ABSTRACT

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                       EXPOSURE TO POLYBROMINATED
                       DIPHENYL ETHERS IN DOMESTIC AND
                       WILD BIRDS.

                       Moira Ann McKernan, PhD, 2008

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Concentrations of polybrominated diphenyl ethers (PBDEs) in eggs of wild birds have increased dramatically over the past 25 years, yet only limited data are available to interpret toxicological consequences of exposure. Embryonic survival, pipping and hatching success, and sublethal biochemical, endocrine, and histological endpoints were examined in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) hatchlings following *in ovo* (air cell) administration of the commercial penta-BDE mixture DE-71 (0.01-20 µg DE-71/g egg). Environmentally realistic concentrations of DE-71 induced ethoxyresorufin-*O*-dealkylase activity, and reduced bursa of Fabricius follicle size and number in chicken hatchlings, but not in other species. Pipping and hatching success decreased in American kestrels receiving 10 and 20 µg DE-71/g egg, but these endpoints were unaffected in other species. Absorption of air cell administered DE-71 (dose = 11.1 µg/g egg) into the contents of eggs varied among species and uptake rate tended to increase during the later half of development (dose absorbed by pipping: chicken 29.6%, kestrel 18.8%). At least six PBDE congeners and two metabolites were detected in DE-71 dosed eggs that were not present in the dosing

solution, suggesting evidence of debromination and methoxylation. Uptake of the

commercial octa-BDE mixture DE-79 (dose = 15.4 µg /g egg) following air cell

administration to avian eggs was found to be low (dose absorbed <6.5%). Based on the

DE-71 uptake rate in kestrels, the lowest observable effect level on pipping and hatching

success may be 1.8 µg total PBDE/g egg, which approaches concentrations detected in

eggs of free-ranging birds. As some PBDE congeners are still increasing in the

environment, the embryotoxic effects observed following DE-71 administration are cause

for concern.

COMPARATIVE EFFECTS OF EMBRYONIC EXPOSURE TO
POLYBROMINATED DIPHENYL ETHERS IN DOMESTIC AND WILD BIRDS.

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List of Abbreviations

BCNH   black-crowned night-heron

BDE   brominated diphenyl ether

CON   control

μg   microgram

ng   nanogram

PBDE   polybrominated diphenyl ether

PCB   polychlorinated biphenyl

PCB 126   polychlorinated biphenyl congener 3,3',4,4',5

pg   picogram

T4   thyroxine

Chapter 1: Introduction

<u>Background</u>

Polybrominated diphenyl ethers (PBDEs) are a chemical class of fire
retardants most commonly used in polymers, textiles, electronics and other materials.
Because PBDEs are physically combined (i.e., not covalently bound) with the product
matrix, they may migrate out of that matrix over time. Environmental release of
PBDEs can occur from production waste streams, municipal waste incineration and
treatment, and migration out of landfills.

Polybrominated diphenyl ethers bioaccumulate in aquatic and terrestrial food
chains, and biomagnify in predators due to consumption of contaminated prey (Hale
et al. 2002). Monitoring studies indicate that PBDE concentrations are increasing in
the environment. Notably, a retrospective study of archived herring gull eggs (*Larus
argentatus*) from the Great Lakes demonstrated that PBDE concentration increased
by one and a half orders of magnitude during 1981-2000, with a concentration
doubling time between 2.6-3.1 years (Norstrom *et al.* 2002) and, as recently reported,
concentrations of these congeners in gull eggs have plateaued (Gauthier *et al.* 2008).
Remarkably, there are limited toxicological effects data for wildlife, especially avian
species.

Considering the ubiquitous nature of PBDEs, and their elevated
concentrations in the environment, there is a genuine need for more complete
ecotoxicological data on these compounds. General toxicity studies should be
conducted with species that may be at high risk of exposure. Developmental and

reproductive effects are of particular concern to ecotoxicologists because of their linkage to higher order effects, although more subtle neurobehavioral and immunological responses are of concern because they may be the early sublethal warning signal of potential higher order effects.

This dissertation reports a series of controlled laboratory exposure studies with domestic chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), American kestrel (*Falco sparverius*), and black-crowned night-heron (*Nycticorax nycticorax*) embryos. These studies will provide embryotoxicity and biotransformation data on four well-characterized avian models from several different feeding guilds (granivorous, omnivorous, carnivorous, piscivorous). Data from the toxicity portion of the study will help establish biological effect thresholds, (e.g., endpoints reflecting growth, development, cytochrome P450, thyroid function, and histopathology), which will be useful in interpreting environmental concentrations of PBDEs in avian eggs. These data will also be useful to applications in avian risk assessments and ultimately in predicting impact to wild populations.

Manufacture and consumer use

During the last 50 years, there have been advances in polymer science, leading to a wide variety of polymers in electronics, clothing, computers, and furniture. The majority of these polymers are petroleum-based and therefore flammable. Flame retardants are added to these polymers to increase fire resistance and to meet fire safety standards. There are four major classes of flame retardants: inorganic,

halogenated organic, organophosphorus, and nitrogen-based flame retardants. Halogenated flame retardants eliminate the capability of a flame to grow by capturing the free radicals produced during the combustion process (Alaee and Wenning, 2002). Brominated fire retardants are used more than other halogenated flame retardants, and their use has increased dramatically since 1965 (Alaee and Wenning, 2002).

Polybrominated diphenyl ethers are a class of fire retardants most commonly used in polymers, textiles, electronics and other materials. Diphenyl ethers contain 10 hydrogen atoms, which can be exchanged for bromine. There are 209 possible PBDE congeners, with individual congeners named according to the IUPAC system used for polychlorinated biphenyls (PCBs) and numbered consecutively from 1 to 209 (Ballschmiter and Zell 1980).

PBDEs are manufactured as three major technical mixtures called penta, octa, and deca. Penta technical mixtures primarily include tetra and penta-brominated congeners, octa mixture is made of primarily hepta-octa congeners. Penta-BDE formulations are mostly used in polyurethane foams. Commercial octa-BDE is used in plastics, especially those that house televisions and computers. Deca is almost exclusively (99%) composed of the fully brominated deca congener. Deca-BDE is used predominantly in plastics and on textiles.

While there has been an increase in PBDE use worldwide, companies in Asia, North America, and Europe have voluntarily phased out production of penta and octa formations in the 1990s and 2000s due to increasing concern about their presence in the environment and potential toxicity. The European Union established a ban on penta- and octa-BDE formulations which began August 2004. The state of California

enacted a law banning the use of the same formulations by June 2006. Hawaii, Maine, New York, and Washington are considering or have passed similar legislation. In 2003, Great Lakes Chemical Corporation, the only U.S. producer of penta- and octa-BDE formulations, announced a voluntary phase out of penta- and octa-BDE formulations by the end of 2004.

Sources and persistence

Environmental PBDE sources vary. The obvious sources include effluents from factories producing PBDEs, flame retardant polymers, and plastics. Other possible emissions include sewage sludge, municipal, hospital, and hazardous waste incinerators, and recycling facilities and landfills. Hale (2002) demonstrated that polyurethane foam containing penta-BDE is a likely source of PBDEs in the environment.

Because PBDEs are lipophilic, there is growing concern about their occurrence in the environment. The lower brominated congeners tend to be more volatile, water soluble, and bioaccumulative. The highly brominated congeners (e.g. BDE-209) tend to accumulate in the sediment near emission sources due to their low volatility, water solubility, and low potential to bioaccumulate (Watanabe and Sakai, 2003b).

Standards for individual PBDEs have been slow to reach the market and also are very expensive, hampering chemists' efforts to detect them accurately in environmental samples. Currently, Accustandard Inc. (New Haven, CT, USA) sells

over 170 individual congeners, several congener mixtures, and hydroxylated and methoxylated metabolites.

Less brominated congeners have been commonly detected in wildlife and humans around the world, specifically, BDEs-47, -99,–100, -153, -154, and -183. These congeners have log K_{ow} (distribution of a chemical between water and octanol, at equilibrium) values similar to those of the most accumulative PCBs. K_{ow} can be a good indicator of the partitioning for a substance from water to lipid, thus predicting its tendency to move from aqueous environments into biota. BDE-209, however has a log K_{ow} of 9.97, which predicts strong sorption to sediments and lower bioavailability (Gustafsson *et al*. 1999).

BDE congeners -47, -99,–100, -153, -154, and -183 may originate from the penta- and octa-BDE formulations, or may be the result of debromination of higher brominated congeners (Stapleton *et al*. 2004). These congeners are volatile and persistent enough to allow for long-range transport in the environment. They also have high lipophilicities (log K_{ow} values ~5-6) which allow for bioaccumulation in biota. The environmental behavior and fate of these congeners is thought to be similar to those of chlorinated organic pollutants such as PCBs. In the early 1980s, PBDEs were detected in tissues of fish-eating birds and marine mammals from the North Sea, Baltic Sea, and Artic ocean, suggesting that PBDEs are a global contaminant of concern (Jansson *et al*., 1987). PBDEs were first reported to be present in North American wildlife, specifically fish-eating birds and their eggs in 1983 (Stafford, 1983).

BDE congener patterns in avian species differ, possibly due to marine or terrestrial exposure. Peregrine falcon eggs are dominated by congeners -153 and -99 (Lindberg *et al.*, 2004). However, guillemot (*Uria algae*), herring gull, osprey (*Pandion haliaetus*), and other marine and aquatic species tissues contain primarily congeners -47 and -99 (Sellstrom *et al.*, 1993; Norstrom *et al.*, 2002; Rattner *et al.*, 2004; Toschik *et al.*, 2005).

While BDE congeners -47 and -99 are dominant in biota, BDE-209 occurs in the abiotic environment (Watanabe and Sakai, 2003). The presence of deca-BDE was first reported in the environment by de Carlo (1979), where PBDEs were measured in soil and sludge samples collected from areas surrounding manufacturing facilities in the United States. While deca-BDE is the most commonly used technical product, it is frequently below detection limits in biota, but sometimes present in high concentrations, in sediments (Watanabe *et al.*, 1987; Allchin *et al.*, 1999). The stability of deca-BDE in the environment is not well understood. However, Stapleton *et al.* (2004) demonstrated that juvenile carp fed 940 ng/day pure BDE 209, the major component of Deca-BDE, debrominated the compound into similar congeners in the Penta-BDE mixtures.

Deca-BDE has recently been reported in peregrine falcons (*Falco peregrinus*) and red fox (*Vulpes vulpes*), both top predators (Vorkamp *et al.*, 2005; Voorspoels *et al.*, 2006; Lindberg *et al.*, 2004). Concentrations of specific congeners reported in peregrine falcon resembled that of other biota, with BDE-47, -99,-153, and -154 dominating the profile and a lower concentration of BDE209. Fox tissues, on the other hand had dominating concentrations of BDE209, and low concentrations of the

congeners that dominate the profiles in other vertebrate studies. This may be indicative of a greater ability of the fox to clear lower brominated congeners, but an inability to debrominate BDE209. The congener profile of the peregrine falcon, however, may indicate a reduced ability to debrominate and metabolize PBDEs compared to the fox.

Temporal and spatial trends of PBDE

Monitoring studies indicate that PBDE concentrations in soil, sediment, humans, and wildlife are increasing in the environment (Noren and Meironyte, 2000; Betts, 2002; Norstrom *et al*., 2002). Congeners most commonly detected in biota continue to include BDE-47, -99,–100, -153, -154, and –183, regardless of the location of sampling. Levels of PBDEs tend to be greater in aquatic and marine ecosystems compared to terrestrial ecosytems (de Wit, 2002).

Spatial trends tend to follow those of PCBs and persistent organchlorine pesticides and their metabolites (e.g. *p,p'*-DDE), and can be observed across all trophic levels (Boon *et al*., 2002). Heavily populated and industrialized areas are generally more contaminated than remote areas. Rattner *et al*. (2004) and Toschik *et al*. (2005) have reported spatial trends in PBDEs in osprey, a piscivorous bird in the Chesapeake and Delaware Bays. The Delaware Bay osprey study documented a river-to-ocean gradient in persistent organochlorine pesticides, PCBs, Hg, perfluorinated compounds, and PBDEs in osprey eggs (Toschik *et al*., 2005). PBDEs present in air, sediment, soil and biota in artic regions far from emissions sites provide evidence of long range transport (de Wit *et al*., 2006).

Sakai *et al*. (2002) reported increasing concentrations of PBDEs from 1986-1989, and then a decreasing trend after 1990 in a sediment core collected from Osaka Bay shows. A sediment core taken from Drammenfjord in Norway showed an increasing trend of PBDEs from the early 1960's to 1999 (Zegers *et al*., 2000). However, cores taken in Germany show a leveling off in PBDE concentrations in the 1970's.

PBDE concentrations in pike (*Esox lucius*) in Sweden increased from 1967 to the early 1980's, and concentrations began to level off from 1982-1996 (Kierkegaard *et al*., 1999).   Japanese sea bass and gray mullet have increasing levels of PBDEs from 1986-1989, and then a drastically decreasing trend after 1990 (Ohta *et al*., 2001).

PBDE concentrations increased in human milk in Sweden collected from 1972 to 1997, with a doubling time of 5 years while the more "classic" contaminants, including PCBs, and dioxins decreased during this period (Noren and Meironyte, 2000). In some areas of Europe, the rate of increase of PBDEs in biota has slowed since the early to mid 1990's (Kierkegaard *et al*., 2004). This may be in part due to the discontinued use of penta and octa formulations of PBDEs. Human breast milk concentrations in North America are relatively high compared to those in Europe and Japan (reported to be 2 orders of magnitude greater), and have a doubling time of 2 years (Betts, 2000).

Blubber samples from hunted ringed seals (*Pusa hispida*) located in Holman Island in the Northwest Territories were collected and analyzed for PBDEs. PBDE concentrations increased exponentially between 1981-2000 (Ikonomou *et al*., 2002).

Increasing trends also have been reported in samples from stranded beluga whales inhabiting the St. Lawrence Estuary, Canada (Lebeuf *et al*., 2004). Concentrations of PBDEs in guillemot eggs from the Baltic Sea increased from the 1970's to the beginning of the 1990's, and concentrations decreased up until 1997 (Kierkegaard *et al*., 1999).

A study of archived herring gull eggs from the Great Lakes demonstrated that total PBDE concentrations increased by one and a half orders of magnitude during 1981-2000, with a concentration doubling time between 2.6-3.1 years (Norstrom *et al*. 2002). Total PBDE concentrations in gull eggs have remained elevated since then (Gauthier *et al*. 2008). Herring gulls at these same sites in the Great Lakes have experienced a decline in egg concentrations of PCBs, DDT, and its metabolites. Despite the fact that North America uses over half the world's production of PBDEs, with steadily rising concentrations of PBDEs in sediment, blood, breast milk and avian eggs, few data are available on environmental levels in for large areas of North America, especially for biotic and abiotic media.

PBDE in Biota

Biotransformation

Polybrominated diphenyl ethers can undergo Phase I and Phase II enzymatic processes. Polybrominated diphenyl ethers are structurally similar to thyroxine (T_4), and some have suggested (Benedict *et al*. 2007) that congeners may undergo debromination using a similar/related pathway to deiodination. While the mechanism of debromination of PBDEs is poorly understood, there is evidence that it occurs in

vertebrates. Stapleton *et al*. (2004) reported debromination of PBDEs in common carp (*Cyprinus carpio*) fed BDE congeners -99 and -183. Congener 99 was debrominated to BDE-47, and congener 183 was debrominated to an unidentified hexa-BDE congener. Stapleton *et al*. (2006) fed BDE-209 to juvenile trout (*Oncorhynchus mykiss*) and detected several hepta-, octa-, and nona-BDE congeners in tissues as a result of debromination. Debromination of PBDEs has also been reported in European starlings (*Sturnus vulgaris*) exposed to BDE-209 using silastic implants as octa- and nona-BDEs were detected in muscle and liver tissues (van den Steen *et al*. 2007).

Mammalian and fish studies implicate Phase I CYP enzymes in hydroxylation of PBDEs (Hakk and Letcher 2003). Hydroxylated and methoxylated PBDE congeners have been detected in free ranging wildlife (Marsh *et al*. 2004; McKinney *et al*. 2006; Verreault *et al*. 2005, 2007; Gebbink *et al*. 2008). This class of flame retardants has also been reported to be metabolized through Phase II conjugative processes (e.g. uridine diphosphate glucoronosyl-transfereases and glutathione S-transferases) (Hakk and Letcher 2003).

Toxicity of PBDE

While the temporal trends seem to vary around the globe, the general increase in many time trend studies has given rise to concern. Environmental toxicologists find it difficult to interpret concentrations detected in biota due to limited toxicological effects data for wildlife. Most of the toxicological literature available is focused on mammalian laboratory models (i.e., rats, mice).

Acute toxicity of PBDE formulations is low in laboratory mice, rats, and rabbits. Some studies have demonstrated changes in immune function, learning behavior, cytochrome P450-associated monooxygenases, endocrine function, and histopathology (Eriksson *et al*., 2001; Fowles *et al*., 1994; Darnerud and Thuvander, 1999; Zhou *et al*., 2001; 2002; Hallgren *et al*., 2001, 2002; von Meyerinck *et al*. 1990; Chen *et al*., 2001). However, there are very limited effects data from chronic exposure studies using environmentally realistic concentrations of PBDEs.

Intermediate-duration oral studies in animals indicate that, similar to acute toxicity studies, the liver and thyroid are the primary targets of repeated exposures to deca-, penta-, and octa-BDEs (Darnerud, 2003). Effects include enlargement and histological alterations in both organs and altered serum thyroid hormone levels, specifically decreased serum thyroxine (T_4). This response may be related to the structural similarity between PBDEs and thyroid hormones. With this structural similarity to thyroid hormones, PBDEs are believed to bind competitively for thyroid transporting proteins, therefore disrupt the normal transport of T_4 to target tissues (Marsh *et al*., 1998).

Fetotoxic effects have been observed in rats and rabbits at much lower doses than the median lethal dose (LD50) values. Effects include skeletal ossification variations, weight decrease, and increased post-implantation loss in rats at maternal octa-BDE doses as low as 10 and 25 mg/kg/day, respectively (U.S. EPA., 1986). Similar skeletal effects were observed in rabbits after maternal Saytex 111 (octa-BDE mixture) doses starting at 2 mg/body weight. (Breslin *et al*., 1989). There is little information on neurotoxic effects of PBDEs. Some changes in spontaneous

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locomotion behavior, learning, and memory were noted in mice two and four months of age following neonatal exposure to single low oral doses of the BDE congeners -47 and -99 (Eriksson *et al.*, 2001).

Research on the immunosuppressive potential of PBDE mixtures has been limited to evidence from acute-duration oral studies in mammals exposed to relatively high doses of pentaBDE. PBDEs have been reported to suppress antibody production following inoculation of sheep red blood cells (SRBC) in laboratory rodents. There have been some immunotoxic effects as a result of PBDE exposure, including suppression of SRBC response and decreased thymus weight after 14 daily oral administrations (72 mg/kg bw) of commercial penta BDE mixture DE-71, and reduced splenocyte numbers after daily oral administrations (18 mg/kg bw) of BDE 47 (Fowles *et al.*, 1994; Darnerud and Thuvander, 1999).

There are conflicting reports about the ability of PBDEs to induce cytochrome P450-associated monooxygenases in mammals. Microsomal enzyme activities of ethoxyresorufin *O*-deethylase (EROD) and methyoxyresorufin *O*-deethylase (MROD) in rats dosed with PBDEs, PCBs and chlorinated paraffins resulted in weakly induced activity in the group dosed with only congener BDE-47. Bromkal 70 (a penta-BDE technical mixture) exposure resulted in a strong enzyme induction in rats (de Wit, 2002). However, this same technical mixture weakly induced EROD activity in rainbow trout larvae. Pentoxyresorufin *O*-deethylase (PROD) activity has been shown to increase in a dose-dependent manner similar to that of PCBs in rats exposed to BDE-47 (Hallgren and Darnerud, 1998).

There is a lack of data on effects of PBDEs in avian species. Only two dosing studies in birds have examined developmental and reproductive effects of environmentally relevant concentrations of PBDEs (Fernie *et al*. 2005a, 2005b, 2006, 2008). In the first study, kestrels were exposed in ovo and through diet to environmentally relevant concentrations of congeners commonly detected in biota. Eggs were injected with 1.5 µg/g egg PBDEs on d19 of incubation. Hatchlings were then fed the same mixture of congeners (15.6 ng/g bw/day) until d29. Hatchability, fledging success, immune function, growth, thyroid hormones, vitamin A, glutathione homeostasis, and oxidative stress were evaluated (Fernie *et al*., 2005a; Fernie *et al*., 2005b; Fernie *et al*., 2006). Polybrominated diphenyl ether-exposed nestlings were larger, gained weight more quickly, and ate more food. Fledgling kestrels exposed to PBDEs had lower thyroxine, plasma retinol, hepatic retinol, and retinyl palmitate concentrations. Polybrominated diphenyl ether exposure induced hepatic oxidative stress. PBDE exposure did not affect hatching or fledging success. Exposure to PBDEs did not affect triiodothyronine concentrations and thyroid gland structure. Bursal and spleen somatic indices were smaller in PBDE-exposed birds. PBDE-exposed birds exhibited a reduced antibody response, and a greater PHA skin response, which is mediated by T-cell-mediated-immunity. This study indicates that environmentally relevant concentrations of PBDEs may induce sublethal effects on growth, immune function, thyroid hormone and vitamin A concentrations in wild birds. In a second study by Fernie and coworkers (2008), reproductively active adult kestrels were fed concentrations (0.3 µg DE-71/g diet or 1.6 µg DE-71/g diet) of a

commercial penta-BDE formulation and changes in reproductive behavior were noted (e.g., fewer bonding behaviors, copulated less, and spent less time in nest box).

Utility of egg injection studies

There are several scientific reasons for choosing avian species to monitor environmental contaminants. Birds are a highly valued natural resource, with both consumptive and nonconsumptive uses. Many avian species are protected by listing through state or federal threatened or endangered species lists and by the International Migratory Bird Treaty Act. Birds are more sensitive than mammals to a broad range of contaminants and pesticides due to several factors, including relatively small liver size, lower cytochrome P450 monooxygenase activity, higher body temperature which requires greater relative food intake, differences in their circulatory system (renal portal system), reabsorption of contaminant metabolites in the cloaca, and transfer of lipophilic compounds directly into eggs (Walker, 1983). In addition, avian embryos are often exposed to greater concentrations of contaminants compared to mammals that have a metabolically active placenta. Birds are often useful in embryotoxicity studies, as this cleidoic system is relatively easy to maintain and manipulate, and responses to environmental contaminants are generally well characterized (Hill and Hoffman, 1984). Embryotoxicity studies with bird eggs appear to effectively mimic maternal deposition of persistent organic pollutants in free-ranging birds.

The avian egg has been used extensively for teratology and toxicity testing (e.g., chemical screening, comparative species sensitivity and mechanistic studies). Its widespread use today can be attributed to the responsiveness of the avian embryo

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to a wide variety of toxic agents, and logistical and economic factors (Hill and Hoffman, 1990). Topical and *in ovo* exposures were first used by Féré (1893-1901) who reported teratogenic effects of alcohols, nicotine and several drugs (Warkany 1977). Use of eggs from domestic species (chicken, *Gallus gallus*; mallard, *Anas platyrhynchos*) to examine the toxicity of metals, pesticides, industrial compounds and petroleum crude oil became commonplace in the second half of the 20th century (Ridgeway and Karnofsky 1952; Hoffman and Albers 1983; Hoffman 1990), and has greatly assisted in ecological risk assessments. In nature, the eggs of birds can be exposed to environmental contaminants by topical transfer (e.g., petroleum crude oil from parental feathers, pesticide sprays) or by maternal deposition (e.g., methylmercury into the albumen, polychlorinated biphenyls into the yolk). In an experimental setting, the toxicity (LD50) of such contaminants to avian embryos can be evaluated by reproductive feeding trials involving maternal transfer into eggs, by topical applications onto the egg, and injection into the air cell, albumen or yolk. Because feeding studies entail maternal deposition of the contaminant into the egg, they more closely approximate natural exposure than do egg injections. However, the logistics and cost of such studies impedes large scale testing of multiple compounds at many dose levels. For some compounds, egg injection studies closely approximate embryotoxicity that occurs following natural exposure and overcome the difficulties of feeding trials.

Summary

The concentrations of certain congeners (BDE 47, 99) may be sufficient to illicit harmful effects in wildlife (de Wit, 2002). Remarkably, there is limited toxicological effects data for wildlife, especially for avian species. Acute toxicity of PBDE formulations is low in laboratory mice, rats, and rabbits. However, there are very limited effects data from chronic exposure studies using environmentally realistic concentrations of PBDEs, especially in wildlife species.

PBDEs are ubiquitous and increasing in the environment. It is therefore important to generate more complete ecotoxicological data on these compounds. Toxicity studies, especially with species at high risk of exposure, should be conducted. Developmental and reproductive stages during an individual's lifetime may be the most sensitive to exposure. Effects at these stages may lead to higher order effects or may be early sublethal warning signals of higher order effects.

This dissertation reports the results of a series of controlled exposure studies to assess PBDE toxicity and uptake following air cell injection in several species of birds. The mallard and the American kestrel were the principal species in this study because they are well characterized and frequently used model species in wildlife toxicology. Because of its greater sensitivity to PCBs and some other polyhalogenated contaminants, the domestic chicken was evaluated for comparative purposes. Due to year-round commercial availability and the need to develop and validate methods, initial studies were conducted in domestic chicken, followed by the mallard and the American kestrel. The black-crowned night-herons was used in the uptake and metabolism portion of the study to represent fish-eating species. Black-

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crowned night-herons have often been used in ecotoxicology studies (Golden *et al*.

2003; Rattner *et al*., 1993, 1994, 1996, 1997).

Chapter 2: Egg incubation position affects toxicity of air cell administered polychlorinated biphenyl 126 (3,3',4,4',5-pentachlorobiphenyl) in chicken (*Gallus gallus*) embryos

Abstract

The avian egg is used extensively for chemical screening and determining the relative sensitivity of species to environmental contaminants (e.g., metals, pesticides, polyhalogenated compounds). The effect of egg incubation position on embryonic survival, pipping, and hatching success was examined following air cell administration of polychlorinated biphenyl (PCB) congener 126 (3,3',4,4',5-pentachlorobiphenyl; PCB 126; 500-2000 pg/g egg) on day 4 of development in fertile chicken (*Gallus gallus*) eggs. Depending on dose, toxicity was found to be up to nine times greater in vertically versus horizontally incubated eggs. This may be due to enhanced embryonic exposure to the injection bolus in vertically incubated eggs compared to more gradual uptake in horizontally incubated eggs. Following air cell administration of PCB 126, horizontal incubation of eggs may more closely approximate uptake and toxicity that has been observed with naturally incorporated contaminants. These data have implications for chemical screening and use of laboratory data for ecological risk assessments.

Introduction

The avian egg has been used extensively for teratology and toxicity testing (e.g., chemical screening, comparative species sensitivity and mechanistic studies).

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Its widespread use today can be attributed to the responsiveness of the avian embryo to a wide variety of toxic agents, and logistical and economic factors (Hill and Hoffman 1990). Topical and in ovo exposures were first used by Féré (1893-1901) who reported teratogenic effects of alcohols, nicotine and several drugs (Warkany 1977). Use of eggs from domestic species (chicken, *Gallus gallus*; mallard, *Anas platyrhynchos*) to examine the toxicity of metals, pesticides, industrial compounds and petroleum crude oil became commonplace in the second half of the 20th century (Ridgeway and Karnofsky 1952; Hoffman and Albers 1983; Hoffman 1990), and has greatly assisted in ecological risk assessments.

In nature, the eggs of birds can be exposed to environmental contaminants by topical transfer (e.g., petroleum crude oil from parental feathers, pesticide sprays) or by maternal deposition (e.g., methylmercury into the albumen, polychlorinated biphenyls into the yolk). In an experimental setting, the toxicity (dose-response relationship, estimated median lethal dose [LD50]) of such contaminants to avian embryos can be evaluated by reproductive feeding trials involving maternal transfer into eggs by topical applications onto the egg and injection into the air cell, albumen or yolk. Because feeding studies entail maternal deposition of the contaminant into the egg, they more closely approximate natural exposure than do egg injections. However, the logistics and cost of such studies impedes large-scale testing of multiple compounds at many dose levels. For some compounds, egg injection studies closely approximate embryotoxicity that occurs following natural exposure and overcome the difficulties of feeding trials.

In nature when eggs are incubated in the nest by the parent, they are usually situated on their sides in a near horizontal position. However, for species that have been domesticated and reared in commercial hatcheries, eggs are artificially incubated vertically with the apical or pointed end of the egg pointing downward and the cap or blunt (air cell) end of the egg upwards.

As a preliminary step to investigations of polybrominated diphenyl ether embryotoxicity in various species of birds, we studied 3,3',4,4',5-pentachlorobiphenyl (PCB 126), that would ultimately serve as a positive control. Surprisingly, when domestic chicken eggs were incubated in the horizontal position, little to only moderate toxicity was observed at doses (500-2000 pg/g egg) previously reported to be highly toxic in vertically incubated chicken eggs (Brunstrom and Andersson 1988; Hoffman *et al.* 1998; Fox and Grasman 1999; Lavoie and Grasman 2007). These observations followed a recent report from Heinz *et al.* [10] that documented greater embryonic mortality in vertically versus horizontally incubated eggs following air cell administration of methylmercury. In view of these findings, a detailed study was undertaken comparing the toxicity of PCB126 in horizontally versus vertically incubated chicken eggs.

Methods

Fertile white leghorn chicken eggs were obtained from CBT Farms (Chestertown, MD, USA). Upon arrival, all eggs were washed in a 40°C 1% Betadine® solution (Purdue, Wilson, NC, USA), weighed, labeled with a number 2 lead pencil, and then stored in a cooler at 13 $^{\circ}$C. Eggs were allowed to equilibrate to

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room temperature for 6 h before placement into incubators. Eggs were set in incubators within 3 d of receipt.

Eggs were artificially incubated (Kuhl Incubator, Flemington, NJ, USA) in trays that were adapted to either turn horizontally oriented eggs 180 degrees or vertically oriented eggs 60 degrees once each hour. Incubators were set at $37.6\,^{\circ}C$, the recommended incubation temperature for chicken eggs. The relative humidity within the incubator was initially set at approximately 40% but was adjusted so egg weight loss by the end of incubation was 14 to 16%. Egg weight loss was determined by weighing eggs at 3 to 4 d intervals during the course of incubation. Eggs were candled at the time of weighing to confirm viability. Unfertilized or dead eggs were removed at the time of candling.

Corn oil (Sigma-Aldrich, St. Louis, MO, USA) was used as the vehicle because it causes relatively low mortality compared to other commonly used solvents administered to 3-d-old chicken embryos regardless of incubation position (Heinz *et al*. 2006). We exposed the eggs to PCB 126 because it is among the most toxic and well studied PCB congeners in avian embryos. In chicken embryos, Brunström and Andersson (1988) reported a 30% mortality when 600 pg/g egg were injected on day 7 of incubation, Fox and Grasman (1999) found a 25% mortality when 130 to 320 pg/g egg were injected before incubation (d 0), and Lavoie and Grasman (2007) reported 69 and 87% mortality when 250 and 500 pg/g egg were injected before incubation. Hoffman et al. (1998) noted that the dose-response curve for PCB 126 is relatively steep in avian embryos.

The injection solution was prepared by dissolving neat PCB126 (AccuStandard, New Haven, CT, USA) in acetone prior to mixing with corn oil. Solutions were set onto stir plates for 3 h after mixing. Each solution, including the corn oil control, contained 1% acetone by volume. Nominal concentrations of PCB126 in dosing solutions were verified analytically by diluting test solutions in isooctane and comparing retention times of two independently obtained PCB 126 standards (AccuStandard and Ultra Scientific, North Kingstown, RI, USA) by gas chromatography/mass spectrometry (Varian 3400 and Varian 4D ion trap; Sugar Land, TX, USA) as described by Chu *et al*. (2003). Actual concentrations of PCB126 were lower than expected, 45.2 to 51.1% of nominal, but consistent.

Chicken eggs were incubated for 4 d prior to injection, at which time they were candled to confirm fertility and viability. Any infertile, non-viable, or cracked eggs were discarded. Eggs were then randomly assigned to groups of uninjected, vehicle-injected, and PCB-treated (*n* =27-28 eggs/group). Vehicle or PCB 126 (500, 1000, or 2000 pg/g egg) was injected as previously described (Hoffman *et al*. 1998; Heinz *et al*. 2006), but an even lower volume was used (0.5 µl/g egg) to minimize potential effects of the vehicle. Briefly, the cap end of each egg was cleaned with an alcohol swab. A 2mm hole was drilled (Dremel, Racine, WI, USA) into the cap end of egg and the dose was injected with an Eppendorf repeat pipettor. After injection, the hole was sealed using a hot glue gun (ethylene vinyl acetate adhesive). Eggs were kept at a vertical position for 30 min post-injection outside the incubator to allow the oil to spread over the air cell membrane. Eggs were then placed in incubator trays in a horizontal or vertical position and returned to the incubator. Eggs were monitored

by candling or with a viability-detecting instrument called the Buddy (Vetronic, Torquay, UK) for survival at 3 to 4 d intervals through 90% of the 21 d incubation period, and the incidence of pipping and hatching success were monitored until the embryos had either hatched or died. Any dead or unhatched eggs were examined for deformities and edema. Approximately 24 h post-hatch, chicks were examined for evidence of teratogenicity (e.g., eye, foot, and bill deformities) and edema. The chicks were then weighed and immediately sacrificed via CO_2 asphyxiation.

Data were analyzed using SAS® (SAS Institute, NC, USA). Survival through 90% of incubation, pipping, and hatching success was statistically compared using contingency table analysis with Bonferroni correction. Survival through 90% incubation is an endpoint that can be useful in that it bypasses what is sometimes a late surge in embryo mortality, related more to the inability of the chick to hatch than to any toxic effect of the test chemical. This late stage mortality is often seen in controls and may relate to the inability of the artificial incubator to mimic natural incubation by the parents. Heinz *et al*. (2006) used this 90% endpoint with studies of injected methylmercury. Body weights were tested for normality with the Shapiro-Wilk (W) statistic and for homogeneity of variance. Differences in body weights among treatments were determined using one-way analysis of variance and Tukey's honestly significant differences method of multiple comparison. Statistical significance was determined as $p < 0.05$.

Results

For uninjected and vehicle-injected eggs incubated horizontally and vertically, survival to 90% of incubation (89.3-100%), pipping (85.7-92.5%), and hatching success (78.6-92.6%) were within an acceptable range. Survival, pipping and hatching data from uninjected and vehicle-injected controls were not statistically different ($p > 0.2$), and were therefore combined for comparisons among treatments.

Regardless of orientation, at PCB 126 doses of 1000 and 2000 pg/g, survival to 90% of incubation, incidence of pipping, and hatching success tended to be less or were significantly ($p < 0.05$) reduced compared to the combined controls (Table 2-1). At 2000 pg/g, survival to 90% of incubation was 64.3% in horizontally incubated eggs compared to only 7.1% in vertically incubated eggs. At this dose, pipping and hatching success were 25.0% and 21.4% in horizontally incubated eggs compared to 0% for both pipping and hatching success in vertically incubated eggs.

The majority of mortality at 500 pg/g PCB 126 occurred between observations on day 18 and day 21 of incubation (Figure 2-1). This same temporal pattern was apparent at 1000 pg/g in horizontally incubated eggs; however, mortality occurred earlier and was more pronounced in vertically incubated eggs. Based on observations at 3 d intervals, mortality occurred predominantly by day 12 and after day 18 of incubation in horizontally incubated eggs that received 2000 pg/g. Most mortality in vertically incubated eggs dosed with 2000 pg/g occurred by day 12 of incubation, which was not the case in horizontally incubated eggs (i.e., 82.1% vs 28.66%; $p < 0.0001$).

The incidence of edema in eggs that failed to hatch was statistically greater in horizontally incubated eggs (32.1%) at 2000 pg/g compared to vertically incubated eggs (10.7%) (Table 2-1). This is presumably due to the small number of vertically incubated embryos that survived past 90% of incubation in that dose. One embryo from the horizontally incubated control group exhibited exencephaly and a crossed bill, and another embryo that received PCB 126 at 2000 pg/g that was incubated horizontally exhibited a shortened beak. Many of the embryos that died prior to 90% of incubation appeared to be lagging in development and stunted in growth.

Of the eggs that did hatch, as many as four hatchlings per treatment exhibited splayed legs and curled toes, but the effects did not appear to be dose-dependent. Body weights of uninjected and vehicle-injected controls were not different ($p > 0.2$), and were thus combined into one group for comparisons to PCB 126 treatments. No differences in body weights were detected among groups.

Discussion

The present study demonstrates that orientation of the egg during incubation has a profound effect on the toxicity of PCB 126 following administration by air cell injection. Toxicity was clearly greater in vertically incubated eggs compared to those oriented horizontally. This great toxicity may be due to differences in initial exposure of the embryo to the injection bolus before it is evenly distributed throughout the albumen, differences in embryonic uptake over the course of incubation, and possibly other factors.

Corn oil volumes of ≥1 µl/g egg administered by air cell injection may result in increased embryonic mortality (DeWitt *et al*. 2005; Heinz *et al*. 2006). In the present study, an injection volume of 0.5 µl/g egg was utilized, and survival, pipping, and hatching success in control groups were relatively high and comparable to that previously reported for vertically incubated eggs (Hoffman *et al*. 1998; DeWitt *et al*. 2005a; 2005b).

Responses to PCB 126 (i.e., stunted growth, edema, and mortality) in both vertically and horizontally incubated eggs were similar to many previous reports (Brunstrom and Andersson 1988; Hoffman *et al*. 1998; Fox and Grasman 1999; Lavoie and Grasman 2007). Percent survival through 90% of incubation, and pipping and hatching success in vertically incubated eggs was similar to other reports (Brunstrom and Andersson 1988; Hoffman *et al*. 1998). However, greater concentrations of PCB 126 were required to evoke these responses in horizontally incubated eggs. Based on the limited number of doses in the present study, and the steep dose-response curve for PCB 126 embryotoxicity (Hoffman *et al*. 1998), only a rough estimate of potency as affected by egg orientation could be determined. At 1000 pg/g egg, the incidence of edema, impaired survival through 90%, pipping and hatching success were 1.34, 1.37, 1.31, and 1.54 times greater in vertically oriented eggs compared to horizontally incubated eggs. At 2000 pg/g egg, PCB 126 potency in vertically incubated eggs appeared to be even greater (incidence of edema was 3.0 and impairment of survival was 9.1 times greater) compared to horizontally incubated eggs, but this dose is at the tail end of the response curve.

Regardless of egg orientation, avian embryos float to the top of the egg during early development. Differences in air cell administered PCB 126 toxicity between vertically and horizontally incubated eggs may be related to the proximity of the embryo to the injection site. In vertically incubated eggs, the embryo is directly beneath the air cell. The greater toxicity of PCB 126 in vertically oriented eggs within a week of injection may be due to the enhanced exposure of the embryo from the injection bolus for hours to days before it diffuses and is distributed throughout the egg. Observations at the 2000 pg/g egg dose support this notion (Fig. 1, 2000 pg/g egg dose). In vertically incubated eggs injected with PCB 126, development and growth were retarded, and substantial mortality was apparent within 8 d of injection. In contrast, for dosed eggs incubated horizontally, growth appeared to be only slightly impaired and mortality was less pronounced within 8 d of injection. Over half of the mortality occurred at the very end of incubation in horizontally incubated eggs, suggesting more gradual uptake and cumulative toxicity. Vertically incubated chicken eggs injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the yolk sac exhibited higher mortality than eggs injected in the air cell on day 0 of incubation (Henshel *et al*. 1997). Embryos from both injection sites died within the first two weeks of incubation, in contrast to the late mortality observed in horizontally incubated eggs in the present study. This late toxicity in horizontally incubated eggs seems to closely approximate uptake of PCBs following yolk sac injections in chickens (de Roode and van den Brink 2002; Maervoet *et al*. 2005) and the increased mortality observed at pipping in herring gulls (*Larus argentatus*) from PCB-polluted field sites (Gilbertson and Fox 1977).

Studies of avian embryo mortality have provided important data for use in ecological risk assessments. The most valuable data for such assessments have been generated in feeding studies using a wide range of doses. In these feeding studies the compound of interest is naturally incorporated into the egg. However, due to economic and logistic constraints, hundreds of compounds have been screened with injection studies using domesticated and wild bird eggs. In many instances, there is good concordance between egg injection and feeding studies (Hoffman *et al*. 1996). The present findings with PCB 126 extend those of Heinz *et al*. (2006) on methylmercury embryotoxicity, and highlight the need to carefully consider exposure methods and egg orientation when using these data in ecological risk assessments.

Tables and Figures

Table 2-1. Effect of egg orientation during incubation on survival, pipping and hatching success and edema in chicken embryos treated with polychlorinated biphenyl congener 126[a]

Egg orientation	Combined control	PCB 126 dose (pg/g egg)		
		500	1000	2000
Survival to 90% Incubation				
Horizontal	53/56 (94.6%)	28/28 (100%)	26/28 (92.9%)	18/28 (64.3%)[b]
Vertical	52/55 (94.5%)	27/27 (100%)	19/28 (67.9%)[bc]	2/28 (7.1%)[bc]
Pipping				
Horizontal	49/56 (87.5%)	24/28 (85.7%)	17/28 (60.7%)	7/28 (25%)[b]
Vertical	50/55 (90.9%)	24/27 (88.9%)	13/28 (46.4%)[b]	0/28 (0%)[bc]
Hatching				
Horizontal	46/56 (82.1%)	23/28 (82.1%)	17/28 (60.7%)	6/28 (21.4%)[c]
Vertical	49/55 (89.1%)	22/27 (81.5%)	11/28 (39.3%)[c]	0/28 (0%)[bc]
Edema				
Horizontal	2/56 (3.6%)	4/28 (14.3%)	8/28 (28.6%)[b]	9/28 (32.1%)[b]
Vertical	2/55 (3.6%)	2/27 (7.4%)	6/28 (21.4%)[b]	3/28 (10.7%)[bc]

[a] Values are response/n (percent).

[b] Indicates significant difference ($p < 0.05$) from corresponding control.

[c] Indicates significant difference ($p < 0.05$) between egg orientation within the same dose.
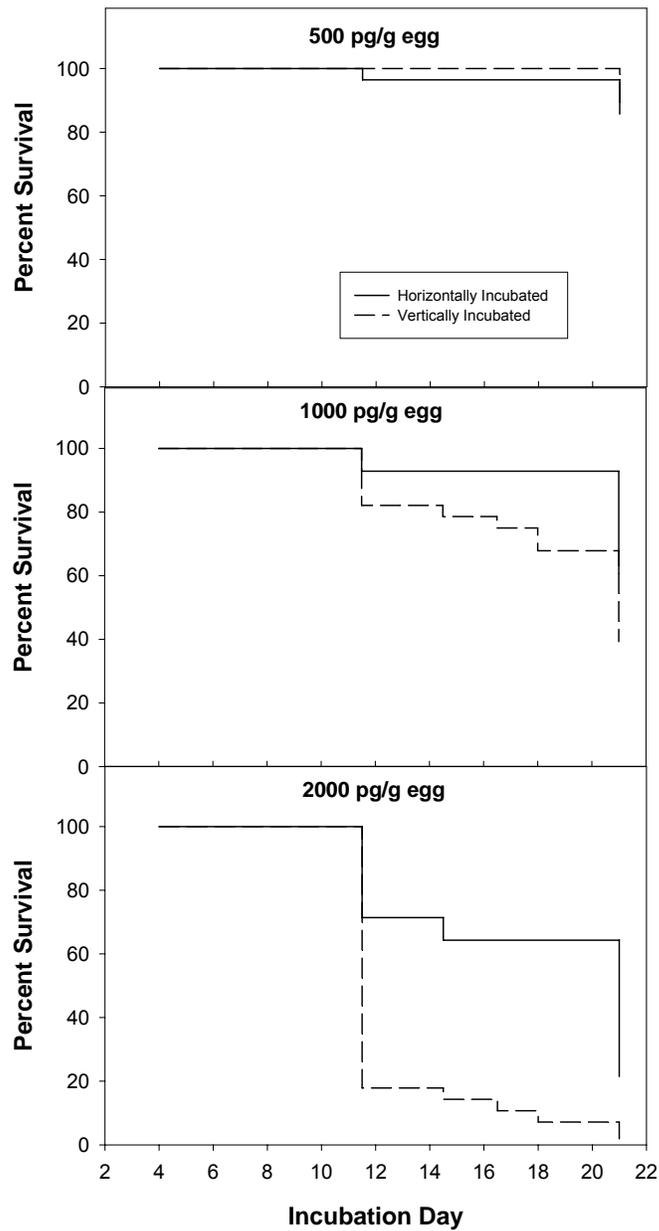
Figure 2-1. Effect of egg orientation during incubation on survival of chicken embryos administered varying doses of (polychlorinated biphenyl congener 126) PCB 126 on day 4 of embryonic development. Percent survival is through 90% of incubation.

Chapter 3: Absorption and biotransformation of polybrominated diphenyl ethers (DE-71 and DE-79) in developing chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), American kestrel (*Falco sparverius*) and Black-crowned night-heron (*Nycticorax nycticorax*) eggs following air cell administration

Abstract

Absorption into egg contents of polybrominated diphenyl ethers (PBDE) was measured following air cell administration of a penta-brominated (penta-BDE) (11.1 μg DE-71/g egg) or an octa-brominated diphenyl ether (octa-BDE) mixture (15.4 μg DE-79/g egg). Uptake of PBDE congeners was measured at 24 h post-injection, midway through incubation, and at pipping in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) egg contents, and at the end of incubation in black-crowned night-herons (*Nycticorax nycticorax*). Absorption of air cell administered penta-BDE and octa-BDE occurred throughout incubation and the uptake rate (relative proportion of administered dose absorbed into the egg) seemed to increase during the latter half of development. Up to 29.6% of penta-BDE was absorbed, but only 1.40-6.48% of octa-BDE was absorbed into egg contents by pipping. Higher brominated congeners appeared to be absorbed more slowly than lower brominated congeners. Eight PBDE congeners and two metabolites were detected in penta-BDE dosed eggs that were not reported in the dosing solution, suggesting debromination and methoxylation in the developing embryo. This study also demonstrates that verifying absorption into the egg following air cell

administration of toxicants is critical for characterizing lowest observable effect levels.

Introduction

There has been considerable interest regarding the increase of polybrominated diphenyl ethers (PBDEs) in the environment over the last 25 years. PBDEs have been commonly used as fire retardants in textiles, electronics, polymers and other materials since the 1970's. Congeners detected in penta- and octa-BDE mixtures bioaccumulate, and biomagnify in food chains (de Wit 2002). There is concern over the elevated concentrations of PBDEs in avian eggs (Norstrom *et al*. 2002; Gauthier *et al*. 2008; Chen *et al*. 2008). In a recent study of toxicological effects of air cell-administered DE-71 (commercial penta-BDE mixture) in avian eggs, the authors sugggested a lowest observable effect level for pipping and hatching success as low as 1.8 µg total PBDE/g ww egg (McKernan *et al*. in press). This approaches concentrations detected in eggs of free-ranging birds (Norstorm *et al*. 2002; Rattner *et al*. 2004; Toschik *et al*. 2005; Chen *et al*. in press). Two other avian studies have examined developmental and reproductive effects of environmentally relevant concentrations of PBDEs (Fernie *et al*. 2005a, 2005b, 2006, 2008). Using a combined egg injection/dietary exposure regime, the authors observed changes in growth (Fernie *et al*. 2006), immune organ structure, and immune function (Fernie *et al*. 2005a). The PBDEs also evoked oxidative stress in 29-d-old kestrel nestlings and apparent changes in plasma thyroxine (T_4), plasma retinol and hepatic retinol (Fernie *et al*. 2005b). In a second study by Fernie and coworkers (2008), exposure to DE-71 led to alterations in reproductive behavior of adults.

Two studies reporting accumulation, distribution and excretion of PBDEs following exposure have demonstrated evidence of debromination in adult chickens and starlings (*Sturnus vulgaris*) (Pirard and de Pauw 2007; van den Steen *et al*. 2007). Studies of PBDE exposure in laboratory mammals and fish have demonstrated Phase I and Phase II metabolism, and debromination of congeners (reviewed by Hakk and Letcher 2003, Stapleton *et al*. 2004, 2006).

The avian egg has been used extensively for toxicity testing. Its use can be attributed to the responsiveness of the avian embryo to a wide variety of toxic agents, as well as logistical and financial factors. Use of eggs from domestic species (chicken, mallard) to examine the toxicity of pesticides, industrial compounds, petroleum crude oil, and metals have provided critical data in support of ecological risk assessments. The toxicity of contaminants to avian embryos has been extensively evaluated by topical application and by injection into the air cell, albumen or yolk. For some compounds (e.g. methylmercury and PCBs), egg injection studies closely approximate embryotoxicity that occurs following natural exposure and overcome the difficulties of feeding trials (Hoffman *et al*. 1996; Heinz *et al*. 2008).

There has been little information on the absorption of air cell injected lipophilic compounds into avian egg contents. The importance of verifying actual exposure of the administered dose to better establish thresholds of toxic responses is well recognized. To address this issue, chicken, mallard, American kestrel and black-crowned night-herons (BCNH) eggs were injected with DE-71 or DE-79, collected and chemically analyzed at selected days of incubation. The goal of this study was to

provide data on the rate of uptake and potential metabolism by embryos from several different species using this convenient exposure approach.

Methods

Egg management and PBDE injection

Fertile mallard eggs were obtained from Whistling Wings (Hanover, IL, USA) and white leghorn chicken eggs were obtained from CBT Farms (Chestertown, MD, USA). American kestrel eggs were collected fresh from the colony at the Patuxent Wildlife Research Center, Laurel, MD USA. Fresh BCNH eggs were collected from Chincoteague Bay, VA, USA (Middle Mouth Marsh; N 37˚56'44 W75˚24'13), a site that has been reported in several studies where BCNH eggs and chicks were relatively free of persistent organic pollutants (e.g., Ohlendorf *et al*. 1978, Rattner *et al*. 1997). All eggs were washed at 40˚C with 1% Betadine® solution (Purdue, Wilson, NC, USA), weighed, and labeled with a number 2 graphite pencil. Eggs were then stored in a cooler at 13°C for up to 3 d. Eggs were allowed to equilibrate to room temperature before placement into incubators.

Eggs were artificially incubated (Kuhl Incubator Company, Flemington, NJ, USA) in trays that were adapted to turn horizontally oriented eggs 180˚ hourly, as previously described (McKernan *et al*. 2007, in press). Chicken eggs were incubated at 37.6 ˚C and all other species were incubated at 37.5˚C. Moisture loss was determined by weighing eggs at 3-4 d intervals. Relative humidity within the incubator was adjusted so mean egg weight loss by the end of incubation was 14-

16%. Eggs were candled at the time of weighing to confirm viability, and unfertilized or dead eggs were removed at that time.

Corn oil (Sigma-Aldrich, St. Louis, MO, USA) was used as the vehicle for this study because it causes relatively low mortality in developing avian embryos (Heinz *et al*. 2006, McKernan *et al*. 2007). A penta- and octa- PBDE mixture (Great Lakes DE-71 and DE-79; LGC Promochem, Teddington, UK) were chosen for this study because they contain congeners commonly reported in avian eggs and tissues. Injection solutions were prepared by dissolving neat PBDE in acetone and following the addition of corn oil vehicle, and were mixed for 3 hr. The final dosing solutions contained 1% acetone by volume.

Chicken eggs were incubated for 4 d, and black-crowned night-heron, mallard and kestrel eggs were incubated until the developmental equivalent of a 4-d-old chicken embryo, at which point they were candled to confirm fertility. Any infertile, non-viable, or cracked eggs were discarded. Eggs were then randomly assigned to groups of uninjected, vehicle-injected, DE-71 or DE-79 treatments. Vehicle or PBDE was injected at a constant volume (0.5 µl/g egg) in the air cell, as previously described (McKernan *et al*. 2007, McKernan *et al*. in press). Briefly, a hole was drilled into the cap end of the egg and the vehicle or PBDE mixture [DE-71 (11.1 µg/g egg) or DE-79 (15.4 µg/g egg)] was injected. Immediately following injection, the hole was sealed with a hot glue gun (ethylene vinyl acetate) and the eggs were kept in a vertical position for 30 minutes to allow the oil to spread over the air cell membrane. Eggs were then placed horizontally in trays and returned to the incubator. Survival throughout incubation was monitored by candling or with a viability-

detecting instrument called the Buddy (Vetronic, Torquay, UK) until the embryos were sampled, had pipped or died.

Sample Collection

In both the DE-71 and DE-79 injection trials, eggs were randomly sampled at three stages in development, 24 hr post-injection (N = 3), midway through incubation (N = 3), and at pipping (N = 3-4) in chicken, mallard and kestrel eggs. Black-crowned night-heron eggs (n = 6) were only sampled at pipping. Eggs were removed from the incubator and weighed. The narrow end of the egg was gently removed so that the contents of the egg could be poured out into chemically-clean jars and weighed. Samples were frozen at -20˚C until chemical analysis.

The distribution of the administered dose of PBDE was also assessed by collecting the air cell membrane (injection site in blunt end of egg) in chicken eggs. Air cell membranes were dissected from the eggs, blotted to remove blood and albumen, weighed, and stored at -20˚C. Air cell membranes were pooled by day of incubation (d5, d10, d21), and these three pools were chemically analyzed.

Analytical methods

All samples were analyzed at the Virginia Institute of Marine Science in the laboratory of Dr. Robert Hale. Concentrations of DE-71 and DE-79 dosing solutions were verified analytically by gas chromatography/mass spectrometry (GC/MS)

(Varian 3400 and Varian 4D ion trap; Sugar Land, TX, USA). Corn oil test solutions

were diluted in hexane. Congener peak areas were compared to that of an internal

standard (p-terphenyl). Congener response factors were determined using authentic

PBDE standards (AccuStandard, Inc.). Detection was in the electron ionization

mode. The GC was equipped with a DB-5 column (60m length x 0.32 mm inner

diameter and a 0.25 μm film thickness; JandW Scientific, Folsom, CA, USA) and the

carrier gas was He. Injections were made in the spitless mode. Congener

identification was achieved by MS in the full-scan, electron-ionization mode.

Quantification was performed by comparison of the sum of the areas of the three

major ions of each PBDE congener (85 congeners for DE-71 and 49 congeners for

DE-79) versus that of the internal standard.

Egg contents were analyzed using previously described methods (Hale *et al*.

(2001) and McKernan *et al*. (in press). Briefly, eggs were lyophilized, and

subsamples were spiked with surrogate standard PCB 204 (Ultra Scientific, North

Kingstown, RI, USA). Blanks were run coincidentally to evaluate possible laboratory

contamination. Egg samples were subjected to enhanced solvent extraction (Dionex

ASE 200, Sunnyvale, CA, USA) with methylene chloride. Large molecular weight

compounds were separated from the PBDEs in the extracts on an Envirosep size-

exclusion chromatography column (350 mm length x 21.2 mm diameter with 60 x

21.1 mm guard column; Phenomenex, Torrance, CA, USA). The PBDE-containing

fraction was then purified on a 2000 mg, silica gel, solid-phase extraction glass

column (Enviroprep, Burdick and Jackson, Muskegon, MI, USA). The PBDEs in the

purified extracts were separated by GC/MS as previously described. Data were

corrected based on the recovery of surrogate standard PCB 204 in each sample. Mean recovery of surrogate PCB 204 from the eggs was 72.9%. The limit of quantification was 100 pg/g wet weight.

Statistical methods

Data was analyzed using SAS (SAS Institute Inc., NC, USA). PBDE concentrations were tested for normality with the Shapiro-Wilk (W) statistic and for homogeneity of variance. For each species, differences in PBDE concentrations among collection times were determined using one-way analysis of variance (ANOVA) and Tukey's HSD method of multiple comparison. Percent uptake of the administered DE-71 and DE-79 doses were arcsine transformed and compared using factorial ANOVA (three species by two PBDE formulations by three incubation stages). Statistical significance was determined as $p < 0.05$.

Results

Background concentrations of PBDE and administered dose

Vehicle treated chicken, mallard and kestrel eggs contained exceedingly low concentrations of PBDE (congeners 28/33, 47, 99, 100, 153, 154, or 183 were detected in 11 of 27 samples with total PBDE ranging up to 83.4 ng/g ww). Of the vehicle treated BCNH eggs, total PBDE averaged 1.37 ± 0.496 ng/g ww (congener 47 was detected in all samples; congeners 99 and 153 were detected in a single egg).

Concentrations of total PBDE in these vehicle control samples were less than 1% of the air cell administered dose of DE-71 or DE-79.

The DE-71 dose was analytically verified to be 11.1 ug/g egg. The dose was composed of 24 individual congeners, with congeners 47, 99 and 100 dominating the profile at 22, 37 and 18%, respectively (Figure 1a and 1c). The DE-79 dose was analytically verified to be 15.4 ug/g egg, and was composed of 13 congeners with 185/175, 204/197, 196, 207, 198/203 dominating the profile at 27, 16, 12, 10 and 8%, respectively (Figure 2a and 2c).

DE-71 in egg contents

Absorption of air cell injected DE-71 continued throughout incubation (Table 3-1). By pipping, 18.8-29.6% of DE-71 was absorbed into the egg contents (yolk, albumen, embryo) in chickens, mallards, kestrels, and BCNHs. The dominant congeners from DE-71 absorbed into egg contents at all three time points were BDE-47, -99, and -100 (Figure 3-1b and 3-1d for chicken, Figure 3-3 for mallard and kestrel, Figure 3-4 for BCNH). Although the absolute concentration of BDE-47 in egg contents increased over time, its relative contribution to total PBDE in egg contents was slightly lower at mid-incubation and pipping as the absorption of BDE-99 and -100 had increased.

A total of eight congeners or metabolites (two to five per species) were detected at mid-incubation or at pipping that were not reported in the dosing solution (Table 3-2). Congeners 88 and 97/118 were detected in most species at pipping, while the other congeners were detected in only one or two samples of some of the

species. Each of these congeners accounted for less than 0.05% of total PBDE measured in egg contents.

Attempts to account for administered DE-71 dose

In preliminary range finding studies, corn oil injected into the air cell of mallard eggs was observed to spread well beyond the perimeter of the air cell, between the inner shell (contacts the albumen) and outer shell membranes (located between inner shell membrane and egg shell). The translucent nature of the eggshell for mallards (but not the chicken and kestrel) probably facilitated this observation. The spreading of the injection solution took several days and seemed to be complete by mid-incubation. Using a surface area equation for eggs from Paganelli *et al*. (1974), the inner shell membrane of our chicken eggs was estimated to be about 73.0 cm^2. At pipping, the air cell membrane averaged 8.65cm^2, while the remaining inner membrane attached to the shell for eggs would account for approximately 64.35 cm^2.

As previously described, DE-71 was administered at a dose of 11.1 µg/g egg. At pipping, 29.6% (196 µg) of administered dose of DE-71 was detected in egg contents (Table 3-1). Total PBDE remaining associated with the air cell membrane accounted for 4.3% (28.9 µg) of the administered dose. Assuming that the DE-71 spread evenly around the inner shell membrane remaining attached to the shell (i.e., 64.35% cm^2 of the total 73.0 cm^2), we can estimate that as much as 34.4% of the administered dose might be associated with the attached inner shell membrane. If this assumption is correct, then we can account for 68.3% of the administered dose.

DE-79 in egg contents

Absorption of injected DE-79 into egg contents increased over the course of incubation (Table 3-3), although concentrations were only significantly greater ($p \leq$ 0.0392) at pipping. The percentage of DE-79 absorbed was markedly less than DE-71 (Tables 3-1 and 3-3; factorial ANOVA, $p < 0.0001$). This is apparent at all three stages of incubation, with concentrations of DE-71exceeding those of DE-79 by five- to ten-fold. A range of 1.40-6.48% of DE-79 dose was absorbed into the contents of chicken, mallard, kestrel, or BCNH eggs at pipping.

Congeners BDE-153 and -183 dominated the congener profiles in all species (Figure 3-4b and 3-4d for chicken, Figure 3-5 for mallard and kestrel, Figure 3-6 for BCNH) and congener 197 was detected only at pipping. Although the absolute concentration of lower brominated congeners increased during incubation, their relative contribution decreased as higher brominated congeners (BDE-183, -197) that were present in the dosing solution were gradually absorbed into the egg.

There was no evidence of in ovo biotransformation of DE-79; all congeners detected in egg contents were also present in the dosing solution. Total PBDE detected in air cell membranes was similar at various stages of incubation, and accounted for a larger percentage of the administered dose of DE-79 (24.9-36.4%) compared to the percentage of the dose retained on air cell membranes of DE-71 treated eggs.

Analytical measurements accounted for 38.7% (2.3% in egg contents and 36.4% in air cell membrane) of the total administered dose of DE-79 in chicken eggs by pipping. Using the DE-71 methodology (see previous section) for estimating

PBDE associated with inner and outer shell membranes, recovery estimates exceeded 250% of administered dose. This overestimate may be attributable to low mobility among the inner and outer shell membranes of higher brominated congeners, as evidenced by their slower absorption into egg contents, and perhaps their limited migration from the air cell.

Discussion

Findings from this study demonstrate that up to 29.6% of air cell injected PBDE is absorbed through the air cell membrane into the contents of chicken, mallard, kestrel, and BCNH eggs. The DE-71 formulation was more readily absorbed than DE-79. There was some evidence of biotransformation of PBDE in the avian egg, as some congeners detected in eggs were not present in the dosing solution or vehicle injected eggs.

Uptake of air cell administered PBDE

Over the course of incubation, the absorption of lower brominated DE-71 was greater than higher brominated DE-79 ($p = 0.0183$) in chickens, mallards, and kestrels. By the time of pipping, 18.8-29.6% of DE-71 and only 1.4-6.5% of DE-79 was absorbed into egg contents. The greater molecular volume and lower water solubility of the higher brominated congeners may render them more difficult to absorb through the air cell membrane. This was also evidenced by more DE-79 being retained by the air cell membrane compared to DE-71. Comparison of findings from

our test system (air cell injected bird eggs) to feeding studies involving more advanced life stages and different vertebrate classes is tenuous, but still worthy of discussion. Burreau *et al*. (1997) fed BDE-47, -99, and -153 to juvenile pike (*Esox lucius*) and found lower uptake efficiency with increasing congener bromination, which is similar to the present findings. In contrast to differential uptake of air cell administered DE-71 and DE-79 in the present study, carcass and liver retention of DE-79 (octa-BDE formulation) was nearly equal to that of Bromkal 70 (penta-BDE formulation) (over 30% vs. 44%, respectively) in male rats following a 21-d dietary exposure (Huwe *et al*. 2002; Huwe *et al*. 2007). Using an exposure regime of egg injection and daily gavage during the nestling period of American kestrels, Drouillard and coworkers (2007) found greater carcass retention of higher brominated congeners, possibly due to preferential metabolism and clearance of lower brominated congeners. Pirard and de Pauw (2007) measured PBDE concentrations in fat of laying chickens fed DE-71 for 14 weeks and found some higher brominated congeners were more readily retained (greater bioconcentration factor) than lower brominated congeners.

The DE-71 and DE-79 congener profiles of egg contents at 24 h post injection are markedly different than those of the dosing solution. As metabolic capabilities are limited in early development (DE-71 metabolites only appeared in egg contents toward the end of incubation), it seems likely that this different profile reflects preferential uptake of lower brominated congeners. At mid-incubation and pipping, congener profiles of DE-71 and DE-79 in egg contents continued to differ from the dosing solutions and this could be due to both preferential uptake and

biotransformation as some unique congeners were detected. The mechanisms (preferential uptake and/or biotransformation) accounting for the differential PBDE congener profiles of the dosing solutions and egg contents might be best resolved using radio-labeled, single component exposures.

Following DE-71 administration into the air cell of chicken and mallard eggs, a the trend in concentration of total PBDEs suggested gradual absorption over the exposure period. The apparent increase in uptake rate between mid-incubation and pipping was suggestive of an exponential relationship. Enhanced absorption during the latter half of incubation could be due to increased density and size of the blood vessels of the chorioallantoic network. The mallard egg appeared to more readily absorb DE-71 and DE-79 compared to other species, although this difference was marginally significant ($p = 0.0657$).   In contrast to chickens and mallards, total DE-71 (but not DE-79) appeared to be absorbed at a greater rate up to mid-incubation in kestrels (i.e., 18.4% of administered dose), and then leveled off. It is not clear if these apparent species differences in PBDE absorption are real or may be a function of small sample size.

Few studies report the relationship between administered dose and actual exposure following treatment of avian or reptilian eggs. De Roode and van den Brink (2002) injected PCBs into the yolk of chicken eggs, and found an exponential increase in uptake into the embryo, with 18% of the administered dose absorbed by d 19 of incubation. Similarly, Maervoet and coworkers (2005) noted an exponential uptake after yolk injection of PCB congeners 77, 153 and 180 into the chicken embryo during the last week of incubation. Portelli *et al.* (1999) reported dose-

dependent uptake of topically administered DDT (dichlorodiphenyltrichloroethane) into snapping turtle (*Chelydra serpentina serpentine*) eggs within 3 d of application, however only a small fraction (1.6 to 20%) of the total dose was absorbed. Gale *et al*. (2002) found that 4 to 10% of topically administered 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3,3',4,4',5-pentachlorobiphenyl was absorbed into egg contents of red-eared sliders (*Trachemys scripta elegans*) over a 16-d period. The fraction of air cell administered DE-71 and DE-79 absorbed into avian egg contents in the present study is similar to the degree of uptake following topical application of environmental contaminants in the above studies using turtle eggs. To the best of our knowledge, ours is the first report describing quantities of air cell administered PBDE absorbed into avian eggs.

Biotransformation

Eight PBDE congeners and two metabolites were detected in egg contents of chicken, mallard, kestrel, or BCNH that were not present or not detectable in the penta-BDE dosing solution or control eggs (Table 3-2). Similarly, Tomy *et al*. (2004) fed juvenile lake trout (*Salvelinus namaycush*) a mixture of PBDEs and detected three congeners (an unknown penta, BDE-140, and an unknown hexa) in carcass that were not present in control fish or spiked food samples. These eight congeners and two metabolites detected in the present study suggest PBDE debromination and methoxylation in mid-incubation and pipping bird embryos. There was no evidence of biotransformation of the DE-79 dose, presumably due to the low absorption into egg contents.

Polybrominated diphenyl ethers can undergo debromination, and better characterized Phase I, and Phase II enzymatic processes. Polybrominated diphenyl ethers are structurally similar to thyroxine (T_4), and some have suggested (Benedict *et al*. 2007) that congeners may undergo debromination using a similar/related pathway to deiodination. While the mechanism of debromination of PBDEs is poorly understood, there is evidence that this process occurs in vertebrates. Stapleton *et al*. (2004) reported debromination of PBDEs in common carp (Cyprinus carpio) fed BDE congeners -99 and -183. Congener 99 was debrominated to BDE-47, and congener 183 was debrominated to an unidentified hexa-BDE congener. Stapleton *et al*. (2006) fed BDE-209 to juvenile trout (*Oncorhynchus mykiss*) and detected several hepta-, octa-, and nona-BDE congeners in tissues. Debromination of PBDEs has also been reported in European starlings (*Sturnus vulgaris*) exposed to BDE-209 using silastic implants, as octa- and nona-BDEs were detected in muscle and liver tissues (van den Steen *et al*. 2007).

Mammalian and fish studies implicate Phase I CYP enzymes in hydroxylation of PBDEs (Hakk and Letcher 2003). Hydroxylated and methoxylated PBDE congeners have been detected in free ranging wildlife (Marsh *et al*. 2004; McKinney *et al*. 2006; Verreault *et al*. 2005, 2007; Gebbink *et al*. 2008). This class of flame retardants has also been reported to be metabolized through Phase II conjugative processes (e.g. uridine diphosphate glucoronosyl-transfereases and glutathione S-transferases) (Hakk and Letcher 2003).

Utility of air cell injection studies

The avian embryo has been used extensively as a toxicity bioassay for environmental contaminants. As a time and cost saving measure, many investigators have administered toxicants by injection into the air cell or yolk, and there is some evidence that such techniques mimic toxicity observed when contaminants are maternally deposited into the egg. For example, embryotoxic responses elicited by egg injection of PCBs compare favorably to those observed following natural exposure (Hoffman 1996). However, methylmercury injected into mallard eggs seems to be more toxic than dietary methylmercury, as air cell administration of 1.6 µg/g egg resulted in comparable toxicity to that observed following maternal deposition at 5.5 µg/g ww (Heinz *et al* 2008). Air cell injected compounds must cross the air cell membrane, blood vessels, albumen, and yolk to reach the embryo. It is unknown if air cell administered compounds mimic the distribution of naturally deposited contaminants in eggs, although they do evoke similar types of toxic responses (e.g., teratogenic effects, edema, histopathology, enzyme induction). However, the migration and metabolism of air cell injected compounds must be carefully characterized to ascertain true exposure.

Clearly, the PBDE dose to which the embryo was exposed was much less than the administered dose. This difference should be taken into account when designing egg injection studies and using findings for risk assessments. Additionally, degree of halogenation (bromination, chlorination) must be considered when using air cell injection as a route of embryonic exposure as larger congeners may not readily cross the air cell membrane. Previous reports of LOELs and LD50s of air cell injections of

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PCBs and other persistent organic pollutants may underestimate toxicity if test compounds are incompletely absorbed. In our previous study, we demonstrated that at an administered concentration of 10 μg DE-71/g egg, kestrel pipping and hatching success was significantly reduced, yet much less PBDE was absorbed into egg contents and thus the estimated LOEL was approximately 1.8 μg/g egg ww (McKernan *et al*. in press). Although air cell administered compounds are not naturally incorporated into the yolk or albumen, determining their concentration in egg contents is critical in predicting toxic effects in eggs of free-ranging birds.

In the present study, we observed up to 36% of the PBDE dose remaining associated with the air cell membrane at pipping. The air cell membrane represents only a portion of the remaining inner shell membrane, outer shell membrane, and porous eggshell where the remaining PBDE may be sequestered. We performed a rough estimate of how much PBDE could have spread between the inner and outer shell membranes in order to account for the remaining DE-71 and DE-79 doses. Using this estimate, we can account for 68.3% of the total DE-71 dose injected, however the same calculations account for over 250% of the DE-79 dose. The overestimate for DE-79 may be due to lower mobility of higher brominated congeners around the inner and outer shell membranes. In future studies, we recommend analyzing the remaining air cell membrane, eggshell, and membranes attached to the eggshell, as well as egg contents, to more accurately estimate mass balance. In addition, researchers may want to consider analyzing specific tissues of the embryo in order to generate information on distribution of air cell injected compounds in the avian embryo.

Biotransformation (e.g., debromination, hydroxylation, methoxylation) of PBDE congeners confounds efforts to measure uptake of parent compounds following dietary administration, or in this case, air cell injections. Increased concentrations of lower brominated congeners (or even differences in ratios of congeners) by pipping may be from differential absorption through the air cell membrane from the dosing solution, or possibly the result of debrominated parent compounds which would supplement lower molecular weight congeners in the dosing solution. Formation of other metabolites that are not analyzed may reduce percent recovery in mass balance studies. In a mass balance study, Huwe *et al*. (2007) reported that 20-40% of the total dose of PBDE fed to rats for 21 d was not recovered, and was attributed to biotransformation. Similarly, in another mass balance study Huwe and coworkers (2002) were only able to recover 48-80% of DE-79 fed to rats for 21 d.

Conclusions

This study demonstrates that there is differential uptake of air cell administered PBDE and that it is important to confirm the actual exposure following air cell administration of compounds, as the entire dose may not be absorbed through the air cell membrane. In addition, evidence is provided that PBDE can be debrominated and methoxylated in the avian embryo. Production of penta- and octa-BDE formulations has been discontinued due to the potential toxicity of lower brominated congeners. However, deca-BDE is still in production. It is being released into the environment and has been detected in tissues of wildlife (Chen *et al*. 2008). Phasing out of penta- and octa-mixtures may not fully protect free ranging wildlife, as

fish and birds have been shown to be capable of debrominating deca-BDE (Stapleton

et al. 2006, van den Steen *et al*. 2007).

Tables and Figures

Table 3-1. Uptake of air cell administered DE-71 during incubation in chicken, mallard, kestrel and black-crowned night-heron eggs.[a]

	Stage of Incubation		
	24 hr post-injection	midway	pip
	Chicken		
DE-71 injected (µg total PBDE/g egg)[b]	0.088 ± 0.0459^A	1.03 ± 0.111^B	4.93 ± 0.994^C
Uptake of DE-71 Dose (%)	0.64 ± 0.356	7.71 ± 0.857	29.6 ± 4.56
	Mallard		
DE-71 injected (µg total PBDE/g egg)	0.345 ± 0.0777^A	2.42 ± 0.272^B	4.58 ± 0.174^C
Uptake of DE-71 Dose (%)	2.62 ± 0.628	16.94 ± 2.077	27.7 ± 1.23
	American kestrel		
DE-71 injected (µg total PBDE/g egg)	0.208 ± 0.1129^A	2.43 ± 0.452^B	2.80 ± 0.498^B
Uptake of DE-71 Dose (%)	1.64 ± 0.871	18.42 ± 3.318	18.8 ± 3.04
	Black-crowned night-heron		
DE-71 injected (µg total PBDE/g egg)	-	-	3.16 ± 0.761
Uptake of DE-71 Dose (%)	-	-	21.8 ± 5.24

[a] DE-71 was administered at a nominal concentration of 20 ug/g egg, which was analytically verifed to be 11.1 ug/g egg.
Values presented are mean $\pm$ SE, n=3/day for chicken, mallard, kestrels, n=6 for black-crowned night-herons

[b] Groups with different capital letter superscripts are significantly different ($p < 0.05$).

Table 3-2. PBDE congeners detected in egg contents that were not detected in DE-71 dosing solution or control eggs.

		Stage of Incubation				
	Congener	24 hr post-injection	midway (ng/g ww ± SE)[A]	% of BDE congeners in sample	pip (ng/g ww ± SE)	% of BDE congeners in sample
Chicken	BDE 120	3ND	3ND		1.7 ± 0.3	0.03%
	BDE 88	3ND	3ND		2ND - 1.9	≤0.03%
	BDE 97/118	3ND	3ND		2ND - 0.7	≤0.02%
Mallard	BDE 104/121	3ND	3ND		2ND, 1.6	≤0.04%
	BDE 120	3ND	3ND		2ND , 1.8	≤0.04%
	BDE 88	3ND	2ND, 1.5	≤0.05%	1ND, 1.7, 2.3	≤0.05%
	BDE 97/118	3ND	3ND		2ND, 1.1	≤0.02%
	5' MeO BDE 100	3ND	3ND		2ND, 1.0	≤0.02%
Kestrel	BDE 88	3ND	1ND, 0.7, 1.2	≤0.04%	3ND, 0.8	≤0.04%
	BDE 97/118	3ND	1ND, 0.4, 1.1	≤0.03%	3ND, 0.6	≤0.04%
Black-crowned Night-heron	BDE 35	-	-		4ND, 0.7, 0.7	≤0.02%
	BDE 37	-	-		5ND, 0.2	≤0.01%
	BDE 71/48	-	-		0.5 ± 0.1	0.02%
	2'MeO BDE 68	-	-		5ND, 0.2	≤0.01%
	BDE 97/118	-	-		1.2 ± 0.4	0.04%

[A] Detection limit (DL) = 100pg/g wet weight. ND = nondetect; n = 3-6 per stage of incubation

Table 3-3. Uptake of air cell administered DE-79 during incubation in chicken, mallard, kestrel and black-crowned night-heron eggs.[a]

	Stage of Incubation		
	24 hr post-injection	midway	pip
	Chicken		
DE-79 injected (μg total PBDE/g egg)[b]	0.008 ± 0.001^A	0.065 ± 0.0141^A	0.532 ± 0.0930^B
Uptake of DE-79 Dose (%)	0.041 ± 0.0100	0.345 ± 0.0748	2.31 ± 0.401
	Mallard		
DE-79 injected (μg total PBDE/g egg)	0.016 ± 0.0038^A	0.162 ± 0.0260^A	1.49 ± 0.584^B
Uptake of DE-79 Dose (%)	0.086 ± 0.2106	0.813 ± 0.1378	6.48 ± 2.524
	American kestrel		
DE-79 injected (μg total PBDE/g egg)	0.011 ± 0.0668^A	0.073 ± 0.0169^A	0.486 ± 0.0734^B
Uptake of DE-79 Dose (%)	0.058 ± 0.0378	0.402 ± 0.0958	2.24 ± 0.346
	Black-crowned night-heron		
DE-79 injected (μg total PBDE/g egg)	-	-	0.286 ± 0.0561
Uptake of DE-79 Dose (%)	-	-	1.40 ± 0.274

[a] Octa-BDE was administered at an analytically verifed dose of 15 ug/g egg.
Values presented are mean $\pm$ SE, n=3/day for chicken, mallard, kestrels, n=6/day for black-crowned night-herons
[b] Groups with different capital letter superscripts are significantly different ($p < 0.05$).
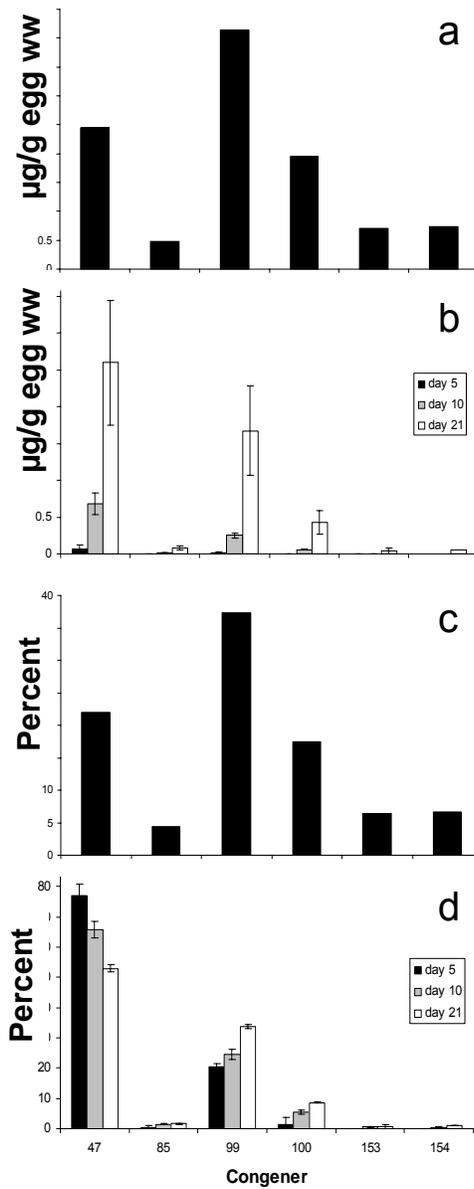
Figure 3-1. (a) Congener concentration in administered dose of DE-71, on a fresh egg weight basis, (b) Congener concentrations in embryonated chicken eggs, c) Relative contribution of congeners in administered dose of DE-71, and (d) Relative contribution of congeners in embryonated chicken eggs.
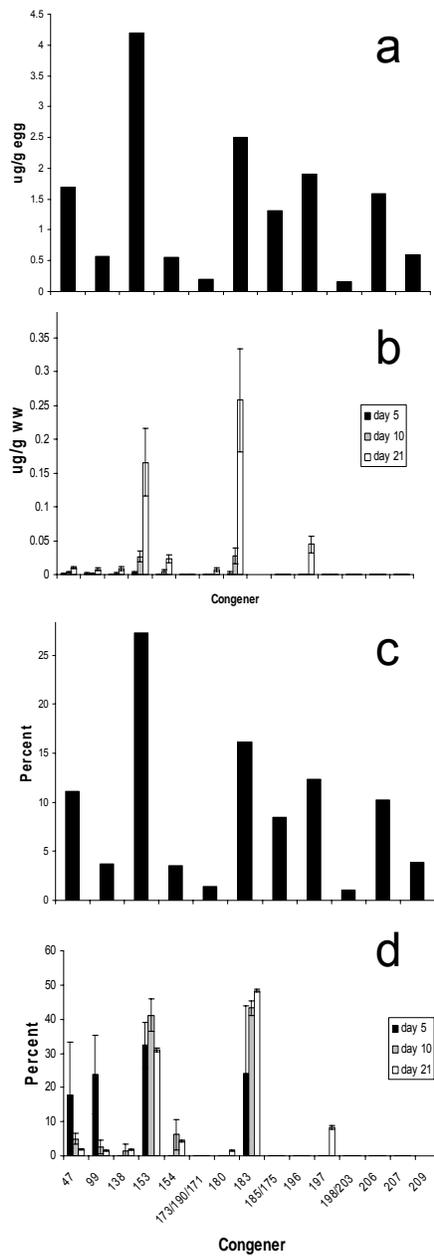
Figure 3-2. (a) Congener concentration in administered dose of DE-79, (b) Congener concentrations in embryonated chicken eggs, (c) Relative contribution of congeners in administered dose of DE-79, and (d) Relative contribution of congeners in embryonated chicken eggs.
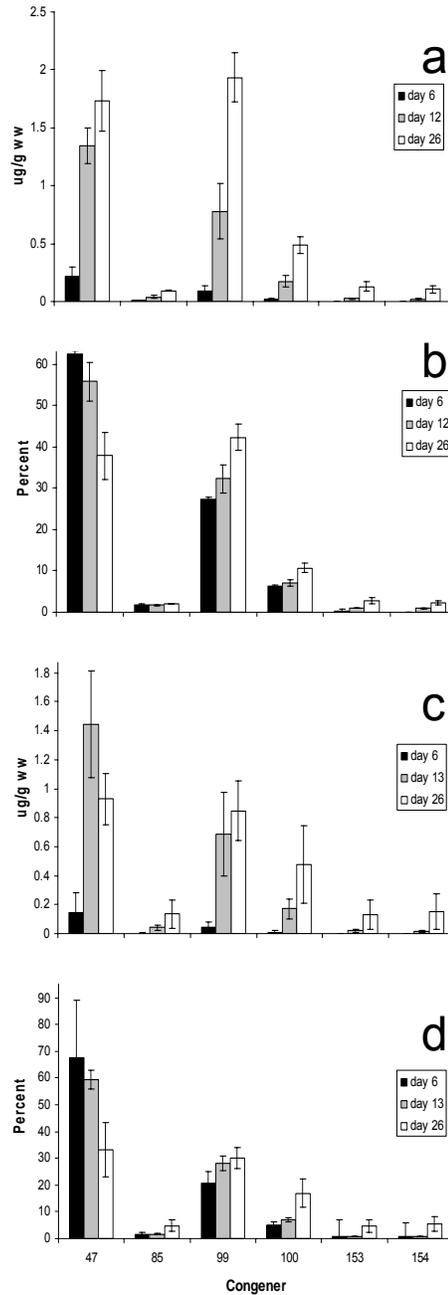
Figure 3-3. (a) Congener concentration in embryonated mallard eggs administered DE-71, (b) Relative contribution of congeners in embryonated mallard eggs, (c) Congener concentration in embryonated kestrel eggs administered DE-71, and (d) Relative contribution of congeners in embryonated kestrel eggs.
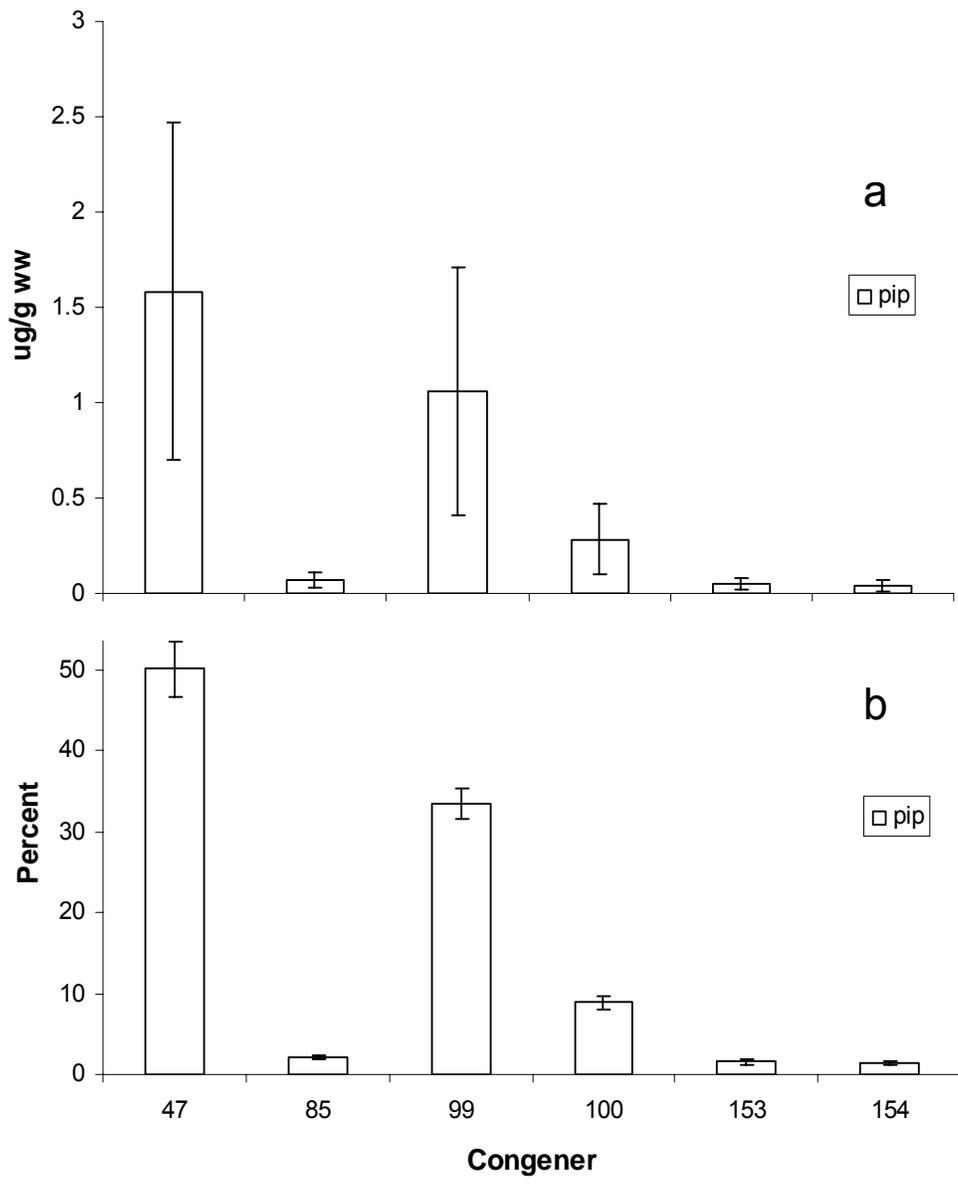
Figure 3-4. (a) Congener concentration in embryonated black-crown night-heron eggs administered DE-71 and (b) Relative contribution of congeners in embryonated black-crown night-heron eggs.
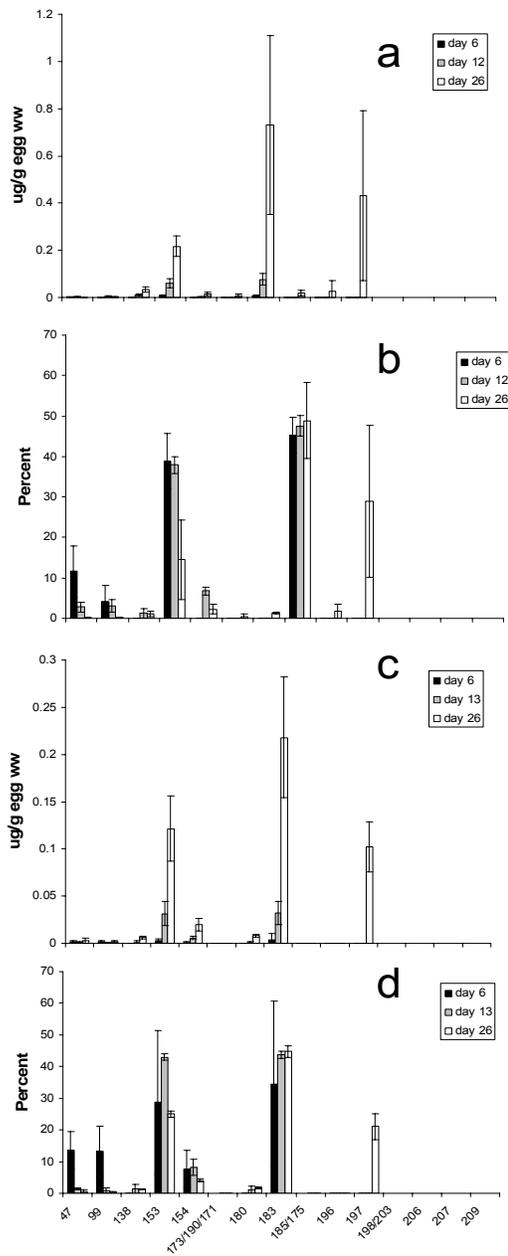
Figure 3-5. (a) Congener concentration in embryonated mallard eggs administered DE-79, (b) Relative contribution of congeners in embryonated mallard eggs, (c) Congener concentration in embryonated kestrel eggs administered DE-79, and (d) Relative contribution of congeners in embyronated kestrel eggs.
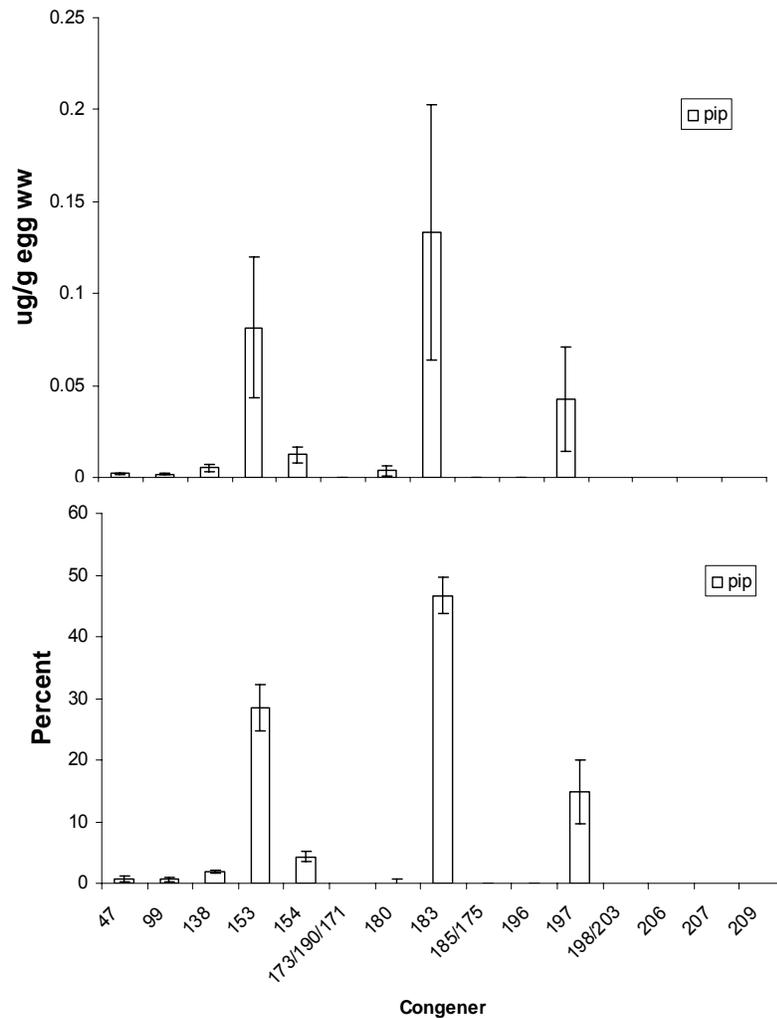
Figure 3-6. (a) Congener concentration in embryonated black-crown night-heron eggs administered DE-79 and (b) Relative contribution of congeners in embryonated black-crown night-heron eggs.

Chapter 4: Toxicity of polybrominated diphenyl ethers (DE-71) in developing chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrels (*Falco sparverius*) embryos and hatchlings

Abstract

Embryonic survival, pipping and hatching success, and sublethal biochemical, endocrine, and histological endpoints were examined in hatchling chickens (*Gallus gallus*), mallards (*Anas platyrhynchos*), and American kestrels (*Falco sparverius*) following air cell administration of a penta-brominated diphenyl ether (penta-BDE) mixture (0.01-20 µg DE-71/g egg) or polychlorinated biphenyl congener 126 (3,3',4,4',5-pentachlorobiphenyl; PCB 126; 2000 pg/g egg). Penta-BDEs decreased pipping and hatching success at concentrations of 10 and 20 µg/g egg in kestrels, but had no effect on survival endpoints in chickens or mallards. Sublethal effects in hatchling chickens included ethoxyresorufin-*O*-dealkylase (EROD) induction and histological changes in the bursa, but these responses were not observed in other species. Polychlorinated biphenyl congener 126 (positive control) reduced survival endpoints in chicken and kestrel embryos, and caused sublethal effects (EROD induction, reduced bursal mass and follicle size) in chickens. Mallards were clearly less sensitive to administered penta-BDE and PCB 126 than the other species. In a second experiment, the absorption of penta-BDE (11.1 µg/g egg air cell administered in early development) into the contents of chicken and kestrel eggs were determined at various intervals (24 h post-injection, mid-incubation, pipping). By pipping, 29%

of the PBDE administered dose was present in the egg contents in chickens and 18%

of the administered dose was present in kestrel egg contents. Based on the uptake

rate in kestrels, the lowest observable effect level on pipping and hatching success

may be as low as 1.8 µg total PBDE/g egg, which approaches concentrations detected

in eggs of free-ranging birds. As some PBDE congeners are still increasing in the

environment, the toxic effects observed in this study are cause for concern in wildlife.

Introduction

Polybrominated diphenyl ethers (PBDEs) have been commonly used as fire

retardants in polymers, textiles, electronics and other materials. These compounds

bioaccumulate in aquatic and terrestrial organisms and biomagnify in food chains (de

Wit 2002). Monitoring studies indicate that PBDE concentrations in the environment

have increased over the past 25 years. A retrospective study of archived herring gull

(*Larus argentatus*) eggs from the Great Lakes demonstrated that individual congeners

(BDE-47, -99, -100) found in the commercial penta-BDE mixture increased by one

and a half orders of magnitude between 1981-2000 (doubling time of 2.6-3.1 years)

(Norstrom *et al*. 2002), and concentrations of these congeners in gull eggs have

remained elevated (Gauthier *et al*. 2008). On a wet weight basis, concentrations of

total PBDEs in avian eggs range up to 1.40 µg/g in herring gulls from the Great Lakes

(Norstrom *et al*. 2002), 0.928 µg/g in ospreys (*Pandion haliaetus*) from Chesapeake

and Delaware Bays (Rattner *et al*. 2004, Toschik *et al*. 2005), 0.804 µg/g in white-

tailed sea eagles (*Haliaeetus albicilla*) from the Norwegian coast (Herzke *et al*.

2005), and 6.60 µg/g in peregrine falcons (*Falco peregrinus*) from the northeast

United States (D. Chen 2008, personal communication). Interpretation of the significance of these concentrations in eggs is not possible as adverse effects thresholds have yet to be adequately established in birds.

Only two studies in birds have examined developmental and reproductive effects of environmentally relevant concentrations of PBDEs (Fernie *et al*. 2005a, 2005b, 2006, 2008). In the initial study by Fernie and coworkers (2005a, 2005b, 2006), American kestrel (*Falco sparverius*) eggs were injected with 18.7 µg total PBDEs (BDE congeners -47, -99, -100, -153) on day 19 of incubation (i.e., 1.43 µg/g egg), and then nestlings were gavaged daily with the same PBDE mixture at 15.6 ng/g body weight through day 29 post-hatch. Using this combined egg injection/dietary exposure regime, there was some evidence of increased growth (i.e., body weight, tarsometatarsus and feather length) (Fernie *et al*. 2006) and structural changes in immune organs (i.e., fewer germinal centers in spleen, reduced apoptosis in bursa, increased macrophages in thymus) (Fernie *et al*. 2005a). As carcass concentrations of BDE-47 and -183 increased, some alterations in immune function (i.e., greater phytohemagglutinin skin response and reduced antibody-mediated response) were detected (Fernie *et al*. 2005a). The PBDE mixture also evoked oxidative stress (i.e., marginal increases in the ratio of oxidized to reduced glutathione, oxidized glutathione, and lipid peroxidation) in kestrel nestlings. Additionally, in 29-d-old nestlings, plasma thyroxine (T4), plasma retinol and hepatic retinol were inversely related to carcass concentrations of BDE-47 and -99 (Fernie *et al*. 2005b). In a second study by Fernie and coworkers (2008), reproductively active adult kestrels were fed concentrations (0.3 µg DE-71/g diet or 1.6 µg DE-71/g diet) of a

commercial penta-BDE formulation and changes in reproductive behavior were noted (e.g., fewer bonding behaviors, copulated less, and spent less time in nest box). Studies of PBDE toxicity in laboratory mice and rats, often using relatively high dosage levels, have demonstrated changes in behavior and memory, impaired immune function, decreased circulating concentrations of T4 , and induction of cytochrome P450-associated monooxygenases (von Meyerinck *et al.* 1990; Fowles *et al.*, 1994, Eriksson *et al.*, 2001; Chen *et al.*, 2001; Zhou *et al.*, 2001).

Considering the ubiquitous nature of PBDEs, and their increasing concentrations in the environment, there is a need for more complete ecotoxicological data on these compounds. General toxicity studies should be conducted with species that may be at high risk of exposure. Developmental and reproductive effects are of particular concern because of their linkage to higher order effects, although more subtle responses also merit consideration as they may serve as early sublethal warning signals of potential higher order effects.

The present investigation examined the effects of penta-BDE exposure in avian embryos (domestic chicken*, Gallus gallus*; mallard, *Anas platyrhynchos*; and American kestrel) following air cell administration of a commercial PBDE mixture. These studies describe biological effects (e.g., embryonic survival and hatching success, and endpoints reflecting development and growth, histopathology, cytochrome P450 induction, and glandular thyroxine content) in three well characterized avian model species from several different feeding guilds (granivorous, omnivorous, carnivorous). These data, in conjunction with findings on absorption of air cell administered penta-BDE into chicken and kestrel egg contents (albumen,

yolk, embryo), will be useful in establishing effect thresholds and may be of value in

ecological risk assessments of these flame retardants.

Methods

Eggs and incubation

All animal procedures were approved by the Institutional Animal Care and

Use Committees of the Patuxent Wildlife Research Center and the University of

Maryland, College Park. Fertile white leghorn chicken eggs were obtained from CBT

Farms (Chestertown, MD, USA) and mallard eggs from Whistling Wings (Hanover,

IL, USA). American kestrel eggs were collected fresh from the colony at the

Patuxent Wildlife Research Center (Laurel, MD, USA). Upon arrival, all eggs were

washed in a 40°C 1% Betadine® solution (Purdue, Wilson, NC, USA), weighed, and

labeled with a number 2 graphite pencil. Eggs were then stored in a cooler at 13°C

for up to 3 d, and were allowed to equilibrate to room temperature before placement

into incubators. Eggs were artificially incubated (Kuhl Incubator Company,

Flemington, NJ, USA) in trays that were adapted to turn horizontally oriented eggs

180 degrees each hour. Incubators were set at the recommended incubation

temperature; 37.6°C for chickens (21 d incubation), and 37.5°C for mallards and

kestrels (27 d incubation). The relative humidity within the incubator was initially set

at about 40% and was adjusted so mean egg weight loss by the end of incubation was

14-16%. Eggs were incubated horizontally rather than vertically to more closely

mimic natural incubation, and presumably increase survival and hatching success of

semi-domesticated (mallard) and wild species (kestrel). Eggs were weighed at 3-4 d

intervals during the course of incubation to determine weight loss. Eggs were

candled at the time of weighing to confirm viability, and any unfertilized or dead eggs

were removed.

Test solutions and administration

Corn oil (Sigma-Aldrich, St. Louis, MO, USA) was used as the vehicle for

this study because many studies have demonstrated that low volumes cause little

mortality in avian embryos in early development (de Wit *et al*. 2005, Heinz *et al*.

2006, McKernan *et al*. 2007). A penta-BDE mixture (DE-71, LGC Promochem,

Teddington, UK) was chosen for this study because it contains congeners commonly

detected in North American bird eggs (Norstrom *et al*., 2002; Rattner *et al*., 2004;

Toschik *et al*., 2005).

Injection solutions were prepared by dissolving neat penta-BDE or PCB 126

(AccuStandard, Inc., New Haven, CT, USA) in acetone prior to mixing with corn oil.

Solutions were stirred for 3 h. Each dosing solution, including the corn oil control,

contained 1% acetone by volume.

Prior to injection, the blunt end of each egg was cleaned with an alcohol swab.

A hole was drilled (Dremel, Racine, WI, USA) into the blunt end of the egg. A

constant volume (0.5 µl/g egg) of vehicle, penta-BDE, or PCB 126 was injected into

the air cell with an Eppendorf repeat pipettor (Heinz *et al*. 2006, McKernan *et al*.

2007). The hole was then sealed with ethylene vinyl acetate adhesive using a hot glue

gun. Injected and uninjected eggs were kept in a vertical position (blunt end up) for

30 min post-injection outside the incubator to allow the oil to spread over the air cell membrane. Eggs were then placed in trays in a horizontal position and returned to the incubator.

Experiment 1: Biological effects of penta-BDEs in chicken, mallard, and kestrel embryos and hatchlings

Penta-BDE doses for chickens and mallards (0.01, 0.1, 1, 10 and 20 µg/g egg) were arranged around environmentally relevant concentrations detected in herring gull and osprey eggs. Due to the limited availability of eggs from the kestrel colony, doses for kestrels ranged from 0.1-20 µg/g egg. Nominal concentrations of penta-BDE dosing solutions were verified analytically by gas chromatography/mass spectrometry (GC/MS) (Varian 3400 and Varian 4D ion trap; Sugar Land, TX, USA). Corn oil test solutions were diluted in hexane. Congener peak areas were compared to that of an internal standard (p-terphenyl). Congener response factors were determined using authentic PBDE standards (AccuStandard, Inc.). Detection was in the electron ionization mode. The GC was equipped with a DB-5 column (60m length X 0.32 mm inner diameter and a 0.25 µm film thickness; JandW Scientific, Folsom, CA, USA) and the carrier gas was He. Injections were made in the spitless mode. Identification was achieved by MS in the full-scan, electron-ionization mode. Quantification was performed by comparison of the sum of the areas of the three major ions of each PBDE congener (BDE-17, -28, -47, -49, -66, -85, -99, -100, -153, -154) in DE-71 versus that of the internal standard. For the 1 µg/g egg dosage, penta-BDE was analytically verified to be 134-152% of the nominal

concentration and for the 10 μg/g egg dosage, penta-BDE was analytically verified to be 96-104% of the nominal concentration.

The well-studied and highly toxic 3,3',4,4',5-pentachlorobiphenyl congener (PCB 126) was used as a positive control. The PCB 126 dose chosen in the present study (2000 pg/g egg) may seem high compared to other studies, however, the toxicity of air cell administered PCB 126 on d 4 of incubation has been shown to be much lower in horizontally incubated eggs (McKernan *et al.* 2007) compared to vertically incubated eggs (complete failure to hatch 500 pg/g egg; Hoffman *et al.* 1998, Lavoie and Grasman 2007). Concentrations of PCB 126 in the dosing solution were verified as described above. Analytically verified concentrations of PCB126 were 45-51% of the nominal concentration.

Chicken eggs were incubated for 4 d, and mallard and kestrel eggs were incubated for 5 d (i.e., the developmental equivalent of a 4-d-old chicken embryo), at which point they were candled to confirm fertility. Any infertile, non-viable, or cracked eggs were discarded. Eggs were then randomly assigned to uninjected control, vehicle control, PCB 126, or penta-BDE groups (chicken $n = 22$-42 eggs/treatment; mallard $n = 26$-27 eggs/treatment; kestrel $n = 18$-20 eggs/treatment), and vehicle or test compounds were administered on this day.

Monitoring survival and sample collection

Embryo viability during incubation was monitored at 3-4 d intervals by candling or with a viability-detecting instrument (Buddy, Vetronic, Torquay, UK). Embryos that died during development or failed to pip were removed from the

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eggshell and evaluated for stage of development and presence of abnormalities. Survival through 90% of the incubation period, incidence of pipping and hatching success were determined.

Day-old hatchlings were examined for evidence of edema and teratogenicity (e.g., eye, foot, bill deformities). Each hatchling was weighed and then sacrificed via decapitation. Immediately, the liver (minus the gall bladder), and the yolk sac were removed and weighed. A small piece of the liver was fixed in formalin for histopathogical examination, and the remaining tissue was placed in a cryovial, snap frozen, and stored at -80˚C for assay of hepatic microsomal P450-asssociated monooxygenase. Sex was determined by examination of the gonads. The weight of paired thyroid glands in chickens and mallards, and the left thyroid in kestrels was measured and then they were frozen for subsequent hormone analysis. The weight of the bursa of Fabricius, spleen, and thymus were also determined.

Skeletal preparations and histopathology

After sacrifice and sample collection, the remaining carcass was labeled and stored in 70% ethanol. Carcasses were cleared, feathers were removed, and skeletons were stained by the method of Karnofsky (1965). Crown-rump, humerus, radius-ulna, femur, tibiotarsus, and metatarsus lengths were measured to the nearest mm.

Formalin-fixed livers, bursa of Fabricius and other tissues were embedded in paraffin, sectioned at 5μm, and stained with hematoxlyin and eosin (American HistoLabs, Gaithersburg, MD, USA). Liver and bursa (2 step sections) were examined by light microscopy for 10 or more individuals for control and dosage

groups (except groups with poor hatching success). Hepatocyte density (number of hepatocytes/10μm length at three locations), incidence of enlarged hepatocytes (narrowing of sinusoids), vacuolation, and other lesions were noted.

Because of qualitative changes in the bursa of chicken hatchlings (but not mallards or kestrels), morphometric measurements (two sections/hatchling) were conducted on the number of follicles and their size. The number of follicles per section was determined by averaging those observed at two locations for each bursa. Images of follicles were digitized using IPLab for Windows (Scanalytics, Inc., Fairfax, VA, USA), and average size was determined by measuring 10 follicles per bursa.

Cytochrome P450

Liver samples were thawed and homogenized (1:4 wt/v) in ice-cold 1.15% KCl in 0.01 M Na/K phosphate buffer at pH 7.4. The homogenate was centrifuged at 9,000 g for 20 min at 4˚C; the supernatant was then centrifuged at 100,000 g for 1 h. The microsomal pellet was resuspended in 0.05 M Na/K phosphate buffer at pH 7.6 containing 0.001 M disodium ethylenediamine tetraacetate at 3-5 mg protein/ml. Ethoxyreorufin-*O*-dealkylase (EROD) was assayed in triplicate on a fluorescence 96 microwell plate scanner (Fluroroscan II; ICN Flow Laboratories, McLean, VA, USA) (Melancon 1996). The assay utilized 1.25 μM ethoxyresorufin (Sigma-Aldrich Chemical) substrate, 0.125 mM NADPH (Sigma-Aldrich Chemical), and microsomal protein, and was brought to a constant volume with 66 mM Tris buffer. Assays were run in a total volume of 260 μl at 37˚C. Microsomal protein (5-30 μg per well) was

optimized for each species to obtain linear reaction rates. Reference mallard

microsomes were included with each plate. Change in fluorescence units over time

were converted to the rate of product formation with the use of a 7 point standard

curve (0.01-0.4µM). Protein was determined using the BCA Protein Assay kit (Pierce

Chemical Company, Rockford, IL, USA). Ethoxyresorufin-O-dealkylase activity was

calculated as picomoles of product formed/min/mg microsomal protein. The

coefficient of variation for hatchling samples ($n = 253$) run in triplicate averaged

15%. Each species' assay was run over a period of 8 days, and the average inter-

assay coefficient of variation for mallard reference microsomes ($n = 12$) assays

averaged 9.56%.

Thyroid hormone

Glandular hormone content was measured by the method of McNabb and

Cheng (1985). Briefly, thyroid tissue was digested in capped microcentrifuge tubes

containing 25 mg of Pronase (Sigma-Aldrich Chemical) in a volume of 350 ul of

distilled water at 37°C for 24 h. One ml of ethanol was added to the digested sample

which was then vortexed. This mixture was held at -20°C for 24 h, and then tubes

were centrifuged at 13,500 g for 5 min. The supernatant was removed and stored at -

20°C for thyroxine (T_4) analysis. Thyroxine concentrations in the extract were

determined in duplicate by radioimmunoassay (RIA) using a Coat-A-Count Canine

Total T_4 assay kit (Diagnostic Products Corporation, Los Angeles, CA, USA). After

sample (25 ul) incubation, the bound and free fractions were separated, and bound

radioactivity remaining in each tube was counted for 1 min using a Wallac 1470

Wizard gamma counter (PerkinElmer, Waltham, MA, USA). Average counts for

duplicate tubes were log transformed and then total T_4 was estimated from a 6 point

(0-15 ng/ml) standard curve. Thyroxine assays were validated for each species prior

to running samples by testing various dilutions of extract samples against the standard

curve for parallelism and by spiking sample extracts with known concentration of T_4

standard (3 µg/dL) prepared in ethanol. Mean T_4 spike recoveries from chicken,

mallard, and kestrel extracts ranged from 81-99%. Assay precision (coefficient of

variation for duplicate determinations) was 9.93% ($n = 284$). Values are expressed as

ng total T_4 /mg thyroid tissue.

Experiment 2: Absorption of air cell administered penta-BDEs into the egg and embryo

As part of a larger PBDE uptake and metabolism study in eggs of several avian species, corn oil vehicle or penta-BDE (analytically determined to be 11.1 µg/g egg) was administered into the air cell of chicken and kestrel eggs at a volume of 0.5 µl/g egg on d 4 and 5 of incubation, respectively. Eggs were randomly sampled 24 h post-injection, midway through incubation, and at pipping. Eggs were removed from the incubator, weighed, and the apex end of the egg was gently cut away so that the contents could be poured into a chemically clean jar. Care was taken not to allow the inner shell membrane to be included with the sample. The embryo was sacrificed and samples were frozen at -20°C until analysis for total PBDE content.

Egg contents were analyzed following the methods of Hale *et al*. (2001) and Rattner *et al*. (2004) at the Virginia Institute of Marine Science in Gloucester Point, VA in the laboratory of Dr. Robert Hale. Briefly, eggs were lyophilized, and subsamples were spiked with surrogate standard PCB 204 (Ultra Scientific, North Kingstown, RI, USA). Blanks were run along with samples to evaluate possible laboratory contamination. Egg samples were subjected to enhanced solvent extraction (Dionex ASE 200, Sunnyvale, CA, USA) with methylene chloride. Large molecular weight compounds were separated from the PBDEs in the extracts on an Envirosep size-exclusion chromatography column (350 mm length x 21.2 mm diameter with 60 x 21.1 mm guard column; Phenomenex, Torrance, CA, USA). The PBDE-containing fraction was then purified on a 2000 mg, silica gel, solid-phase extraction glass column (Enviroprep, Burdick and Jackson, Muskegon, MI, USA).

The PBDEs in the purified extracts were separated by GC/MS as previously described. Data were corrected based on the recovery of surrogate standard PCB 204 in each sample. Mean recovery of surrogate PCB 204 from the eggs was 72.9%. The limit of quantification was 100 pg/g wet weight.

Data analyses

Data were analyzed using SAS (SAS Institute Inc., NC, USA). For each species, survival through 90% of incubation, pipping, and hatching success were compared using contingency analysis with Bonferroni correction. Uninjected and vehicle injected controls were initially compared, and if there were no statistically significant differences ($p > 0.05$) then these groups were combined as a single control.

Continuously distributed variables (Experiment 1: body and organ weight, organ to body weight ratio, bone length, histological measurements, EROD activity, glandular T_4 concentration; Experiment 2: PBDE concentration in eggs) were examined for homogeneity of variance and tested for normality with the Shapiro-Wilk (W) statistic. Uninjected and vehicle injected controls were compared using a Student's t-test, and if there were no differences, these groups were combined as a single control group. Differences among measurement endpoints were determined using one-way analysis of variance (ANOVA) and Tukey's HSD method of multiple comparison.

Results

Experiment 1: Survival, pipping, and hatching success following administration of PCB 126 and penta-BDEs

Embryonic survival through 90% of incubation, and pipping and hatching success of uninjected and vehicle controls were within an acceptable range (chicken: 90-93%, 79-91%, 79-86%; mallard: 86-96%, 68 and 68%, 63-68%; kestrel: 90-95%, 75-95%, 75-85%). These endpoints did not differ between uninjected and vehicle groups ($p = 0.08$ to 1), and were thus combined into a single control group. Administration of PCB 126 (positive control) elicited mortality resulting in reduced survival, pipping and hatching success in chickens and kestrels (Table 4-1). Mallards were less sensitive as 2000 pg PCB 126/g egg did not affect these endpoints. At doses up to 20 µg/g egg, penta-BDE did not affect embryonic survival, pipping or hatching success in chickens or mallards. In kestrels, doses of penta-BDE up to 20 µg/g egg had no effect on survival through 90% of incubation, however, dose-dependent decreases in pipping and hatching success were apparent at 1, 10, and 20 µg/g egg, with significant differences ($p < 0.05$) occurring at 10 and 20 µg/g egg.

Edema, deformities, organ weights, and bone lengths

Head and neck edema were observed in many embryos that failed to hatch (Table 4-1). Subcutaneous edema in the rump area was observed in one control

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kestrel and one mallard that received 1 µg penta-BDE/g egg. In chickens, edema was

frequently observed in PCB 126-treated eggs that failed to hatch, but this was not the

case with penta-BDE-treated embryos. The incidence of edema in embryos that

failed to hatch was greater in mallards and kestrels. One unhatched control mallard

embryo exhibited exencephaly, and three hatchlings had splayed legs (a chicken that

received 20 µg penta-BDE/g egg, a control mallard, and a kestrel that received 0.1 µg

penta-BDE/g egg). Many of the kestrel embryos that died appeared developmentally

stunted and physically small relative to their incubation age, but this tendency was not

observed in chicken or mallard embryos.

Crown-rump length, body weight (without yolk sac), and liver to body weight

ratio did not differ between uninjected and vehicle-injected groups for chickens,

mallards and kestrels ($p > 0.15$). However, liver to body weight ratio did differ

between the mallard uninjected and vehicle control groups ($p < 0.001$). With minor

exception, there were no differences in crown-rump length, body weight, liver to

body weight ratio, thyroid to body weight ratio, and bone lengths in hatchling

chickens, mallards, or kestrels that had been treated with PCB 126 or penta-BDE.

Compared to controls, femur length appeared to be shorter in PCB 126 treated birds,

but this was only significant ($p = 0.0086$, Student's t-test) for the right femur in

kestrels (mean ± SE, 9.49 ± 0.11 mm vs. 8.74 ± 0.31 mm).

Bursa to body weight ratio of the six surviving chicken hatchlings treated with

PCB 126 in ovo were markedly smaller (52%; $p = 0.002$) compared to controls, but

weights of other immune organs were unaffected. There were no differences in the

organ to body weight ratios of spleen, bursa, or right thymus in penta-BDE exposed chickens, mallards, or kestrel hatchlings.

Histopathology

Hepatic lipidosis, associated with yolk assimilation, was observed in all species. No lesions were seen in liver sections of chicken hatchlings that had been treated in ovo with PCB 126 or penta-BDE. Because of some qualitative observations suggesting changes in hepatocyte cellularity in chickens (but not mallards or kestrels), the number of hepatocytes/10μm length was determined, but did not differ among treatments ($p > 0.3$). Liver samples from a small number of kestrel and mallard hatchlings exhibited focal necrosis in control, PCB and penta-BDE treatment groups. However the incidence of these lesions in treated groups were not different from controls ($p > 0.3$).

Bursal follicle size was smaller ($p < 0.02$) in PCB 126 exposed chickens compared to controls. Bursal follicle size of chicken hatchlings in all penta-BDE treatment groups, including the lowest doses (0.01 and 0.1 μg/g) seemed consistently smaller than controls (Figure 4-1). Based on this observation and preliminary statistical findings, additional chicken bursa samples at all dose levels were processed. Bursal follicle size of chicken hatchlings was found to be consistently smaller in all penta-BDE treatment groups compared to controls (24-42%; $p = 0.057$ to 0.001). Mallard and kestrel bursa and follicle size did not differ among PCB 126, 20 μg/g penta-BDE, and the combined control groups ($p > 0.3$).

Hepatic microsomal ethoxyresorufin-O-dealkylase activity

For mallard and kestrel hatchlings, hepatic EROD activity of uninjected and vehicle-injected controls did not differ within species (kestrel: $p = 0.6008$; mallard: $p = 0.6409$). However, EROD activity in chickens was significantly different between uninjected and vehicle-injected controls ($p = 0.0379$), and therefore only the vehicle-injected group was used for comparisons with PCB and penta-BDE treatment groups. In chicken hatchlings, log transformed EROD activity was induced over 35-fold in PCB 126-exposed embryos, 5 times greater at 1 µg/g penta-BDE, 21 times greater at 10 µg/g penta-BDE, and 22 times greater at 20 µg/g penta-BDE ($p < 0.0001$) (Figure 4-2). Activity of EROD was neither induced by PCB 126 or penta-BDEs in kestrels and mallards.

Glandular thyroxine content

Because of heterogeneity of variance, thyroid T_4 content data were log transformed prior to comparisons among treatments. Thyroxine content did not differ among treatments in hatchling chickens ($p > 0.2$) or mallards ($p > 0.7$). Glandular T_4 content was lower ($p < 0.001$) in kestrel hatchlings exposed to PCB 126, but unaffected by penta-BDE (Table 4-2).

Experiment 2: Penta-BDE absorption into chicken and kestrel eggs

The administered dose of penta-BDE (analytically verified to be 11.1 µg/g egg) was gradually absorbed through the air cell membrane over the exposure period

in chickens (Table 4-3). This was evidenced by an increase in total PBDE concentration in contents of eggs ($p < 0.048$) over the course of incubation. Of the dose administered in chicken eggs, 0.64% was absorbed after 24 h, 7.71% was absorbed after 6 d, and 29.6 % had been absorbed by the time of pipping (i.e., 17 d after administration). On a lipid weight basis, PBDE absorbed into chicken eggs was 0.855 ± 0.459, 10.0 ± 0.78, and 47.0 ± 6.12 µg/g lipid on d 5, 10 and 21 of incubation, respectively.

In kestrels, there was a significant increase in PBDE uptake between injection and midway through incubation ($p < 0.008$) (1.64% was absorbed after 24 h, 18.4% after 8 d). Uptake rate was seemingly not sustained through the last half of incubation (18.6% at pipping, d 26 of incubation), although this is based on a small sample size ($n = 4$) and uptake rates were variable (13.3 to 24.8%). On a lipid weight basis, PBDE absorbed into kestrel eggs was 3.10 ± 1.64, 37.2 ± 6.91 and 54.4 ± 10.6 µg/g lipid on d 6, 13 and 26 of incubation, respectively.

Discussion

In chickens, in ovo administration of penta-BDE reduced the number and size of bursal follicles at administered doses as low as 0.01 µg/g egg, and induced hepatic EROD activity starting at doses of 1 µg/g egg. No effects of penta-BDE were observed on embryonic survival, or pipping and hatching success in chickens. However, pipping and hatching success in kestrels appeared to be lower at an administered dose of 1 µg/g egg, and adverse reproductive effects were statistically

significant at 10 and 20 µg/g egg. The reproductive effects of penta-BDE observed in kestrels, but not in chickens, are in contrast to the extreme sensitivity of the chicken embryo to coplanar PCBs (present study, and Brunström 1988, Brunström and Halldin 1998, Hoffman *et al*. 1998). Although PBDEs and PCBs have some structural similarities, their toxicity (histology of the bursa, cytochrome P450 induction, lethality) appears to be markedly different in chickens and kestrels.

Survival, pipping, and hatching success

Administration of PCB 126 (positive control) impaired embryonic survival, pipping and hatching success in chickens and kestrels. Clearly, PCB 126 was most toxic in chickens, where only 20% of injected eggs hatched. Mallard embryos appeared far more tolerant to PCB 126 than chickens as previously noted for this congener and other coplanar PCBs (Brunström and Reutergardh 1986, Brunström 1988, Jin *et al*. 2001). Decreased embryonic survival and hatching success has been reported in chickens receiving air cell administered PCB 126 at doses as low as 250 pg/g egg (Hoffman *et al*. 1998, Lavoie and Grasman 2007), and the estimated median lethal dose of this congener may be as high as 3100 pg/g egg when injected on d 7 of incubation (Brunström and Halldin 1998). The dose used in the present study (2000 pg/g egg) is high compared to previously reported toxicity thresholds for chickens (Hoffman *et al*. 1998, Fox and Grasman 1999, Lavoie and Grasman 2007). However, air cell administration of PCB 126 (McKernan *et al*. 2007), and presumably other compounds (e.g., methylmercury, Heinz *et al*. 2006), is considerably less embryotoxic in eggs incubated horizontally compared to those incubated vertically

(Fox and Grasman 1999, Hoffman *et al*. 1998, Lavoie and Grasman 2007). In

vertically incubated eggs, the embryo is situated directly under the air cell, and due to

its proximity to the injection site, the embryo may be more directly exposed to

administered PCB 126 (McKernan *et al*. 2007).

Of the three species tested, pipping and hatching success were only affected in

kestrels, with decreases in these endpoints seemingly starting at a dose of 1 µg/g egg,

and becoming definitive at 10 and 20 µg/g egg. The metabolism of lipid soluble

xenobiotic compounds depends partly upon the action Phase I enzymes and the

activity of some of these (e.g., cytochrome P450-associated monooxygenases) are

lower in fish-eating birds and raptors than in other groups of birds and mammals

(Walker 1980). The reduced ability of kestrels to metabolize persistent organic

pollutants such as PBDEs may contribute to sustained exposure and thus greater

toxicity. In addition, kestrels are semi-altricial, and the structural and metabolic

capabilities of the liver and kidneys are relatively less developed during incubation

and at hatch than precocial species (chicken and mallard).

In a study by Fernie and coworkers (2006), hatching success of an air cell

administered dose of a 1.5 µg PBDE formulation/g to kestrel eggs on day 19 of

incubation did not differ from that of vehicle injected controls. However, their

treatment regime only permitted an 8 day exposure period before pipping, unlike our

22 day exposure period. In addition, hatching success of their control group was

quite low compared to the present study (i.e., 53.6% versus 80.0%). The 1.5 µg

PBDE/g dose approaches environmentally relevant wet weight concentrations in eggs

of fish eating birds, including herring gulls (1.4 µg/g) (Norstrom *et al*. 2002) and

ospreys (0.928 µg/g) (Rattner *et al*. 2004, Toschik *et al*. 2005). Wet weight

concentrations of PBDEs in eggs of peregrine falcons, a terrestrial predatory species,

have been reported to average 0.23 µg/g in northern Sweden (Lindberg *et al*. 2004).

Recently, analysis of 114 unhatched peregrine falcon eggs from the northeast United

States revealed an average of 0.59 µg PBDE/g wet weight, with 8.8% of the samples

exceeding 1 µg/g and values ranging up to 6.6 µg/g (D. Chen 2008, personal

communication). These values in eggs from the once endangered peregrine falcon

are within the range of the lowest observable effect levels (LOEL) for pipping and

hatching success in kestrels. These concentrations are of concern as PBDE values in

bird eggs have increased for 20 years (Norstrom *et al*. 2002), although values appear

to have leveled off possibly due to a decrease in production of penta-BDE and octa-

BDE formulations (Gauthier *et al*. 2008).

Incidence of edema and deformities, hatchling organ weights, bone lengths

As previously noted (Brunström and Andersson 1988, Hoffman *et al* 1998,

Fox and Grasman 1999, Lavoie and Grasman 2007), air cell administration of PCB

126 resulted in stunted growth and edema in many of the chicken and kestrel embryos

that failed to hatch. However, greater concentrations of PCB 126 were required to

evoke these responses, presumably due to the horizontal position of incubating eggs

(McKernan *et al*. 2007). In the present study, neither body weight nor liver to body

weight ratio were affected by PCB 126 in day-old hatchlings. This is similar to

observations in air cell administered PCBs in chickens, but in contrast to findings in

kestrels (decreased body and liver weight) (Hoffman *et al*. 1998). Some evidence of

reduced growth (i.e., shorter femur length) was observed in kestrel hatchlings following administration of PCB 126, which is similar to findings by Hoffman and coworkers (1998). Notably, yolk sac administration of higher doses of PCB 126 (3200 pg/g egg) have been reported to result in lower body weight of hatchlings, and to increase the ratios of brain, heart, and liver to body weight (but not absolute weights of these organs) (Powell *et al*. 1996).

Stunted growth and edema were observed in most penta-BDE treated kestrel embryos that failed to hatch. Most of the mallard embryos that failed to hatch, including *both* controls and penta-BDE groups, exhibited edema (but not stunted growth). This is attributed to technical difficulties in artificial incubation of mallard eggs, perhaps associated with retention of the waxy eggshell cuticle and apparent retention of fluid (i.e., failure to attain 14-16% moisture loss throughout incubation). Body weight, crown-rump length, liver to body weight ratio, and length of bones were not affected in any of the species in the present study. In the study by Fernie and coworkers (2006) growth and tarsometatarsus bone length were marginally greater in PBDE-treated nestling and fledgling kestrels. Larger liver masses have been reported in mice, rats, and mink exposed to PBDEs (IPCS 1994, Martin *et al*. 2007). However, these effects occurred in animals that were exposed repeatedly to much higher doses of PBDEs.

Liver EROD activity and histopathology

Toxic effects of dioxins and dioxin-like compounds are principally mediated through binding to a cytosolic aryl hydrocarbon receptor (AhR), and this ligand

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activated factor increases or decreases transcription of mRNAs and translation of proteins. Induction of cytochrome P450 1A (CYP1A), which is mediated by AhR, is a well- characterized response to dioxin-like compounds. The induction of CYP1A-associated monooxygenases, specifically EROD activity, has been extensively used as an exposure biomarker of dioxin-like compounds (e.g., coplanar PCB congeners) in birds (Brunström 1988, Bosveld *et al*. 1993, Rattner *et al*. 1994, Hoffman 1998). In agreement with other studies, our findings (Figure 4-2) illustrate that EROD activity is induced by PCB 126 in chickens. In contrast, doses at least 50 times greater are required to induce EROD in mallards and kestrels (Brunström and Halldin 1998, Hoffman *et al*. 1998). This greater sensitivity in the chicken is apparently due to the presence of two amino acids (isoleucine-325 and serine-381) in the ligand binding domain of the AhR in chickens for which there are substitutions in some avian species (less sensitive species, alanine-381; insensitive species, valine-325 and alanine-381) (Karchner *et al*. 2006, Head *et al*. 2007).

Polybrominated diphenyl ethers are structurally similar to polyhalogenated aromatic hydrocarbons that bind to AhR and induce EROD activity, although PBDE mixtures and individual congeners are less potent (10^{-2} to 10^{-5}) than dioxin (Chen *et al*. 2001). There have been conflicting reports about the ability of PBDEs to induce EROD activity in mammals and mammalian hepatocyte cultures (Chen *et al*. 2001, Chen and Bunce 2003, Peters *et al*. 2004, 2006, Martin *et al*. 2007). Induction in some studies may be due to dioxin and dibenzofuran impurities in the technical mixture. Martin *et al*. (2007) analyzed DE-71, the technical mixture used in the present study, and did not detect dioxins or dibenzofurans (detection limit < 30 pg/g).

The failure to detect dioxins or dibenzofurans would suggest that the observed EROD induction in chickens in the present study may not have been caused by impurities. Despite reproductive effects in kestrels, EROD activity was not induced. Based on this observation, it would seem that the toxic effects of PBDEs in kestrels may not be principally mediated through the AhR.

Despite the induction of EROD activity in chicken hatchlings following the administration of PCB 126, there was no evidence of liver lesions in survivors. The absence of liver pathology may be due to a "survivor effect"; that is, by the time of hatching, any embryos that might have had hepatic lesions had died, while the least affected chicken embryos hatched. Notably, Rifkind *et al.* (1984) described narrowing of hepatic sinusoids in d 18 chicken embryos 24 h after administration of approximately 22500 pg/g egg of PCB congener 77. This congener is one-half the toxic potency of PCB 126 in birds (van den Berg *et al.* 1998). Thus, the dose used in the present study (2000 pg PCB 126/g egg) was less than one-fifth the LOEL observed in the Rifkind study (approximately equivalent to 11,250 pg PCB 126/g egg). Administration of PCB 126 in kestrel eggs evoked mortality, but no histopathological lesions were observed. Hoffman *et al.* (1996) reported multifocal necrosis in liver of kestrel hatchlings exposed repeatedly to PCB 126 from d 1 to 10 post-hatch, receiving a total administered dose of approximately 87,000 ng. In the present study, kestrel eggs were injected once and received a total of about 30 ng. Again, the effect level observed by Hoffman *et al.* (1996) resulted from administration of a much greater dose. Fox *et al.* (2007) observed periportal hepatitis in livers of adult herring gulls from the Great Lakes. Concentrations of PCBs in liver

tissue averaged 13,400 ng/g. Clearly, the single early exposure regime in the present study was well below the threshold eliciting microscopic effects in liver tissue of surviving embryos that hatched.

Thyroid gland and hormones

Alterations to the thyroid system can affect metabolism, growth, and thermoregulation. In the current study, exposure to PCB126 resulted in lower glandular T_4 content in kestrel hatchlings, but not in the other species tested. As previously discussed, American kestrels are semi-altricial, and are less developed at hatch than precocial species. Based upon the number and size of follicles, colloid staining characteristics, and T_4 content, the thyroid is less developed in altricial embryos and hatchlings compared to precocial species (McNabb and McNabb 1977, McNabb and King 1992, McNabb 2006). The difference in development in kestrels may render the thyroid gland more sensitive to in ovo exposure to thyroid-disrupting chemicals such as PCBs. Notably, pipping herring gull embryos and chicks (semi-precocial species) from PCB contaminated sites in the Great Lakes have been reported to have lower glandular T_4 content than gulls from reference sites (McNabb and Fox 2003).

Compared to circulating concentrations of T_4 and triiodothyronine, and thyroid weight, glandular T_4 content has been reported to be a more sensitive indicator of decreased thyroid function in studies of perchlorate and PCB toxicity in birds (McNabb and Fox 2003; McNabb *et al*. 2004a; 2004b). Toxic effects of PBDEs on thyroid function are incompletely known. In laboratory studies in mice and rats,

PBDE exposure decreases plasma T_4 concentrations (Fowles *et al*. 1994; Hallgren *et al* 2001; Zhou *et al*. 2001), may alter plasma transport of thyroxine through competitive binding mechanisms (Hallgren and Darnerud 2002), and induce enzymatic degradation of thyroid hormones through induction of hepatic T_4-glucuronidation activity (Zhou *et al*, 2001, Richardson *et al*. 2008). Fernie *et al*. (2005b) suggested that PBDE exposure results in slightly lower plasma T4 concentrations in kestrels, although no alterations in thyroid histology were observed. In the present study, the absence of effects on thyroid weight and glandular T_4 content in chicken, mallard, and kestrel embryo suggests that thyroid function may not be altered by in ovo exposure to PBDEs at the doses tested.

Immune organs and histology

In birds, the thymus, bursa of Fabricius and spleen are all recognized as integral parts of the immune system. The bursa is a primary lymphoid organ which is unique to birds, and is necessary for normal development of the humoral immune system. In the present study, bursal weights were significantly lower in PCB 126 treated chicken hatchlings. This and other AhR active congeners have been previously reported to induce atrophy of the bursa in chickens exposed in ovo (Fox and Grasman 1999; Goff *et al*. 2005; Lavoie and Grasman 2007). Bursal somatic index decreased with increasing concentrations of PBDE congener 47 in kestrel hatchlings exposed in ovo and post-hatching (Fernie *et al*. 2005a). Nonetheless, this change was not seen in penta-BDE treated chickens, mallards and kestrels exposed in ovo. However, the number of follicles per bursa and follicle size were consistently

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lower in chickens treated with PCB126 and all doses of penta-BDE. Fernie *et al.*

(2005a) reported reduced antibody-mediated response in kestrels exposed to PBDEs

in ovo and post-hatching. Thus, results from both of these studies indicate that the

bursa may be sensitive to embryonic PBDE exposure. It also has been demonstrated

that immune organ cellularity may be a more sensitive indicator of PCB induced

atrophy than organ mass (Goff *et al.* 2005, Lavoie and Grasman 2007). Lavoie and

coworkers (2007) suggest that the immune system may recover from in ovo exposure

to PCBs, and this could be the case for PBDEs. Therefore, more studies on the

immune system with multiple endpoints in birds chronically exposed to PCBs and

PBDEs seem warranted.

Penta-BDE absorption into the egg and implications on toxicity thresholds

Artificial treatment of eggs by air cell or yolk sac injection is believed to

approximate the toxicity of maternally deposited compounds. Embryotoxic responses

(e.g., cytochrome P450, edema, deformities, mortality) evoked by egg injection of

PCBs compare favorably to those observed following natural exposure (Hoffman *et*

al. 1996). However, at equal concentrations, air cell injected methylmercury seems to

be more toxic than naturally incorporated methylmercury (Heinz *et al.* 2008). Air

cell injected compounds must cross the air cell membrane, albumen, blood vessels,

and yolk to reach the embryo. It is unknown if air cell administered or yolk injected

compounds mimic the distribution of naturally deposited contaminants in eggs, but

these techniques are frequently employed to circumvent the difficulties and expense

of studying such effects in feeding trials. With these caveats in mind, the actual

concentrations absorbed into egg contents were determined, and can be tentatively related to embryotoxic effects.

De Roode and van den Brink (2002) injected PCBs into the yolk of chicken eggs prior to incubation and measured an exponential increase in uptake by the embryo, with 18% of the administered dose absorbed into the embryo by d19. Maervoet and coworkers (2005) noted a similar exponential uptake after yolk injection of PCB congeners 77, 153 and 180 into the embryo during the last week of incubation. The penta-BDEs injected into the air cell of chicken eggs were gradually absorbed over the 17 d exposure period, with an apparent increase in uptake rate into egg contents between d 10 and pipping, perhaps suggestive of an exponential absorption relationship. This increase in absorption rate could be due to increased size and density of vitelline blood vessels and the vast growth of the blood vessels of the chorioallantoic network under the inner shell membrane. Compared to chickens, penta-BDE appeared to be absorbed at a greater rate through mid-incubation in kestrels (i.e., 18.4% vs 7.71% of administered dose). Thereafter, PBDE absorption in kestrels appeared to level off, although sample size was small.

Based on the concentrations of total PBDEs absorbed into chicken and kestrel eggs (Table 4-3), effects in the present study are occurring at substantially lower concentrations than those in dosing solutions administered into the air cell. We observed up to 29.6% of the analytically verified dose of 11.1 µg penta-BDE/g egg in the chicken egg by pipping. Sublethal effects were noted in chicken hatchlings in the present study, but, from an ecological perspective, the most important effects were on pipping and hatching in kestrels, a toxicological model species often used in risk

assessments for raptorial birds. During the first half of incubation, over twice as much PBDE was absorbed in kestrel eggs compared to chicken eggs. This could have influenced mortality rate, however the majority of mortality occurred at the end of incubation. By the end of incubation, findings in Experiment 2 indicate that 18% of the administered dose was absorbed in kestrels. Adverse reproductive effects were observed in kestrels receiving 10 μg/g egg of this technical mixture into the air cell, and at an 18% absorption rate, the LOEL associated with impaired pipping and hatching success could be as low as 1.8 μg/g egg wet weight. This exposure is only about two times greater than the concentrations of total PBDEs reported in osprey eggs (Rattner *et al*. 2004, Toschik *et al*. 2005), and well within the range of total PBDE concentrations detected peregrine falcon eggs from the northeast United States (D. Chen Virginia Institute of Marine Science, Gloucester Point, VA, personal communication). On a lipid weight basis, the LOEL for impaired pipping and hatching success would be approximately 32 ug PBDE/g egg lipid weight.

Conclusion

This study demonstrates that air cell administration of environmentally realistic concentrations of penta-BDE induced EROD activity and reduced bursal follicle size and number in chicken hatchlings, and survival endpoints were affected in kestrel embryos. As previously demonstrated, PCB 126 reduced survival endpoints in both chicken and kestrel embryos. Mallards were less sensitive to both PCB 126 and penta-BDEs than chickens or kestrels. The observed effects are cause for concern in free-ranging avian predators and other wildlife exposed to PBDEs.

Although the concentrations of penta-BDE congeners commonly detected in the environment seem to have plateaued in herring gull eggs in the Great Lakes, levels of higher brominated congeners are still increasing (Gauthier *et al*. 2008). Further effect studies with higher brominated congeners in top predators are suggested.

Tables and Figures

Table 4-1. Effects of polychlorinated biphenyl congener 126 (PCB 126) and penta-brominated diphenyl ether (penta-BDE; DE-71) on chicken, mallard, and American kestrel embryos through hatching[a]

		Dose					
	PCB 126 (pg/g egg)	penta-BDE (µg/g egg)					
	Combined control	2000	0.01	0.1	1	10	20
Chicken							
Survival to day 18	65/71 (91.5%)	17/30 (56.7%)*	25/30 (83.3%)	32/38 (84.2%)	19/22 (86.4%)	24/30 (80.0%)	26/30 (86.7%)
Pipped	61/71 (85.9%)	8/30 (26.7%)*	23/30 (76.7%)	28/38 (73.7%)	16/22 (72.7%)	23/30 (76.7%)	22/30 (73.3%)
Hatched	59/71 (83.1%)	6/30 (20.0%)*	22/30 (73.3%)	28/38 (73.7%)	16/22 (72.7%)	20/30 (66.7%)	22/30 (73.3%)
Edema/Failed to hatch	2/12	9/24	2/8	1/10	1/6	1/10	3/8
Mallard							
Survival to day 24	49/54 (90.7%)	24/28 (85.7%)	22/27 (81.5%)	27/27 (100%)	23/27 (85.2%)	25/27 (92.6%)	21/27 (77.8%)
Pipped	36/54 (66.7%)	17/28 (60.7%)	13/27 (48.1%)	15/27 (55.6%)	20/27 (74.1%)	15/27 (55.6%)	13/27 (48.1%)
Hatched	35/54 (64.8%)	17/28 (60.7%)	12/27 (44.4%)	15/27 (55.6%)	19/27 (70.4%)	14/27 (51.9%)	13/27 (48.1%)
Edema/Failed to hatch	9/19	6/9	10/15	9/12	3/8	12/13	8/14
Kestrel							
Survival to day 24	37/40 (92.5%)	13/20 (65.0%)*		18/18 (100%)	18/20 (90.0%)	16/20 (80.0%)	16/20 (80.0%)
Pipped	34/40 (85.0%)	9/20 (45.0%)*		16/18 (88.9%)	13/20 (65.0%)	11/20 (55.0%)*	11/20 (55.0%)*
Hatched	32/40 (80.0%)	9/20 (45.0%)*		14/18 (77.8%)	12/20 (60.0%)	9/20 (45.0%)*	9/20 (45.0%)*
Edema/Failed to hatch	2/8	1/11		4/4	5/8	7/11	6/11

[a] Values are response/n (percent). Asterick (*) indicates significant difference ($p < 0.05$) from combined control for a given species

Table 4-2. Glandular Thyroxine content (T4) in chickens, mallards, and American kestrels exposed to polychlorinated biphenyl congener 126 (PCB 126) and penta-brominated diphenyl ether (penta-BDE; DE-71) in ovo[a]

		PCB 126 (µg/g egg)	PBDE (µg/g egg)				
	Combined control	0.002	0.01	0.1	1	10	20
Chicken							
n	30	6	13	14	12	14	12
Paired Thyroid Wt (mg)	6.39 ± 0.22	7.83 ± 0.79	5.82 ± 0.37	5.88 ± 0.38	6.00 ± 0.32	5.81 ± 0.34	6.66 ± 0.26
T4 (ng/mg thyroid)	440 ± 29.7	484 ± 52.8	645 ± 64.2	459 ± 69.5	491 ± 84.4	448 ± 46.4	538 ± 75.8
T4 (ng/paired thyroids)	2758 ± 171	3619 ± 212	3493 ± 186	2551 ± 317	2649 ± 374	2540 ± 250	2515 ± 391
Mallard							
n	23	12	11	11	12	12	11
Paired Thyroid Wt (mg)	6.45 ± 0.29	6.39 ± 0.27	7.77 ± 0.42	7.58 ± 0.40	7.16 ± 0.34	6.51 ± 0.33	6.99 ± 0.55
T4 (ng/mg thyroid)	317 ± 28.0	313 ± 68.0	212 ± 19.9	283 ± 29.4	232 ± 25.3	284 ± 24.8	298 ± 28.7
T4 (ng/paired thyroids)	2023 ± 200	1933 ± 359	1701 ± 240	2133 ± 233	1628 ± 225	1898 ± 240	2060 ± 159
Kestrel							
n	30	9		13	9	8	9
Paired Thyroid Wt (mg)	1.19 ± 0.09	1.39 ± 0.21	-	1.30 ± 0.12	1.04 ± 0.14	1.32 ± 0.15	1.28 ± 0.19
T4 (ng/mg thyroid)	464 ± 64.4	144 ± 48.2*	-	333 ± 70.4	378 ± 54.8	371 ± 64.9	405 ± 80.3
T4 (ng/left thyroid)	438 ± 29.3	159 ± 23.8*	-	350 ± 35.3	351 ± 48.9	443 ± 53.8	403 ± 29.1

[a] Untransformed values are mean ± SE and n. Asterick (*) indicates significant difference ($p < 0.001$) difference from corresponding control

Table 4-3. Uptake of air cell administered penta-brominated diphenyl ether (penta-BDE; DE-71) during incubation in chicken eggs[a]

| | Sampling day | | |
	24 hr post-injection	Mid-incubation	Pipped
Chicken			
Vehicle injected (μg total PBDE/g egg)	ND	ND	0.00042 ± 0.0002
Penta-BDE injected (μg total PBDE/g egg ww)b	$0.084 \pm 0.0459A$	$1.03 \pm 0.111B$	$4.93 \pm 0.994C$
Uptake of analytically verified penta-BDE Dose (%)	0.64 ± 0.356	7.71 ± 0.857	29.6 ± 4.56
Kestrel			
Vehicle injected (μg total PBDE/g egg)	0.00043 ± 0.000216	1ND - 0.00232	0.00038 ± 0.000198
Penta-BDE injected (μg total PBDE/g egg ww)b	$0.208 \pm 0.1129A$	$2.43 \pm 0.452B$	$2.80 \pm 0.498B$
Uptake of analytically verified penta-BDE Dose (%)	1.64 ± 0.871	18.4 ± 3.32	18.8 ± 3.04

[a] Penta-BDE was administered at an analytically verified dose of 11.1 μg/g egg. Values presented are mean $\pm$ SE, chickens n = 3/sampling day; kestrels n = 3-4 in penta-BDE treated eggs/ sampling day and n = 2-3 in vehicle treated eggs/sampling day; ND = below detection limit

[b] Groups with different capital letter superscripts are significantly different ($p < 0.05$).
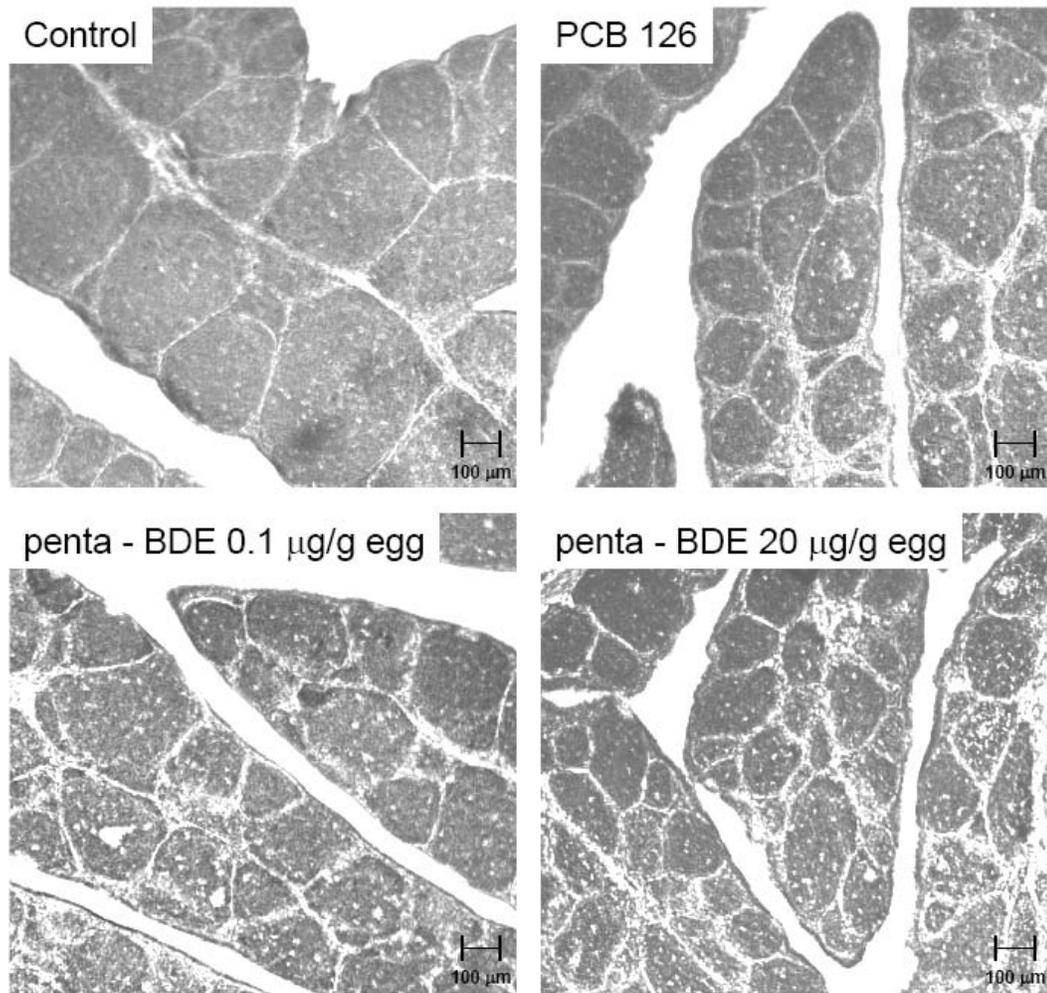
Figure 4-1. Low magnification photomicrographs of bursa of Fabricius demonstrating smaller follicle size of chicken hatchlings that had been treated with polychlorinated biphenyl 126 and penta-brominated diphenyl ether in ovo.
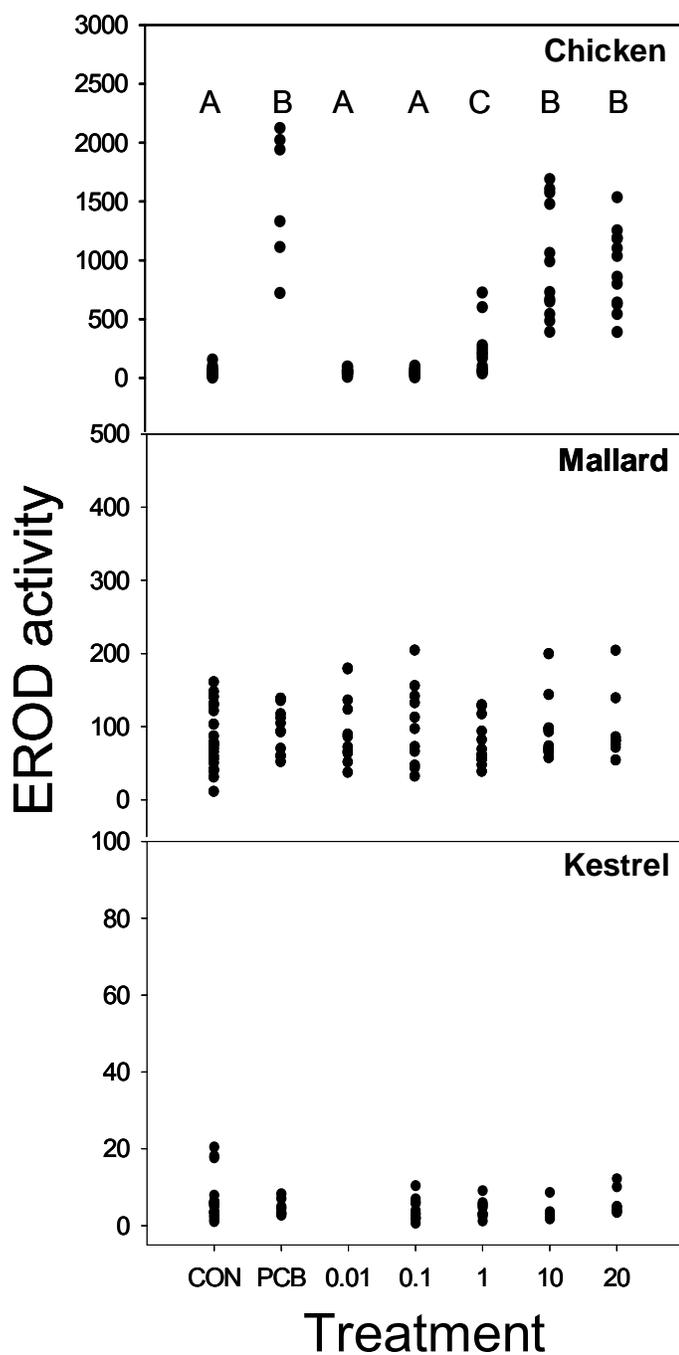
Figure 4-2. Hepatic ethoxyresorufin-*O*-dealkylase activity (picomoles product/min/mg microsomal protein) of chicken, mallard, and American kestrel control (CON) hatchlings or hatchlings that had been treated with 2000 pg polychlorinated biphenyl 126/g egg (PCB) and penta-brominated diphenyl ether (0.01-20 µg/g egg) in ovo. For chickens, groups with different capital letters are significantly different ($p < 0.05$).

Chapter 5: Conclusions

PBDE production, release, and alternatives

Although use of PBDE is intended to protect property and lives, there have been unintended potential consequences to the environment, wildlife, and human health. Monitoring programs have detected PBDEs in human breast milk, fish, birds, mammals, and measureable quantities in the environment worldwide. Lower brominated PBDEs (i.e., the dominant congeners in the penta-BDE formulations) are most frequently detected in wildlife and humans, although higher brominated congeners are currently being detected in biota. The exact pathways by which the PBDEs end up in the abiotic and biotic environments are not fully understood. Possible sources include releases from manufacturing sites or processing of the chemicals into products like plastics or textiles (Figure 5-1). In addition, product aging and wear may also lead to the presence of these compounds in the environment and coincident environmental exposure.

The European Union banned penta-BDE and octa-BDE in all products, effective August 15, 2004. The Great Lakes Chemical Corporation was the sole producer of the penta-BDE formulation in the United States. In November 2003, Great Lakes announced a voluntary phase out of penta- and octa-BDE by the end of 2004. The EPA has since been coordinating with chemical manufacturers and end users to facilitate an orderly switch to safer flame retardant substitutes. The State of California has enacted a law banning use of penta-BDE and octa-BDE by January 2008 and other states (Michigan, Massachusetts, Maine, Hawaii, Washington, and New York) are also considering or have passed similar legislation.

Potential replacements for PBDEs are currently being sought. These products must be evaluated for flame retardant performance and potential human and environmental toxicity. There are several PBDE flame retardant alternatives on the market; however, toxicity data for these products are scarce, and the best available information is generated for many of these substitutes are from quantitative structure activity relationships (QSARs). The penta-BDE formulation replacements include melamine and phosphorus-containing compounds.

Effects of PBDE in avian embryos and hatchlings

Results from these studies demonstrated that air cell administration of environmentally realistic concentrations of penta-BDE induced EROD activity and reduced bursal follicle size and number in chicken hatchlings. Further, survival endpoints were adversely affected in kestrel embryos (Figure 5-2). The reproductive effects of penta-BDE observed in kestrels, but not in chickens are in contrast to the extreme sensitivity of the chicken embryo to coplanar PCBs. Although PBDEs and PCBs have some structural similarities, their toxicity (histology of the bursa, cytochrome P450 induction, lethality) appears to be markedly different in chickens and kestrels (Table 5-1). Further studies on the function of the immune system in avian species, especially hatchlings, are warranted. Mallards were less sensitive to both PCB 126 and penta-BDEs than chickens or kestrels. The observed effects of penta-BDE are cause for concern in free-ranging avian predators and other wildlife exposed to PBDEs. Although the measured concentrations of penta-BDE congeners commonly detected in the environment have plateaued in herring gull eggs in the

Great Lakes, levels of higher brominated congeners are still increasing (Gauthier *et al*. 2008). Furthermore, effect studies with higher brominated congeners in top predators are needed in order to better understand exposure pathways and toxic effects in wildlife.

Biotransformation and bioavailability

The data from these studies demonstrate that PBDEs are metabolized, specifically debrominated and methoxylated in the avian embryo. Penta- and octa-BDE production has been discontinued due to the potential environmental and human health toxicity of lower brominated congeners. However, deca-BDE is still in production and release into the environment may lead to debromination and thus continued exposure of the more toxic congeners to wildlife. Phasing out of penta- and octa-mixtures may not fully protect free ranging wildlife, as fish and birds have been shown to be capable of debrominating deca-BDE.

Methods – Utility of egg injections

A compelling reason for using egg injections is to the ability to compare response to selected compounds across a variety of species, while not having to necessarily maintain a captive colony for every species of interest. Additionally, egg injection studies are designed to mimic maternal exposure of either lipophilic or aqueous compounds into the yolk or albumin, respectively. Researchers can order eggs from game farms and breeders to obtain chicken, duck and quail eggs. Alternatively, if other species are needed, then eggs can be collected from wild birds'

nests from relatively clean sites. Egg collections from colonial nesting species such as herons or gulls generally are the least labor-intensive. Egg injection studies also allow for greater sample size. Feeding and maternal transfer studies are expensive, and number of breeding pairs would be limited. The researcher has better control over exposure and other factors such as incubation with egg injection studies. A consistently applied dose can be given to every egg within a treatment as opposed to maternally transferred doses, which can be different within clutches. A dose can be applied at different stages in embryonic development to help pinpoint effects. Therefore, egg injection studies are an excellent alternative to reproductive feeding studies in order to obtain an assessment of potential impact of a compound, in addition to being less expensive and time consuming, allow for increased sample sizes and greater ability to compare sensitivity between species, and precise control over dose and stage at exposure.

However, there are several disadvantages in using egg injections to evaluate embryotoxicity of contaminants. Hatching success is often lower with injected eggs, and with eggs that are incubated artificially, making a vehicle control critical with every injection study. Also, there is some evidence that maternal transfer may not be as toxic as injected compounds (Heinz *et al*. 2008). Additionally, if hens are allowed to incubate the eggs, a more complete picture of effects on reproductive success can be obtained.

It is clear from our results that several aspects of air cell injections must be considered before embarking on studies. First, the method of incubation can alter the toxicity of air cell injected compounds. In early incubation, embryos float at the top

of the egg. If eggs are incubated vertically, i.e. directly underneath the air cell, embryos are in close proximity with any air cell injected compound, thus increasing the potential toxicity due to more direct concentrated exposure. In addition, it is important to consider that eggs are naturally incubated horizontally. In order to minimize mortality from anything other than the injected compound, it is best to mimic natural incubation as much as possible.

Dose vs. exposure

It is also clear from these studies that the air cell injected dose is not the same as internal embryonic exposure. By pipping, only up to 29.6% of the penta-BDE was absorbed into the avian egg, and much less of the octa-BDE was absorbed. Researchers must verify how much of an air cell injected compound is getting into egg contents and the embryo. This study demonstrates the importance of confirming actual uptake following air cell administration of compounds as the entire dose may not be absorbed through the air cell membrane. In addition, in order to maximize exposure, injections should be conducted near the beginning of incubation, to allow the dose to absorb into egg contents.

Conclusions

Results from these studies illustrate that environmentally realistic concentrations of a penta-BDE formulation can adversely affect survival endpoints in developing American kestrels. This is a cause for concern as the LOEL generated for

these effects (1.8 µg/g ww) are within the range of concentrations detected in eggs of free-ranging wildlife. In addition, air cell administration of toxicants can be a valuable tool in evaluating the effects of toxicants on avian embryos. However, conditions of artificial incubation must be considered when employing these methods. While air cell administered compounds are not naturally incorporated into the yolk or albumen, determining their concentration in egg contents would seemingly allow one to better predict toxic effects in eggs of free-ranging birds.

Tables and Figures

Table 5-1. Overview of significant effects on chicken, mallard, and American kestrel

embryos and hatchlings exposed to penta-BDE in ovo.

|	Chicken	Mallard	Kestrel
Survival to 90%	-	-	-
Pipping	-	-	**+**
Hatching	-	-	**+**
Edema	-	-	-
Deformities	-	-	-
Bone Lengths	-	-	-
Histopathology	**+**	-	-
EROD	**+**	-	-
Thyroid	-	-	-
Penta Uptake	29.6%	27.7%	18.8%
Octa Uptake	2.31%	6.48%	2.24%
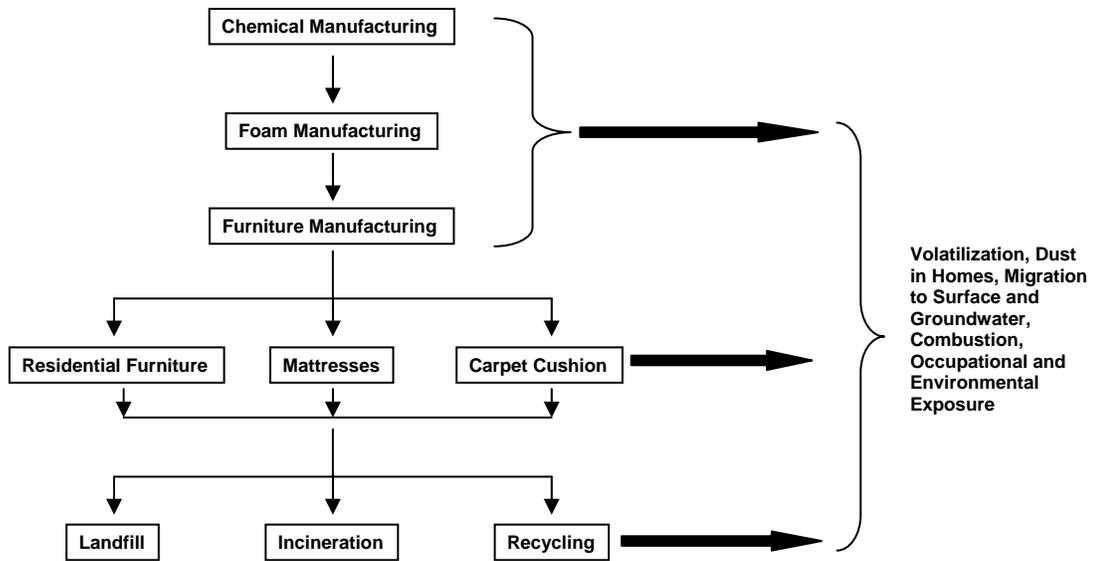
Figure 5-1. Potential routes for human and environmental release and exposure to polybrominated diphenyl ethers.
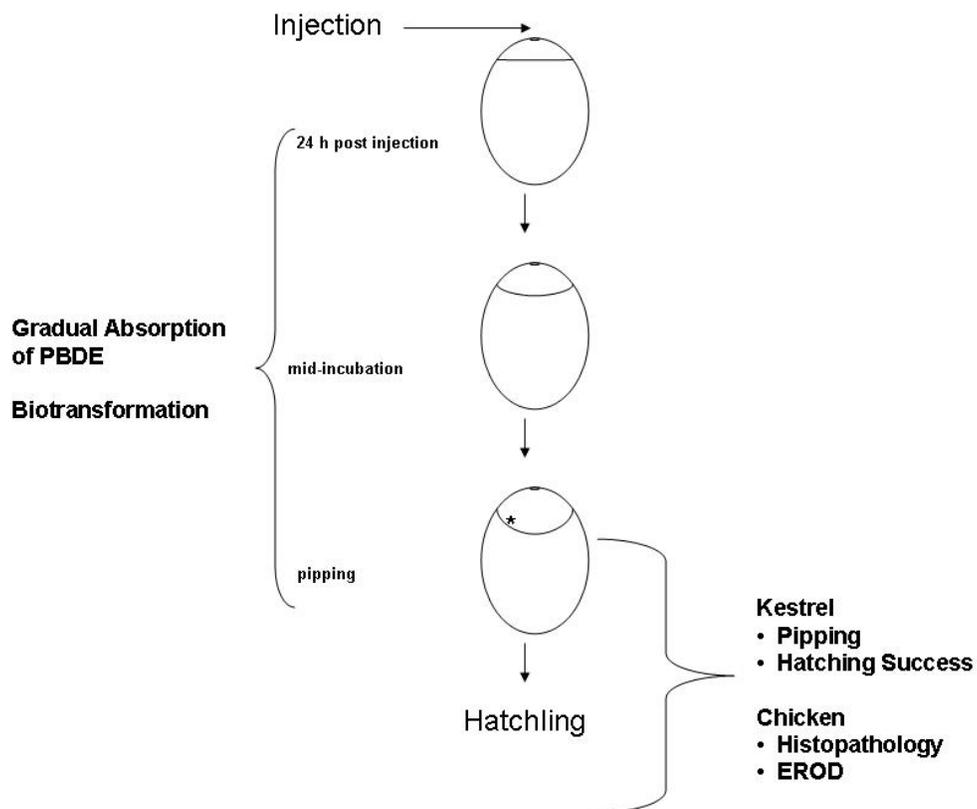
Figure 5-2.   Significant findings after air cell injection of a penta-BDE mixture into avian eggs.

Appendices

List of PBDE congeners analyzed in avian eggs.

IUPAC Congener Name	Structure	IUPAC Congener Name	Structure
BDE 1	2 mono-BDE	BDE 180	2,2',3,4,4',5,5' hepta-BDE
BDE 2	3 mono-BDE	BDE 181	2,2',3,4,4',5,6 hepta-BDE
BDE 3	4 mono-BDE	BDE173/190	2,2',3,3',4,5,6 & 2,3,3',4,4',5,6hepta-BDE
BDE 10	2,6 di-BDE	BDE 171	2,2',3,3',4,4',6 hepta-BDE
BDE 7	2,4 di-BDE	BDE 202	2,2',3,3',5,5',6,6' octa-BDE
BDE 4	2,2' di-BDE	BDE 201	2,2',3,3',4,5,6,6' octa-BDE
BDE 11	3,3' di-BDE	BDE 204	2,2',3,4,4',5,6,6'octa-BDE
BDE 8	2,4' di-BDE	BDE 197	2,2',3,3',4,4',6,6'octa-BDE
BDE 12/13	3,4&3,4'di-BDE	BDE 198	2,2',3,3',4,5,5',6 octa-BDE
BDE 15	4,4' di-BDE	BDE 203	2,2',3,4,4',5,5',6 octa-BDE
BDE 30	2,4,6 tri-BDE	BDE 196	2,2',3,3',4,4',5,6' octa-BDE
BDE 32	2,4',6 tri-BDE	BDE 205	2,3,3',4,4',5,5',6 octa-BDE
BDE 17)	2,2',4-tri-BDE	BDE 194	2,2',3,3',4,4',5,5' octa-BDE
BDE 25	2,3',4 tri-BDE	BDE 208	2,2',3,3',4,5,5',6,6' nona-BDE
BDE 28/33	2,4,4'&2',3,4 tri-BDE	BDE 207	2,2',3,3',4,4',5,6,6' nona-BDE
BDE 35	3,3',4 tri-BDE	BDE 206	2,2',3,3',4,4',5,5',6 nona-BDE
BDE 37	3,4,4' tri-BDE	BDE 209	deca-BDE
BDE 75	2,4,4',6 tetra-BDE		
BDE 51	2,2',4,6' tetra-BDE		
BDE 49	2,2',4,5' tetra-BDE		
BDE 71/48	2,3',4,6' & 2,2'4,5 tetra-BDE		
BDE 47	2,2',4,4' tetra-BDE		
BDE 74	2,4,4',6 tetra-BDE		
BDE 66/42	2,3',4,4' & 2,2'3,4' tetraBDE		
2'MeO BDE 68	2'methoxy 2,3',4,5'tetra-BDE		
BDE 104/121	2,2',4,6,6'&2,3',4,5',6 tetra-BDE		
6-MeO BDE 47	6-methoxy 2,2',4,4'tetra-BDE		
BDE 102	2,2',4,5,6' penta-BDE		
5 MeO BDE 47	5 methoxy 2,2',4,4' tetra-BDE		
BDE 100	2,2',4,4',6 penta-BDE		
BDE 101	2,2',3,5,5' penta-BDE		
4' MeO BDE 49	4'methoxy 2,2',4,5' tetra-BDE		
BDE 120	2,3',4,5,5' penta-BDE		
BDE 119	2,3',4,4',6 penta-BDE		
BDE 88	2,2',3,4,6 penta-BDE		
BDE 99	2,2',4,4',5 penta-BDE		
BDE 116	2,3,4,5,6 penta-BDE		
BDE 97/118	2,2',3',4,5 & 2,3',4,4',5 penta-BDE		
5' MeO BDE 100	5'methoxy 2,2',4,4',6 penta-BDE		
4' MeO BDE 103	4'methoxy 2,2',4,5',6 penta-BDE		
BDE 85	2,2',3,4,4'penta-BDE		
BDE 126	3,3',4,4',5 penta-BDE		
BDE 155 ()	2,2',4,4',6,6' hexa-BDE		
BDE 154	2,2',4,4',5,6'hexa-BDE		
5' MeO BDE 99	5'methoxy 2,2',4,4',5 penta-BDE		
4' MeO BDE 101	4'methoxy 2,2',4,5,5' penta-BDE		
BDE 144	2,2',3,4,5',6 hexa-BDE		
BDE 153	2,2',4,4',5,5'hexa-BDE		
BDE 139	2,2',3,4,4',6 hexa-BDE		
BDE 140	2,2',3,4,4',6' hexa-BDE		
BDE 138	2,2',3,4,4',5',6 hexa-BDE		
BDE 156	2,3,3',4,4',5 hexa-BDE		
BDE 188	2,2',3,4',5,6,6' hepta-BDE		
BDE 184	2,2',3,4,4',6,6' hepta-BDE		
BDE 179	2,2',3,3',5,6,6' hepta-BDE		
BDE 128	2,2',3,3',4,4' hexa-BDE		
BDE 175	2,2',3,3',4,5,6 hepta-BDE		
BDE 183	2,2',3,4,4',5',6 hepta-BDE		
BDE 182	2,2',3,4,4',5,6' hepta-BDE		
BDE 185	2,2',3,4,5,5',6 hepta-BDE		
BDE 192	2,3,3',4,5,5',6 hepta-BDE		
BDE 191	2,3,3',4,4',5',6 hepta-BDE		

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