henshel DS, Hehn B, Vo MT, Steeves JD. 1993. A short-term test for dioxin teratogenicity using chicken embryos, Environmental Toxicology and Risk Assessment, Vol 2. STP 1216. American Society for Testing and Materials, Philadelphia, PA, USA, pp 159–174.

Henshel DS. 1994. Early embryonic teratogenic changes induced by in ovo exposure to TCDD. The Toxicologist 14:383.

Henshel DS, Sparks DW. 2006. Site Specific PCB-correlated interspecies differences in organ somatic indices. Ecotoxicol 15:9-18.

Hervé JC, Crump D, Jones SP, Mundy LJ, Giesy JP, Zwernick MJ, Bursian SJ, Jones PD, Wiseman SB, Wan Y, Kennedy SW. 2010. Cytochrome P450A induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. Toxicol Sci 113:380-391.

Hill EF, Hoffman DJ. 1984. Avian models for toxicity testing. J Am Coll Toxicol 3:357.

Hirokawa K. 1972. A quantitative study on pre and postnatal growth of human heart. Acta Path Jpn 22:613–624.

Hoffman JIE. 1995. Incidence of congenital heart disease: II. Prenatal incidence. Pediat Cardiol 16:155-165.

Hoffman DJ, Melancon MJ, Klein PN, Rice CP, Eisemann JD, Hines RK, Spann JW, Pendleton GW. 1996. Developmental toxicity of PCB 126(3, 3', 4, 4', 5-pentachlorobiphenyl in nestling American kestrels (Falco sparverius). Fundam Appl Toxicol 34:188-200.

Hogers B, de Ruiter MC, Baasten AMJ, Gittenberger-de Groot AC, Poelmann RE. 1995. Intracardiac flow patterns related to the yolk sac circulation of the chick embryo. Circ Res 76:871–877.

Hontela A, Rasmussen JB, Audet C, Chevalier G. 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Environ Contam Toxicol 22:278-283.

Hord NG, Perdew GH. 1994. Physiochemical and immunochemical analysis of aryl hydrocarbon receptor nuclear translocator characterization of two monoclonal antibodies of the aryl hydrocarbon receptor nuclear translocator. Mol Pharm 46:18-24.

Hsu PC, Huang W, Yao WJ, Guo YL, Lambert GH. 2003. Sperm changes in men exposed to polychlorinated biphenyls and dibenzofurans. JAMA 289:2943-2944.

Icardo JM, Fernandez-Teran A. 1987. Morphologic study of ventricular trabeculation in the embryonic chick heart. Act Anat 130:264 –274.

Icardo JM. 1996. Developmental biology of the vertebrate heart. J Exp Zool 275:144-161.

Jacobson JL, Jacobson SW. 1996. Intellectual impairment in children exposed to polychlorinated biphenyls in utero. N Engl J Med 335:783-789.

Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, Elixson M, Warnes CA, Webb CL. 2007. Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American heart association council on cardiovascular disease in young: endorsed by the academy of pediatrics. Circulation 115:2995-3014.

Jenni, R, Oechslin, E, Schneider, J. 2001. Echocardiographic and pathoanatomical characteristics of isolated left ventricular non-compactions: a step towards classification as a distinct cardiomyopathy. Heart 86: 666.

Jeter JR, Cameron II. 1971. Cell proliferation patterns during cyto-differentiation in embryonic chick tissues: liver, heart and erythrocytes. J Embryol Exp Morphol 25:405–422.

Johnson BL, Hicks HE, Cibulas W, Faroon O, Ashizawa AE, DeRosa CT, Cogliano J, Clark M. 2000. Public health implications of exposure to polychlorinated biphenyls (PCBs). Agency for Toxic Substances and Disease Registry, Div of Toxicol, Atlanta, GA, USA.

Jones SP, Kennedy SW. 2009. Chicken embryo cardiomyocyte cultures: a new approach for studying effects of halogenated aromatic hydrocarbons in the avian heart. Toxicol Sci 109:66-74.

Kazlauskas A, Poellinger L, Pongratsz I. 1999. Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon receptor). J Biol Chem 274:13519-13524.

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 141:214-230.

Kimbrough RD, Squire RA, Linder RE, Strandberg JD, Montali RJ, Burse VW. 1975. Induction of liver tumors in Sherman strain female rats by polychlorinated biphenyl Aroclor 1260. J Nat Cancer Inst 55:1453-1459.

Kimbrough RD. 1985. Laboratory and human studies on polychlorinated biphenyls (PCBs) and related compounds. Environ Health Perspect 59:99-106.

King AS, McLelland J. 1986. Birds: their structure and function. Baillere Tindal, London, UK.

Kirby ML. 2007. Cardiac Development. Oxford University Press, New York, NY, USA.

Knowles R, Griebsch I, Dezateuz C, Brown J, Bull C, Wren C. 2005. Newborn screening for congenital heart defects: a systematic review and cost-effectiveness analysis. Health Tech Assess 9:1-152.

Komarek V, Malinovsky L, Lemez L, eds. 1982. Anatomia avium domesticarum et embryologia galli. Bratislava: Piroda.

Koopman-Essenboom C, Morse DC, Weisglas-Kuperus N, Lutkeschipholt IJ, Van der Paauw CG, Tuinstra LG. 1994. Effects of dioxins and polychlorinated biphenyls on thyroid hormone status of pregnant women and their infants. Pediat Res 36:468-473.

Kopf PG, Walker MK. 2009. Overview of developmental heart defects by dioxins, PCBs, and pesticides. Environ Sci Health 27: 276-285.

Kovacevic R, Vojinovic-Miloradov M, Teodorovic I, Andric S. 1995. Effect of PCBs on androgen production by suspension of adult rat Leydig cells in vitro. J Steroid Biochem Mol Biol 52:595-597.

Kreiss K, Zack MM, Kimbrough RD, Needham LL, Smrek AL, Jones BT. 1981. Association of blood pressure and polychlorinated biphenyl levels. JAMA 245: 25050–2509.

Kudyakov R, Baibergenova A, Zdeb M, Carpenter DO. 2004. Respiratory disease in relation to patient residence near to hazardous waste sites. Pediat18:249-257.

Kuratsune M. Yusho. In: Kimbrough RD. 1980. Halogenated Biphenyls, Terphenyls, Napththalenes, Dibenzodioxins and Related Products. Amsterdam, Elsevier North Holland. 287–292.

Lahvis GP, Wells RS, Kuehl DW, Stewart JL, Rhinehart HL, Via CS. 1995. Decreasing lymphocyte responses in free ranging bottlenose dolphins (Tursiops truncates) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ Health Perspect 103:67-72.

Larsen WJ. 1993. Human embryology. Churchill-Livingstone, New York, NY, USA.

Lavoie ET, Grasman KA. 2007. Effects of in ovo exposure to PCBs 126 and 77 on mortality, deformities, and post-hatch immune function in chickens. Toxicol Environ Health, Part A 70:547-558.

Leonard H, Derrick G, O'Sullivan J, Wren C. 2000. Natural and unnatural history of pulmonary atresia. Heart 84:499-503.

Linask KK, Han MD, Artman M, Ludwig CA. 2001. Sodium-calcium exchanger (NCX-1) and calcium modulation: NCX protein expression patterns and regulation of early heart development. Dev Dyn 221:249-264.

Linask KK. 2003. Regulation of heart morphology: current molecular and cellular perspectives on coordinated emergence of cardiac form and function. Birth Defects Res 69:14-24.

Lind PM, Orberg J, Edlund UB, Sjoblom L, Lind L. 2004. The dioxin like pollutant PCB 126 (3,3',4,4',5-pentachlorobiphenyl) affects risk factors for cardiovascular disease in female rats. Toxicol lett 150:293-299.

Lindebro MC, Poellinger L, Whitelaw ML. 1995. Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-ARNT transcription factor complex. J Emb 14:3528-3539.

Longnecker MP, Klebanoff MA, Brock JW, Zhou H. 2001. Polychlorinated biphenyl serum levels in pregnant subjects with diabetes. Diabetes Care 24:1099–1101.

Lonky E, Reihman J, Darvill T, Mather J, Daly H. 1996. Neonatal behavioral assessment scale performance in humans influenced by maternal consumption of environmentally contaminated Lake Ontario fish. J Great Lakes Res 22:198-212.

Love OP, Shutt LJ, Silfies JS, Bortolotti GR, Smits JEG, Bird DM. 2003. Effects of dietary PCB exposure on adrenocortical function in captive American kestrels (Falco sparverius). Ecotoxicol 12:199-208.

Manner J. 2000. Cardiac looping in chick embryo: a morphological review with specific reference to terminological and biomechanical aspects of the looping process. Anat Rec 259:248-262.

Martineau D, Lemberger K, Dallaire A, Labelle P, Lipscomb TP, Michel P, Mikaelian I. 2002. Cancer in wildlife, a case study: beluga from the St. Laurence estuary, Quebec, Canada. Environ Health Perspect 110:285-292.

Martinsen BJ. 2005. Reference Guide to Stages of Chick Heart Embryology. Dev Dyn 233:1217-1237.

Mayes BA, McConnell EE, Neal BH, Brunner MJ, Hamilton SB, Sullivan TM. 1998. Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclors 1016, 1242, 1254, 1260. Toxicol Sci 41:62-76.

McCarty JP, Secord AL. 1999. Reproductive ecology of tree swallows (Tachycineta bicolor) with high levels of polychlorinated biphenyl contamination. Environ Toxicol Chem 18:1433-1439.

McDevitt TC, Laflamme MA, Murry CE. 2005. Proliferation of cardiomyocytes derived from human embryonic stem cells is mediated via the IGF/PI 3-kinase/Akt signaling pathway. J Mol Cell Cardiol. 39:865-873.

McKinney JD, Chae K, Gupta BN, Moore JA, Goldstein JA. 1976. Toxicological assessment of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. Toxicol Appl Pharmacol 36:65-80.

McNabb A, Fox G. 2003. Avian thyroid development in chemically contained environments: Is there evidence of alterations in thyroid function and development? Evol Dev 5:76-82.

McNabb FM. 2005. Biomarkers for the assessment of avian thyroid disruption by chemical contaminants. Avian Poult Biol Rev 16:3-10.

Meilhac SM, Kelly RG, Rocancourt D, Eloy-Trinquet S, Nicolas JF, Buckingham ME. 2003. A retrospective clonal analysis of myocardium reveals two phases of clonal growth in the developing mouse heart. Development 130:3877-3889.

Meeker JD, Hauser R. 2010. Exposure to polychlorinated biphenyls (PCBs) and male reproduction. Sys Biol Repro Med 56:122-131.

Meyer BK, Pray-Grant MG, Heuvel JP, Perdew GH. 1998. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol Cell Biol 18: 978-988.

Mochizuki H, Oda H, Yokogoshi H. 1998. Amplified effect of taurine on PCB-induced hypercholesterolemia in rats. Adv Exp Med Biol 1998. 442: 285– 290.

Mochizuki H, Oda H, Yokogoshi H. 2000. Dietary taurine alters ascorbic acid metabolism in rats fed diets containing polychlorinated biphenyls. J Nutr 130: 873–876.

Morgan DP, Lin LI, Saikaly HH. 1980. Morbidity and mortality in workers ccupationally exposed to pesticides. Environ Contam Toxicol 9:349–382.

Moysich KB, Ambrosone CB, Mendola P, Kostyniak PJ, Greizerstein HB, Vena JE. 2002. Exposures associated with serum organochlorine levels among postmenopausal women from western New York State. Am J Ind Med 41: 102–110.

Naert C, Van Peteghem C, Kupper J, Jenni L, Naegeli H. 2007. Distribution of polychlorinated biphenyls and polybrominated diphenyl ethers in birds of prey from Switzerland. Chemosphere 68:977-987.

Nebeker AV, Puglisi FA, DeFoe DL. 1974. Effect of polychlorinated biphenyl compounds on survival and reproduction of the fathead minnow and flagfish. Trans Am Fish Soc 103:562.

Norstrom RJ. 1988. Patterns and trends of PCB contaminations in Canadian wildlife. Crine JP, ed. Hazards, decontamination and replacement of PCB. Plenum, New York, NY, USA pp 85-100.

Oda YS, Tsunoda S, Doue T, Iida Y, Matsubara H. 2005. Clinical characteristics and morphological features of isolated noncompaction of the ventricular myocardium: electrocardiogram-gated magnetic resonance imaging can elucidate more accurate biventricular structures. Heart Vessels 20:126-127.

Oechslin EN, Attenhofer-Jost CH, Rojas JR, Kaufmann PA, Jenni R. 2000. Long-term follow-up of 34 adults with isolated left ventricular noncompaction: a distinct cardiomyopathy with poor prognosis. J Am Coll Cardiol 36:493-500.

Oenga GN, Spink DC, Carpenter DO. 2004. TCDD and PCBs inhibit breast cancer cell proliferation in vitro. Toxicol in vitro. 18:811-819.

Osius N, Karmaus W, Kruse H, Witten J. 1999. Exposure to polychlorinated biphenyls and levels of thyroid hormones in children. Environ Health Perspect 107:843-849.

Oskam I, Ropstad E, Lie E, Derocher A, Wilg O, Dahl E, Larsen S, Skaare JU. 2004. Organochlorines affect the steroid hormone cortisol in free-ranging polar bears (Ursus maritimus) at Svalbard, Norway. Toxicol Environ Health 67:959-977.

Peakall DB, Peakall ML. 1973. Effects of polychlorinated biphenyl on reproduction of artificially and naturally incubated dove eggs. J Appl Ecol 10:863-868.

Pierpont ME, Basson CT, Benson DW, Gelb BD, Giglia TM, Goldmuntz E, McGee G, Sable CA, Srivastava D, Webb CL. 2007. Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American heart association congenital cardiac defects committee, council on cardiovascular disease in the young: endorsed by the American academy of pediatrics. Circulation 115:3015-3038.

Pollenz RS, Sattler CA, Poland A. 1994. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol 45:428-438.

Portigal CL, Cowello SP, Feboruk MN, Butler CM, Rennie PS, Nelson CC. 2002. Polychlorinated biphenyls interfere with androgen induced transcriptional activation and hormone binding. Toxicol Appl Pharmacol 179:185-194.

Potts JD, Dagle JM, Walder JA, Weeks DL, Runyon RB. 1991. Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor B3. Proc Natl Acad Sci 88:1516-1520.

Powell DC, Aulerich RJ, Meadows JC, Tillitt DE, Giesy JP, Stromborg KL, Bursian SL. 1996. Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) injected into the yolks of chicken (Gallus domesticus) eggs prior to incubation. Environ ContamToxicol 31:404-409.

Reijnders PJH. 1986. Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature 324:456.

Render JA, Aulerich RJ, Bursian SJ, Nachreiner RF. 2000. Proliferation of maxillary and mandibular periodontal squamous cells in mink fed 3,3',4,4' 5-pentachlorobiphenyl (PCB126). J Vet Diagn Inves 12:477-479.

Restum, JC, Giesy JP, Render JA, Shipp EB, Verbrugge DA, Aulerich RJ. 1998. Multigenerational study of the effects of consumption of PCB-contaminated carp from Saginaw Bay, Lake Huron, on Mink. 1. Effects on mink reproduction, kit growth and survival, and selected biological parameters. Toxicol Environ Health 54:345-375.

Rier SE, Turner WE, Martin DC, Morris R, Lucier GW, Clark GC. 2001. Serum levels of TCDD and dioxin like chemicals in rhesus monkeys chronically exposed to dioxin: correlation of increased serum PCB levels with endometriosis. Toxicol Sci 59:147-159.

Ritchthoff J, Rylander L, Jonsson BA, Akesson H, Hagmar L, Nilsson-Ehle P. 2003. Serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CP-153) in relation to markers of reproductive function in young males from the general Swedish population Environ Health Perspect 111:409-413.

Rogan WJ, Galden BC, McKinney JD, Carreras N, Hardy P, Thullen J. 1986. Neonatal effects of transplacental exposure to PCBs and DDE. J Pediat 109:335-341.

Rosenquist GC. 1970. Location and movements of cardiogenic cells in the chick embryo: the heart-forming portion of the primitive streak. Dev Biol 22:461-475.

Ross P, DeSwart R, Addison R, Loveren HV, Vos J, Osterhaus A. 1996. Contaminant-induced immunotoxicity in harbor seals: Wildlife at risk? Toxicol 112:157-169.

Ross PS, Ellis GM, Ikonomou MG, Barett-Lennard LG, Addison RF. 2000. High PCB concentrations in free-ranging Pacific killer whales, orcinus orca: effects of age, sex, and dietary preference. Marine Pollut Bull 40:504-515.

Rufer ES, Hacker TA, Flentke GR, Drake,VJ, Brody MJ, Lough J, Smith SM. 2009. Altered cardiac function and ventricular septal defect in avian embryos exposed to low-dose trichloroethylene. Toxicol Sci 113: 444-452.

Rushneck DR, Beliveau A, Fowler B, Hamilton C, Hoover D, Kaye K, Berge M, Smith T, Telliard WA, Roman H, Ruder E, Ryan L. 2004. Concentrations of dioxin-like PCBs in unweather Aroclors by HRGC/HRMS using EPA method 1668A. Chemosphere 54:79-87.

Rychter Z, Rychterova V. 1981. Angio- and myoarchitecture of the heart wall under normal and experimentally changed conditions. In: Pexieder T, ed. Perspectives in cardiovascular research, Vol. 5, mechanisms of cardiac morphogenesis and teratogenesis. Raven, New York, New York, USA pp 431–452.

Rychterova V. 1971. Principle of growth in thickness of the heart ventricular wall in the chick embryo. Folia Morphol 19:262– 272.

Rychterova V. 1978. Development of proliferation structure of ventricular heart wall in the chick embryo between 6th and 14th day of embryogenesis. Folia Morphol 26:131-143.

Safe SH. 1994. Polychlorinated biphenyls (PCBs). Environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87-149.

Sanders E, de Groot IJ, Geerts WJ, de Jong F, van Horssem AA, Los JA, Moorman AF. 1986. The local expression of adult chicken heart myosins during development. II. Ventricular conducting tissue. Anat Embryol (Berl) 174:187–193.
Schaefer KS, Doughman YQ, Fisher SA, Watanabe M. 2004. Dynamic patterns of apoptosis in the developing chicken heart. Dev Dyn 229:489-499.

Schantz, SL, Levin ED, Bowman RE. 1991. Long-term neurobehavioral effects of perinatal polychlorinated biphenyl (PCB) exposure in monkeys. Environ Toxicol Chem 10:747-756.

Schantz SL, Gasior DM, Polverejan E, McCaffrey RJ, Sweeny AM, Humphrey HE. 2001. Impairments of memory and learning in older adults exposed to polychlorinated biphenyls via consumption of Great Lakes fish. Environ Health Perspect 109:605-611.

Schell LM, DeCaprio A, Gallo M, Hubicki L. 2002. Polychlorinated biphenyls and thyroid function in adolescents of the Mohawk Nation at Akwesansne. In Gill G, Schell LM, Benson L, eds. Human Growth from Conception to Maturity, Chapter 30. Smith-Gordon, London, UK pp 289-296.

Schmidt JV, Bradfield CA. 1996. Ah receptor signaling pathways. Ann Rev Cell Dev Biol 12:55-89.

Schuur AG, Brouwer A, Bergman A, Coughtrie MW, Visser TJ. 1998. Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polychlorinated biphenyls. Chem Biol Interact 109:293-297.

Schwacke LH, Voit EO, Hansen LJ, Wells RS, Mitchum GB, Hohn AA, Fair PA. 2002. Probabilistic risk assessment of reproductive effects of polychlorinated biphenyls on bottlenose dolphins (Tursiops truncatus) from the southest United States coast. Environ Toxicol Chem 21:2752-2764.

Secord AL, McCarty JP, Echols KR, Meadows JC, Gale RW, Tillitt DE. 1999. Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from Upper Hudson River, New York State, USA. Environ Toxicol Chem 18:2519-2525.

Sedmera DM, Reckova M, Bigelow MR, Dealmeida A, Stanley CP, Mikawa T, Gourdie RG, Thompson RP. 2004. Developmental transitions in electrical activation patterns in chick embryonic heart. Anat Rec 280: 1001-1009.

Sedmera D and McQuinn T. 2008. Embryogenesis of heart muscle. Heart Fail Clin 4: 235-245.

Sergeev A V, Carpenter DO. 2005. Hospitalization rates for coronary heart disease in relation to residence near areas contaminated with persistent organic pollutants and other pollutants. Environ Health Perspect 113:756-761.

Shen ES, Whitlock JP. 1992. Protein-DNA interactions at a dioxin-responsive enhancer. Mutational analysis of the DNA-binding site for the liganded Ah receptor. J Biol Chem 267:6815-6819.

Silberhorn EM, Glauert HP, Robertson LW. 1990. Carcinogenicity of Polyhalogenated biphenyls: PCBs and PBBs. Cri Rev Toxicol 20:440-496.

Sinks T, Steele G, Smith AB, Watkins K, Shults RA. 1992. Mortality among workers exposed to polychlorinated biphenyls. Am J Epidem 136:389-398.

Smits JE, Wayland ME, Miller MJ, Liber K, Trudeau S. 2000. Reproductive, immune, and physiological end points in tree swallows on reclaimed oil sands mine sites. Environ Toxicol Chem 19:2951-2960.

Somi S, Klein AT, Houweling AC, Ruijter JM, Buffing AAM, Moorman AFM, van den Hoff MJB. 2006. Atrial and ventricular myosin heavy-chain expression in the developing chicken heart: strengths and limitations of non-radioactive in situ hybridization. Histochem Cytochem 54:649-664.

Sommer RJ, Hume AJ, Ciak JM, Vannostrand JJ, Figgens M, Walker MK. 2005. Early developmental 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure decrease chick embryo heart chronotropic response to isoproterenol but not to agents affecting signaling downstream of beta-adrenergic receptor. Toxicol Sci 83:363-371.

Stickel WH, Stickel LF, Dyrland RA, Hughes DL. 1997. Aroclor 1254 residues in birds: lethal levels and loss rates. Environ Contam Toxicol 13:7-13.

Tabuchi M, Veldhoen N, Dangerfield N, Jeffries S, Helbing CC, Ross PS. 2006. PCB-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging harbor seals (Phoca vitulina). Environ Health Perspect 114:10924-1031.

TAMS/Gradient. 1991. Phase 1 Report, Review Copy, Interim Characterization and Evaluation, Hudson River PCB Reassessment RI/FS. USEPA Work Assignment No. 013-2N84. Prepared for USEPA by TAMS Consultants, Inc. and Gradient Corporation.

Tapia C, Kutzner H, Mentzel T, Savic S, Baumhoer D, Glatz K. 2006. Two mitosis-specific antibodies, MPM-2 and phophohistone H3 (Ser28), allow rapid and precise determination of mitotic activity. Amer J Surg Path 30:83-89.

Taylor PR, Lawrence CE, Hwang HL, Laulson AS. 1984. Polychlorinated biphenyls: influence on birth weight and gestation. Amer J Public Health 74: 1153-1154.

Tharappel JC, Lee EY, Robertson LW, Spear BT, Glauert HP. 2002. Regulation of cell proliferation, apoptosis, and transcription factor activities during prootion of liver carcinogenesis by polychlorinated biphenyls. Toxicol Appl Pharmacol 179:172-184.

Thomas PT, Hinsdill RD. 1978. Effects of polychlorinated biphenyls on the immune responses of rhesus monkeys and mice. Toxicol Appl Pharmacol 44:41-51.

Thompson RP, Abercrombie V, Wong M. 1987. Morphogenesis of the truncus arteriosus of the chick embryo heart: movements of autographic tattoos during septation. Anat Rec 218:434-440.

Tillitt DE, Zajicek JL, Nicks DK. 2011. Preparation of individual and custom PCB mixture dosing solutions for avian egg injection studies associated with injury determinations under Hudson River NRDA. USCG Biochemistry and Physiology Branch. Final Report. Hudson River Natural Resource Trustees, New York.

Tilson HA, Jacobson JL, Rogan WJ. 1990. Polychlorinated biphenyls and the developing nervous system: Cross-species comparisons. Neurotox Teratol 12:239-248.

Tilson HA, Kodavanti PS, Mundy WR, Bushnell PJ. 1998. Neurotoxicity of environmental chemicals and their mechanism of action. Toxicol Lett 102:631-635.

Tokuyasu KT, Maher PA. 1987. Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. II. Generation of alpha-actinin dots within titin spots at the time of the first myofibril formation. JCB 105:2795-2801.

Tomanek RJ, Hu N, Phan B, Clark EB. 1999. Rate of coronary vascularization during embryonic chicken development is influences by rate of myocardial growth. Cardio Research 41:663-671.

Toxic Substances Control Act. (TSCA).1976. Public Law 94-469.

Trombitas K, Greaser ML,Pollack GH. 1997. Interaction between titin and thin filaments in intact cardiac muscle. J Muscle React Cell Motil 18:345-351.

Tryphonas H. 1995. Great Lakes health effects: immunotoxicity of PCBs (Aroclors). Environ Health Perspect 103:33-46.

U.S. Environmental Protection Agency. 1992a. Estimating exposure to dioxin-like compounds. EPA 600-6-88-005B. Office of Research and Development, Washington, DC.

U.S. Environmental Protection Agency. 1992b. Framework for ecological risk assessment. EPA 630-R-92-001. Risk assessment forum, Washington, DC.

U.S. EPA. 1996. PCBs: Cancer Dose-Response Assessment and Application to Environmental Mixtures. EPA 600-P-96-001F. Office of Research and Development, National Center for Environmental Assessment, Washington, DC.

U.S. Environmental Protective Agency. 2002. Hudson River PCBs Site New York: Record of Decision (USEPA ROD). Washington, DC.

Van den Berg KJ. 1990. Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin, and thyroid binding globulin. Chem Biol Interacti 76:63-75.

Van den Berg M, Birbaum LS, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson PRE, Poellinger L, Safe S, Schrenk D, Tillitt DE, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg M, Birnbaum LS, Denison M, DeVito, M, Farland W, Feeley W, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. 2005. The 2005 world health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93:223-241.

Van den Heuvel RL, Koppen G, Staessen JA, Hond ED, Verheyen G, Nawrot TS. 2002. Immunologic biomarkers in relation to exposure markers of PCBs and dioxins in Flemish adolescents (Belgium). Environ Health Perspect 110:595-600.

Van den Hoff MJB, Kruithof BP, Moorman AF, Markwald RR, Wessels A. 2001. Formation of myocardium after the initial development of the linear heart tube. Dev Biol 240:61-76.

Van Mierop LHS, Kutsche LM. 1984. Comparative anatomy and embryology of the ventricles and arterial pole of the vertebrate heart. In: Nora JJ, Takao A, eds. Congenital heart disease: causes and processes. Futura Publishing Co, New York, NY, USA pp 459–479.

Wakimoto K, Kobayashi K, Kuroo M, Yao A, Iwamoto T, Yanaka N, Kita S, Nishida A, Azuma S, Toyoda Y, Omori K, Imahie H, Oka T, Kudoh S, Kohmoto O, Yazaki Y, Shigekawa M, Imai Y, Nabeshima Y, Komuro I. 2000. Targeted disruption of Na^+/Ca^{2+} exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. J Biol Chem 275:36991-36998.

Waldo KL, Kumiski DH, Wallis KT, Stadt HA, Hutson MR, Platt DH, Kirby ML. 2001. Conotruncal myocardium arises from secondary heart field. Development 128:3179-3188.

Waldo KL, Hutson MR, Ward CC, Zdanowicz M, Stadt HA, Kumiski D, Abu-Issa R, Kirby ML. 2005. Secondary heart field contributes myocardium and smooth muscle to the arterial pole of the developing heart. Dev Biol 281:78-90.

Walker MK, Pollenz RS, Smith SM. 1997. Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with tetrachlorodibenzo-p-dioxin induced heart defects. Toxicol Appl Pharmacol 107:1075-1083.

Walker MK, Catron TF. 2000. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early embryo development. Toxicol Appl Pharmacol 167:210-221.

Waller BF, Smith ER, Blackbourn BD. 1980. Congenital hypoplasia of portions of both right and left ventricular myocardial walls. Clinical necropsy observations in two patients with parchment heart syndrome. Am J Cardiol 46:885.

Wang SM, Greaser ML. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. J Muscle Res Cell Motil 6:293-312.

Wang SM, Greaser ML, Schultz E, Bulinski JC, Lin JJ, and Lessard JL. 1988. Studies on cardiac myofibrillogenesis with antibodies to titin, actin, tropomyosin, and myosin. JCB 107:1075-1083.

Weisglas-Kuperus N, Sas TC, Koopman EBC, van der Zwan CW, DeRidder MA, Beisbuizen A. 1995. Immunologic effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. Pediat Res 38:404-410.

Weisglas-Kuperus N, Patandin S, Berbers GA, Sas TC, Mulder PG, Sauer PJ. 2000. Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. Environ Health Perspect 108:1203-1207.

Weisglas-Kuperus N, Vreugdenhil HJ, Mulder PG. 2004. Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. Toxicol Lett 149:281-285.

Whitlock JP. 1999. Induction of cytochrome P450A1. Ann Rev Pharm Toxicol 39:103-125.

Wilsgaard T, Jacobsen BK, Schirmer H, Thune I, Lochen ML, Njolstad I. 1981. Tracking of cardiovascular risk factors. Am J Epidemiol 154: 418–426.

Wilson L, Curtis A, Korenberg JR, Schipper RD, Allan L, Chenevix-Trench G, Stephenson A, Goodship J, Burn J. 1993. A large, dominant pedigree of atrioventricular septal defect (AVSD): exclusion from the Down syndrome critical region on chromosome 21. Amer J Hum Gen 53:1262-1268.

Wilson PD, Loffredo CA, Correa-Villasenor A, Ferencz C. 1998. Attributable fraction for cardiac malformations. Am J Epidem 148:414-423.

Wilson PD, Loffredo CA, Correa-Villasenor A, Ferencz C. 1998. Attributable fraction for cardiac malformations. Am J Epidem 148:414-423.

Wren C, Birrell G, Hawthorne G. 1993. Cardiovascular malformations in infants of diabetic mothers. Heart 89:1217-1220.

Wren C, Reinhardt Z, Khawaja K. 2008. Twenty year tends in diagnosis of life threatening neonatal cardiovascular malformations. Arch Dis Child Fetal Neonat Ed 93:F33-F35.

Yang QH, Khoury MJ, Mannino D. 1997. Trends and patterns of mortality associated with birth defects and genetic diseases in the United States, 1972-1992: an analysis of multiple-cause mortality data. Genet Epidem 14:493-505.

Yang X, Dormann D, Munstergberg A, Weijer CJ. 2002. Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8. Dev Cell 3:425-437.

Yitalo GM, Stein JE, Hom T, Johnson LL, Tilbury KL, Hall AJ, Rowles, T, Greig D, Lowenstine LJ, Gulland FMD. 2005. The role of organochlorines in cancer-associated mortality in California sea lions (Zalophus californianus). Marine Poll Bull 50:30-39.

Yu ML, Hsu CC, Guo YL, Lai TJ, Chen SJ, Luo JM. 1994. Disordered behavior in early-born Taiwan Yucheng children. Chemosphere 29:2413-2422.