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IMMUNOLOGICAL AND DEVELOPMENTAL EFFECTS OF POLYBROMINATED DIPHENYL ETHERS (PBDES) AND 2,3,7,8-TETRACHLORO-P-DIOXIN (TCDD) IN BIRDS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

Randall Troy Stetzer
B.S., University of Michigan, Ann Arbor, 1999

2007
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School of Graduate Studies

July 25th, 2007

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Randall Troy Stetzer ENTITLED Immunological and Developmental Effects of polybrominated diphenyl ethers (PBDEs) and 2,3,7,8-tetrachloro-*p*-dioxin (TCDD) in Birds BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Stetzer, Randall Troy. MS., Department of Biological Sciences, Wright State University, 2007. Immunological and Developmental Effects of polybrominated diphenyl ethers (PBDEs) and 2,3,7,8-tetrachloro-*p*-dioxin (TCDD) in Birds.

This thesis contains two separate and distinct research projects with a common overall theme – the study of immunotoxicity associated with the developmental exposure of young birds to halogenated chemicals posing environmental concern. These chemicals share many chemical characteristics, including environmental ubiquity and longevity, one has been studied extensively since the 1970s, and one is a relatively new environmental contaminant with few studies pertaining to toxicological affects on humans or wildlife. Specific summaries of each study begin each project chapter.

The first study was entitled "Developmental and Immunological Effects of a Commercial Mixture of PBDE Flame Retardants in Chicken Embryos." The primary objective of this study was to determine if chicken embryos exposed *in ovo* to a commercial mixture of polybrominated diphenyl ethers (PBDEs), a halogenated flame retardant sharing many characteristics with PCBs and dioxins, presented indication of toxicity by way of increased mortality, developmental abnormalities, or immunotoxicity. Chicken (*Gallus gallus*) embryos were exposed to a commercial PBDE mixture *in ovo* via air cell injection at environmentally relevant concentrations. This study resulted in minimal impacts by PBDEs with respect to mortality and immunotoxicity at the doses tested, but interesting, however non-significant results with respect to deformities.

The second study, entitled "Immunotoxic Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the Wood Duck (*Aix sponsa*): A Sensitive Indicator Species?", was conducted in cooperation with the US Fish and Wildlife Service in Raleigh, NC. The objective of this research was to determine if wood ducks exposed *in ovo* to environmentally relevant concentrations of TCDD expressed signs of immunotoxicity. Previous research has shown that wood ducks may be extremely sensitive to low dioxin levels. Accounting for high sensitivity to dioxin-like chemicals and certain life history and behavioral patterns associated with wood ducks, results of previous research has suggested that wood ducks may be an ideal indicator species for environmental contamination and remediation. The study herein described effects by wood duck embryos exposed to environmentally relevant concentrations of TCDD *in ovo* via yolk injection. While the present study exhibited high background mortality, no statistically significant toxicity was revealed in terms of increased dose-related mortality or immunotoxicity characteristic of dioxin toxicity.

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Chapter 1: LITERATURE REVIEW

1 - 1 Introduction

Continual debates exist concerning the benefits of anthropogenic chemicals and the potential risks associated with their use. In 1948, Paul Müller of Switzerland won the Nobel Prize in physiology (medicine) for his discovery of the pesticide dichlorodiphenyltrichloro-ethane (DDT). The use of his discovery, known simply as DDT, has been suggested to have aided in the virtual disappearance of malaria in the western world (reviewed in Bate, 2000). Environmental degradation was not widely attributed to DDT until 1962 when Rachel Carson wrote her best selling novel *Silent Spring*, which resulted in a focal environmental preservation campaign worldwide. Possibly as a direct result of Carson's book, DDT was banned in the United States in 1972; yet, its breakdown products persist in the environment more than 30 years later. Historically, most industrial, agricultural, and household chemicals, such as DDT, are provided minimal consideration prior to their introduction with respect to environmental impact. Of the more than 100,000 chemicals in use today, only the most blatant impacts on humans or wildlife populations brought chemical hazards into the limelight. Whether they are in the form of insecticides, herbicides, coolants, liquid insulators, or other industrial uses, chemicals have become an integral part of everyday life (Danish Environmental Protection Agency, 1999). The environment is often overlooked in exchange for the increased comforts, decreased labor, and added benefits associated with the use synthetic chemicals.

1 - 2 Background

Halogenated aromatic hydrocarbons (HAHs) comprise a group of toxic environmentally persistent chemicals. HAHs have aromatic molecular structures with one or more attached halogens such as chlorine or bromine. Chlorinated HAHs include polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzodioxins (PCDDs). Furans and dioxins are produced as unintentional industrial by-products, especially in the production of chlorinated herbicides and in paper bleaching. Governmental regulations restrict unintentional dioxin production in the United States and EU nations. PCBs have been used since the 1930s for a variety of industrial and electrical purposes, primarily to insulate electrical components and make products more heat resistant. Their chemical nature and stability made them excellent heat insulators and dielectric fluids for electric capacitors and transformers. PCB production reached its peak in 1970, when 85 million pounds of Aroclors, commercial mixtures of PCBs, were produced in the U.S. PCB production and use was limited in the United States in 1977, and banned altogether in 1979, in accordance with the Toxic Substances Control Act (TSCA) and the Resource Conservation and Recovery Act (RCRA) (ATSDR, 2000).

HAHs comprise a group of anthropogenic chemicals that share many physiochemical properties. These chemicals are extremely stable and often persist in the environment for years upon release. HAHs are extremely resistant to extremes in pH and temperature, and thus do not break down under normal environmental conditions. These chemicals are lipophilic compounds, meaning they do not dissolve well in water, resulting in precipitation and storage in sediments. The highly lipophilic nature of these compounds allows them to cross readily through cell membranes, and become stored in adipose tissues, creating a magnification trend up food chains. These contaminants reach maximum concentrations at the highest trophic levels, where the majority of impacts are detected. As fat is mobilized for reproduction, females often also free stored HAHs, making them more bioavailable and passing them to offspring through yolk fats, across the placenta, and (or) through breast milk. The most devastating and extreme effects often are observed when high exposure occurs during crucial developmental periods (Law *et al.*, 2003; Tomy *et al.*, 2004).

While there are strict controls and bans on many HAHs throughout much of the world, their production and use has by no means vanished. While most chlorinated HAHs are no longer used in developed countries, brominated versions, which are primarily used as flame-retardants, have become increasingly widespread. With all that is known about the environmental relevance and toxicity effects of the organochlorines, relatively little data has been collected on the effects of brominated aromatic hydrocarbons.

1 - 2.1 PBDEs

1 - 2.1.1 History

Throughout history, humans have attempted to increase material safety and longevity by reducing flammability. The ancient Egyptians as early as 450BC used alum to decrease flammability of wood. The Romans added vinegar to the flame retardant around 200BC. Gypsum and clay were used in 1600s as flame retardant additives on theater curtains (Hindersinn, 1990). Joseph Gay-Lussac was the first to use a halogen as a flame retardant, where, in 1821, he used ammonium chloride with ammonium phosphate and borax to reduce flammability of linen (IPCS, 1997). During World War II, chlorinated paraffins were used to create flame-proofed canvas. With the increased use of synthetic and petroleum-based products in textiles and building materials in the 1960s and 70s, newer, cheaper, and more effective flame-retardant chemicals were needed (Hindersinn, 1990; Alaee *et al.*, 2003). The World Health Organization reports that of the 175 flame retarding chemicals in use today, halogenated formulas account for 25% by volume of the worldwide production (IPCS, 1997).

1 - 2.1.2 Chemistry

All the natural halogens (including chlorine, fluorine, iodine, and bromine) could be used as flame retardants based on their chemical nature. Combustion occurs as a flammable gas is exposed to oxygen and an ignition source. Halogens in a gas phase are extreme oxidizing agents, readily taking up available free radicals. If halogens are released to a gas form upon heating, the oxygen free radicals required for flame propagation become bound, limiting ignition, thereby decreasing combustibility. While all the halogens have similar flame-retardant properties, only chlorine and bromine are suitable for most situations. Fluorine is extremely stable, requiring extremely high temperatures to change to a gaseous phase. On the other hand, iodine is not stable, and readily disassociates to gas forms just above room temperatures (Sellström *et al.*, 1993; Darnerud *et al.*, 2001; Alaee *et al.*, 2003).

As halogenated aromatic hydrocarbon and flame retardants, polybrominated diphenyl ethers (PBDEs) are often compared to their chlorinated counterparts. PBDEs (Figure 1a) have similar chemical structure to organochlorines such as PCBs (Figure 1b) and dioxins (Figure 1c), with many similar chemical characteristics. PBDEs have 209 potential congeners, similar to PCBs, each differing by the number and location of bromine atoms around the two phenyl rings. The individual PBDE congeners are numbered like PCBs according to the IUPAC system (Darnerud *et al.*, 2001; de Wit, 2002; Harju *et al.*, 2002; Alaee *et al.*, 2003).

While PBDEs may share many physiochemical properties with their chlorinated cousins, it is the differences that may present differences in toxicity. It has been shown that toxicity of chlorinated HAHs increases with planarity. With respect to PCBs, while some rotation is usually allowed between the phenyl rings, the more sterically hindered the congener, in most cases, the more toxic. Thus, as planarity increases, so does the compound's toxicity, through the most molecularly planar and the most toxic HAH, TCDD. The ether group in PBDEs, however, reduces molecular planarity, allowing rotation between phenyl rings. In addition, bromine is a significantly larger atom than chlorine, increasing the molecular bond lengths, making PBDEs much larger molecules than their chlorinated counterparts. This may burden receptor-route toxicity as with many chlorinated HAHs (Chen *et al.*, 2001).

1 - 2.1.3 Commercial Mixtures

Three commercial PBDE flame retardant mixtures are currently produced. Each commercial mixture is named according to its primary congeners. The decabromodiphenyl ether (Deca-BDE) mixture makes up over 80% of the global PBDE market (Darnerud, 2003). It is sold as a white powder and contains 83% bromine by weight. Deca-BDE contains 97-98% BDE-209 and a small amount of nona-BDEs (Ikonomou *et al.*, 2002; Alaee *et al.*, 2003). Deca-BDE is a general purpose flame retardant, primarily used in resins such as polyvinyl chloride and rubber, and in high impact polystyrene for electronic enclosures (Hardy, 2002b; Alaee *et al.*, 2003). Encapsulated in latex, this mixture is also used to coat fabrics for furniture upholstery (Hardy, 2002b).

The second commercial mixture, octabromodiphenyl ether (Octa-BDE) also is produced in the lowest quantity, partially due to a European Union marketing ban (Alaee *et al.*, 2003). This mixture, purchased as a white powder, contains 67% bromine, and consists of an average mixture of 62% hexabromodiphenyl ethers (HxBDE) and 34% octabromodiphenyl ethers (OcBDE) (Ikonomou *et al.*, 2002; Alaee *et al.*, 2003). This formulation is primarily used to flame retard business equipment composed of acrylonitrile-butadiene-styrene (ABS) plastics (Hardy, 2002b; Alaee *et al.*, 2003).

Pentabromodiphenyl ether (Penta-BDE), the third commercial mixture, contains the congeners found in the greatest bioavailable concentrations in the environment, and has thus been banned in E.U. nations and in California. Thus, the chemical producers of these flame retardants have primarily withdrawn penta-BDE mixtures from the open market (Watanabe and Sakai 2001; European Union, 2003; Great Lakes Chemical Corporation, 2003; Tullo, 2003). Penta-BDEs are purchased as a highly viscous liquid containing 70% bromine by weight (Hardy, 2002b; Alaee *et al*, 2003). This mixture consists of 41-42% tetra-BDEs (primarily BDE-47), 44-45% penta-BDEs (chiefly BDE-99 and less significantly BDE-100), and 6-7% hexa-BDE congeners (mainly BDE-153 and -154) (Alaee *et al.*, 2003). One of the commercial Penta-BDE mixtures, Bromkal 70-DE, is said to contain six additional congeners: BDE-17, -28, -66, -85, -138, and 183. The penta-BDE mixtures are primarily used in North America as flame retardants in polyurethane foams, furniture, and automobile textiles (Manchester-Neesvig *et al.*, 2001; Alcock *et al.*, 2003; Alaee *et al.*, 2003).

Deca-BDE has extremely poor water solubility. This larger congener precipitates from the water column and accumulates in sediments. The lack of water solubility took some focus off the deca-congener, as the swift removal from the water column as precipitate prompted the belief that they were minimally bioavailable. Research has recently increased in relation to this congener, as concentrations have been found in wildlife and human tissues (Kierkegaard *et al.*, 1999; Law *et al.*, 2003; Schecter *et al.*, 2003; Lindberg *et al.*, 2004). A study comparing PBDE concentrations in human breast milk from nursing mothers in Texas showed concentrations of the deca-congener as high as nine ng/g-lipid (Schecter *et al.*, 2003).

1 - 2.1.4 Environmental Concentrations and Trends

Flame retardants have been attributed to saving numerous lives and money from fire in the United States and the world. Fire in the United States costs Americans an average of nearly $11 billion annually, and accounted for around 5,000 deaths in 2001 (not including the 2,791 deaths from the World Trade Center disaster). Flame retardant chemicals have been attributed with a 20% reduction in flame-related deaths in Europe over the last 10 years, and an estimated 3,000 saved lives from 1988-2000 (Geneva Association, World Fire Statistics, 2004). The worldwide demand for flame retardant chemicals was near 204,000 metric tons in 1999, significantly higher than the 150,000 metric tons produced in 1992. In 1999, PBDEs accounted for one-third of the total flame retardant demand. The Americas were accountable for 50% of the global PBDE market in 1999, demanding nearly 34,000 metric tons (Renner, 2000; Law *et al.*, 2003). Since 1970, an estimated 100,000 metric tons of the penta-BDE commercial mixture have been produced globally (Alcock *et al.*, 2003). Unlike many other flame retardant chemicals, PBDEs are applied to materials after production. Thus, these chemicals are not chemically bound to the materials to which they are applied, and are prone to environmental leaching (Sellström *et al.*, 1993; deWit, 2002).

Fish analyzed from Swedish rivers in the early 1980s resulted in the first reports of PBDEs in the environment (Anderson and Blomkvist, 1981; Lindström *et al.*, 1999). Since the first reports, environmental PBDEs have increased globally, both in concentration and in ubiquity. Traces of PBDEs have been found in tissues of a variety of organisms from some of the most remote regions of the globe and have been shown to biomagnify through increasing trophic levels (deWit, 2002; Law *et al.*, 2003; Tomy *et al.*, 2004; Wolkers *et al.*, 2004). Hepatopancreases of Dungeness crabs (*Cancer magister*) found along the coast of Vancouver, British Columbia, reportedly had 4.2 ng total PBDEs/g-lipid from even the most pristine sites, and ranged from 200-480 ng total PBDEs/g-lipid from suburban and industrial sites. Liver samples from English sole from the similar parts of Canada averaged concentrations around 150 ng PBDEs/g-lipid (Ikonomou *et al.*, 2002). Spring Baltic herring and Atlantic char found along the coast of Sweden in the late-1980s contained average concentrations of tetra-BDE congeners around 450 ng/g-lipid (Sellström *et al.*, 1993). Salmonids caught from Lake Michigan in the fall of 1996 averaged 2,440 ng total PBDEs/g-lipid (Manchester-Neesvig *et al.*, 2001). White crappie (*Pomoxis annularis*), bluegill (*Lepomis macrochirus*), and common carp (*Cyprinus carpio*) caught in Hadley Lake, about 1.3 km from a PBDE production facility in Indiana, had total PBDE concentrations of 2,400 ng/g-lipid.

Pisciverous birds obtain biomagnifying contaminant loads from the fish they consume. Livers of black guillemots (*Cepphus grylle*) from two remote areas of Greenland had average total PBDE concentrations of around 70 ng/g-lipid (Vorkamp *et al.*, 2004). Great cormorants (*Phalacrocorax carbo*) from the United Kingdom had liver PBDE concentrations from 300-6,400 ng/g-lipid (reviewed in deWit, 2002). As females mobilize fats for yolk production, lipid-stored HAHs such as PBDEs are made biologically available, often polluting eggs and potentially affecting embryonic development (Law *et al.*, 2003). Total PBDE concentrations in Forster's tern (*Sterna forsteri*) eggs in 2002 from the San Francisco area came close to 12,000 ng/g-lipid (She *et al.*, 2004). Concentrations of PBDEs extracted from herring gull (*Larus argentatus*) eggs from multiple sites around the Laurentian Great Lakes from 1981-2000 ranged from just below 2,000 to near 16,500 ng/g-lipid (Norstrom *et al.*, 2002). Peregrine falcon (*Falco peregrinus*) eggs obtained from multiple sites throughout

Sweden from 1987-1999 contained total PBDE concentrations as high as 39,000 ng/g-lipid (Lindberg *et al.*, 2004). Thus, contamination concentrations biomagnify in the higher trophic groups of birds as they consume aquatic prey with high PBDE concentrations.

Popular interest has increased, however, as traces of PBDEs are being reported in mammals and humans. The first report of PBDEs in marine mammals was issued in 1987. It was found that the PBDE concentrations in ringed and harbor seals (*Phoca hispida* and *Phoca vitulina*, respectively) collected in the Baltic, Arctic, and North Seas, measured at 90, 40, and 10 ng/g-lipid (total PBDEs) respectively (Jansson *et al.*, 1987; Lindström *et al.*, 1999). Pilot whale (*Globicephala macrorhynchus*) blubber sampled in 1996 from the Faroe Islands just below the Artic Circle had total PBDE concentrations of more than 1,000 - 3,000 ng/g-lipid (Lindström *et al.*, 1999). Blubber from bottlenose dolphins (*Tursiops truncatus*) sampled from the Gulf of Mexico had total PBDE concentrations as high as 8,000 ng/g-lipid (Kuehl and Haebler, 1995). Human breast milk samples extracted from women near Austin, Texas, had summed PBDE concentrations higher than 400 ng/g-lipid (Schecter *et al.*, 2003).

Congener concentrations in environmental samples are relative to congener distributions in commercially used mixtures, with some exceptions. These exceptions are based on a variety of break down and availability variables, such as natural degradation properties, water solubility, and differences of chemical metabolism in exposed organisms (IPCS, 1994; de Wit, 2002; Wolkers *et al.*, 2004). Bromkal 70-5DE, the penta-mixture used in Europe, is a mixture of 37% BDE-47 (2,2',4,4'-tetraBDE), 35% BDE-99 (2,2',4,4',5-pentaBDE), 6.8% BDE-100 (2,2',4,4',6-pentaBDE), 3.9% BDE-153 (2,2',4,4',5,5'-hexaBDE), 2.5% BDE-154 (2,2',4,4',5,6'-hexaBDE), 1.6% BDE-85 (2,2',3,4,4',-pentaBDE), and 0.41% BDE-138 (2,2',3,4,4',5'-hexaBDE). Congeners found in the Great Lakes were similarly distributed:

43% BDE-47, 26% BDE-99, 13% BDE-100, 11% BDE-153, 4% BDE-154 (Norstrom *et al.*, 2002). The average congener profile measured in humans (samples from blood, adipose, and milk) exposed primarily through foodstuff remains somewhat consistent with the commercial mixture profiles: 54.9% BDE-47, 20.2% BDE-153, 14.4% BDE-99, 9.7% BDE-100, and 5.0% BDE-154 (Hites, 2004).

Temporal trends of environmental concentrations of PBDEs vary based on locale and organism. Two long-term studies on birds, one looking at guillemots from Sweden and another looking at herring gulls from the North American Great Lakes, show different increasing environmental concentrations. The guillemot study (1970-1989), showing increasing trends of PBDEs from the earliest production, showed a PBDE doubling at a rate of every 5.8 years (Sellström *et al.*, 1993; reviewed in Hites, 2004). The Great Lakes herring gull study (1981-2000) revealed a much faster contaminant-doubling rate of 3.4 years, possibly due to a closer proximity to manufacturing plants and high use areas (Norstrom *et al.*, 2002; reviewed in Hites, 2004). The rates of increases in PBDE concentrations of these two areas are consistent with human studies from the same regions (reviewed in Hites, 2004).

Similar to chlorinated HAHs, PBDEs appear to have different bioaccumulative properties based on the species exposed. A study looking at changes in PBDE concentrations through Arctic food chains shows that PBDE congeners vary from organism to organism, and between sexes and ages. Polar bears were able to metabolize and excrete virtually all PBDE congeners. Females of both beluga whales and polar bears had the lower PBDE concentrations than males, probably due to the mobilization of fats for birthing. Calves in both species had the highest respective congener concentrations (Wolkers *et al.*, 2004).

As organochlorines such as DDT and PCBs in the environment have generally plateaued over the last decade, concentrations of PBDEs are increasing rapidly. PBDEs, on the other hand, are rapidly increasing with their continued use. From 1972 though 1997, concentrations of PBDEs in human milk have doubled every five years (Norén and Meironyté, 2000). The European Union, the Canadian government, and areas of the United States have enacted guidelines to phase out PBDEs, primarily with respect to the tetra-mixtures. Plants producing PBDEs have begun to halt production of the penta-BDE mixtures in an effort to curb environmental concerns.

1 - 2.2 TCDD

Dioxins are produced as byproducts of industrial processes such as combustion and incineration of chlorinated wastes, chlorine-based bleaching of pulp and paper, and manufacturing of other chlorinated chemicals, including a variety of pesticides and herbicides. Dioxins are a group of up to 75 different chemicals, sharing a structure of two phenyl rings held in planar conformation by two ether groups on adjoining carbons, and varying by the number of chlorine groups surrounding the benzene rings. Like other HAHs, dioxins are lipophilic, environmentally persistent, and bioaccumulative. Structurally locked in a planar configuration, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has the highest affinity of known compounds to the aryl hydrocarbon receptor (AhR), and is thus considered the most toxic anthropogenic chemical to living organisms through the AhR pathway.

1 - 3 Toxicology

1 - 3.1 AhR Affinity and TEFs

The AhR is a cytosolic protein that, once bound to its ligand, migrates into the cell nucleus and elicits DNA unwinding and binding on a portion of the gene called the dioxin response element (DRE). The result is DNA transcription yielding induction of mRNA for a number of proteins, many of them for the cytochrome P450 (CYP1A) family of metabolic enzymes. Toxicity of HAHs, as a rule, is usually dependent on their affinity to the AhR, with the more planar HAHs having the highest binding affinities (Safe, 1986; Safe, 1990).

Toxic equivalency factors (TEFs) are used to compare toxicity of a HAH to that of the most toxic, TCDD. TEF values are determined by a comparison of toxic effect concentrations of each HAH to that of TCDD for a series of *in vivo* and *in vitro* toxicity endpoints. The resultant equivalency factor provides a relative toxicity value to compare HAH potencies. Thus, the most potent HAH, TCDD, would has a TEF equal to one, and other HAHs would be represented by a fraction based on it's relative comparable toxicity to that of TCDD. Although the toxicity of PBDEs remains largely unknown, evidence is uncertain as to their primary route of toxicity. Research has begun to determine PBDE affinity to the AhR, and thus its toxicity related to dioxin.

Three PBDE commercial mixtures and 18 synthesized congeners were compared to TCDD for AhR affinity and CYP1A induction in an effort to formulate TEF values for PBDEs. Using hepatocytes from rat, chicken embryo, rainbow trout, and human *in vitro*, studies show that PBDEs have relative AhR binding affinities at least two orders of magnitude lower than TCDD in all cases (Chen et al, 2001; Behnisch *et al.*, 2001; Behnisch *et al.*, 2002; Chen and Bunce, 2003; Brown *et al.*, 2004). Of the congeners tested, BDE-119 had the highest AhR activation to a DRE-binding form, similar to TCDD at a 10^{-8}M, but at a 10^{-5}M concentration. BDE congeners -77 and -126 stimulated a maximum AhR activation to a DRE-binding form only ~60 and 80% respectively when compared to that of TCDD, again at much higher concentrations. Congeners 47 and 99, two of the most environmentally relevant congeners, remained almost completely unbound to the AhR, as were all commercial mixtures (Chen and Bunce, 2003; Brown *et al.*, 2004).

When tested in conjunction with TCDD, many PBDE congeners have been shown to alter normal TCDD/AhR reactions. Four PBDE congeners appeared solely agonistic, activating the AhR (BDE-32, -153, -166, and -190). BDE congeners 85, 99, and 119 can act as either an agonist or an antagonist depending on the concentration used. Nine congeners (i.e. BDE-15, -28, 47, 77, and 138) were AhR antagonistic, out-competing TCDD for binding sites but failing to induce CYP1A induction (Behnisch *et al.*, 2001; Behnisch *et al.*, 2002; Boon *et al.*, 2002; de Wit, 2002; Chen and Bunce, 2003). With the most environmentally relevant congeners' ability to act antagonistically to the AhR, effects from exposure to mixtures containing these congeners may appear depressed.

Much of the data collected regarding possible environmental contamination and toxicity was obtained using commercial mixtures. While analyses of commercial PBDE mixtures somewhat represents what is found in the environment, toxicity results from these mixtures can also be misleading. Congener components in combination may present some antagonistic or synergistic effects, similar to that found in research dealing with other mixtures (such as shown in studies using commercial PCB mixtures) (Zhou *et al.*, 2001; Carpenter *et al.*, 2002; Darnerud, 2003). For example, data related to AhR binding and induction of active cytochrome P450 (CYP1A), a primary metabolic enzyme related to the activation of the AhR, was collected using a series of individual PBDE congeners and three commercial brominated flame retardant mixtures. While some individual congeners had some induction of active CYP1A, the same congeners in the mixtures did not provide the same results (Chen *et al.*, 2001).

1 - 3.2 HAH Toxicity

HAHs such as PCBs and TCDD have been shown to have toxicological effects on physiological, reproductive, and behavioral endpoints in both laboratory and field studies (Powell *et al.*, 1996; Hoffman *et al.*, 1998). HAHs toxicity can directly or indirectly affect metabolism, kidney and liver function, body and organ masses, and mortality (Blankenship *et al.*, 2003; Grasman *et al.*, 1998; Rifkind *et al.*, 1985). Sensitivity to HAH toxicity varies by organism, age, and sex (Nikolaidis *et al.*, 1988; Kennedy et al; 1996).

Exposure to HAHs often results in increased embryonic mortality, as seen in a variety of colonial waterbirds (reviewed in Gilbertson *et al.*, 1991). Avian toxicity effects from HAHs have been documented since the 1960s, and include eggshell thinning from DDT and its derivatives, as well as decreased hatching success and growth retardation from dioxins and PCBs. Pisciverous birds such as double-crested cormorants (*Phalacrocorax auritus*), black-crowned night herons (*Nicticorax nycticorax*), Caspian terns (*Sterna caspia*), and herring gulls (*Larus argentatus*) from HAH-contaminated portions of the Laurentian Great Lakes have been observed with a series of abnormalities and deformities entitled Great Lakes Embryo Mortality, Edema, and Deformities Syndrome (GLEMEDS). Chicks with GLEMEDS have high incidences of extreme edema; cross bill, missing eye, and clubfoot deformities; male feminization; decreasing body and organ masses; lengthened incubation and yolk absorption periods; and poor piping and hatching success. Biochemical indications of GLEMEDS also include altered vitamin A storage and metabolism, increased metabolic liver enzymes (i.e. porphyrins and cytochome P450), and hormone alterations (reviewed in Gilbertson *et al.*, 1991; Ludwig *et al.*, 1996).

Embryonic malformations and increased mortality from avian exposure to PCBs, (GLEMEDS in wild birds or chick edema disease in chickens) are probably the most pronounced consequences of HAH toxicity. Developmental abnormalities associated with this group of syndromes include crossed bills, missing eyes, malformed vertebrae and appendages, and high incidences of edema, hemorrhages. Gastroschisis and exencephally (GI tract and brain outside body respectively) are also characteristics of GLEMEDS (Rifkind *et al.*, 1985; Gilbertson *et al.*, 1991; Fox *et al.*, 1991; Sanderson *et al.*, 1994; Ludwig *et al.*, 1996; Grasman *et al.*, 1998). While symptoms of GLEMEDS originate from observations of wild birds exposed to high HAH concentrations around the Great Lakes, these effects have been observed in the lab following HAH exposure experiments (Powell *et al.*, 1997; Hoffman *et al.*, 1998; Fox and Grasman, 1999; Grasman and Whitacre, 2001; Blankenship *et al.*, 2003; Goff *et al.*, 2005).

Many HAHs have similar molecular structure to natural steroid and thyroid hormones, thereby competing for hormone receptor binding sites resulting in altered or impeded receptor function or altered hormone bloodstream concentrations. HAH exposure often results in altered thyroid and sex steroid function, potentially resulting in developmental abnormalities, sterility, and behavioral irregularities. Thyroid hormones play crucial roles in embryonic and juvenile development, primarily concerned with neural development and body growth. Thyroid hormones also help to regulate body temperature and oxidative metabolism, heart rate and circulation volume, and hair, feather, and nail growth. The active thyroid hormone comes in two forms: thyroxine (T_4) and triiodothyronine (T_3). While both forms are active hormones, T_3 has as much as four times the affinity to the thyroid hormone receptor (THR). T_4 is a more stable form of the hormone, with a longer half-life, and is thus considered more a storage form for cell delivery via blood plasma. A large extent of the circulating hormone is bound to one of two plasma proteins, thyroxine-binding globulin and thyroxine binding prealbumin (or transthyretin)., extending hormone half-life and increasing solubility in blood At the target cell, T_4 is deiodinated to the T_3 form for binding to the THR (Norris, 1997).

HAHs directly influence thyroid homeostasis. There are a number of mechanisms by which HAHs affect free and bound thyroid hormones in the bloodstream. Some HAHs are remarkably similar in molecular structure to both thyroid hormones, with an iodine group replaced with a halogen such as chlorine or bromine. The similar structure to thyroid hormones can result in HAH competing for and binding to thyroid binding proteins in bloodstream (Goldey and Crofton, 1998; Chauhan *et al.*, 2000). Replacement of the normal hormone on plasma binding proteins results in a shortage of T_4, primarily due to its short half-life and increased glomerular removal in its free state, resulting in hypothyroidism (Debier *et al.*, 2005). Another mechanism for thyroid disruption by HAHs is through the production of metabolic enzymes through AhR activation. These metabolic enzymes, such as CYP1A, are believed to break down normal enzymes used in thyroid metabolism and deiodinization. Without these enzymes, T_4 is not converted to T_3, again resulting in hypothyroidism (Chauhan *et al.*, 2000; reviewed in McNabb and Fox, 2003; Debier *et al.*, 2005). HAHs, such as PCBs and their breakdown products, have also been shown to induce the phase II liver enzyme UDP-glucuronosyltransferase (UDP-GT), which increases T_4 excretion from the body, thus decreasing circulating bound and free thyroxine concentrations (Barter and Klassen, 1994; Van Birgelen *et al.*, 1995; Morse *et al.*, 1996). Regardless of the mechanisms involved, HAH exposure has been directly correlated with alterations in thyroid hormone concentrations. T_3 was negatively correlated with HAH concentration in wild sea lions from the California coast (Debier *et al.*, 2005). Oral administration of Aroclor 1254, a commercial mixture of PCBs, to pregnant rats resulted in decreased circulating T_4 concentrations in the offspring (Barter and Klassen, 1994; Van Birgelen *et al.*, 1995; Morse *et al.*, 1996). Correspondingly, pups from rats exposed to Aroclor had decreased body weight, early eye opening, reduced motor activity, decreased startle responses, and increased hearing loss. When subcutaneous T_4 was supplemented to pregnant dams exposed to the PCBs, effects on motor skills, startle responses, and hearing loss were significantly reduced (Goldey and Crofton, 1998).

1 - 3.3 Effects on the Immune System

The primary role of the immune system is to protect an organism from invading, potentially harmful microorganisms. The humoral immune response protects against extracellular antigens such as bacteria and viruses, while the cellular response defends against microorganisms that invade cells (Janeway *et al.*, 2001). When the immune system is compromised, the organism could be mortally susceptible by any invading pathogen.

Since the early 1970s, HAHs have been known to affect the immune system (Friend and Trainer, 1970; Vos *et al.* 1973). HAHs are now known to affect both humoral and cellular immune responses. Multiple tissue targets within the immune system have been recognized in field and laboratory studies (Grasman et al, 1996 and Kerkvliet *et al.*, 2002 respectively). HAHs are known to suppress antibody (humoral) responses in lab and field studies. In mice, TCDD suppressed antibody responses to sheep red blood cell (SRBC) by 50% at

0.7μg/kg (Kerkvliet *et al.*, 1990; Silkworth *et al.*, 1993). In rats, 30μg/kg was needed to produce the same results (Smialowicz *et al.*, 1994). IgM was suppressed following exposure to TCDD in AhR+ B cells *in vitro* (Holsapple *et al.*, 1991; Sulentic *et al.*, 1998). Significant dose-dependent atrophy and resulting decrease in lymphocyte cellularity of the bursa of Fabricius was evident in lab chicken studies following PCB treatment *in ovo* (Fox and Grasman, 1999; Whitacre and Grasman, 2001; Goff *et al.*, 2005). IgG concentrations were reduced in herring gulls from sites in the Great Lakes found to be high in HAH concentrations (Grasman *et al.*, 1996).

While the immunotoxicity of HAHs is often dependent on the activation of the AhR, the exact targets and/or mechanisms within the immune system remain somewhat enigmatic. While lymphocytes do have measurable AhR proteins, they have relatively low amounts when compared to other tissues of the body. Epithelial cells of the thymus, which do contain relatively large concentrations of AhR proteins, are the location for a series of selection steps that result in T-cell maturation (Nohara *et al.*, 2000). Positive selection, the first step in the T-cell maturation process, is where immature double positive T-cells (CD4+/CD8+) from the bone marrow are selected for their ability to bind to particular forms of major histocombatability (MHC) proteins. Those immature cells that bind to either of the MHC proteins proliferate and differentiate (CD4+ T cells, or T helper cells, recognize MHCII glycoproteins; CD8+ T cells, or cytotoxic T cells, recognize MHC I glycoproteins). The rest die of neglect or apoptosis. The second step leading to T-cell maturation in the thymus is negative selection, where T-cells that do not strongly react to self-antigens are selected for proliferation, and the rest are allowed die off (Janeway *et al.*, 2001; Laiosa *et al.*, 2002). This step is to maintain a high effectiveness of pathogen elimination with minimal healthy tissue damage (Bachmann and Kopf, 2002; Vorderstrasse *et al.*, 2003). Thymic epithelial cells are a known target of HAHs. Increased apoptosis in thymic epithelium cells from HAHs can result in alterations in thymocyte maturation and modifications in mature T-cell counts (Laiosa *et al.*, 2002; Greenlee *et al.*, 1985).

Prenatal exposure to TCDD produces thymic atrophy and may affect T cell maturation (Goff *et al.*, 2005; Laiosa *et al.*, 2002; Grasman *et al.*, 1996; Andersson *et al.*, 1991; Benjamini *et al.*, 1991). Thymic atrophy is characterized by reduced cellularity of the thymic cortex, particularly of thymic lymphocytes, and is reversible over time (De Heer *et al.*, 1994; Vos *et al.*, 1997/1998). Therefore, exposure in developing organisms is more harmful than exposure in adults. TCDD given orally to female rats at does of 1 or 2 μg/kg significantly reduced thymic mass and cellularity and skewed the ratio of CD4:CD8 cells toward the latter, possibly illustrating effects on T-cell maturation (Nohara *et al.*, 2000). TCDD has also been shown to affect T-cells directly, providing they contain AhR proteins (Kerkvleit *et al.*, 2002).

While the AhR is believed to play a crucial role in HAH immunotoxicity, the exact mechanisms by which HAHs affect the immune system are not completely understood. The importance of AhR in immune suppression was shown using mice that were altered to be either responsive or non-responsive to AhR. Responsive mice were an order of magnitude more sensitive to HAH immunotoxicity than the non-responsive strain, and heterozygous mice showed intermediate sensitivity (Poland and Glover, 1980). AhR knock-out mice showed that this receptor is not involved in normal immune system development, but is necessary for HAH induced immune effects (Kerkvliet *et al.*, 1990; Vorderstrasse *et al.*, 2001; Kerkvliet *et al.*, 2002; Laiosa *et al.*, 2002). TCDD exposure has been shown to initiate inappropriate activation of cells, leading to anergy and cell death rather than inducing immune suppression directly (Kerkvliet *et al.*, 2002). PCBs elicit immunotoxicity through the same mechanisms as TCDD. The immunotoxicity of individual HAH congeners are dependent on planarity, and thus AhR affinity (Tanabe *et al.*, 1987; Safe, 1990; Kerkvliet 2002).

1 - 3.4 Immunotoxic Effects of PBDEs

Four studies have been conducted to determine immunologic effects of PBDEs in comparison to other HAHs: two of them *in vivo* involving rodents (Fowles *et al.*, 1994; Thuvander and Darnerud, 1999), one *in vitro* using human lymphocytes (Fernlöf *et al.*, 1997), and one involving American kestrel (*Falco sparverius*) nestlings (Fernie *et al.*, 2005). While the results of these studies may shed some light as to PBDEs ability to present some aspects of immunotoxicity, their effects on immune function at this point remain largely unknown.

An *in vivo* study using in C57BL/6J mice exposed acutely and sub-chronically to concentrations of DE-71, a commercial penta-BDE mixture, found only a moderate immune suppression at the longest duration of sub-chronic exposure. While no other immune alterations were observed, a 37% plaque reduction response to sheep red blood cells in the longest sub-chronic exposure was significantly different from that of controls. CYP1A and 2B were significantly induced in the sub-chronic group (2B1 [PROD] > 1A1 [EROD]), but not in the acute group. Liver weights significantly increased in the sub-chronic group and in the highest acute dose group when compared to controls (Fowles *et al.*, 1994).

The researchers also monitored endocrine effects, noting changes in total and free thyroxine (T_4) and corticosterone (CS) concentrations. Significant reductions were noted in free and total serum T_4 concentrations. These reductions were observed in all groups except the 100 mg/kg acute dose, although reductions were not dose-dependent. CS concentrations increased with dose concentrations, but also correlated with order of kill. This may be indicative of a result bias, as stress levels increased as cage disturbances increased and cage-mates disappeared (Fowles *et al.*, 1994).

A second immunotoxicity study was conducted directly dosing human lymphocytes *in vitro* with two PBDE congeners, a tetra-BDE (BDE-47) and a penta-BDE (BDE-85) and three PCBs (CB-77, -118, and -153). No significant differences were found between controls and exposed lymphocytes (using pokeweed mitogen (PWM) and phytohemagglutinin (PHA) assays) indicating mitogen-induced proliferation and IgG synthesis were not affected by the PBDE or PCB congeners used (Fernlöf *et al.*, 1997). The lack of observed immune effects by the PCBs, however, may suggest some inefficacies in the model system used.

A third study on PBDE immunotoxicity was completed to compare immunological effects of: i.) commercial mixtures of PBDEs and PCBs (Bromkal 70-5 DE and Aroclor 1254 respectively), ii.) an individual congener of each a PBDE and a PCB (BDE-47 and PCB-105 respectively), iii.) species differences between rats and mice *in vivo*. Similar to the first study, liver weights significantly increased in both rats and mice for all dose groups (except the rats in the lower PBDE mixture group) when compared to controls. However, minimal immunological alterations were observed in rats. Exposure to the tetra-PBDE congener caused a significant 25% decrease in spleen cell numbers in mice. This decrease was also seen in both PCB groups, but not in the Bromkal group. Flow cytometric analyses of splenic lymphocytes revealed significant reductions in CD4+ and CD8+ T-cells in all mouse dose groups except the PBDE mixture. B-cells (surface marker CD45RA) also showed similar reductions in the mouse tetra-BDE dose group only. While the proportions of these cells were not altered, 25% reductions to those of controls were observed in absolute numbers in all these cell types, meaning that the PBDE congener equally suppressed murine splenic T- and B-cells, while PCBs only affected T-cell populations (Thuvander and Darnerud, 1999).

Although thymic mass showed decreasing trends in all dose groups when compared to controls, the only significant decrease was with Aroclor exposure in rats. The Aroclor mixture also resulted in an increased proportion of CD8+ thymocytes and a decreased proportion double negative (CD4-CD8-) immature thymocytes, characteristic of HAH toxicity through the AhR. However, no significant changes in thymic weight or cellularity were observed in any other dose group. Thymic atrophy and altered thymocyte cellularity are characteristic effects of HAH toxicity via the AhR. The researchers concluded that AhR is probably only minimally bound by PBDEs and the single PCB chosen for this study (Fernlöf *et al.*, 1997).

Exposure to Aroclor and the highest dose of Bromkal resulted in significantly decreased IgG synthesis when compared to that of controls in human lymphocyte cultures *in vitro* (Fernlöf *et al.*, 1997). This result is similar to Fowles *et al.* (1994), where their PFC assay demonstrated decreased antibody response to SRBCs, although Fowles *et al.* used near twice the dose of DE-71. Previous studies have shown that coplanar PCB congeners, such as Aroclor 1254 components PCB-77 and -118, can inhibit antibody synthesis, but less coplanar isomers will not. AhR affinity increases from coplanar HAH configurations, and thus adds to the idea of the AhR mechanism in this type of antibody inhibition. The ether group in PBDEs prevents strict coplanar configurations, and the larger bromine atom hampers AhR affinity (de Wit, 2002; Hardy, 2002a). Some PBDE congeners do illicit weak or moderate AhR binding affinity. As the concentration of these more active congeners increases with higher doses, the probability of AhR binding increases, potentially resulting in some decreases in IgG. Other congeners with poor AhR binding affinity (e.g. BDE-47) show no antibody alterations. This also answers why PBDE mixtures may be less potent immunotoxins than PCB mixtures.

Several studies (Fowles *et al.*, 1994; Hallgren *et al.*, 2001; Zhou *et al.*, 2001; Zhou *et al.*, 2002) have shown that individual PBDE congeners and mixtures decrease free and total T_4 in the bloodstream. Effects are repeatedly observed in T_4 concentrations, but T_3 and TSH concentrations are rarely affected. These observations probably rule out direct toxicant effect on the hypothalamic-pituitary-thyroid axis feedback system. While the exact mechanism is still not known, research is pointing to a PBDE-directed inhibition or displacement of the T_4-binding protein transthyrethin (TTR) which allows transport and longer half-life of the thyroid hormone.

1 - 3.5 TCDD and Wood Ducks

Bayou Meto, a major drainage system and wood duck habitat in central Arkansas, was contaminated by TCDD from an industrial plant producing the herbicide 2,4,5-T. The U.S Environmental Protection Agency (US EPA) included this area on the national priorities list of hazardous waste sites in 1982, and the Arkansas Game Commission reported elevated TCDD concentrations in fish and waterfowl from the area. Sediment TCDD concentrations were found to be has high as 12,400 pg/g, and concentrations in fish were found to be as high as 1,900 pg/g wet weight (Johnson *et al.*, 1996). Wood ducks in Bayou Meto began to suffer from reproductive failure similar to that seen in field studies of other aquatic birds exposed to dioxins in the Great Lakes (White and Seginak, 1994 and Gilbertson, 1983, respectively). Preliminary tests by the EPA in 1985-87 showed residues of TCDD in wood duck tissues up to 510 pg/g wet weight (White and Seginak, 1994).

Follow-up studies in the years following resulted in significant reductions in reproductive success in wood ducks with egg TCDD concentrations as low as 14-36 pg/g (White and Seginak, 1994; White and Hoffman, 1995). Subsequent avian field studies (Elliott *et al.*, 1996; Johnson *et al.*, 1996; Woodford *et al.*, 1998; Custer *et al.*, 2005) suggest that wood ducks may be significantly more susceptible to the effects of dioxin than any other wild bird species studied (Kennedy *et al.*, 1996; Sanderson *et al.*, 1998).

1 - 4 Justification for Thesis Research

1 - 4.1 PBDEs and Chicken Embryos

The effects of PBDEs on immune or endocrine endpoints have yet to be tested in avian species. The chicken embryo would be the best-suited model to study such developmental and immunotoxic effects of PBDEs. Chickens are extremely sensitive to the effects of other halogenated aromatic hydrocarbons (HAHs) such as dioxins and PCBs. Immunotoxicity associated with PCB exposure has been well documented in previous studies from this lab, with impacted endpoints including reduced thymic masses, decreased T cell proliferation, and overall reductions in T-cell populations in hatching chicks of PCB toxicity in chicken embryos (Grasman and Whitacre, 2001). Chicken embryos have been used extensively in developmental toxicity studies, as incubation parameters and development schedules of chicken embryos are known and well documented. Injecting chicken eggs with these flame retardant compounds would simulate maternal transmission of PBDEs in the wild. Chickens also present valid models for how these chemicals may affect wild avian species as PBDE concentrations continue to increase.

PBDE concentrations, as with those of other persistent HAHs have been on the rise worldwide. High concentrations have been reported in water systems and biota of North America and Europe. Concentrations as high as 2,300 ng/g lipid of PBDE congeners have been found in Dungeness crabs, Harbor porpoises, and English sole from various sites along the West coast of Canada (Ikonomou *et al.*, 2002). PBDE congener concentrations as high as 25 ng/g wet weight have been reported in Glaucous gulls inside the Arctic Circle (Herzke *et al.*, 2003). Summed PBDE concentrations have been found as high as 16,500 ng/g lipid in Herring gull eggs in the Great Lakes (samples ranged from 1981-2000) and 63,300 ng/g lipid in Forster tern eggs from the San Francisco Bay area in 2003 (Norstrom *et al.*, 2002). Similarly high concentrations have been documented in human tissues and breast milk in the US and Europe (de Wit, 2002; Alaee *et al.*, 2003; Darnerud, 2003). PBDEs continue to leach from household furniture, electronics, and the food we eat, bioaccumulating in humans.

Research and the media continue to publicize potential effects of these substances as accumulative trends become more apparent. The ongoing global rise in environmental PBDE concentrations only foreshadows the more serious ecological effects of increasing environmental ubiquity, persistence, and biomagnification in wildlife and humans. More research is needed on what exactly these compounds are doing to living systems and how they will inevitably influence the global ecological balance.

1 - 4.1.1 Specific Aim I

To determine, via an egg injection study, if a commercial mixture of PBDEs, DE-71, causes increased mortality, developmental abnormalities, and immunotoxicity in chicken embryos when compared to vehicle controls.

1 - 4.1.2 Hypothesis I

The hypothesis of this study is that a chicken embryo exposed to a commercial mixture of polybrominated diphenyl ethers (PBDEs) will show evidence of characteristic HAH toxicity. Such evidence would include, but not be limited to, increased mortality, developmental abnormalities, and signs of immunosupression (altered primary immune organ mass and cellularity) when compared to vehicle controls.

1 - 4.2 TCDD and Wood Ducks

Wood ducks inhabit much of the United States, with extensive breeding grounds throughout the Mississippi and Atlantic Flyways. Historic point-source discharges of dioxins from paper and wood pulp mills often overlap on wood duck habitats. Studies from Bayou Meto, Arkansas, where wood duck habitats juxtaposed sites with heavy dioxin contamination, led researchers toward conclusions of high wood duck sensitivity to dioxin toxicity. Similar locations of dioxin contamination overlapping waterfowl habitats have also been documented along the lower Roanoke River and its tributaries, where low dioxin levels were found in blue heron eggs (Beeman *et al.*, 1993).

Dioxins are carcinogenic, mutagenic, teratogenic, and immunotoxic. TCDD has been said to be one of the most toxic synthetic compounds ever produced by humans (Poland and Kende, 1976; ATSDR, 1998). The known most sensitive avian species to the effects of TCDD (and TCDD-like chemicals) is the chicken, where concentrations of only 150 pg/g TCDD injected in eggs resulted in 50% mortality (Powell *et al.*, 1996). If wood ducks are even close to as sensitive as chickens, it would create the perfect early indicator sentinel species, as they readily inhabit artificial nest boxes and feed in areas surrounding their nests. Further research is required to verify the sensitivity of wood ducks.

1 - 4.2.1 Specific Aim II

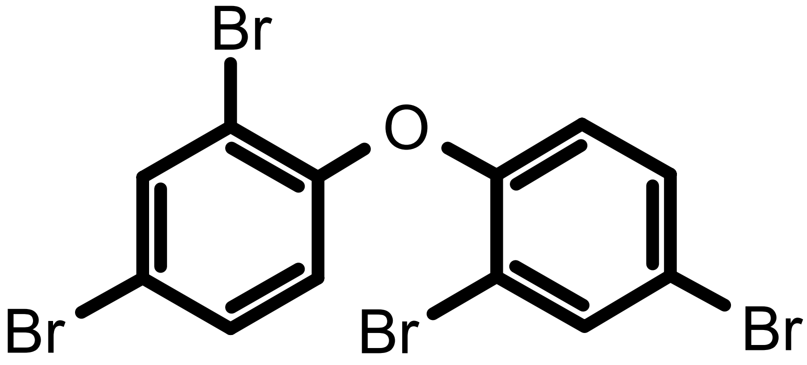
To compare relationships between immune variables and TCDD concentrations in wood duck embryos.

1 - 4.2.2 Hypothesis II

The hypothesis of this study is that wood ducks embryos exposed to environmentally relevant concentrations of TCDD will show signs of immunosupression (altered primary immune organ mass and cellularity in wood duck embryos and decreased T lymphocyte-mediated immunity responses to the phytohemagglutinin-P (PHA-P) skin test in chicks) when compared to vehicle controls.

Figure 1. Molecular structure of (a) 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47), (b) 3,3',4,4',5-pentachlorobiphenyl (PCB 126), (c) 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD), and (d) Thyroid hormone Thyroxine (T4).

(a)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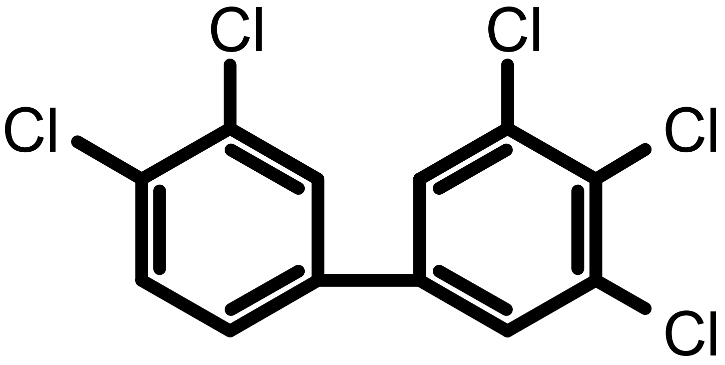
(b)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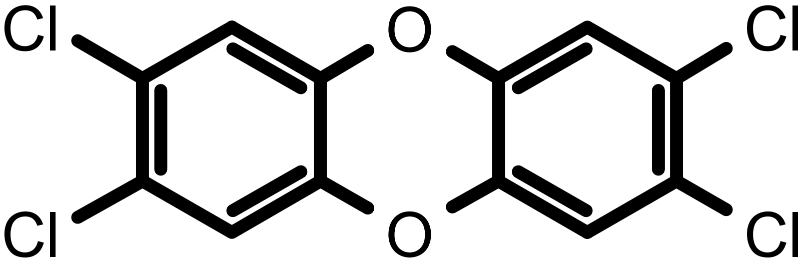
(c)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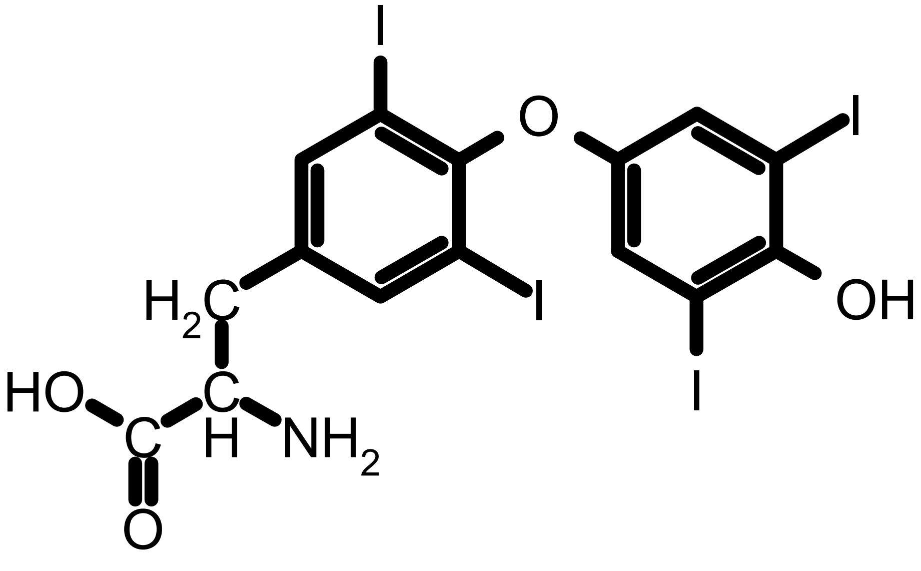
(d)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Chapter 2: DEVELOPMENTAL AND IMMUNOLOGICAL EFFECTS OF A COMMERCIAL MIXTURE OF PBDE FLAME RETARDANTS IN CHICKEN EMBRYOS

2 - 1 Summary

Polybrominated flame retardants, primarily polybrominated diphenyl ethers (PBDEs), share chemical characteristics with other HAHs and are suspected to alter thyroid function. The objective of this study was to determine if a commercial mixture of PBDEs, DE-71, causes mortality, developmental abnormalities, and immunotoxicity in chicken embryos. Fertilized chicken eggs were injected on embryonic day 0 with DE-71 dissolved in sunflower oil at dose concentrations of 0, 0.64, 1.6, 4, 10, and 25 μg/g egg. A dose of 0.8 ng/g egg polychlorinated biphenyl (PCB) 126 was used as a positive control because it causes embryonic mortality and immunotoxicity. Immunotoxicity was determined by examination of the mass and cellularity of the thymus and bursa of Fabricius, the sites of T and B lymphocyte maturation, respectively. One day before hatch, the thymus and bursa were weighed and homogenized, and lymphoid cellularity and viability were assessed using trypan blue exclusion and a hemacytometer. PCB 126 produced the expected significant increase in percent mortality (24%), decrease in viable thymocytes in the left lobe of the thymus (26%), and decrease in bursal lymphoid cell density (35%). PCB 126 also increased deformities and subcutaneous and abdominal edema as expected for a dioxin-like chemical. The doses of PBDEs tested did not significantly increase mortality. One embryo in the 0.64 μg/g PBDE group had a crossed bill and missing eyes, deformities commonly associated to dioxin-like chemicals. PBDEs did not significantly affect the mass or lymphoid cellularity of either the

thymus or bursa of Fabricius. While this PBDE mixture was not immunotoxic in chicken embryos at the levels tested, the potential endocrine and teratogenic effects, as well as a potential for interactions with other HAHs, indicate a need for further research.

2 - 2 Introduction

Organochlorines (OCs) such as PCBs, used for decades primarily as thermal insulators in electrical transformers, became chemicals of concern in the 1970s due to their high stability in the environment and ability to biomagnify up trophic levels. Relatively low-to-moderate doses of coplanar PCBs have been shown to result in a multitude of physiological impacts in wildlife and humans, prompting the U.S. EPA to ban the use of PCBs in 1979 (US EPA, 1979). polybrominated diphenyl ethers (PBDEs) are brominated hydrocarbons used as flame retardant additives to plastics and foams, and are found in a variety of household products from furniture to computers (Darnerud *et al.*, 2001; Law *et al.*, 2003). PBDEs have numerous characteristics in common with PCBs and other halogenated aromatic hydrocarbons (HAHs), including high chemical stability, similar molecular structure, high lipophillicity, low vapor pressure, low water solubility, and high environmental longevity. Similar to PCBs, there are 209 possible molecular congeners of PBDEs, differing by the number and location of halogen groups on two phenyl rings adjoined by two ether groups. Although these compounds have been in use only since the late 1970s, extensive use of brominated flame retardants will lead to continued release into the environment for years to come, even with current mandatory and voluntary production limitations in place in the United States and the European Union (Rahman *et al.*, 2001; de Wit, 2002; Alcock *et al.*, 2003; Benisch *et al.*, 2003).

While concentrations of other HAHs in the environment have primarily been leveling off or slowly decreasing since the 1980s (Pekarik and Weseloh, 1998; Scheider *et al.*, 1998; Aguilar and Borrell, 2005), environmental concentrations of PBDEs are rising exponentially (Norstrom *et al.*, 2002; She *et al.*, 2004). For the past two decades, levels of PBDEs in the environment have increased exponentially with their continued use (Norstrom *et al.*, 2002; Alcock *et al.*, 2003; Hites, 2004). Much like PCBs, PBDEs biomagnify in adipose tissue and have been found in organisms in remote areas of the world, far away from human activities. Yet, with the increasing trends with respect to concentration and ubiquity, little is known regarding toxicological mechanisms and effects of PBDEs on living organisms (Darnerud, 2003; Law *et al.*, 2003).

Measurements of PBDEs in human breast milk from Sweden have illustrated doubling levels every five years from 1972 through 1997 (Norén *et al.*, 2000). PBDE levels in herring gull eggs from the Great Lakes have increased at even faster rates, with levels doubling every 2.8 years. Researchers unexpectedly found that PBDE concentrations in the Great Lakes were higher than most other organohalogen chemicals, including chlordanes, chlorobenzenes, and hexachlorocyclohexanes (HCHs), all considered as environmental chemicals of concern for decades, and were doubling at nearly twice the rate seen in previous lake trout studies. Great Lakes herring gull eggs were found to have twice the levels of the congener BDE-47, the congener of the greatest concern and the highest levels in the environment, than in guillemot (*Uria aalga*) eggs from the Baltic Sea. Concentrations of BDE-99 measured in Great Lakes herring gull eggs in 2000 were higher than ever recorded over the past 20 years in the Baltic region of Europe (Norstrom *et al.*, 2002).

While PBDEs share physiochemical characteristics with PCBs and other HAHs, a great deal is known regarding the toxicological mechanisms and effects of the latter. A variety of developmental abnormalities has been attributed to PCB exposure in wildlife and laboratory animals. Effects of PCB toxicity include increased mortality, reduced reproductive success, and higher incidence of disease and abnormalities directly and indirectly associated with the chemicals themselves. Other abnormalities observed in wildlife exposed to PCBs and other HAHs include an overall wasting syndrome resulting in significant loss of body mass; severe subcutaneous edema in the head and neck; internal edema in the heart, lungs, and abdomen; and crossed bill, clubbed feet, and missing or malformed eyes (Gilbertson, 1991; Ludwig *et al.*, 1996).

Developmental toxicity, immune suppression and endocrine disruptions are well-documented affects of PCB and dioxin toxicity (Kennedy *et al.*, 1996; Ludwig *et al.*, 1996; Hoffman *et al.*, 1998; Fox and Grasman, 1999; Grasman and Whitacre, 2001; Blankenship *et al.*, 2003; Goff *et al.*, 2005). Planar PCBs and dioxins have been shown to have detrimental effects on both humoral and cellular immune responses, thus drastically affecting an organism's ability to fight infectious microorganisms and parasites (Vorderstrasse *et al.*, 2003). Multiple tissue targets within the immune system have been recognized in laboratory studies (Kerkvliet *et al.*, 2002). Exposure to planar HAHs causes atrophy of both the thymus, which is responsible for T-cell maturation, and the bursa of Fabricius, the avian organ for B-cell maturation (Fox and Grasman, 1999; Whitacre and Grasman, 2001, Goff *et al.*, 2005). Lymphoid cells themselves, as well as the production of antibodies, have been shown to be negatively impacted following HAH exposure (Sulentic *et al.*, 1998).

Planar HAHs are classified as endocrine disrupting compounds due to their ability to disturb hormone production, level, metabolism, and (or) action. Compounds such as PCBs and dioxins alter unbound thyroid hormones in the bloodstream by competing for thyroid-binding proteins, resulting in increased natural elimination of thyroxine (T_4) and triiodothyronine (T_3), ensuing hypothyroidism (Chauhan *et al.*, 2000; Debier *et al.*, 2005). Prenatal disruption of thyroid hormone can be particularly harmful, as this hormone is responsible for growth, neural development, cardiac development, metabolism, and temperature regulation (Goldey and Crofton, 1998).

Previous research has shown that chickens (*Gallus gallus*) are drastically more sensitive to the toxic effects of other HAHs (i.e., PCBs and TCDD) than most any other avian species (Blankenship *et al.*, 2003; Grasman and Whitacre, 2001; Fox and Grasman, 1999; Hoffman *et al.*, 1998; Nikolaidis *et al.*, 1988). Thus, chicken embryos exposed to DE-71, a commercial PBDE mixture, may show signs of immunotoxicity where rodents have not. The objective of this study was to determine if exposure to environmentally relevant levels of a commercial PBDE mixture during development would result in increased mortality and abnormalities, as well as any indication of immunotoxicity, similar to that previously observed with PCBs, in chicken embryos.

2 - 3 Materials and Methods

2 - 3.1 Chemicals

A commercial PBDE-based flame retardant mixture, DE-71 (Great Lakes Chemical, Indianapolis, IN), was injected into chicken eggs at the following doses: 0, 0.64, 1.6, 4, 10, and 25 μg of DE-71 per gram of chicken egg. Doses were constructed based on congener profiles of PBDE concentrations in herring gull eggs from the Great Lakes, as well as projected environmental trends for the next decade (Norstrom *et al.*, 2002).

DE-71 was dissolved into a sunflower oil vehicle, which also served as the injected control, in amber glass jars and mixed using a Teflon-coated magnetic stir-bar for 24 hours. DE-71 appeared to be dissolved in the sunflower oil after one hour of agitation; however, the solution was allowed to continue to mix for 24 hours to ensure consistent dispersal. The mixture was then allowed to sit undisturbed for another 24 hours to observe any potential precipitation, but none was observed. The mixture was then serially diluted to provide lower doses. The ortho-polychlorinated biphenyl congener PCB 126, at a dose of 0.8 ng/g of chicken egg, was used for comparison as a positive control for PCB-related increases in mortality and immunotoxicity (Fox and Grasman, 1999; Grasman and Whitacre, 2001).

2 - 3.2 Egg Injection, Incubation, and Embryo Assessment

White Leghorn Chicken eggs (CBT Farms, Chestertown, MD) were stored at 15^{o}C for up to five days from laying date until injection (and incubation) date. Eggs were inspected and candled upon arrival. Eggs were weighed, and the location of the air was determined and marked in pencil on the shell on the day of injection, or embryonic day 0 (ED 0). The shell adjacent to the air cell was sterilized with ethanol, and a flame-sterilized probe was used to make a small hole in the shell. Injection protocols were consistent with previous work in this lab (Fox and Grasman, 1999; Grasman and Whitacre, 2001; Lavoie and Grasman, 2005).

Twenty eggs were injected for each dose group except the PCB positive control group, which had 27 to account for the predicted 35-40% mortality. The PBDE- or PCB-carrier mixtures and the carrier control were injected into the air cell in aliquots of 0.1 μL/g egg via 10ul glass Hamilton syringes (Hamilton No.: 80330). Immediately following injection, the air cell hole was sealed with melted paraffin wax, and the eggs placed pointed-end in a cabinet incubator (Model 1202, G.Q.F. Manufacturing Co., Savannah, GA) set at 37.5°C and 48-59% humidity. Eggs were turned every six hours, until day 19, when they were turned horizontal until dissection at ED 20, one day prior to hatch. Incubators were monitored daily for temperature and humidity. Eggs were candled on days 4, 11, and 18, and non-viable eggs were opened, examined for deformities, and staged according to Hamburger and Hamilton (1951).

2 - 3.3 Tissue Collection and Lymphoid Cell Quantification

Embryos were euthanized by decapitation on ED 20. Embryos were inspected for signs of deformities and (or) edema. Body and yolk masses, tarsus length, head length, and head width were measured.

Ten embryos from each dose group were chosen randomly for organ preservation. The liver, heart, brain, and thyroid were removed, weighed, and preserved for further analyses.

Both lobes of the thymus and the bursa of Fabricius were removed and weighed (Fox and Grasman, 1999; Grasman and Whitacre, 2001). The right thymus lobe was cryopreserved for further studies. The left lobe of the thymus and the bursa were homogenized in separate Kontes tissue grinders with phosphate-buffered saline (PBS). Homogenate was diluted and live and dead lymphoid cells were counted using trypan blue and a hemacytometer under 400x magnification.

2 - 3.4 Statistical Analyses

One-way analysis of variance (ANOVA) was used to test for differences between treatment groups in cell counts and body masses. If ANOVA was significant, Dunnett's test was used to compare the mean of each treatment group to that of the negative control. All statistical analyses were conducted using JMP 5.1 (SAS Institute, Cary, NC). Organ masses (except thyroid due to small masses) were divided by yolk-free body mass and multiplied by 100 to provide an indexed value. Non-injected controls were pooled with vehicle control eggs if no significant differences were found. Mortality data were expressed as the % of dead embryos relative to fertile eggs. Mortality was analyzed statistically using a one-way Fisher's exact test (ToxCalc Ver. 5.0.20, Tidepool Scientific Software, McKinleyville, CA).

2 - 4 Results

No significant differences were observed between non-injected and carrier controls for any measured variable. Therefore, data from these two groups were combined for the following analyses.

2 - 4.1 Mortality, Body Mass, and Abnormalities

PBDEs did not seem to directly affect mortality, as the number of eggs that did not survive to hatch differed little from controls. Less than 2% of the eggs showed no evidence of development and were therefore considered infertile and excluded from all analyses. Mortality was 0% in the 1.6 and 4 μg/g dose groups; 3-5% in the negative control and the 10 μg/g dose groups, and 10-15% in the 0.64 and 25 μg dose groups (Figure 2). A significantly elevated mortality rate of 26% was observed in the PCB positive control group (Fisher's Exact p=0.02), slightly less than expected from this dose range based on previous experiments from this lab, (Fox and Grasman, 1999; Grasman and Whitacre, 2001).

Some developmental deformities were observed in embryos exposed to PBDE, but PBDEs did not affect body mass. Of the total 154 embryos, 15 showed signs of edema and (or) deformities consistent with planar HAH toxicity (Table 1). The breakdown of deformities within this study showed four of these birds had gastroschisis (two in the PCB treatment group and two in the negative control). One low dose (0.64 µg/g) PBDE embryo had major physical deformities previously seen with planar HAHs, including missing eyes and crossed bill. A comparison of body masses between all PBDE treatment groups showed no significant differences from controls (F=1.99, n=140, p=0.07) (Figure 3). Body masses from the PCB positive control group were nonsignificantly lower than negative control.

2 - 4.2 Immune Organ Masses

Although some differences were observed in the comparison of immune organ masses, this study failed to show any statistically significant differences as a result of PBDE exposure. The mass of the left thymic lobe (indexed for body mass) showed significant differences between treatment groups (F=3.26, n=137, p=0.005) (Figure 4). However, only the positive control PCB 126 dose group was significantly lower (12%) than the negative control (Dunnett's test, p=0.006). No significant differences in bursal indices were found with between dose groups (F=0.62, n=139, p=0.7) (Figure 5).

2 - 4.3 Lymphoid Cellularity

Embryos exposed to the PBDE mixture did not yield any significant differences from controls in lymphoid densities (number of lymphoid cells found/organ mass) for both the thymus (F=1.59, n=133, p=0.15) and bursa (F=1.80, n=135, p=0.1). Thymocyte density showed a non-significant decreasing trend in higher PBDE treatment groups following an initial decrease in the lowest dose group from that of the negative control (Figure 6). PBDE effect on bursa density showed a non-significant increasing trend. PCB-126, however, resulted in a non-significant 35% reduction in bursal density (Figure 7).

The PBDE mixture did not significantly alter the cellularity or lymphoid density of primary lymphoid organs. Total viable thymocytes differed significantly among groups (F=2.55, n=136, p=0.02), but no single group differed significantly than the negative control. Total thymocyte numbers were non-significantly26% lower in the PCB 126 group then negative controls (Figure 8). Percent thymocyte viability was between 97 and 100 percent among all dose groups. Bursal lymphoid numbers showed no statistical differences (F=1.42, n=136l, p=0.21) but showed a 29% decrease in bursal lymphoid cells in the PCB 126 group (Figure 9).

2 - 4.4 Liver, Heart, and Thyroid Mass

Few changes were observed in mean liver masses (indexed for body mass) between negative controls and PBDE treatment groups (F=1.23, n=140, p=0.3) (Figure 10). Mean heart masses (indexed for body mass) of PBDE treatment groups showed no significant differences from negative controls; however, embryos exposed to PCB 126 embryos showed significant 18% increase in heart mass from negative controls (F=2.87, n=139, p=0.01, Dunnett's test - p=0.008) (Figure 11).

No significant differences were found when comparing thyroid masses of embryos exposed to PBDEs and that of negative controls (F=1.52, n=126, p=0.18). The lowest PBDE treatment group resulted in a non-significant mean reduction thyroid mass; however, increasing doses yielded dose-dependent increases in thyroid masses, with the highest dose returning to a mean mass consistent with negative control. PCB 126 exposure yielded mean thyroid masses nonsignificantly lower (25%) than negative controls (Dunnett's p=0.27) (Figure 12).

2 - 5 Discussion

Little has been demonstrated on the potential toxic effects of PBDEs on living systems. Thus, this study of possible effects of PBDE exposure on developmental and immunological endpoints is particularly pertinent biologically, especially with the increasing concentrations and ubiquity paralleling historical trends of chlorinated HAHs, at least from an environmental standpoint. The present study, however biologically significant, failed to demonstrate statically significant effects as a result of exposure to a commercial PBDE mixture at the neither concentrations nor endpoints tested.

2 - 5.1 PBDEs

The concentrations used in this study were environmentally relevant, consistent with the concentrations presently found and expected in the future in herring gull eggs in the Great Lakes (Norstrom *et al.,* 2002). At the concentrations used in this study, no significant effects were observed in chicken embryos exposed to DE-71 *in ovo*. Previous studies on male Sprague-Dawley rats found oral an LD_{50} of DE-71 at as high as 7400 mg/kg. DE-71 also had no teratogenic effects when given to pregnant female rats at levels up to 200 mg/kg (ICPS, 1994). No mortality studies with respect to PBDEs were found related to avian species.

Some deformities were observed in PBDE treated embryos in this study that showed remarkable similarity to characteristic HAH-related deformities. However, this study was not designed to analyze deformity rates, and the sample sizes were not sufficient to maintain any statistically significant changes. One chick from the lowest PBDE dose group had numerous physical abnormalities commonly associated with HAH toxicity. This chick, surviving only until the egg was opened, had gross head deformities, including missing eyes and crossed bill, as well as severe abdominal edema.

This study did not reveal any statistically significant immunotoxic effects with the exposure of the commercial PBDE mixture DE-71 at doses the doses described on chicken embryos *in ovo*. The overall lack statistically significant DE-71 effects on thymus and bursa are in agreement with other studies (Darnerud and Thuvander, 1999; Fernie *et al.*, 2005), supporting the idea that these brominated compounds, at least in a commercial mixture, have weak interaction with the Aryl hydrocarbon Receptor (AhR), a cytosolic protein believed the immunotoxic pathway for other HAHs (such as with PCBs). Some studies have shown limited immunotoxic effects of PBDEs on exposed organisms. One such study found reduced cellular and humoral responses to applied antigens, but these appeared associated with levels of specific congeners (BDE-47 and BDE-183 respectively), and not the commercial mixture (Fernie *et al.*, 2005). The exposed kestrels also showed some signs of humoral suppression, although again not statically significant, by way of antibody-related antigen analysis. The present study found a similar 9% increase in thymocyte numbers, precursors to T-cells, in the three middle PBDE doses, similar to the previous study findings, and possibly illustrating a result of some biological importance. Analyses of the bursa and bursal lymphocytes, responsible for antibody production after maturity and exposure to a potential antigen, found an initial non-significant decrease in lymphocyte numbers at the lowest PBDE dose. This non-significant bursal lymphocyte reduction is followed by a gradual increase back to that of control in, what appears to be, a dose-related trend.

Lymphocytes of the spleen were also significantly reduced in another PBDE study, were mice and rats were exposed to BDE-47 *in vivo*. Yet, consistent with the present study, no such effect was observed in the commercial mixture (Thuvander and Darnerud, 1999). Yet another PBDE congener study found no effects of two individual PBDE congeners (BDE-47 and -85) on human lymphocytes *in vitro* (Fernlöf *et al.*, 1997). Thus, the present study follows previous reports by Fowles (1994) and Thuvander and Darnerud (1999) in illustrating that PBDEs, at least in commercial mixtures, do not appear to be potent immunotoxins.

This study presented slightly elevated liver masses in the chicken embryos exposed to DE-71, but not significantly outside the range of the negative control. However slight, the higher liver mass is consistent across all PBDE treatment groups, except the very lowest dose, suggesting links to potential hepatic enzyme induction. Thuvander and Darnerud (1999) suggest liver enzymes may be a more sensitive indicator of PBDE exposure than immune endpoints based on increases in these enzymes in previous studies.

PBDEs can disrupt various aspects of the endocrine system, particularly with respect to thyroxine depletion (Darnerud *et al.*, 2001). While there is some debate as the exact mechanism of thyroid hormone depletion, studies have shown that some PBDE congeners increase hepatic phase II metabolic enzymes (uridinediphosphate-glucuronosyltransferase [UDPGT]), thus increasing T_4 metabolism resulting in hypothyroxinemia (Brouwer *et al.*, 1998; Zhou *et al.*, 2001; Hallgren and Darnerud, 2002). A hypothyroid state activates feedback systems whereby thyroid-stimulating hormone (TSH) levels increase because of low T_3/T_4 levels. Interestingly, PBDE exposure in this study yielded an increasing dose-dependent trend in the mass of the left thyroid. An increase in TSH has been shown to cause enlargement of the thyroid gland, possibly illustrated by PBDE treatments in this study (Norris, 1997).

2 - 5.2 PCBs

The purpose of a PCB in the present study was two fold. First, as PBDEs share many physiochemical characteristics to other chlorinated HAHs, a PCB positive control provided beneficial emphasis to compare toxicity effects. Second, as PCB-126 has been tested extensively in this lab in similar studies, a PCB positive control group only added importance as a measure of quality control. As such, the PCB 126 dose in this study was successful as a quality control standard, as the toxicity results of exposed chicken embryos were comparable to previous research from this lab and others.

Chickens, which are especially sensitive to the effects of dioxin-like chemicals, have demonstrated direct links between exposure to PCBs and significant increases in mortality, abnormalities and immunotoxicity (Ludwig *et al.*, 1996; Kennedy *et al.*, 1996; Hoffman *et al.*, 1998; Fox and Grasman, 1999; Grasman and Whitacre, 2001; Blankenship *et al.*, 2003; Goff *et al.*, 2005). Previous studies of chicken embryos exposed to PCB 126 *in ovo* from this lab reported 48% mortality at the concentration used as the positive control in the current study, as well as 33% observed deformities (Fox and Grasman, 1999, Grasman and Whitacre, 2001; Goff *et al.*, 2005). This is not far from the current study with an observed 27% mortality and 19% abnormalities.

PCB 126 provided some immunotoxicity in exposed chicken embryos as illustrated in previous research (Fox and Grasman, 1999, Grasman and Whitacre, 2001). The PCB 126 positive control treatment resulted in statistically significant decreases in thymic mass and viable thymocyte cellularity. Similar differences were observed in mass and lymphocyte cellularity of the bursa, although these results were not statistically significant.

PCB chicks exposed minimal differences from negative controls with respect to body mass and most organ masses. PCBs, however, showed signs of liver atrophy with mean masses lower than those of controls. While previous studies relate inconsistent results with respect to PCB effects on liver mass, the finding of slight decreases of liver mass in this study has previously been reported (Powell *et al.*, 1996). The PCB 126 positive control group showed a statistically significant increase heart mass (indexed for body mass) when compared to negative control (Dunnett's test p=0.008), similar to results found in previous studies (Powell *et al.*, 1996; Walker and Catron, 2000).

2 - 6 Conclusions and Future Research

Exposure to a commercial mixture of PBDEs at the doses tested in this study did not result in significant immune suppression in chicken embryos *in ovo*. Studies related to organism exposure to Aroclors, the PCB commercial mixtures, have had a tendency to present imprecise results, as congeners within can act differently in the presence of other ingredients. While particular congeners within the mixture may actively bind to AhR and promote DNA transcription, other inactive congeners within the mixture may compete for AhR binding sites, possibly with higher affinities, thereby blocking the potent action of the counterpart. Thus, different congener profiles will impact accurate measures of potential toxicity, and will not truthfully allow comparison to individual congener profiles found in the environment or in an organism (Carpenter *et al.*, 2002). Thus, future research should include studies of congener interactions in mixtures. Similarly, initial studies have shown that PBDEs may actually antagonize dioxin effects when exposed in a mixture (Kuiper *et al.*, 2004; Peters *et al.*, 2006). Because both of these groups of compounds co-exist in the environment, future research in the effects of parallel exposure could provide beneficial in real-world studies.

Future research on endocrine effects of PBDEs may also be of great benefit. Although no significant effects were observed in this study, individual PBDE congeners have been shown to reduce thyroid hormone levels in the bloodstream, yet exactly how and to what extent remains largely unknown. Theoretical links between thyroid hormone and immunity have been documented, and PBDE exposure at high enough levels in the environment may lead to immune impairment through this pathway (Bachman and Mashaly, 1987; Fabris *et al.*, 1995; Dorshkind and Horseman, 2001; Silberman *et al.*, 2002; Zhou *et al.*, 2002; McNabb and Fox, 2003).

Studies aimed at confirming teratogenesis often must reach epidemiological proportions, testing thousands of organisms, to find significant deformity numbers due to exposure to a single or small group of compounds (Summer *et al.*, 1996). In the present study of 134 fertile treated eggs, a single embryo from a lowest level DE-71 treatment was found with a serious set of deformities consistent with AhR activated toxicity: crossed malformed bill, no eyes, and mass of only 40% of the average embryo. This study was not designed to establish if a commercial mixture of PBDEs are potentially teratogenic. Previous studies shed some doubt on the possible teratogenicity of PBDEs, at least in mechanisms comparable to that of dioxins. Yet, the finding of some deformities in this study, while not statistically significant, should be pursued by further research.

Table 1. Abnormalities and mortality in chicken embryos exposed *in ovo* to a commercial mixture of PBDEs or a PCB 126 between ED 0 and 20.

Summary Statistics	Negative Control	0.64 µg DE-71 /g egg	1.6 µg DE-71 /g egg	4 µg DE-71 /g egg	10 µg DE-71 /g egg	25 µg DE-71 g egg	0.8 ng PCB 126/g egg
Total Eggs	30	20	20	20	20	20	27
# Infertile Eggs	1	0	0	2	0	0	0
# Fertile Eggs	29	20	20	18	20	20	27
# Dead Embryos	1	2	0	0	1	3	7
# Viable Embryos	28	18	20	18	19	17	20
# Embryos w/Abnormalities	3	3	1	2	1	0	5
Fertile Eggs w/Abnormalities (%)	10%	15%	5%	11%	5%	0%	19%
Abnormality Categories *(single embryo may have multiple deformities listed below)*							
Head/Neck Edema	1	2	1	2	1		1
Neck Edema		1					
Abdominal Edema							5
Thoracic/Cardiac Edema							2
Eye Deformity		1					
Bill Deformity		1					
Gastroschisis	2						2
TOTAL DEFORMITIES	**3**	**5**	**1**	**2**	**1**	**0**	**10**

NOTE: Negative control group consisted of both vehicle control and non-injected control.

Figure 2. Mortality of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs or 0.8 ng/g PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs).

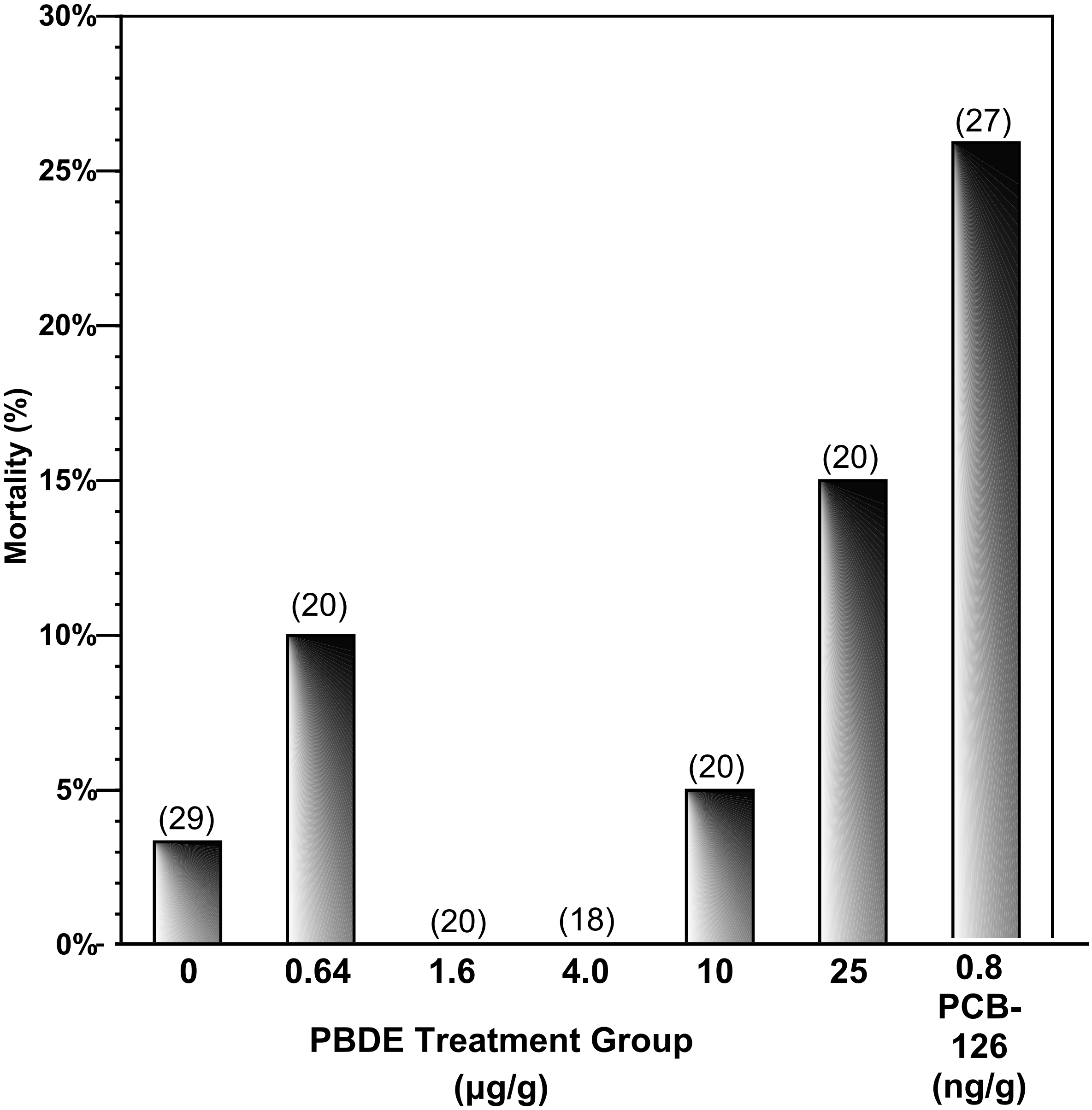

30%
25%
20%
15%
10%
5%
0%
Mortality (%)
(29)
(20)
(20)
(18)
(20)
(20)
(27)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 3. Mean body mass of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or 0.8 ng/g PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.

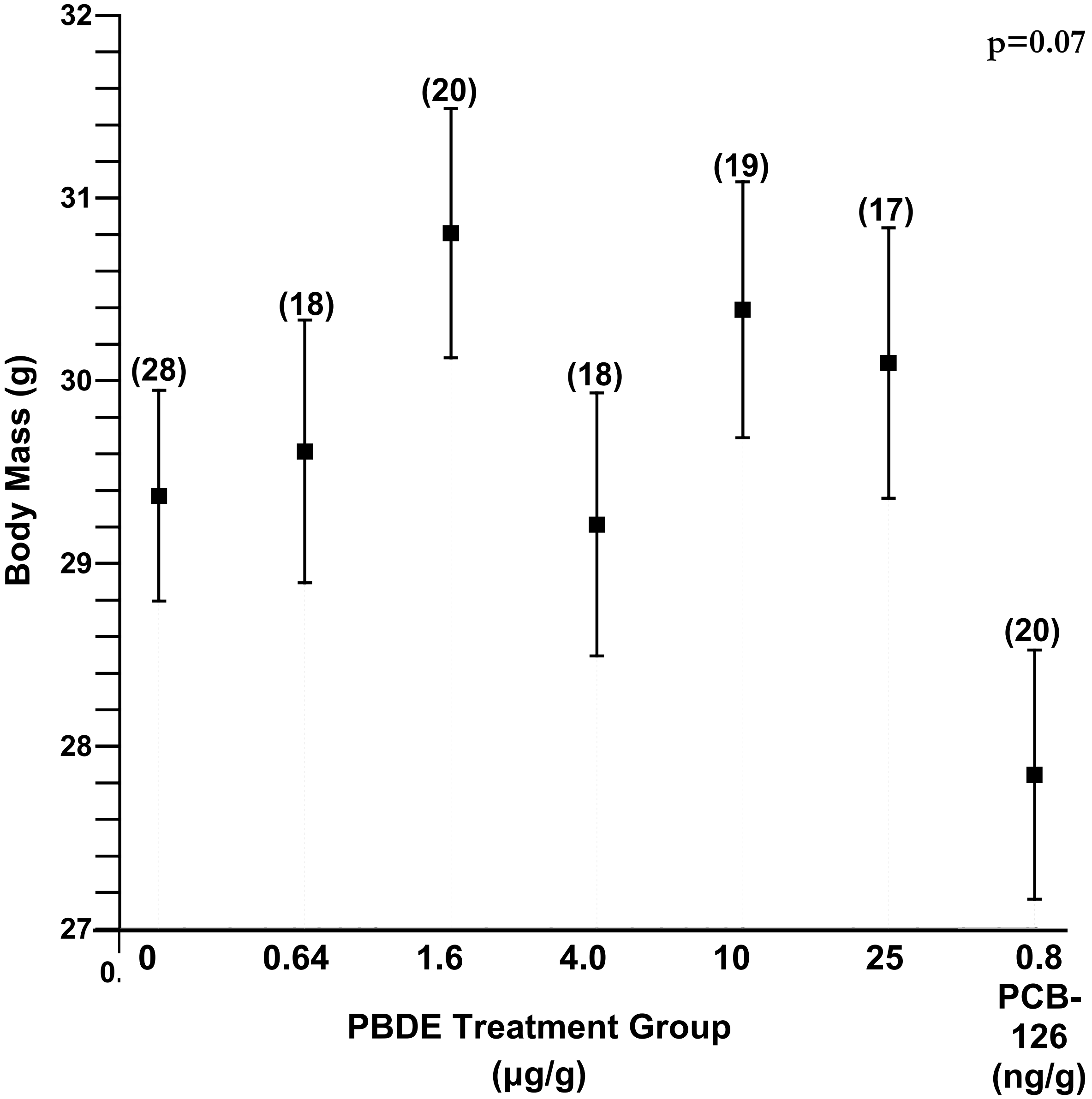

p=0.07
Body Mass (g)
32
31
30
29
28
27
0.
(28)
(18)
(20)
(18)
(19)
(17)
(20)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 4. Left thymus lobe mass (divided by body mass for comparability) of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean. Asterisk indicates group significantly different from control (Dunnett's p<0.05).

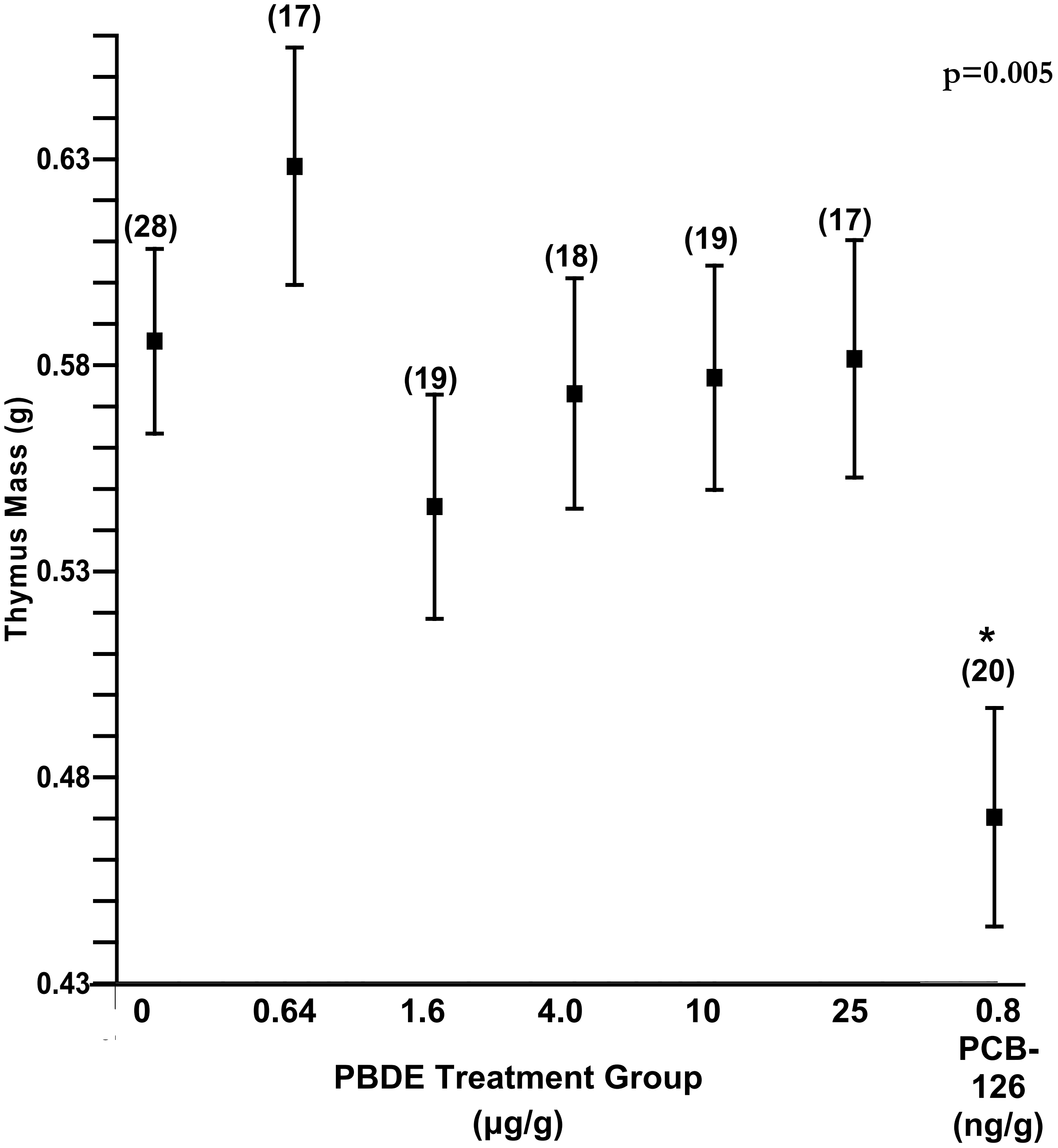
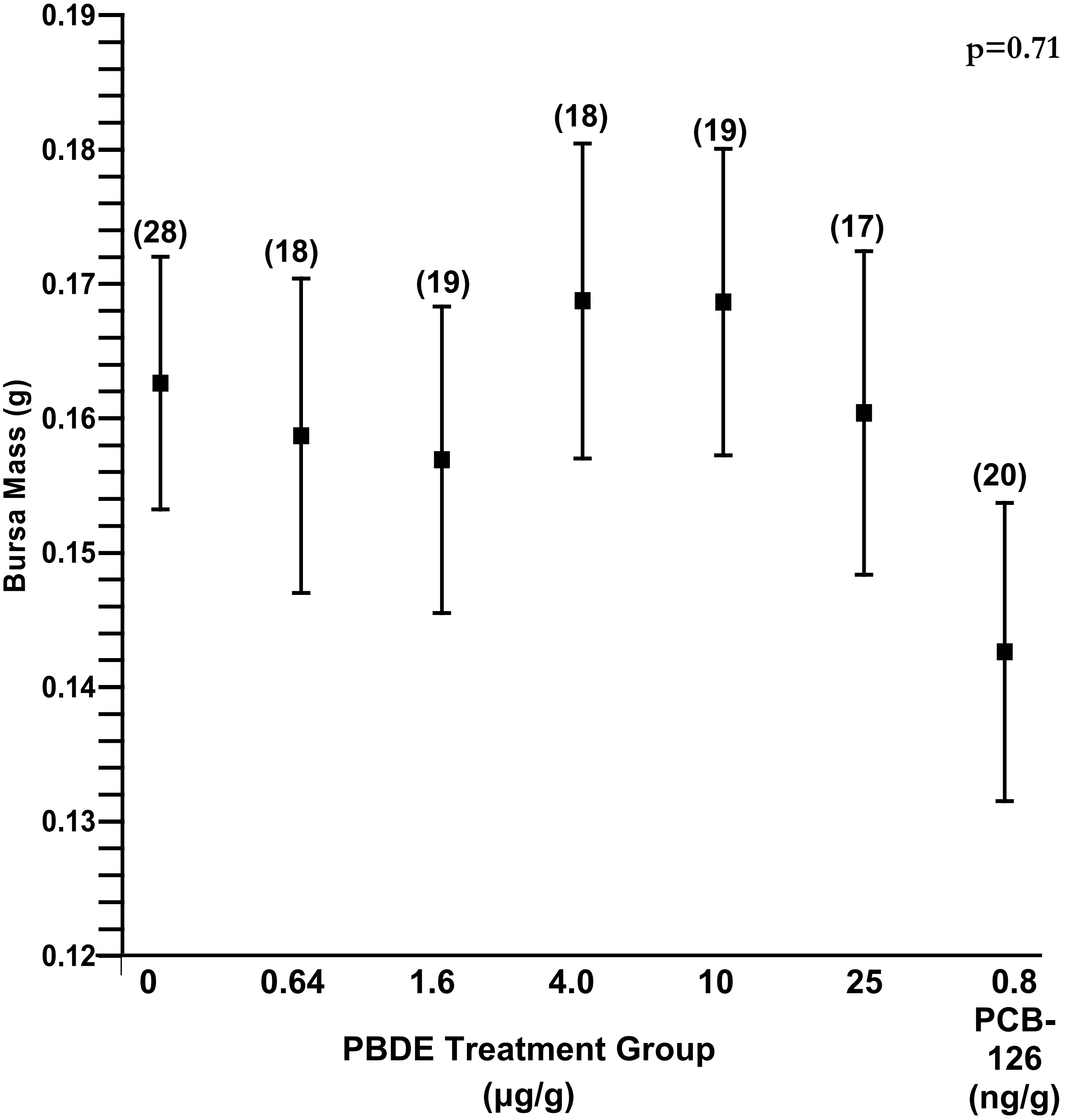

p=0.005
Thymus Mass (g)
0.63
0.58
0.53
0.48
0.43
(28)
(17)
(19)
(18)
(19)
(17)
*
(20)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 5. Bursa mass (divided by body mass for comparability) of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.



p=0.71
Bursa Mass (g)
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
(28)
(18)
(19)
(18)
(19)
(17)
(20)
0
0.64
1.6
4.0
10
25
0.8 PCB-126 (ng/g)
PBDE Treatment Group (µg/g)

Figure 6. Thymocyte density of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.

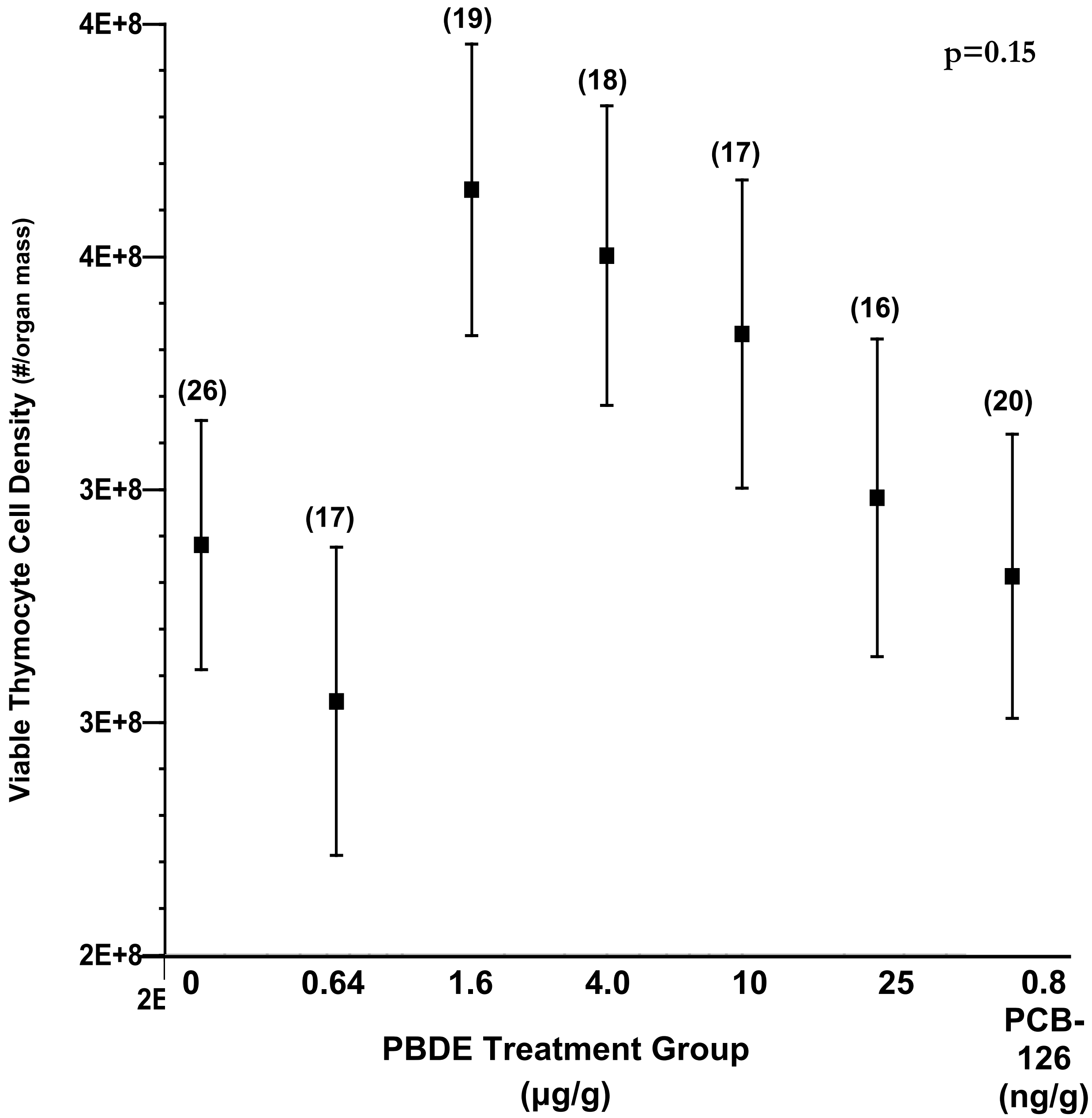
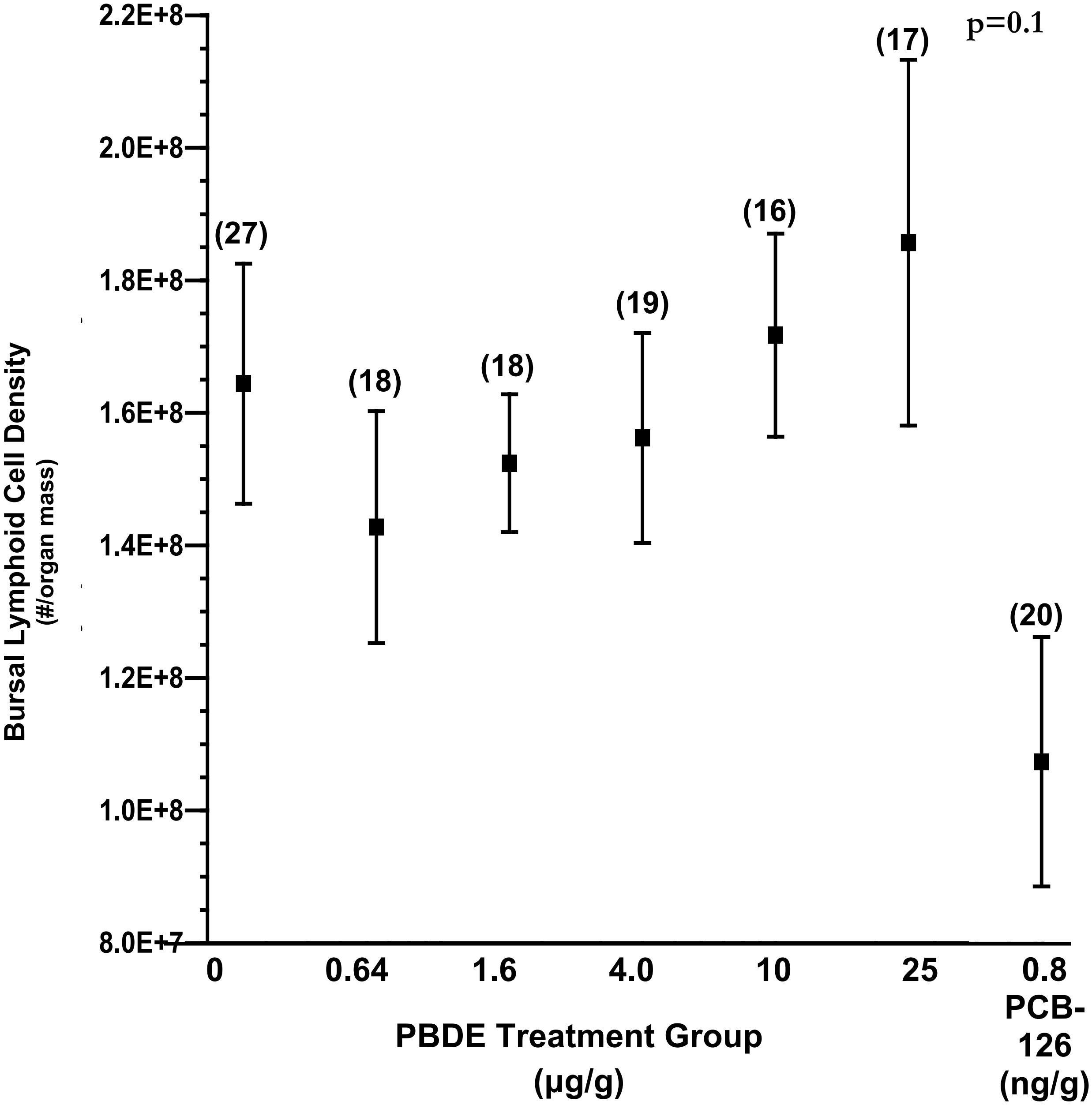
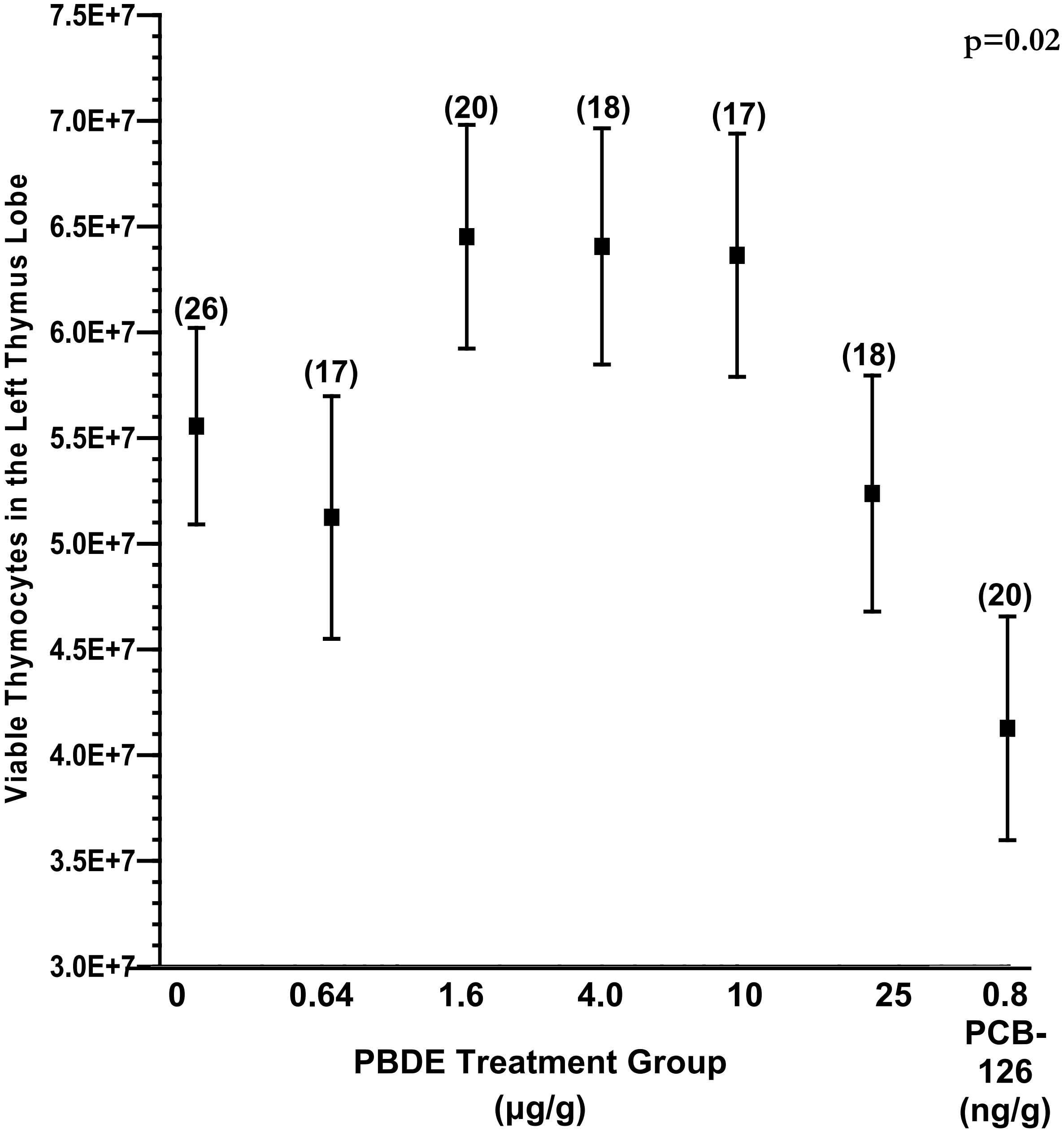

Viable Thymocyte Cell Density (#/organ mass)
4E+8
4E+8
3E+8
3E+8
2E+8
2E
p=0.15
(26)
(17)
(19)
(18)
(17)
(16)
(20)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 7. Bursa lymphoid density of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.



2.2E+8
2.0E+8
1.8E+8
1.6E+8
1.4E+8
1.2E+8
1.0E+8
8.0E+7
Bursal Lymphoid Cell Density
(#/organ mass)
(27)
(18)
(18)
(19)
(16)
(17)
(20)
p=0.1
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 8. Viable thymocytes from the left lobe of the thymus of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.



Viable Thymocytes in the Left Thymus Lobe
7.5E+7
7.0E+7
6.5E+7
6.0E+7
5.5E+7
5.0E+7
4.5E+7
4.0E+7
3.5E+7
3.0E+7
p=0.02
(26)
(17)
(20)
(18)
(17)
(18)
(20)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 9. Viable lymphoid cells from the bursa of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.

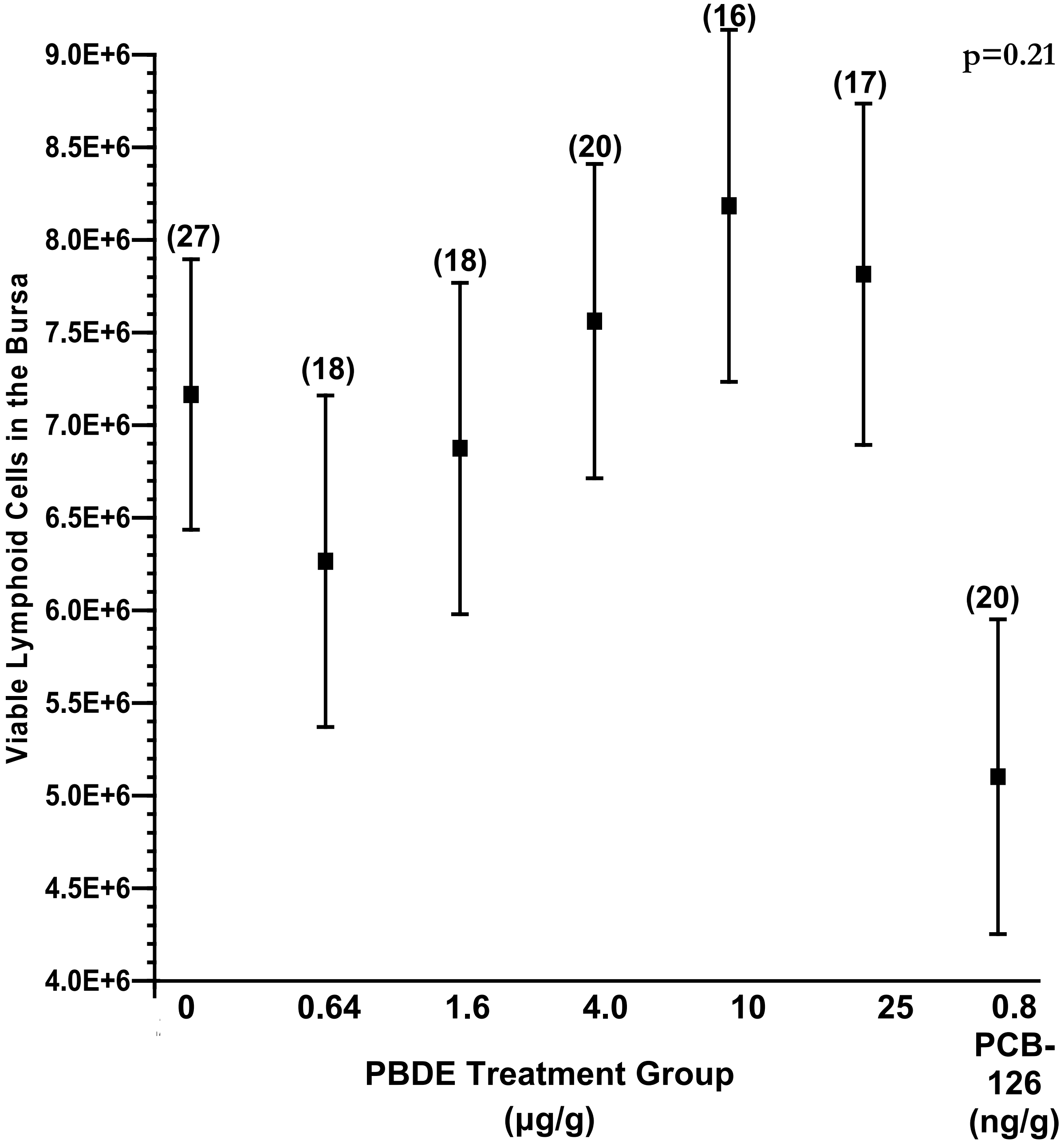

Viable Lymphoid Cells in the Bursa
9.0E+6
8.5E+6
8.0E+6
7.5E+6
7.0E+6
6.5E+6
6.0E+6
5.5E+6
5.0E+6
4.5E+6
4.0E+6
0
0.64
1.6
4.0
10
25
0.8 PCB-126 (ng/g)
PBDE Treatment Group (µg/g)
(27)
(18)
(18)
(20)
(16)
(17)
(20)
p=0.21

Figure 10. Liver mass (divided by body mass for comparability) of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.

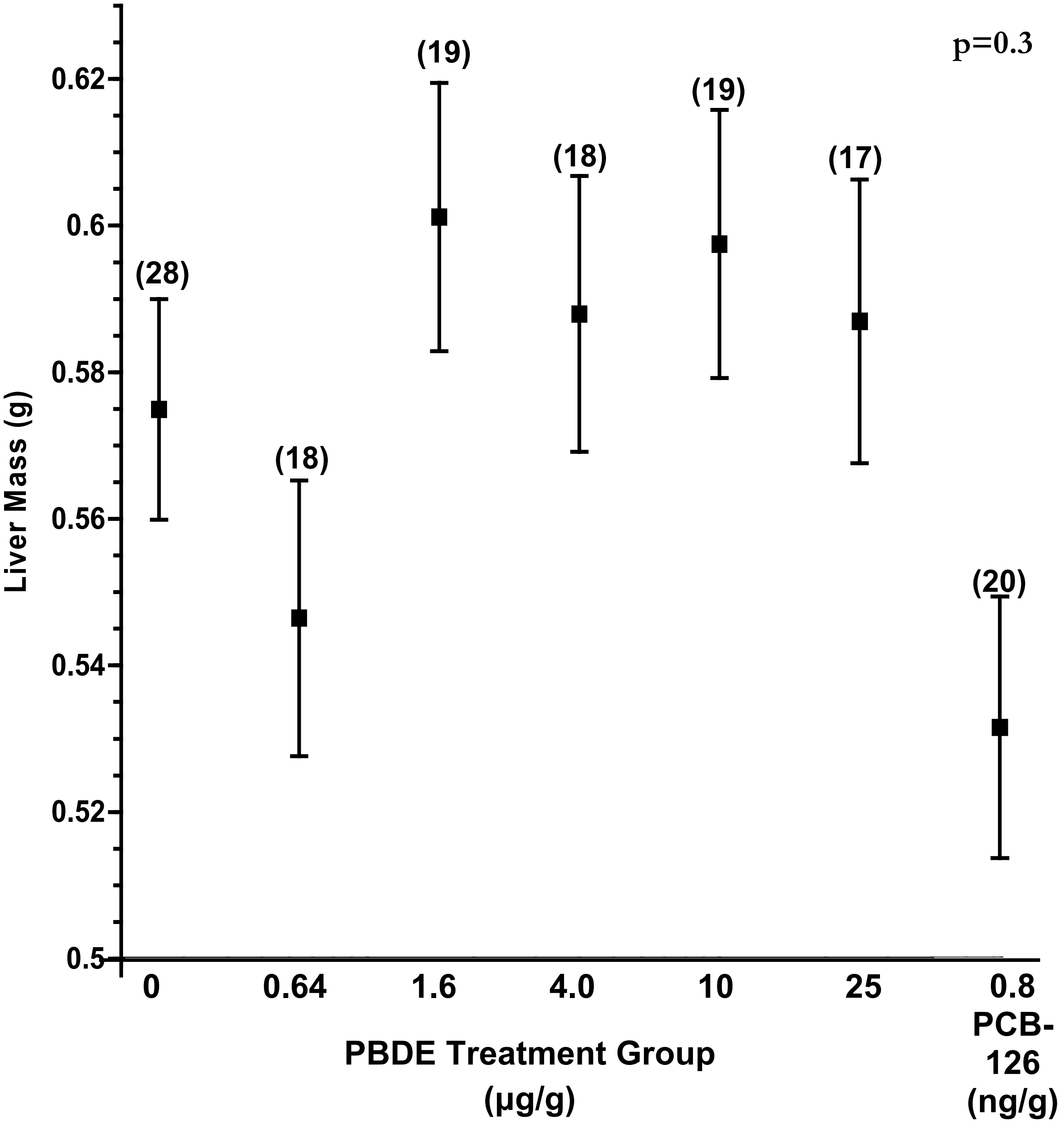

p=0.3
Liver Mass (g)
0.62
0.6
0.58
0.56
0.54
0.52
0.5
(28)
(18)
(19)
(18)
(19)
(17)
(20)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Figure 11. Heart mass (divided by body mass for comparability) of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean. Asterisk indicates group significantly different from control (Dunnett's $p<0.05$).

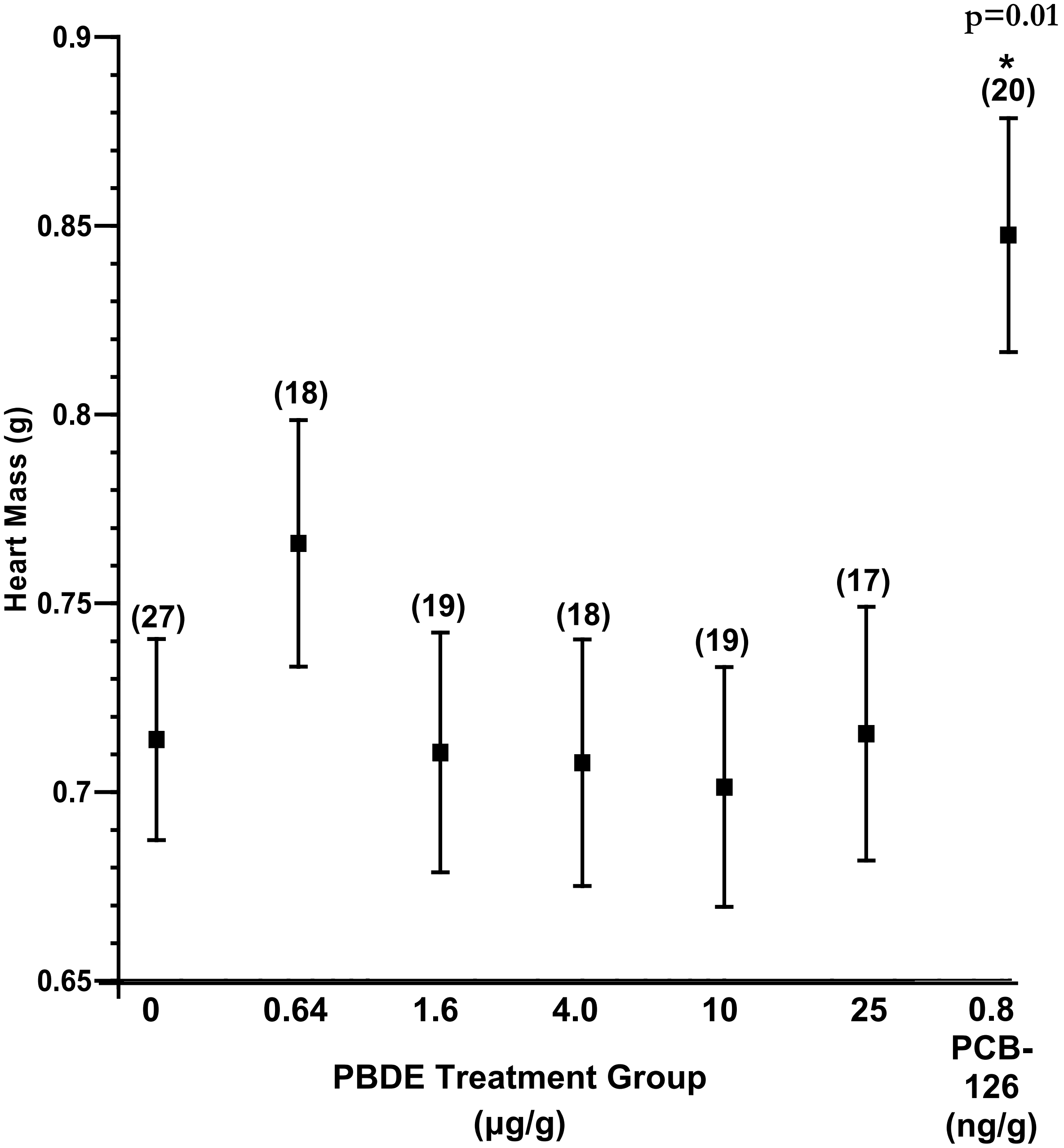
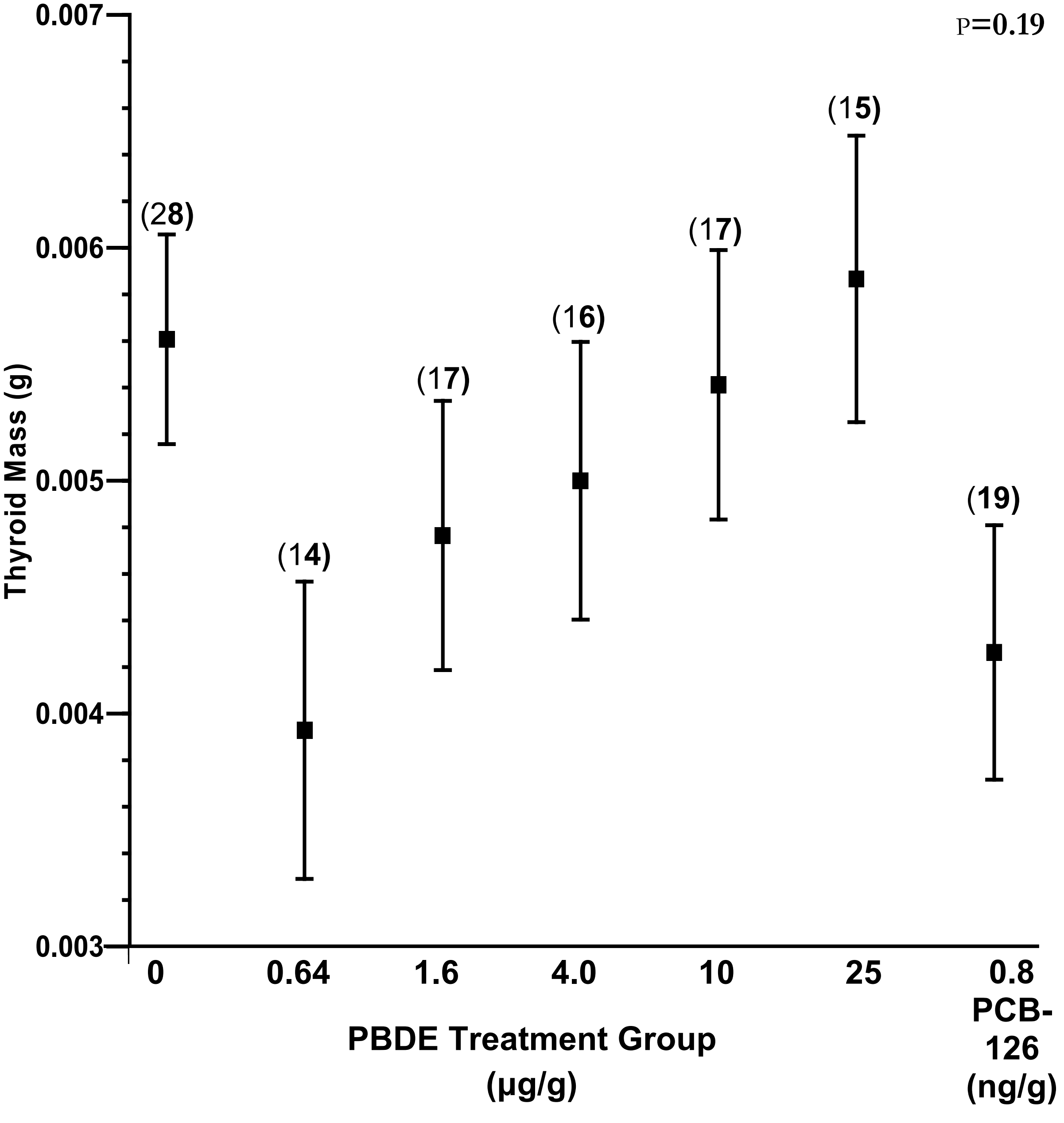

p=0.01
*
(20)
(18)
(27)
(19)
(18)
(19)
(17)
0.9
0.85
0.8
0.75
0.7
0.65
Heart Mass (g)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(μg/g)

Figure 12. Left thyroid mass of chicken embryos exposed *in ovo* to either commercial mixture of PBDEs, or PCB 126 (positive control). The negative control group (dose 0) is pooled non-injected and vehicle control. Numbers in parentheses show number of eggs injected (not including infertile eggs). Error bars indicate standard error of the mean.



P=0.19
Thyroid Mass (g)
0.007
0.006
0.005
0.004
0.003
(28)
(14)
(17)
(16)
(17)
(15)
(19)
0
0.64
1.6
4.0
10
25
0.8
PCB-
126
(ng/g)
PBDE Treatment Group
(µg/g)

Chapter 3: IMMUNOTOXIC EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) IN WOOD DUCKS (*AIX SPONSA)*: A SENSITIVE INDICATOR SPECIES?

3 - 1 Summary

Wood ducks (*Aix sponsa*) are one of North America's most widely distributed endemic species, inhabiting forested wetlands throughout most of the continental United States. While wood ducks breed throughout most of their habitat range, regions of heaviest wood duck reproduction occur along the Mississippi Valley through the lower Great Lakes, often overlapping areas of heavy industrial pollution. Numerous studies have shown that wood ducks may be one of the most sensitive organisms to the toxic effects of chlorinated dioxins, second only to the domestic chicken (*Gallus gallus*). The present study examined the effects of *in ovo* exposure to TCDD (0, 50, 200, 800, or 3200 pg TCDD/g egg) on immune function in wood ducks two weeks after hatch. Hypothesized changes included increased mortality and characteristic dioxin-induced immunotoxicity, including atrophy of thymus and bursa of Fabricius, decreased lymphocyte numbers, and decreased T cell function (phytohemagglutinin (PHA) skin response). Due to high mortality from unknown causes, treatment groups were pooled (non-injected and vehicle controls = control; 50 and 200 pg TCDD/g egg = Low Dose (LD) and 800 and 3200 pg TCDD/g egg = High Dose (HD)) to increase sample sizes. No differences between TCDD treatment groups and controls were found. At the doses tested, no characteristic TCDD-induced immunosupression was

observed, as no mean significant differences were observed between any treatment group and control in any immune parameter tested. Thus, this study suggests, contrary to previous studies, that wood ducks are not particularly sensitive to TCDD at the concentrations tested.

3 - 2 Introduction

TCDD is primarily produced as an inadvertent byproduct of the use, disposal, and combustion of chlorinated compounds, including those associated with the bleaching process of pulp and paper, production of pesticides and herbicides, and disposal of hazardous wastes. Reports of environmental contamination are often localized downstream of plant effluents. A chemical plant responsible for the manufacture of the herbicide 2,4,5-T, was identified by the U.S. Environmental Protection Agency (EPA) to have contaminated a wetland in central Arkansas with dioxins though much of the 1970s (Johnson *et al.*, 1996). In 1982, the EPA placed this area, named Bayou Meto, on the national priorities list of hazardous waste sites (White and Hoffman, 1995; Johnson *et al.*, 1996).

In years following the chemical release at Bayou Meto, researchers have attributed decreased reproductive success in a variety of waterfowl in the area to high 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentrations. Wood ducks (*Aix sponsa*), common throughout the region, had wet tissue mass concentrations of TCDD up to 510 pg/g, and even higher concentrations of other tetra-dioxin homologues. Reports of extremely high reproductive impairments in exposed wood ducks were released from studies of wildlife impacts downriver from the herbicide plant, leading researchers to conclude that this species was significantly more sensitive to the effects of TCDD than those of any other wild bird species, second only to the domestic chicken (*Gallus gallus*) (White and Seginak, 1994; White and Hoffman, 1995).

The lower Roanoke River, from Plymouth, NC, to Albemarle Sound has a history of dioxin release from pulp and paper mills using chlorinated bleaching processes. While these plants were retrofitted in the 1990s and dioxin release has been eliminated since, concentrations remain in sediment and biota in contaminated areas. In the 1990s, wood duck eggs in the lower Roanoke were found to have dioxin levels equivalent to that observed in Bayou Meto, Arkansas, with geometric mean concentrations as high as 502 pg/g wet weight. By the next decade, however, with the elimination of dioxin discharge, egg concentrations fell to only as high as 31pg/g. Unlike what was previously observed in Arkansas, no significant impacts on reproductive success were observed at the lower concentrations measured within the Roanoke watershed (Augspurger *et al.*, 2004).

Relatively minute doses of TCDD, considered the most toxic of the 75 potential dioxin congeners, are enough to increase mortality, decrease body mass, and decrease reproductive success in exposed organisms. Adult Ringed turtle doves (*Streptopelia risoria)*, Mourning doves (*Zenaida macroura)*, and Forster's terns (*Sterna forsteri*) exposed to PCBs and other dioxin-like chemicals have been shown to have neurobehavioral alterations, which often prevent proper mating and (or) parental care behaviors (Peakall and Peakall, 1973; Kubiak *et al.*, 1989; Tori and Peterle, 1983). Avian offspring exposed to planar HAHs *in ovo* often possess harmful deformities and severe edema, severely impacting hatching success and thus increasing mortality. These deformities have been observed in laboratory chickens exposed to dioxin-like chemicals, as well as a variety of wild fish-eating birds, such as herring gulls and cormorants, from areas contaminated with similar chemicals (Gilbertson *et al.*, 1991; Sanderson et al, 1994; Powell *et al.*, 1996; Grasman *et al.*, 1998; Hoffman *et al.*, 1998; Woodford et al, 1998).

Dioxins and other planar halogenated aromatic hydrocarbons have repeatedly been shown to illicit toxic effects on the immune system in laboratory chickens and a variety of wild pisciverous bird species. Immunotoxicity from planar HAHs is believed to occur through a cytosolic protein called the aryl hydrocarbon receptor (AhR). Ligand binding to the AhR and resulting DNA transcription induces the production of the metabolic enzyme family cytochrome P450 (reviewed in Mandal, 2005). The AhR pathway is the primary route by which dioxin exposure can result in immunosupression, exhibiting atrophy of the thymus (responsible for T-cell maturation) and bursa of Fabricius (responsible for B-cell maturation in birds) and decreased lymphocyte numbers.

Confirmation of extraordinary sensitivity of wood ducks to the effects of dioxin exposure may present an ideal sentinel species. Wood ducks will nest in human-made nest boxes and will gain fat reserves prior to reproduction at or near the nest site. This allows nest placement near contaminated areas, where females will forage before egg laying, eliminating variables found in other migratory indicator species. Confirmation of this sensitivity would validate the use of a lower trophic species, rather than the previously used species higher on the food chain and thus more susceptible to biomagnification effects (Dugger and Fredrickson, 1992).

A non-pisciverous sentinel for the presence and severity of environmental dioxin contamination, as well as in the continuing study of how dioxins effect wild populations, may be beneficial. Typically, pisciverous sentinel species primarily see effects from dioxin contamination at higher trophic levels where exposure concentrations climax. A sensitive insectivore or vegetarian sentinel may provide an earlier warning indicator, as well as an effective tool in cleanup strategies.

This study was designed in conjunction with the U.S. Fish and Wildlife Service to confirm high wood duck sensitivity to TCDD. The goal of this study was to investigate whether TCDD causes immune suppression in wood ducks exposed *in ovo* at environmentally relevant concentrations. Wood duck hatchlings, exposed to TCDD via yolk injection prior to incubation, were tested for immunosupression by way of altered T-cell mediated immune responses, T- and B-cell numbers, and primary immune organ masses to those of controls. The outcome of this study was intended to add validation or annul previous conclusions toward the sensitivity of wood ducks exposed to TCDD.

3 - 3 Materials and Methods

3 - 3.1 Egg Collection and Experimental Design

Wood duck eggs were collected from nest boxes from the Patuxent Research Refuge in Maryland in April, 2005 and transported to North Carolina State University in Raleigh in foam-lined cardboard boxes at ambient temperature. Eggs were then candled, and eggs showing signs of damage or developing embryos were removed. Remaining eggs were weighed, labeled, and assigned to a dose group. Eggs were then allowed to sit horizontal in the lab overnight (~20-25^{o}C) to allow the germ spot to rise in effort to avoid injury during injection. Incubators were disinfected prior to use with warm water and commercial incubator disinfectant containing halogenated tertiary amines (Brinsea, Melbourne, FL), and then wiped with 70% isopropyl alcohol. The eggs were then injected into the yolk the following day with a dose of TCDD (0, 50, 200, 800, or 3200 pg TCDD/g egg), using treolein as vehicle, as per Powell *et al.* (1996). The hole was sealed, and the eggs were incubated in a poultry incubator (Brinsea Octagon 250, Melborne, FL) at 37.2^{o} (±0.3) C, maintaining a relative humidity of 30% (adjusted to allow 15% mass reduction over the 28 day incubation period), and rotated 90° each 90 minutes. Eggs were candled weekly though incubation and those not showing signs of growing embryos were removed. On day 26, remaining eggs were placed in a NatureForm Hatchery Systems (Jacksonville, FL) model UT230N hatcher, kept horizontal in hardware cloth hatching baskets, and maintained at a temperature of 37.0° (±0.3) C with a relative humidity of ≥ 75%. Ducklings were weighed and banded at hatch and separated by dose group. Hatchlings were kept in a Petersime (Gettysburg, OH) brooder at North Carolina State University Dearstyne Avian Isolation Facility, where heating elements were placed at one end to allow ducklings a temperature range. Ducklings were fed poultry starter chow *ad libidum* and were provided fresh drinking water daily.

Hatchlings were euthanized by cervical dislocation between day 11 and 15 and a series of tests were performed to determine potential immune suppression between doses. These tests included phytohemagglutinin (PHA skin) test, which measured both T and B cell responses, as well as primary immune organ mass and organ lymphocyte cellularity comparisons between doses.

3 - 3.2 PHA Skin Response

The PHA skin test (Grasman and Fox, 2001) was conducted from 11-14 days after hatch. Wing webs were measured with pressure sensitive calipers (Dyer, Lancaster, PA). A 30 μL subcutaneous injection was made 0.1 mg PHA (Sigma-Aldrich, St. Louis, MO) in 0.03 mL sterile phosphate-buffered saline (PBS) using a 29 gauge insulin needle. Sterile PBS was injected into the control wing. Wing web thickness was again measured after 24±3 hours, and a stimulation index was calculated for each bird by subtracting the increase in thickness in the control wing web from the increase in thickness in the PHA wing web.

3 - 3.3 Immune Organ Assessment

The thymus and bursa of Fabricius were removed and weighed. The left lobe of the thymus and the bursa were homogenized in a Kontes tissue grinder in PBS. Homogenate of each was diluted and the live and dead cells were counted using trypan blue exclusion and a hemacytometer under 400x magnification (Grasman and Whitacre, 2001).

3 - 3.4 Statistics

One-way analysis of the variance (ANOVA) was used to examine the differences between treatment groups. If ANOVA revealed significant effects ($p < 0.05$), Dunnett's test was used to define significant statistical difference of treated groups from controls. Organ masses were divided by body mass and multiplied by 100 for a comparable indexed value. Due to limited treatment numbers, doses were pooled for comparison as follows: non-injected and vehicle controls = controls (C), 50 and 200 pg TCDD/g egg = Low Dose (LD), and 800 and 3,200 pg TCDD/g egg in the high dose (HD) treatment groups. Comparison between dose groups and organ masses were expressed as the mean ± standard error of the mean (SEM).

3 - 4 Results and Discussion

3 - 4.1 Mortality and Body Mass

Considerable mortality was observed from all dose groups related to unknown factors. Previous attempts at this study yielded similar mortality trends, leading researchers to blame possible microbial infection. Extra precautionary actions were implemented in this study with respect to egg treatment and husbandry to virtually eliminate risk of microbial infection, and no signs of infection were observed. On the same note, the incubation parameters used in this study have been previously used with wood duck eggs, thus eliminating the source of increased mortality related to temperature, humidity, rotation, or other incubation parameters. Background mortality appeared indiscriminate, as no differences were observed between treatment groups and essentially did not differ from controls: 29% fertile non-injected control eggs survived to hatch (5/17), 36% fertile vehicle control eggs survived to hatch (5/16), 19% of the eggs in 50pg TCDD/g egg treatment group survived to hatch (3/16), 31% of the eggs in the 200 pg TCDD/g egg treatment group survived to hatch (4/13), 6% of the eggs in the 800 pg TCDD/g egg treatment group survived to hatch (1/16), and 31% of the eggs in the 3,200 pg TCDD/g egg treatment group survived to hatch (5/16)(see Table 1). Some increased mortality is evident when treatment groups are pooled (32% fertile control eggs surviving to hatch (10/33), 24% of the low dose (LD) fertile treatment eggs surviving to hatch (7/32), and 19% of the high dose (HD) fertile eggs surviving to hatch (6/32)), but mortality related specifically to TCDD-exposure may be impossible to differentiate (Table 2).

Previous studies on wild wood ducks in contaminated areas led researchers to conclude that TCDD exposure may result in significant reproductive impairment in levels as low as 20-50 pg/g egg. This is second only to tests on TCDD-injected chicken eggs, where increased mortality and deformities associated with chick edema disease increased significantly at levels as low as 10 pg/g egg (White and Seginak, 1994; White and Hoffman, 1995). Great blue heron (*Ardea herodias*) eggs with concentrations of TCDD as high as 211 ng/g egg did not show increased mortality from that of controls (Hart *et al.*, 1991). Studies of tree swallow eggs (*Tachycineta bicolor*) taken from a TCDD-contaminated river in outside of Providence, RI, determined a 50% reproductive failure (LD_{50}) at 1,700 pg TCDD/g egg, more than 10 times the LD_{50} of chickens (150 pg/g)(Powell *et al.*, 1996; Custer *et al.*, 2005). Double-crested cormorants (*Phalacrocorax auritus*) are believed to have an LD_{50} near or greater than 4,000 pg TCDD/g egg (Powell *et al.*, 1997). If conclusions of previous studies are valid, wood ducks are significantly more sensitive than other species to the adverse effects of dioxin-like compounds. This study, however, shows no evidence that the TCDD concentrations tested yielded any increased mortality when compared to background mortality.

Mean body masses of treated groups were lower than those of controls, characteristic of 'wasting syndrome' often associated with exposure to dioxin-like chemicals; however, no statistically significant differences were observed at the doses used in this study (F=2.40, n=23, p=0.12) (Figure 13). Chicken embryos exposed to TCDD *in ovo* showed statistically significant body mass decreases after one-week post-hatch with doses as little as 20 ng TCDD/g egg (Bruggeman *et al.*, 2003). Without continued TCDD exposure, however, chicks may have regained some body mass post-hatch. No characteristic deformities commonly associated with exposure to dioxin-like chemicals were observed at the doses tested in this study.

3 - 4.2 PHA Skin Response

The phytohemagglutinin (PHA) skin test is a non-invasive method of testing cell-mediated immune function (Grasman *et al.*, 1996). PHA is a T-cell mitogen which, when injected subcutaneously, results in a measurable inflammation. When PHA is compared to a control injection of sterile saline, a cell-mediated response factor can be determined for comparison between treatment groups and controls. A stimulation index is determined by subtracting any increase in skin thickness caused by a control saline injection from the increase caused by the PHA.

The single congener TCDD used in this study at treatments up to 3,200 pg/g egg showed no statistically significant differences with the PHA skin test (F=0.33, n=23, p=0.72) (Figure 14). The mean skin inflammatory response (stimulation index) to PHA initially showed a substantial 37% decrease with the low TCDD dose group, as expected with following dioxin-like chemical exposure, but the high dose group mean appears no different from that of controls.

Wildlife studies have confirmed reduced T-cell function in Caspian turns (*Sterna caspia*) and herring gulls (*Larus argentatus*) using the PHA skin test, where stimulation indices maintained significant decreasing trends with increasing dioxin-like pollution. This decrease in cell-mediated functionality was observed in gulls at TCDD-equivalent levels (TEQs) of 2,600 to over 17,500 pg/g egg and in terns exposed to TEQs from 1,260 to higher than 6,800 pg/g egg (Grasman *et al.*, 1996). While chemical mixtures associated with true environmental contamination can react differently than single congener egg injections in the laboratory, concentration comparisons can illustrate true references of characteristic suppression of t-cell-mediated immunosupression by dioxin-like chemicals.

3 - 4.3 Immune Organ Mass and Lymphoid Cellularity

A primary target of toxicity from dioxin-like chemicals is the immune system, illustrated by characteristic primary immune organ atrophy. In this study, however, no differences were observed with respect to thymus mass (F=2.88, n=23, p=0.08) or bursa mass (standardized for body mass) (F=0.71, n=23, p=.051) differences from those of controls (Figures 15 and

16 respectively). Both humoral (B-cell and antibody) as well as cellular (T-cell) immunity have been shown to be affected by planar aromatic hydrocarbons, such as TCDD and some PCBs. Atrophy of the thymus gland, responsible for T-cell maturation, and impacts on B-cells and antibody production are characteristic immunotoxic effects of dioxin exposure (reviewed by Kerkvliet, 2002). Thymus and bursal atrophy were observed in a chicken egg injection study using PCB-126, where levels equivalent to 6.4 pg TCDD/g egg showed significant atrophy in both organs (Goff *et al.*, 2005).

No statistical differences were observed in relation to lymphocyte densities of the thymus (F=0.88, n=21, p=0.43) (Figure 17) and bursa (F=0.31, n=23, p=0.73) (Figure 18) as well as the total number of viable lymphoid cells in both immune organs to that of controls (F=1.59, n=21, p=0.23 for the thymus; F=0.28, n=23, p=0.76 for the bursa) (Figures 19 and 20 respectively). Immune organ lymphocyte densities and viability numbers from the wood ducks are consistent with chickens as reported from previous studies from this lab (Fox and Grasman, 1999). Any changes in lymphoid cellularity of primary immune organs were minimal at best, and normal immune homeostasis was evident with reciprocal decreases in mean bursal lymphoid cell number and density with increases in mean thymocyte numbers. In previous studies exposing chicken embryos to PCB-126, lymphoid cellularity of primary immune organs was a more sensitive indicator of dioxin-like toxicity than organ masses, showing reductions in lymphocyte cellularity at 13 pg TCDD-equivalents/g egg (Fox and Grasman, 1999).

3 - 5 Conclusion

At the concentrations tested, TCDD had no apparent effects on the immune endpoints measured in this experiment. Although the sample sizes were small due to increased mortality probably related to incubation issues, the overall lack of characteristic toxicity associated with dioxin exposure in wood ducks may indicate potentially exaggerated wood duck sensitivity in previous studies. Previous conclusions of higher sensitivity of wood ducks to dioxins could possibly have resulted from additional environmental variables or from unaccounted for contamination compounds resulting in reduced reproductive success.

This study shows a definite need for further research in a variety of areas. Not only does the sensitivity of wood ducks to dioxins need further study, but the conditions associated with successful incubation of this and other wild species also need further exploration.

Table 2. Numbers of wood duck (*Aix sponsa*) eggs surviving to hatch following injection of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) [non-pooled breakdown].

Treatment Group	Eggs Injected	Infertile	Sample Size (minus infertile)	Survival to Hatch	% Hatch
Non-injected Control	17	0	17	5	29%
Vehicle Control	16	2	14	5	36%
50 pg TCDD/g egg	16	0	16	3	19%
200 pg TCDD/g egg	16	3	13	4	31%
800 pg TCDD/g egg	16	0	16	1	6%
3,200 pg TCDD/g egg	16	0	16	5	31%
TOTAL	**97**	**5**	**92**	**23**	**25%**

Table 3. Numbers of wood duck (*Aix sponsa*) eggs surviving to hatch following injection of TCDD [pooled breakdown].

Treatment Group	Eggs Injected	Infertile	Sample Size (minus infertile)	Survival to Hatch	% Hatch
Control	33	2	31	10	32%
Low Dose (LD)	32	3	29	7	24%
High Dose (HD)	32	0	32	6	19%
TOTAL	**97**	**5**	**92**	**23**	**25%**

Figure 13. Effect of *in ovo* TCDD exposure on body mass on wood ducks (*Aix sponsa)*. TCDD was injected into the yolk prior to visible embryo growth. Masses were measured 11-14 days post hatch. Squares indicate the mean response of each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.

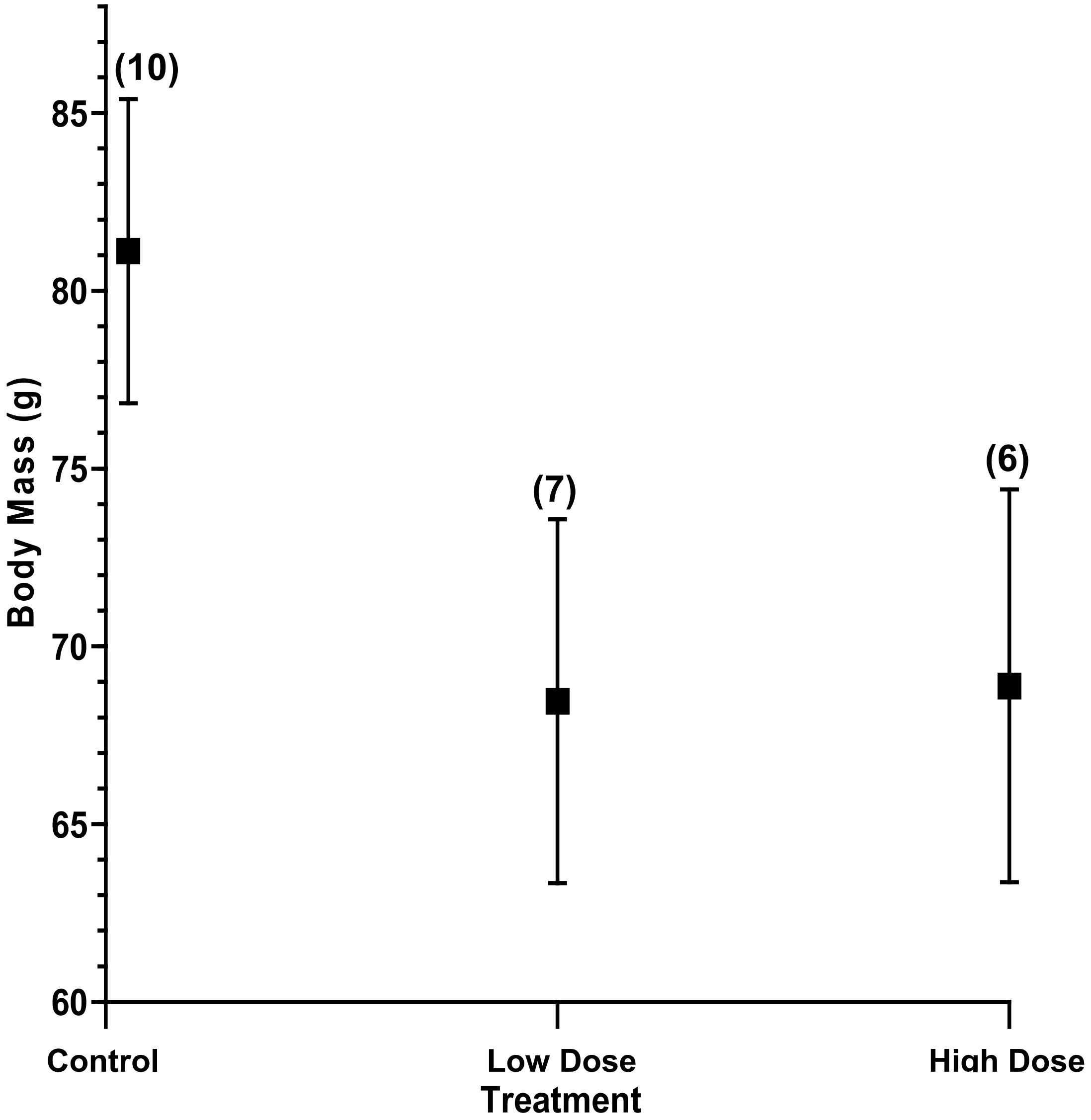
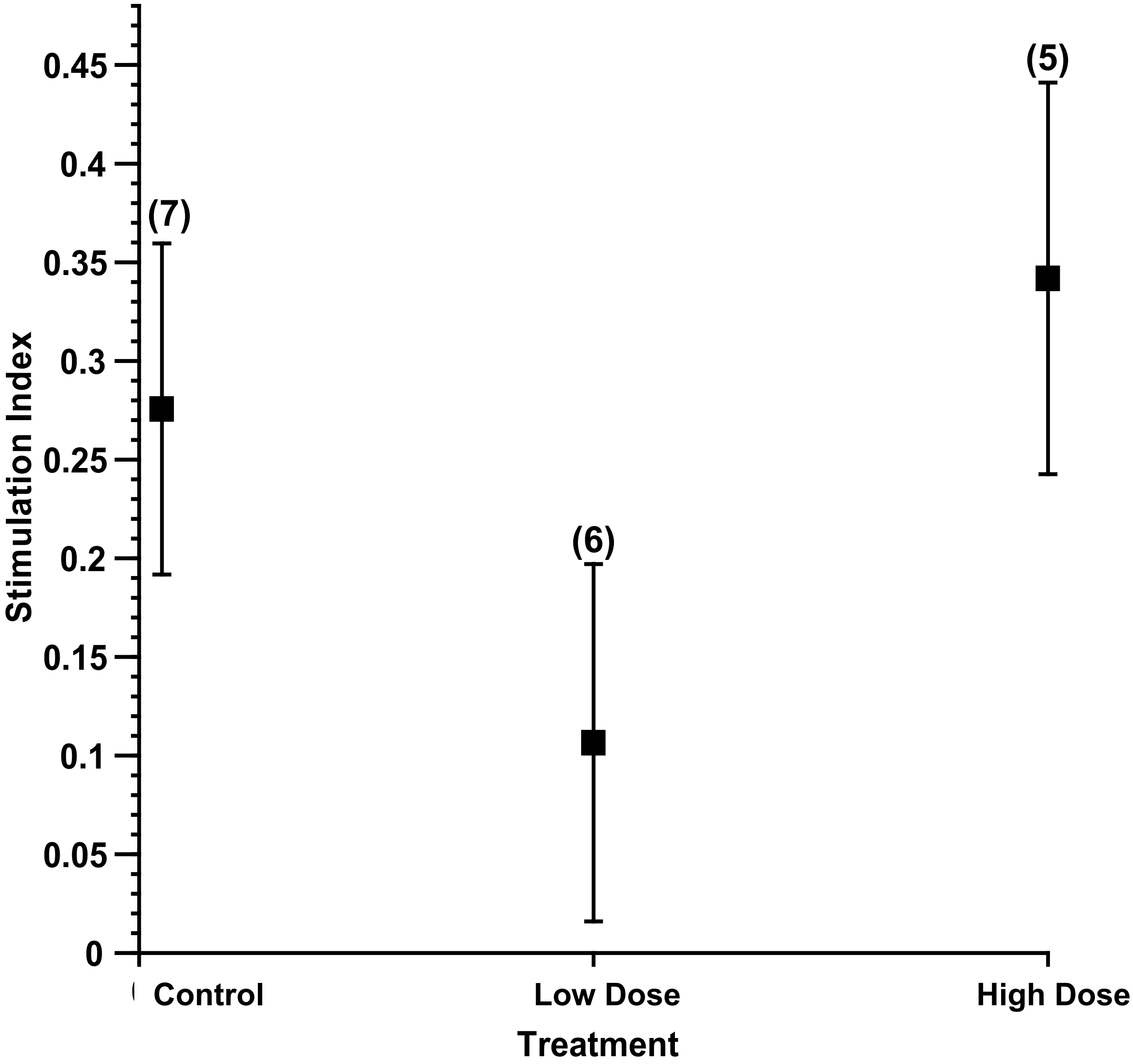

Body Mass (g)
60
65
70
75
80
85
(10)
(7)
(6)
Control
Low Dose
High Dose
Treatment

Figure 14. Effect of *in ovo* TCDD exposure on T-cell-mediated immunity (PHA skin test) on wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Stimulation index (representing measurement of inflammation from PHA minus measurement of inflammation by the saline control) was measured 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.



Stimulation Index
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
(7)
(6)
(5)
Control
Low Dose
High Dose
Treatment

Figure 15. Effect of *in ovo* TCDD exposure on thymus mass of wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Thymus mass (divided by body mass for increased comparability) was measured 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.

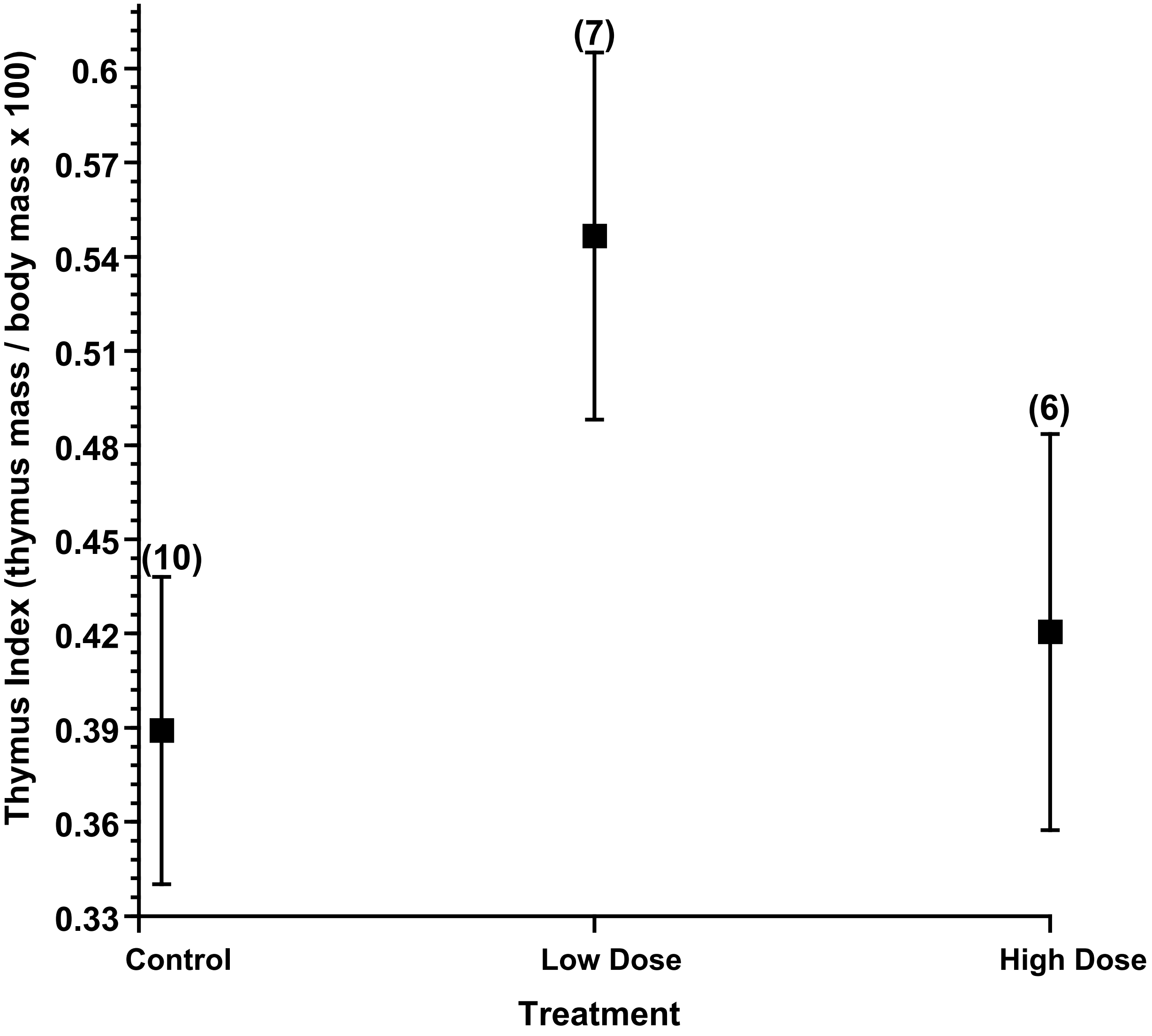

Thymus Index (thymus mass / body mass x 100)
0.6
0.57
0.54
0.51
0.48
0.45
0.42
0.39
0.36
0.33
(10)
(7)
(6)
Control
Low Dose
High Dose
Treatment

Figure 16. Effect of *in ovo* TCDD exposure on mass of the bursa of Fabricius of wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Bursa mass (divided by body mass for increased comparability) was measured 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.

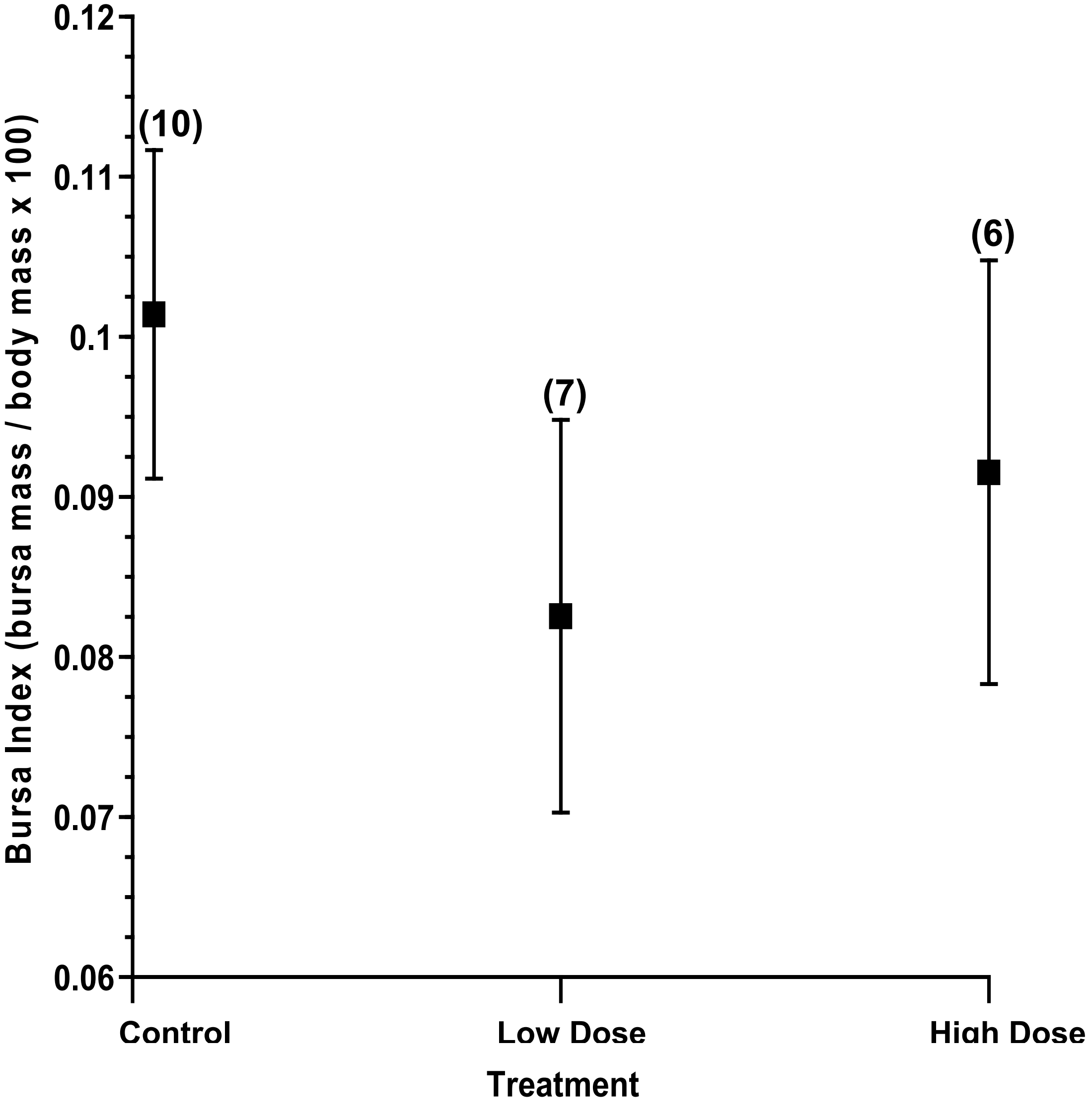

0.12
0.11
0.1
0.09
0.08
0.07
0.06
Bursa Index (bursa mass / body mass x 100)
(10)
(7)
(6)
Control
Low Dose
High Dose
Treatment

Figure 17. Effect of *in ovo* TCDD exposure on lymphocyte density in the thymus of wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Lymphocyte density (determined by the number of lymphocytes in the left lobe of the thymus divided by the mass of the left lobe of the thymus) was determined on 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.

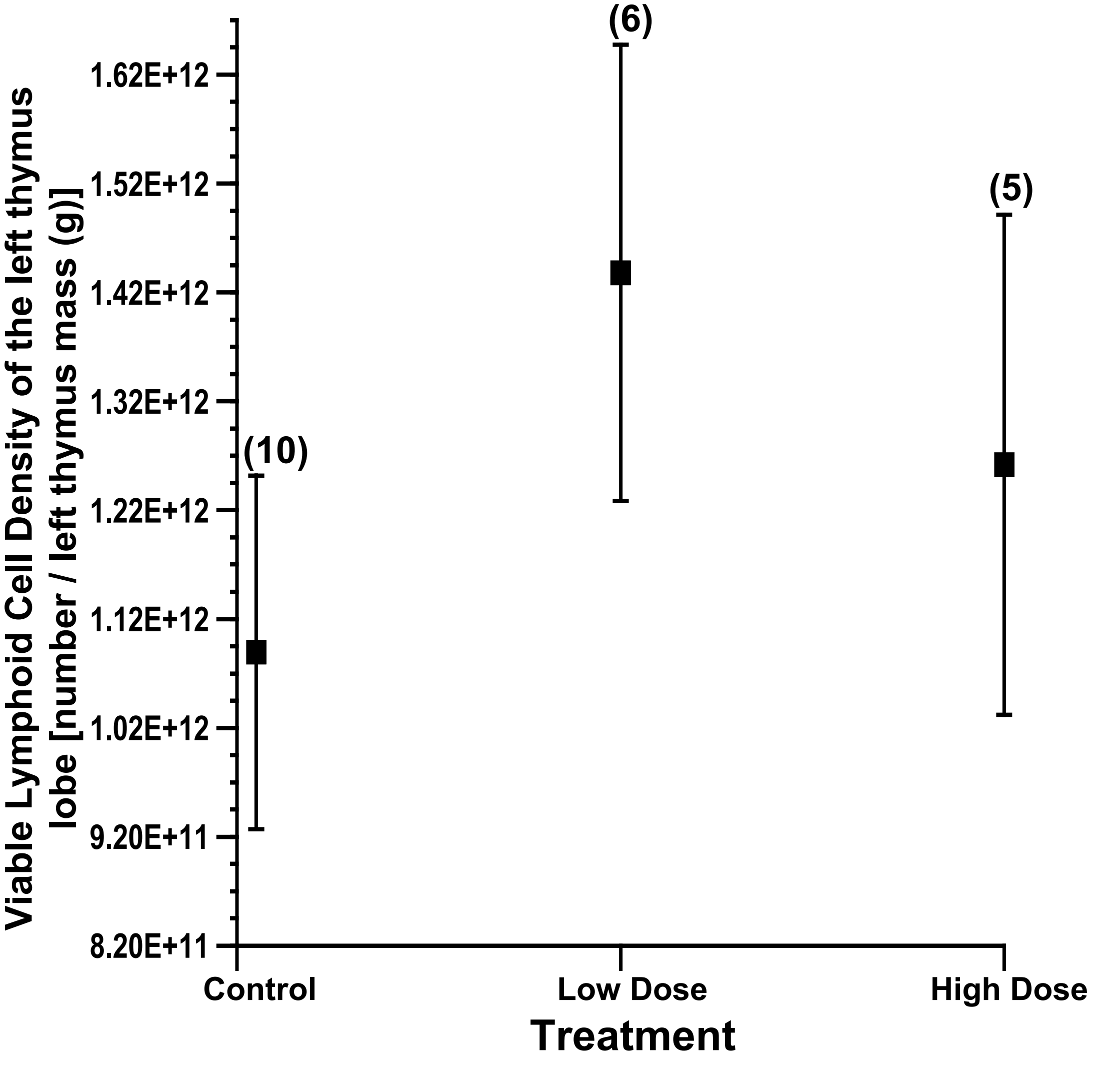

Viable Lymphoid Cell Density of the left thymus lobe [number / left thymus mass (g)]
1.62E+12
1.52E+12
1.42E+12
1.32E+12
1.22E+12
1.12E+12
1.02E+12
9.20E+11
8.20E+11
(10)
(6)
(5)
Control
Low Dose
High Dose
Treatment

Figure 18. Effect of *in ovo* TCDD exposure on lymphocyte density in the bursa of Fabricius of wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Lymphocyte density (determined by the number of lymphocytes in the bursa divided by the mass of the left lobe of the thymus) was determined on 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.

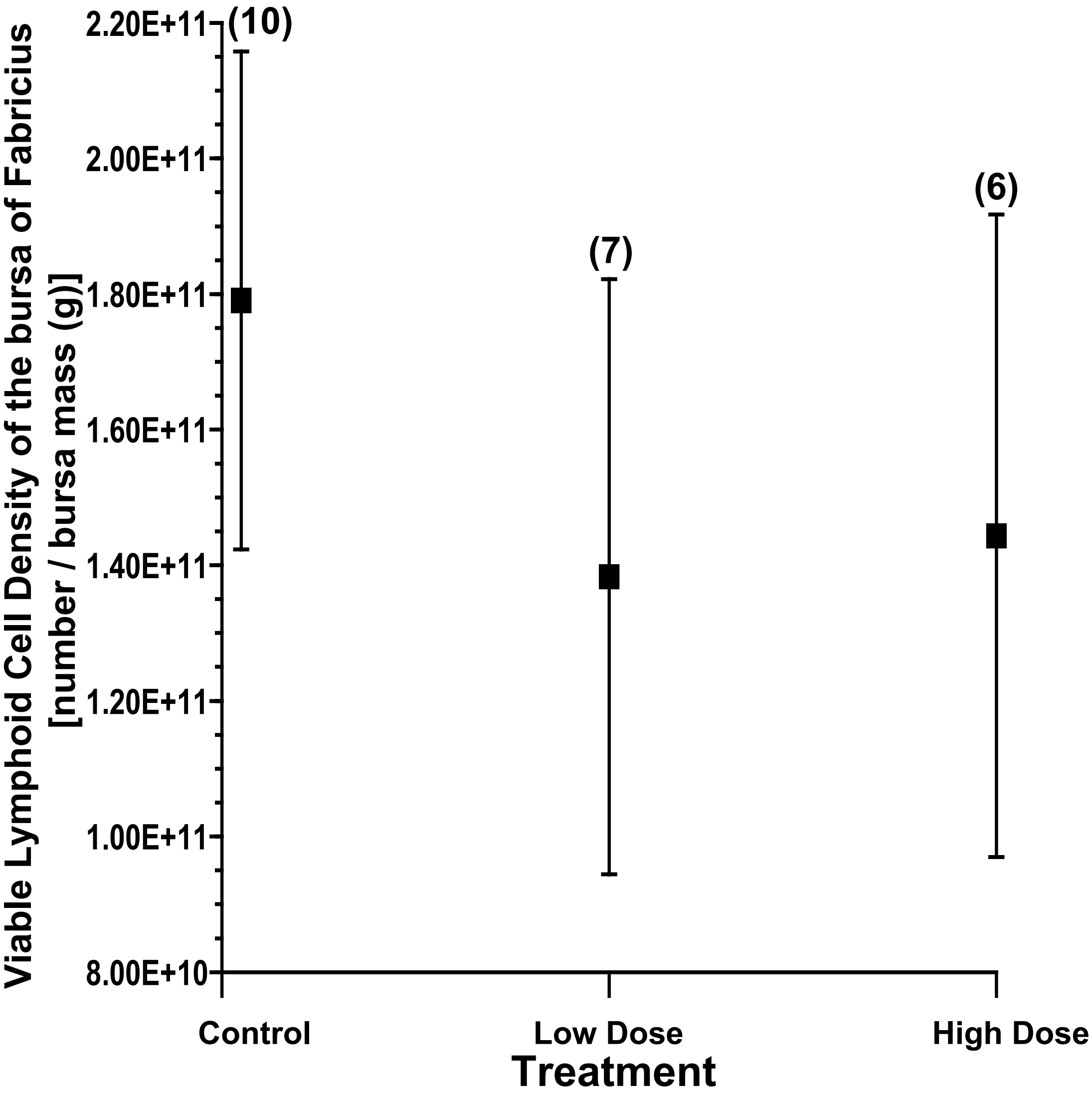
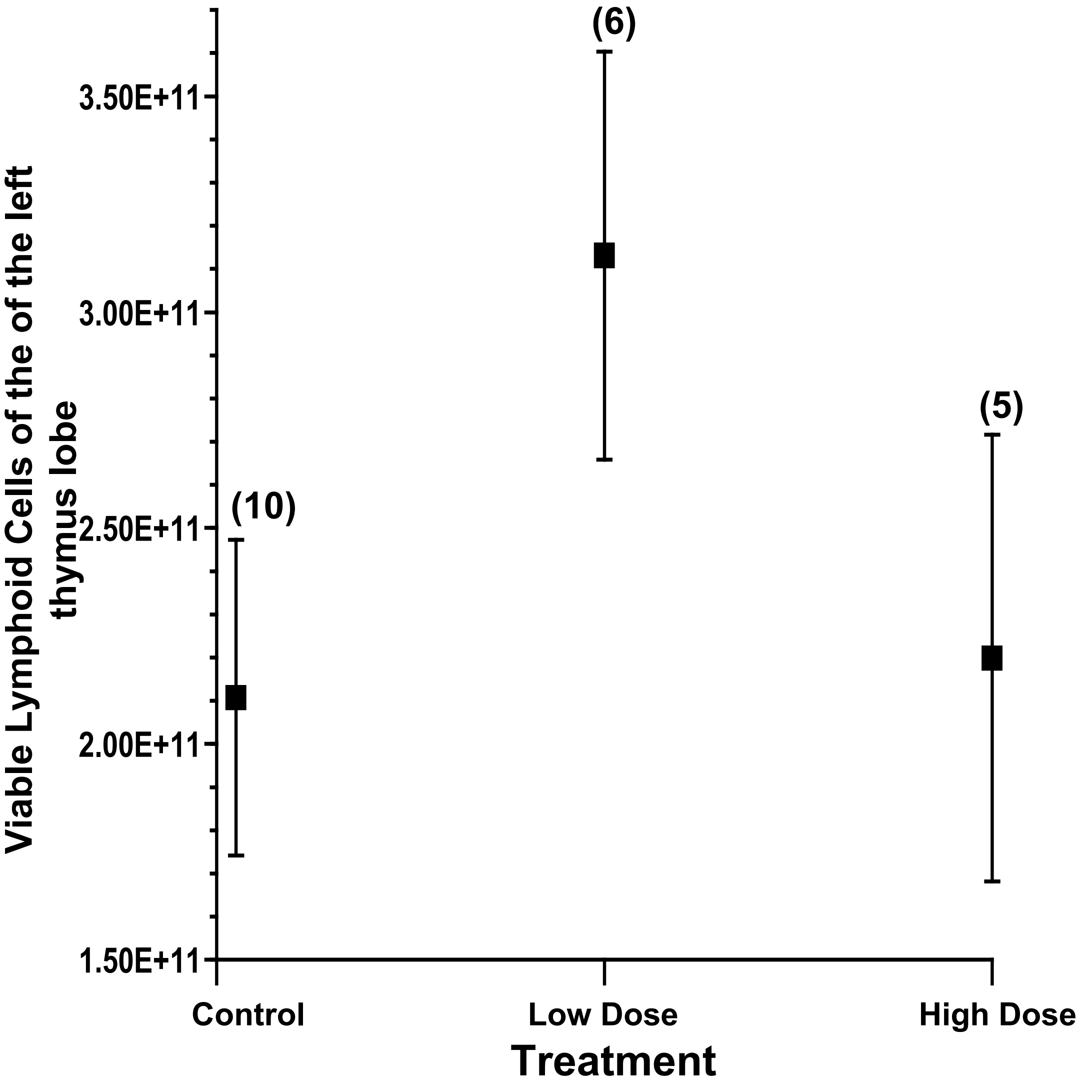
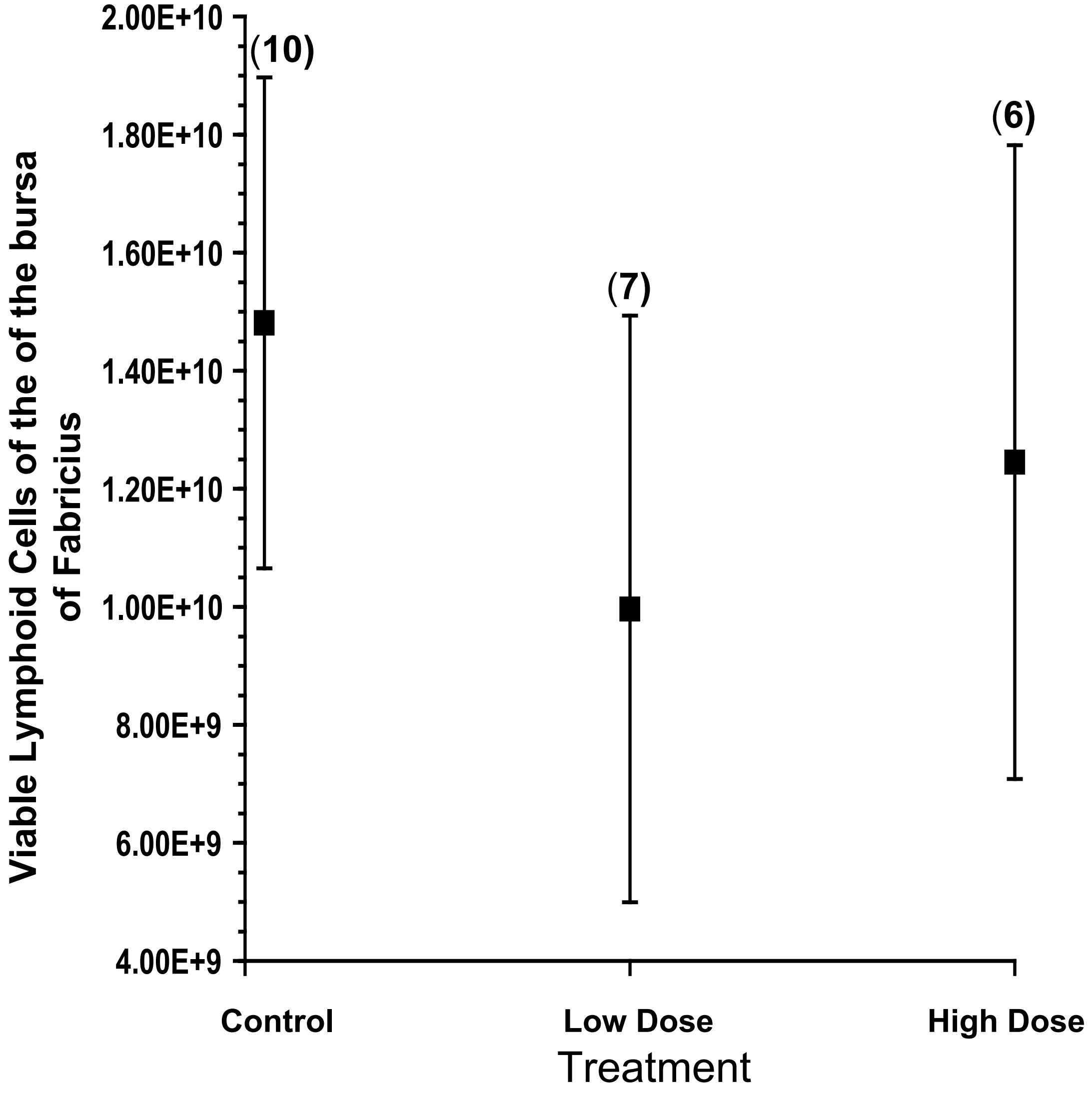

Viable Lymphoid Cell Density of the bursa of Fabricius [number / bursa mass (g)]
2.20E+11
2.00E+11
1.80E+11
1.60E+11
1.40E+11
1.20E+11
1.00E+11
8.00E+10
(10)
(7)
(6)
Control
Low Dose
High Dose
Treatment

Figure 19. Effect of *in ovo* TCDD exposure on viable lymphocyte numbers in the left lobe of the thymus of wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Total viable lymphocyte numbers were calculated on 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.



Viable Lymphoid Cells of the of the left thymus lobe
3.50E+11
3.00E+11
2.50E+11
2.00E+11
1.50E+11
(10)
(6)
(5)
Control
Low Dose
High Dose
Treatment

Figure 20. Effect of *in ovo* TCDD exposure on viable lymphocyte numbers in the bursa of Fabricius of wood ducks (*Aix sponsa*). TCDD was injected into the yolk prior to visible embryo growth. Total viable lymphocyte numbers were calculated on 11-14 days post hatch. Squares indicate the mean response from each dose group. Error bars designate one standard error of the mean. Numbers in parentheses represent sample sizes.


Viable Lymphoid Cells of the of the bursa of Fabricius
2.00E+10
1.80E+10
1.60E+10
1.40E+10
1.20E+10
1.00E+10
8.00E+9
6.00E+9
4.00E+9
(10)
(7)
(6)
Control
Low Dose
High Dose
Treatment

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