Early Exposure to a Brominated Flame Retardant (BDE-99): An Assessment of Long-term Effects Using an Integrated Laboratory and Field Avian Model System

by

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Abstract

Polybrominated diphenyl ethers (PBDEs) are a group of hydrophobic and bioaccumulative chemicals that have been widely used as additive flame retardants(Hites 2004). 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) is one of the major constituents of the commercial penta-BDE flame retardant mixture, and is consistently found in avian tissue and egg samples throughout the world. It is known to cause a wide range of adverse effects in mammals, yet its effects in birds are not well known due to a lack of toxicological literature. Developmental life stages tend to be the most sensitive to environmental contaminants, and effects of early exposure may not be evident until reproductive maturity, necessitating long term studies to assess fitness implications of contaminant exposure. The objectives of this project were to develop an integrated field and lab avian monitoring system to assess the long-term effect of early exposure to BDE-99, using the zebra finch (*Taeniopygia guttata*) in the lab and the European starling (*Sturnus vulgaris*) in the field as model passerine species. I exposed nestlings to sub-lethal BDE-99 concentrations, and birds were raised to reproductive maturity to assess a range of endpoints, including neuroanatomy, behavior, physiology, growth, survival, reproductive development, and reproductive success. I also measured concentrations of persistent organic pollutants in free-living starling eggs and related concentrations to adult and nest conditions. In addition, I characterized the maternal transfer of BDE-99 from mothers to eggs in the zebra finch. Behavior was the most sensitive BDE-99 exposure, with the number of males that participated in courtship or singing behaviour being significantly reduced in the highest dose groups. There were few other overt effects of BDE-99 exposure in either zebra finches or starlings. PBDE concentrations in free-living starlings were highly variable, but at the observed concentrations there was no relationship between PBDEs and nest condition or reproductive success. Maternal transfer of BDE-99 is related to individual variation in BDE-99 plasma burden and lipid status, and involves a saturable transport process. Overall, these studies provide insight into the long-term effects of nestling exposure to BDE-99 in passerine birds, the concentrations and effects in free-living terrestrial passerines, and the maternal transfer process.

Keywords: Polybrominated diphenyl ethers; BDE-99; passerine birds; developmental effects; maternal transfer

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1. General Introduction

1.1. Developmental exposure to environmental contaminants

There is considerable evidence that early developmental life stages of organisms are more sensitive than adult stages to anthropogenic contaminants (e.g. Peterson et al. 1993, Hutchinson et al. 1998, Gore 2008). However, the toxicological effects of developmental exposure may not be evident until later in life. In birds, many components critical to normal physiology and behavior of adults are formed during the embryonic and early post-hatch period, such as the hypothalamic-pituitary-gonadal (HPG) axis (Silver et al. 1992, Silverin and Sharp 1996, Ottinger and Abdelnabi 1997), the hypothalamic-pituitary-thyroid (HPT) axis (McNabb 2007), and the brain structures and neural pathways that make up the song-control system (Bottjer et al. 1985, Mooney and Rao 1994). There are several avian examples of exposure to anthropogenic contaminants during these critical development periods having significant effects, many of which are not be manifested until later in life when exposed individuals reach sexual maturity (e.g. Fernie et al. 2001, Holm et al. 2006, Iwaniuk et al. 2006, Hoogesteijn et al. 2008, Quinn et al. 2008, Marteinson et al. 2010). In birds, anthropogenic contaminants in the embryonic environment are of maternal origin, with females transferring some of their own contaminant burden to their eggs during egg production (Bargar et al. 2001, Drouillard and Norstrom 2001). However, the specific mechanisms of maternal transfer and the factors that might affect the rate or amount of contaminant transfer from the mother to the egg are not well understood. In the post-hatch period, nestlings can continue to be exposed to contaminants through their food and environment. Given the sensitivity of developing life stages and the potential for delayed effects of contaminant exposure, studies investigating long-term effects of early exposure are critical for assessing the ecological risk of contaminants. In addition, because embryonic exposure

occurs through maternal transfer, characterizing maternal transfer of contaminants is of value.

1.2. Polybrominated diphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are a group of hydrophobic and bioaccumulative chemicals that have been widely used as additive flame retardants in textiles, plastics, foams and electronic circuitry. Because they are not chemically bonded to the polymers that contain them, they can easily leach into the environment. That, in combination with their persistent and bioaccumulative nature, has resulted in their ubiquitous distribution in environmental, human, and wildlife samples (de Wit 2002, Hites 2004). PBDEs are of relatively recent concern, as monitoring studies indicate that they have only emerged in the North American environment over the past 30 years (de Wit 2002, Law et al. 2003, Chen and Hale 2010). The manufacture and use of PBDEs has been largely restricted in Europe and North America since the mid 2000s (EEC 2003, US EPA 2006, Canada Gazette 2008). However, PBDEs persist in the environment and will continue to leach from existing products that are in use or have been disposed of in landfills. PBDEs have been found in avian tissue and egg samples throughout the world (Chen and Hale 2010). The majority of avian PBDE monitoring and research has been in aquatic birds and top predators (Chen and Hale 2010), but recent studies have shown that small invertebrate-feeding passerine birds can accumulate concentrations of PBDEs that are in the range of or greater than those reported for higher trophic level birds (Sun et al. 2012, Chen et al. submitted).

There are 209 possible PBDE congeners, but only a few have been used in commercial mixtures. One of the most pervasive congeners in birds and other wildlife is 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) (Darnerud et al. 2001, Hites 2004, Chen and Hale 2010), which is a main component of the technical penta-BDE mixture (La Guardia et al. 2006). While BDE-99 has been reported to cause a wide range of adverse effects in mammals (e.g. Hakk et al. 2002, Viberg et al. 2004, Talsness et al. 2005, Viberg et al. 2005, Lilienthal et al. 2006, Kuriyama et al. 2007, Albina et al. 2010, Alonso et al. 2010, Belles et al. 2010), its effects in birds are not well known due to a lack of pertinent toxicological literature. There have been some avian exposure studies using

penta-BDE mixtures (e.g. Fernie et al. 2006, Fernie et al. 2008, McKernan et al. 2009, Van den Steen et al. 2009, Marteinson et al. 2010, Van den Steen et al. 2010). However, the use of mixtures makes it difficult to identify the specific components responsible for any putative toxic effects, and the contribution of BDE-99 to any observed effects of penta-BDE mixtures is not clear. To our knowledge, the only study that has exposed birds solely to the BDE-99 congener was a study of vitamin status in captive mallards (Murvoll et al. 2005). Given the scarcity of information on BDE-99 for avian species, particularly passerines, further toxicological studies of BDE-99 within the context of developmental exposure, long-term effects, and maternal transfer are warranted in both captive and free-living passerine species.

1.3. Integrated laboratory and field avian model system

We developed an integrated laboratory and field avian model system for assessing contaminant effects on passerine birds using the zebra finch (*Taeniopygia guttata*) and the European starling (*Sturnus vulgaris*) as model species. The zebra finch is a useful model to monitor effects of contaminants under controlled laboratory conditions, as it readily breeds in captivity, has a short generation time (4 months), has been extensively studied in avian neuroscience and endocrinology (Ball et al. 2002), and has been widely used in toxicological dosing studies (e.g. Gill et al. 2004, Albert et al. 2008, Hoogesteijn et al. 2008, Kitulagodage et al. 2011). Captive studies generally use individuals living in ideal conditions (controlled climate, provisioned food, absence of predators) and observed effects may not necessarily reflect those that would be seen in free-living individuals facing environmental and anthropogenic stressors (e.g. Lambrechts et al. 1999, Spalding et al. 2000, Bardo and Bird 2009). It is therefore important to also study free-living individuals to corroborate the results found in the lab. European starlings are an important model species for comparative studies of contaminant effects in free-living passerines, as well as for monitoring terrestrial contamination. Starlings have a broad geographic distribution and are found in several habitats. They have been established as a terrestrial wildlife monitoring species both at an intercontinental scale (Van den Steen et al. 2012), and across Canada (Environment Canada 2011, Chen et al. submitted). Starlings readily use nest boxes, which makes

monitoring, manipulations, and sample collection for the purpose of linking contaminant exposure with biological responses relatively easy.

1.4. Objectives and contents of thesis

The objectives of this thesis were to develop and use an integrated laboratory and field avian model system to assess the long-term effects of developmental exposure to a representative contaminant, BDE-99, in passerine birds, as well as to monitor local contamination in terrestrial passerines, and to characterize the transfer of BDE-99 from the mother to the egg. In Chapter 2 we describe a tissue residue study in which we orally dosed zebra finch nestlings with a range of BDE-99 doses to validate that our dosing methods and concentrations resulted in dose-dependent body burdens of BDE-99 that fell within the range of concentrations that have been reported in free-living birds. Following the tissue residue study, we conducted a full-scale study to examine the effects of nestling exposure to BDE-99 on the song-control system and male mating behavior in sexually mature zebra finches. Chapter 3 follows the same cohort of zebra finches to investigate the effects of early exposure to BDE-99 on non-behavioral endpoints including measures of growth, physiology, and reproduction. The focus of chapter 4 was to assess the individual nest variation in background levels of persistent organic pollutants (PBDEs, polychlorinated biphenyls, and organochlorine pesticides) in European starlings at an agricultural site, and to relate PBDE exposure in starlings to reproductive success and to measures of condition in the mothers and offspring. Chapter 5 describes the development of the European starling model system for assessing short- and long-term effects of early exposure to anthropogenic contaminants under ecological conditions, using BDE-99 as a representative contaminant. In Chapter 6, we characterize the maternal transfer of BDE-99 to eggs using the zebra finch as a model songbird species. In chapter 7 I briefly summarize the main results from this thesis, discuss the implications, and describe future research.

1.5. References

Albert, C., T. D. Williams, C. A. Morrissey, V. W. M. Lai, W. R. Cullen, and J. E. Elliott. (2008). Tissue uptake, mortality, and sublethal effects of monomethylarsonic acid (MMA(V)) in nestling zebra finches (*Taeniopygia guttata*). *Journal of Toxicology and Environmental Health-Part a-Current Issues* 71:353-360.

Albina, M. L., V. Alonso, V. Linares, M. Belles, J. J. Sirvent, J. L. Domingo, and D. J. Sanchez. (2010). Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. *Toxicology* 271:51-56.

Alonso, V., V. Linares, M. Belles, M. L. Albina, A. Pujol, J. L. Domingo, and D. J. Sanchez. (2010). Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food and Chemical Toxicology* 48:2206-2211.

Ball, G. F., L. V. Riters, and J. Balthazart. (2002). Neuroendocrinology of song behavior and avian brain plasticity: Multiple sites of action of sex steroid hormones. *Frontiers in Neuroendocrinology* 23:137-178.

Bardo, L. and D. M. Bird. (2009). The use of captive American kestrels (*Falco sparverius*) as wildlife models: a review. *Journal of Raptor Research* 43:345-364.

Bargar, T. A., G. I. Scott, and G. P. Cobb. (2001). Maternal transfer of contaminants: Case study of the excretion of three polychlorinated biphenyl congeners and technical-grade endosulfan into eggs by white leghorn chickens (*Gallus domesticus*). *Environmental Toxicology and Chemistry* 20:61-67.

Belles, M., V. Alonso, V. Linares, M. L. Albina, J. J. Sirvent, J. L. Domingo, and D. J. Sanchez. (2010). Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99. *Toxicology Letters* 194:1-7.

Bottjer, S. W., S. L. Glaessner, and A. P. Arnold. (1985). Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. *Journal of Neuroscience* 5:1556-1562.

Canada Gazette. (2008). Part II. Polybrominated diphenyl ether regulations. 142:1663-1682.

Chen, D. and R. C. Hale. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International* 36:800-811.

Chen, D., P. Martin, N. M. Burgess, L. Champoux, J. E. Elliott, D. Forsyth, A. Idrissi, and R. J. Letcher. (submitted). European starlings (*Sturnus vulgaris*) indicate that landfills are an important source of bioaccumulative flame retardants to Canadian terrestrial ecosystems. *Environmental Science & Technology*.

Darnerud, P. O., G. S. Eriksen, T. Johannesson, P. B. Larsen, and M. Viluksela. (2001). Polybrominated diphenyl ethers: Occurrence, dietary exposure, and toxicology. *Environmental Health Perspectives* 109:49-68.

de Wit, C. A. (2002). An overview of brominated flame retardants in the environment. *Chemosphere* 46:583-624.

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

EEC. (2003). Directive2003/11/EC of the European Parliament and of the Council of 6 February 2003 amending for the 24th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (pentabromodiphenyl ether, octabromodiphenyl ether). *Official Journal of the European Union* L 42:45-46.

Fernie, K. J., J. L. Shutt, R. J. Letcher, J. I. Ritchie, K. Sullivan, and D. M. Bird. (2008). Changes in reproductive courtship behaviors of adult American kestrels (Falco sparverius) exposed to environmentally relevant levels of the polybrominated diphenyl ether mixture, DE-71. *Toxicological Sciences* 102:171-178.

Fernie, K. J., J. L. Shutt, I. J. Ritchie, R. J. Letcher, K. Drouillard, and D. M. Bird. (2006). Changes in the growth, but not the survival, of American kestrels (*Falco sparverius*) exposed to environmentally relevant polybrominated diphenyl ethers. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 69:1541-1554.

Fernie, K. J., J. E. Smits, G. R. Bortolotti, and D. M. Bird. (2001). In ovo exposure to polychlorinated biphenyls: Reproductive effects on second-generation American kestrels. *Archives of Environmental Contamination and Toxicology* 40:544-550.

Gill, H., T. D. Williams, C. A. Bishop, K. M. Cheng, and J. E. Elliott. (2004). Effects of azinphos-methyl on cholinergic responses and general health in zebra finches (*Taeniopygia guttata*) after previous treatment with p,p '-DDE. *Archives of Environmental Contamination and Toxicology* 48:118-126.

Gore, A. C. (2008). Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Frontiers in Neuroendocrinology* 29:358-374.

Hakk, H., G. Larsen, and E. Klasson-Wehler. (2002). Tissue disposition, excretion and metabolism of 2,2 ',4,4 ',5-pentabromodiphenyl ether (BDE-99) in the male Sprague-Dawley rat. *Xenobiotica* 32:369-382.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environmental Science & Technology* 38:945-956.

Holm, L., A. Blomqvist, I. Brandt, B. Brunstrom, Y. Ridderstrale, and C. Berg. (2006). Embryonic exposure to o,p '-DDT causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. *Environmental Toxicology and Chemistry* 25:2787-2793.

Hoogesteijn, A. L., G. V. Kollias, F. W. Quimby, A. P. De Caprio, D. W. Winkler, and T. J. DeVoogd. (2008). Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by Aroclor 1248. *Environmental Toxicology and Chemistry* 27:2071-2075.

Hutchinson, T. H., J. Solbe, and P. J. Kloepper-Sams. (1998). Analysis of the ECETOC aquatic toxicity (EAT) database - III - Comparative toxicity of chemical substances to different life stages of aquatic organisms. *Chemosphere* 36:129-142.

Iwaniuk, A. N., D. T. Koperski, K. M. Cheng, J. E. Elliott, L. K. Smith, L. K. Wilson, and D. R. W. Wylie. (2006). The effects of environmental exposure to DDT on the brain of a songbird: Changes in structures associated with mating and song. *Behavioural Brain Research* 173:1-10.

Kitulagodage, M., W. A. Buttemer, and L. B. Astheimer. (2011). Adverse effects of fipronil on avian reproduction and development: maternal transfer of fipronil to eggs in zebra finch Taeniopygia guttata and in ovo exposure in chickens Gallus domesticus. *Ecotoxicology* 20:653-660.

Kuriyama, S. N., A. Wanner, A. A. Fidalgo-Neto, C. E. Talsness, W. Koerner, and I. Chahoud. (2007). Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology* 242:80-90.

La Guardia, M. J., R. C. Hale, and E. Harvey. (2006). Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used penta-, octa-, and deca-PBDE technical flame-retardant mixtures. *Environmental Science & Technology* 40:6247-6254.

Lambrechts, M. M., P. Perret, M. Maistre, and J. Blondel. (1999). Do experiments with captive non-domesticated animals make sense without population field studies? A case study with blue tits' breeding time. *Proceedings of the Royal Society B-Biological Sciences* 266:1311-1315.

Law, R. J., M. Alaee, C. R. Allchin, J. P. Boon, M. Lebeuf, P. Lepom, and G. A. Stern. (2003). Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. *Environment International* 29:757-770.

Lilienthal, H., A. Hack, A. Roth-Harer, S. W. Grande, and C. E. Talsness. (2006). Effects of developmental exposure to 2,2 ',4,4 ',5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environmental Health Perspectives* 114:194-201.

Marteinson, S. C., D. M. Bird, J. L. Shutt, R. J. Letcher, I. J. Ritchie, and K. J. Fernie. (2010). Multi-generation effects of polybrominated diphenylethers exposure: embryonic exposure of male American kestrels (*Falco sparverius*) to DE-71 alters reproductive success and behaviors *Environmental Toxicology and Chemistry* 29:1740-1747.

McKernan, M. A., B. A. Rattner, R. C. Hale, and M. A. Ottinger. (2009). Toxicity of polybrominated diphenyl ethers (DE-71) in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) embryos and hatchlings. *Environmental Toxicology and Chemistry* 28:1007-1017.

McNabb, F. M. A. (2007). The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Critical Reviews in Toxicology* 37:163-193.

Mooney, R. and M. Rao. (1994). Waiting periods versus early innervation - the development of axonal connections in the zebra finch song system. *Journal of Neuroscience* 14:6532-6543.

Murvoll, K. M., B. M. Jenssen, and J. U. Skaare. (2005). Effects of pentabrominated diphenyl ether (PBDE-99) on vitamin status in domestic duck (*Anas platyrhynchos*) hatchlings. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 68:515-533.

Ottinger, M. A. and M. A. Abdelnabi. (1997). Neuroendocrine systems and avian sexual differentiation. *American Zoologist* 37:514-523.

Peterson, R. E., H. M. Theobald, and G. L. Kimmel. (1993). Developmental and reproductive toxicity of dioxins and related compounds - Cross-species comparisons. *Critical Reviews in Toxicology* 23:283-335.

Quinn, M. J., C. L. Summitt, and M. A. Ottinger. (2008). Consequences of in ovo exposure to p,p '-DDE on reproductive development and function in Japanese quail. *Hormones and Behavior* 53:249-253.

Silver, R., C. Ramos, H. Machuca, and B. Silverin. (1992). Immunocytochemical distribution of GnRH in the brain of adult and posthatching great tit *Parus major* and ring dove *Streptopelia roseogrisea*. *Ornis Scandinavica* 23:222-232.

Silverin, B. and P. Sharp. (1996). The development of the hypothalamic-pituitary-gonadal axis in juvenile great tits. *General and Comparative Endocrinology* 103:150-166.

Spalding, M. G., P. C. Frederick, H. C. McGill, S. N. Bouton, L. J. Richey, I. M. Schumacher, C. G. Blackmore, and J. Harrison. (2000). Histologic, neurologic, and immunologic effects of methylmercury in captive great egrets. *Journal of Wildlife Diseases* 36:423-435.

Sun, Y. X., X. J. Luo, L. Mo, Q. Zhang, J. P. Wu, S. J. Chen, F. S. Zou, and B. X. Mai. (2012). Brominated flame retardants in three terrestrial passerine birds from South China: Geographical pattern and implication for potential sources. *Environmental Pollution* 162:381-388.

Talsness, C. E., M. Shakibaei, S. N. Kuriyama, S. W. Grande, A. Sterner-Kock, P. Schnitker, C. de Souza, K. Grote, and I. Chahoud. (2005). Ultrastructural

changes observed in rat ovaries following in utero and lactational exposure to low doses of a polybrominated flame retardant. *Toxicology Letters* 157:189-202.

US EPA. (2006). Certain Polybrominated Diphenylethers; Significant New Use Rule. *Federal Register* 71:34015-34021. http://www.epa.gov/fedrgstr/EPA-TOX/2006/June/Day-13/t9207.htm

Van den Steen, E., M. Eens, A. Covaci, A. C. Dirtu, V. L. B. Jaspers, H. Neels, and R. Pinxten. (2009). An exposure study with polybrominated diphenyl ethers (PBDEs) in female European starlings (*Sturnus vulgaris*): Toxicokinetics and reproductive effects. *Environmental Pollution* 157:430-436.

Van den Steen, E., M. Eens, A. Geens, A. Covaci, V. M. Darras, and R. Pinxten. (2010). Endocrine disrupting, haematological and biochemical effects of polybrominated diphenyl ethers in a terrestrial songbird, the European starling (*Sturnus vulgaris*). *Science of the Total Environment* 408:6142-6147.

Viberg, H., A. Fredriksson, and P. Eriksson. (2004). Neonatal exposure to the brominated flame-retardant, 2,2',4,4',5-pentabromodiphenyl ether, decreases cholinergic nicotinic receptors in hippocampus and affects spontaneous behaviour in the adult mouse. *Environmental Toxicology and Pharmacology* 17:61-65.

Viberg, H., A. Fredriksson, and P. Eriksson. (2005). Deranged spontaneous behaviour and decrease in cholinergic muscarinic receptors in hippocampus in the adult rat, after neonatal exposure to the brominated flame-retardant, 2,2 ',4,4 ', 5-pentabromodiphenyl ether (PBDE 99). *Environmental Toxicology and Pharmacology* 20:283-288.

2. Early exposure to BDE-99: An assessment of effects on the song-control system and mating behavior in zebra finches

2.1. Abstract

2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) is a brominated flame retardant congener that has pervaded global food chains, being reported in avian egg and tissue samples throughout the world. Its effects on birds are not well known, but there is evidence in exposed mammals that it directly mediates and causes neurotoxicity, alters thyroid hormone homeostasis, and lowers sex steroid hormone concentrations. In birds, those processes could disrupt the song-control system and male mating behavior. In this study, the effects of nestling exposure to sub-lethal concentrations of BDE-99 were assessed in a model songbird species, the zebra finch (*Taeniopygia guttata*). A tissue residue study in which zebra finch nestlings were orally exposed to 0, 2.5, 15.8, or 50.7 ng BDE-99/g bw/day over the 21-day nesting period validated dosing methods and confirmed dose concentrations were within the range of concentrations reported for free-living birds (332.7 ± 141.0 to 4450.2 ± 1396.2 ng/g plasma lipid). A full scale study exposing nestlings to 0, 2.5, 15.8, 50.7 or 173.8 ng BDE-99/g bw/day was carried out to investigate long-term effects of BDE-99 on the adult song-control nuclei volumes, song quality, and male mating behavior. Early exposure to BDE-99 had significant effects on male mating behavior and the response of clean experienced females to exposed males. There was no effect on male song-control nuclei or song quality, and there were non-dose-dependent effects on female song-control nuclei. The results demonstrate that early exposure to concentrations BDE-99 within the range of concentrations reported in free living birds affects the behavior of zebra finches.

2.2. Introduction

Polybrominated diphenyl ethers (PBDEs), formulated as several technical mixtures, find wide commercial use as additive flame retardants in a variety of textiles, plastics, foams and electronic circuitry. They have become ubiquitous in environmental, human, and wildlife samples (Darnerud et al. 2001) and are consistently found in avian tissue and egg samples throughout the world (Chen and Hale 2010). 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) is one of the most pervasive congeners (Darnerud et al. 2001, Chen and Hale 2010) and causes a range of toxicological effects in mammals; however, there is a lack of pertinent toxicological literature concerning its effects in birds. In mammals, exposure to BDE-99 causes a range of adverse effects, including decreased neurotransmitter receptor densities (Viberg et al. 2004, 2005), aberrations in spontaneous behavior (Viberg et al. 2004, 2005), disruption of thyroid hormone homeostasis (Hakk et al. 2002, Kuriyama et al. 2007), and lowered concentrations of sex steroid hormones (Lilienthal et al. 2006, Alonso et al. 2010). If BDE-99 has similar effects in birds, exposure could significantly alter, for example, the brain regions controlling the learning and production of bird song (song-control system) and male mating behavior.

The brain structures and neural pathways that make up the song-control system develop primarily in the first few months posthatch (Bottjer et al. 1985, Mooney and Rao 1994), making the song-control system sensitive to early environmental conditions. There is evidence in songbirds that exposure to halogenated organic contaminants (dichlorodiphenyltrichloroethane (DDT) and its metabolites, and polychlorinated biphenyls (PCBs)) at early life stages results in smaller song-control nuclei volumes (Iwaniuk et al. 2006, Hoogesteijn et al. 2008). Nuclei volumes may also increase following exposure to estrogen mimics (e.g. 17-β estradiol, dioctylphthalate, bisphenol A, and dibutylphthalate; Markman et al. 2008). If BDE-99 lowers neurotransmitter receptor densities in birds, the song-control system may be directly affected, as concentrations of nicotinic and muscarinic cholinergic receptors are present within and at the boundaries of song-control nuclei (Ryan and Arnold 1981, Watson et al. 1988), and there is evidence of cholinergic input and modulation of the song-control circuits (Li and Sakaguchi 1997, Salgado-Commissariat et al. 2004, Shea et al. 2010). The role of

thyroid hormones in the development of the song-control system is not well known, but there is evidence that many of the neurons that project into the song-control nuclei have thyroxine (T4) receptors and exposure to abnormally elevated T4 concentrations increases cell death in song-control nuclei of zebra finches (Tekumalla et al. 2002). Disruption of thyroid hormone homeostasis by BDE-99 could therefore alter the cell number and volume of the song-control nuclei. The song-control nuclei could also be affected by any changes in sex steroid hormones caused by BDE-99, as several of the song-control nuclei contain androgen and estrogen receptors, and decreased concentrations of sex steroids can result in smaller nuclei (Ball et al. 2002). Despite evidence that BDE-99 disrupts many of the mechanisms that can affect the song-control system, there have been no studies investigating possible PBDE-induced effects on the song-control system of birds.

Reduction in song-control nuclei volume as a result of BDE-99 exposure could lead to lowered song quality and/or song perception, as the volumes of key song-control nuclei correlate with male song characteristics, such as song complexity and duration (Garamszegi and Eens 2004), and the song-control nuclei can also play a role in song perception in females (Brenowitz 1991). Singing is an important aspect of reproduction in birds, serving to define territories and attract females (e.g. Kroodsma 1976, Krebs et al. 1978, Searcy 1992). Learned features of song can act as an honest signal of male quality (Nowicki et al. 2002) and females of many species prefer males with higher song rate (Collins et al. 1994), and more complex and longer songs (Clayton and Prove 1989). Developmental conditions that result in smaller song-control nuclei, and reduced song quality in males, or altered song perception in females, could ultimately disrupt pair formation and lower reproductive success.

If BDE-99 lowers sex steroid hormones in birds as it does in mammals, then we would also expect exposed male birds to exhibit decreased mating behavior, as androgens and estrogens play an important role in mediating sexual behaviors in birds (Ball and Balthazart 2004). There are reports suggesting that penta-BDE technical mixtures containing BDE-99 may reduce reproductive behavior and circulating testosterone in male birds (Fernie et al. 2008, Marteinson et al. 2011), but no studies have specifically looked at the BDE-99 congener.

We investigated the effect of early developmental exposure to concentrations of BDE-99 relevant to exposure in free-living birds, using the zebra finch (*Taeniopygia guttata*) as a model songbird species. The zebra finch is a well established model species that has been extensively studied in avian neuroscience and endocrinology (e.g. Ball et al. 2002), and has been successfully used in toxicological dosing studies (e.g. Hoogesteijn et al. 2008).Our objectives were to first assess relationships between oral dosing concentrations of BDE-99 and plasma and lipid tissue residues, and to then conduct a full-scale study to examine the effects of BDE-99 exposure on the song-control system and male mating behavior in sexually mature adult birds. We hypothesize that adult birds that had been exposed to BDE-99 early in development in the nest have (1) smaller song-control nuclei, (2) decreased song quality (i.e. song rate, song phrase duration, and syllable repertoire size), and (3) decreased mating behavior.

2.3. Materials and methods

2.3.1. Animals and husbandry

This study was conducted on a captive colony of zebra finches maintained at the Simon Fraser University Animal Care Facility located in Burnaby, British Columbia. Zebra finches were housed in a controlled environment (temperature 19-23°C; humidity 35-55%; photoperiod 14 hours light to 10 hours dark; lights on at 07:00). All birds were provided with mixed seed (panicum and white millet 1:2; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate by dry mass), water, grit, and cuttlefish bone (calcium) *ad libitum* plus a multivitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (864B-08) in accordance with guidelines from the Canadian Committee on Animal Care.

For all breeding and chick rearing, the same basic protocol was followed. Experienced adult zebra finches were randomly paired and housed in individual breeding cages (51 × 39 × 43cm) equipped with an external nest box (14 × 14.5 × 20 cm). In addition to the *ad libitum* seed diet, breeding pairs were provided with an egg-food supplement (20.3% protein:6.6% lipid) daily from pairing to clutch completion (2

days after the last egg was laid) and then again during the chick-rearing stage. Nest boxes were checked daily between 09:00 and 11:00 for egg laying, and new eggs were numbered in consecutive order and weighed (0.001 g). Breeding pairs that did not produce eggs within 15 days following pairing were separated and classified as "nonbreeders." Nest boxes were then checked again daily toward the end of the 12- to 14-day incubation period to determine hatching dates. Nestlings were weighed daily and tarsus length was measured every 5 days using digital calipers (to the nearest 0.01mm) to assess body condition.

2.3.2. Dosing procedure

All dosing was done with technical grade BDE-99 (>98% purity, Cambridge Isotope Labs, Andover, MA, USA). BDE-99 was analyzed via gas chromatography (GC)-mass spectrometry (MS) (electron capture negative ionization mode [ECNI]) (see "Chemical Analysis" section below), and the only bromide ion that was quantifiable was BDE-99. The BDE-99 was dissolved in safflower oil (Spectrum organics, Boulder, CO) and the µl amounts of BDE-99 dissolving solvent (nonane) were evaporated off using a steady stream of purified nitrogen gas. There were four dose concentrations (2.5, 15.8, 50.7 and 173.8 ng BDE-99/g body weight [bw] per day) and a safflower oil only control group. Within 24 h of hatching, individual chicks within each nest were marked with feather tract removal for identification, and dose concentrations were randomly assigned within the nest to account for any heritable effects. Nestlings were orally dosed daily from 24hrs after hatching (day 1) until fledging (day 21), using a micropipette. Doses were adjusted daily according to chick mass, with the dose volume being 10 µl/g bw. All surviving nestlings were banded at 10 days of age. Nestlings were returned to the nests immediately after handling and dosing. There were no observed adverse effects with the dosing approach on chick growth or survival.

2.3.3. Tissue residue study

An initial study was conducted to assess relationships between oral dosing concentrations of BDE-99 and plasma and lipid tissue residues. Twenty nestlings (n=5/dose group) were dosed with 0, 2.5, 15.8 or 50.7 ng/g bw/day for 21 days. At 30 days of age, birds were euthanized via anesthetic (50:50 ketamine:rompun) followed by

exsanguination. Plasma and adipose tissue samples were collected. All plasma samples (n = 20), a subset of adipose tissue samples (n = 6) and dosing solutions (n = 4) were analyzed for BDE-99 and six less brominated PBDE congeners (see “Chemical Analysis” sub-section).

2.3.4. Full-scale study

Following the tissue residue study, a full-scale study was carried out using 48 male and 29 female nestlings and 5 dose groups (male control n = 13, 2.5 ng/g n = 9, 15.8 ng/g n = 9, 50.7 ng/g n = 9, 173.8 ng/g n = 8; female control n = 6, 2.5 ng/g n = 6, 15.8 ng/g n = 5, 50.7 ng/g n = 6, 173.8 ng/g n = 6). Birds were orally dosed between day 1 – 21 post-hatching. Once young were independent from parents (day 30) they were placed into cages (102 × 39 × 43 cm) as juvenile groups, and separated by sex once adult plumage and bill color started to form. Two adult male song tutors were placed in each juvenile cage containing males, and birds were not visually or acoustically isolated from birds in adjacent cages. Blood samples were collected at 30 and 90 days of age.

Once birds reached sexual maturity (day 90), male mating trials were conducted. For each exposed male, two mating trials were conducted over two separate days. At the start of each mating trial, an experienced, clean wild-type female was randomly chosen from a pool of 60 females and placed in a cage for 5 minutes to acclimate alone. Different females that were novel to the experimental male were chosen for each male and trial. The cage contained two perches, grit, a cuttlefish bone, but no water or food inside. A microphone was positioned in the upper right corner of the cage. For each trial, an experimental male was placed in the cage with the experienced female, and the behaviors of both the male and the female were recorded for 10 min by an observer blind to treatment. All of the courtship trials were performed between 09:00 and 12:00 h. The following typical male courtship behaviors (described in Zann 1996) were recorded during the trial: invitation (Y or N), bill wiping (number of wipes against perch), head or tail twisting (scored per left to right cycle), following (number of times the male followed the female), number of copulation attempts, number of successful copulations, and time in seconds to initial copulation attempt. The female response to the male was also recorded (scored 1 to 5; 1 = no response, 5 = solicitation of copulation). All songs were

digitally recorded during the male mating trials using a Sennheiser ME62 microphone with a K6 power module, connected to a laptop computer.

Following the completion of breeding and mating trials, birds were anesthetized and then exsanguinated. Brains were immediately dissected from the cranium, weighed (0.001g), and fixed by immersing in buffered 4% paraformadehyde (pH 8.5) for two weeks. After fixation, the brains were cryoprotected in 30% sucrose for 24 hours, then frozen on pulverized dry ice, and stored at –80°C until further processing.

2.3.5. Song analysis

Digital song recordings were measured using Syrinx-PC software (J. Burt, Seattle, WA). For each mating trial we measured three variables of song quality: (1) the song rate (number of song phrases/hour), (2) the song phrase duration, and (3) the syllable repertoire size (number of different syllables types). Songs were analyzed based on methods in Airey and DeVoogd (2000). Duration and repertoire were estimated based on 10 song phrases for each recording period, and measures were averaged over both recordings. Birds that did not sing during either recording attempt (N = 21) were not included in the song analysis. All mating behaviors and song measurements were collected and analyzed blind to the treatment group of the males.

2.3.6. Tissue processing and neuroanatomical measurements

Using a cryostat (–20°C), we sectioned brains into 40 µm coronal sections and collected these in 0.1*M* PBS (pH 7.5). Tissue sections were then mounted onto Superfrost Plus microscope slides (VWR). Sections were Nissl stained with thionin, serially dehydrated in ethanol, cleared in solvent and then protected with coverslips affixed with Permount (Fisher Scientific). An observer (MLE) blind to subject and treatment group then made all measurements. Slides were examined with a bright field microscope (Leici DM5500 B) equipped with a digital microscope camera (DFC420 C). Images of sections of the song-control regions, HVC (proper name, not an acronym), Area X and RA (robust nucleus of the arcopallium), were captured (Fig. 2.1). The song nuclei were all readily defined by darkly stained cells relative to surrounding tissue. Telencephalon images were captured using a high resolution (2400 dpi) flatbed scanner. The software ImageJ (version 1.42q, National Institutes of Health) was used to trace the

outlines of these regions and measure their area. We then combined these areas using the formula for the volume of a cone frustum to estimate the total volume of each structure. Volume estimates were based on the areas of every 10th section for the telencephalon (400 µm intervals) and every second section for the song-control regions (80 µm intervals). Poor staining or tissue damage prevented volume reconstruction of RA and Area X in 3 male brains, and HVC and RA in 3 female brains.

2.3.7. Chemical analysis

All PBDE standards (BDE-17, -28/33, -47, -49, -66, -85 and -99) were purchased from Wellington Laboratories (Guelph, ON, Canada). Plasma, adipose tissue, and dosing solutions were analyzed for BDE-17, -28/33, -47, -49, -66, -85 and -99.

Plasma samples (0.14 to 0.37 g), adipose tissue samples (0.05 to 0.17 g) and dosing solutions (150 µl) were accurately weighed, and neutral fractions were extracted and cleaned up using established methodologies (Verreault et al. 2005, Gauthier et al. 2008). In brief, plasma samples were spiked with 50ng of each of the internal standards (BDE-30 and -156), acidified, denatured, and liquid-liquid extracted with 50% (v/v) methyl tert-butyl ether (MtBE)/ hexane. The organic phase layer containing the PBDEs was separated and collected. Lipid content of plasma was determined colorimetrically using olive oil as the calibration standard (Frings et al. 1972). Adipose tissue samples were ground with ~25 g of anhydrous sodium sulphate and extracted with 50% dichloromethane (DCM)/hexane using an accelerated solvent extraction system (Dionex ASE 200). The extraction columns were spiked with 20 ng of each internal standard. The column extraction eluant was concentrated to 10 mL and a 10% portion was removed for gravimetric lipid determination. The remaining extracts were cleaned by gel permeation chromatography (GPC) and eluted from the GPC column with 50% DCM/hexane. The first fraction (140 ml) containing lipids and biogenic material was discarded, and the second fraction (200 ml) containing PBDEs was concentrated to a volume of ~4 ml. Dosing solution samples were initially processed with GPC and did not go through ASE extraction. The dosing solutions were spiked with 20 ng of each internal standard.

All samples were cleaned up using a silica solid phase extraction (SPE) column (J.T. Baker). The column was conditioned with successive washes of 10% (vol/vol)

methanol (6 ml) in DCM and then 8 ml of 5% DCM in hexane. The sample was then loaded onto the cartridge and eluted with 8 ml of 5% DCM/hexane. The eluant was then concentrated and solvent exchanged with isooctane to a final volume of approximately 175 µl. The exact mass of each sample was recorded and the final volume determined by dividing by the density of 2,2,4-trimethylpentane (0.69 g/ml).

PBDEs in the isolated chemical fractions were analyzed using gas chromatography-mass spectrometry working in electron capture negative ionization mode (GC/ECNI-MS). Analytes were separated and quantified on an Agilent 6890 series GC equipped with a 5973 quadrupole MS detector (Agilent technologies, Palo Alto, CA). The analytical column was a 15 m × 0.25 mm × 0.10 µm DB-5HT fused-silica column (J & W Scientific, Brockville, ON, Canada). Helium and methane were used as the carrier and reagent gases, respectively. A sample volume of 1 µl was introduced to the injector operating in pulsed-splitless mode (injection pulse at 25.0 psi until 0.50 min; purge flow to split vent of 96.4 ml/min to 2.0 min; gas save flow of 20 ml/min at 2.0 min), with the injector held at 240°C. The GC oven ramping temperature program was as follows: initial 100°C for 4.0 min, 25°C/min. until 260°C, 2.5°C/min until 280°C for 10.0 min, 25°C/min until 325°C and hold for a final 7.0 min. The GC to MS transfer line was held at 280°C, ion source temperature was 200°C, and the quadrupole temperature was 150°C.

PBDE congeners were monitored using the bromine anions of *m/z* 79 and 81. Analytes were identified by comparison of retention times and ECNI mass spectra to those of the authentic standards.

Mean internal standard recoveries for the BDE-30 was 97% ± 3% SE for plasma analysis and 73% ± 2 for the adipose tissue and dosing solution analysis. Analytes were quantified using an internal standard approach; thus, all reported values were inherently recovery-corrected. The method limits of quantification (MLOQ) for BDE-99, based on a signal-to-noise ratio of 10, was 0.05 ng/g wet weight (ww) for plasma analysis and 1.1 ng/g ww for adipose and dosing solution analysis. Method blanks (n = 4) were included for each sample batch to assess background interference and possible contamination, and a blank subtraction was done for BDE-28/33, -47, -49, -66 and -99. BDE-17, -28/33, -47, -49, -66 and -85 were generally below the MLOQ or at sub ng/g ww concentrations,

and thus essentially not present in the plasma or adipose samples. Duplicate analysis of the samples was not possible as all plasma and adipose tissue was consumed to ensure quantifiable analyte concentrations. In-house standard reference material (polar bear [*Ursus maritimus*] plasma for plasma analysis and double-crested cormorant [*Phalacrocorax auritus*] egg for adipose and dosing solution analysis) was also included in each sample batch to ensure consistency of data acquisition (within 2 SD of in-house mean).

2.3.8. Statistical analysis

Before analysis, all non-parametric continuous variables were natural log-transformed to more closely approximate normal distributions and to homogenize variance. An index of body condition of the tissue residue study birds was estimated as the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde et al. 2005). Male and female brains were analyzed separately because of prominent sexual differences in anatomy (Nottebohm and Arnold 1976), and because in females the borders of Area X are poorly defined and were not measured. Left and right brain hemispheres were compared for HVC, RA, Area X and telencephalon using paired *t*-tests, and no evidence of anatomical lateralization was found, so all the analyses were performed on summed left and right volumes. Allometric effects were tested for using linear regression on the volume of each nucleus against the volume of the telencephalon. Male Area X and female RA were significantly related to telencephalon volume ($F_{1,39} = 6.01$, $p = 0.019$ and $F_{1,27} = 10.05$, $p = 0.004$, respectively), so the residuals from the regression were used to represent the relative size of those nuclei. The effect of dose was assessed using generalized linear models for continuous variables, and *post-hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey–Kramer method. Fisher's exact probability test was used to assess effects of dose for categorical variables. To determine the repeatability of mating behaviors across the two mating trials, nested ANOVA was used following Lessells and Boag (1987). Correlations were measured by Pearson's correlation coefficient. All statistical analyses were done using SAS 9.1.3 (SAS Institute 2003).

2.4. Results

2.4.1. Tissue residue study

We measured PBDE concentrations in 20 zebra finch plasma samples (n = 5/dose level) and 6 adipose samples. There was a strong dose-dependent relationship for plasma BDE-99 concentrations at 30 days of age among the control and dose groups, with concentrations ranging from 332.7 ± 141.0 to 4450.2 ± 1396.2 ng/g lipid weight (lw) (Fig. 2.2). In addition, adipose tissue concentrations were significantly correlated with plasma concentrations (for lipid normalized values, r = 0.981, p = 0.0005), and were related to each other according to the equation [(adipose BDE-99 lw concentration) = 1.16*(plasma BDE-99 lw concentration) + 86.98]. The BDE-99 dose concentrations in the initial tissue residue study had no effect on body condition ($F_{3,37}$ = 0.14, p = 0.934) or survival (p = 0.255, Fisher's exact test, FET), and so we added an additional dose group of 173.8 ng BDE-99/g bw/day in the full-scale study. Using the significant relationship between dose group and known plasma concentrations (r = 0.724 , p = 0.0003; plasma concentration = 78.38*dose + 453.57), it was estimated that the additional 173.8 ng/g dose group would result in plasma concentrations of approximately 14079.7 ng/g lw (Fig. 2.2).

2.4.2. Full scale study

In male zebra finches, we found no effect of dose on the size of any of the song-control nuclei or on total brain mass (Table 2.1). In females, HVC volume was significantly larger in the control group than the 2.5 and 50.7 ng/g dose groups (p = 0.016, Table 2.1). Female RA volume and brain mass were not significantly different across dose groups (Table 2.1).

Of the song characteristics, duration and repertoire size were repeatable within individual males across mating trails, with individual explaining 64.8% of the variation in phrase duration ($F_{16,17}$ =4.68, p = 0.001) and 76.9% of the variation in repertoire size ($F_{16,17}$ = 10.13, p <0.0001). Song rate was not repeatable ($F_{16,17}$ = 0.53, p = 0.897, 0%), and so was not considered in further analysis. BDE-99 dosing had no effect on phrase duration ($F_{4,22}$ = 0.75, p = 0.568) or repertoire size ($F_{4,22}$ = 1.06, p = 0.398).

Male behaviors that were repeatable across mating trials include the number of copulation attempts ($F_{47,48} = 3.46$, $p < 0.0001$, 55.2%), the number of billwipes ($F_{47,48} = 5.58$, $p < 0.0001$, 69.6%), whether the male invited the female ($F_{47,48} = 3.89$, $p < 0.0001$, 59.1%), and whether a male sang ($F_{47,48} = 3.85$, $p < 0.0001$, 58.7%). The response of different clean experienced females to the same male was also repeatable ($F_{47,48} = 3.10$, $p < 0.001$, 51.1%). Other mating behaviors were not repeatable and not considered in subsequent analysis.

BDE-99 exposure affected whether males engaged in courtship behavior ($p = 0.013$, FET), with significantly fewer males in the 173.8 ng/g dose group inviting females than expected (Fig. 2.3a). Whether a male sang or not was also significantly affected by BDE-99 exposure ($p = 0.023$, FET), with the 50.7 and 173.8 ng/g dose groups having a lower than expected proportion of birds that sang (Fig. 2.3b). Female response to exposed males was significantly reduced in the highest dose group (Fig. 2.3c; $F_{4,43} = 3.35$, $p = 0.018$). The number of bill wipes and copulation attempts were not significantly affected by BDE-99 exposure ($F_{4,43} = 1.65$, $p = 0.179$ and $F_{4,43} = 1.22$, $p = 0.315$, respectively). Within each dose group, there was no effect of female used on any of the mating trial variables ($p > 0.760$ for all variables)

2.5. Discussion

In this study, we found that early exposure to BDE-99 caused significant effects in female HVC volume, male mating behavior, and the effectiveness of male courtship (as assessed by female response to exposed males), at concentrations that fall within those reported for free-living birds. However, there were no significant effects of BDE-99 on the size of song-control regions of the brain or on the song quality of male birds.

In our tissue residue study, we demonstrated that birds were being exposed in a dose-dependent manner, and that oral exposure to BDE-99 served as a valid dosing method. Control birds had detectable levels of BDE-99, however lipid normalized concentrations of BDE-99 in control birds were approximately 2.5x lower than the lowest dose group, and 42x lower than the highest dose group. The safflower control oil had no detectable PBDEs, so the BDE-99 in control birds was either due to possible cross-

contamination between treatment groups within the nest, or background levels of BDE-99. Plasma BDE-99 was significantly and positively correlated with adipose tissue burdens. In future studies, this close relationship can be used to estimate body burdens from non-lethal plasma sample concentrations of BDE-99.

With respect to environmental relevance, the plasma BDE-99 concentrations in the lowest dose groups are in the same range as plasma samples collected from wild birds. There are reported plasma BDE-99 concentrations of up to 638 ng/g l.w (4.15 ng/g ww, 0.65% avg. lipid) in bald eagle (*Haliaeetus leucocephalus*) nestlings on the North American west coast (McKinney et al. 2006), and of up to 566.23 ng/g lw (8.72 ng/g ww, 1.54% avg. lipid) in Glaucous gulls (*Larus hyperboreus*) in the Norwegian arctic (Verreault et al. 2005). In comparison, the 2.5 ng/g bw/day dose group in our zebra finch study averaged 843.33 ng/g plasma lipid. In peregrine falcon (*Falco peregrinus*) eggs, concentrations of up to 9200 ng BDE-99/g lw have been reported (Lindberg et al. 2004).The nestling plasma concentrations in the highest dose group in the present zebra finch study were estimated at 14079.7 ng BDE-99/g lw. In zebra finches, the lipid normalized BDE-99 concentration is typically four times higher in day-old nestlings compared with eggs from the same mother (M.L.E., unpubl. data), therefore the nestling concentrations in our highest dose group are comparable to reported concentrations of BDE-99 in wild bird eggs. We did not measure BDE-99 in brain tissue, however BDE-99 is able to cross the blood-brain barrier and accumulate in the brain tissue of birds (Naert et al. 2007). It is therefore possible that the effects that we observed may be the result of direct brain exposure to BDE-99, as well as indirect effects of endocrine disruption. Further studies examining the species specific accumulation of BDE-99 in the brain would be informative for determining the underlying mechanisms of effects.

We observed no effects of early exposure to BDE-99 on neuroanatomy of the song-control system or song quality in reproductively mature male zebra finches, which was contrary to our hypotheses. BDE-99 exposure was limited to the nestling period, and there was no *in ovo* exposure in our study. In studies that have observed effects of halogenated organic contaminants on the song-control nuclei, *in ovo* exposure was present (Iwaniuk et al. 2006, Hoogesteijn et al. 2008). Under natural conditions, birds are exposed to environmental contaminants *in ovo* via maternal transfer, and it is possible we did not see effects in male neuroanatomy and song quality due to a lack of *in ovo*

exposure. However significant development of the song-control system occurs post hatch (Bottjer et al. 1985, Mooney and Rao 1994); therefore, there was a window for potential effects of nestling exposure to BDE-99. Future studies examining the effect of *in ovo* exposure to BDE-99 on the song-control system would help identify whether the lack of significant effect in males in our study was the result of timing of exposure. In addition, future studies should examine cellular properties of the song-control regions of exposed birds. Although we found no effect on the size of song-control nuclei in this study, we cannot rule out potential effects on cellular phenotypes.

Although there was no effect of BDE-99 exposure on measured male neuroanatomy, females in the lowest exposure and second highest exposure groups had significantly smaller HVC volumes compared with the control group. As these effects were not dose dependent, it is not clear whether smaller HVC volumes were the result of BDE-99 exposure. Further studies in females examining *in ovo* exposure and cellular properties of the song-control system could help clarify whether there are BDE-99 effects on female neuroanatomy. Sex differences in effects of BDE-99 on the song-control system would not be surprising, as it has been well established that there are profound sex differences in the connectivity, volume, and cellular properties of this system in zebra finches (Nottebohm and Arnold 1976, Ball and MacDougall-Shackleton 2001). There is also some evidence of sex differences in the sensitivity of T4 and sex steroid hormone homeostasis to BDE-99 exposure (Lilienthal et al. 2006, Kuriyama et al. 2007), which could potentially result in sex differences in the song-control system. All existing studies comparing male and female PBDE-induced changes are in mammals, and sex differences in effects of BDE-99 exposure should be further explored in avian systems.

Early exposure to BDE-99 appeared to decrease motivation of zebra finch males to mate, as the proportion of males that participated in courtship behavior was significantly lower in the highest dose group, and the proportion of males that sang was significantly lower in the two highest dose groups. This reduction in mating behavior might be connected with a reduction in sex steroid hormones, as male sexual behavior is mediated by androgens and estrogens (Ball and Balthazart 2004), and BDE-99 has been shown to reduce levels of these hormones in mammals (Lilienthal et al. 2006, Alonso et al. 2010). There are reports in birds that congeners in penta-BDE technical

mixtures may reduce reproductive behavior and circulating testosterone levels (Fernie et al. 2008, Marteinson et al. 2011). In the present zebra finch study, male mating behavior was affected by BDE-99 exposure, but the male song-control system was not, this suggests that the neural circuits that underlie the expression of male sexual behavior may be more sensitive to changes in sex steroids than the song-control nuclei. Future work should examine the effects of early BDE-99 exposure on brain regions controlling sexual motivation and the motivation to sing, such as the medial preoptic area of the hypothalamus (Riters and Ball 1999).

In mating trials, just the males had been exposed to BDE-99, but there were still effects of treatment on female behavior. Clean experienced female zebra finches paired with BDE-99 exposed males responded the least to the highest dose group, which is likely a consequence of the reduced singing and courtship behavior exhibited by males in the higher dose groups. The behavioral changes caused by BDE-99 could potentially lead reduced reproductive success. Observations in captive American kestrels (*Falco sparverius*) studies have shown that unexposed females lowered their investment in the number and size of eggs laid when paired with penta-BDE exposed males that exhibited reduced reproductive behavior, such as fewer copulations and fewer mating calls (Marteinson et al. 2010).

In conclusion, this study shows that early exposure to BDE-99 has adverse long-term effects on the behavior of zebra finches. Although previous studies in birds have looked at the effects of PBDEs on growth, physiology and reproduction (e.g. Fernie et al. 2005, Fernie et al. 2006, Marteinson et al. 2011), ours was the first to look at possible effects of a PBDE on neuroanatomical measures and song quality, and to assess mating behavior using repeatable variables from observer-blind mating trails. Male mating behavior was significantly reduced by BDE-99 exposure, and female response to exposed males was lowest in the highest dose group. Male behavior was more sensitive to BDE-99 exposure than male neuroanatomy, which highlights the importance of considering behavior when assessing biological effects of contaminant exposure. Our study has shown that current environmental concentrations of BDE-99 are high enough to have significant effects on mating behavior in birds, and may therefore be impacting the reproductive rates of free-living birds. These results warrant further investigation in birds into the mechanisms of action of BDE-99 and the consequent effects on behavior

and reproductive success, in order to quantify the importance of BDE-99 exposure in free-living bird populations.

2.6. References

Airey, D. C. and T. J. DeVoogd. (2000). Greater song complexity is associated with augmented song system anatomy in zebra finches. *Neuroreport* 11:2339-2344.

Alonso, V., V. Linares, M. Belles, M. L. Albina, A. Pujol, J. L. Domingo, and D. J. Sanchez. (2010). Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food and Chemical Toxicology* 48:2206-2211.

Ball, G. F. and J. Balthazart. (2004). Hormonal regulation of brain circuits mediating male sexual behavior in birds. *Physiology & Behavior* 83:329-346.

Ball, G. F. and S. A. MacDougall-Shackleton. (2001). Sex differences in songbirds 25 years later: What have we learned and where do we go? *Microscopy Research and Technique* 54:327-334.

Ball, G. F., L. V. Riters, and J. Balthazart. (2002). Neuroendocrinology of song behavior and avian brain plasticity: Multiple sites of action of sex steroid hormones. *Frontiers in Neuroendocrinology* 23:137-178.

Bottjer, S. W., S. L. Glaessner, and A. P. Arnold. (1985). Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. *Journal of Neuroscience* 5:1556-1562.

Brenowitz, E. A. (1991). Altered perception of species-specific song by female birds after lesions of a forebrain nucleus. *Science* 251:303-305.

Chen, D. and R. C. Hale. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International* 36:800-811.

Clayton, N. and E. Prove. (1989). Song discrimination in female zebra finches and Bengalsese finches. *Animal Behaviour* 38:352-354.

Collins, S. A., C. Hubbard, and A. M. Houtman. (1994). Female mate choice in the zebra finch - the effect of male beak color and male song. *Behavioral Ecology and Sociobiology* 35:21-25.

Darnerud, P. O., G. S. Eriksen, T. Johannesson, P. B. Larsen, and M. Viluksela. (2001). Polybrominated diphenyl ethers: Occurrence, dietary exposure, and toxicology. *Environmental Health Perspectives* 109:49-68.

Fernie, K. J., J. L. Shutt, R. J. Letcher, J. I. Ritchie, K. Sullivan, and D. M. Bird. (2008). Changes in reproductive courtship behaviors of adult American kestrels (*Falco sparverius*) exposed to environmentally relevant levels of the polybrominated diphenyl ether mixture, DE-71. *Toxicological Sciences* 102:171-178.

Fernie, K. J., J. L. Shutt, G. Mayne, D. Hoffman, R. J. Letcher, K. G. Drouillard, and I. J. Ritchie. (2005). Exposure to polybrominated diphenyl ethers (PBDEs): Changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicological Sciences* 88:375-383.

Fernie, K. J., J. L. Shutt, I. J. Ritchie, R. J. Letcher, K. Drouillard, and D. M. Bird. (2006). Changes in the growth, but not the survival, of American kestrels (*Falco sparverius*) exposed to environmentally relevant polybrominated diphenyl ethers. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 69:1541-1554.

Frings, C. S., C. A. Queen, R. T. Dunn, and T. W. Fendley. (1972). Improved determination of total serum-lipids by sulfo-phospho-vanillin reaction *Clinical Chemistry* 18:673-674.

Garamszegi, L. Z. and M. Eens. (2004). Brain space for a learned task: strong intraspecific evidence for neural correlates of singing behavior in songbirds. *Brain Research Reviews* 44:187-193.

Gauthier, L. T., C. E. Hebert, D. V. C. Weseloh, and R. J. Letcher. (2008). Dramatic changes in the temporal trends of polybrominated diphenyl ethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982-2006. *Environmental Science & Technology* 42:1524-1530.

Hakk, H., G. Larsen, and E. Klasson-Wehler. (2002). Tissue disposition, excretion and metabolism of 2,2 ',4,4 ',5-pentabromodiphenyl ether (BDE-99) in the male Sprague-Dawley rat. *Xenobiotica* 32:369-382.

Hoogesteijn, A. L., G. V. Kollias, F. W. Quimby, A. P. De Caprio, D. W. Winkler, and T. J. DeVoogd. (2008). Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by Aroclor 1248. *Environmental Toxicology and Chemistry* 27:2071-2075.

Iwaniuk, A. N., D. T. Koperski, K. M. Cheng, J. E. Elliott, L. K. Smith, L. K. Wilson, and D. R. W. Wylie. (2006). The effects of environmental exposure to DDT on the brain of a songbird: Changes in structures associated with mating and song. *Behavioural Brain Research* 173:1-10.

Krebs, J., R. Ashcroft, and M. Webber. (1978). Song repertories and territory defence in great tit. *Nature* 271:539-542.

Kroodsma, D. E. (1976). Reproductive development in a female songbird - differential stimulation by quality of male song. *Science* 192:574-575.

Kuriyama, S. N., A. Wanner, A. A. Fidalgo-Neto, C. E. Talsness, W. Koerner, and I. Chahoud. (2007). Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology* 242:80-90.

Lessells, C. M. and P. T. Boag. (1987). Unrepeatable repeatabilities - a common mistake. *Auk* 104:116-121.

Li, R. and H. Sakaguchi. (1997). Cholinergic innervation of the song control nuclei by the ventral paleostriatum in the zebra finch: a double-labeling study with retrograde fluorescent tracers and choline acetyltransferase immunohistochemistry. *Brain Research* 763:239-246.

Lilienthal, H., A. Hack, A. Roth-Harer, S. W. Grande, and C. E. Talsness. (2006). Effects of developmental exposure to 2,2 ',4,4 ',5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environmental Health Perspectives* 114:194-201.

Lindberg, P., U. Sellstrom, L. Haggberg, and C. A. de Wit. (2004). Higher brominated diphenyl ethers and hexabromocyclododecane found in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Environmental Science & Technology* 38:93-96.

Markman, S., S. Leitner, C. Catchpole, S. Barnsley, C. T. Muller, D. Pascoe, and K. L. Buchanan. (2008). Pollutants increase song complexity and the volume of the brain area HVC in a songbird. *Plos One* 3:6.

Marteinson, S. C., D. M. Bird, J. L. Shutt, R. J. Letcher, I. J. Ritchie, and K. J. Fernie. (2010). Multi-generation effects of polybrominated diphenylethers exposure: embryonic exposure of male American kestrels (*Falco sparverius*) to DE-71 alters reproductive success and behaviors *Environmental Toxicology and Chemistry* 29:1740-1747.

Marteinson, S. C., S. Kimmins, D. M. Bird, J. L. Shutt, R. J. Letcher, I. J. Ritchie, and K. J. Fernie. (2011). Embryonic exposure to the polybrominated diphenyl ether mixture, DE-71, affects testes and circulating testosterone concentrations in adult American kestrels (*Falco sparverius*). *Toxicological Sciences* 121:168-176.

McKinney, M. A., L. S. Cesh, J. E. Elliott, T. D. Williams, D. K. Garcelon, and R. J. Letcher. (2006). Brominated flame retardants and halogenated phenolic compounds in North American west coast bald eaglet (*Haliaeetus leucocephalus*) plasma. *Environmental Science & Technology* 40:6275-6281.

Mooney, R. and M. Rao. (1994). Waiting periods versus early innervation - the development of axonal connections in the zebra finch song system. *Journal of Neuroscience* 14:6532-6543.

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

Nottebohm, F. and A. P. Arnold. (1976). Sexual dimorphism in vocal control areas of songbird brain. *Science* 194:211-213.

Nowicki, S., W. A. Searcy, and S. Peters. (2002). Brain development, song learning and mate choice in birds: a review and experimental test of the "nutritional stress hypothesis". *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* 188:1003-1014.

Riters, L. V. and G. F. Ball. (1999). Lesions to the medial preoptic area affect singing in the male European starling (*Sturnus vulgaris*). *Hormones and Behavior* 36:276-286.

Ryan, S. M. and A. P. Arnold. (1981). Evidence for cholinergic participation in the control of bird song - acetylcholinesterase distribution and muscarinic receptor autoradiography in the zebra finch brain. *Journal of Comparative Neurology* 202:211-219.

Salgado-Commissariat, D., D. B. Rosenfield, and S. A. Helekar. (2004). Nicotine-mediated plasticity in robust nucleus of the archistriatum of the adult zebra finch. *Brain Research* 1018:97-105.

Schulte-Hostedde, A. I., B. Zinner, J. S. Millar, and G. J. Hickling. (2005). Restitution of mass-size residuals: Validating body condition indices. *Ecology* 86:155-163.

Searcy, W. A. (1992). Song repertoire and mate choice in birds. *American Zoologist* 32:71-80.

Shea, S. D., H. Koch, D. Baleckaitis, J. M. Ramirez, and D. Margoliash. (2010). Neuron-specific cholinergic modulation of a forebrain song control nucleus. *Journal of Neurophysiology* 103:733-745.

Tekumalla, P. K., M. Tontonoz, M. A. Hesla, and J. R. Kirn. (2002). Effects of excess thyroid hormone on cell death, cell proliferation, and new neuron incorporation in the adult zebra finch telencephalon. *Journal of Neurobiology* 51:323-341.

Verreault, J., G. V. Gabrielsen, S. G. Chu, D. C. G. Muir, M. Andersen, A. Hamaed, and R. J. Letcher. (2005). Flame retardants and methoxylated and hydroxylated polybrominated diphenyl ethers in two Norwegian Arctic top predators: Glaucous gulls and polar bears. *Environmental Science & Technology* 39:6021-6028.

Viberg, H., A. Fredriksson, and P. Eriksson. (2004). Neonatal exposure to the brominated flame-retardant, 2,2',4,4',5-pentabromodiphenyl ether, decreases cholinergic nicotinic receptors in hippocampus and affects spontaneous behaviour in the adult mouse. *Environmental Toxicology and Pharmacology* 17:61-65.

Viberg, H., A. Fredriksson, and P. Eriksson. (2005). Deranged spontaneous behaviour and decrease in cholinergic muscarinic receptors in hippocampus in the adult rat, after neonatal exposure to the brominated flame-retardant, 2,2 ',4,4 ', 5-pentabromodiphenyl ether (PBDE 99). *Environmental Toxicology and Pharmacology* 20:283-288.

Watson, J. T., E. Adkins-Regan, P. Whiting, J. M. Lindstrom, and T. R. Podleski. (1988). Autoradiographic localization of nicotinic acetylcholine-receptors in the brain of the zebra finch (*Poephila guttata*) *Journal of Comparative Neurology* 274:255-264.

Zann, R. A. (1996). The Zebra Finch: A Synthesis of Field and Laboratory Studies. Oxford University Press, New York.

Table 2.1. ***Brain region volumes (mean mm^3 ± standard error (SE)) and mass (mean g ± SE) for reproductively mature zebra finches exposed to 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) during the nestling period.***

	Dose (ng BDE-99/g body weight/day)						
	0	**2.5**	**15.8**	**50.7**	**173.8**	**F**	***p***
Male							
HVC	0.682 ± 0.041	0.733 ± 0.026	0.734 ± 0.042	0.824 ± 0.058	0.690 ± 0.043	$F_{4,43}$ = 1.70	0.167
RA	0.467 ± 0.023	0.477 ± 0.030	0.473 ± 0.023	0.528 ± 0.024	0.473 ± 0.020	$F_{4,40}$ = 1.08	0.379
Relative AreaX	-0.110 ± 0.093	-0.146 ± 0.163	0.031 ± 0.210	0.432 ± 0.164	-0.211 ± 0.233	$F_{4,36}$ = 2.21	0.088
Mass	0.444 ± 0.008	0.446 ± 0.006	0.452 ± 0.007	0.454 ± 0.008	0.454 ± 0.012	$F_{4,43}$ = 0.39	0.813
Female							
HVC	0.077 ± 0.01[a]	0.036 ± 0.008[b]	0.055 ± 0.01[ab]	0.044 ± 0.006[b]	0.063 ± 0.007[ab]	$F_{4,21}$ = 3.88	0.016
Relative RA	0.008 ± 0.007	-0.004 ± 0.003	0.000 ± 0.003	-0.007 ± 0.003	0.001 ± 0.005	$F_{4,21}$ = 1.26	0.316
Mass	0.418 ± 0.013	0.416 ± 0.013	0.422 ± 0.014	0.402 ± 0.012	0.404 ± 0.007	$F_{4,24}$ = 0.56	0.697

[ab]Significant difference between groups are indicated by different lower case letters ($p < 0.05$)

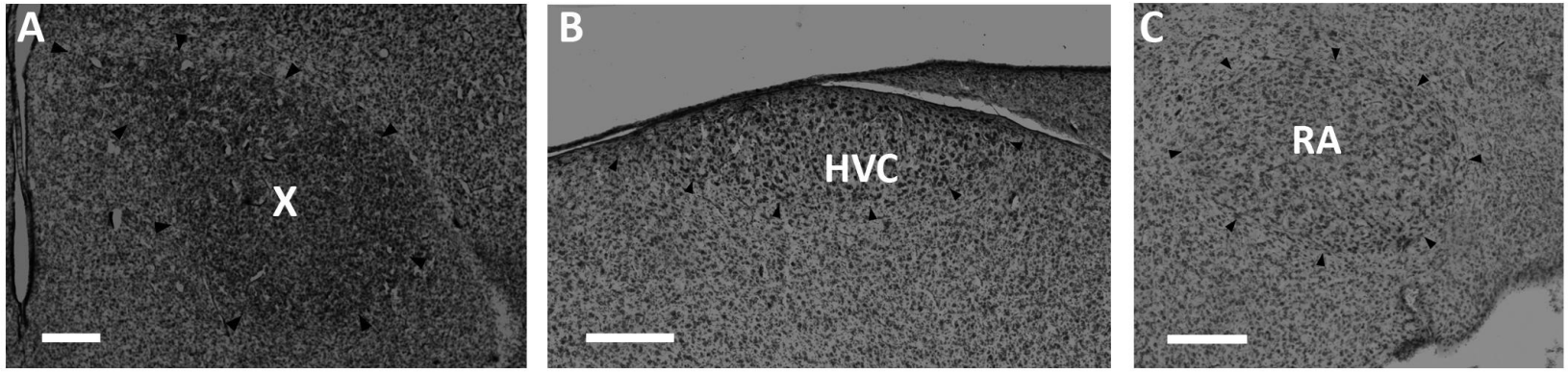

Figure 2.1. ***Photomicrographs showing the three song nuclei measured, Area X (A), HVC (B) and the robust nucleus of the arcopallium (C). The solid triangles indicate the borders delineating each region. Abbreviations refer to the following: RA - robust nucleus of the arcopallium; X - Area X. (Scale bars: 300 µm)***

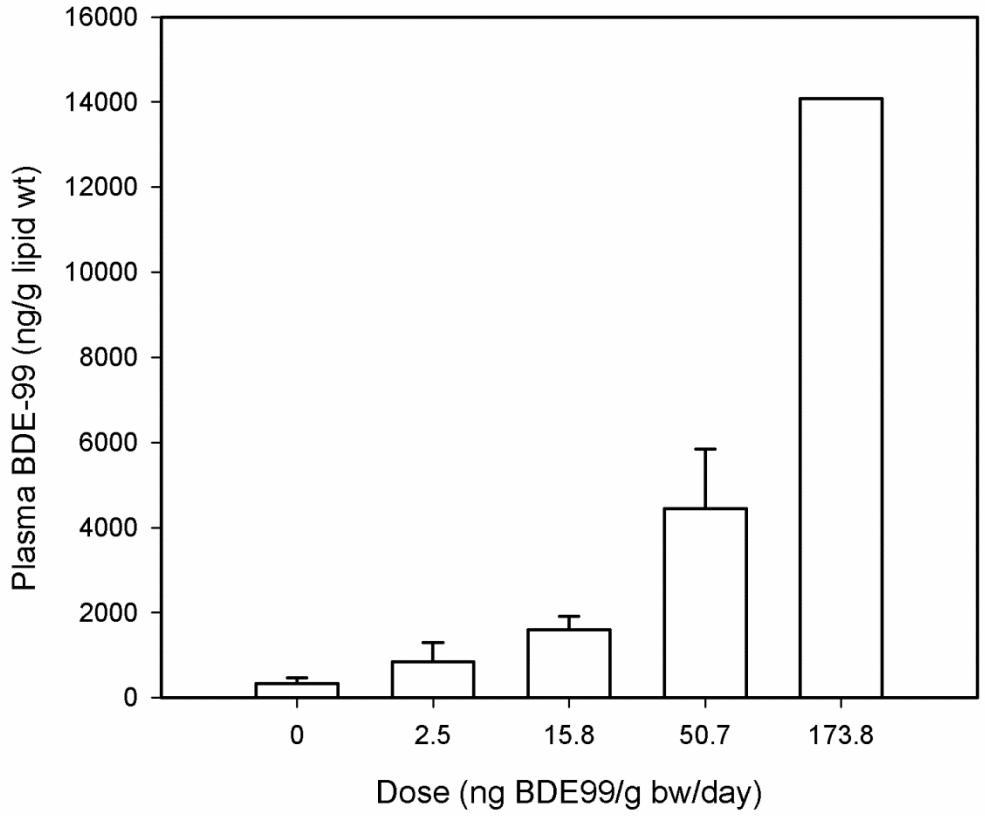

Figure 2.2. ***2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) concentrations measured in zebra finch plasma on day 30 of the tissue residue study following oral exposure to 0 to 50.7 ng BDE-99 over the 21-day nesting period, and predicted plasma concentration of the 173.8 ng/g bw/day dose group.***

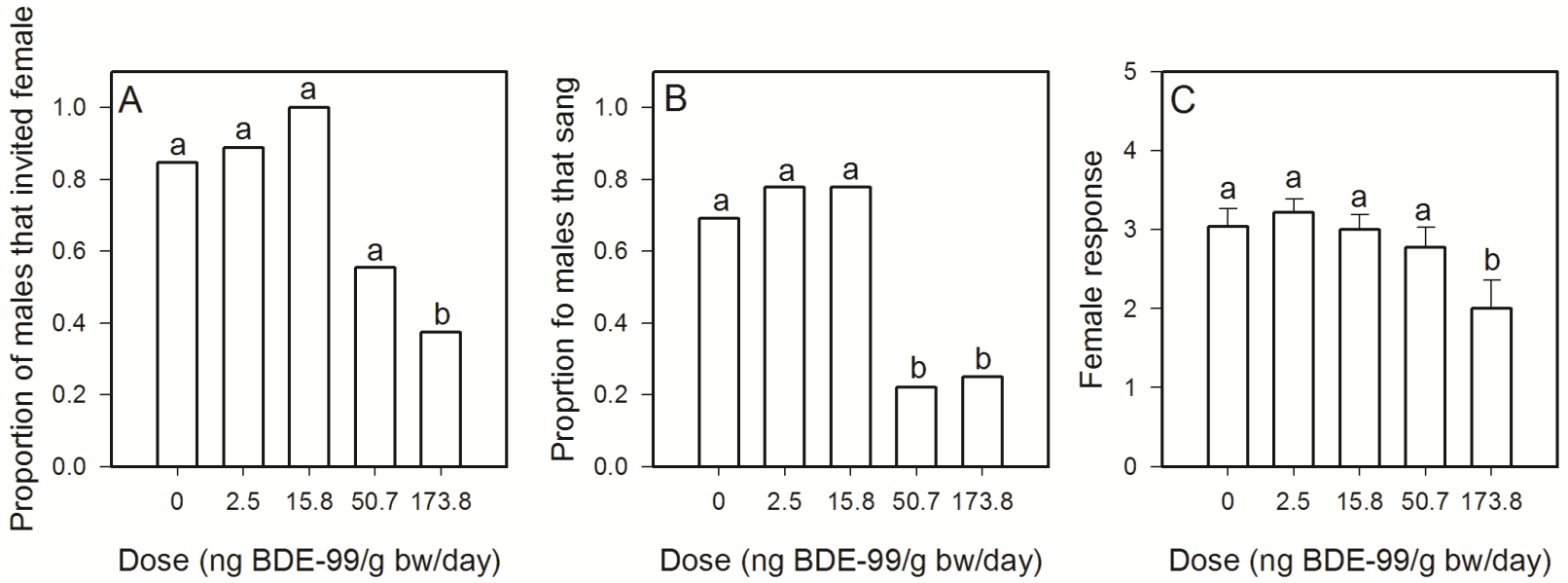

Figure 2.3. ***Results from zebra finch mating trials between reproductively mature males that were orally exposed to 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) over the 21 day nesting period, paired with clean experienced females. (A) The proportion of males that engaged in courtship behavior was significantly lower than expected in the highest dose group (p = 0.013). (B) The proportion of males that sang during mating trials was significantly lower than expected in the two highest dose groups (p = 0.023). (C) The response of clean experienced females to BDE-99 exposed males was significantly reduced in the highest dose group (p = 0.018). Sample size (n) = 13, 9, 9, 9 and 8 for the control, 2.5, 15.8, 50.7 and 173.8 ng/g treatment groups, respectively. Significant difference between groups are indicated on the graph with different lower case letters (p < 0.05).***

3. Early exposure to BDE-99: An assessment of effects on physiology, growth, and reproduction in the zebra finch

3.1. Abstract

Mixtures of polybrominated diphenyl ether (PBDE) congeners have been widely used as additive flame retardants, and 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) is one of the most predominant congeners found in the environment. BDE-99 has been reported in avian egg and tissue samples throughout the world, yet knowledge of its toxicity to birds is minimal. There is evidence in mammals that BDE-99 can cause a wide range of effects, including oxidative stress, thyroid hormone disruption, and changes in reproductive hormones and development. In the present study, the short- and long-term effects of nestling exposure to sub-lethal concentrations of BDE-99 were assessed in a model passerine species, the zebra finch (*Taeniopygia guttata*). Nestlings were orally exposed to 0, 2.5, 15.8, 50.7 or 173.8 ng BDE-99/g bw/day for 21 days, and then raised to reproductive maturity to investigate long-term effects. Early exposure to BDE-99 did not affect hematocrit, oxidative stress, or thyroid hormone concentrations in either the juvenile or adult stages, and there were no effects on chick growth or survival. There was some evidence that BDE-99 exposure caused a dose-dependent delay in timing of reproduction, with the two highest dose groups having more individuals that delayed egg laying, but there were no other effects on reproductive success. In zebra finches, endpoints related to reproductive behavior appear to be the most sensitive to BDE-99 exposure. However, passerines overall appear to be less sensitive than birds of prey or mammals to PBDE exposure.

3.2. Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of hydrophobic and bioaccumulative chemicals that find commercial use as flame retardants, and have become ubiquitous in environmental, human, and wildlife samples (Hites, 2004). 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) is one of the most pervasive congeners (Hites, 2004). In mammals, exposure to BDE-99 has been shown to cause a wide range of adverse effects, including disruption of thyroid hormone homeostasis (Hakk et al., 2002; Kuriyama et al., 2007), oxidative stress (Albina et al., 2010; Belles et al., 2010), and interference in reproductive development and behavior (Talsness et al., 2005; Lilienthal et al., 2006).

BDE-99 is consistently found in avian tissue and egg samples throughout the world (Chen and Hale, 2010), yet its effects in birds are not well known due to a lack of pertinent toxicological literature. There have been some avian exposure studies using penta-BDE mixtures, but these show considerable variation in the sensitivity of bird species to PBDEs. American kestrels (*Falco sparverius*) exposed during development to a penta-BDE mixture containing 27.2% BDE-99 were reported to exhibit some mild effects on growth (Fernie et al., 2006), thyroid hormones and oxidative stress (Fernie et al., 2005), and kestrels exposed in ovo through maternal transfer showed effects on reproductive success (Marteinson et al., 2010). In ovo exposure to a penta-BDE mixture decreased pipping and hatching success in American kestrels, but had no effect on embryo survival endpoints in chickens (*Gallus gallus*) or mallard ducks (*Anas platyrhynchos*) (McKernan et al., 2009). Adult female European starlings (*Sturnus vulgaris*) exposed to a penta-BDE mixture through subcutaneous implants showed limited reproductive and endocrine disruption effects, and no biochemical or hematological effects (Van den Steen et al., 2009; Van den Steen et al., 2010). To our knowledge only one study has exposed birds to BDE-99: in captive mallards, in ovo exposure to BDE-99 altered the vitamin status of chicks (Murvoll et al., 2005). The use of penta-BDE mixtures makes it difficult to identify the specific components responsible for any putative toxic effects. Specifically the contribution of BDE-99, a dominant congener found in the environment and wildlife, to any observed effects of penta-BDE mixtures is

not clear so further studies are required to assess the specific effects of PBDE-99 in birds.

There is considerable evidence in oviparous organisms that early developmental life stages are more sensitive than adult stages to contaminant exposure (e.g. Peterson et al., 1993; Hutchinson et al., 1998). However, the toxicological effects of developmental exposure may not be evident until the individual reaches reproductive maturity, necessitating long-term studies to assess fitness implications of early, developmental exposure to contaminants. Here, we investigate the long-term effects of early developmental exposure to BDE-99 at concentrations relevant to exposure in free-living birds, using the zebra finch (*Taeniopygia guttata*) as a model passerine species. The zebra finch is a useful model to monitor effects of contaminants under controlled laboratory conditions, as it readily breeds in captivity, has a short generation time (4 months), and has been widely used in toxicological dosing studies (Gill et al., 2004; e.g. Albert et al., 2008; Hoogesteijn et al., 2008; Kitulagodage et al., 2011). We have previously shown that reproductively mature male zebra finches exposed to BDE-99 as nestlings exhibit altered mating behavior (Eng et al., 2012). The objectives of the present study were to investigate the effects of early exposure to BDE-99 in the same cohort of zebra finches on non-behavioral endpoints including growth, physiology, and reproduction.

3.3. Materials and methods

3.3.1. Animals and husbandry

The present study was conducted on a captive colony of zebra finches maintained at the Simon Fraser University Animal Care Facility located in Burnaby, British Columbia. Zebra finches were housed in a controlled environment (temperature 19-23°C; humidity 35% to 55%; photoperiod 14 hours light to 10 hours dark; lights on at 07:00). All birds were provided with mixed seed (panicum and white millet 1:2; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate by dry mass), water, grit, and cuttlefish bone (calcium) *ad libitum* plus a multivitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University

Animal Care Committee permit (864B-08) in accordance with guidelines from the Canadian Committee on Animal Care.

For all breeding and chick rearing, the same basic protocol was followed. Experienced adult zebra finches were randomly paired and housed in individual breeding cages (51 × 39 × 43cm) equipped with an external nest box (14 × 14.5 × 20 cm). In addition to the *ad libitum* seed diet, breeding pairs were provided with an egg-food supplement (20.3% protein:6.6% lipid) daily from pairing to clutch completion (2 days after the last egg was laid) and then again during the chick-rearing stage. Nest boxes were checked daily between 09:00 and 11:00 for egg laying, and new eggs were numbered in consecutive order and weighed (0.001 g). Nest boxes were then checked again daily toward the end of the 12- to 14-day incubation period to determine hatching dates. Nestlings were weighed daily and tarsus length was measured using digital callipers (to the nearest 0.01mm) every 5 days until day 30, and again at day 90.

3.3.2. Experimental protocol

Prior to this experiment, a tissue residue study was carried out which validated oral dosing methods and confirmed environmental relevance of doses (see Eng et al. 2012 or Chapter 2 for details). All dosing was done with technical grade BDE-99 (>98% purity, Cambridge Isotope Labs, Andover, MA). The BDE-99 was dissolved in safflower oil (Spectrum organics, Boulder, CO) and the microliter amounts of BDE-99 dissolving solvent (nonane) were evaporated off using a steady stream of purified nitrogen gas. Dosing solutions were analyzed via gas chromatography (GC)-mass spectrometry (MS) (electron capture negative ionization mode [ECNI]), and a full description of the procedures used for the extraction and determination of PBDEs in the dosing solutions have been described elsewhere (Eng et al. 2012). The only bromide ion that was quantifiable was BDE-99. There were four dose levels (2.5, 15.8, 50.7, and 173.8 ng BDE-99/g body weight [bw]/day) and a safflower oil only control group. Within 24 h of hatching, individual chicks within each nest were marked with feather tract removal for identification, and dose levels were randomly assigned within the nest to account for any heritable effects. Nestlings were orally dosed daily from 24hrs after hatching (day 1) until fledging (day 21), using a micropipette. Doses were adjusted daily according to chick mass, with the dose volume being 10 µl/g bw. All surviving nestlings were banded at 10

days of age. Nestlings were returned to the nests immediately after handling and dosing. Once young were independent from parents (day 30) they were placed into cages (102 × 39 × 43 cm) as juvenile groups and separated by sex once adult plumage and bill color started to form. Blood samples were collected at 30 and 90 days of age. All birds were blood-sampled from the brachial vein following puncture with a 26G needle and blood was collected into heparinized hematocrit tubes. Blood samples were centrifuged at 3000g for 10 minutes to separate plasma from the red blood cells, and hematocrit was measured by packed cell volume. Plasma was then stored frozen (-80°C) until analysis.

Once birds reached sexual maturity (day 90), female breeding trials were conducted. Each exposed female was paired with an experienced, clean male and bred following the standard protocol as described above. Following the completion of breeding trials, birds were anesthetized and then exsanguinated, and blood was centrifuged and stored as for day 30 blood samples.

3.3.3. Plasma analysis

Total antioxidant capacity (TAC) was measured using a colorimetric method adapted from Erel (2004), where the colored 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{*+}$) is decolorized by antioxidants in the plasma according to their concentrations and antioxidant capacities. This reaction can be monitored spectrophotometrically, and the final absorbance is inversely related to the TAC of the sample. The reaction is calibrated using Trolox for the standard curve, and results are expressed as mmol Trolox equivalent/L. The total oxidant status (TOS) was measured using a colorimetric method adapted from Erel (2005), where oxidants in the plasma oxidize the ferrous ion–*o*-dianisidine complex to the ferric ion, which then reacts with xylenol orange to make a colored complex. The color intensity can be measured spectrophotometrically, and is proportional to the total amount of oxidant molecules in the plasma sample. The assay is calibrated using hydrogen peroxide (H_2O_2) for the standard curve, and the results are expressed in mmol H_2O_2 equivalent/L. The oxidative status index (OSI) was calculated as the ratio of TOS:TAC for each individual, with high ratios reflecting high oxidative stress. A hen plasma pool was used as an avian standard to compare inter-assay variation. The TAC assay had an average intra-assay coefficient of variation (CV) of 3.8% (n = 2 replicates) and the inter-assay CV was 3.3% (n = 2

assay plates). For TOS the average intra-assay CV was 5.1% (n = 2 replicates) and the inter-assay CV was 3.5% (n = 2 assay plates).

Total and free thyroxine (T4 and FT4) and total and free triiodothyronine (T3 and FT3) concentrations in plasma samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225-300, 1225-300, 125-300, and 1325-300, Lake Forest CA). Kits were validated for parallelism (see Plikaytis et al. 1994 for methods) and recovery using plasma from non-experimental zebra finches. Hen plasma from a plasma pool was included in each plate to assess reproducibility and intra-assay precision, as well as quality control standards (Monobind ML-300) that were verified to be in the expected range. The T4 assay had an average intra-assay coefficient of variation (CV) of 12.0% (n = 2 replicates) and the inter-assay CV was 7.7% (n = 6 assay plates). For FT4 the average intra-assay CV was 12.8% (n = 2 replicates) and the inter-assay CV was 11.4% (n = 3 plates). The average intra-assay CV for T3 was 5.4% (n = 2 replicates) and the inter-assay CV was 5.9% (n = 4 plates). For FT3 the average intra-assay CV was 8.5% (n = 2 replicates) and the inter-assay CV was 8.3% (n = 3 plates).

3.3.4. Statistical analysis

All statistical analyses were carried out using SAS 9.1.3 (SAS Institute 2003). Data were tested for normality and homogeneity of variance following Shapiro-wilk and Levene's tests, and by inspecting q-q plots. Sample sizes of birds that survived to 90 days consisted of 21 (8 female, 13 male), 20 (8 female, 12 male), 19 (5 female, 14 male), 20 (6 female, 14 male), and 20 (12 female, 8 male) nestlings exposed to control oil, 2.5, 15.8, 50.7, and 173.8 ng BDE-99/g bw/day, respectively. To assess the effect of dose on each endpoint, initially sex and dose were both included for each analysis and if there was no effect or interaction with sex, sex was removed from the model. Contingency tables and fisher's exact test (FET) for small sample sizes were used to assess effects of BDE-99 dose on survival. An index of body condition was estimated as the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde et al., 2005). The effect of BDE-99 dose on growth from 0-90 days was assessed using the REPEATED statement in the MIXED procedure. The effect of BDE-99 dose on body condition and physiological variables was assessed using generalized linear models (GLM procedure), and *post-hoc* tests for differences between means were adjusted for

multiple comparisons following the Tukey–Kramer method. A linear regression was used to test whether laying interval increased with dose. Variables that were not parametric were log transformed before analysis. Treatment effects on binary variables (hatching and fledging success) were tested with generalized linear models (GENMOD procedure) in a two level structure with individual eggs nested within broods, using a binomial distribution and the logit link function.

3.4. Results

BDE-99 tissue residue values following oral exposure for the 21-day nestling period have been previously reported in Eng et al. (2012). Based on the tissue residue study, the estimated 30-day plasma concentrations were 332.7 ± 141.0, 843.4 ± 454.0, 1597.3 ± 314.5 and 4450.2 ± 1396.2 ng/g lipid weight (lw) for the 0, 2.5, 15.8, and 50.7 ng BDE-99/g bw/day dose groups, respectively. The predicted concentration for the 173.8 dose group based on the tissue residue data was 14079.69 ng/g lw. Control birds had detectable levels of BDE-99, however lipid normalized concentrations of BDE-99 in control birds were approximately 2.5x lower than the lowest dose group, and 42x lower than the highest dose group. The safflower control oil had no detectable PBDEs, so the BDE-99 in control birds was either due to possible cross-contamination between treatment groups within the nest, or background levels of BDE-99.

There was no effect of BDE-99 dose on day 30 ($F_{4,95}$ = 0.63, p = 0.645) or adult ($F_{4,95}$ = 1.06, p = 0.383) hematocrit levels (Table 1), and there was no effect of sex on day 30 ($F_{1,90}$ = 0.96, p = 0.330) or adult ($F_{1,90}$ = 0.19, p = 0.663) hematocrit levels.

On day 30 there was no effect of BDE-99 dose on TAC ($F_{4,51}$ = 0.61, p = 0.655), TOS ($F_{4,51}$ = 0.93, p = 0.456) or OSI ($F_{4,46}$ = 1.04, p = 0.399; Table 1). Similarly in adults, there was no effect of dose on TAC ($F_{4,70}$ = 1.21, p = 0.312), TOS ($F_{4,83}$ = 0.88, p = 0.478) or OSI ($F_{4,78}$ = 0.81, p = 0.521). Sex had no effect on 30-day TAC or TOS, or on adult TOS (p > 0.082). The 30-day OSI was significantly higher in females than in males ($F_{1,54}$ = 4.19, p = 0.046), but there was no interaction between dose and sex for OSI ($F_{4,46}$ = 0.81, p = 0.524). In adults, males had significantly higher TAC than females ($F_{1,79}$ = 8.12, p = 0.006), and no sex*dose interaction (F4,79 = 1.26, p = 0.295) for TAC. The

adult OSI was significantly higher in females than in males ($F_{1,78} = 5.44$, $p = 0.022$), but there was no interaction between sex and dose ($F_{4,78} = 1.21$, $p = 0.312$).

There was no effect of BDE-99 dose on day 30 T4 ($F_{4,90} = 0.57$, $p = 0.683$), or on adult T4 ($F_{4,88} = 0.46$, $p = 0.765$), T3 ($F_{4,93} = 0.93$, $p = 0.449$), FT4 ($F_{4,70} = 0.38$, $p = 0.487$) or FT3 ($F_{4,51} = 2.09$, $p = 0.095$; Table 1). There was no effect of sex on thyroid hormone concentrations for day 30 T4, or for adult T3 or FT3 ($p > 0.226$). Adult males had significantly higher T4 and FT4 than adult females ($F_{1,88} = 6.89$, $p = 0.010$ and $F_{1,70} = 53.18$, $p < 0.0001$, respectively), but there was no sex*dose interaction for T4 or FT4 ($F_{4,88} = 0.43$, $p = 0.785$ and $F_{4,70} = 0.87$, $p = 0.487$, respectively).

Chick mass over time was not affected by BDE-99 dose ($F_{4,95} = 0.31$, $p = 0.868$) and chick growth was not affected by dose as there was no interaction between dose and age ($F_{28,665} = 0.72$, $p = 0.855$; Fig. 1). There was also no effect of dose on body condition on day 30 ($F_{4,95} = 0.19$, $p = 0.945$) or day 90 ($F_{4,95} = 1.47$, $p = 0.218$). Sex had no effect on body condition or growth ($p > 0.149$). The overall survival of BDE-99 exposed nestlings to reproductive maturity was 93.5%, and there was no effect of dose on survival ($p = 0.755$, FET).

Among experimental females dosed with BDE-99 as nestlings, 89.7% successfully laid eggs when paired with clean experienced males at 90 days of age, and there was no effect of dose on whether a female laid eggs or not ($p = 0.385$, FET). Of the females that laid eggs, there was also no significant difference between dose groups in the clutch size ($F_{4,30} = 0.69$, $p = 0.604$), average egg mass ($F_{4,30} = 0.28$, $p = 0.889$), hatching success ($p > 0.140$), fledging success ($p > 0.123$), or the laying interval (number of days between pairing and laying of the first egg) ($F_{4,30} = 1.57$, $p = 0.207$; Table 2). However, the average laying interval did increase in a dose dependent manner (Table. 2), and there was a significant positive relationship between BDE-99 dose and laying interval ($F_{1,33} = 6.09$, $p = 0.019$, adj. $R^2 = 0.130$; Fig. 2). There was an average delay of approximately 3 days in the 50.7 ng/g bw/day dose group, and about 6 days in the 173.8 ng/g bw/day dose group compared to the control group.

3.5. Discussion

In this study we assessed the long-term effects of early developmental exposure to the polybrominated diphenyl ether congener BDE-99 on selected physiological variables, growth, and reproduction in zebra finches. As previously demonstrated through tissue residue analysis (Eng et al., 2012), our doses resulted in plasma and adipose burdens that fall within the range of concentrations reported in free-living birds (e.g. Lindberg et al., 2004; McKinney et al., 2006; Voorspoels et al., 2007). At these ecologically-relevant dose concentrations, there were no effects on a range of endpoints including hematocrit, oxidative stress, thyroid hormone homeostasis, chick growth and survival. There was also no effect on female reproductive success in adults, although there was evidence that females exposed to higher concentrations of BDE-99 during early development were more likely to delay onset of egg laying. Overall we observed very few effects in 30-day-old or adult birds following early developmental exposure to BDE-99, similar to the results of a study of PBDE exposure in European starlings, which concluded that passerine species may be relatively less sensitive to the effects of PBDEs (Van den Steen et al., 2010).

It has been suggested that hematocrit can be used as an indicator of whether a pollutant has caused changes in the red blood cell volume or number (Handy and Depledge, 1999). In the present study, 30-day and adult hematocrit levels were unaffected by nestling exposure to BDE-99. Other studies that have looked at the effect of BDE-99 or penta-BDE exposure in birds also found no effect on hematocrit (Murvoll et al., 2005; Van den Steen et al., 2010; Winter et al., in press). In contrast, there is some evidence in mammals that PBDE exposure has hematological effects. In free living harbor seals (*Phoca vitulina*) the red blood cell count was inversely related to ΣPBDEs in the blood (Neale et al., 2005), and in ranch mink (*Mustela vison*) hematocrit decreased following exposure to a commercial PBDE mixture (Martin et al., 2007). However, at least in the mink study the dose groups that showed an effect had tissue PBDE concentrations approximately three orders of magnitude higher than in our study (18,505 µg/g lw and 27,909 µg/g lw versus up to 14079.69 ng/g lw in the present study). Alternatively, it is possible that the red blood cells in birds may be less sensitive to PBDE exposure than in mammals, as the hematological cells differ between birds and

mammals. Notably, avian erythrocytes are nucleated whereas mammalian erythrocytes are not (Glomski and Pica, 2011).

Oxidative stress results from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, and an increase in ROS can potentially damage cell components or activate specific signaling pathways (Finkel and Holbrook, 2000). Oxidative stress has been proposed as one of the potential mechanisms for the toxic effects of BDE-99 (Albina et al., 2010; Belles et al., 2010). Adult rats acutely exposed to BDE-99 (single dose of 0, 600 or 1200 ng/g bw) showed decreased antioxidant capacity in the brain, kidney and liver (Albina et al., 2010; Belles et al., 2010), and altered oxidative stress markers in the serum and erythrocytes (Alonso et al., 2010). Similarly, an in vitro study in a human cell line found that BDE-99 exposure increased markers of ROS formation and lipid peroxidation (Tagliaferri et al., 2010). In contrast, we found that BDE-99 exposed zebra finches did not show changes in plasma oxidative stress compared to control birds at either the juvenile (day 30) or adult stages. Other studies in birds have also found limited evidence supporting an effect of PBDEs on oxidative state. A study in American kestrels reported that in ovo and nestling exposure to a penta-BDE mixture (18.7 µg PBDE per egg, 15.6 ng/g bw for 29 days post-hatch) increased markers of oxidative stress in the liver (Fernie et al., 2005); however, the increase was not significant (p = 0.09). In a passerine species, the European starling, adults exposed to a penta-BDE mixture through subcutaneous implants (~1740 ng/g bw) showed no significant differences from control birds in the total plasma antioxidative capacity (Van den Steen et al., 2010), which is consistent with our results. Overall, there is little evidence that PBDE exposure in birds causes oxidative stress, which suggests that birds may produce fewer ROS following PBDE exposure than mammals.

BDE-99 and its hydroxylated (OH) metabolites are structurally similar to thyroid hormones, and have the potential to disrupt thyroid hormone homeostasis through various mechanisms, such as competitive displacement of thyroid hormones from thyroid hormone transport proteins (Ucan-Marin et al., 2009). There is evidence in mammals that BDE-99 can alter T4 concentrations (Hakk et al., 2002; Kuriyama et al., 2007). However, studies of free living birds have found that in bald eagles (*Haliaeetus leucocephalus*) and glaucous gulls (*Larus hyperboreus*), there were no associations between PBDE concentrations and thyroid hormone concentrations (Verreault et al.,

2007; Cesh et al., 2010), although circulating T3 concentrations in nestling bald eagles were positively correlated with OH-PBDEs (Cesh et al., 2010). In laboratory studies of birds, there is limited evidence that penta-BDE mixtures can affect thyroid hormones. Adult female European starlings exposed to a penta-BDE mixture through subcutaneous implants showed no differences in T3 and T4 concentrations between control and exposed groups in the six months after implantation. When egg-laying starlings were excluded from these results, there was a non-significant trend for lower T3 two weeks after implantation, which disappeared by two months after implantation (Van den Steen et al., 2010). American kestrels exposed to a penta-BDE mixture through egg injection and nestling gavage had lower circulating T4 than controls, although the difference was not significant, and their T3 concentrations and thyroid glandular structure were unaffected by exposure (Fernie et al., 2005). There were also no effects on T3 or T4 concentrations in American kestrels exposed to penta-BDEs through maternal transfer (low exposure 289 ng/g ww, high exposure 1131 ng/g ww; Marteinson et al., 2011). Thyroid hormones in zebra finches exposed in ovo to BDE-99 (10, 100 or 1000 ng/g ww) showed no significant differences between dosed and control birds (Winter et al., in press). Similarly in our study, thyroid hormone homeostasis was unaffected by early exposure to BDE-99. Overall, the majority of field and laboratory studies in birds have not found significant effects of PBDE exposure on thyroid hormone homeostasis. The lack of significant effects may be because PBDE concentrations are too low to disrupt thyroid hormone binding to transport proteins or receptor sites. It has recently been demonstrated that OH-PBDE metabolites bind to gull thyroid hormone transport proteins (transthyretin and albumin) with a higher affinity than T3 or T4 do, but non-OH-PBDEs have a lower binding affinity than T3 and T4, and reported environmental concentrations of OH-PBDEs are likely too low to have a substantial effect on T3 or T4 binding (Ucan-Marin et al., 2009; Ucan-Marin et al., 2010).

In our study, the chick growth and survival of zebra finches exposed as nestlings to BDE-99 was not affected. In immature birds, thyroid hormones are important for growth and development (McNabb, 2007); therefore, in the present study the absence of effect of BDE-99 exposure on thyroid hormone homeostasis is consistent with the lack of effect on growth. In ovo exposure to BDE-99 in zebra finch had no effect on growth in the first-generation, BDE-treated chicks (Winter et al., in press). However, the offspring

from the high-dose group of these in ovo exposed birds had significantly lower body weight as juveniles, although this effect disappeared by sexual maturity (Winter et al., in press). Therefore, we might have observed effects of BDE-99 exposure in nestlings if we had followed multiple generations. Alternatively, zebra finches may be more sensitive to in ovo exposure than nestling exposure. Additionally, in contrast to the decreased weight observed in the offspring of zebra finches exposed in ovo to BDE-99 by Winter et al. (in press), penta-BDE exposed female kestrels were reported to grow faster and were larger than control birds (Fernie et al., 2006). In the kestrels, the BDE-99 congener was not positively associated with weight gain. The absence of an effect of BDE-99 on survival in the present study is consistent with a study in zebra finches that assessed the long-term effects of in ovo exposure to BDE-99 (Winter et al., in press), as well as with a penta-BDE exposure study of American kesterels exposed in ovo and as nestlings (Fernie et al., 2006), that also found no effect of exposure on survival.

We did find some effect of early developmental BDE-99 exposure on the timing component of reproduction in these same birds as adults: as BDE-99 dose increased, the laying interval increased. Mean laying interval was not statistically different among dose groups, likely due to the large variability in laying interval in the two highest dose groups. Our results agree with those from zebra finches exposed to BDE-99 in ovo, which also showed a trend of longer laying intervals in exposed birds compared to control birds (Winter et al., in press). If BDE-99 were to similarly delay onset of egg laying in free-living birds this could have important consequences since timing of breeding is one of the main determinants of reproductive success and lifetime fitness (Williams, 2012). Evidence that PBDEs increase laying interval is variable from other experimental studies of captive birds. Following exposure to a penta-BDE mixture, American kestrels exhibited longer laying intervals (Fernie et al., 2009), while European starlings did not (Van den Steen et al., 2009). Nevertheless, our data suggest that mechanisms related to timing of breeding should be included as an important endpoint in future studies of effects of PBDEs.

Other than the effect on timing of breeding, we did not observe adverse effects of early exposure to BDE-99 on any other reproductive endpoints including breeding propensity, clutch size, egg mass, and hatching or fledging success. In contrast, zebra finches that were exposed to BDE-99 in ovo had significantly smaller clutch sizes than

control birds (Winter et al., in press), which again suggests that the impact of BDE-99 might depend on the timing of exposure relative to offspring development. In adult European starlings exposed to penta-BDEs, exposed birds laid larger eggs than controls, and there was a non-significant trend for exposed birds to lay less frequently (Van den Steen et al., 2009), but there were no effects on any other reproductive measures or on sex steroid hormones (Van den Steen et al., 2010). In American kestrels, both in ovo and adult exposure to PBDEs have been reported to cause negative effects on reproductive success and mating behavior (Fernie et al., 2008; Fernie et al., 2009; Marteinson et al., 2010; Marteinson et al., 2011). In free living birds, a negative relationship between ΣPBDEs in eggs and average brood size was reported for peregrine falcons (*Falco peregrinus*; Johansson et al., 2009) , and there is correlative evidence that high concentrations (>1000 ng/g ww in eggs) of ΣPBDEs may negatively affect reproductive productivity in ospreys (*Pandion haliaetus*; Henny et al., 2009). BDE-99 exposure also has significant effects on reproductive variables in mammals. Gestational and lactational exposure to BDE-99 (single dose to the mother of 60 or 300 ng/g bw) resulted in reproductive tract changes and lower fertility in female rats (Talsness et al., 2005). In male rats exposed to BDE-99 (single dose 600 or 1200 ng/g bw) as adults, circulating sex steroids were significantly decreased (Alonso et al., 2010). A separate study of developmental BDE-99 exposure also showed a decrease in circulating sex steroids and evidence of feminization in male rats, and a decreased number of ovarian follicles in female rats, although doses were relatively high (1000 or 10 000 ng/g bw injected gestational days 10-18; Lilienthal et al., 2006). Overall, studies in passerines have reported fewer reproductive effects relative to birds of prey and mammals, which again suggests that passerines may be less sensitive to PBDE exposure. Further studies of toxicity of developmental exposure to BDE-99 or related chemicals that examine additional reproductive endpoints such as sex steroid hormone concentrations, histology of the reproductive tract, and female mating behavior could be informative for understanding effects on avian reproduction.

In conclusion, early developmental exposure to BDE-99 at concentrations relevant to free-living birds had very few long-term negative effects in zebra finches. Similarly, a study in European starlings concluded passerines were less sensitive than other bird species to PBDE exposure (Van den Steen et al., 2010). Another possibility for

the lack of observed effects in our study may be that while our doses covered a range of concentrations comparable to those reported in free-living birds, higher concentrations or mixtures of PBDE congeners may be needed to cause more overt adverse effects. Alternatively, the embryonic life stage may be more sensitive than the nestling period, and *in ovo* exposure may be necessary to see effects on physiology and reproduction. Future studies looking at higher concentrations and timing of exposure would be needed to confirm this. We did observe negative effects on laying behavior in female zebra finches exposed to BDE-99 as nestlings, and have previously reported that BDE-99 exposure reduced the sexual behavior of male zebra finches (Eng et al., 2012). Endpoints related to reproduction, particularly behavior, might therefore be the most sensitive to BDE-99 exposure in zebra finches and should be included as endpoints in future studies. There is also now a body of evidence in mammals and other bird species that exposure to BDE-99 or PBDE mixtures can have negative consequences. All together, these results suggest that wild birds, particularly birds of prey, in environments with high PBDE exposure could suffer from negative effects on reproductive performance.

3.6. References

Albert, C., T. D. Williams, C. A. Morrissey, V. W. M. Lai, W. R. Cullen, and J. E. Elliott. (2008). Tissue uptake, mortality, and sublethal effects of monomethylarsonic acid (MMA(V)) in nestling zebra finches (*Taeniopygia guttata*). *Journal of Toxicology and Environmental Health-Part a-Current Issues* 71:353-360.

Albina, M. L., V. Alonso, V. Linares, M. Belles, J. J. Sirvent, J. L. Domingo, and D. J. Sanchez. (2010). Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. *Toxicology* 271:51-56.

Alonso, V., V. Linares, M. Belles, M. L. Albina, A. Pujol, J. L. Domingo, and D. J. Sanchez. (2010). Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food and Chemical Toxicology* 48:2206-2211.

Belles, M., V. Alonso, V. Linares, M. L. Albina, J. J. Sirvent, J. L. Domingo, and D. J. Sanchez. (2010). Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99. *Toxicology Letters* 194:1-7.

Cesh, L. S., K. H. Elliott, S. Quade, M. A. McKinney, F. Maisoneuve, D. K. Garcelon, C. D. Sandau, R. J. Letcher, T. D. Williams, and J. E. Elliott. (2010). Polyhalogenated aromatic hydrocarbons and metabolites: Relation to circulating thyroid hormone and retinol in nestling bald eagles (*Haliaeetus leucocephalus*). *Environmental Toxicology and Chemistry* 29:1301-1310.

Chen, D. and R. C. Hale. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International* 36:800-811.

Eng, M. L., J. E. Elliott, S. A. MacDougall-Shackleton, R. J. Letcher, and T. D. Williams. (2012). Early exposure to 2,2 ',4,4 ',5-Pentabromodiphenyl ether (BDE-99) affects mating behavior of zebra finches. *Toxicological Sciences* 127:269-276.

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry* 37:277-285.

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry* 38:1103-1111.

Fernie, K. J., J. L. Shutt, R. J. Letcher, I. J. Ritchie, and D. M. Bird. (2009). Environmentally relevant concentrations of DE-71 and HBCD alter eggshell thickness and reproductive success of American kestrels. *Environmental Science & Technology* 43:2124-2130.

Fernie, K. J., J. L. Shutt, R. J. Letcher, J. I. Ritchie, K. Sullivan, and D. M. Bird. (2008). Changes in reproductive courtship behaviors of adult American kestrels (*Falco sparverius*) exposed to environmentally relevant levels of the polybrominated diphenyl ether mixture, DE-71. *Toxicological Sciences* 102:171-178.

Fernie, K. J., J. L. Shutt, G. Mayne, D. Hoffman, R. J. Letcher, K. G. Drouillard, and I. J. Ritchie. (2005). Exposure to polybrominated diphenyl ethers (PBDEs): Changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicological Sciences* 88:375-383.

Fernie, K. J., J. L. Shutt, I. J. Ritchie, R. J. Letcher, K. Drouillard, and D. M. Bird. (2006). Changes in the growth, but not the survival, of American kestrels (*Falco sparverius*) exposed to environmentally relevant polybrominated diphenyl ethers. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 69:1541-1554.

Finkel, T. and N. J. Holbrook. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408:239-247.

Gill, H., T. D. Williams, C. A. Bishop, K. M. Cheng, and J. E. Elliott. (2004). Effects of azinphos-methyl on cholinergic responses and general health in zebra finches (*Taeniopygia guttata*) after previous treatment with p,p '-DDE. *Archives of Environmental Contamination and Toxicology* 48:118-126.

Glomski, C. A. and A. Pica. (2011). The Avian Erythrocyte: Its Phylogenetic Odyssey. Science Publishers.

Hakk, H., G. Larsen, and E. Klasson-Wehler. (2002). Tissue disposition, excretion and metabolism of 2,2 ',4,4 ',5-pentabromodiphenyl ether (BDE-99) in the male Sprague-Dawley rat. *Xenobiotica* 32:369-382.

Handy, R. D. and M. H. Depledge. (1999). Physiological responses: Their measurement and use as environmental biomarkers in ecotoxicology. *Ecotoxicology* 8:329-349.

Henny, C. J., J. L. Kaiser, R. A. Grove, B. L. Johnson, and R. J. Letcher. (2009). Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology* 18:802-813.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environmental Science & Technology* 38:945-956.

Hoogesteijn, A. L., G. V. Kollias, F. W. Quimby, A. P. De Caprio, D. W. Winkler, and T. J. DeVoogd. (2008). Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by Aroclor 1248. *Environmental Toxicology and Chemistry* 27:2071-2075.

Hutchinson, T. H., J. Solbe, and P. J. Kloepper-Sams. (1998). Analysis of the ECETOC aquatic toxicity (EAT) database - III - Comparative toxicity of chemical substances to different life stages of aquatic organisms. *Chemosphere* 36:129-142.

Johansson, A. K., U. Sellstrom, P. Lindberg, A. Bignert, and C. A. de Wit. (2009). Polybrominated diphenyl ether congener patterns, hexabromocyclododecane, and brominated biphenyl 153 in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Environmental Toxicology and Chemistry* 28:9-17.

Kitulagodage, M., W. A. Buttemer, and L. B. Astheimer. (2011). Adverse effects of fipronil on avian reproduction and development: maternal transfer of fipronil to eggs in zebra finch Taeniopygia guttata and in ovo exposure in chickens Gallus domesticus. *Ecotoxicology* 20:653-660.

Kuriyama, S. N., A. Wanner, A. A. Fidalgo-Neto, C. E. Talsness, W. Koerner, and I. Chahoud. (2007). Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology* 242:80-90.

Lilienthal, H., A. Hack, A. Roth-Harer, S. W. Grande, and C. E. Talsness. (2006). Effects of developmental exposure to 2,2 ',4,4 ',5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environmental Health Perspectives* 114:194-201.

Lindberg, P., U. Sellstrom, L. Haggberg, and C. A. de Wit. (2004). Higher brominated diphenyl ethers and hexabromocyclododecane found in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Environmental Science & Technology* 38:93-96.

Marteinson, S. C., D. M. Bird, J. L. Shutt, R. J. Letcher, I. J. Ritchie, and K. J. Fernie. (2010). Multi-generation effects of polybrominated diphenylethers exposure: embryonic exposure of male American kestrels (*Falco sparverius*) to DE-71 alters reproductive success and behaviors *Environmental Toxicology and Chemistry* 29:1740-1747.

Marteinson, S. C., S. Kimmins, D. M. Bird, J. L. Shutt, R. J. Letcher, I. J. Ritchie, and K. J. Fernie. (2011). Embryonic exposure to the polybrominated diphenyl ether mixture, DE-71, affects testes and circulating testosterone concentrations in adult American kestrels (*Falco sparverius*). *Toxicological Sciences* 121:168-176.

Martin, P. A., G. J. Mayne, S. J. Bursian, G. Tomy, V. Palace, C. Pekarik, and J. Smits. (2007). Immunotoxicity of the commercial polybrominated diphenyl ether mixture DE-71 in ranch mink (*Mustela vison*). *Environmental Toxicology and Chemistry* 26:988-997.

McKernan, M. A., B. A. Rattner, R. C. Hale, and M. A. Ottinger. (2009). Toxicity of polybrominated diphenyl ethers (DE-71) in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) embryos and hatchlings. *Environmental Toxicology and Chemistry* 28:1007-1017.

McKinney, M. A., L. S. Cesh, J. E. Elliott, T. D. Williams, D. K. Garcelon, and R. J. Letcher. (2006). Brominated flame retardants and halogenated phenolic compounds in North American west coast bald eaglet (*Haliaeetus leucocephalus*) plasma. *Environmental Science & Technology* 40:6275-6281.

McNabb, F. M. A. (2007). The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Critical Reviews in Toxicology* 37:163-193.

Murvoll, K. M., B. M. Jenssen, and J. U. Skaare. (2005). Effects of pentabrominated diphenyl ether (PBDE-99) on vitamin status in domestic duck (*Anas platyrhynchos*) hatchlings. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 68:515-533.

Neale, J. C. C., F. M. D. Gulland, K. R. Schmelzer, J. T. Harvey, E. A. Berg, S. G. Allen, D. J. Greig, E. K. Grigg, and R. S. Tjeerdema. (2005). Contaminant loads and hematological correlates in the harbor seal (*Phoca vitulina*) of San Francisco Bay, California. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 68:617-633.

Peterson, R. E., H. M. Theobald, and G. L. Kimmel. (1993). Developmental and reproductive toxicity of dioxins and related compounds - Cross-species comparisons. *Critical Reviews in Toxicology* 23:283-335.

Plikaytis, B. D., P. F. Holder, L. B. Pais, S. E. Maslanka, L. L. Gheesling, and G. M. Carlone. (1994). Determination of parallelism and nonparallelism in bioassay dilution curves. *Journal of Clinical Microbiology* 32:2441-2447.

Schulte-Hostedde, A. I., B. Zinner, J. S. Millar, and G. J. Hickling. (2005). Restitution of mass-size residuals: Validating body condition indices. *Ecology* 86:155-163.

Tagliaferri, S., A. Caglieri, M. Goldoni, S. Pinelli, R. Alinnovi, D. Poli, C. Pellacani, G. Giordano, A. Mutti, and L. G. Costa. (2010). Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells. *Toxicology In Vitro* 24:116-122.

Talsness, C. E., M. Shakibaei, S. N. Kuriyama, S. W. Grande, A. Sterner-Kock, P. Schnitker, C. de Souza, K. Grote, and I. Chahoud. (2005). Ultrastructural changes observed in rat ovaries following in utero and lactational exposure to low doses of a polybrominated flame retardant. *Toxicology Letters* 157:189-202.

Ucan-Marin, F., A. Arukwe, A. Mortensen, G. W. Gabrielsen, G. A. Fox, and R. J. Letcher. (2009). Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species (*Larus argentatus* and *Larus hyperboreus*). *Toxicological Sciences* 107:440-450.

Ucan-Marin, F., A. Arukwe, A. S. Mortensen, G. W. Gabrielsen, and R. J. Letcher. (2010). Recombinant albumin and transthyretin transport proteins from two gull species and human: Chlorinated and brominated contaminant binding and thyroid hormones. *Environmental Science & Technology* 44:497-504.

Van den Steen, E., M. Eens, A. Covaci, A. C. Dirtu, V. L. B. Jaspers, H. Neels, and R. Pinxten. (2009). An exposure study with polybrominated diphenyl ethers (PBDEs) in female European starlings (*Sturnus vulgaris*): Toxicokinetics and reproductive effects. *Environmental Pollution* 157:430-436.

Van den Steen, E., M. Eens, A. Geens, A. Covaci, V. M. Darras, and R. Pinxten. (2010). Endocrine disrupting, haematological and biochemical effects of polybrominated diphenyl ethers in a terrestrial songbird, the European starling (*Sturnus vulgaris*). *Science of the Total Environment* 408:6142-6147.

Verreault, J., C. Bech, R. J. Letcher, E. Ropstad, E. Dahl, and G. W. Gabrielsen. (2007). Organohalogen contamination in breeding glaucous gulls from the Norwegian Arctic: Associations with basal metabolism and circulating thyroid hormones. *Environmental Pollution* 145:138-145.

Voorspoels, S., A. Covaci, V. L. B. Jaspers, H. Neels, and P. Schepens. (2007). Biomagnification of PBDEs in three small terrestrial food chains. *Environmental Science & Technology* 41:411-416.

Williams, T. D. (2012). Physiological Adaptations for Breeding in Birds. Princeton University Press, Princeton.

Williams, T. D. and J. K. Christians. (2003). Experimental dissociation of the effects of diet, age and breeding experience on primary reproductive effort in zebra finches *Taeniopygia guttata*. *Journal of Avian Biology* 34:379-386.

Winter, V., T. D. Williams, and J. E. Elliott. (in press). A three-generational study of in ovo exposure to PBDE-99 in the zebra finch. *Environmental Toxicology and Chemistry*.

Table 3.1. Physiological variables in juvenile (30-day old) and adult zebra finches that were orally exposed to BDE-99 for the 21 day nesting period. Mean (SE). There was no significant effect of dose on any variable (p ≥ 0.095). TAC = total antioxidant capacity, TOS = total oxidant status, OSI = oxidative status index, T4 = total thyroxine, T3 = total triiodothyronine, FT4 = free thyroxine, FT3 = free triiodothyronine.

	Dose (ng/g BDE-99/g bw/day)				
	0	**2.5**	**15.8**	**50.7**	**173.8**
Day 30					
Hematocrit	0.53 (0.01)	0.52 (0.01)	0.51 (0.02)	0.52 (0.01)	0.51 (0.01)
TAC (m*M* Trolox)	1.33 (0.1)	1.29 (0.07)	1.16 (0.07)	1.31 (0.07)	1.29 (0.15)
TOS (m*M* H_2O_2)	0.47 (0.06)	0.5 (0.07)	0.42 (0.05)	0.36 (0.04)	0.43 (0.06)
OSI	0.39 (0.07)	0.41 (0.08)	0.37 (0.04)	0.29 (0.04)	0.37 (0.07)
T4 (nmol/L)	11.03 (1.05)	10.72 (1.25)	14.36 (2.35)	12.74 (2.98)	11.4 (1.3)
Adult					
Hematocrit	0.54 (0.01)	0.53 (0.01)	0.53 (0.01)	0.53 (0.01)	0.55 (0.01)
TAC (m*M* Trolox)	1.53 (0.09)	1.3 (0.09)	1.54 (0.08)	1.48 (0.12)	1.24 (0.12)
TOS (m*M* H_2O_2)	0.68 (0.08)	0.55 (0.06)	0.48 (0.06)	0.57 (0.11)	0.69 (0.13)
OSI	0.48 (0.07)	0.49 (0.09)	0.32 (0.04)	0.51 (0.13)	0.95 (0.39)
T4 (nmol/L)	5.07 (0.99)	5.83 (1.29)	5.22 (0.87)	7.18 (1.33)	5.75 (1.15)
T3 (nmol/L)	1.3 (0.07)	1.29 (0.12)	1.33 (0.09)	1.21 (0.08)	1.47 (0.11)
FT4 (pmol/L)	2.81 (0.5)	2.69 (0.4)	2.89 (0.46)	2.67 (0.41)	2.09 (0.43)
FT3 (pmol/L)	2.58 (0.13)	2.94 (0.19)	2.47 (0.19)	2.49 (0.26)	3.21 (0.27)

Table 3.2. Reproductive variables in adult zebra finches that were orally exposed to BDE-99 for the 21 day nesting period. Mean (SE). There was no significant effect of dose for any variable (p > 0.123).

	Dose (ng/g BDE-99/g bw/day)				
Reproductive variable	**0**	**2.5**	**15.8**	**50.7**	**173.8**
Laying interval	7.13 (0.74)	7.83 (0.98)	8.50 (0.65)	10.67 (3.31)	13.00 (2.46)
Clutch size	6.25 (0.53)	5.17 (0.70)	5.25 (0.25)	5.83 (0.31)	5.36 (0.53)
Average egg mass (grams)	1.06 (0.04)	1.01 (0.03)	1.05 (0.05)	1.02 (0.03)	1.02 (0.03)
Proportion of eggs to hatch per clutch	0.58 (0.14)	0.74 (0.15)	0.65 (0.22)	0.25 (0.16)	0.58 (0.12)
Proportion of hatched eggs to fledge per brood	0.89 (0.07)	0.9 (0.07)	0.81 (0.10)	0.38 (0.38)	0.82 (0.13)

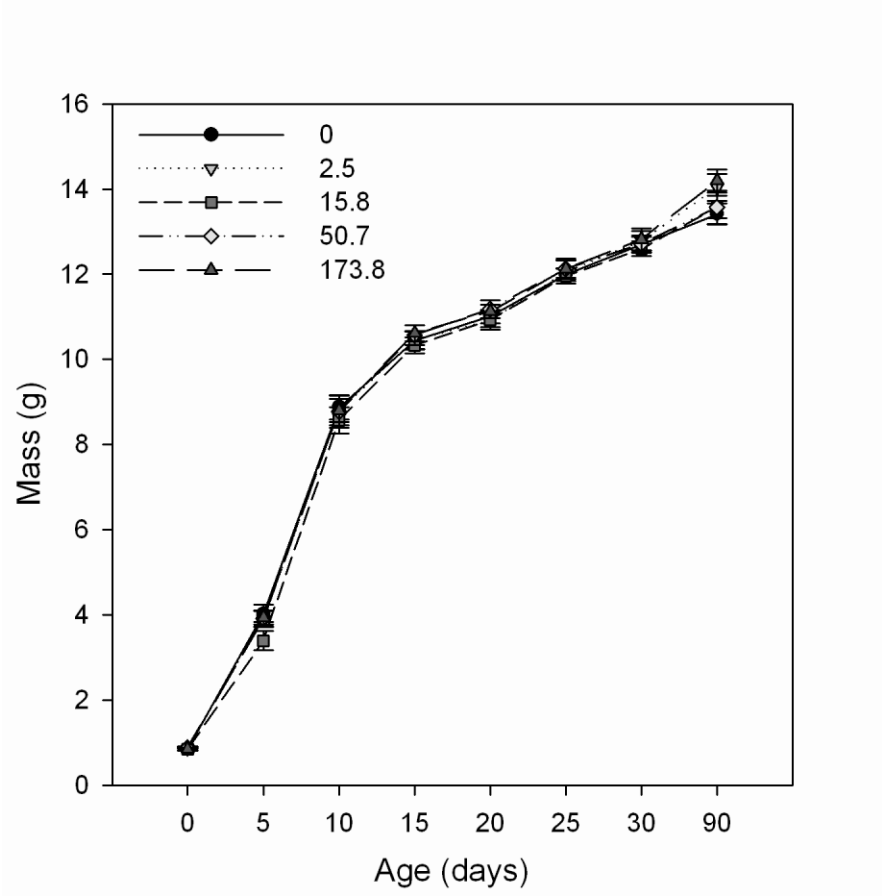

Figure 3.1. ***Growth of zebra finches orally exposed to 0, 2.5, 15.8, 50.7, or 173.8 ng BDE-99/g bw/day for the 21-day nesting period. There was no interaction between dose and age ($F_{28,665} = 0.72$, $p = 0.855$)***

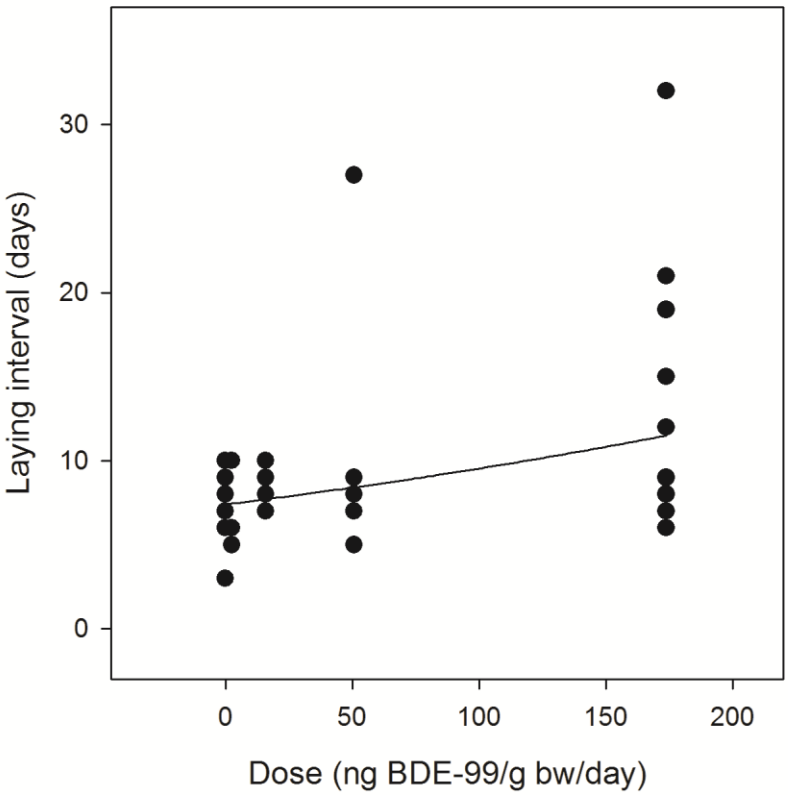

Figure 3.2. ***Laying interval (number of days between pairing and laying of the first egg) of reproductively mature female zebra finches that were exposed to BDE-99 for the 21-day nestling period. Laying interval increases as dose increases according to the equation: laying interval = $10^{(0.8693+0.0011*dose)}$***

4. Assessment of concentrations and effects of organohalogen contaminants in a terrestrial passerine, the European starling

4.1. Abstract

European starlings (*Sturnus vulgaris*) are a valuable model species for the assessment of concentrations and effects of environmental contaminants in terrestrial birds. Polybrominated diphenyl ethers (PBDEs) are found in birds throughout the world, but relatively little is known of their concentrations or effects in free-living terrestrial passerines. The objectives of this study were to use a nest box population of European starlings in Langley, British Columbia, to 1) measure the variation in egg concentrations of persistent organohalogen contaminants at an agricultural site, and 2) assess whether individual nest variation in PBDE exposure was related to reproductive parameters, as well as maternal or nestling characteristics including body condition, thyroid hormones, oxidative stress, and hematocrit. Exposure to the primary contaminants was extremely variable over this relatively small study area. Geometric mean wet weight concentrations (range in brackets) of the major contaminants were 36.5 (12-174) ng/g ΣDDT, 10.9 (2-307) ng/g ΣPBDEs. ΣPCBs at 3.58 (1.5-6.4) ng/g were lower and less variable. There were low concentrations of other organochlorine pesticides such as dieldrin (2.02 ng/g), chlordanes (1.11 ng/g) and chlorobenzenes (0.23 ng/g). The only form of DDT detected was the principle metabolite, *p,p*'-DDE. The congener profiles of PBDEs and PCBs reflect those of industrial mixtures. For all of the contaminant classes, concentrations detected in eggs at our study site were below concentrations previously reported to cause effects. We saw no relationship between PBDE concentrations in starling eggs and reproductive success, maternal condition, or nestling condition in the corresponding nests.

4.2. Introduction

Polybrominated diphenyl ethers (PBDEs) have been widely used as additive flame retardants in plastics, textiles, foams, and electronic circuitry and are persistent and bioaccumulative, which contributes to their ubiquitous distribution in environmental, wildlife, and human samples (de Wit 2002, Law et al. 2003). PBDEs have been found in avian tissue and egg samples throughout the world (Chen and Hale 2010) but are only of relatively recent concern, as monitoring studies indicate that concentrations in the North American environment have increased over the past 30 years (de Wit 2002, Law et al. 2003, Chen and Hale 2010). In Canada, regulations prohibit the manufacture of all PBDEs and restrict the use of penta-BDE and octa-BDE mixtures (Canada Gazette 2008). However, PBDEs persist in the environment and will continue to leach from existing products that are in use or have been disposed of in landfills. Legacy persistent organic pollutants that have been heavily restricted in North America since the 1970s, such as polychlorinated biphenyl ethers (PCBs), dichlorodiphenyltrichloroethane (*p,p*'-DDT) and its metabolite 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p*'-DDE), and other organochlorine pesticides (OCs), are also commonly detected in the environment. The presence of PBDEs, PCBs and OCs in the environment is a concern, as they are known to cause a wide range of toxicological effects in birds (Chen and Hale 2010, Blus 2011, Elliott and Bishop 2011, Harris and Elliott 2011).

In the North American environment, there are several long-term datasets that have assessed the temporal and spatial variation of organohalogens in aquatic birds and top predators (e.g. Elliott et al. 2005, Braune et al. 2007, Gauthier et al. 2008); however, much less is known about organohalogen concentrations in terrestrial passerines in North America, particularly with respect to PBDEs. Our understanding of the toxic effects of PBDEs in birds is also limited relative to our knowledge of DDT and PCB toxicity. Studies that have assessed the effects of PBDE exposure in birds have primarily been in captive individuals (e.g. Fernie et al. 2005, McKernan et al. 2009, Van den Steen et al. 2009a) or in top predators (e.g. Verreault et al. 2007, Henny et al. 2009, Cesh et al. 2010), and effects in free-living terrestrial passerines have received little attention.

The European starling (*Sturnus vulgaris*) is a useful model species for both monitoring local contamination and assessing consequent effects in terrestrial free-living

birds. European starlings have a widespread distribution across several habitats (urban, suburban, rural, agricultural) and throughout many regions of the world (Europe, North America, parts of Asia, Africa, and Australasia), which allows for within-species comparisons of contaminant concentrations across many different spatial scales. European starlings have been used to monitor organohalogen contaminants over an intercontinental scale (Van den Steen et al. 2012), and Environment Canada has recently selected the European starling as a terrestrial wildlife monitoring species across Canada (Environment Canada 2011, Chen et al. submitted). Starlings readily use nest boxes, which makes monitoring and sample collection for the purpose of linking contaminant exposure with biological responses relatively easy. Starlings have successfully been used as biological indicators of PCB effects (Arenal et al. 2004). Contaminant concentrations can be measured in eggs, and previous studies of organohalogens in birds have shown that a single egg can be used as an indicator of nest contaminants (Custer et al. 1990, Van den Steen et al. 2006). By only collecting a single egg per nest, the growth, physiology and survival of remaining offspring in the nest can be monitored, and the productivity and condition of the individual nest can be related back to the contaminant concentrations in the egg. The objectives of our study were to 1) measure the concentrations of PBDEs, PCBs and OCs in European starling eggs to assess the individual nest variation in background contaminant concentrations at an agricultural site, and 2) to relate PBDE exposure to reproductive success and to measures of condition in the mothers and offspring, including body condition, thyroid hormones, oxidative stress, and hematocrit.

4.3. Materials and methods

4.3.1. Study site

This research was carried out from May to June of 2009 at Wind's Reach Farm in Langley, British Columbia (BC), Canada (49° 9' 16"N, 122° 28' 22"W) under a Simon Fraser University Animal Care Committee permit (864B-08) in accordance with guidelines from the Canadian Committee on Animal Care. The site consists of approximately 60 wooden next boxes on farm buildings, fence posts, and trees, and is within the BC Agricultural Land Reserve.

4.3.2. Monitoring

All boxes were checked daily to determine clutch initiation and completion dates, laying sequence of the eggs, and hatching and fledgling success. We monitored 14 occupied nest boxes. The nestling period is typically 21 days. Each egg was weighed (to the nearest 0.01 g) on the day it was laid. Nestlings were weighed and measured (tarsus length to the nearest 0.1 mm) at 0, 5, 10 and 15 days after hatching, and at 10 days of age nestlings were metal-banded. For each occupied nest box we recorded the number of parental nest visits for 30 minutes each day when nestlings were aged 6, 7 and 8 days, between 10:00 and 13:00. Provisioning rates were calculated per nestling per hour and based on the mean brood size of the nest for the 3-day observation period.

4.3.3. Blood sampling and egg collection

We caught female starlings (n = 14) while they roosted in their nest box at approximately 30 minutes before sunrise during late incubation (day 8 of incubation) to minimize possibility of nest abandonment. All birds were blood sampled (≤ 700 µl) from the brachial vein following puncture with a 26G needle within 3 min of capture. All females were measured (tarsus length and mass), and banded with metal and color bands. We returned birds to their nest boxes following sampling. Blood was collected into heparinized capillary tubes and stored on ice. Samples were centrifuged within 2 hours to separate plasma from the red blood cells, and hematocrit was measured by packed cell volume. Plasma was stored frozen at 20°C until analysis. Nestlings were blood sampled between 13:00 and 16:00 h on day 15 after hatching following the same methods, and returned to the nest box.

The second egg from the first clutch of each nest (n = 14) was collected to use as an indicator of nest contaminant concentration. We consistently collected the same egg to control for any possible laying order effects when comparing across nests. In addition, it has been previously shown in birds that the variation in contaminant concentration within clutches is less than among, and a single egg can be used as an indicator of nest contaminants (Custer et al. 1990, Van den Steen et al. 2006). Eggs were collected on the first day of incubation, using warmth as an indicator of incubation initiation. Whole eggs were removed from the shell and stored frozen (-80°C) in chemically cleaned glass vials.

4.3.4. Plasma analysis

Total and free thyroxine (T4 and FT4) and total and free triiodothyronine (T3 and FT3) concentrations in plasma samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225-300, 1225-300, 125-300, and 1325-300, Lake Forest CA). We validated kits for parallelism and recovery using European starling plasma. In each plate we included hen plasma from a plasma pool to assess reproducibility and intra-assay precision, as well as quality control standards (Monobind ML-300) that we verified were in the expected range. The T4 assay had an average intra-assay coefficient of variation (CV) of 11.8% (n = 2 replicates) and the inter-assay CV was 8.2% (n = 3 assay plates). For FT4 the average intra-assay CV was 8.1% (n = 2 replicates) and the inter-assay CV was 11.8% (n = 2 plates). The average intra-assay CV for T3 was 1.8% (n = 2 replicates) and the inter-assay CV was 15.1% (n = 2 plates). For FT3 the average intra-assay CV was 12.7% (n = 2 replicates) and the inter-assay CV was 4.8% (n = 2 plates).

Total antioxidant capacity (TAC) was measured using a colorimetric method adapted from Erel (2004), where the colored 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{*+}$) is decolorized by antioxidants in the plasma according to their concentrations and antioxidant capacities. This reaction can be monitored spectrophotometrically, and the final absorbance is inversely related to the TAC of the sample. The reaction is calibrated using Trolox for the standard curve, and results are expressed as mmol Trolox equivalent/L. The total oxidant status (TOS) was measured using a colorimetric method adapted from Erel (2005), where oxidants in the plasma oxidize the ferrous ion–*o*-dianisidine complex to the ferric ion, which then reacts with xylenol orange to make a colored complex. The color intensity can be measured spectrophotometrically, and is proportional to the total amount of oxidant molecules in the plasma sample. The assay is calibrated using hydrogen peroxide (H_2O_2) for the standard curve, and the results are expressed in mmol H_2O_2 equivalent/L. The oxidative status index (OSI) was calculated as the ratio of TOS:TAS, with high ratios reflecting high oxidative stress. A hen plasma pool was used as an avian standard to compare intra- and inter-assay variation. The TAC assay had an average intra-assay coefficient of variation (CV) of 6.4% (n = 2 replicates) and the inter-assay CV was 11.9% (n = 4 assay

plates). For TOS the average intra-assay CV was 8.7% (n = 2 replicates) and the inter-assay CV was 11.4% (n = 4 assay plates).

4.3.5. Chemical analysis

Egg samples were allowed to thaw at room temperature and homogenized using an Ultra Turrax High Performance disperser. All eggs (n = 14) were analyzed for 46 PBDE congeners, and a subset of eggs (n = 6) was analyzed for 74 PCB congeners and 21 different OC pesticide compounds. Details on the individual congeners/compounds determined are provided in footnotes a through j in Table 4.1. All PBDE, OC and PCB standards were purchased from Wellington Laboratories (Guelph, ON, Canada). Egg samples were accurately weighed, and neutral fractions were extracted and cleaned up using established methodologies (Gauthier et al. 2008). In brief, Approximately 1 g of egg homogenate was subsampled and ground with 8-10 g of diatomaceous earth, and then extracted with 50% DCM/hexane using an accelerated solvent extraction system (Dionex ASE 200). The extraction columns were spiked with 20 µl of each internal standard (BDE-30, BDE-156, $^{13}C_{12}$-BDE-209, ^{13}C PCB and ^{13}C OC). The column extraction eluant was concentrated to 10 ml and a 10% portion was removed for gravimetric lipid determination. The remaining extracts were cleaned by gel permeation chromatography (GPC) and eluted from the GPC column with 50% DCM/hexane. The first fraction (140 ml) containing lipids and biogenic material was discarded, and the second fraction (200 ml) containing PBDEs, PCBs and OCs was concentrated to a volume of ~4 ml.

All samples were cleaned up using a silica solid phase extraction (SPE) column (J.T. Baker, USA). The column was conditioned with successive washes of 10% (v/v) methanol (6 ml) in DCM and then 8 ml of 5% DCM in hexane. The sample was then loaded onto the cartridge and eluted with 8 ml of 5% DCM/hexane. The eluant was then concentrated and solvent exchanged with isooctane to a final volume of approximately 200 µl. The exact mass of each sample was recorded and the final volume determined by dividing by the density of 2,2,4-trimethylpentane (0.69 g/ml).

PBDEs in the isolated chemical fractions were analyzed using gas chromatography-mass spectrometry working in electron capture negative ionization

mode (GC/ECNI-MS). Analytes were separated and quantified on an Agilent 6890 series GC equipped with a 5973 quadrupole MS detector (Agilent technologies, Palo Alto, CA). The analytical column was a 15 m × 0.25 mm × 0.10 μm DB-5HT fused-silica column (J & W Scientific, Brockville, ON, Canada). Helium and methane were used as the carrier and reagent gases, respectively. A sample volume of 1 µl was introduced to the injector operating in pulsed-splitless mode (injection pulse at 25.0 psi until 2 min; purge flow to split vent of 96.4 ml/min), with the injector held at 240°C. The GC oven ramping temperature program was as follows: initial 100°C for 2.0 min, 25°C/min. until 250°C, 1.5°C/min until 260°C for 10.0 min, 25°C/min until 325°C and hold for a final 7.0 min. The GC to MS transfer line was held at 280°C, ion source temperature was 250°C, and the quadrupole temperature was 150°C. Forty-eight BDE congeners were monitored using the bromine anions of *m/z* 79 and 81. Ions of *m/z* 487 and 489 were used for BDE-207, -208 and -209. Ions of *m/z* 485 and 487 were used to monitor BDE-197, -201 and -202. Ions of *m/z* 495 and 497 were used for the internal standard of $^{13}C_{12}$-labelled BDE-209.

OCs and PCBs in the isolated chemical fractions were analyzed using gas chromatography-mass spectrometry working in electron ionization mode (GC/EI-MS). Analytes were separated and quantified on an Agilent 6890 series GC equipped with a 5973 quadrupole MS detector The analytical column was a 30 m × 0.25 mm × 0.25 μm DB-5HT fused-silica column (J & W Scientific, Brockville, ON, Canada). Helium and methane were used as the carrier and reagent gases, respectively. For OCs, a sample volume of 1 µl was introduced to the injector operating in pulsed-splitless mode (injection pulse at 40.0 psi until 1 min; purge flow to split vent of 23.5 ml/min), with the injector held at 250°C. The GC oven ramping temperature program was as follows: initial 100°C for 3.0 min, 20°C/min until 180°C, 5°C/min until 300°C. The GC to MS transfer line was held at 280°C, ion source temperature was 230°C, and the quadrupole temperature was 150°C. For PCBs, a sample volume of 1 µl was introduced to the injector operating in splitless mode (purge flow to split vent of 53.7 ml/min to 1.5 min), with the injector held at 250°C. The GC oven ramping temperature program was as follows: initial 100°C for 3.0 min, 20°C/min until 180°C, 2.5°C/min until 300°C. The GC to MS transfer line was held at 280°C, ion source temperature was 230°C, and the quadrupole temperature was 150°C.

The analytes were identified on the basis of their retention times on the DB-5HT column, and verified by matching retention times with those of authentic standard mixtures. Mean internal standard recoveries for BDE-30, -156 and $^{13}C_{12}$-labeled BDE-209 were 80.7±5%, 74.7±11% and 53.5±12%, respectively. Mean internal standard recoveries for $^{13}C_{12}$-labeled PCB-28, -52, -118, -153, -180 and -194 were 84.5±5%, 89.5±5% and 90.2±9%, 92.2±5%, 97.4±7% and 112.7±7%, respectively. The average recovery values for the internal standards $^{13}C_{12}$-labeled 1,2,4,5-tetrachlorobenzene, pentachlorobenzene, hexachlorobenzene, α-hexachlorobenzene, β-hexachlorobenzene, γ-hexachlorobenzene, octachlorostyrene, heptachloroepoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *p,p'*-DDE, dieldrin, *p,p'*-DDD, *cis*-nonachlor, *p,p'*-DDT and mirex were 52.2±6%, 58.4±5%, 64.8±4%, 84.0±9%, 116.3±29%, 104.4±12%, 83.9±9%, 108.0±12%, 101.9±10%, 99.8±7%, 99.5±6%, 97.7±6%, 107.3±6%, 95.5±11%,127.7±15%, 103.4±7%, 146.2±22% and 96.6±7%, respectively. Analytes were quantified using an internal standard approach, thus all reported values were inherently recovery-corrected.

The limits of detection (LOD) for PBDE, OC and PCB analysis, based on a signal-to-noise ratio of 3, were 0.2, 0.1 and 0.06 ng/g wet weight (ww), respectively, with the exception of a LOD of 0.6 ng/g ww for Dieldren. Method blanks were included for each sample batch to assess background interference and possible contamination, and a blank subtraction was done for BDE-2, 7, 10, 15, 17, 28, 47, 49, 54, 66, 71, 77, 99, 100, 153, 154, 155, 183, 195, 196, 197, 203, 206 and 209, as well as for PCB-66, 99, 101/90, 118, 138, 153, 170/190, 180 and 187, and a blank subtraction was done for hexachlorobenzene and *p,p'*-DDE. In-house standard reference material (double-crested cormorant [*Phalacrocorax auritus*] egg and Lake Michigan fish tissue) was also included in each sample batch to ensure consistency of data acquisition (within two SD of in-house mean).

4.3.6. Statistical analysis

All statistical analyses were carried out using SAS 9.1.3 (SAS Institute 2003). Data were tested for normality following Shapiro-wilk's tests, and by inspecting q-q plots. Variables that did not approximate the normal distribution were log-10 transformed prior to analysis to approximate a normal distribution. There was no correlation between egg

contaminants and gravimetrically determined lipid content ($p \geq 0.084$), so the contaminant concentrations were not lipid normalized. Where appropriate, contaminants were analyzed as sums of closely related congeners and compounds based on their chemical structure. The contaminants quantified in this study were categorized as follows: ΣPBDE (n = 46 congeners), ΣPCB (n=74 congeners), chlorinated benzene (ΣCBz, n=4 congeners), chlordanes (ΣCHL, n=6 compounds), DDT (ΣDDT, n=3 compounds), hexachlorocyclohexane (ΣHCH, n=3 isomers), mirex (n = 2 compounds), tris(4-chlorophenyl)methanol (TCPM), octachlorostyrene (OCS), and dieldrin. For statistical purposes to avoid missing values, the samples with concentrations below the LOD were assigned a random value between zero and the compound-specific LOD. An index of body condition was estimated as the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde et al. 2005). The relationships between ΣPBDE concentrations and adult characteristics were assessed using generalized linear models (proc GLM). The relationships between ΣPBDE and chick characteristics were assessed using nest means, and brood size and provisioning rate of the nest were included as covariates in the GLMs. Due to small sample sizes, we did not assess the relationship between ΣPCBs or ΣOCs and adult or chick characteristics.

4.4. Results

The contaminant class with the highest geometric mean egg concentration was ΣDDT, followed by ΣPBDEs, then ΣPCBs, then other organochlorine pesticides (Table 4.1). All of the ΣDDT was made up of *p,p*'-DDE, with *p,p'*-DDT and *p,p'*-DDD not being detected. The major PBDE congeners in starling eggs, in descending order, were BDE-99, -47, and -100 which were found in all of the eggs, followed by BDE-153, -154, and -209 (Table 4.2). All other PBDE congeners were either not detected or contributed less than 1% of the ΣPBDEs. CB-153, -138, -187, -180, and -146 were the most abundant PCB congeners (22%, 19%, 15%, 8%, and 6% of the ΣPCBs, respectively). Of the chlordanes, heptachloroepoxide, oxychlordane, *trans*-nonachlor and *cis*-nonachlor were detected. Hexachlorobenzene was the only chlorobenzene detected. Many of the organochlorine pesticides (HCH, mirex, TCPM, OCS) were not detected in the starling eggs. None of the organochlorine pesticide classes were correlated with either ΣPBDEs ($p \geq 0.102$) or ΣPCBs ($p \geq 0.191$). There was a correlation between ΣPBDEs and ΣPCBs

(r = 0.876, p = 0.022), although this is not significant after Bonferroni correction (α = 0.05/15 = 0.0033).

Among the contaminant classes, ΣPBDEs had the broadest range of concentrations (Table 4.1), with most eggs (79%) having less than 10 ng/g ww, and three eggs (21%) having more than 50 ng/g ww. ΣPBDEs in the egg were not related to egg lipid (r = 0.478, p = 0.084) or egg mass (r = -0.323, p = 0.261). ΣPBDE concentrations in the eggs from European starlings were not related to any of the measured maternal characteristics (Fig. 4.1), including body condition ($F_{1,12}$ = 0.76, p = 0.399), thyroid hormones ($p \geq 0.097$), oxidative stress ($F_{1,12}$ = 0.45, p = 0.516), or hematocrit ($F_{1,12}$ = 1.23, p = 0.289). ΣPBDEs in eggs were also not related to any of the measured reproductive parameters, including lay date, clutch size, clutch mass, average egg mass, or the number of fledglings per brood ($p \geq 0.513$). Similarly, PBDE concentrations in eggs were not related to characteristics of chicks from corresponding nests, including body condition at day 15 ($F_{1,10}$ = 0.86, p = 0.376), thyroid hormones ($p \geq$ 0.122), oxidative stress ($F_{1,10}$ = 0.05, p = 0.829), or hematocrit ($F_{1,10} < 0.01$, p = 0.981).

4.5. Discussion

In the present study, we found detectable concentrations of PBDEs, DDTs and PCBs in every European starling egg that we measured. The mean concentration of ΣDDTs was higher than ΣPBDEs and ΣPCBs, which is likely related to the agricultural landscape in which our field site is located. Concentrations of PCBs and PBDEs in passerines have been previously linked to urban and industrialized areas, while DDT and other OCs have been linked to rural and agricultural areas (Van den Steen et al. 2008, Van den Steen et al. 2009b, Sun et al. 2012).

Comparing organohalogen concentrations in starling eggs from our study to reported values for other passerines in British Columbia, starling eggs had significantly lower mean *p,p*'-DDE concentrations (geometric mean [GM] = 36.53 ng/g ww) than American robin (*Turdus migratorius*) eggs in both agricultural sites in the Okanagan Valley (GM = 39,300 ng/g ww), and in non-agricultural sites in the same geographic region as our study (GM = 1,100 ng/g ww; Gill et al. 2003), but somewhat higher

concentrations than American dipper (*Cinclus mexicanus*) eggs in southern BC rivers (GM = 9.4ng/g ww; Morrissey et al. 2010a). DDT has been banned in North America since the 1970s, and we did not detect the parent compound, *p,p'*-DDT, in any of the starling eggs. The only form detected was the principle metabolite *p,p'*-DDE, which indicates it is from a historic origin rather than a recent release. It is unlikely that *p,p'*-DDE concentrations at our study site would negatively affect productivity in starlings, as the effect level in the apparently most sensitive avian species to DDT was 3 µg *p,p'*-DDE/g ww in the eggs (Blus 2011), and in American robins, reproductive success was not affected by mean ΣDDT concentrations of 48 µg/g ww (Gill et al. 2003). Concentrations of other OC pesticides in starling eggs in our study (~ 0.2 to 2 ng/g ww) were also well below those reported to cause adverse effects in birds (Elliott and Bishop 2011)

ΣPCB concentrations in our study (GM = 3.58 ng/g ww) were lower than in robin eggs from agricultural (GM = 49.2 ng/g ww) and non-agricultural areas (GM = 79.5 ng/g ww; Gill et al. 2003), as well as dipper eggs in river basins (GM = 21.2 ng/g ww; Morrissey et al. 2010a).

The main PCB congeners detected in starling eggs were all major components of commercially used PCB mixtures. CB-138 and 153 were major congeners in Aroclors 1254, 1260 and 1262, CB-187 and -180 were major congeners in Aroclor 1260 and 1262, and CB-146 was a major congener in Aroclor 1260 (Frame 1997). It is not likely that ΣPCB concentrations found in starling eggs would have effects on productivity, as they were far below the suggested critical threshold for behavioral effects of 1 to 30 µg ΣPCB s/g ww in eggs (Harris and Elliott 2011).

Although our study site covered a relatively small geographic area (ca. 3.6 ha), there was a relatively high variability in PBDE burden in eggs (2 to 307 ng/g ww). This variation could be due to differences in diet or feeding locations among individuals, and suggests that in our agricultural region there are point sources of PBDE contaminants. Starling eggs at our study site had similar ΣPBDE concentrations (GM = 10.9 ng/g ww) compared to American dipper eggs in British Columbia (GM = 14.2 ng/g ww; Morrissey et al. 2010a), and their ΣPBDE concentrations fell within the range of ΣPBDE values reported for passerines outside of British Columbia. Starling eggs had approximately 10

times more PBDEs than the highest mean values reported for great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) across Europe (Van den Steen et al. 2009b, Van den Steen et al. 2010b). They had similar ΣPBDE concentrations to cliff swallow (*Petrochelidon pyrrhonota*) carcasses in agricultural areas of Texas (GM = 18 ng/g ww), but significantly lower concentrations than cliff swallows in heavily industrialized areas (GM = 258 ng/g ww and 196 ng/g ww; Mora et al. 2012). In passerine birds in China, the highest concentrations of ΣPBDEs were near electrical-waste sites, with concentrations in pectoral muscle up to 15,000 ng/g lipid weight (lw), followed by urban, then suburban, then rural sites (Sun et al. 2012). Our concentrations in European starling eggs (median 186.5 ng/g lw) were the most similar to concentrations measured at suburban locations in China (median values from 160 to 350 ng/g lw). Concentrations of ΣPBDEs in starling eggs also fell within the range of values reported for top predators in British Columbia. Arithmetic mean ΣPBDEs were from 1.78 to 8.49 ng/g ww in bald eagle nestling (*Haliaeetus leucocephalus*) plasma (~1% lipid) on the west coast (McKinney et al. 2006), 47.9 ng/g ww in great blue heron (*Ardea herodias*) eggs on Vancouver Island, 62.5 ng/g ww in double-crested cormorant eggs (*Phalacrocorax auritus*) on the west coast, 185 ng/g ww in osprey (*Pandion haliaetus*) eggs from southern river systems, and 455 ng/g ww in heron eggs on the southwest coast (Elliott et al. 2005). It has been suggested that trophic level can influence PBDE accumulation, and $\delta^{15}N$ was positively related to PBDE concentrations in passerines in China (Sun et al. 2012) and in Cooper's hawks in British Columbia (Elliott et al. 2010), although not in a high trophic level raptor, the bald eagle (Elliott et al. 2009). Overall, accumulation of PBDEs appears to be more influenced by the level of urbanization or industrialization and resulting contaminant concentrations in the local environment, rather than by trophic level, as birds within similar trophic levels can have a broad range of PBDE burdens, and PBDE burdens overlap across trophic levels.

Although the lower brominated BDEs were regulated in Canada in 2006, they still made up over 95% of the ΣPBDEs in starling eggs. The PBDE congener profile in starling eggs (BDE-99 > -47 > -100 > -153 > -154) matches the profile of DE-71, which was the major penta-BDE technical flame-retardant mixture manufactured in North America (La Guardia et al. 2006). Low concentrations of BDE-209, which is the main component of the deca-BDE technical mixture (Saytex 102E), were also detected in

most (78.6%) of the starling eggs. The presence of BDE-209 in starling eggs likely indicates recent exposure, as it has been shown to have a relatively short half life (13 days) in European starlings (Van den Steen et al. 2007). The major components of the octa-BDE technical mixture (i.e. BDE-183, -197, -207, -196) made up less than 1% of the ΣPBDEs in starling eggs. PBDE congener profiles in other bird species in British Columbia are also generally dominated by the penta-BDEs (e.g. Elliott et al. 2005, McKinney et al. 2006, Morrissey et al. 2010b, Wilson et al. 2010).

The concentrations of ΣPBDEs in starling eggs were not related to reproductive success or any measures of condition in the mothers or offspring of the corresponding nests. This is not surprising as concentrations in starling eggs were generally lower than those reported to cause effects in birds. In free-living osprey (*Pandion haliaetus*), effects on productivity were not observed until ΣPBDEs were greater than 1000 ng/g ww in eggs (Henny et al. 2009). In bird eggs exposed to a penta-BDE mixture through egg injection, the lowest concentration to affect hatching success was 1.8 µg/g ww in American kestrels, and doses up to 20 µg/g ww had no effect on embryo survival endpoints in chicken (*Gallus gallus*) or mallard (*Anas platyrhynchos*) eggs (McKernan et al. 2009). European starlings that were exposed to a penta-BDE mixture through subcutaneous implants (~1740 ng/g bw) and produced eggs with ΣPBDEs from 130 to 220 ng/g ww showed some effects on egg size, but there were otherwise minimal effects on reproductive, endocrine disrupting, haematological and biochemical endpoints (Van den Steen et al. 2009a, Van den Steen et al. 2010a). Zebra finches (*Taeniopygia guttata*) exposed to BDE-99 via egg injection showed reduced clutch sizes at concentrations as low as 10 ng/g ww (Winter et al. *accepted*), which is similar to the ΣPBDE concentrations seen in starling eggs. However, the clutch size effects in zebra finches could not be seen until *in ovo* exposed birds reached reproductive maturity, and to assess whether such effects occur in the field, long term studies that link *in ovo* exposure to adult productivity are needed.

In conclusion, we found that European starlings in a rural agricultural area of British Columbia are being exposed to PBDEs, PCBs, DDTs, and other OCs at concentrations below those of concern. European starlings are found globally, and are exposed to a broad range of contaminant concentrations (Van den Steen et al. 2012). While we did not see effects in birds at an agricultural site with relatively low contaminant

burdens, the European starling would be a useful species for assessing contaminant effects in terrestrial passerines at sites with higher levels of pollution.

4.6. References

Arenal, C. A., R. S. Halbrook, and M. Woodruff. (2004). European starling (*Sturnus vulgaris*): Avian model and monitor of polychlorinated biphenyl contamination at a Superfund site in southern Illinois, USA. *Environmental Toxicology and Chemistry* 23:93-104.

Blus, L. (2011). DDT, DDD and DDE in Birds. Pages 425-444 *Environmental Contaminants in Biota*. CRC Press.

Braekevelt, E., S. A. Tittlemier, and G. T. Tomy. (2003). Direct measurement of octanol-water partition coefficients of some environmentally relevant brominated diphenyl ether congeners. *Chemosphere* 51:563-567.

Braune, B. M., M. L. Mallory, H. G. Gilchrist, R. J. Letcher, and K. G. Drouillard. (2007). Levels and trends of organochlorines and brominated flame retardants in Ivory Gull eggs from the Canadian Arctic, 1976 to 2004. *Science of the Total Environment* 378:403-417.

Canada Gazette. (2008). Part II. Polybrominated diphenyl ether regulations. 142:1663-1682.

Cesh, L. S., K. H. Elliott, S. Quade, M. A. McKinney, F. Maisoneuve, D. K. Garcelon, C. D. Sandau, R. J. Letcher, T. D. Williams, and J. E. Elliott. (2010). Polyhalogenated aromatic hydrocarbons and metabolites: Relation to circulating thyroid hormone and retinol in nestling bald eagles (*Haliaeetus leucocephalus*). *Environmental Toxicology and Chemistry* 29:1301-1310.

Chen, D. and R. C. Hale. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International* 36:800-811.

Chen, D., P. Martin, N. M. Burgess, L. Champoux, J. E. Elliott, D. Forsyth, A. Idrissi, and R. J. Letcher. (submitted). European starlings (*Sturnus vulgaris*) indicate that landfills are an important source of bioaccumulative flame retardants to Canadian terrestrial ecosystems. *Environmental Science & Technology*.

Custer, T. W., G. Pendleton, and H. M. Ohlendorf. (1990). Within-clutch and among-clutch variation of organochlorine residues in eggs of black-growned night-herons. *Environmental Monitoring and Assessment* 15:83-89.

de Wit, C. A. (2002). An overview of brominated flame retardants in the environment. *Chemosphere* 46:583-624.

Elliott, J. E. and C. Bishop. (2011). Cyclodiene and Other Organochlorine Pesticides in Birds. Pages 447-475 *Environmental Contaminants in Biota*. CRC Press.

Elliott, J. E., L. K. Wilson, and K. Drouillard. (2010). Accumulation of PBDEs and legacy POPs in urban raptors from British Columbia, Canada, 1999-2009. Society of Environmental Toxicology and Chemistry North America 31st Annual Meeting, Portland, OR.

Elliott, J. E., L. K. Wilson, and B. Wakeford. (2005). Polyhrominated diphenyl ether trends in eggs of marine and freshwater birds from British Columbia, Canada, 1979-2002. *Environmental Science & Technology* 39:5584-5591.

Elliott, K. H., L. S. Cesh, J. A. Dooley, R. J. Letcher, and J. E. Elliott. (2009). PCBs and DDE, but not PBDEs, increase with trophic level and marine input in nestling bald eagles. *Science of the Total Environment* 407:3867-3875.

Environment Canada. (2011). Environmental monitoring and surveillance in support of the Chemicals Management Plan.

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry* 37:277-285.

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry* 38:1103-1111.

Fernie, K. J., J. L. Shutt, G. Mayne, D. Hoffman, R. J. Letcher, K. G. Drouillard, and I. J. Ritchie. (2005). Exposure to polybrominated diphenyl ethers (PBDEs): Changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicological Sciences* 88:375-383.

Frame, G. M. (1997). A collaborative study of 209 PCB congeners and 6 Aroclors on 20 different HRGC columns .2. Semi-quantitative Aroclor congener distributions. *Fresenius Journal of Analytical Chemistry* 357:714-722.

Gauthier, L. T., C. E. Hebert, D. V. C. Weseloh, and R. J. Letcher. (2008). Dramatic changes in the temporal trends of polybrominated diphenyl ethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982-2006. *Environmental Science & Technology* 42:1524-1530.

Gill, H., L. K. Wilson, K. M. Cheng, and J. E. Elliott. (2003). An assessment of DDT and other chlorinated compounds and the reproductive success of American robins (*Turdus migratorius*) breeding in fruit orchards. *Ecotoxicology* 12:113-123.

Harris, M. and J. E. Elliott. (2011). Effects of Polychlorinated Biphenyls, Dibenzo-p-Dioxins and Dibenzofurans and Polybrominated Diphenyl Ethers in Wild Birds. Pages 477-528 *Environmental Contaminants in Biota*. CRC Press.

Henny, C. J., J. L. Kaiser, R. A. Grove, B. L. Johnson, and R. J. Letcher. (2009). Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology* 18:802-813.

La Guardia, M. J., R. C. Hale, and E. Harvey. (2006). Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used penta-, octa-, and deca-PBDE technical flame-retardant mixtures. *Environmental Science & Technology* 40:6247-6254.

Law, R. J., M. Alaee, C. R. Allchin, J. P. Boon, M. Lebeuf, P. Lepom, and G. A. Stern. (2003). Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. *Environment International* 29:757-770.

McKernan, M. A., B. A. Rattner, R. C. Hale, and M. A. Ottinger. (2009). Toxicity of polybrominated diphenyl ethers (DE-71) in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) embryos and hatchlings. *Environmental Toxicology and Chemistry* 28:1007-1017.

McKinney, M. A., L. S. Cesh, J. E. Elliott, T. D. Williams, D. K. Garcelon, and R. J. Letcher. (2006). Brominated flame retardants and halogenated phenolic compounds in North American west coast bald eaglet (*Haliaeetus leucocephalus*) plasma. *Environmental Science & Technology* 40:6275-6281.

Mora, M. A., J. L. Sericano, and C. Baxter. (2012). Swallows as indicators of environmental pollution of the Rio Grande/Rio Bravo Basin: Are persistent organic pollutants a concern? *Archives of Environmental Contamination and Toxicology* 62:512-518.

Morrissey, C. A., J. E. Elliott, and S. J. Ormerod. (2010a). Diet shifts during egg laying: Implications for measuring contaminants in bird eggs. *Environmental Pollution* 158:447-454.

Morrissey, C. A., J. E. Elliott, and S. J. Ormerod. (2010b). Local to continental influences on nutrient and contaminant sources to river birds. *Environmental Science & Technology* 44:1860-1867.

Schulte-Hostedde, A. I., B. Zinner, J. S. Millar, and G. J. Hickling. (2005). Restitution of mass-size residuals: Validating body condition indices. *Ecology* 86:155-163.

Sun, Y. X., X. J. Luo, L. Mo, Q. Zhang, J. P. Wu, S. J. Chen, F. S. Zou, and B. X. Mai. (2012). Brominated flame retardants in three terrestrial passerine birds from South China: Geographical pattern and implication for potential sources. *Environmental Pollution* 162:381-388.

Van den Steen, E., A. Covaci, V. L. B. Jaspers, T. Dauwe, S. Voorspoels, M. Eens, and R. Pinxten. (2007). Accumulation, tissue-specific distribution and debromination of decabromodiphenyl ether (BDE 209) in European starlings (*Sturnus vulgaris*). *Environmental Pollution* 148:648-653.

Van den Steen, E., T. Dauwe, A. Covaci, V. L. B. Jaspers, R. Pinxten, and M. Eens. (2006). Within- and among-clutch variation of organohalogenated contaminants in eggs of great tits (Parus major). *Environmental Pollution* 144:355-359.

Van den Steen, E., M. Eens, A. Covaci, A. C. Dirtu, V. L. B. Jaspers, H. Neels, and R. Pinxten. (2009a). An exposure study with polybrominated diphenyl ethers (PBDEs) in female European starlings (*Sturnus vulgaris*): Toxicokinetics and reproductive effects. *Environmental Pollution* 157:430-436.

Van den Steen, E., M. Eens, A. Geens, A. Covaci, V. M. Darras, and R. Pinxten. (2010a). Endocrine disrupting, haematological and biochemical effects of polybrominated diphenyl ethers in a terrestrial songbird, the European starling (*Sturnus vulgaris*). *Science of the Total Environment* 408:6142-6147.

Van den Steen, E., V. L. B. Jaspers, A. Covaci, T. Dauwe, R. Pinxten, H. Neels, and M. Eens. (2008). Variation, levels and profiles of organochlorines and brominated flame retardants in great tit (*Parus major*) eggs from different types of sampling locations in Flanders (Belgium). *Environment International* 34:155-161.

Van den Steen, E., R. Pinxten, M. Bateson, C. Carere, P. Clergeau, D. Costantini, Z. Dolenec, J. E. Elliott, J. Flux, H. Gwinner, R. Halbrook, P. Heeb, T. Mazgajsk, A. Moksnes, V. Polo, J. J. Soler, R. Sinclair, J. Veiga, A. Covaci, and M. Eens. (2012). International monitoring study confirms the usefulness of starling eggs as a biomonitoring tool of organohalogenated contaminants. *Environment International accepted*.

Van den Steen, E., R. Pinxten, A. Covaci, C. Carere, T. Eeva, P. Heeb, B. Kempenaers, J. T. Lifjeld, B. Massa, A. C. Norte, M. Orell, J. J. Sanz, J. C. Senar, A. Sorace, and M. Eens. (2010b). The use of blue tit eggs as a biomonitoring tool for organohalogenated pollutants in the European environment. *Science of the Total Environment* 408:1451-1457.

Van den Steen, E., R. Pinxten, V. L. B. Jaspers, A. CovaCi, E. Barba, C. Carere, M. Cichon, A. Dubiec, T. Eeva, P. Heeb, B. Kempenaers, J. T. Lifjeld, T. Lubjuhn, R. Mand, B. Massa, J. A. Nilsson, A. C. Norte, M. Orell, P. Podzemny, J. J. Sanz, J. C. Senar, J. J. Soler, A. Sorace, J. Torok, M. E. Visser, W. Winkel, and M. Eens. (2009b). Brominated flame retardants and organochlorines in the European environment using great tit eggs as a biomonitoring tool. *Environment International* 35:310-317.

Verreault, J., C. Bech, R. J. Letcher, E. Ropstad, E. Dahl, and G. W. Gabrielsen. (2007). Organohalogen contamination in breeding glaucous gulls from the Norwegian Arctic: Associations with basal metabolism and circulating thyroid hormones. *Environmental Pollution* 145:138-145.

Wilson, L. K., M. L. Harris, S. Trudeau, M. G. Ikonomou, and J. E. Elliott. (2010). Properties of blood, porphyrins, and exposure to legacy and emerging persistent organic pollutants in surf scoters (*Melanitta perspicillata*) overwintering on the south coast of British Columbia, Canada. *Archives of Environmental Contamination and Toxicology* 59:322-333.

Winter, V., T. D. Williams, and J. E. Elliott. (*accepted*). A three-generational study of in ovo exposure to PBDE-99 in the zebra finch. *Environmental Toxicology and Chemistry*.

Table 4.1. Arithmetic mean (AM), geometric mean (GM), standard error (SE) and range of lipid percentage and sum (Σ) concentrations (ng/g wet weight) of organohalogen contaminants in European starling eggs.

	AM	GM	SE	Min.	Max.
% Lipid	3.57	3.42	0.27	1.74	5.24
ΣDDT[a]	54.46	36.53	24.68	11.94	174.3
ΣPBDE[b]	46.88	10.88	25.63	2.03	307.4
ΣPCBs[c]	4.05	3.58	0.78	1.47	6.40
Dieldrin	2.04	2.02	0.12	1.51	2.34
ΣCHL[d]	1.16	1.11	0.15	0.65	1.62
ΣCBz[e]	0.24	0.23	0.03	0.15	0.33
ΣHCH[f]	nd[j]	nd	-	-	-
Σmirex[g]	nd	nd	-	-	-
ΣTCPM[h]	nd	nd	-	-	-
ΣOCS[i]	nd	nd	-	-	-

[a]Σdichloro-diphenyl-trichloroethane (ΣDDT): *p,p*'-DDE, *p,p'*-DDT, *p,p'*-DDD

[b]Σpolybrominated diphenyl ether (ΣPBDE): BDE-1, 2, 3, 7, 10, 15, 17, 28, 47, 49, 54, 66, 71, 77, 85, 99, 100, 119, 138, 139, 140, 153, 154, 155, 170, 171, 179, 180, 181, 182, 183, 184, 188, 190, 191, 194, 195, 196, 197, 201, 202, 203, 205, 206, 207, 208, 209

[c]Σpolychlroinated biphenyl (ΣPCB): CB-16/32, 17, 18, 22, 28, 31, 33/20, 42, 44, 47/48, 49, 52, 56/60, 64/41, 66, 70/76, 74, 85, 87, 92, 95, 97, 99, 101/90, 105, 110, 114, 118, 128, 130, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 167, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 201, 202, 205, 206, 207, 208, 209

[d]Σchlordanes (ΣCHL): Heptachloroepoxide, Oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor

[e]Σchlorinated benzene (ΣCBz): 1,2,4,5-tetrachlorobenzene, 1,2,3,4,-tetrachlorobenzene, Pentachlorobenzene, Hexachlorobenzene

[f]hexachlorocyclohexane (ΣHCH): α-hexachlorocyclohexane, β-hexachlorocyclohexane, γ-hexachlorocyclohexane

[g]Σmirex: mirex, photomirex

[h]Σtris(4-chlorophenyl)methanol (ΣTCPM)

[i]Σoctachlorostyrene (ΣOCS)

[j]Not detected. Limit of detection 0.1 ng/g ww.

Table 4.2. Arithmetic mean (AM), geometric mean (GM), standard error (SE) and range of concentrations (ng/g wet weight) of PBDE congeners that made up greater than 1% of the sum (Σ) PBDEs in European starling eggs. Limit of detection (LOD) for PBDEs was 0.2 ng/g ww.

	AM	GM	SE	Min.	Max.	% of ΣPBDE (based on AM)	% of ΣPBDE (based on GM)	% n > LOD	Log K_{ow}[a]
ΣPBDE	46.9	10.9	25.6	2.0	307.4			100	
BDE-99	28.2	4.7	16.2	0.8	183.2	60.2	43.0	100	7.3
BDE-47	7.1	2.0	3.9	0.5	52.5	15.2	18.2	100	6.8
BDE-100	5.9	1.1	3.3	0.2	40.3	12.6	9.9	100	7.2
BDE-153	2.2	0.4	1.2	<0.2	13.4	4.7	4.0	57.1	7.9
BDE-154	1.3	0.3	0.7	<0.2	8.7	2.8	2.9	35.7	7.8
BDE-209	1.0	0.7	0.2	<0.2	2.3	2.2	6.6	78.6	10.3

[a]Log octanol-water coefficient values from Braekevelt et al (2003)

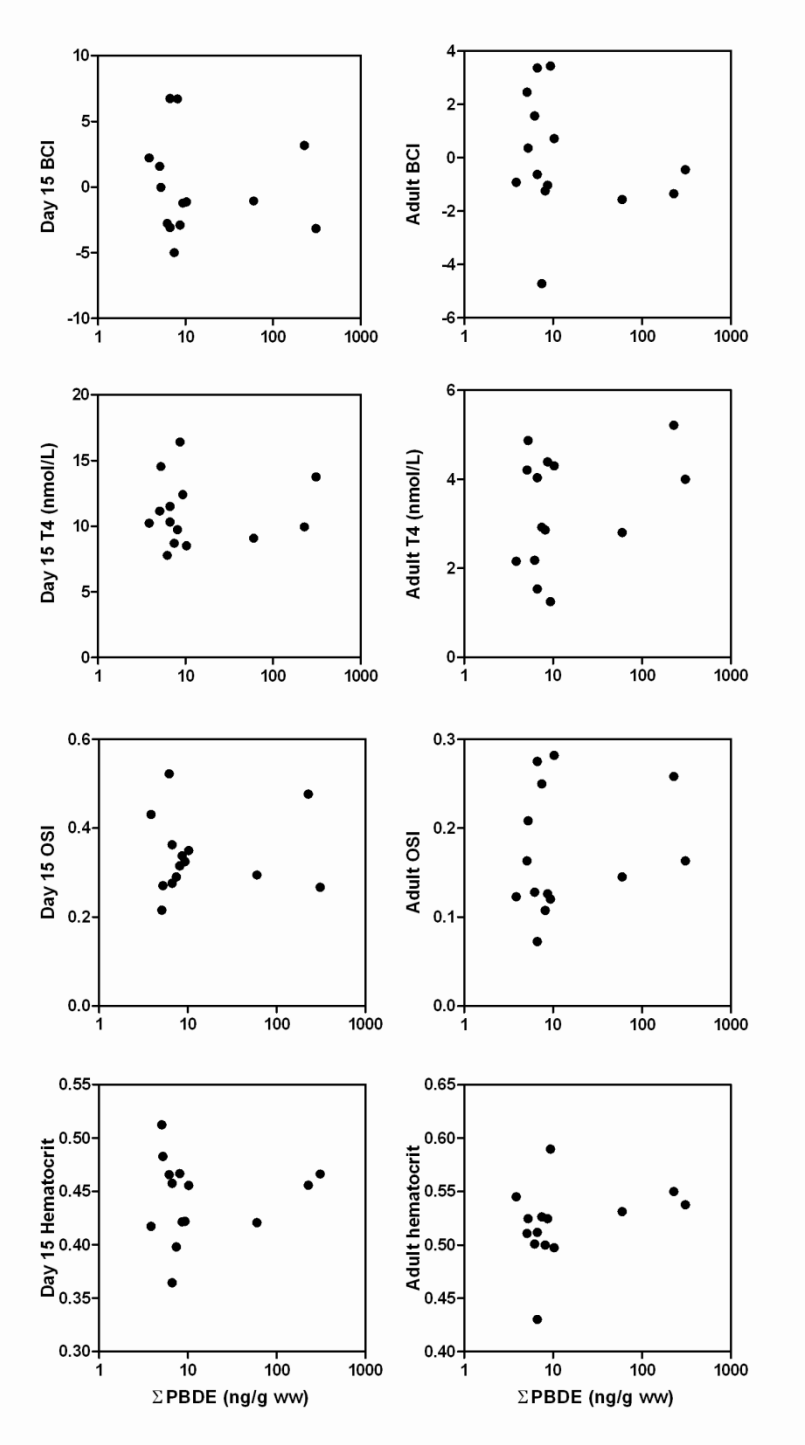

Figure 4.1. ***Relationship between ΣPBDEs in European starling eggs and measures of condition in 15 day-old nestlings and adults. BCI = body condition index, T4 = plasma thyroxine, OSI = oxidative status index.***

5. Methods for assessing developmental toxicity in a free living passerine: BDE-99 effects on growth, physiology, neuroanatomy, and photo-induced reproductive development

5.1. Abstract

In birds, the nestling period can be sensitive to environmental conditions, such as exposure to contaminants. Effects of such exposure may not be evident until reproductive maturity, necessitating long term studies to assess fitness implications. The objectives of this study were to develop a method to examine the long-term effects of early exposure to a representative chemical contaminant, 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99) under ecological conditions in a model passerine species, the European starling (*Sturnus vulgaris*). BDE-99 is one of the major constituents of the commercial penta-BDE flame retardant mixture, and has been reported in avian egg and tissue samples throughout the world. We orally dosed free-living nestlings for the duration of the nesting cycle to sublethal concentrations of BDE-99 (0-173.8 ng/g bw/day). To monitor long-term effects of exposure, birds were brought into captivity prior to fledging and raised to reproductive maturity using photoperiod manipulations. We took fledgling (day 30) and adult blood samples to measure hematocrit, oxidative stress, and thyroid hormones. At reproductive maturity we measured neuroanatomy of the song-control system in male birds. To assess development we monitored growth, molt rate, bill color, and testes development. We found some evidence of thyroid hormone disruption, but there were no effects on any other measures of physiology or development. Our results are consistent with studies in captive passerines, which overall suggests that passerines may be less sensitive to PBDE exposure than mammals and some other bird species are reported to be.

5.2. Introduction

Effects of environmental contaminants in birds are typically assessed either through dosing studies in captive birds (e.g. Fernie et al. 2005, Hoogesteijn et al. 2008, Eng et al. 2012), or through correlative wildlife monitoring studies (e.g. Verreault et al. 2007, Henny et al. 2009, Cesh et al. 2010). Captive studies generally use individuals living in ideal conditions (controlled climate, provisioned food, absence of predators) and observed effects may not necessarily reflect those that would be seen in free-living individuals facing environmental and anthropogenic stressors (e.g. Lambrechts et al. 1999, Spalding et al. 2000, Bardo and Bird 2009). It is important to also study free-living individuals to corroborate the results found in the lab. However, field monitoring studies can be limited by a lack of control over the range of contaminant exposure. Experimentally administering prescribed concentrations of contaminants to free-living birds can help put results of lab studies into ecological context, and at the same time ensure that exposure of toxicological interest is achieved. In the present study, we exposed free-living European starling (*Sturnus vulgaris*) nestlings that were living under ecological conditions to prescribed concentrations of representative contaminant, 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99), and then monitored the long-term effects.

Polybrominated diphenyl ethers (PBDEs) are a group of hydrophobic and bioaccumulative chemicals that have been widely used as additive flame retardants in textiles, plastics, foams and electronic circuitry. They are ubiquitous in environmental, human, and wildlife samples (Hites 2004), and are consistently found in avian tissue and egg samples throughout the world (Chen and Hale 2010). BDE-99 is one of the most dominant congeners in birds and other wildlife samples (Hites 2004, Chen and Hale 2010). It is known to cause a wide range of adverse effects in mammals, yet its effects in birds are not well known due to a lack of pertinent toxicological literature. Reported effects of BDE-99 exposure in mammals include disruption of thyroid hormone homeostasis (Hakk et al. 2002, Kuriyama et al. 2007), oxidative stress (Albina et al. 2010, Belles et al. 2010), and interference in reproductive development and behavior (Talsness et al. 2005, Lilienthal et al. 2006).

Understanding the long-term effects of early exposure is important for assessing the ecological risk of a chemical, as developmental life stages are typically more sensitive than adults to contaminant exposure (e.g. Peterson et al. 1993, Hutchinson et al. 1998), and the toxicological effects of developmental exposure may not be evident until the individual reaches reproductive maturity. Assessing long-term effects in free-living birds can be challenging, as once birds fledge from the nest they disperse from the breeding area and can rarely be relocated. Therefore, in order to assess the long-term physiological effects of BDE-99 in field-exposed chicks that had completed their growth and development under natural, ecological conditions, we brought them into captivity just prior to fledging. We then used photoperiod manipulation to induce gonadal cycles (Dawson and Goldsmith 1983, Williams et al. 1989) and assess effects of PBDEs on reproductive development in these field-exposed birds. European starlings are born in a photorefractory state, and must undergo a short-day winter-like period to become photosensitive, following which long spring-like days will be photostimulatory and induce reproductive development (Williams et al. 1989, McNaughton et al. 1992). Typically in photoperiod experiments, photosensitive birds are abruptly shifted from short days (~8 hours of light) to long days (~13 to 18 hours of light) to induce development. In the present study, we gradually increased day length based on the natural spring photoperiods in our study location to more closely mimic natural conditions during reproductive development.

European starlings (*Sturnus vulgaris*) are an important model species for comparative studies of contaminant effects in free-living passerines. Starlings have a broad geographic distribution and are found in several habitats. They have been established as a terrestrial wildlife monitoring species both at an intercontinental scale (Van den Steen et al. 2012), and across Canada (Environment Canada 2011, Chen et al. submitted). Starlings readily use nest boxes, which makes monitoring, manipulations, and sample collection relatively easy. In the present study we exposed European starlings to concentrations of BDE-99 relevant to exposure in wild birds during nestling development under ecological conditions, and assessed the short- and long-term effects on a range of endpoints. In addition to assessing effects of BDE-99 on offspring growth directly (mass, skeletal size) we measured hematocrit, oxidative stress (antioxidant capacity, oxidant status) and thyroid hormone function in fledgling birds at independence

(30 days of age). We then assessed long-term effects of early BDE-99 exposure in these same individuals as adults (205 days of age), using the same physiological endpoints, as well as neurobiological (brain nuclei of the song-control system), reproductive (gonadal development, androgen-dependent bill colour) and adult development endpoints (feather moult). The objectives of this study were to develop a method for examining the long-term effects of early exposure to anthropogenic contaminants in passerine birds under ecological conditions.

5.3. Materials and methods

5.3.1. Field monitoring and captive housing

Field research was carried out April of 2010 at Wind's Reach Farm in Langley, British Columbia (BC), Canada (49° 9' 16"N, 122° 28' 22"W) under a Simon Fraser University Animal Care Committee permit (864B-08) in accordance with guidelines from the Canadian Committee on Animal Care. The site consists of approximately 60 wooden next boxes on farm buildings, fence posts, and trees, and is within the BC Agricultural Land Reserve.

All boxes were checked daily to determine clutch initiation and completion dates, laying sequence of the eggs, and hatching success. We monitored 60 nestlings (27 male, 33 female) from 13 occupied nest boxes. The nestling period is typically 21 days. Each egg was weighed (to the nearest 0.01 g) on the day it was laid. At 10 days of age nestlings were color-banded. In order to continue monitoring of experimental individuals, it was necessary to bring them into captivity prior to fledging (day 15, Apr 29/30). We transferred them to the Simon Fraser University Animal Care Facility, where they were held indoors in metal cages (103 x 40.5 x 35.5 cm) with one nest group per cage (typically 4-5 birds). Chicks were be hand fed every 3 hours during daylight hours with a soft food mixture of poultry starter crumbles (Whole Earth 22% protein, Otter Co-op), hard boiled eggs, pureed carrots, multivitamin powder (Hagen Prime), and water. For each feeding, birds received food until they were no longer begging (typically

~10-20mL). Birds were weaned starting at day 20 by spoon feeding, as well as by placing dishes of food and water *ad libitum* on the floor of their cages. During weaning, bird mass was monitored between feedings to determine which birds had started self-feeding. Birds were self feeding by 25 to 30 days of age, at which point they were provided with dry poultry starter crumbles (Whole Earth 26% protein, Otter Co-op) and water *ad libitum,* and a supplement of ~10mL soft food mixture per bird once per day.

5.3.2. Dosing protocol

Prior to this experiment, a tissue residue study was carried out which validated oral dosing methods and confirmed environmental relevance of doses (see Eng et al. 2012 or Chapter 2 for details). All dosing was done with technical grade BDE-99 (>98% purity, Cambridge Isotope Labs, Andover, MA). The BDE-99 was dissolved in safflower oil (Spectrum organics, Boulder, CO) and the microliter amounts of BDE-99 dissolving solvent (nonane) were evaporated off using a steady stream of purified nitrogen gas. Dosing solutions were analyzed via gas chromatography (GC)-mass spectrometry (MS) (electron capture negative ionization mode [ECNI]), and a full description of the procedures used for the extraction and determination of PBDEs in the dosing solutions have been described elsewhere (Eng et al. 2012). The only bromide ion that was quantifiable was BDE-99. There were two dose levels (15.8 and 173.8 ng BDE-99/g body weight [bw]/day) and a safflower oil only control group. Within 24 h of hatching, individual chicks within each nest were marked with feather tract removal for identification, and dose levels were randomly assigned within the nest to account for any heritable effects. Nestlings were orally dosed daily from 24hrs after hatching (day 1) for the duration of the 21-day nestling period, using a micropipette. Doses were adjusted daily according to chick mass, with the dose volume being 10 µl/g bw. Nestlings were returned to the nests immediately after handling and dosing.

5.3.3. Photoperiod manipulations

After birds were brought into captivity, they were initially held at approximately natural day length (15 hours light [L]:9 hours dark [D]). Once all birds were self-feeding and maintaining body mass (at 35 days old, May 21), photoperiod was reduced to mimic winter day lengths to break photorefractoriness and induce photosensitivity. Birds were acclimated to shorter day lengths by reducing the length of light exposure by one hour each day over 7 days. Mass was monitored over the acclimation period to ensure birds were not losing mass. At the end of 7 days (May 28), all birds were exposed to short, winter-like day lengths (8L:16D), which was maintained until completion of their first prebasic molt from juvenile to adult plumage. All birds had completed their molt into adult plumage after 11 weeks at 8L:16D (17 weeks of age, Aug 13). At that point, day length was gradually increased in order to induce reproductive development. Increases were based on the natural day length (using civil twilight) in Langley, BC, from the equivalent of January 9th (9:42L:14:18D), to mimic the transition from winter to spring. The experiment was terminated after 12 weeks of increasing day length, at which point photoperiod was equivalent to April 3rd in Langley, BC (14:05L:9:55D).

5.3.4. Monitoring and sample collection

Chick mass (± 0.01 g), wing length (± 0.5 mm), and tarsus length (± 0.1 mm) were measured every 5 days until day 30 to monitor growth. Fledgling birds were blood sampled between 13:00 and 16:00 h at independence (~day 30). All birds were blood-sampled from the brachial vein following puncture with a 26G needle and blood was collected into heparinized hematocrit tubes. Blood samples were centrifuged at 3000g for 10 minutes to separate plasma from the red blood cells, and hematocrit was measured by packed cell volume. Plasma was then stored frozen (-20°C) until analysis. Molt of the primary feathers was recorded every 7 days from the start of the 8L:16D photoperiod until molt was complete. Each primary feather was given a score of 0 to 5 depending on its

stage of growth (Newton 1966), and the molt score was calculated as the sum of scores for each wing. Starlings have nine primary feathers in each wing, and a bird that has not yet started molt will have a score of 0, and a fully molted bird has a score of 45. During spring-like photoperiod we monitored changes in bill color from black to yellow using a 4 point scale, with 0 = entirely black, 1 = one quarter yellow, 2 = half yellow, 3 = yellow except for just the tip or just the base, and 4 = entirely yellow.

Testes development was estimated based on testicular volume measurements from captive first year starlings that were held in outdoor aviaries and exposed to natural changes in day length and temperature in Cambridgeshire, England (Dawson 2003; see Fig. 5.1). Sunrise/sunset tables were used to calculate the hours of light associated with reported testicular volumes. Starlings become spermatogenic when testes are approximately 125 mm^3, which happens during the third week of March in Cambridgeshire, England, when hours of light are approximately 13:20L:10:40D (Dawson 2003). Laparotomies were conducted on Oct. 22, at which point photoperiod was 13:13L:10:47D (equivalent to March 20 in Langley, BC). Testes volume is estimated to peak at approximately 320-400 mm^3 when hours of light are between 13:54 to 16:07. The experiment was terminated during the estimated time of peak testis size, on Nov 5th after 12 weeks of increased day length, at which point photoperiod was 14:05L:9:55D (equivalent to April 3rd in Langley, BC).

Laparotomies were conducted under general anesthesia. Birds were given a premed dose of ketamine (30 mg/kg) and torbugesic (0.3 mg/kg), followed by masking with isoflurane. Isoflurane was administered at a flow rate of 2L/min of oxygen, using 3% isoflurane to induce general anesthesia, and 2 to 2.5% isoflurane during surgery. A small incision was made in the body wall between the last pair of ribs, and the dimensions of the left testis were measured to the nearest 0.5 mm. After measuring the gonads, the incision was closed with small

sutures, and birds were placed in a dark box for recovery. Testes volume was calculated from $4/3*\pi*a^2*b$, where *a* is the radius of the testis at its widest point and *b* is half of the long axis.

At the estimated peak of photo-induced development birds were euthanized via exsanguinations with anesthesia (1:1 rompun:ketmaine). Blood was centrifuged and the plasma was separated and frozen. Brains were immediately dissected from the cranium, weighed (0.001g), and fixed by immersing in buffered 4% paraformadehyde (pH 8.5) for two weeks. After fixation, the brains were cryoprotected in 30% sucrose for 24 hours, then frozen on pulverized dry ice, and stored at –80°C until further processing. Testes were weighed and measured, and ovaries and oviducts were weighed.

5.3.5. Plasma analysis

Total antioxidant capacity (TAC) was measured using a colorimetric method adapted from Erel (2004), where the colored 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{*+}$) is decolorized by antioxidants in the plasma according to their concentrations and antioxidant capacities. This reaction can be monitored spectrophotometrically, and the final absorbance is inversely related to the TAC of the sample. The reaction is calibrated using Trolox for the standard curve, and results are expressed as mmol Trolox equivalent/L. The total oxidant status (TOS) was measured using a colorimetric method adapted from Erel (2005), where oxidants in the plasma oxidize the ferrous ion–*o*-dianisidine complex to the ferric ion, which then reacts with xylenol orange to make a colored complex. The color intensity can be measured spectrophotometrically, and is proportional to the total amount of oxidant molecules in the plasma sample. The assay is calibrated using hydrogen peroxide (H_2O_2) for the standard curve, and the results are expressed in mmol H_2O_2 equivalent/L. The oxidative status index (OSI) was calculated as the ratio of TOS:TAS, with high ratios reflecting high oxidative stress. A hen plasma pool

was used as an avian standard to compare inter-assay variation. The TAC assay had an average intra-assay coefficient of variation (CV) of 5.7% (n = 2 replicates) and the inter-assay CV was 10.7% (n = 4 assay plates). For TOS the average intra-assay CV was 5.3% (n = 2 replicates) and the inter-assay CV was 10.5% (n = 4 assay plates).

Total and free thyroxine (T4 and FT4) and total and free triiodothyronine (T3 and FT3) concentrations in plasma samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225-300, 1225-300, 125-300, and 1325-300, Lake Forest CA). Kits were validated for parallelism (see Plikaytis et al. 1994 for methods) and recovery using European starling plasma. Hen plasma from a plasma pool was included in each plate to assess reproducibility and intra-assay precision, as well as quality control standards (Monobind ML-300) that were verified to be in the expected range. The T4 assay had an average intra-assay coefficient of variation (CV) of 13.3% (n = 2 replicates) and the inter-assay CV was 15.7% (n = 4 assay plates). For FT4 the average intra-assay CV was 6.8% (n = 2 replicates) and the inter-assay CV was 14.2% (n = 4 plates). The average intra-assay CV for T3 was 4.3% (n = 2 replicates) and the inter-assay CV was 6.1% (n = 4 plates). For FT3 the average intra-assay CV was 9.5% (n = 2 replicates) and the inter-assay CV was 8.4% (n = 4 plates).

5.3.6. Brain tissue processing and neuroanatomical measurements

Using a cryostat (–20°C), brains were sectioned into 40 µm coronal sections and collected these in 0.1 M PBS (pH 7.5). Tissue sections were then mounted onto Superfrost Plus microscope slides (VWR). Sections were Nissl stained with thionin, serially dehydrated in ethanol, cleared in solvent and then protected with cover-slips affixed with Permount (Fisher Scientific). An observer (MLE) blind to subject and treatment group then made all measurements. Slides were examined with a bright field microscope (Leici DM5500 B) equipped with a

digital microscope camera (DFC420 C). Images of sections of the song-control regions, HVC (proper name, not an acronym), Area X and RA (robust nucleus of the arcopallium), were captured. The song nuclei were all readily defined by darkly stained cells relative to surrounding tissue. Telencephalon images were captured using a high resolution (2,400 dpi) flatbed scanner. The software ImageJ (version 1.42q, National Institutes of Health) was used to trace the outlines of these regions and measure their area. These areas were then combined using the formula for the volume of a cone frustum to estimate the total volume of each structure. Volume estimates were based on the areas of every 10th section for the telencephalon (400 µm intervals) and every second section for the song-control regions (80 µm intervals). Poor staining or tissue damage prevented volume reconstruction of RA in 2 brains, and HVC in 1 brain.

5.3.7. Molecular Sexing

Starling nestlings were sexed using a polymerase chain reaction (PCR) amplification process based on established techniques (Griffiths et al. 1998). DNA was isolated from red blood cells using Insta-Gene Matrix (Bio-Rad, Hercules, CA) following manufacturers' protocols. PCR amplification was then carried out in a total volume of 10 µl and run using the P2 (5′-TCTGCATCGCTAAATCCTTT) and CW (5′-AGAAATCATTCCAGAAGTTCA) primers followed by digestion with HAE III Enzyme. PCR products were run on a 1.5% agarose gel containing ethidium bromide for 1.5 h before examination under ultraviolet light.

5.3.8. Statistical analysis

All statistical analyses were carried out using SAS 9.1.3 (SAS Institute 2003). Data were tested for normality and homogeneity of variance following Shapiro-wilk and Levene’s tests, and by inspecting q-q plots. Before analysis, all non-parametric continuous variables were log-transformed to more closely approximate normal distributions and to homogenize variance. Sample sizes were 21 (12 female, 9 male), 20 (11 female, 9 male), and 19 (10 female, 9 male) nestlings exposed to control oil, 15.8, and 173.8 ng BDE-99/g bw/day,

respectively. An index of body condition was estimated as the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde et al. 2005). The effect of BDE-99 dose on fledgling and adult variables was assessed using generalized linear models (GLM procedure), and *post-hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey–Kramer method. To assess the effect of dose on each endpoint, initially sex and dose were both included for each analysis and if there was no effect or interaction with sex, sex was removed from the model. The effect of BDE-99 dose on growth, bill color change, molt, and testes size was assessed using the REPEATED statement in the MIXED procedure. Left and right brain hemispheres were compared for HVC, RA, Area X and telencephalon using paired t-tests, and no evidence of anatomical lateralization was found, so all the analyses were performed on summed left and right volumes. Contingency tables and fisher's exact test (FET) for small sample sizes were used to assess effects of BDE-99 dose on survival.

5.4. Results

BDE-99 tissue residue values following oral exposure for the 21-day nestling period have been previously reported in Eng et al. (2012). Based on the tissue residue study, the estimated 30-day plasma concentrations were 332.7 ± 141.01 and 1597.33 ± 314.51 ng/g lipid weight (lw) for the 0 and 15.8 ng BDE-99/g bw/day dose groups, respectively. The predicted 30-day plasma BDE-99 concentration for the 173.8 ng BDE-99/g bw/day dose group based on the tissue residue study was 14079.69 ng/g lw.

Chick growth was not affected by BDE-99 dose (Fig. 5.2A), as there was no interaction between age and dose for mass ($F_{12,280} = 0.94$, $p = 0.505$). Dose did not affect mass over time ($F_{2,55} = 0.80$, $p = 0.454$). There was also no effect of dose on body condition on day 15 ($F_{2,49} = 0.30$, $p = 0.742$) or fledgling ($F_{2,48} =$

1.30, p= 0.281). There was a significant effect of sex on mass over time ($F_{1,55}$ = 7.55, p = 0.0081), as males were on average heavier at each time point. There was no interaction between sex and age ($F_{6,280}$ = 1.46, p = 0.191).

Overall, 85% of male (23/27), and 85% of female (28/33) offspring survived to 30 days, and 81% of males (22/27) and 79% of females (26/33) survived to the end of the experiment. There was no effect of BDE-99 treatment or dose on the probability of survival to 30 days (Fisher's exact test, p = 0.295) or to the end of the experiment (Fisher's exact test, p = 0.152)

Hematocrit: There was no effect of BDE-99 dose on fledgling ($F_{2,49}$ = 0.11, p = 0.896) or adult ($F_{2,46}$ = 0.57, p = 0.571) hematocrit levels (Table 5.1), and there was no effect of sex on fledgling ($F_{1,46}$ = 2.63, p = 0.112) or adult ($F_{1,43}$ = 2.75, p = 0.104) hematocrit levels, and no sex*dose interaction for fledglings ($F_{2,46}$ = 2.76, p = 0.074) or adults ($F_{2,43}$ = 0.02, p = 0.978).

Oxidative stress: In fledglings there was no effect of BDE-99 dose on TAC ($F_{2,48}$ = 0.03, p = 0.971) TOS ($F_{2,48}$ = 0.19, p = 0.831) or OSI ($F_{2,48}$ = 0.27, p = 0.763; Table 5.1). Similarly in adults, there was no effect of dose on TAC ($F_{2,42}$ = 0.21, p = 0.808), TOS ($F_{2,39}$ = 0.97, p = 0.388) or OSI ($F_{2,39}$ = 1.20, p = 0.312). Sex had no effect on fledgling TAC or TOS, OSI or on adult TAC (p > 0.475). In adults, males had higher TOS ($F_{1,39}$ = 7.92, p = 0.008) and OSI than females ($F_{1,79}$ = 8.12, p = 0.006). ($F_{1,39}$ = 4.16, p = 0.048) There was no sex*dose interaction (p > 0.081) for any oxidative stress measure.

Thyroid hormone function: Fledgling FT4 was significantly elevated in BDE-99 treated birds compared to the control group ($F_{2,49}$ = 3.68, p = 0.032; Table 5.1). However, there was no effect of BDE-99 dose on fledgling T4 ($F_{2,46}$ = 0.21, p = 0.808), T3 ($F_{2,46}$ = 0.11, p = 0.893) or FT3 ($F_{2,49}$ = 1.27, p = 0.289). There was also no effect of dose on adult T4 ($F_{2,41}$ = 0.09, p = 0.914), T3 ($F_{2,42}$ = 1.24, p = 0.297), FT4 ($F_{2,34}$ = 0.17, p = 0.841) or FT3 ($F_{2,36}$ = 0.29, p = 0.750; Table 5.1). Fledgling males had lower T4 than females ($F_{1,46}$ = 8.47, p = 0.006).

There was no effect of sex on thyroid hormone concentrations for fledgling T3, FT4 and FT3 ($p > 0.071$) and there were no interactions between dose and sex ($p > 0.208$). There was also no effect of sex on any of the adult thyroid hormones ($p > 0.065$) or any interaction between sex and dose ($p > 0.173$).

Song control nuclei: BDE-99 exposure had no effect on male HVC ($F_{2,15} = 1.32$, $p = 0.296$), RA ($F_{2,14} = 1.63$, $p = 0.230$) or AreaX ($F_{2,16} = 0.67$, $p = 0.523$), and there was no effect of dose on brain mass ($F_{2,32} = 0.51$, $p = 0.606$) (Table 5.1). Female brains were significantly smaller than male brains ($F_{1,32} = 33.23$, $p < 0.0001$), but there was no interaction between dose and sex ($F_{2,32} = 0.89$, $p = 0.419$).

Adult moult, androgen dependent-traits and reproductive development: There was no significant effect of dose on molt score over time ($F_{2,46} = 0.32$, $p = 0.729$) or the rate of molt (non significant time*dose interaction $F_{20,470} = 0.54$, $p = 0.767$; Fig. 5.2B). There was an effect sex ($F_{1,46} = 5.46$, $p = 0.024$) on molt score and a significant effect of the interaction between time and sex on molt score ($F_{10,460} = 4.24$, $p = 0.005$), with male molt score increasing sooner than female molt score. Similarly, there was no effect of dose on bill color over time ($F_{2,44} = 0.03$, $p = 0.972$) or the rate of bill color change (non significant time*dose interaction $F_{16,352} = 0.73$, $p = 0.764$; Fig. 5.2C). There was a significant effect sex ($F_{1,44} = 44.23$, $p <0.0001$) on bill color over time, and a significant effect of the interaction between day and sex on bill color ($F_{8,352} = 7.69$, $p < 0.0001$), with male bill color being more yellow and changing faster than female bill color.

There was no significant effect of dose on testes size ($F_{2,16} = 0.45$, $p = 0.645$) and no significant change over time ($F_{1,16} = 0.05$, $p = 0.833$), and there was no interaction between time and dose (dose*age $F_{2,16} = 0.45$, $p = 0.644$; Fig. 5.3). There was also no effect on adult female ovary ($F_{2,15} = 1.10$, $p = 0.357$) oviduct ($F_{2,15} = 2.88$, $p = 0.088$) mass. No yolky (vitellogenic) follicles were observed in any of the females (Table 5.1).

5.5. Discussion

In the present study, we have shown how European starlings can be used as a model for developmental effects of xenobiotics in free-living passerines. This approach has the potential to extend an adverse outcome pathway (AOP; Ankley et al. 2010) to effects with reproductive and, therefore, population level consequences. European starling nestlings were exposed to a representative contaminant (BDE-99) at concentrations relevant to exposure in wild birds under natural, ecological conditions during the period of chick growth and development in the nest. Through the use of photoperiod manipulations we were then able to assess the long-term effects of early exposure to BDE-99 in these same individuals as adults. There was some evidence of thyroid hormone disruption in BDE-99 treated fledglings, but there was no effect of BDE-99 on offspring growth or survival, hematocrit, oxidative stress (TAC, TOS, OSI), adult thyroid hormones (free and total T3 and T4), neuroanatomy of the song-control system, adult development (molt rate), or reproductive development (gonadal development, bill color). Our results suggest that passerines may be less sensitive to BDE-99 exposure than mammals.

The absence of effects of early BDE-99 exposure on hematocrit in starlings, in either fledglings or adults, is consistent with the results of studies in zebra finches exposed to BDE-99 as nestlings or *in ovo* (Winter et al. in press, Chapter 3), as well as a study in ducks (*Anas platyrhynchos*) exposed to BDE-99 (Murvoll et al. 2005), and a study in European starlings exposed to a PBDE mixture (Van den Steen et al. 2010). In contrast, there is evidence in humans and other mammals that PBDE exposure can have hematological effects (Neale et al. 2005, Martin et al. 2007, Leijs et al. 2009). A possible reason for the discrepancy could be that the red blood cells in birds are less sensitive to PBDE exposure than in mammals, as the hematological cells differ between birds and mammals (Glomski and Pica 2011).

We also found no effect of early BDE-99 exposure on any measure of oxidative stress in fledgling or adult starlings, which also corresponds with the lack of effect observed in zebra finches exposed to BDE-99 as nestlings (Chapter 3). European starling adults exposed to a PBDE mixture through subcutaneous implants also showed no significant differences from control birds in the total plasma antioxidative capacity

(Van den Steen et al. 2010). Captive American kestrels (*Falco sparverius*) exposed to PBDE mixture *in ovo* and as nestlings exhibited no significant effects ($\alpha = 0.05$, $p = 0.09$) on markers of oxidative stress in the liver (Fernie et al. 2005). In contrast to the general lack of effect of PBDEs on oxidative stress observed in birds, BDE-99 exposure significantly altered oxidative stress markers in rats and a human cell line (Albina et al. 2010, Alonso et al. 2010, Belles et al. 2010, Tagliaferri et al. 2010). Those results suggest that birds may produce fewer reactive oxygen species following PBDE exposure than mammals.

PBDEs and their hydroxylated (OH) metabolites are structurally similar to thyroid hormones, and have the potential to disrupt thyroid hormone homeostasis through various mechanisms, including disruption of thyroid hormone production or metabolism, thyroid receptor binding, and competitive displacement of thyroid hormones from thyroid hormone transport proteins (Miller et al. 2009, Ucan-Marin et al. 2009). The only effect of BDE-99 on thyroid hormone homeostasis that we observed was seen in fledgling FT4, which was significantly higher in exposed birds than control birds. While this effect is not significant if corrected for multiple comparisons, it is consistent with proposed mechanisms of thyroid hormone disruption. If BDE-99 or its metabolites were to competitively bind with thyroid hormone transport proteins, we would expect that bound T4 or T3 would be displaced and there would be an increase in circulating (free) T4 or T3. It has been shown in birds that thyroid hormone transport proteins (transthyretin [TTR] and albumin[ALB]) have a higher binding affinity for T3 than T4, and so T4 would be more susceptible to competitive displacement by exogenous ligands (Chang et al. 1999, Ucan-Marin et al. 2009, Ucan-Marin et al. 2010). In addition, recently exposed individuals would be more likely to exhibit effects, which could explain why we saw higher FT4 in exposed birds when they were fledglings but not when they were adults. The results are also consistent with most other studies that show that juvenile birds are more sensitive to contaminant perturbation of the thyroid system than adults (Cesh et al 2010). Overall, however, the evidence in birds for thyroid hormone disruption by PBDEs is equivocal. Zebra finches exposed to BDE-99 *in ovo* or as nestlings showed no signs of thyroid hormone disruption (Winter et al. in press, Chapter 3), and thyroid hormones in captive American kestrels exposed to PBDEs via maternal transfer were also unaffected (Marteinson et al. 2011). There were non-significant trends for lower T3 in European

starlings and lower T4 in American kestrels exposed to PBDEs (Fernie et al. 2005, Van den Steen et al. 2010), and T3 was positively correlated with OH-PBDEs in free-living bald eagle (*Haliaeetus leucocephalus*) nestlings (Cesh et al. 2010). The lack of consistency of reported effects may be related to the complex nature of thyroid hormone homeostasis. In addition, based on gull TTR and ALB competitive binding assays with PBDEs and their metabolites, it is thought that current environmental concentrations of PBDEs and OH-PBDEs are likely too low to have a substantial effect on T3 or T4 binding (Ucan-Marin et al. 2010). In developing birds, thyroid hormones are important for normal growth and development (McNabb 2007). Although there were higher concentrations of FT4 in fledglings, we did not observe any effects of BDE-99 on growth; therefore, any potential thyroid hormone disruption by BDE-99 was not enough to affect growth. Zebra finches exposed to BDE-99 as nestlings and *in ovo* also did not show effects in growth (Winter et al. in press, Chapter 3).

The brain structures and neural pathways that make up the song-control system develop primarily in the first few months post-hatch (Bottjer et al. 1985, Mooney and Rao 1994), making the song-control system sensitive to early environmental conditions. Previous studies have shown that exposure to halogenated organic contaminants (dichlorodiphenyltrichloroethane (DDT) and its metabolites, and polychlorinated biphenyls (PCBs)) at early life stages can result in smaller song-control nuclei volumes (Iwaniuk et al. 2006, Hoogesteijn et al. 2008). Exposure to BDE-99 reduces sex steroids in mammals (Lilienthal et al. 2006, Alonso et al. 2010), and decreased concentrations of sex steroids can result in smaller song-control nuclei (Ball et al. 2002). However, we found that early exposure to BDE-99 had no effect on male song-control nuclei or on brain mass in adults. Similarly, there was no effect on song-control nuclei in male zebra finches exposed to BDE-99 as nestlings (Eng et al. 2012). It is possible that BDE-99 does not have the same effect on sex steroids in birds as it does in mammals, and in turn does not have effects on song-control nuclei. Alternatively, higher concentrations or embryonic (*in ovo*) exposure to BDE-99 may be required to see effects. European starlings exposed to a PBDE mixture as adults showed no differences in testosterone or estradiol (Van den Steen et al. 2010), but male American kestrels exposed to a PBDE mixture *in ovo* showed a trend ($p = 0.056$) of reduced circulating testosterone during their breeding period (Marteinson et al. 2011).

Molt and change in bill colour are both responses to hormonal changes that are brought on by seasonal variation, and can be used as indicators of post-fledging development in adult birds. In European starlings the onset of the prebasic moult is an indicator of photorefractoriness, and is associated with decreasing plasma prolactin (Dawson 2006). Molt occurs at the end of the breeding season in both juvenile and adult birds. The change in starling bill color from black to yellow early in the breeding season has been demonstrated to be a response to increasing androgens in photosensitive birds (Witschi and Miller 1938). While molt rate and bill color were not affected by BDE-99 exposure, birds did moult completely into adult plumage following the breeding season, and bill color changed from black to yellow in response to photostimulation, which indicates that our photoperiod manipulations were successful at inducing development.

We found no effect of BDE-99 exposure on gonadal development in starlings. While the average male testes size in each treatment group did not change over time (Fig. 5.3A), in individuals there were changes in testes size (Fig. 5.3B). Some individuals showed increases and some decreases in testes volume, but the pattern of change was not related to treatment group. Female ovary and oviduct size were not affected by BDE-99 exposure. As is typical for captive female starlings, there was no yolky follicle development in any of the birds. In contrast to our results, other studies have found effects of PBDEs on the reproductive tract. In mammals gestational and lactational exposure to BDE-99 (single dose to the mother of 60 or 300 ng/g bw) resulted in reproductive tract changes and lower fertility in female rats (Talsness et al. 2005), and gestational exposure to BDE-99 exposure decreased number of ovarian follicles in female rats, although doses were relatively high (1000 or 10000 ng/g bw injected gestational days 10-18; Lilienthal et al. 2006). In a study of captive American kestrels, male birds exposed to high concentrations (1131 ng/g ww) of a BDE mixture *in ovo* through maternal transfer had significantly different testicular structure. Possible reasons why we did not see effects could include differences in BDE-99 sensitivity among species, the level of exposure, or timing of exposure (nestling vs. *in ovo*). In addition, components other than BDE-99 in the PBDE mixture may be responsible for observed effects in kestrels.

Overall, we did not observe any overt effects of developmental exposure to BDE-99 in European starlings. There was some suggestion that BDE-99 disrupted fledgling thyroid hormones; however, if there was thyroid hormone disruption, it was not severe enough to have any effects on growth or development measures. Our results are consistent with other studies in passerines, and suggest that passerines may be less sensitive to PBDE exposure than mammals or other bird species studied to date. In zebra finches (*Taeniopygia guttata*) exposed to BDE-99 as nestlings, there were effects on mating behavior and timing of breeding, but no effects on neuroanatomy, physiology, growth, or reproductive success (Eng et al. 2012 or Chapter 2, Chapter 3). A study in adult European starlings exposed to a PBDE mixture also found minimal reproductive, endocrine disrupting, hematological or biochemical effects, and similarly concluded that passerines may be relatively less sensitive to the effects of PBDEs than other bird species (Van den Steen et al. 2009, Van den Steen et al. 2010). Another possibility for the lack of observed effects in our study may be that while our doses covered a range of concentrations relevant to avian exposure, higher concentrations are needed to cause adverse effects. Alternatively, the embryonic life stage may be more sensitive than the nestling period, and *in ovo* exposure may be necessary to see effects on physiology and reproduction.

Using the European starling, we developed and validated methods for assessing potential long-term effects resulting from early exposure to contaminants. In this model system, developmental exposure of free-living birds to xenobiotics could be via dosing of nestlings or egg injection. A broad range of endpoints can be assessed, from biochemical markers in the blood-stream, to development of the reproductive system. Future studies assessing contaminant effects in free-living starlings would be valuable for further validating this approach.

5.6. References

Albina, M. L., V. Alonso, V. Linares, M. Belles, J. J. Sirvent, J. L. Domingo, and D. J. Sanchez. (2010). Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. *Toxicology* 271:51-56.

Alonso, V., V. Linares, M. Belles, M. L. Albina, A. Pujol, J. L. Domingo, and D. J. Sanchez. (2010). Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food and Chemical Toxicology* 48:2206-2211.

Ankley, G. T., R. S. Bennett, R. J. Erickson, D. J. Hoff, M. W. Hornung, R. D. Johnson, D. R. Mount, J. W. Nichols, C. L. Russom, P. K. Schmieder, J. A. Serrrano, J. E. Tietge, and D. L. Villeneuve. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry* 29:730-741.

Ball, G. F., L. V. Riters, and J. Balthazart. (2002). Neuroendocrinology of song behavior and avian brain plasticity: Multiple sites of action of sex steroid hormones. *Frontiers in Neuroendocrinology* 23:137-178.

Bardo, L. and D. M. Bird. (2009). The use of captive American kestrels (*Falco sparverius*) as wildlife models: a review. *Journal of Raptor Research* 43:345-364.

Belles, M., V. Alonso, V. Linares, M. L. Albina, J. J. Sirvent, J. L. Domingo, and D. J. Sanchez. (2010). Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99. *Toxicology Letters* 194:1-7.

Bottjer, S. W., S. L. Glaessner, and A. P. Arnold. (1985). Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. *Journal of Neuroscience* 5:1556-1562.

Cesh, L. S., K. H. Elliott, S. Quade, M. A. McKinney, F. Maisoneuve, D. K. Garcelon, C. D. Sandau, R. J. Letcher, T. D. Williams, and J. E. Elliott. (2010). Polyhalogenated aromatic hydrocarbons and metabolites: Relation to circulating thyroid hormone and retinol in nestling bald eagles (*Haliaeetus leucocephalus*). *Environmental Toxicology and Chemistry* 29:1301-1310.

Chang, L., S. L. A. Munro, S. J. Richardson, and G. Schreiber. (1999). Evolution of thyroid hormone binding by transthyretins in birds and mammals. *European Journal of Biochemistry* 259:534-542.

Chen, D. and R. C. Hale. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International* 36:800-811.

Chen, D., P. Martin, N. M. Burgess, L. Champoux, J. E. Elliott, D. Forsyth, A. Idrissi, and R. J. Letcher. (submitted). European starlings (*Sturnus vulgaris*) indicate that landfills are an important source of bioaccumulative flame retardants to Canadian terrestrial ecosystems. *Environmental Science & Technology*.

Dawson, A. (2003). A comparison of the annual cycles in testicular size and moult in captive European starlings *Sturnus vulgaris* during their first and second years. *Journal of Avian Biology* 34:119-123.

Dawson, A. (2006). The role of prolactin in the regulation of molt. *Journal of Ornithology* 147:58-58.

Dawson, A. and A. R. Goldsmith. (1983). Plasma prolactin and gonadotropins during gonadal development and the onset of photorefractoriness in male and female starlings (*Sturnus vulgaris*) on artificial photoperiods. *Journal of Endocrinology* 97:253-260.

Eng, M. L., J. E. Elliott, S. A. MacDougall-Shackleton, R. J. Letcher, and T. D. Williams. (2012). Early exposure to 2,2 ',4,4 ',5-Pentabromodiphenyl ether (BDE-99) affects mating behavior of zebra finches. *Toxicological Sciences* 127:269-276.

Environment Canada. (2011). Environmental monitoring and surveillance in support of the Chemicals Management Plan.

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry* 37:277-285.

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry* 38:1103-1111.

Fernie, K. J., J. L. Shutt, G. Mayne, D. Hoffman, R. J. Letcher, K. G. Drouillard, and I. J. Ritchie. (2005). Exposure to polybrominated diphenyl ethers (PBDEs): Changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicological Sciences* 88:375-383.

Glomski, C. A. and A. Pica. (2011). The Avian Erythrocyte: Its Phylogenetic Odyssey. Science Publishers.

Griffiths, R., M. C. Double, K. Orr, and R. J. G. Dawson. (1998). A DNA test to sex most birds. *Molecular Ecology* 7:1071-1075.

Hakk, H., G. Larsen, and E. Klasson-Wehler. (2002). Tissue disposition, excretion and metabolism of 2,2 ',4,4 ',5-pentabromodiphenyl ether (BDE-99) in the male Sprague-Dawley rat. *Xenobiotica* 32:369-382.

Henny, C. J., J. L. Kaiser, R. A. Grove, B. L. Johnson, and R. J. Letcher. (2009). Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology* 18:802-813.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environmental Science & Technology* 38:945-956.

Hoogesteijn, A. L., G. V. Kollias, F. W. Quimby, A. P. De Caprio, D. W. Winkler, and T. J. DeVoogd. (2008). Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by Aroclor 1248. *Environmental Toxicology and Chemistry* 27:2071-2075.

Hutchinson, T. H., J. Solbe, and P. J. Kloepper-Sams. (1998). Analysis of the ECETOC aquatic toxicity (EAT) database - III - Comparative toxicity of chemical substances to different life stages of aquatic organisms. *Chemosphere* 36:129-142.

Iwaniuk, A. N., D. T. Koperski, K. M. Cheng, J. E. Elliott, L. K. Smith, L. K. Wilson, and D. R. W. Wylie. (2006). The effects of environmental exposure to DDT on the brain of a songbird: Changes in structures associated with mating and song. *Behavioural Brain Research* 173:1-10.

Kuriyama, S. N., A. Wanner, A. A. Fidalgo-Neto, C. E. Talsness, W. Koerner, and I. Chahoud. (2007). Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology* 242:80-90.

Lambrechts, M. M., P. Perret, M. Maistre, and J. Blondel. (1999). Do experiments with captive non-domesticated animals make sense without population field studies? A case study with blue tits' breeding time. *Proceedings of the Royal Society B-Biological Sciences* 266:1311-1315.

Leijs, M. M., J. G. Koppe, K. Olie, W. M. C. van Aalderen, P. de Voogt, and G. W. ten Tusscher. (2009). Effects of dioxins, PCBs, and PBDEs on immunology and hematology in adolescents. *Environmental Science & Technology* 43:7946-7951.

Lilienthal, H., A. Hack, A. Roth-Harer, S. W. Grande, and C. E. Talsness. (2006). Effects of developmental exposure to 2,2 ',4,4 ',5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environmental Health Perspectives* 114:194-201.

Marteinson, S. C., S. Kimmins, D. M. Bird, J. L. Shutt, R. J. Letcher, I. J. Ritchie, and K. J. Fernie. (2011). Embryonic exposure to the polybrominated diphenyl ether mixture, DE-71, affects testes and circulating testosterone concentrations in adult American kestrels (*Falco sparverius*). *Toxicological Sciences* 121:168-176.

Martin, P. A., G. J. Mayne, S. J. Bursian, G. Tomy, V. Palace, C. Pekarik, and J. Smits. (2007). Immunotoxicity of the commercial polybrominated diphenyl ether mixture DE-71 in ranch mink (*Mustela vison*). *Environmental Toxicology and Chemistry* 26:988-997.

McNabb, F. M. A. (2007). The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Critical Reviews in Toxicology* 37:163-193.

McNaughton, F. J., A. Dawson, and A. R. Goldsmith. (1992). Juvenile photorefractoriness in starlings, *Sturnus vulgaris*, is not caused by long days after hatching. *Proceedings of the Royal Society B-Biological Sciences* 248:123-128.

Miller, M. D., K. M. Crofton, D. C. Rice, and R. T. Zoeller. (2009). Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. *Environmental Health Perspectives* 117:1033-1041.

Mooney, R. and M. Rao. (1994). Waiting periods versus early innervation - the development of axonal connections in the zebra finch song system. *Journal of Neuroscience* 14:6532-6543.

Murvoll, K. M., B. M. Jenssen, and J. U. Skaare. (2005). Effects of pentabrominated diphenyl ether (PBDE-99) on vitamin status in domestic duck (*Anas platyrhynchos*) hatchlings. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 68:515-533.

Neale, J. C. C., F. M. D. Gulland, K. R. Schmelzer, J. T. Harvey, E. A. Berg, S. G. Allen, D. J. Greig, E. K. Grigg, and R. S. Tjeerdema. (2005). Contaminant loads and hematological correlates in the harbor seal (*Phoca vitulina*) of San Francisco Bay, California. *Journal of Toxicology and Environmental Health-Part a-Current Issues* 68:617-633.

Newton, I. (1966). Moult of the bullfinch *Pyrrhula pyrrhula Ibis* 108:41-67.

Peterson, R. E., H. M. Theobald, and G. L. Kimmel. (1993). Developmental and reproductive toxicity of dioxins and related compounds - Cross-species comparisons. *Critical Reviews in Toxicology* 23:283-335.

Plikaytis, B. D., P. F. Holder, L. B. Pais, S. E. Maslanka, L. L. Gheesling, and G. M. Carlone. (1994). Determination of parallelism and nonparallelism in bioassay dilution curves. *Journal of Clinical Microbiology* 32:2441-2447.

Schulte-Hostedde, A. I., B. Zinner, J. S. Millar, and G. J. Hickling. (2005). Restitution of mass-size residuals: Validating body condition indices. *Ecology* 86:155-163.

Spalding, M. G., P. C. Frederick, H. C. McGill, S. N. Bouton, L. J. Richey, I. M. Schumacher, C. G. Blackmore, and J. Harrison. (2000). Histologic, neurologic, and immunologic effects of methylmercury in captive great egrets. *Journal of Wildlife Diseases* 36:423-435.

Tagliaferri, S., A. Caglieri, M. Goldoni, S. Pinelli, R. Alinnovi, D. Poli, C. Pellacani, G. Giordano, A. Mutti, and L. G. Costa. (2010). Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells. *Toxicology In Vitro* 24:116-122.

Talsness, C. E., M. Shakibaei, S. N. Kuriyama, S. W. Grande, A. Sterner-Kock, P. Schnitker, C. de Souza, K. Grote, and I. Chahoud. (2005). Ultrastructural changes observed in rat ovaries following in utero and lactational exposure to low doses of a polybrominated flame retardant. *Toxicology Letters* 157:189-202.

Ucan-Marin, F., A. Arukwe, A. Mortensen, G. W. Gabrielsen, G. A. Fox, and R. J. Letcher. (2009). Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species (*Larus argentatus* and *Larus hyperboreus*). *Toxicological Sciences* 107:440-450.

Ucan-Marin, F., A. Arukwe, A. S. Mortensen, G. W. Gabrielsen, and R. J. Letcher. (2010). Recombinant albumin and transthyretin transport proteins from two gull species and human: Chlorinated and brominated contaminant binding and thyroid hormones. *Environmental Science & Technology* 44:497-504.

Van den Steen, E., M. Eens, A. Covaci, A. C. Dirtu, V. L. B. Jaspers, H. Neels, and R. Pinxten. (2009). An exposure study with polybrominated diphenyl ethers (PBDEs) in female European starlings (*Sturnus vulgaris*): Toxicokinetics and reproductive effects. *Environmental Pollution* 157:430-436.

Van den Steen, E., M. Eens, A. Geens, A. Covaci, V. M. Darras, and R. Pinxten. (2010). Endocrine disrupting, haematological and biochemical effects of polybrominated diphenyl ethers in a terrestrial songbird, the European starling (*Sturnus vulgaris*). *Science of the Total Environment* 408:6142-6147.

Van den Steen, E., R. Pinxten, M. Bateson, C. Carere, P. Clergeau, D. Costantini, Z. Dolenec, J. E. Elliott, J. Flux, H. Gwinner, R. Halbrook, P. Heeb, T. Mazgajsk, A. Moksnes, V. Polo, J. J. Soler, R. Sinclair, J. Veiga, A. Covaci, and M. Eens. (2012). International monitoring study confirms the usefulness of starling eggs as a biomonitoring tool of organohalogenated contaminants. *Environment International accepted*.

Verreault, J., C. Bech, R. J. Letcher, E. Ropstad, E. Dahl, and G. W. Gabrielsen. (2007). Organohalogen contamination in breeding glaucous gulls from the Norwegian Arctic: Associations with basal metabolism and circulating thyroid hormones. *Environmental Pollution* 145:138-145.

Williams, T. D., A. Dawson, and T. J. Nicholls. (1989). Sexual maturation and molt in juvenile Starlings *Sturnus vulgaris* in response to different day lengths. *Ibis* 131:135-140.

Winter, V., T. D. Williams, and J. E. Elliott. (in press). A three-generational study of in ovo exposure to PBDE-99 in the zebra finch. *Environmental Toxicology and Chemistry*.

Witschi, E. and R. A. Miller. (1938). Ambisexuality in the female starling. *Journal of Experimental Zoology* 79:475-487.

Table 5.1. Measures of fledgling (30-day old) and adult European starlings that were orally exposed to BDE-99 for the 21 day nesting period. Mean (SE). TAC = total antioxidant capacity, TOS = total oxidant status, OSI = oxidative status index, T4 = total thyroxine, T3 = total triiodothyronine, FT4 = free thyroxine, FT3 = free triiodothyronine, RA = robust nucleus of the arcopallium.

	Dose (ng BDE-99/g bw/day)		
	0	**15.8**	**173.8**
Fledgling			
Hematocrit	0.47 (0.01)	0.46 (0.02)	0.46 (0.01)
TAC (m*M* Trolox)	2.31 (0.11)	2.29 (0.17)	2.33 (0.13)
TOS (m*M* H_2O_2)	0.27 (0.03)	0.26 (0.02)	0.25 (0.02)
OSI	0.12 (0.01)	0.12 (0.01)	0.11 (0.01)
T4 (nmol/L)	9.94 (1.45)	11.28 (1.12)	10.81 (1.30)
T3 (nmol/L)	1.38 (0.19)	1.48 (0.24)	1.36 (0.14)
FT4 (pmol/L)	5.44 (0.46)[a]	7.34 (0.65)[b]	7.27 (0.55)[b]
FT3 (pmol/L)	3.40 (0.27)	3.80 (0.26)	3.26 (0.22)
Adult			
Hematocrit	0.48 (0.01)	0.48 (0.01)	0.49 (0.01)
TAC (m*M* Trolox)	2.82 (0.26)	2.87 (0.16)	2.7 (0.19)
TOS (m*M* H_2O_2)	0.16 (0.02)	0.14 (0.01)	0.15 (0.01)
OSI	0.06 (0.01)	0.05 (0.00)	0.06 (0.00)
T4 (nmol/L)	4.69(1.07)	5.39(1.50)	5.08(0.68)
T3 (nmol/L)	0.62(0.07)	0.75(0.05)	0.69(0.04)
FT4 (pmol/L)	6.80(0.53)	7.30(0.64)	6.98(0.52)
FT3 (pmol/L)	2.78(0.13)	2.88(0.32)	2.66(0.16)
HVC volume (mm^3)	2.66 (0.14)	3.10 (0.36)	3.12 (0.18)
RA volume (mm^3)	1.07 (0.06)	1.09 (0.08)	1.24 (0.09)
Area X volume (mm^3)	7.40 (0.23)	6.84 (0.35)	7.10 (0.37)
Brain mass (g)	1.70 (0.03)	1.74 (0.04)	1.72 (0.03)
Ovary mass (g)	0.11 (0.03)	0.07 (0.01)	0.07 (0.01)
Oviduct mass (g)	0.32 (0.11)	0.09 (0.02)	0.15 (0.07)

[ab]Significant difference between groups are indicated by different lower case letters ($p < 0.05$)

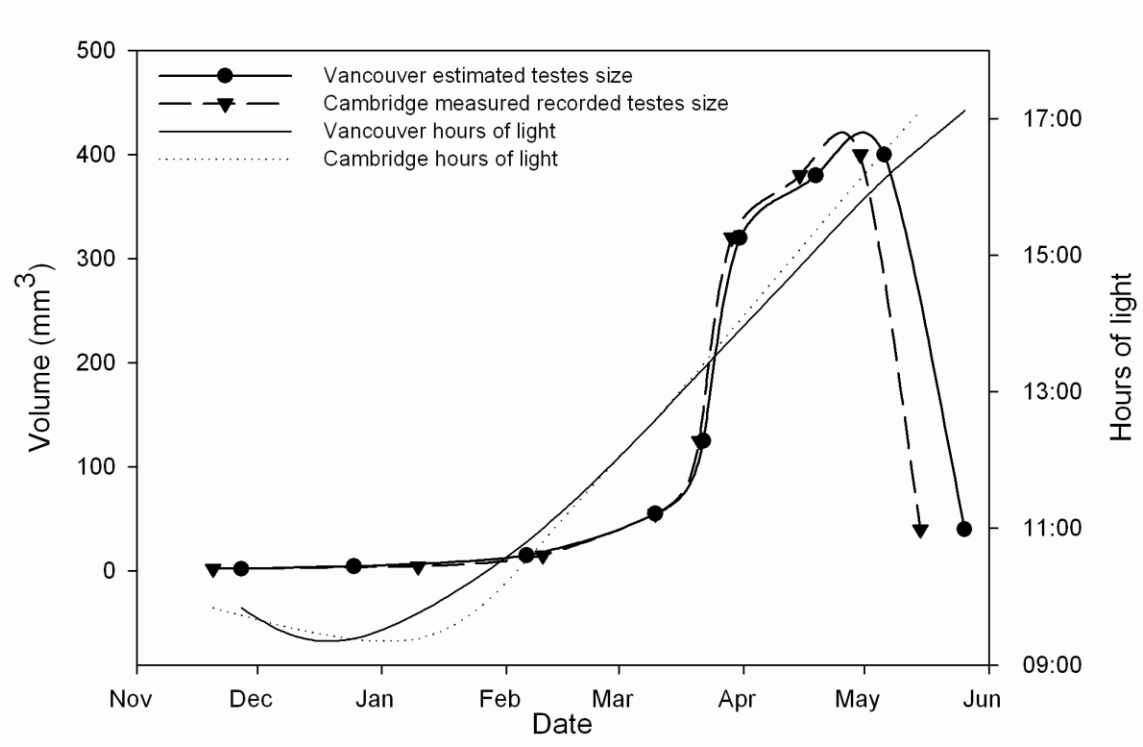

Figure 5.1. ***Seasonal changes in European starling testes development and photoperiod. Measured values of testes volume from European starlings in outdoor aviaries in Cambridgeshire, UK (Dawson 2003), and estimated testes volume for Langley BC based on equivalent photoperiods. Testes size was measured by laparotomies on the equivalent of March 20th at 13:13 hours light, and again at termination on the equivalent of April 3rd at 14:05 hours of light***

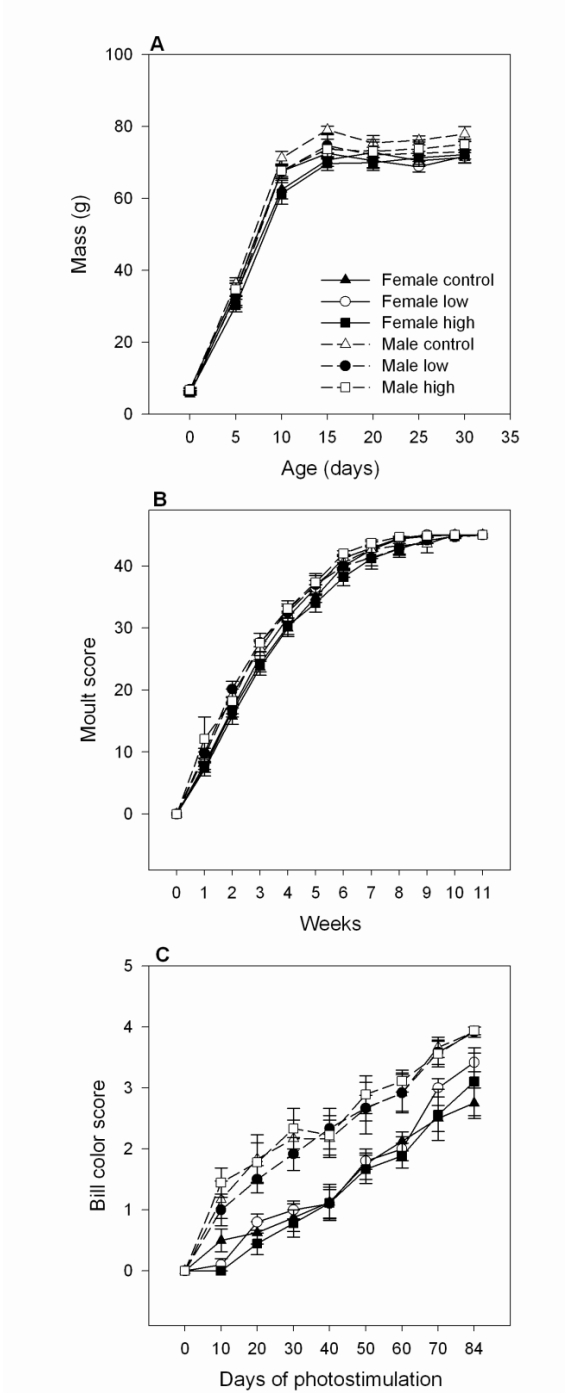

Figure 5.2. ***Developmental measures (mean ± SE) of European starlings exposed to control oil, low (15.8 ng/g bw/day) or high (173.8 ng/g bw/day) concentrations of BDE-99. (A) Chick growth, (B) rate of primary feather molt during the first prebasic molt, and (C) rate of bill color change during photostimulation.***

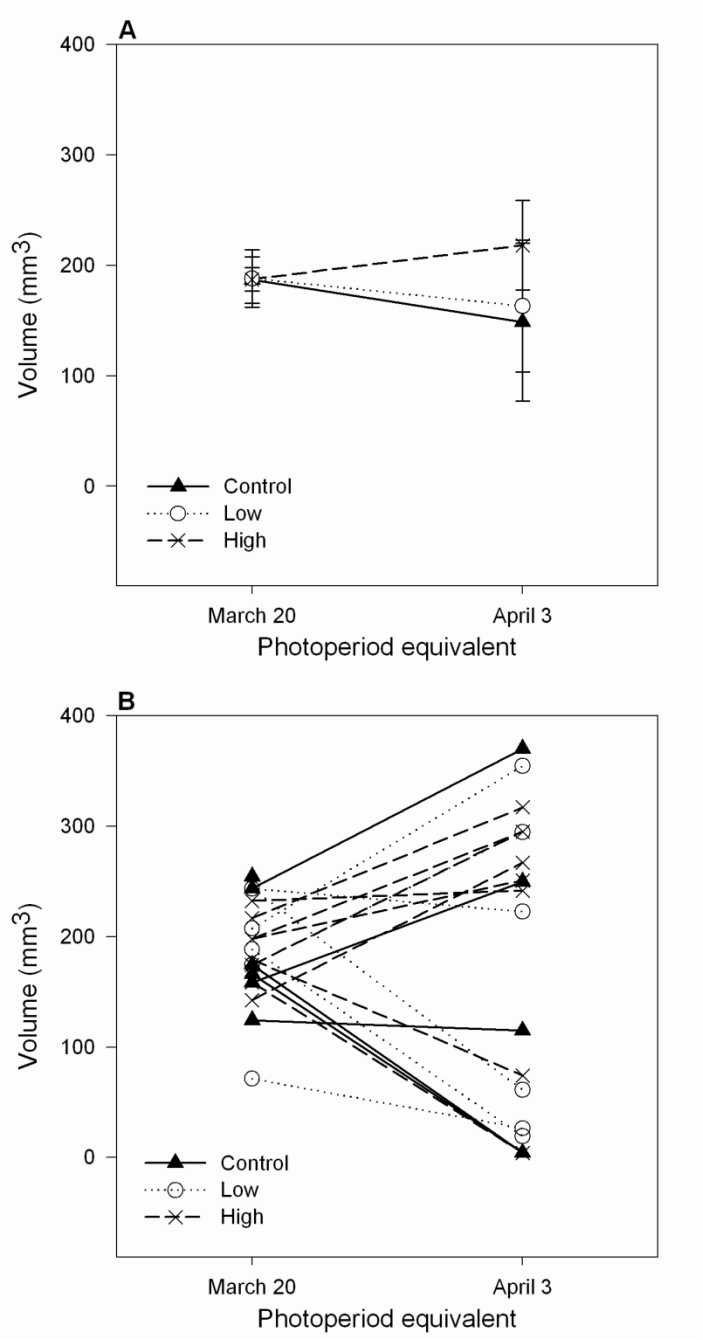

Figure 5.3. Starling testes volume measured by laparotomies when photoperiod was equivalent to March 20th, and measured by dissection when photoperiod was equivalent to April 3rd for (A) treatment groups means, error bars represent SE, and (B) individual birds.

6. Individual variation in body burden, lipid status and reproductive investment is related to maternal transfer of BDE-99 to eggs in the zebra finch

6.1. Abstract

Avian eggs are exposed to hydrophobic contaminants through maternal transfer. There is a lack of understanding of how maternal transfer of contaminants within a species is influenced by individual variation in characteristics such as body burden, yolk precursor levels, or reproductive investment. We investigated sources of variation in the maternal transfer of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) in zebra finches (*Taeniopygia guttata*). We dosed adult female zebra finches with concentrations of BDE-99 relevant to exposure in wild birds (0, 33.7 or 173.8 ng/g bw/day) for three weeks prior to pairing. Maternal BDE-99 and very-low-density lipoprotein (VLDL) in plasma were measured during egg formation and at clutch completion, and BDE-99 was measured in the corresponding egg. As the maternal burden increased, the slope of the lipid-normalized egg-to-maternal tissue BDE-99 relationship decreased. Individual variation in maternal VLDL was related to BDE-99 transfer to the eggs when BDE-99 was at background concentrations in control birds, but not when BDE-99 was elevated in dosed birds. The decrease in maternal plasma BDE-99 over the laying period was only significant ($p < 0.05$) in the high dose birds. Finally, the decrease in BDE-99 in maternal plasma during egg-laying was significantly positively correlated with clutch mass in the high dose group. These results suggest that the relationship between maternal and egg contaminant concentrations can be highly variable. This has significant implications for using eggs as indicators of adult or environmental concentrations, as egg contaminant concentrations from highly exposed birds could underestimate adult concentrations.

Hydrophobic contaminants can accumulate in the lipid-rich yolk of the avian egg, and consequently avian eggs are often used as a biomonitor for concentrations, distribution, and long-term trends of hydrophobic contaminants in the environment (e.g. Norstrom et al. 2002, Elliott et al. 2005, Van den Steen et al. 2010). It is clear that contaminants found in eggs are of maternal origin, with females 'off-loading' some of their own contaminant burden to their eggs during egg production (Bargar et al. 2001, Drouillard and Norstrom 2001). However, the specific mechanisms of this maternal transfer of contaminants and the factors that might affect the rate or amount of contaminant transfer from the mother to the egg are not well understood. This information is important when using contaminant concentrations in eggs to infer contaminant concentrations in the environment, at the sampling site, or as a proxy of maternal body burdens, as variation in egg contaminant concentrations could reflect differences in the physiological state or reproductive effort of individual females that laid the sampled eggs rather than variation in site or maternal contaminant residues per se.

If transfer of hydrophobic contaminants is solely regulated by a passive partitioning process among lipid-rich tissue compartments, the lipid normalized egg-to-maternal tissue contaminant ratio is expected to be one (Russell et al. 1999). However, in birds there is significant variability in the lipid-normalized egg-to-maternal tissue contaminant ratios among species, and ratios often deviate from one (Russell et al. 1999, Drouillard and Norstrom 2001). It has been suggested that this variability in maternal transfer among species might be related to the differences in reproductive strategies and level of egg investment (Drouillard and Norstrom 2001). Although there have been some within-species studies on the effects of laying order and chemical structure of the contaminant on maternal transfer (Bargar et al. 2001, Verreault et al. 2006, Van den Steen et al. 2009), the influence of individual variation in characteristics such as body burden and yolk precursor levels on maternal transfer has received less attention. During egg production the lipid status of laying females changes dramatically due to the hepatic synthesis and secretion into plasma of large amounts of the lipid-rich yolk precursors (vitellogenin and very-low-density lipoproteins [VLDL]) that serve as the main source of yolk lipids (Burley and Vadehra 1989). For example, plasma lipid concentrations increase from approximately 3 mg/ml in non-laying turkeys (*Meleagris gallopavo*) to 21 mg/ml in laying turkeys (Bacon et al. 1974), and similar changes have

been documented in free-living birds (Vanderkist et al. 2000, Challenger et al. 2001, Gorman et al. 2009). Importantly, there is marked inter-individual variation (8- to 10-fold) in plasma yolk precursor levels for any given egg or follicle size (Williams 2012). In addition, in some species at least, there can be two- to four-fold variation in the size and number of eggs laid among females within populations, and among years (Christians 2002). Thus, in addition to differences in maternal body burden of contaminants both the lipid status and level of reproductive investment among females could affect the dynamics of maternal transfer of contaminants to eggs.

In the present study, we investigated sources of variation in the maternal transfer of the polybrominated diphenyl ether (PBDE) congener 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) to eggs using the zebra finch (*Taeniopygia guttata*) as a model songbird species. PBDEs are a group of hydrophobic and bioaccumulative chemicals that find use as flame retardants, and have become ubiquitous in environmental, human, and wildlife samples (Hites 2004). BDE-99 is one of the most pervasive congeners, and has been detected in avian tissue and egg samples throughout the world (Chen and Hale 2010). Female zebra finches were exposed to BDE-99 at three different dose levels (control, low, high) prior to breeding. We then measured a) BDE-99 and VLDL levels in maternal plasma during egg formation, b) the BDE-99 concentration in the corresponding egg formed at the time the plasma sample was obtained, and c) the BDE-99 and VLDL levels in maternal plasma at clutch completion to assess the extent to which maternal plasma burden was depleted during egg laying. The objectives of the present study were therefore, 1) to examine how individual variation in body burden and yolk precursor levels influences the transfer of BDE-99 from the mother to the egg, 2) to determine the lipid normalized egg-to-maternal plasma contaminant relationship, and 3) to determine how dosing level (i.e. body burden) interacts with individual variation in reproductive investment (clutch mass) to influence the reduction in maternal plasma BDE-99 over the laying period. Establishing the relationship between chemical concentrations in the mother and egg for a species can be useful for predicting the exposure of wild birds based on egg contaminant data. The relationship can also be useful in embryo toxicity studies, where embryos could be dosed through maternal transfer, and the embryo exposure could be predicted from data on maternal body burden.

6.2. Materials and methods

6.2.1. Animals and husbandry

The present study was conducted on a captive colony of zebra finches maintained at the Simon Fraser University Animal Care Facility located in Burnaby, British Columbia. Zebra finches were housed in a controlled environment (temperature 19-23°C; humidity 35 % to 55%; photoperiod 14 hours light to 10 hours dark; lights on at 07:00). Non-breeding birds were held in single sex cages 102 × 39 × 43 cm. Breeding birds were housed in individual breeding cages (51 × 39 × 43 cm) equipped with an external nest box (14 × 14.5 × 20 cm). All birds were provided with mixed seed (panicum and white millet 1:2; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate by dry mass), water, grit, and cuttlefish bone (calcium) *ad libitum* plus a multivitamin supplement in the drinking water once per week. Breeding pairs were also provided with an egg-food supplement (20.3% protein:6.6% lipid) daily from pairing to clutch completion (2 days after the last egg was laid). Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (864B-08) in accordance with guidelines from the Canadian Committee on Animal Care.

6.2.2. Experimental protocol

All dosing was done with technical grade BDE-99 (>98% purity, Cambridge Isotope Labs). BDE-99 was analyzed via gas chromatography (GC)-mass spectrometry (MS) (electron capture negative ionization mode [ECNI]) (see “Chemical analysis” section below), and the only bromide ion that was quantifiable was BDE-99. The BDE-99 was dissolved in safflower oil (Spectrum organics) and the microliter amounts of BDE-99 dissolving solvent (nonane) were evaporated off using a steady stream of purified nitrogen gas. Females were treated with BDE-99 prior to breeding at two dose levels that we predicted would result in environmentally relevant body burdens based on a previous study in zebra finches (Eng et al. 2012): 33.7 ng/g body weight [bw]/day (n = 14) and 173.8 ng/g bw/day (n = 14). A safflower oil only control group comprised n = 15 females. BDE-99 levels in the safflower oil group were below the detection limits of our quantification method, and no PBDE congeners other than BDE-99 were detected in any of the dosing solutions. Adult females were randomly assigned to a dose group. Dose

groups were kept in separate cages to prevent cross contamination. Birds were orally dosed with BDE-99 or safflower oil daily for 21 days using a micropipette. Doses were adjusted daily according to female mass, with the dose volume being 10 µl/g bw.

Following the completion of the 21 day dosing period, females were paired with randomly selected males. All birds used in the breeding trials were experienced individuals that had previously produced at least one clutch. Nest boxes were checked daily between 09:00 and 11:00 for egg laying, and new eggs were numbered in consecutive order and weighed (0.001 g).

All plasma and egg samples were collected between 09:00 and 11:00. A blood sample was collected from each female on the day she laid her first egg (1E). All birds were blood-sampled from the brachial vein following puncture with a 26G needle and blood was collected into heparinized capillary tubes. Blood samples were centrifuged at 3000*g* for 10 minutes to separate plasma from the red blood cells, and plasma was then stored frozen (-80°C). In zebra finches, the day and night after a female lays her first egg corresponds with the deposition of yolk for the third-laid egg (Haywood 1993), and so the third egg was collected. Eggs were collected the morning that they were laid, i.e. before significant incubation of each egg, so embryo development should not have affected egg composition. The collected egg was replaced by a dummy egg in the nest. Whole eggs were removed from the shell and stored frozen (-80°C) in chemically cleaned glass vials. A second blood sample was taken from each female at clutch completion (CC) defined as two days after the last egg was laid.

6.2.3. Lipid and yolk precursor analysis

Total lipid content of plasma was determined colorimetrically using olive oil as the calibration standard (Frings et al. 1972). The average intra-assay CV was 3.7% (n = 2 replicates) and the inter-assay CV was 2.6% (n = 2 assay plates). Plasma triglyceride was measured as an index of total plasma VLDL using an analytical assay for free glycerol and total glycerol (Sigma-Aldrich). This index of VLDL was developed in domestic fowl (Mitchell and Carlisle 1991) and has been validated in zebra finches (Williams and Martyniuk 2000). Plasma triglyceride was calculated as the difference between total glycerol and free glycerol. Average intra-assay coefficient of variation (CV)

was 3.5% (n = 2 replicates), and inter-assay CV was 4.5% (n = 3 assay plates) for plasma triglyceride. Total egg lipid content was determined gravimetrically from a 1 ml aliquot of the dichloromethane (DCM)/hexane sample extract (see “Chemical Analysis”section below). The extract was placed on a pre-weighed aluminum dish and the solvent was then evaporated, and the dish reweighed to determine the total mass of the lipid.

6.2.4. Chemical analysis

All PBDE standards (BDE-17, -28/33, -47, -49, -66, -85 and -99) were purchased from Wellington Laboratories. Plasma, eggs, and dosing solutions were analyzed for BDE-17, -28, -47, -49, -54, -66, -71 and -99.

Plasma samples (0.038 to 0.120 g), egg samples (0.48 to 1.03g), and dosing solutions (200 µl) were accurately weighed, and neutral fractions were extracted and cleaned up using established methodologies (Gauthier et al. 2008, Chen et al. 2012). In brief, plasma samples were spiked with 50 ng of each of the internal standards (BDE-30 and -156), acidified, denatured, and liquid-liquid extracted with 50% (vol/vol) methyl tert-butyl ether (MtBE)/hexane. The organic phase layer containing the PBDEs was separated and collected. Egg samples were ground with approximately 25g of anhydrous sodium sulphate and extracted with 50% DCM/hexane using an accelerated solvent extraction system (Dionex ASE 200). The extraction columns were spiked with 20 ng of each internal standard. The column extraction eluant was concentrated to 10 ml and a 10% portion was removed for gravimetric lipid determination. The remaining extracts were cleaned by gel permeation chromatography (GPC) and eluted from the GPC column with 50% DCM/hexane. The first fraction (140 ml) containing lipids and biogenic material was discarded, and the second fraction (200 ml) containing PBDEs was concentrated to a volume of approximately 4 ml. Dosing solution samples were initially processed with GPC and did not go through ASE extraction. The dosing solutions were spiked with 20 ng of each internal standard.

All samples were cleaned up using a silica solid phase extraction (SPE) column (J.T. Baker). The column was conditioned with successive washes of 10% (v/v) methanol (6 ml) in DCM and then 8 ml of 5% DCM in hexane. The sample was then

loaded onto the cartridge and eluted with 8 ml of 5% DCM/hexane. The eluant was then concentrated and solvent exchanged with isooctane to a final volume of approximately 175 µl. The exact mass of each sample was recorded and the final volume determined by dividing by the density of 2,2,4-trimethylpentane (0.69 g/ml).

PBDEs in the isolated chemical fractions were analyzed using gas chromatography-mass spectrometry working in electron capture negative ionization mode (GC/ECNI-MS). Analytes were separated and quantified on an Agilent 6890 series GC equipped with a 5973 quadrupole MS detector (Agilent technologies). The analytical column was a 15 m × 0.25 mm × 0.10 μm DB-5HT fused-silica column (J & W Scientific). Helium and methane were used as the carrier and reagent gases, respectively. A sample volume of 1 µl was introduced to the injector operating in pulsed-splitless mode (injection pulse at 25.0 psi until 0.50 min; purge flow to split vent of 96.4 ml/min to 2.0 min; gas save flow of 20 ml/min at 2.0 min), with the injector held at 240°C. The GC oven ramping temperature program was as follows: initial 100°C for 4.0 min, 25°C/min. until 260°C, 2.5°C/min until 280°C for 10.0 min, 25°C/min until 325°C and hold for a final 7.0 min. The GC to MS transfer line was held at 280°C, ion source temperature was 200°C, and the quadrupole temperature was 150°C.

PBDE congeners were monitored using the bromine anions of *m/z* 79 and 81. Analytes were identified by comparison of retention times and ECNI mass spectra to those of the authentic standards.

Mean internal standard recoveries for the BDE-30 was 95 ± 2% SE for plasma analysis and 89 ± 2% SE for egg analysis. Mean internal standard recovery for BDE-156 was 110 ± 4% SE for plasma analysis, and 95 ± 4% SE for egg analysis.

Analytes were quantified using an internal standard approach, thus all reported values were inherently recovery-corrected. The method limits of quantification (MLOQ) for BDE-99, based on a signal-to-noise ratio of 10, was 0.07 ng/g wet weight (w.w) for plasma analysis, and 0.02 ng/g ww for egg analysis. Method blanks (n = 19) were included for each sample batch to assess background interference and possible contamination, and a blank subtraction was done for BDE-28, -47, -49, and -99. Duplicate analysis of the samples was not possible as all plasma and egg tissue were

consumed to ensure quantifiable analyte levels. In-house standard reference material (polar bear [*Ursus maritimus*] plasma for plasma analysis, double-crested cormorant [*Phalacrocorax auritus*] egg for egg analysis) was also included in each sample batch to ensure consistency of data acquisition (within two SD of in-house mean).

6.2.5. Statistical analysis

All statistical analyses were carried out using SAS 9.1.3 (SAS Institute 2003). Data were found to meet assumptions of normality and homogeneity of variance following Shapiro-Wilk's and Levene's tests, and by inspecting q-q plots and residual plots. The effect of dose was assessed using generalized linear models, and *post-hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey–Kramer method. Correlations were measured by Pearson's correlation coefficients. The lipid-normalized egg-to-maternal plasma BDE-99 relationship was analyzed using linear and nonlinear regressions (REG and NLIN procedures). We assessed the relationship between egg BDE-99 and maternal 1E VLDL or plasma lipid using linear regression, and we controlled for the effect of maternal BDE-99 on egg BDE-99 by using the residuals from the linear regression of egg BDE-99 on maternal BDE-99. We analyzed difference in maternal plasma from 1E to CC stage using the REPEATED statement in the MIXED procedure, controlling for clutch mass as a covariate. Unless otherwise stated, all analysis was done on lipid-normalized BDE-99 concentrations.

6.3. Results

6.3.1. Influence of individual variation in body burden and yolk precursor levels on maternal transfer

Maternal treatment with BDE-99 had a significant dose-dependent effect on maternal plasma concentrations, which were significantly higher in the high dose group compared to the control and low dose group at both the 1E and CC stages (Table 6.1). High dose birds had approximately 8x more BDE-99 than the low dose birds at the 1E stage, and approximately 10x more at the CC stage. Egg concentrations were also significantly affected by dose, but not to the same extent as maternal concentrations,

because a smaller proportion of the maternal plasma burden was transferred to eggs in the high dose group compared to the low dose group. The high dose group eggs had approximately 2.3x more BDE-99 than low dose group eggs (Table 6.1).

The lipid normalized egg-to-maternal plasma BDE-99 ratio was assessed by looking at the relationship between the BDE-99 concentration in the third egg (ng/g lipid weight[lw]) and the BDE-99 concentration in 1E maternal plasma (ng/g lw) for each dose group. The control and high dose group had a significant positive egg-to-maternal plasma BDE-99 relationship (Control: $r^2 = 0.604$, $p = 0.0007$, slope = 0.93; high dose: $r^2 = 0.693$, $F_{1,12} = 27.12$, $p = 0.0002$, slope = 0.321) , and the low dose group had a marginally non-significant positive relationship, which may have improved if sample size were increased ($r^2 = 0.262$, $p = 0.061$, slope = 0.709). The slope of the egg-to-maternal plasma relationship in the control and low dose groups was not significantly different from one ($t = 0.330$, $p = 0.748$ and $t = 0.847$, $p = 0.414$, respectively), and in the high dose group the slope was significantly lower than one ($t = 11.029$, $p < 0.0001$). Because the slope of the egg-to-maternal plasma BDE-99 relationship decreased from control to low dose and from low to high dose, we tested the overall fit of an equation based on a saturation binding curve (Egg concentration = [A*Maternal plasma concentration]/[B+Maternal plasma concentration] where A is the maximum egg concentration, and B is the maternal plasma concentration needed to achieve half of the maximum egg concentration) to the full data set (Fig. 6.1). The parameter estimates for the non-linear regression were A = 7506 ± 674.2 SE and B = 3488 ± 844.0 SE. The point of the curve at which the slope is 1 is when the maternal plasma BDE-99 concentration is 1629 ng/g lw. Both the non linear and linear regressions significantly fit the full data set (adjusted $R^2 = 0.879$, $F_{2,41} = 351.95$, $p < 0.0001$ and adj. $r^2 = 0.823$, $F_{1,41} = 196.19$, $p < 0.0001$, respectively), however the fit of the non-linear relationship was significantly better than the linear relationship ($F_{1,41} = 20.26$, $p < 0.0001$).

Maternal 1E plasma VLDL was negatively related to egg BDE-99 (ng/g ww) in the control group, but had no relationship with egg BDE-99 in either dose group (Table 6.2, Fig. 6.2). Egg lipid was not correlated with BDE-99 concentration of the third egg (ng/g ww) in any dose group ($r < 0.125$, $p > .0671$), and was also not correlated with maternal plasma lipid or plasma VLDL at the 1E stage ($r = -0.175$, $p = 0.256$ and $r = -0.222$, $p =$

0.148, respectively). Mean maternal plasma lipid or plasma VLDL at the 1E stage were not significantly different between dose groups ($p > 0.070$).

6.3.2. Effect of dosing level and reproductive investment on maternal reduction in BDE-99

There was a significant decrease in maternal plasma BDE-99 over the laying period based on a repeated measures analysis, comparing 1E and clutch completion, including all doses and controlling for clutch size ($p = 0.001$). However, the interaction between dose group and plasma sample stage was highly significant ($p = 0.001$). While there was a decrease in the maternal plasma concentrations of BDE-99 from the 1E to CC stage in all dose groups (Table 6.1), the decrease was only significant for the high dose group ($p < 0.0001$; Fig. 6.3).

Total clutch mass (g) did not vary significantly with dose ($p = 0.709$). The decrease in maternal plasma BDE during egg-laying (i.e. the difference between 1E maternal plasma and CC maternal plasma BDE-99 concentrations) was significantly positively correlated with clutch mass in the high dose group ($r^2 = 0.395$, $p = 0.016$) but not in the low ($r^2 = 0.275$, $p = 0.054$) or control dose groups ($r^2 = 0.007$, $p = 0.644$) (Fig. 6.4). It is possible that the decrease in maternal plasma BDE-99 over the laying period is due to metabolism of BDE-99 rather than transfer to eggs. However, there were no significant differences in the BDE-47:BDE-99 ratio between the 1E and CC stages in any of the doses ($p > 0.131$ for all doses), something that would not be expected if metabolism of BDE-99 was responsible for the decrease.

6.4. Discussion

In the present study, we found that individual variation in maternal plasma contaminant burden, yolk precursor levels and reproductive effort (clutch mass) was related to maternal transfer of contaminants to eggs. Our dosing protocol resulted in plasma burdens relevant to exposure in wild birds, with concentrations of up to 8818.21ng/g lw in plasma, and up to 5034.44 ng/g lw in eggs. There are reported concentrations of BDE-99 in free-living birds of up to 9200 ng/g lw in peregrine falcon

(*Falco peregrinus*) eggs (Lindberg et al. 2004) and up to 26600 ng/g lw in the liver of sparrowhawks (*Accipiter nisus*) (Voorspoels et al. 2007).

The lipid-normalized relationship between maternal plasma BDE-99 at the timing of yolk deposition and the BDE-99 in the corresponding egg across all individuals followed a saturation curve, with the maximum predicted egg concentration being approximately 7506 ng/g lw. This relationship could have consequences for using eggs as indicators of adult contaminant burdens, as egg contaminant concentrations from highly exposed birds could underestimate adult concentrations. The mechanisms controlling egg:mother contaminant ratios are not well understood. Russell et al. (Russell et al. 1999) proposed that in oviparous organisms, transport of hydrophobic chemicals from the maternal tissues to the eggs is a passive process, and that the lipid-normalized egg-to-maternal tissue concentration ratio would equal 1. In birds, yolk precursors are transported by the plasma to the highly vascularized walls of the developing follicle, where they are then deposited into the developing oocyte via receptor-mediated endocytosis (Burley and Vadehra 1989). Being small, neutral and hydrophobic molecules, PBDEs theoretically should be able to freely diffuse across biological membranes, such as the oocyte plasma membrane (Camenisch et al. 1996). Because of the close association of the yolk with the blood stream, and because of the physicochemical properties of BDE-99, we would expect that the lipid-normalized egg contaminant and maternal plasma levels would be in equilibrium and have a 1:1 relationship. However, at high maternal BDE-99 concentrations the slope of the egg-to-mother relationship was significantly less than one. One possible explanation for the relationship being less than one is that the transfer of BDE-99 to the eggs is not an entirely passive process. Contaminant molecules could bind to other lipophilic particles, such as the yolk precursors, and then be actively transported into the yolk “piggy-back” fashion complexed with VTG or VLDL molecules via receptor-mediated endocytosis (MacLachlan et al. 1994). If contaminant transfer to the egg is a receptor-mediated process, higher concentrations of BDE-99 could be saturating relative to lower concentrations, which corresponds with the relationship that we observed. Alternatively, if BDE-99 transfer to the eggs is entirely through passive diffusion, a possible explanation for egg-to-mother contaminant ratios less than 1 could be that during the rapid growth phase of the yolk there is insufficient time for BDE-99 to reach equilibrium

across compartments. This is not likely the case as in the control and low dose groups the egg-to-maternal plasma BDE-99 relationship was able to reach a slope similar to 1. It has also been proposed that lipid-normalized egg-to-maternal tissue contaminant ratios less than 1 are the result of dilution of egg lipid contaminants from maternal dietary lipids or newly synthesized lipids from the liver (Norstrom et al. 1986, Braune and Norstrom 1989). However, we measured circulating maternal BDE-99 rather than storage tissue BDE-99, which should account for any dilution from additional lipids. Overall, our data suggest that the maternal transfer of BDE-99 is at least partially through a saturable transport process, rather than exclusively passive diffusion.

It has also been suggested that the extent of egg contaminant dilution is related to reproductive investment (Drouillard and Norstrom 2001), and birds that invest low quantities of maternal lipids in eggs will have ratios less than one, while birds that invest large quantities of maternal lipids in eggs and use more endogenous sources of lipid for yolk formation will have ratios closer to one (Norstrom et al. 1986, Drouillard and Norstrom 2001). However in our study, reproductive investment (clutch mass) was the same across dose groups, yet we still observed differences in the egg:mother contaminant ratios. Maternal plasma burden rather than reproductive investment influenced the egg-to-maternal tissue contaminant ratio in our zebra finches, and the lipid-normalized egg-to-maternal tissue contaminant ratio decreased with increasing maternal burden. In contrast with our findings, a study of maternal transfer of contaminants in white leghorn chickens (*Gallus domesticus*) found that the lipid-normalized egg:mother polychlorinated biphenyl (PCB) ratio was not affected by body burden (Bargar et al. 2001). These contrasting results may be accounted for by differences in dosage, as our cumulative administered low and high dose was 707.7 and 3649.8 ng/g bw (33.7 and 173.8 ng/g bw/day for 21 days), whereas in the leghorn chicken study total dosage ranged from 312.5 to 937.5 µg, which in a 1.5 kg hen would be equivalent to 208 to 625 ng/g bw. It is possible that the dosage in the leghorn chicken study was not high enough for saturation to occur and the egg-to-maternal tissue ratio to start decreasing. Additional studies with different species examining the effect of individual variation in maternal burden and reproductive investment on the egg-to-maternal tissue contaminant ratios are needed to identify if the saturating relationship we

saw with BDE-99 is observed for other hydrophobic contaminants and in other species, which could help elucidate potential mechanisms of contaminant transfer.

To further investigate the relationship between maternal and egg BDE-99, we considered the effects of maternal yolk precursor (VLDL) levels on egg BDE-99 at the time of yolk deposition within each dose group. Mothers with more plasma VLDL transferred less BDE-99 in the control group, but there was no relationship between maternal VLDL and egg BDE-99 when maternal BDE-99 concentrations were elevated above background concentrations. Avian VLDL is 87% lipid and 13% protein, and is the main source for yolk lipids (Burley and Vadehra 1989). VLDL is taken up by the plasma membrane of growing oocytes via receptor-mediated endocytosis (Nimpf and Schneider 1991). When VLDL levels in the plasma have saturated the transport mechanisms, increased plasma VLDL does not result in increased egg lipids. In this situation, as maternal plasma VLDL levels increase, the relative proportion of lipids in the plasma compared to the eggs would increase, and fewer hydrophobic contaminants would be transferred to the egg if contaminant transfer is passive. We found no correlation between maternal VLDL, or plasma lipids, with egg lipids. This is consistent with previous studies that have failed to find strong relationships between individual variation in plasma yolk precursor levels and egg composition (Challenger et al. 2001, Salvante and Williams 2002), and suggests that levels of VLDL are typically above those needed to saturate the transport mechanisms in zebra finches. The negative relationship between maternal VLDL and egg BDE-99, such as would be expected if BDE-99 transfer was passive, was only observed in the control group when BDE-99 was at background concentrations, and the relationship dissociated at elevated BDE-99 concentrations. This pattern supports the idea that at higher concentrations the egg-to-mother BDE-99 relationship is not solely driven by passive partitioning, as we have suggested above.

We found that maternal dose group affected the extent of the reduction in maternal plasma BDE-99 from the 1E to CC stage. Maternal plasma BDE-99 significantly decreased from the 1E to the CC stage in the high dose group but not in the low dose group. Thus, while the total reduction in plasma BDE-99 was greater in the high dose group (3129.76 ng/g in high dose, 511.95 ng/g lw in low dose), the proportion of BDE-99 lost was greater in the low dose group (35.5% in high dose, 47.2% in low dose), which corresponds with the pattern of high dose birds transferring proportionally

less BDE-99 to their eggs. Maternal plasma BDE-99 stayed at low concentrations throughout the laying period in control birds. We also found that maternal dose group affected whether or not the reduction in maternal plasma burden over the laying period was related to reproductive investment (measured by clutch mass), as only the high dose group showed a significantly positive relationship. The reduction in maternal plasma BDE-99 over the 3- to 7-day laying period is most likely due to elimination through maternal transfer, as there is evidence in birds that BDE-99 is persistent with an estimated half-life of 100 to 175 days (Norstrom et al. 2002, Drouillard et al. 2007). In addition we observed no increase in the BDE-47:BDE-99 ratio, which suggests that metabolism by debromination did not contribute significantly to the reduction in maternal plasma BDE-99 burden. Several studies that report lower concentrations of contaminants in females than in male birds suggest that it is due to the female having an additional contaminant elimination route through egg laying (Peterson and Ellarson 1978, Tanabe et al. 1998, Donaldson and Braune 1999). However, differences in male and female contaminant concentrations are not always observed (Lundstedt-Enkel et al. 2005, Luo et al. 2009). Our results show that while females can eliminate large amounts of their burden to the eggs, the reduction may not be significant in birds with low initial body burdens or small clutch masses. That could explain why differences in male and female contaminant burdens are not always observed. The relationships of contaminant exposure level and clutch mass with the reduction in maternal burden over the laying period could also have consequences on laying order effects, if the decreasing burden results in declining egg concentrations. Reviews of studies on laying order effects on egg contaminant concentrations have found that there is no consistent pattern for egg contaminants over the laying sequence, and the majority of studies show no significant laying order effects (Van den Steen et al. 2009, Custer et al. 2010). That is not surprising as individual variation in maternal contaminant burden and reproductive investment could result in variation in laying order effects among individuals. In addition, if the saturating egg-to-mother contaminant relationship that we observed for BDE-99 occurs for other contaminants, proportionally less contaminants will be transferred to the egg at higher maternal burdens, which could also minimize laying order effects.

In summary, we found a significant effect of plasma burden on maternal transfer, with more highly exposed birds transferring proportionally less BDE-99 to their eggs.

These data suggest that maternal transfer of BDE-99 involves a saturable transport process. This has significant implications for using eggs as indicators of adult or environmental concentrations, as even within a species the egg-to-maternal tissue relationship can vary significantly, and eggs from highly exposed birds may underestimate adult exposure. Maternal burden also affects whether individual variation in female lipid status, as measured by yolk precursor levels of the mother, can influence contaminant transfer to the egg. Mothers with higher plasma VLDL levels transfer less BDE-99 to their eggs only when BDE-99 is at background levels. Finally, the decreases in maternal plasma BDE-99 burden over the laying period, as contaminants are transferred to eggs, was affected by dose level (i.e. body burden) and reproductive investment, with only the high dose group showing a significant reduction burden, and the reduction being greater in birds with larger clutches. These results suggest that the presence of sex differences in adult contaminants or laying order effects on egg contaminants can be highly variable, even within a species.

6.5. References

Bacon, W. L., M. A. Musser, and K. I. Brown. (1974). Plasma free fatty acid and neutral lipid concentrations in immature, laying and broody turkey hens. *Poultry Science* 53:1154-1160.

Bargar, T. A., G. I. Scott, and G. P. Cobb. (2001). Maternal transfer of contaminants: Case study of the excretion of three polychlorinated biphenyl congeners and technical-grade endosulfan into eggs by white leghorn chickens (*Gallus domesticus*). *Environmental Toxicology and Chemistry* 20:61-67.

Braune, B. M. and R. J. Norstrom. (1989). Dynamics of organochlorine compounds in Herring-Gulls - .3. Tissue distribution and bioaccumulation in Lake-Ontario gulls. *Environmental Toxicology and Chemistry* 8:957-968.

Burley, R. W. and D. V. Vadehra. (1989). The Avian Egg: Chemistry and Biology. Wiley, New York.

Camenisch, G., G. Folkers, and H. van de Waterbeemd. (1996). Review of theoretical passive drug absorption models: historical background, recent developments and limitations. *Pharmaceutica acta Helvetiae* 71:309-327.

Challenger, W. O., T. D. Williams, J. K. Christians, and F. Vezina. (2001). Follicular development and plasma yolk precursor dynamics through the laying cycle in the European starling (*Sturnus vulgaris*). *Physiological and Biochemical Zoology* 74:356-365.

Chen, D. and R. C. Hale. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International* 36:800-811.

Chen, D., R. J. Letcher, N. M. Burgess, L. Champoux, J. E. Elliott, C. E. Hebert, P. Martin, M. Wayland, D. V. Chip Weseloh, and L. Wilson. (2012). Flame retardants in eggs of four gull species (Laridae) from breeding sites spanning Atlantic to Pacific Canada. *Environmental Pollution* 168:1-9.

Christians, J. K. (2002). Avian egg size: variation within species and inflexibility within individuals. *Biological Reviews* 77:1-26.

Custer, C. M., B. R. Gray, and T. W. Custer. (2010). Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallws, *Tachycineta bicolor Environmental Toxicology and Chemistry* 29:909-921.

Donaldson, G. M. and B. M. Braune. (1999). Sex-related levels of selenium, heavy metals, and organochlorine compounds in American white pelicans (*Pelecanus erythrorhyncos*). *Archives of Environmental Contamination and Toxicology* 37:110-114.

Drouillard, K. G., K. J. Fernie, R. J. Letcher, L. J. Shutt, M. Whitehead, W. Gebink, and D. A. Bird. (2007). Bioaccumulation and biotransformation of 61 polychlorinated biphenyl and four polybrominated diphenyl ether congeners in juvenile American kestrels (*Falco sparverius*). *Environmental Toxicology and Chemistry* 26:313-324.

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

Elliott, J. E., L. K. Wilson, and B. Wakeford. (2005). Polyhrominated diphenyl ether trends in eggs of marine and freshwater birds from British Columbia, Canada, 1979-2002. *Environmental Science & Technology* 39:5584-5591.

Eng, M. L., J. E. Elliott, S. A. MacDougall-Shackleton, R. J. Letcher, and T. D. Williams. (2012). Early exposure to 2,2 ',4,4 ',5-Pentabromodiphenyl ether (BDE-99) affects mating behavior of zebra finches. *Toxicological Sciences* 127:269-276.

Frings, C. S., C. A. Queen, R. T. Dunn, and T. W. Fendley. (1972). Improved determination of total serum-lipids by sulfo-phospho-vanillin reaction *Clinical Chemistry* 18:673-674.

Gauthier, L. T., C. E. Hebert, D. V. C. Weseloh, and R. J. Letcher. (2008). Dramatic changes in the temporal trends of polybrominated diphenyl ethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982-2006. *Environmental Science & Technology* 42:1524-1530.

Gorman, K. B., D. Esler, R. L. Walzem, and T. D. Williams. (2009). Plasma yolk precursor dynamics during egg production by female greater scaup (*Aythya marila*): Characterization and indices of reproductive state. *Physiological and Biochemical Zoology* 82:372-381.

Haywood, S. (1993). Sensory control of clutch size in the Zebra Finch (*Taeniopygia guttata*). *Auk* 110:778-786.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environmental Science & Technology* 38:945-956.

Lindberg, P., U. Sellstrom, L. Haggberg, and C. A. de Wit. (2004). Higher brominated diphenyl ethers and hexabromocyclododecane found in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Environmental Science & Technology* 38:93-96.

Lundstedt-Enkel, K., A. K. Johansson, M. Tysklind, L. Asplund, K. Nylund, M. Olsson, and J. Orberg. (2005). Multivariate data analyses of chlorinated and brominated contaminants and biological characteristics in adult guillemot (Uria aalge) from the Baltic Sea. *Environmental Science & Technology* 39:8630-8637.

Luo, X. J., J. Liu, Y. Luo, X. L. Zhang, J. P. Wu, Z. Lin, S. J. Chen, B. X. Mai, and Z. Y. Yang. (2009). Polybrominated diphenyl ethers (PBDEs) in free-range domestic fowl from an e-waste recycling site in South China: Levels, profile and human dietary exposure. *Environment International* 35:253-258.

MacLachlan, I., J. Nimpf, and W. J. Schneider. (1994). Avian riboflavin binding protein binds to lipoprotein receptors in association with vitellogenin. *Journal of Biological Chemistry* 269:24127-24132.

Mitchell, M. A. and A. J. Carlisle. (1991). Plasma zinc as an index of vitellogenin production and reproductive status in the domestic fowl. *Comparative Biochemistry and Physiology a-Physiology* 100:719-724.

Nimpf, J. and W. J. Schneider. (1991). Receptor-mediated lipoprotein transport in laying hens. *Journal of Nutrition* 121:1471-1474.

Norstrom, R. J., T. P. Clark, D. A. Jeffrey, H. T. Won, and A. P. Gilman. (1986). Dynamics of organochlorine compounds in Herring-Gulls (*Larus Argentatus*) .1. Distribution and clearance of C-14 DDE in free-living Herring-Gulls (*Larus Argentatus*). *Environmental Toxicology and Chemistry* 5:41-48.

Norstrom, R. J., M. Simon, J. Moisey, B. Wakeford, and D. V. C. Weseloh. (2002). Geographical distribution (2000) and temporal trends (1981-2000) of brominated diphenyl ethers in Great Lakes herring gull eggs. *Environmental Science & Technology* 36:4783-4789.

Peterson, S. R. and R. S. Ellarson. (1978). p,p'-DDE, polychlorinated biphenyls, and endrin in old squaws in North America 1969-73 *Pesticides Monitoring Journal* 11:170-181.

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

Salvante, K. and T. D. Williams. (2002). Vitellogenin dynamics during egg-laying: daily variation, repeatability and relationship with reproductive output. *Journal of Avian Biology* 33:391-398.

Tanabe, S., K. Senthilkumar, K. Kannan, and A. N. Subramanian. (1998). Accumulation features of polychlorinated biphenyls and organochlorine pesticides in resident and migratory birds from South India. *Archives of Environmental Contamination and Toxicology* 34:387-397.

Van den Steen, E., V. L. B. Jaspers, A. Covaci, H. Neels, M. Eens, and R. Pinxten. (2009). Maternal transfer of organochlorines and brominated flame retardants in blue tits (*Cyanistes caeruleus*). *Environment International* 35:69-75.

Van den Steen, E., R. Pinxten, A. Covaci, C. Carere, T. Eeva, P. Heeb, B. Kempenaers, J. T. Lifjeld, B. Massa, A. C. Norte, M. Orell, J. J. Sanz, J. C. Senar, A. Sorace, and M. Eens. (2010). The use of blue tit eggs as a biomonitoring tool for organohalogenated pollutants in the European environment. *Science of the Total Environment* 408:1451-1457.

Vanderkist, B. A., T. D. Williams, D. F. Bertram, L. Lougheed, and J. P. Ryder. (2000). Indirect, physiological assessment of reproductive state and breeding chronology in free-living birds: an example in the Marbled Murrelet (*Brachyramphus marmoratus*). *Functional Ecology* 14:758-765.

Verreault, J., R. A. Villa, G. W. Gabrielsen, J. U. Skaare, and R. J. Letcher. (2006). Maternal transfer of organohalogen contaminants and metabolites to eggs of Arctic-breeding glaucous gulls. *Environmental Pollution* 144:1053-1060.

Voorspoels, S., A. Covaci, V. L. B. Jaspers, H. Neels, and P. Schepens. (2007). Biomagnification of PBDEs in three small terrestrial food chains. *Environmental Science & Technology* 41:411-416.

Williams, T. D. (2012). Physiological Adaptations for Breeding in Birds. Princeton University Press, Princeton.

Williams, T. D. and C. J. Martyniuk. (2000). Tissue mass dynamics during egg-production in female Zebra Finches *Taeniopygia guttata*: dietary and hormonal manipulations. *Journal of Avian Biology* 31:87-95.

Table 6.1. Lipid normalized BDE-99 concentrations (ng/g lw) in maternal plasma at the first egg (1E) and clutch completion (CC) stage, and in the third egg laid. Mean (SE).

		Dose (ng BDE-99/g bw/day)				
Sample	**% lipid**	**0**	**33.7**	**173.8**	**$F_{2,43}$**	***p***
1E plasma	2.31	35.53 (9.91)[a]	1083.70 (143.62)[a]	8818.21 (1061.14)[b]	64.58	<0.0001
CC plasma	2.00	29.50 (3.66) [a]	571.75 (61.01) [a]	5688.45 (646.60)[b]	74.48	<0.0001
third egg	7.35	68.20 (11.88) [a]	2148.37 (206.49)[b]	5034.44 (408.78)[c]	94.00	<0.0001

[abc]Significant difference between groups are indicated by different lower case letters ($p < 0.05$)

Table 6.2. The relationship between egg BDE-99 (ng/g ww) and maternal plasma VLDL (mg triglyceride/ml) controlling for the effect of maternal 1E plasma BDE-99 on egg BDE-99.

	Unstandardized coefficients		Standardized coefficients			
Dose group	**b**	**SE**	**β**	***t***	***p***	**R^2**
Control	-0.289	0.131	-0.522	-2.2	0.046	0.272
Low	0.556	1.413	0.113	0.39	0.701	0.013
High	4.828	8.026	0.171	0.6	0.559	0.029

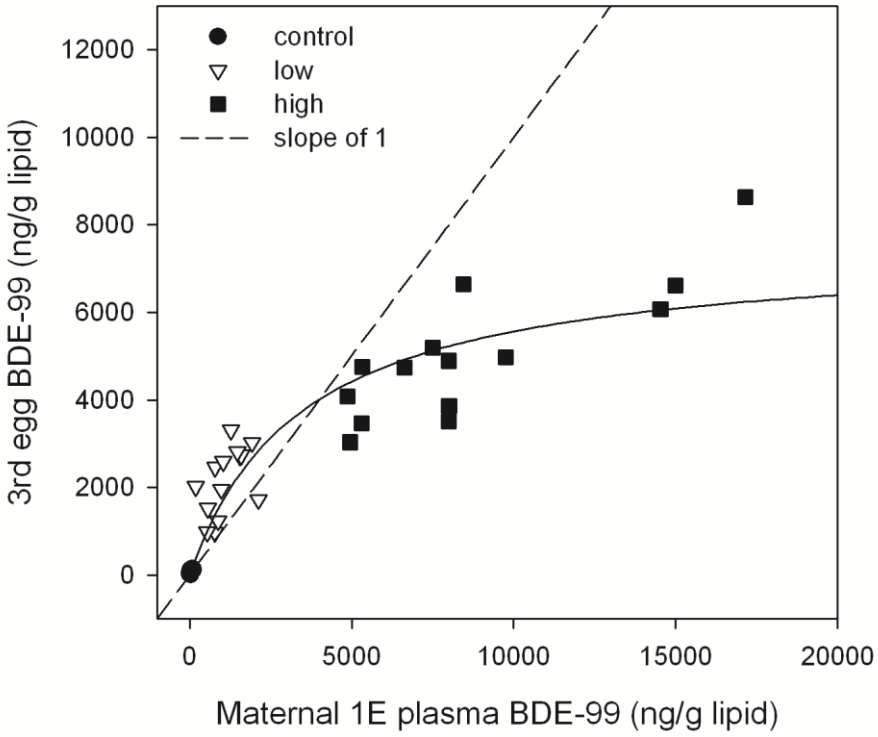

Figure 6.1. ***Relationship between lipid-normalized BDE-99 concentration in the third egg, and the maternal plasma on the day that the first egg was laid (1E). The timing of yolk deposition for the third egg corresponds with the timing of the 1E blood sample. The lipid-normalized egg-to-maternal tissue BDE-99 relationship is described by the equation: Egg concentration = (7506*Maternal concentration)/(3488+Maternal concentration).***

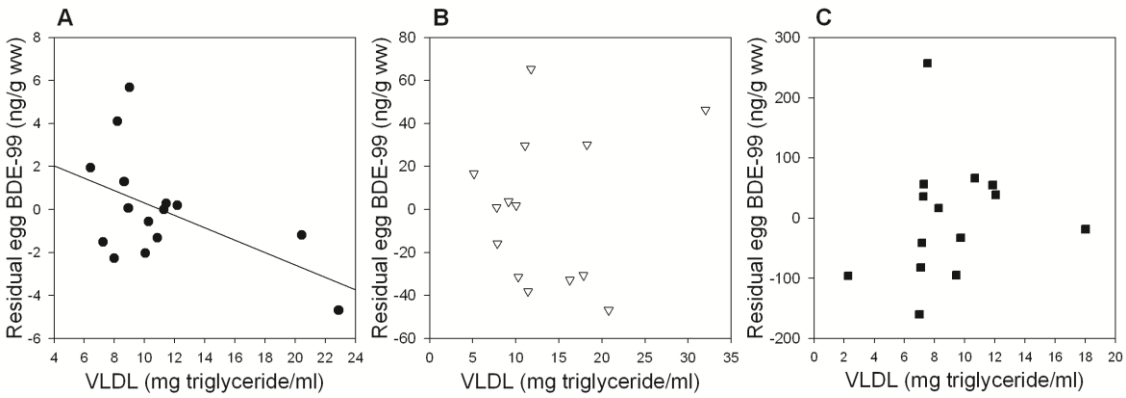

Figure 6.2. Relationship between BDE-99 in the third egg (ng/g ww) and maternal plasma VLDL (mg triglyceride/ml) for each dose group, controlling for the effect of maternal 1E plasma BDE-99 on egg BDE-99. The slope (unstandardized coefficient) and associated probability for each relationship is given in Table 2. Egg BDE-99 was negatively related to maternal plasma VLDL in the control group (A), and had no relationship with egg BDE-99 in either the low (B) or high (C) dose group.

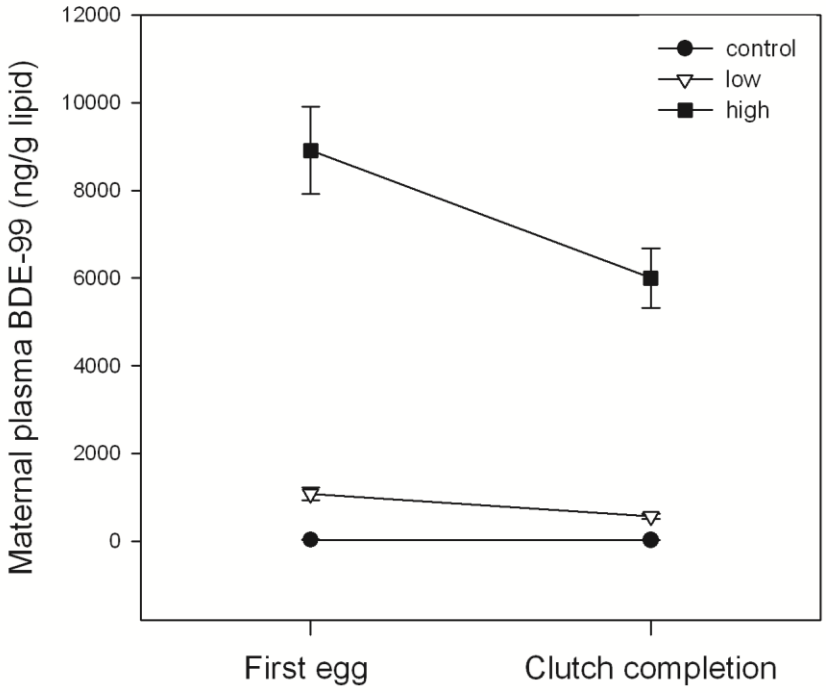

Figure 6.3. ***Decrease in maternal plasma burden over laying period. Maternal plasma BDE-99 (ng/g lw) concentration the day the first egg (1E) was laid and at clutch completion (CC). There is a significant decrease in plasma concentration over the laying period, controlling for clutch size (p = 0.001). The interaction between dose and plasma sample is significant (p = 0.001). Maternal plasma BDE-99 did not differ significantly from the 1E to CC stage in the control or low dose groups (p = 0.991 and p = 0.352, respectively), but there were significant differences between 1E and CC plasma BDE-99 concentrations in the high dose group (p < 0.0001). Error bars represent SE.***

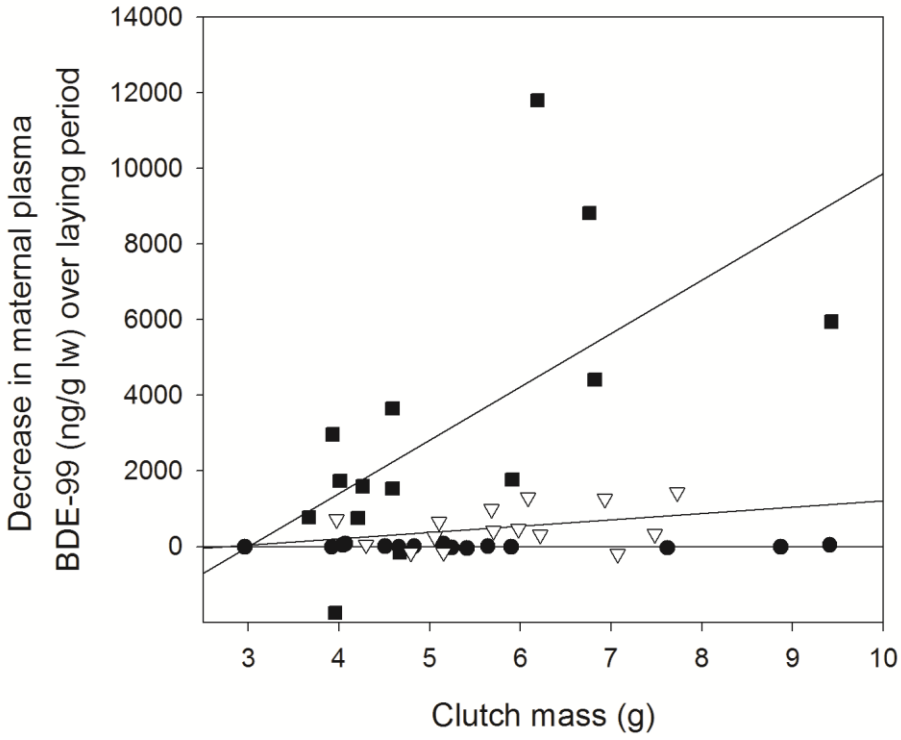

Figure 6.4. ***Effect of clutch mass (total mass of all eggs laid) on the decrease in maternal plasma BDE-99 (ng/g lw) over laying period (from the day the first is egg laid to clutch completion). The high dose group (solid squares) had a significant positive relationship between clutch mass and maternal plasma BDE-99 reduction (r^2 = 0.395, p = 0.016 slope = 1408.750), and the low dose (open triangles) had a weak positive relationship (r^2 = 0.275, p = 0.054, slope = 245.466).There was no relationship in the control group (solid circles, r^2 = 0.007, p = 0.644, slope = -1.638).***

7. General Conclusions

7.1. Zebra finches and European starlings as model species

We successfully developed an integrated laboratory and field avian model system, and found that zebra finches and European starlings make very efficient and effective models for linking contaminant exposure with biological responses. Due to the short generation time of zebra finches and their willingness to breed in captivity, we were able to assess long-term effects of developmental exposure to contaminants on measures such as mating behavior and reproductive success, which are difficult to study in free-living species. The broad distribution of European starlings and their use of nest boxes makes monitoring and manipulation of contaminant exposure under ecological conditions relatively easy, and through the use of photoperiod manipulations, we were able to assess long-term developmental effects of exposure. We have shown that oral dosing is a valid method for exposing nestlings to contaminants, and that in zebra finches it is relatively easy to expose embryos to contaminants of interest via maternal transfer. A recent study validated the use of egg injection for embryotoxicity studies in the zebra finch (Winter et al. 2013), and exogenous compounds have also successfully injected into starling eggs (e.g. Love and Williams 2008). In future studies using this model system, egg injection as well as oral dosing of the nestlings could serve as potential exposure routes in both species, and maternal transfer could be used to expose zebra finch embryos. Other benefits of this system include that both the zebra finch and the European starling can be easily blood sampled at the nest, which allows for non-terminal assessment of physiological condition and plasma contaminant burden, and that chick growth and survival is also easily measured in both species.

7.2. Long-term effects of early exposure to BDE-99 in passerines

Overall, we found that the long-term effects of early exposure to BDE-99 at concentrations relevant to exposure in wild birds are subtle in passerines, with behaviour being the most sensitive endpoint. In nestling-exposed zebra finches we observed a significant reduction in male mating behaviour and in the effectiveness of male courtship (as measured by the response of unexposed females to exposed males). Female zebra finches exposed to high concentrations of BDE-99 as nestlings were more likely to delay the onset of egg laying. If similar effects occur in free-living birds as in captive zebra finches, then current environmental concentrations of BDE-99 may be high enough to impact reproduction and lifetime fitness in those birds. In European starlings, there was some evidence that BDE-99 exposure disrupted fledgling thyroid hormones; however, whether this was a biologically real effect was not clear. Our results demonstrate that in addition to measures of overt effects, such as growth and survival, it is important to also measure more subtle effects, such as changes in reproductive behavior or biochemical qualities of the plasma, to effectively assess the ecological risk of contaminant exposure. Based on observed effects in our studies, mechanisms related to mating behavior, timing of breeding, and thyroid hormone homeostasis should be included as important endpoints in future studies of effects of PBDEs.

In both zebra finches and starlings exposed to BDE-99 as nestlings, there were few other observed effects on any measure of growth, survival, physiology, neuroanatomy, or reproduction, which suggests that passerines may be less sensitive to BDE-99 exposure than mammals or other bird species are reported to be. Similarly, a study in adult European starlings exposed to a PBDE mixture also found minimal reproductive, endocrine disrupting, hematological or biochemical effects, and also concluded that passerines may be less sensitive than other bird species to PBDE exposure (Van den Steen et al. 2009, Van den Steen et al. 2010). Another possibility for the lack of observed effects in our study may be that while our doses covered a range of concentrations comparable to those reported in free-living birds, higher concentrations are needed to cause adverse effects. Alternatively, the embryonic life stage may be more sensitive than the nestling period, and *in ovo* exposure may be necessary to see effects on physiology and reproduction. In agreement with that, zebra finches exposed to

BDE-99 as embryos rather than as nestlings did show significant reductions in clutch size (Winter et al. in press) and song-control nuclei volumes (V. Winter, unpublished data).

7.3. Organohalogen contaminants in terrestrial passerines at an agricultural site

We found that European starlings in a rural agricultural area of British Columbia are being exposed to PBDEs, PCBs, DDTs, and other OCs at concentrations below those of concern, and we did not observe any relationships between PBDE concentrations in eggs and reproductive or condition measures of the corresponding nest. The only form of DDT detected was the principle metabolite, p,p'-DDE, indicating historical agricultural use, rather than current release. The congener profiles of PBDEs and PCBs reflect those of commercially used mixtures. These results contribute to our relatively limited knowledge of organohalogen concentrations in terrestrial passerines, and also demonstrate how egg collection and nest box monitoring in European starlings can be used to relate individual variation in contaminant burden with individual measures of condition and reproductive success at the nest level.

7.4. Maternal transfer of BDE-99 in a passerine species

We found that individual variation in maternal plasma contaminant burden, yolk precursor levels and reproductive effort (clutch mass) was related to maternal transfer of BDE-99 to eggs in zebra finches. More highly exposed birds transferred proportionally less BDE-99 to their eggs, which suggests that maternal transfer of BDE-99 involves a saturable transport process. Mothers with higher plasma yolk lipid precursors transferred less BDE-99 to their eggs when BDE-99 was at background levels, but not when BDE-99 was elevated. These results indicate that whether or not individual variation in maternal yolk precursors is related to the amount of contaminant transfer is influenced by maternal body burden. The degree to which mothers offloaded BDE-99 to eggs was related to both body burden and reproductive investment, with the total reduction in maternal plasma burden from egg laying only being significant in highly exposed birds,

and being greater in birds with larger clutches. Individual variation in contaminant burden and reproductive investment could contribute to the high variability in published studies of whether sex differences in adult contaminants and laying order effects on egg contaminants are observed or not. This characterization of BDE-99 transfer from mothers to eggs contributes to our currently limited understanding of the mechanisms of maternal transfer of hydrophobic molecules.

7.5. Future directions

We plan to use the avian model system that we have developed here to assess chemicals identified as priorities by the government of Canada's Chemical Management Plan (CMP), specifically organophosphorus flame retardants (OPFRs). We also plan to further develop the zebra finch and starling models following the Adverse Outcome Pathway (AOP) approach, which has a goal of linking mechanistic data, such as molecular or biochemical measures, to adverse outcomes at levels that are more relevant to ecological risk, such as effects on survival, development, and reproduction, which by extension are measures of impacts of chemicals on populations (Ankley et al. 2010). Selection of specific chemicals and endpoints that we will examine in whole organism studies using zebra finches and starlings will be informed by results from high throughput *in vitro* assays in avian cell cultures. The use of zebra finches and starlings can extend an adverse outcome pathway to effects with reproductive and, therefore, population level consequences. This research has the potential to link in vitro effects to in vivo effects observed in a laboratory species to field effects observed in free-living birds.

7.6. References

Ankley, G. T., R. S. Bennett, R. J. Erickson, D. J. Hoff, M. W. Hornung, R. D. Johnson, D. R. Mount, J. W. Nichols, C. L. Russom, P. K. Schmieder, J. A. Serrrano, J. E. Tietge, and D. L. Villeneuve. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry* 29:730-741.

Love, O. P. and T. D. Williams. (2008). The adaptive value of stress-induced phenotypes: Effects of maternally derived corticosterone on sex-biased investment, cost of reproduction, and maternal fitness. *American Naturalist* 172:E135-E149.

Van den Steen, E., M. Eens, A. Covaci, A. C. Dirtu, V. L. B. Jaspers, H. Neels, and R. Pinxten. (2009). An exposure study with polybrominated diphenyl ethers (PBDEs) in female European starlings (*Sturnus vulgaris*): Toxicokinetics and reproductive effects. *Environmental Pollution* 157:430-436.

Van den Steen, E., M. Eens, A. Geens, A. Covaci, V. M. Darras, and R. Pinxten. (2010). Endocrine disrupting, haematological and biochemical effects of polybrominated diphenyl ethers in a terrestrial songbird, the European starling (*Sturnus vulgaris*). *Science of the Total Environment* 408:6142-6147.

Winter, V., J. E. Elliott, R. J. Letcher, and T. D. Williams. (2013). Validation of an egg-injection method for embryotoxicity studies in a small, model songbird, the zebra finch (*Taeniopygia guttata*). *Chemosphere* 90:125-131.

Winter, V., T. D. Williams, and J. E. Elliott. (in press). A three-generational study of in ovo exposure to PBDE-99 in the zebra finch. *Environmental Toxicology and Chemistry*.