Validation of an Egg-injection Method for Testing *in ovo* Toxicity of PBDE-99 in a Small Songbird Model Species, the Zebra Finches (*Taeniopygia guttata*)

by

Viktoria Khamzina

B.Sc. (Agriculture and Environmental Science), McGill University, 2007

PROJECT SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF ENVIRONMENTAL TOXICOLOGY

in the
Department of Biology
Faculty of Science

SIMON FRASER UNIVERSITY

Fall 2011

Approval

Name: **Viktoria Khamzina**

Degree: **Master of Environmental Toxicology**

Title of Thesis: ***Validation of an egg-injection method for testing in ovo toxicity of PBDE-99 in a small songbird model species, the zebra finches (Taeniopygia guttata)***

Examining Committee:

Chair: Firstname Surname, Position

Tony D. Williams
Senior Supervisor
Professor

Chris J. Kennedy
Supervisor
Professor

John E. Elliott
Supervisor
Adjunct Professor

Firstname Surname
Internal Examiner
Assistant/Associate/Professor
School/Department or Faculty

Date Defended/Approved: November 25, 2011

Declaration of
Partial Copyright Licence

SFU SIMON FRASER UNIVERSITY
THINKING OF THE WORLD

STATEMENT OF ETHICS APPROVAL

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

(a) Human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

(b) Advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

(c) as a co-investigator, collaborator or research assistant in a research project approved in advance,

or

(d) as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Simon Fraser University
Burnaby, BC, Canada

Last update: Spring 2010

Abstract

Egg injection was validated for studies of *in ovo* exposure to xenobiotics in the zebra finch (*Taeniopygia guttata*). Puncturing eggs and injecting DMSO had no effect on embryo development or hatching success, but DMSO negatively affected post-hatching growth in males. *In ovo* injection was used to test the long-term, inter-generational effects of embryonic exposure to the polybrominated diphenyl ether PBDE-99. Eggs were dosed at 10, 100 and 1000 ng/egg and chicks, their offspring and grand-offspring were followed (three generations). *In ovo* PBDE exposure did not affect hatching success, chick growth, thyroid hormone levels or hematology (measured at 30 and 90 days of age). However, there were effects of PBDE treatment on adult phenotype of *in ovo* exposed bird: reduced clutch size, longer laying intervals. Second generation chicks of PBDE-exposed parents had decreased growth, but there were no longer-term effects on adult reproductive phenotype of second-generation offspring, or growth of their (third-generation) offspring.

Keywords: Clutch size; egg injection method; development; multigenerational studies; PBDE-99; zebra finch

Acknowledgements

The author thanks Margaret Eng for use, guidance, and help with the zebra finches at the Animal Care facility, Simon Fraser University (BC, Canada), Tony Williams (Simon Fraser University, BC, Canada), for academic and technical support, John Elliott (Pacific Wildlife Research Centre, BC, Canada) for inspiration, Robert Letcher and Luke Periard for chemical analyses of bird tissues and dose validation in Organic Contaminants Research Laboratory at National Wildlife Research Centre (ON, Canada), and Peer Winter (BC, Canada) for assistance and moral support. This research was supported by a Chemical Management Plan Grant, Environment Canada.

Table of Contents

Article 1.
Vehicle test for an egg-injection method for a small songbird species, the zebra finch (*Taeniopygia guttata*)

1.1. Introduction

The increasing presence of polybrominated diphenyl ethers (PBDEs) in the environment and biota has become a concern in recent years (de Wit 2002, Henny et al. 2009, Chen & Hale 2010) due to bioaccumulation in aquatic and terrestrial organisms. PBDEs belong to the group of flame retardants commonly used in polymers, textiles, electronics, and other materials. Among 209 different PBDE congeners the most common ones found in biota are BDE-47, -99 (also known as penta-BDE) and -153 (Darnerud et al. 2001; 2003). With low water solubility and high binding affinity to suspended particles, these lipophilic persistent compounds steadily accumulate in soil and sediments and become bioavailable for aquatic and terrestrial organisms, and therefore, biomagnify at the top of food web (Sellstrom et al. 2005, de Wit 2002). For example, studies of the North Sea food web revealed that the mean concentration of PBDE-99, lipid normalized, was up to two times higher in herring liver (12 ng/g) than in shrimp (5.1 ng/g), while in marine mammals liver (715 ng/g) it was up to 60 times higher than in herring (Boon et al. 2002, Law et al. 2003). Concentrations of penta- and octa-BDEs increased exponentially in herring gull eggs at the Great Lakes basin from 1981 to 2006 (Gauthier et al. 2006, Norstrom et al. 2002). In British Columbia, the concentration of penta-BDEs increased 305-fold from 1.3 to 455 µg/g with doubling time every 5.4 years in the eggs of great blue herons from 1983 to 2002 (Elliott et al. 2005). The biological significance of these increased concentrations of PBDEs in the environment is largely unknown because adverse effect thresholds and toxicological or physiological effects in birds remains poorly studied (McKernan et al. 2009).

Concentrations of xenobiotics in bird eggs and nestlings can accurately reflect contamination in the environment, because females transfer or 'excrete' contaminants which have accumulated in lipid-rich tissues in their body, to their eggs during egg formation (Dauwe et al. 2006). For that reason, bird eggs are commonly used as a biomonitoring tool for detecting levels, distribution and long-term trends in environmental contamination with persistent organic pollutants (Elliott et al. 2005, Norstrom et al. 2002, Van den Steen et al. 2010). Although quite a few studies have investigated the processes of maternal transfer of xenobiotics to eggs in birds (Bargar et al. 2001, Drouillard and Norstrom 2001, Van den Steen et al. 2009, Verreault et al. 2006), there is a lack of experimental works related to direct effects of this process on embryo development. It is widely assumed that maternally transferred contaminants can cause adverse effects on the embryo, considering that this developmental stage appears to be among the most vulnerable periods in the life cycle (Ottinger et al. 2008). For example, malformations, associated with the most common persistent organic pollutants, such as polychlorinated biphenyls (PCBs), polychlorinated diphenyl ethers (PCDE), PBDEs, and organochlorine pesticides in guillemot (*Uria aalge*) eggs from the Baltic Sea and Atlantic Ocean, included edema, hemorrhaging, open body cavities, anopthalmia, deformed bills, and aberrant limbs, both in living and in dead embryos (de Roode et al. 2002).

The effect of maternally-derived xenobiotics on offspring development can be studied either indirectly by treatment of the mother or by direct egg injection of contaminants into the air cell or yolk sac of the egg. It is not known if egg injection techniques correctly imitate the natural distribution of xenobiotics in eggs (McKernan et al. 2009). However, by directly dosing eggs with known, specific amounts of xenobiotic, egg injection can avoid problems associated with the indirect exposure of chicks via dosing of egg-laying mothers such as differential food intake, uptake and accumulation, or transfer to eggs. Direct injection into the egg therefore has the main advantage over feeding trials in that it enables the researcher to know absolute dose levels and exposure which can be directly related to embryotoxic effects (McKernan et al. 2010).

Only four avian studies have evaluated developmental and reproductive effects of environmentally relevant concentrations of PBDE-mixtures using *in ovo* exposure via egg injection (Fernie et al. 2006, Fernie et al. 2009, McKernan et al. 2009, Marteinson et al. 2010). Furthermore, all studies to date have been on relatively large birds, with

correspondingly large egg sizes (15-60g), which might not provide a good model for *in ovo* effects in small songbirds. To our knowledge, no studies have validated and utilized the egg injection method for small songbirds, perhaps due to technical difficulties associated with injection/incubation of the small eggs. Here we validate the use of egg injection for studies of effects of *in ovo* exposure to xenobiotics for a small songbird model species, the zebra finch (*Taeniopygia guttata*), where egg weight averages only 1 g. This was part of a larger study designed to investigate the effect on *in ovo* exposure to PBDE-99 on embryo development, hatchability, chick development and adult phenotype (see Article 2). In a first experiment we investigated the effect of puncturing eggs with or without vehicle (DMSO) injection on egg fate (hatched, infertile, incomplete embryo development, missing eggs), chick hatching success and subsequent growth to 90 days (sexual maturity). In a second larger experiment we investigated the effects of two vehicle solutions (DMSO and safflower oil) on embryo and chick growth, as well as repeating the punctured-only treatment, in relation to that of non-manipulated eggs within each clutch.

1.2. Material and methods

1.2.1. Animal care

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light/dark schedule, 14L:10D, lights on at 07.00). All birds were provided with a mixed seed (millet) diet, water, grit, and cuttlefish bone (calcium) *ad libitum,* and received a multivitamin supplement in the drinking water once per week. Breeding pairs also received 6 g of egg food supplement (20.3% protein: 6.6% lipid) per day between pairing and clutch completion, and again during the chick-rearing period. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit no. 864B-08 in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

1.2.2. Breeding and egg-injection protocol

Experiment 1. For the initial validation experiment, twenty four (24) male and female birds were assigned to breeding pairs at random. All birds used in this experiment had bred (laid eggs) at least once previously, and were more than 6 months

old. Breeding pairs were housed individually in cages (50 x 41 x 37 cm) each with an external nest box (14.5 x 14.5 x 20 cm). On the day of pairing, body mass (± 0.01 g) and tarsus length (± 0.01 mm) of all birds was recorded, and females were weighed on the day they laid their first egg and when the clutch was completed (two days after the last egg was laid). Nest boxes were checked daily between 9.00 am and 12.00 pm and all new eggs were weighed (± 0.001 g), and numbered, to obtain data on egg size, clutch size, and laying interval (the time between pairing and laying of the first egg). All eggs were injected within 24 hours of being laid (day 0). Eggs were randomly assigned to either the punctured-only group (*n* = 32 eggs) or DMSO-injected group (*n* = 32 eggs), so that each clutch contained the eggs from both treatments (to control for variation among females, and genetic background).

Before puncture of the shell or injection, the upper side of each egg was cleaned with 70% ethyl alcohol, using a swab. A hole was made with a sterile needle (26 ½ gauge) into the pointed end of the egg from the side. For the punctured-only group eggs received a sham injection, where the sterile needle was pushed through the shell and into the egg approximately 7 mm deep at a 45° angle, to reach the yolk, but no solution was injected into the egg. For DMSO-injected eggs, a constant volume (5 µl/egg) of DMSO was injected into the yolk sac using a Hamilton syringe. In both cases the hole was then sealed with Vetbond (1469SB). Injected eggs were kept in a vertical position for 5 min to allow the seal to dry, and were then placed back into the nest-box for incubation by the parent birds.

Experiment 2. For the vehicle test experiment, we used *n* = 42 breeding pairs. Birds were maintained and bred as described above, but eggs were randomly assigned to four treatments within each clutch. The treatments included a DMSO-injected group (*n* = 45 eggs), safflower oil – injected group (*n* = 43 eggs), punctured-only group (*n* = 45 eggs), and non-injected or non-manipulated eggs which were only handled and replaced in the nest (*n* = 44 eggs). For sealing, Vetbond was replaced with Loctite Super Glue gel (cyanoacrylate) to achieve pin-point control of sealed surface (topical application volume was approximately 1 µl). Injected eggs were kept in a vertical position for 10 min to allow the seal to dry before they were put back to the nest.

Following egg treatments, in both experiments, nest boxes were monitored daily to record “missing” or “broken” eggs. We considered eggs as missing if they disappeared during the period of incubation. Eggs were classified as “broken” if they were accidentally broken during injection or if they were found broken by birds, and they were excluded from analysis. Eggs laid within two days of pairing and eggs lighter than 0.600 g were removed from the nest and excluded from the experiment for likeliness of infertility. Prior to hatching, nest boxes were checked three times a day (09.00, 13.00, 17.00) to determine hatching success of each egg. All newly hatched chicks were then weighed within 24 hours of hatching. Newly hatched chicks were individually marked initially by clipping down feathers from specific feather tracts using a unique combination to identify each chick within a brood. At day 8 post-hatching all chicks were permanently banded using numbered metal bands. Hatching success per treatment was recorded, and all chicks were weighed at day 0, 7, 14, 21 (= fledging), 30 (= independence), and 90 (= sexual maturity) post-hatching. Tarsus length was measured at day 90.

1.2.3. Statistical analysis

All statistical analyses were conducted using the SAS statistical computing system, version 9.1.3 (SAS Institute 2003). Effect of treatment on egg fate and hatchability was tested using χ-square test (proc Freq), with the Fisher’s Exact test for small sample sizes.

Treatment effects on chick growth were analyzed using generalized mixed linear models (proc MIXED) to compare chicks from DMSO-, oil-, sham-injected and control eggs, with “brood” as a random factor, and with other covariates included in the model as necessary (e.g. egg mass, number of chicks per brood; see Results). We then used post-hoc multiple comparisons with Bonferroni correction to identify specific differences among treatments. Values are presented as least square means ± SEM unless otherwise stated.

1.3. Results

1.3.1. Experiment 1

There was no difference in mean egg mass for eggs subsequently assigned to DMSO (1.085 ± 0.030 g) or sham treatments (1.078 ± 0.030 g; $F_{1,48.9} = 0.07$, $P > 0.7$). Similarly, mean egg laying sequence did not differ by treatment (DMSO, 2.45 ± 0.25 vs. Sham, 2.55 ± 0.24; $F_{1,63} = 0.07$, $P > 0.7$). In other words, eggs were randomly assigned to either the DMSO-injection or sham-injection groups with respect to mass and laying sequence.

There was no effect of treatment on the distribution of different egg/embryo fates (Fisher's Exact $\chi^2 = 0.006$, $P > 0.80$; Table 1). However, many cells had expected counts <5 so we pooled data to compare "hatched" versus all "not hatched" eggs. There was no effect of treatment on overall hatching success: DMSO, 25.8% hatched vs. Sham, 24.2% hatched ($\chi^2 = 0.021$, $P > 0.80$; Table 1).

Hatching mass was strongly positively correlated with egg mass $F_{1, 9} = 27.63$, $P<0.001$). However, there was no effect of treatment on hatching mass, controlling for egg mass or brood size at hatching ($F_{1, 8.87} = 0.23$, $P > 0.60$). Sample sizes for post-hatching chicks were small and there was no significant effect on treatment on chick mass at either 7, 14, 21, or 30 days of age ($P > 0.05$ in all cases; Table 2).

1.3.2. Experiment 2

There was no difference in mean egg mass for eggs subsequently assigned to DMSO (1.062 ± 0.020 g), Oil (1.063 ± 0.021 g), Sham (1.073 ± 0.020 g), or Control treatments (1.058 ± 0.021 g; $F_{3, 137} = 0.36$, $P > 0.7$). Similarly, mean egg laying sequence did not differ by treatment (DMSO, 3.38 ± 0.26, Oil, 3.14 ± 0.26, Sham, 3.27 ± 0.26, Control, 3.41 ± 0.26; $F_{3, 1.99} = 0.66$, $P > 0.2$). In other words, eggs were randomly assigned to either DMSO-injection, oil-injection, sham-injection, or control groups with respect to mass and laying sequence.

There was no effect of treatment on the distribution of different egg/embryo fates ($\chi^2 = 14.0$, df = 12, $P > 0.30$; Table 1) although 40% of cells had expected counts <5. We ran Fishers Exact test by deleting "missing eggs" and pooling fertile eggs and those with

more developed embryos. This confirmed no effect of treatment on egg fate comparing infertile eggs, hatched eggs and non-hatched eggs which were fertile or with some embryo development eggs (Fisher's Exact $\chi^2 = 9.98$, $P > 0.05$). We also pooled data to compare "hatched" versus all "not hatched" eggs. There was no effect of treatment on overall hatching success: DMSO, 43.5% hatched, Oil, 34.8% hatched, Sham, 42.6% hatched, Control, 53.3% hatched ($\chi^2 = 3.26$, $P > 0.30$; Table 1). Pooling DMSO and sham treatments, overall hatching success was higher for experiment 2 (39%) compared with experiment 1 (25%, $\chi^2 = 3.56$, $P = 0.06$).

Hatching mass was strongly positively correlated with egg mass in Experiment 2 ($F_{1, 47.4} = 16.75$, $P < 0.001$). However, there was no effect of treatment on hatching mass, controlling for egg mass or brood size at hatching ($F_{3, 55.6} = 0.59$, $P > 0.60$). In an overall analysis of chick mass with age the age*treatment interaction was not significant ($F_{12,264} = 1.20$, $P > 0.20$) however, there was a highly significant effect of treatment ($F_{3,52.1} = 5.21$, $P < 0.01$) and, not surprisingly, of age ($F_{4,264} = 5134.2$, $P < 0.001$). We included sex into the model, and found that sex also had a highly significant effect ($F_{1,53.1} = 6.74$, $P < 0.01$), with sex*treatment interaction ($F_{3,50.8} = 3.20$, $P < 0.03$). We therefore compared the effect of treatment on chick mass for each age and sex separately, controlling for effects of egg mass and brood size at hatching. There was no significant effect of treatment on growth in females, from hatching day to day 90. However, there was a significant effect of treatment on growth in males. At day 7, males treated with oil and DMSO showed a significantly lower body mass than control ($t_{119} = 2.10$, $P < 0.04$); $t_{131} = 2.53$, $P < 0.02$); Table 3(a)). This body mass difference disappeared by day 14 in the oil-treated male group but stayed significant for DMSO-treated males at day 14 ($t_{131} = 2.40$, $P < 0.02$), day 21 ($t_{131} = 2.47$, $P < 0.02$), day 30 ($t_{131} = 2.51$, $P < 0.02$), and day 90 ($t_{131} = 3.26$, $P < 0.001$; Fig. 1.1 top).

All pair-wise contrasts between control, oil and sham chicks within female group were non-significant (Table 3 (b); Fig. 1.1 bottom).

There was also a significant effect of treatment on tarsus length measured at day 90. Males treated with DMSO had shorter tarsus length than the control group ($t_{15.2} = 3.13$, $P < 0.007$; Table 4). Female groups had no significant differences in tarsus length (Table 4).

1.4. Discussion

In our first experiment hatching success was similar for eggs that were punctured but not injected compared with DMSO-treated eggs. However, hatching success was relatively low (25%) compared to typical hatchability of non-manipulated eggs in our breeding colony (~48%, T.D. Williams, unpub. data). This suggests that simply puncturing the shell has a negative effect on hatchability, but introducing vehicle into the egg did not cause an additional reduction in hatchability. Since hatchability of eggs was higher in our second experiment (see below) we believe that the abnormally low hatching success was related to technical or methodological problems in the first experiment, e.g. multiple use of one needle for up to five different injections, and the use of Vetbond as a sealing material. Although multiple injections were used successfully for *in ovo* hormone treatment in European starling (*Sturnus vulgaris*) (O. Love; in personal communication) when each needle was used up to five times, we switched to single-use, disposable sterile needles to avoid any potential problem with cross-contamination. Vetbond was rejected after our first experiment due to its highly flowable properties which meant that it was easy to accidentally cover a large surface of the egg, potentially inhibiting gas exchange for the developing embryo, and probably, causing pre-hatching death. Vetbond was replaced with Loctite Ultra Gel Super Glue. This also minimized the possibility of eggs sticking to nest material or other eggs (O. Love, in personal communication). The gel form allowed us to achieve pin-point sealing with minimum amounts of glue. In addition, this type of glue resists warming, which was important during incubation. Loctite also required more time to dry out completely, so that we increased the drying time to 10 minutes. We believe that these changes in technique were responsible for the higher average hatchability in the second experiment (43%) similar to normal hatchability of non-manipulated eggs in our colony as described above (48%).

The key result from our second experiment was that DMSO had a significant inhibitory effect of male chick growth as assessed by body mass at 7-, 14-, 21- and 30 days of age. We cannot rule out the possibility that DMSO had negative effects *in ovo* during embryo development, producing poorer quality chicks which subsequently grew more slowly. However, there was no effect of DMSO on hatching mass. Thus, DMSO appeared to have a negative effect on post-hatching male chick growth late in the

nestling development period. This occurred despite the fact that our captive-breeding zebra finches had *ad lib* access to food and were provided with a high protein egg food supplement during chick-rearing. This negative effect of DMSO itself is of concern as DMSO is a widely-used vehicle in toxicological studies.

DMSO (Dimethyl sulfoxide, CAS 67-68-5) is known for its excellent solvent properties, and it is widely used in the topical administration of drugs, the application of pesticides, as an antifreeze, hydraulic fluid, etc. (Gad 2005). In vitro, DMSO is also known as cryoprotective agent (Casali et al. 1999). DMSO is readily absorbed by animals and humans by dermal and oral routes. It can persist in serum for more than 2 weeks after a single exposure (Gad 2005). DMSO is metabolized by oxidation to dimethyl sulfone or by reduction to dimethyl sulfide, which are excreted in the urine and feces. No residual accumulation of DMSO has been reported in animals or humans who have received DMSO treatment for the long period of time, regardless of route of dose administration. (Gad 2005).

Although, in addition to its penetration properties, DMSO has a potential to inhibit or stimulate enzymes and to cause histamine release from mast cells, its acute toxicity has been reported to be quite low in animals (Gad 2005). In earlier studies the properties and toxicity of DMSO were extensively investigated in non-avian species. Vogin et al. (1969) evaluated DMSO toxicity in rhesus monkeys (*Macaca mulatta*) over an 18-month period, and found no toxicological or pathologic changes attributable to oral administration of 1 or 3 ml/kg or topical application of 1, 3, or 9 ml/kg of 90% DMSO. Later, Dresser et al. (1992) exposed embryos of the South African clawed frog, *Xenopus laevis*, for 96 h to ethanol, dimethyl sulfoxide (DMSO), formamide and glycerol formal. They found DMSO to be the least toxic or teratogenic solvent examined, with a pooled LC_{50} of 1.92%, and a pooled EC_{50} (malformation) of 1.57%. Guerre et al. (1999) investigated acute and chronic toxicity of DMSO on the number of species, and estimated acute oral toxicity for chicken within range of 11-16.5 g/kg for 5 days. No mortality was observed in rats, received intravenous injection of 5 g/kg/d during 6 weeks (Guerre et al. 1999), which is compatible to our study, where we used the same proportion (5 µl/g) for the single injection only.

Hallare et al. (2004) used DMSO as a solvent to study diclophenac toxicity to zebrafish (*Danio rerio*) and found that after the 96-h exposure, the stress protein (heat shock protein 70) was expressed in DMSO control, and its level was positively correlated with DMSO concentration. Although results showed no significant inhibition in the normal development until the end of 96 h for all exposure groups, Hallare et al. (2004) warned against potential use of DMSO as solvent in toxicity assays.

Fewer studies appear to have directly tested for DMSO toxicity in avian species. Scialli et al. (1994) found DMSO to be very toxic for chicken embryo, when injecting 50 µl per egg in to the air sac, on day 1 and day 2 of artificial incubation (although this study did not include a non-manipulated or sham-control so other methodological issues could have contributed to embryo lethality). In contrast DMSO has been used successfully (no sham-control either) as a solvent for chicken egg injection, in volume 1 µl/g, or 50 µl/egg (O'Brien et al. 2009), which is relatively less than the amount used in our experiment (5 µl/g, or 5 µl/egg). Heinz et al. (2006) evaluated DMSO as a solvent for methylmercury injection in the chicken eggs, and found that DMSO itself had little if any toxicity when injected just before the start of incubation. DMSO was injected on day 0 into the air sac at the volume 0.5 µl/g, or approximately 25 µl per egg. At the end of incubation, 8 out of 10 DMSO-injected group survived vs 10 out of 10 uninjected control eggs. However, again, this study did not include a sham-injected group so the slightly lower survival might be due to DMSO solvent or to the injection itself. There was no information of possible DMSO toxicity on a later growth stage, because in both studies mentioned above chicks were euthanized immediately after hatching.

The "delayed" effect of DMSO injection on the chick mass, which was observed during our experiment, might be related not to DMSO itself, but to its primary metabolite, dimethyl sulfone ($DMSO_2$). $DMSO_2$ persists in the blood for five times as long as DMSO after percutaneous application in humans (Goldstein et al. 1992). It was evaluated as a more potent cytotoxic drug than DMSO, with less reversible effect, and as more potent growth inhibitor on smooth muscle cells and endothelial cells (Layman 1987). As we observed, the first difference in mass between DMSO and other treatment group occurred around 7 days. Because DMSO can stay in the serum more than for two weeks (Gad 2005), we can hypothesize that this period passes through during incubation and rapid growth stage, so that by 7 days of chick life, initial DMSO amount becomes

replaced by its metabolite, $DMSO_2$. This hypothesis can explain an approximate temporary difference in 0.5 g in chick weight between control and DMSO-treated group, which started around day 7. We can also suggest that male birds are more sensitive to both DMSO than females. However, the mechanism of this gender sensitivity remains unknown and requires further studies.

In conclusion, we think that an egg injection method validated in our study, could be used to test a wide variety of organic pollutants for a small songbird species. However, the suitability of DMSO as a vehicle remains under question suggesting the need to thoroughly investigate its potential toxicity on the later stages of chick development. We would recommend repeating the egg injection experiment using various volume of DMSO, i.e. 1, 2, 3 and 5 µl per egg. Besides the use of an appropriate, non-toxic vehicle, the most important conditions for hatching success with this *in ovo* method are injection sterility and appropriate sealing material. A good practice would be to remove all eggs laid within two days after pairing (most likely, they are infertile), and all eggs lighter than 0.600 g, for the same reason. We would also stress the importance of optimizing the nest-checking time, to avoid any excessive disturbance of the nesting/incubating parents.

1.5. References

Bargar, T. A., Scott, G. I., & Cobb, G. P. (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*(1), 61-67.

Boon, J. P., Lewis, W. E., Tjoen-A-Choy, M. R., Allchin, C. R., Law, R. J., de Boer, J., et al. (2002). Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web. *Environmental Science & Technology, 36*(19), 4025-4032. doi:10.1021/es0158298

Casali, F., & Guerre, P. (1999). Dimethylsulfoxide (DMSO): Properties and permitted uses. *Revue De Medecine Veterinaire, 150*(3), 207-220.

Chen, D., & Hale, R. C. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International, 36*(7), 800-811. doi:10.1016/j.envint.2010.05.013

Darnerud, P. O. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environment International, 29*(6), 841-853. doi:10.1016/S0160-4120(03)00107-7

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

Dauwe, T., Jaspers, V. L. B., Covaci, A., & Eens, M. (2006). Accumulation of organochlorines and brominated flame retardants in the eggs and nestlings of great tits, *Parus major*. *Environmental Science & Technology, 40*(17), 5297-5303. doi:10.1021/es060747a

Dresser, T., & Finch, R. (1992). Teratogenic assessment of 4 solvents using the frog embryo teratogenesis assay - Xenopus (FETAX). *Journal of Applied Toxicology, 12*(1), 49-56.

Drouillard, K. G., & Norstrom, R. J. (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*(3), 561-567.

Elliott, J. E., Wilson, L. K., & Wakeford, B. (2005). Polybrominated diphenyl ether trends in eggs of marine and freshwater birds from British Columbia, Canada, 1979-2002. *Environmental Science & Technology, 39*(15), 5584-5591. doi:10.1021/es050496q

Fernie, K. J., Shutt, J. L., Letcher, R. J., Ritchie, I. J., & Bird, D. M. (2009). Environmentally relevant concentrations of DE-71 and HBCD alter eggshell thickness and reproductive success of American kestrels. *Environmental Science & Technology, 43*(6), 2124-2130. doi:10.1021/es8027346

Fernie, K. J., Shutt, J. L., Ritchie, I. J., Letcher, R. J., Drouillard, K., & Bird, D. M. (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*(16), 1541-1554. doi:10.1080/15287390500468753

Gad, S.E. (2005). Dimethyl sulfoxide. In P. Wexler (Ed.), *Encyclopedia of Toxicology* (pp. 51-52). Amsterdam: Elsevier.

Gauthier, L. T., Hebert, C. E., Weseloh, D. V. C., & Letcher, R. J. (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*(5), 1524-1530. doi:10.1021/es702382k

Goldstein, P., Magnano, L., & Rojo, J. (1992). Effects of dimethyl sulfone ($DMSO_2$) on early gametogenesis in *Caenorhabditis elegans* - ultrastructural aberrations and loss of synaptonemal complexes from pachytene nuclei. *Reproductive Toxicology, 6*(2), 149-159.

Guerre, P., & Casali, F. (1999). Dimethylsulfoxide (DMSO): Experimental uses and toxicity. *Revue De Medecine Veterinaire, 150*(5), 391-412.

Hallare, A. V., Kohler, H. R., & Triebskorn, R. (2004). Development toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvents, DMSO. *Chemosphere, 56*(7), 659-666. doi:10.1016/j.chemosphere.2004.04.007

Heinz, G. H., Hoffman, D. J., Kondrad, S. L., & Erwin, C. A. (2006). Factors affecting the toxicity of methylmercury injected into eggs. *Archives of Environmental Contamination and Toxicology, 50*(2), 264-279. doi:10.1007/s00244-005-1002-y

Henny, C. J., Kaiser, J. L., Grove, R. A., Johnson, B. L., & Letcher, R. J. (2009). Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology, 18*(7), 802-813. doi:10.1007/s10646-009-0323-4

Layman, D.L. (1987). Growth inhibitory effect of dimethyl sulfoxide and dimethyl sulfone on vascular smooth muscle cells and endothelial cells *in vitro*. *In Vitro Cell and Developmental Biology*, *23*(6), 422-428.

Law, R. J., Alaee, M., Allchin, C. R., Boon, J. P., Lebeuf, M., Lepom, P., et al. (2003). Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. *Environment International, 29*(6), 757-770. doi:10.1016/S0160-4120(03)00110-7

Marteinson, S. C., Bird, D. M., Shutt, J. L., Letcher, R. J., Ritchie, I. J., & Fernie, K. J. (2010). Multi-generational 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*(8), 1740-1747. doi:10.1002/etc.200

McKernan, M. A., Rattner, B. A., Hale, R. C., & Ottinger, M. A. (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*(5), 1007-1017.

McKernan, M. A., Rattner, B. A., Hatfield, J. S., Hale, R. C., & Ottinger, M. A. (2010). Absorption and biotransformation of polybrominated diphenyl ethers DE-71 and DE-79 in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), American kestrel (*Falco sparverius*) and black-crowned night-heron (*Nycticorax nycticorax*) eggs. *Chemosphere, 79*(1), 100-109. doi:10.1016/j.chemosphere.2009.12.023

Norstrom, R. J., Simon, M., Moisey, J., Wakeford, B., & Weseloh, D. V. C. (2002). Geographical distribution (2000) and temporal trends (1981-2000) of brominated diphenyl ethers in Great Lakes herring gull eggs. *Environmental Science & Technology, 36*(22), 4783-4789. doi:10.1021/es025831e

O'Brien, J. M., Crump, D., Mundy, L. J., Chu, S., McLaren, K. K., Vongphachan, V., et al. (2009). Pipping success and liver mRNA expression in chicken embryos exposed in ovo to C-8 and C-11 perfluorinated carboxylic acids and C-10 perfluorinated sulfonate. *Toxicology Letters, 190*(2), 134-139. doi:10.1016/j.toxlet.2009.07.004

de Roode, D. E., Gustavsson, M. B., Rantalainen, A. L., Klomp, A. V., Koeman, J. H., & Bosveld, A. T. C. (2002). Embryotoxic potential of persistent organic pollutants extracted from tissues of guillemots (*Uria aalge*) from the Baltic Sea and the Atlantic ocean. *Environmental Toxicology and Chemistry, 21*(11), 2401-2411.

Scialli, A. R., Desesso, J. M., & Goeringer, G. C. (1994). Taxol and embryonic-development in the chick. *Teratogenesis Carcinogenesis and Mutagenesis, 14*(1), 23-30.

Sellstrom, U., De Wit, C. A., Lundgren, N., & Tysklind, M. (2005). Effect of sewage-sludge application on concentrations of higher-brominated diphenyl ethers in soils and earthworms. *Environmental Science & Technology, 39*(23), 9064-9070. doi:10.1021/es051190m

van den Steen, E., Pinxten, R., Covaci, A., Carere, C., Eeva, T., Heeb, P., et al. (2010). The use of blue tit eggs as a biomonitoring tool for organohalogenated pollutants in the European environment. *Science of the Total Environment, 408*(6), 1451-1457. doi:10.1016/j.scitotenv.2009.12.028

van den Steen, E., Jaspers, V. L. B., Covaci, A., Neels, H., Eens, M., & Pinxten, R. (2009). Maternal transfer of organochlorines and brominated flame retardants in blue tits (*Cyanistes caeruleus*). *Environment International, 35*(1), 69-75. doi:10.1016/j.envint.2008.08.003

Verreault, J., Villa, R. A., Gabrielsen, G. W., Skaare, J. U., & Letcher, R. J. (2006). Maternal transfer of organohalogen contaminants and metabolites to eggs of Arctic-breeding glaucous gulls. *Environmental Pollution, 144*(3), 1053-1060. doi:10.1016/j.envpol.2005.10.055

Vogin, E., & Rubin, L. (1970). Chronic toxicity of DMSO in primates. *Toxicology and Applied Pharmacology, 16*(3), 606-612. doi:10.1016/0041-008X(70)90065-7

de Wit, C. (2002). An overview of brominated flame retardants in the environment. *Chemosphere, 46*(5), 583-624. doi:10.1016/S0045-6535(01)00225-9

1.6. Tables

Table 1.1. Egg fate by treatment from Experiment 1 and Experiment 2 in number and percentage (in parentheses). The egg is considered hatched if there was a chick found in the nest (dead or alive). Other fate is determined when the eggs were opened artificially, in 21 days after incubation started. We consider the egg fate to be embryo, if there was a distinct embryo (wt>0.1g) inside. Fertile means some visible sign of fertilization, or indistinct embryo (wt<0.1g). Infertile means no visible sign of fertilization. Missing eggs are those that disappeared (probably, eaten or destroyed by parents) during incubation time.

Experiment	Treatment	Egg fate					
		Hatched	Embryo	Fertile	Infertile	Missing	Total
1	DMSO	8 (25.8)	3 (9.7)	2 (6.5)	15 (45.2)	3 (9.7)	31
1	Sham	8 (24.2)	3 (9.1)	3 (9.1)	18 (58.1)	1 (3.0)	33
2	DMSO	20 (43.5)	8 (17.4)	6 (13.0)	11 (23.9)	1 (2.2)	46
2	Oil	16 (34.8)	3 (6.5)	7 (15.2)	17 (37.0)	3 (6.5)	46
2	Sham	20 (42.6)	5 (10.6)	6 (12.7)	14 (29.8)	2 (4.3)	47
2	Control	24 (53.3)	1 (2.2)	2 (4.4)	17 (37.8)	1 (2.2)	45

Table 1.2. Chick mass (g) at hatching, and at 7, 14, 21, and 30 days of age in relation to egg injection treatment for Experiment 1.

Chick age (days)	DMSO	Sham
0 (hatching)	0.78 ± 0.04	0.82 ± 0.05
7	6.60 ± 0.92	6.54 ± 0.93
14	10.05 ± 0.42	12.01 ± 0.46
21	11.28 ± 1.11	12.72 ± 0.83
30	13.09 ± 0.84	14.16 ± 0.63

Table 1.3. Chick mass (g) at hatching, and at 7, 14, 21, 30 and 90 days of age in relation to egg injection treatment for Experiment 2.

(a) Males

Chick age (days)	DMSO	Sham	Oil	Control
0 (hatching)	0.74 ± 0.23	0.98 ± 0.28	0.79 ± 0.33	0.96 ± 0.23
7	6.01 ± 0.29	6.81 ± 0.38	6.27 ± 0.44	6.85 ± 0.30
14	10.97 ± 0.25	11.29 ± 0.32	11.44 ± 0.37	11.86 ± 0.25
21	11.46 ± 0.18	11.71 ± 0.23	12.65 ± 0.27	12.21 ± 0.18
30	12.91 ± 0.23	12.91 ± 0.32	13.65 ± 0.38	13.55 ± 0.24
90	13.39 ± 0.24	14.37 ± 0.29	14.81 ± 0.33	14.72 ± 0.25

(b) Females

Chick age (days)	DMSO	Sham	Oil	Control
0 (hatching)	0.72 ± 0.31	0.89 ± 0.24	0.86 ± 0.25	0.76 ± 0.26
7	6.64 ± 0.42	6.87 ± 0.34	6.69± 0.31	6.82± 0.37
14	11.79 ± 0.36	12.14 ± 0.31	11.99 ± 0.29	11.70 0.33
21	12.17 ± 0.34	12.44 ± 0.28	12.53 ± 0.26	12.53 ± 0.29
30	13.55 ± 0.26	13.67 ± 0.22	13.71 ± 0.21	13.88 ± 0.23
90	15.05 ± 0.52	15.67± 0.41	14.96 ± 0.37	14.73± 0.43

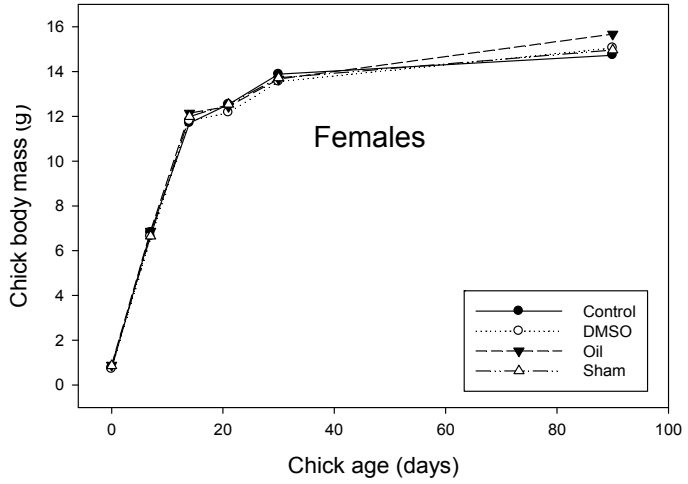
Table 1.4. Tarsus length (mm) at 90 days of age in relation to egg injection treatment for Experiment 2.

Sex	DMSO	Sham	Oil	Control
Males	13.45 ± 0.27	14.37 ± 0.31	14.76 ± 0.37	14.66 ± 0.27
Females	14.97 ± 0.63	15.65 ± 0.43	14.91 ± 0.42	14.69 ± 0.45

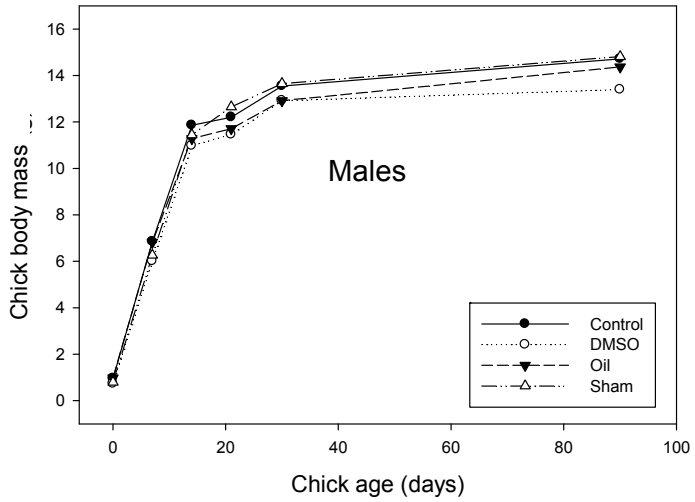
1.7. Figures

Figure 1.1. Growth curves for a) males chicks and b) female chicks exposed *in ovo* to DMSO or oil, sham injected or non-manipulated controls. In males body mass was significantly lower ($P < 0.05$) than controls in oil-treated chicks at day 7, and in DMSO-treated at days 7, 14, 21, 30 ($P < 0.05$) and 90 ($P < 0.005$). No differences were significant in female chicks.

(a)



(b)



Article 2.
A three-generational study of *in ovo* exposure to PBDE-99 in zebra finch (*Taeniopygia guttata)*

2.1. Introduction

The increasing presence of polybrominated diphenyl ethers (PBDEs) in the environment and biota is becoming a growing concern (de Wit 2002, McKernan et al. 2009). These compounds are persistent, they bioaccumulate, and have potential for toxicity in humans and wildlife (Darnerud 2008, Birnbaum & Staskal 2004). Polybrominated flame retardants (PBDEs) are added to high impact polystyrene, electronic housings, furniture foams, and fabrics to increase the temperature of combustion (Harrad et al. 2006). Until recent restrictions on their usage PBDEs belonged to the largest market group of flame retardants because of their low cost and high efficiency (Birnbaum & Staskal 2004). Recent reports showed that PBDEs are widespread in the environment and are found far from the industrial or urban centers where they are produced or used, and their occurrence and concentrations are rapidly increasing in humans, wildlife and in the environment (Alaee and Wenning 2002). For example, relatively high concentrations of PBDEs (410–5960 µg/kg lipid) have been reported in Arctic-breeding seals (Ikonomou et al. 2002), in North sea harbour porpoise and harbour seals (Boon et al. 2002), in St. Lawrence estuary beluga whales and harbour seals (Frouin & Fournier 2011; Lebeuf et al. 2004), and in dolphins near Brazil (Dorneles et al. 2010).

PBDEs comprise 209 different congeners, numbered by the position of bromines on the carbon ring. The most common PBDE congeners found in biota are BDE-47, -99 (also known as penta-PDE) and -153 (Frouin & Fournier 2011; Darnerud et al. 2001). The higher brominated compounds tend to be less mobile in the environment, whereas lower brominated compounds (four to seven bromines) are less lipophilic and therefore,

more bioavailable. (Darnerud 2008, Watanabe and Sakai 2003, de Wit 2002). Bioaccumulation is reflected in increasing concentration in animals higher up the food chain, with the lowest PBDE concentration in invertebrates, levels being higher in fish, and the highest levels being recorded in marine mammals (Alaee et al. 2000) or avian top predators (Chen & Hale 2010; Jaspers et al., 2006). Several studies have estimated biomagnification factors (BMF) for PBDEs in avian food chains, i.e. “the increase in tissue contaminant concentrations in higher trophic levels as a result of dietary accumulation” (Hayes 2001). Voorspoels et al. (2007) found BMF to be equal to 17 in the passerine-sparrowhawk food chain and 14 in the rodent-buzzard food chains, and Chen et al. (2010) calculated BMF in fish to osprey eggs (James River, Virginia, U.S.) to be equal to 23.7. De Wit (2002) determined BMF of several individual PBDE compounds, and reported rates of 19 for BDE-47, 17 for BDE-99 and 7.1 for BDE-100, from herring to guillemot eggs.

In addition to short-term biomagnification effects there is growing evidence of long-term trends for increasing levels of PBDE in the environment, e.g. in bird eggs. There was an exponential increase in PBDE levels in herring gull (*Larus smithsonianus*) eggs in the Great Lakes basin from 1981 to 2006 (Gauthier et al. 2008). Total BDE-47,-99, -100 concentrations were 5-12 µg/kg (wet weight) in 1981-1983 and then increased to 400-1100 µg/kg over the next 17 years (Norstrom et al. 2002). Doubling times were 2.6 years in Lake Michigan, 3.1 years in Lake Huron and 2.8 years in Lake Ontario (Norstrom et al. 2002). Total PBDE concentration doubled every 5.4 years in the eggs of great blue herons (*Ardea herodias*) from 1983 to 2002 in British Columbia, Canada (Elliott et al. 2005). In great blue heron eggs, for these two decades the concentration of total BDEs increased 305-fold from 1.3 to 455 µg/kg (Elliott et al. 2005). According to Norstrom et al. (2002) the exponential increase in total BDE concentrations observed in the Great Lakes aquatic ecosystem seem to be connected with the penta-BDE commercial mixture.

In this study we focus on a major component of the penta-BDE commercial mixture, a lower brominated PBDE congener, PBDE-99 (five bromines). PBDE-99 is a viscous liquid used primarily in textiles as an additive (up to 30% of weight) to polyurethane foams (Hale et al. 2002). Being physically blended but not bonded chemically to polymeric materials, PBDE-99 leaches into the environment where its

persistence poses risks of contamination for humans and biota (Harrad et al. 2006). Currently, the use of penta-BDE has decreased due to a commercial ban in Europe and North America (Chen & Hale 2010) but high concentrations remain in the environment. Penta-BDE was classified as very toxic for aquatic organisms (Wollenberger et al. 2005). Eriksson et al. (2001), and Alm et al. (2006) reported developmental neurotoxicity in mice with this congener. Branchi et al. (2002), studying the perinatal effect of PBDE-99 in mice, found that 6 mg/kg/day dose decreased the number of pups per litter whereas the higher dose (30 mg/kg/day) led to a delay in sensory motor development. Viberg et al. (2005) reported that neonatal exposure to PBDE-99 in rats can affect both spontaneous behaviour and cholinergic system in adult animals. Penta-BDEs have also been suggested to be endocrine disrupters associated with decreases in thyroid hormones (Darnerud 2008, Fernie et al. 2005, Stoker et al. 2003). Fernie et al. (2005) suggested that DE-71, a commercial mixture with PBDE-99 as a major component, lowered the plasma thyroxin (T4) levels in American kestrel*, Falco sparverius.* Nevertheless, elevated levels of total PBDEs have been found in the bird tissues (Lindberg et al. 2004; Jaspers et al. 2006, Chen et al. 2008, Johansson et al. 2009), there is relatively little toxicokinetic data available for birds, especially on possible reproductive effects and multi-generation effects of these substances associated with effects of PBDEs on embryo and chick development.

Marteinson et al. (2010) examined effect of embryonic exposure to DE-71 (a commercial PBDE mixture) on reproduction in male American kestrels. Males were exposed *in ovo* by direct maternal transfer to low (288 ng/g wet weight), high (1130 ng/g wet weight) and control (3 ng/g) environmentally relevant DE-71 concentrations. After one year, these males were paired with unexposed females. Reproductive success was lower in pairs with high-exposed males, and female partners of high-exposed males laid smaller clutches, and produced smaller eggs with reduced fertility. In contrast to studies indirectly exposing offspring via maternal dosing, only a few previous studies have evaluated developmental and reproductive effects of environmentally relevant concentrations of PBDE-mixtures using direct *in ovo* exposure via egg injection, and previous studies have all involved relatively large body mass avian species. Fernie et al. (2006a,b) injected American kestrel (*Falco sparverius*) eggs into the air sac with PBDE-mixture (2.1 µg/µl, or 16-27 µl per egg) on day 19 of incubation, and then fed nestlings daily with the same PBDE mixture through day 29 post-hatch, with the mean daily dose

equivalent to 15.6 ng/g/day. There was no significant effect on hatching and fledgling success but PBDE-exposed kestrels were heavier at day 21 and at fledging (day 27), and they gained weight more quickly than control birds. McKernan et al. (2009) examined the effects of penta-BDE exposure in avian embryos (domestic chicken, mallard, and American kestrel) through air cell administration of a commercial PBDE mixture, and found that the penta-BDE decreased pipping and hatching success at concentrations of 10 and 20 µg/g egg in kestrels but had no effect on survival endpoints in chickens or mallards. They also found, that, on a lipid-weight basis, the LOAEL for impaired pipping and hatching success could be as low as 1.8 µg/g egg wet weight (McKernan et al. 2009).

To our knowledge, no studies have investigated PBDEs embryotoxic effect in a small passerine species using the egg injection method, and followed effects of PBDE-99 exposure on embryo and chick growth and development, and adult phenotype and reproductive success over multiple generations. Here we use an egg-injection method (described in Article 1) to study the effect of *in ovo* exposure PBDE-99 on offspring development and adult phenotype over three generations in the zebra finch (*Taeniopygia guttata*). First, we conducted a literature review of studies that have reported concentration of PBDEs in avian eggs, in order to determine an ecologically-relevant range of dosing for *in ovo* injection. We then dosed eggs (average mass, 1 g) in the first (parental) generation via *in ovo* injection at low (2 ng/µl, or 10 ng/egg), medium (20 ng/µl, or 100 ng/egg) and high (200 ng/µl, or 1000 ng/egg) dose levels. Subsequently we recorded, a) embryo development and egg fate (e.g. infertile, hatching, not hatching), b) chick growth during the nestling period, mass and size at fledging (30 days of age) and sexual maturity (90 days of age), and c) female reproductive performance (breeding propensity, egg and clutch size, breeding success). We then reared chicks from these dosed, first-generation female parents and repeated the assessment of long-term inter-generational effects of PBDE-99 on embryo and chick growth and adult mating and reproductive phenotype in the second (offspring) and third generation (grand-offspring) birds which were unmanipulated but whose parents and grandparents had been dosed *in ovo*, respectively.

2.2. Materials and methods

2.2.1. Animal care

Zebra finches were maintained in controlled environmental conditions described in Article 1, with the same diet and supplementary egg food schedule. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit no. 864B-08 in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

2.2.2. Dosing solution

For the egg injection, we used three different concentrations of PBDE-99 (CAS No. 60348-60-9) dissolved in 5 µl of DMSO. Doses were based on environmentally relevant PBDE-99 concentrations (Appendix 1) for small passerine birds (Dauwe et al. 2005, Dauwe et al. 2009). The nominal concentrations of the three dosing solutions were 2 ng/µl, or 10 ng/egg (low dose), 20 ng/µl, or 100 ng/egg (medium) and 200 ng/µl, or 1000 ng/egg (high) dose levels. Chemical analysis confirmed the actual concentrations of these dosing solutions as 1.9 ng/µl, 18.8 ng/µl, and 229.3 ng/µl for the low, medium and high solutions respectively. Control birds received DMSO injection only. PBDE-99 (2,2',4,4',5-PentaBDE) was obtained from Cambridge Isotope Laboratories, Inc., dissolved in nonane, in concentration 50 µg/ml, 1.2 ml per vial. To prepare the highest concentration, we evaporated nonane in the fumehood with a low stream of nitrogen from one vial, added the content of the second vial, and evaporated again. Next, we added 600 µl of DMSO and vortexed well. For the medium concentration, we used the dried content of one vial, adding 3 ml of DMSO. The low concentration was prepared by adding 1.8 ml of DMSO to 0.2 ml of medium PBDE-99 concentration. All solutions were prepared and stored in the chemically cleaned (hexane rinse followed by acetone rinse) glass bottles, under room temperature, covered with foil, to protect from light.

2.2.3. Breeding and egg-injection protocol

Fifty (50) pairs of birds were randomly assigned to breeding with initial measurements and breeding conditions as described in Article 1. Nest boxes were checked daily between 09.00 and 12.00 PST, and all new eggs were weighed (± 0.001 g), and numbered, to obtain data on egg size, clutch size, and laying interval (the time between pairing and laying of the first egg). Newly laid eggs were randomly assigned to either control DMSO (*n* = 57) or low (*n* = 57), medium (*n* = 59), and high (*n* = 59) PBDE dose injection, so that each clutch contained eggs with all four different treatments. All eggs were injected on the day of laying (day 0).

Eggs were injected as described in Article 1. Briefly the shell at the injection site was sealed using Loctite cyano-acrylate glue in gel form and eggs were left in a vertical position for 10 min to dry before being placed back in the nest box for incubation by the parents. Nest boxes were monitored daily to record missing or broken eggs. Prior to hatching, nest boxes were checked three times a day (09.00, 13.00, 17.00 PST) to determine hatching success, and the exact duration of incubation, of each egg. All newly hatched chicks were weighed within 24 hours of hatching and were individually marked by clipping down feathers from specific feather tracts using a unique combination to identify each chick within a brood. At day 8 post-hatching all chicks were permanently banded using numbered metal bands. Hatching success per treatment was recorded, and all chicks were weighed at day 0, 7, 14, 21 (= fledging age), 30 (= age of independence) and 90 days (= sexual maturity) post-hatching. At 30 and 90 day, blood samples were obtained for hematocrit and hemoglobin analyzes.

2.2.4. Egg exposure validation

After the reproduction phenotype test (see below), birds from the exposed group (first generation) were sacrificed and kept frozen at -20° C. Bird carcasses, males and females in equal proportion, from control (*n* = 8), low (*n* = 8), and high (*n* = 8) group of first generation (exposed *in ovo*) were randomly sampled for chemical analyses of the liver. To evaluate chemical metabolism of PBDE-99 and PBDE-47 (as a primary metabolite of PBDE-99) at the early stage, we completed an additional egg-injection trial with the same doses. Eggs from control, low, medium and high groups were randomly sampled at two stages of development: 3rd day of incubation (*n* = 3 per group), and at

hatching (3 per group). Samples were frozen at -80° C and sent to the Organic Contaminants Research Laboratory, at National Wildlife Research Centre, Ottawa, for chemical analyses of PBDE congeners. Chemical analysis was performed using an Agilent (USA) 6890 gas chromatograph (GC) equipped with a 5973 quadrupole mass spectrometer (MS) detector.

2.2.5. Measurements of reproductive phenotype and 2nd generation effect

Chicks were separated from their parents at 30 days of age, were maintained in non-breeding groups until they could be sexed with appearance of sexually dimorphic plumage, and thereafter were kept in single-sex groups. At sexual maturity (90 days of age) we then assessed reproductive phenotype or individual quality of these second-generation offspring of the *in ovo* treated females by breeding.

Females were bred at 90 days of age with an experienced (successfully bred), clean, unrelated adult male and laying interval, clutch size and egg mass were recorded. If no eggs were laid within 14 days of pairing the male was replaced, and females were given the second trial with a new mate. Chicks were individually marked and maintained as described above, and weighed at day 0, 7, 30 and 90.

After completion of breeding, all birds from the first, parental, *in ovo* treated generation were sacrificed, to collect blood samples for thyroid hormone analysis.

2.2.6. Measurement of reproductive phenotype and 3rd generation effect

At sexual maturity (90 days of age) we assessed reproductive phenotype or individual quality of the third-generation, grand-offspring of the *in ovo* treated females using mating trials in males, and by breeding in females, as described above. Chicks from this third generation were individually marked and maintained as described above, and weighed at day 0, 7, 30 and 90. No birds from the second or third generation were blood sampled or sacrificed.

2.2.7. Hematological assay

Hematocrit (Hct; %) was measured as packed cell volume using a centrifuge (3·min at 13·000·g). Hemoglobin (Hb; $g.dl^{-1}$) was measured by the cyanomethemoglobin method (Drabkin & Austin, 1932) with a microplate spectrophotometer using 5·µl of whole blood diluted in 1.25·ml Drabkin's reagent (D5941 Sigma_Aldrich Canada Ltd., Oakville, Ontario) with absorbance measured at 540·nm. Intra and inter-assay coefficients were 1.53% – 2.25% and 4.38%, respectively.

2.2.8. Thyroid hormone assay

Plasma total (TT4 and TT3) and free (fT4 and fT3) concentrations of thyroid hormones were measured using Accu-Bind ELISA Microwells test system (225-300 Monobind Inc. Lake Forest, CA 92630, USA), following the standard protocol. This method is based on a competition reaction between the enzyme conjugate and thyroid hormones for a limited number of antibody combining sites immobilized on the microplate well. The assays were first validated using pooled hen plasma. All samples were randomly distributed and analyzed within a two assay kits for each type of assay. The detection limits for the assay were 0.15 µg/dl for TT4, 0.03 ng/ml for TT3, 0.03 ng/dl for fT4, and 0.44 pg/ml for fT3.

Average coefficients of variation based on two quality standards across each type of assay were 1.85 % – 4.14 % for TT4, 5.48 % – 1.96 % for TT3, 3.70 % – 7.59 % for fT4, and 1.10 % – 4.35 % for fT3. Intra-assay coefficients calculated as an average coefficient of variation based on samples from the same plate were 6.97 % – 8.66 % for TT4, 6.46 % – 7.08 % for TT3, 5.50 % – 6.23 % for fT4, and 5.22 % – 5.85 % for fT3. All sample tests were done in duplicates. Readings with a coefficient of variation > 15% were excluded from the final data set (Verrault et al. 2004).

2.2.9. Statistical analysis

All statistical analyses were conducted using the SAS statistical computing system, version 9.1.3 (SAS Institute 2003). Effect of treatment on egg fate and hatchability was tested using χ-square test (proc Freq), with the Fisher's Exact test for small sample sizes. Treatment effects on chick growth were analyzed using generalized

mixed linear models (proc MIXED) to compare chicks from DMSO, oil, sham injected and control eggs, with “brood” as a random factor, and with other covariates included in the model as necessary (e.g. egg mass, number of chicks per brood; see Results). We then used post-hoc multiple comparisons with Bonferroni correction to identify specific pair-wise differences among treatments. Values are presented as least square means ± SEM unless otherwise stated.

2.3. Results

2.3.1. Egg exposure validation

Concentrations of PBDE-99 in the eggs on the 3rd day of incubation was equal to 0.40 ± 6.50 (control), 1.46 ± 5.31 (low), 27.76 ± 6.50 (medium), and 114.29 ± 5.31 (high) ng/g wet weight ($F_3 = 96.45$, $P < 0.0001$). At the hatching stage, on 12th day of incubation, concentration of PBDE-99 was 0.59 ± 33.69 (control), 1.33 ± 33.69 (low), 22.80 ± 33.69 (medium), and 190.84 ± 33.69 (high) ng/g wet weight ($F_3 = 7.44$, $P < 0.0106$). In the livers of 150 day birds concentration was equal to 0.31 ± 0.28 (control), 0.34 ± 0.26 (low), and 1.87 ± 0.26 (high) ng/g wet weight ($F_2 = 10.16$, $P < 0.0014$).

Concentrations of PBDE-47 in the eggs on 3rd day of incubation was equal to 0.57 ± 0.15 (control), 0.13 ± 0.12 (low), 0.19 ± 0.14 (medium), and 1.02 ± 0.12 (high) ng/g wet weight ($F_3 = 11.27$, $P < 0.0071$). At hatching, concentration was equal to 0.52 ± 0.32 (control), 0.00 ± 0.32 (low), 0.76 ± 0.32 (medium), and 1.77 ± 0.32 (high) ng/g wet weight ($F_3 = 5.37$, $P < 0.0255$). In the livers of 150 day birds concentration was equal to 0.04 ± 0.05 (control), 0.20 ± 0.04 (low), and 0.28 ± 0.05 (high) ng/g wet weight ($F_2 = 6.43$, $P < 0.0089$).

We did not observe either sex or sex*treatment effect on PBDE-99 and PBDE-47 concentrations within an adult group of birds. However, treatment effect was still significant ($F_2 = 10.12$, $P < 0.0014$; $F_2 = 6.09$, $P < 0.0108$; Fig. 2.1, 2.2).

2.3.2. Parental, in ovo-treated, birds (generation 1)

There was no difference in mean egg mass for eggs subsequently assigned to control (1.033 ± 0.018 g) or low (1.047 ± 0.018 g), medium (1.047 ± 0.018 g) or high PBDE treatments (1.040 ± 0.018 g; $F_{3,179} = 0.63$, $P > 0.59$). Similarly, mean egg laying

sequence of injected eggs did not differ by treatment (control, 3.4 ± 0.3, low, 3.6 ± 0.3, medium, 3.8 ± 0.3, high, 3.7 ± 0.3; $F_{3,179} = 0.36$, $P > 0.78$). In other words, eggs were randomly assigned to either DMSO or PBDE99 treatment with respect to mass and laying sequence.

2.3.2.1. Egg/embryo fate and hatching success

There was no effect of treatment on the distribution of different egg/embryo fates ($\chi^2 = 8.74$, df = 12, $P > 0.72$; Table 2.1). Since cell samples sizes were small for some fates we pooled data to compare "hatched" versus all "not hatched" eggs. There was no effect of treatment on overall hatching success ($\chi^2 = 1.67$, df = 3, $P > 0.64$; Table 2.1), and pooling all treatments hatching success was 45%. There was no effect of treatment on offspring sex ratio ($\chi^2 = 0.89$, df = 3, $P > 0.82$): overall there were 33 (41.7%) males and 46 females (58.2%).

2.3.2.2. Hatching mass and chick growth

Hatching mass was strongly positively correlated with egg mass ($F_{1,73.6} = 76.56$, $P < 0.0001$). However, there was no effect of treatment on hatching mass, controlling for egg mass or brood size at hatching ($F_{3,63.5} = 0.52$, $P > 0.67$).

We compared patterns of chick growth from 0-90 days using chick mass as the dependent variable, age, sex and treatment as main effects, and brood as a random factor. Not surprisingly, there was a highly significant effect of age ($F_{3,213} = 4160.3$, $P < 0.001$) and egg mass ($F_{1,54.2} = 8.06$, $P < 0.007$) on chick mass. Treatment*sex*age ($F_{12,213} = 1.51$, $P > 0.13$) and treatment*age interactions ($F_{9,213} = 1.22$, $P > 0.29$) were not significant and there was no overall effect of treatment ($F_{3,59.2} = 0.31$, $P > 0.82$).

There was a significant effect of sex in the model ($F_{1,69.7} = 6.00$, $P < 0.017$), on average female had higher body mass than males. We therefore compared the effect of treatment on chick mass with age for each sex separately, controlling for effects of egg mass and brood size at hatching. There was no significant effect of treatment on chick mass from hatching day to day 90 in either females ($F_{3,151} = 2.12$, $P > 0.1$) or males ($F_{3,22.8} = 0.92$, $P > 0.45$. Similarly, there was no effect of treatment on tarsus length measured at 90 days of age ($F_{3,59.3} = 1.00$, $P > 0.40$).

We excluded sex from the model and found no treatment effect on hatching mass ($F_{3,69}$ = 0.50, P > 0.68), 7 day mass ($F_{3,71}$ = 1.33, P > 0.27), 30 day mass ($F_{3,67}$ = 0.17, P > 0.91), and 90 day mass ($F_{3,74}$ = 0.32, P > 0.80; Table 2.1, Fig. 2.3 (a)).

2.3.2.3. Hematocrit and plasma hemoglobin

There was no significant effect of treatment on hematocrit at 90 days of age ($F_{3,61.3}$ = 0.49, P > 0.69): control, 54.2 ± 2.1%; low PBDE, 54.6 ± 1.7%; medium PBDE, 53.1 ± 1.7%; high PBDE, 52.5 ± 1.8%. Similarly, there was no effect of treatment on plasma hemoglobin at 30 days and at 90 days of age ($F_{3,64.3}$ = 0.08, P > 0.97): control, 17.11 ± 0.86 $g.dl^{-1}$, low: 16.813 ± 0.708 $g.dl^{-1}$, medium: 16.705 ± 0.703 $g.dl^{-1}$, and high: 17.040 ± 0.741 $g.dl^{-1}$.

2.3.2.4. Plasma thyroid hormones

There was no treatment effect on total T4 level ($F_{3,45.4}$ = 2.18, P > 0.10), total T3 level ($F_{3,41.6}$ = 1.04, P > 0.38), free T4 level ($F_{3,28}$ = 0.18, P > 0.90), and free T3 level ($F_{3,10.1}$ = 1.78, P > 0.21). Similarly, there was no treatment effect on the free T4/total T4 ratio ($F_{3,21.4}$ = 0.32, P > 0.80), or on the free T3/total T3 ratio ($F_{3,20}$ = 1.81, P > 0.17; Table 2.2). We also did not observe any significant sex (P > 0.44) or treatment*sex interaction effect (P > 0.09).

2.3.2.5. Adult female reproductive phenotype

Laying interval, clutch size, mean egg mass. There was no difference in mean egg mass for eggs laid by female from control (1.050 ± 0.035 g), low (1.070 ± 0.030 g), medium (1.037 ± 0.026 g) or high treatment group (1.083 ± 0.028 g; F_3 = 0.52, P > 0.67). There was no significant treatment effect on laying interval (χ^2 = 3.23, df = 3, P > 0.35, Fig. 2.4 (a)). However, there was a significant effect of treatment on the clutch size, controlling for laying interval (F_1= 6.04, P = 0.02; Table 2.4, Fig. 2.4 (a)). We did not observe any significant treatment effect on the mean incubation duration (approximately 13 days for each group) and on the brood size at hatching and fledgling stages (3-4 chicks per brood for each group).

Hatching success. There was a significant effect of treatment on the distribution of different egg/embryo fates (χ^2 = 28.72, df = 12, P < 0.005; Table 2.1(b)). (May not be valid because of 60% of the cells has count <5). We pooled data to compare “hatched”

versus all “not hatched” eggs. There was a significant effect of treatment on overall hatching success (χ^2 = 13.93, df = 3, P > 0.003; Table 2.1). Pooling all treatments, overall hatching success was 52%. There was no effect of treatment on offspring sex ratio (χ^2 = 2.64, df = 3, P > 0.44): overall there were 44 (51.8%) males and 41 females (48.2%).

2.3.3. Growth and reproductive phenotype of offspring of in ovo-treated females (generation 2)

2.3.3.1. Hatching mass and chick growth

Hatching mass was strongly positively correlated with egg mass in Generation 2 ($F_{1,92}$ = 17.63, P < 0.001). However, there was a significant effect of parental treatment on hatching mass, controlling for egg mass or brood size at hatching ($F_{3, 65.1}$ = 4.92, P < 0.004). In an overall analysis of chick mass with age and sex the age*treatment interaction was significant ($F_{9,231}$ = 2.03, P < 0.037), but the sex*treatment interaction was not ($F_{3, 71.1}$ = 1.44, P > 0.24). We found that sex, again, had a significant effect ($F_{1,74,4}$ = 5.52, P < 0.02), whereas treatment did not ($F_{3,19.3}$= 2.13, P > 0.13). There was a highly significant age effect ($F_{3,231}$= 3565.79, P < 0.001). We compared the effect of treatment on chick mass for each age and sex separately, controlling for effects of egg mass and brood size at hatching. There was no overall significant effect of treatment on females ($F_{3,16}$ = 0.83, P > 0.50), and marginally significant treatment effect on males ($F_{3,15.6}$ = 2.80, P > 0.075). There was no significant effect of treatment on tarsus length measured at day 90 between control (16.81 ± 0.192 mm), low (16.708 ± 0.188 mm), medium (16.536 ± 0.198 mm) or high treatments (16.929 ± 0.207 mm; $F_{3,20.5}$ = 0.62, P > 0.60).

We found significant treatment effect on hatching mass ($F_{3,57.5}$ = 3.83, P < 0.02), due to the fact that chicks from high-treated group had lower mass compared with controls but Bonferroni correction did not show any significant difference (P = 0.12). However, on a day 7, the treatment effect became highly significant ($F_{3,18.8}$ = 7.27, P < 0.002) Chicks from high-treated parents showed significantly lower mass than control group (Bonferroni adjusted P = 0.0026) and chicks from the low-dosed parents (Bonferroni adjusted P = 0.0315). This effect became marginally significant by day 30

($F_{3,17.3}$ = 2.68, P > 0.07, and disappeared by day 90 ($F_{3,21.7}$ = 0.43, P > 0.73; Table 2.1, Fig. 2.3 (b)).

2.3.3.2. Adult female reproductive phenotype

Laying interval, clutch size, mean egg mass. There was no difference in mean egg mass for eggs laid by female from control (1.082 ± 0.022 g), low (0.998 ± 0.044 g), medium (1.074 ± 0.025 g) or high treatment group (1.026 ± 0.025 g; F_3 = 1.66, P > 0.19). There was no significant treatment effect on laying interval (χ^2 = 2.82, df = 3, P > 0.41, Fig. 2.4 (b)). There was no significant effect of treatment on the clutch size, controlling for laying interval (F_1= 3.03, P > 0.09; Table 2.4, Fig. 2.4 (b)). We did not observe any significant treatment effect on the mean incubation duration (approximately 12 days for each group) and on the brood size at hutching and fledgling stages (3-4 chicks per brood for each group).

There was no difference in mean egg mass for eggs laid by female from control (1.080 ± 0.025 g), low (1.020 ± 0.037 g), medium (1.074 ± 0.024 g) or high treatment group (1.026 ± 0.037 g; $F_{3,37.9}$ = 1.30, P > 0.28). There was no effect of treatment on the distribution of different egg/embryo fates (χ^2 = 13.77, df = 12, P > 0.31; Table 2.1). We pooled data to compare “hatched” versus all “not hatched” eggs. There was no effect of treatment on overall hatching success (χ^2 = 2.38, df = 3, P > 0.49; Table 2.1). Pooling all treatments, overall hatching success was 55%. There was no effect of treatment on the sex ratio (χ^2 = 0.52, df = 3, P > 0.91): overall there were 48 (57.8%) males and 35 females (42.2%).

2.3.4. Growth of grand-offspring of in ovo-treated females (generation 3)

Hatching mass was strongly positively correlated with egg mass in Generation 3 ($F_{1,75.7}$ = 26.45, P<0.001). There was no effect of treatment on hatching mass, controlling for egg mass or brood size at hatching ($F_{3,\ 16.9}$ = 2.34, P > 0.11). In an overall analysis of chick mass with age and sex either sex*treatment interaction ($F_{3,282}$ = 1.53, P > 0.20), or the age*treatment interaction was not significant ($F_{9,279}$ = 0.89, P > 0.54), with significant age effect ($F_{3,279}$ = 126.62, P < 0.0001), non-significant egg mass effect ($F_{1,72.5}$ = 1.83, P > 0.18), and neither significant effect of treatment ($F_{3,18.9}$ = 1.11, P > 0.37), nor significant

effect of sex ($F_{1,291}$ = 0.98, P > 0.32). As before, we compared the effect of treatment on chick mass for each age and sex separately, controlling for effects of egg mass and brood size at hatching. There was no significant effect of treatment on females ($F_{3,10.4}$ = 1.17, P > 0.36), and no significant effect on males ($F_{3,18}$ = 0.39, P > 0.75).

We excluded sex from the model and found no treatment effect on hatching mass ($F_{3,17.2}$ = 0.82, P > 0.49), 7 day mass ($F_{3,27}$ = 0.91, P > 0.44), 30 day mass ($F_{3,18}$ = 2.02, P > 0.14), and 90 day mass ($F_{3,26.5}$ = 1.50, P > 0.23; Table 2.1, Fig. 2.3 (c)).

2.3.5. Egg exposure validation

Concentrations of PBDE-99 in the eggs on 3rd day of incubation was equal to 0.40 ± 6.50 (control), 1.46 ± 5.31 (low), 27.76 ± 6.50 (medium), and 114.29 ± 5.31 (high) ng/g wet weight (F_3 = 96.45, P < 0.0001). At the hatching stage, on 12th day of incubation, concentration of PBDE-99 was 0.59 ± 33.69 (control), 1.33 ± 33.69 (low), 22.80 ± 33.69 (medium), and 190.84 ± 33.69 (high) ng/g wet weight (F_3 = 7.44, P < 0.0106). In the livers of 150 day birds concentration was equal to 0.31 ± 0.28 (control), 0.34 ± 0.26 (low), and 1.87 ± 0.26 (high) ng/g wet weight (F_2 = 10.16, P < 0.0014).

Concentrations of PBDE-47 in the eggs on 3rd day of incubation was equal to 0.57 ± 0.15 (control), 0.13 ± 0.12 (low), 0.19 ± 0.14 (medium), and 1.02 ± 0.12 (high) ng/g wet weight (F_3 = 11.27, P < 0.0071). At hatching, concentration was equal to 0.52 ± 0.32 (control), 0.00 ± 0.32 (low), 0.76 ± 0.32 (medium), and 1.77 ± 0.32 (high) ng/g wet weight (F_3 = 5.37, P < 0.0255). In the livers of 150 day birds concentration was equal to 0.04 ± 0.05 (control), 0.20 ± 0.04 (low), and 0.28 ± 0.05 (high) ng/g wet weight (F_2 = 6.43, P < 0.0089).

We did not observe either sex or sex*treatment effect on PBDE-99 and PBDE-47 concentrations within an adult group of birds. However, treatment effect was still significant (F_2 = 10.12, P < 0.0014; F_2 = 6.09, P < 0.0108; Fig. 2.1, 2.2).

2.4. Discussion

We used an egg-injection method to study the effect of *in ovo* exposure to PBDE-99 on offspring development and adult phenotype over three generations of the zebra finch (*Taeniopygia guttata*). We found no evidence for immediate- or short-term effects of PBDE on *in ovo* exposed embryos or offspring in terms of hatching success, chick growth, or thyroid hormone levels and hematological traits (measured at sexual maturity at 90 days of age). However, there were significant effects of PBDE treatment on *adult* phenotype of *in ovo* exposed bird: clutch size was significantly smaller in all PBDE dosed birds (low, medium and high) compared with controls. There was also a trend for longer laying intervals in PBDE-dosed birds (13-14 days) compared to control birds (8 days). In addition, there was a significant effect of PBDE on growth of the second-generation offspring of *in ovo* treated females: body mass was significantly lower in the high-PBDE dosed birds compared with controls from hatch through to fledging (day 30). Finally, we found no evidence of longer-term multigenerational effect either in adult, reproductive phenotype of the second-generation offspring of *in ovo* treated birds, or in the growth of their (third-generation) offspring.

In this study we sought to identify a possible effect of single PBDE-exposure *in ovo* during the critical embryo development stage, using environmentally relevant doses, to approximate embryotoxicity which occurs with natural exposure through maternal transfer of contaminants. We have chosen an egg injection method because it ensures that PBDE does get into egg and, provided it is not rapidly metabolized, must be present during at least the initial stages of embryo development. If early embryo development is critical and particularly sensitive to PBDE, we could probably see long-term effects even of only short-term “acute” *in ovo* exposure. In addition, the egg injection method can overcome difficulties with feeding trials (McKernan et. al 2007) and costs of exposing large number of adult healthy breeders. However, it brings uncertainty in the rate of uptake, absorption, and chemical metabolism (McKernan et al. 2010). To address this issue, we chemically analyzed the content of injected eggs at 3 days of incubation, body carcasses at hatching, and livers of adult birds, at 150 days, after reproduction phenotype tests.

BDE-99 was quantifiable in all samples from exposed birds, and also BDE-47, which indicates that metabolism of BDE-99 to BDE-47 occurred in these birds, which is the only possible source of BDE-47 (R. Letcher; in personal communication). The PBDE-99 concentration detected in the control group at all three stages most likely reflects the chemicals accumulated from the diet. Both unexposed parents and exposed adult group were given supplementary egg food during reproduction period. Negligible small PBDE-47 quantities found in the livers of control adult group probably indicate that PBDE-99 was received recently by this group and did not metabolize. Higher quantities of PBDE-47 in low and highly exposed groups most likely reflect the fact that these groups were dosed by PBDE-99 initially, and therefore, metabolites had more time to occur. These results are consistent with Fernie et al. (2006), where small quantities of PBDE-99 (0.97 ng/g) but no PBDE-47 were found in control group of American kestrels after exposure *in ovo* to safflower oil and being fed by cockerels for 29 days after hatching. Therefore, an egg injection method enables us to expose an embryo to environmentally relevant chemical concentrations on the early developmental stage (Fig. 2.1; Appendix 1).

Two previous studies have evaluated developmental and reproductive effects of environmentally relevant concentrations of PBDE-mixtures using *in ovo* exposure via egg injection, all in relatively large avian species. Fernie et al. (2006 a,b) injected American kestrel (*Falco sparverius*) eggs into the air sac with a PBDE-mixture of BDE-47, -99, -100, and -153 dissolved in sufflower oil (2.1 µg/µl, or 16-27 µl per egg) on day 19 of incubation, and then fed nestlings daily with the same PBDE mixture through day 29 post-hatch, with the mean daily dose equivalent to 15.6 ng/g/day. There was no significant effect on hatching and fledgling success but PBDE-exposed kestrels were heavier at day 21 and at fledging (day 27), and they gained weight more quickly than control birds. McKernan et al. (2009) examined the effects of penta-BDE exposure in avian embryos of domestic chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel through air cell administration of a commercial PBDE mixture, and found that the penta-BDE decreased pipping and hatching success at concentrations of 10 and 20 µg/g egg in kestrels, but had no effect on survival endpoints in chickens or mallards. They also found, that, on a lipid-weight basis, the LOAEL for impaired pipping and hatching success could be as low as 1.8 µg/g egg wet weight (McKernan et al. 2009). For the small passerine birds, the environmentally relevant dose was significantly lower than those of causing effect on American kestrels. We have grounded our dosing

on the quantities reported for the great tits (*Parus major*) eggs (Appendix 1). Our highest dose did not affect either pipping or hatching stage. At the same time, even the lowest dose in our experiment influenced the clutch size of breeding birds, which means that for small passerine birds, concentration in the egg equal or higher than 10 ng/g (wet weight) can negatively impact reproduction.

PBDE-99 is considered to work as an endocrine-disrupting chemical possibly through effects on the thyroid hormone system (Kuriyama et al. 2007, Darnerud et al. 2003). However, we observed no significant differences between the control and exposed groups for any of the thyroid hormones we measured in plasma. Our plasma values for T3 and T4 fell within the known range for passerine birds (van den Steen et al. 2010), and they correspond to other results from our laboratory (M. Eng, unpub. data). Van den Steen et al. (2009) studied the effect of environmentally relevant PBDE-mixture dose on European starlings (*Sturnus vulgaris*), where adult female birds had received an implantation dose of about 150 µg (~1740 µg/kg bodyweight). Plasma concentrations of testosterone, estradiol, triiodothyronine (T3) and thyroxine (T4) were measured before and after implantation (14 days, 2 months and 6 months after implantation) with no significant differences between the control and exposed groups. However, 14 days after implantation, T3 concentrations tended to be lower in the exposed group compared to the control group. Exposure of American kestrels before and after hatching to different PBDE congeners was associated with a decrease in plasma thyroxine (T4) levels, but plasma triiodothyronine (T3) remained unaffected (Fernie et al., 2005). However, that study was done on young kestrels at the fledgling age, whereas in our study and that of van der Steen et al. (2009) only reproductively matured adults were sampled. Due to the small size of a bird, and relatively small numbers per group, we did not collect any blood sample for thyroid hormone assay before the end of the reproductive phenotype test for the exposed generation. Therefore, we do not know whether or not thyroid hormones level in exposed group was affected at earlier developmental stage, such as fledgling, etc.

We found no effect of *in ovo* PBDE exposure on hematocrit or on hemoglobin, which is consistent with van der Steen et al.'s (2009) study on European starlings but in contrast with some previous studies in different species, including humans. In humans, Leijs et al. (2009) have found a positive correlation between serum levels of hemoglobin

and serum concentrations of PBDEs. Hematocrit in ranch mink (*Mustela vison*) exposed to a PBDE-mixture, was reported to be significantly lower compared to control mink (Martin et al., 2007). Neale et al., (2005) found erythrocyte level to be inversely correlated to PBDE concentrations in harbour seal (*Phoca vitulina*). Based on those contradictory results for birds and mammals, we suggest that the blood system of bird may be less sensitive to the effects of PBDE exposure. At the same time, we need to take into account the difference in hematological cells between avian and mammalian species (van den Steen et al. 2009).

Analyzing the longer term effect, we can highlight two main findings. One of them relates to the reproductive phenotype of PBDE-exposed females, while another occurs within the second generation, i.e. it is an inter-generational effect.

All treatment groups, regardless of the dose, showed significantly lower average clutch size (1.8-1.2 eggs less) than the control group. Clutch size and laying interval are negatively correlated, in zebra finches: the longer the time between pairing and laying of the first egg, the smaller the clutch size (Willaims 1996). The mean laying interval within our control group around 8-9 days corresponds to the typical laying interval for a 3-month inexperienced bird (Williams & Christians 2003), whereas mean laying intervals for all PBDE-exposed groups fell into the range of 13-14 days. The main typical clutch size for inexperienced 3-month zebra finch female was found to be 5.7 eggs (Williams and Christians 2003), which is close to the control group clutch in our experiment, and one egg larger on average than those of PBDE-exposed birds. The reduced clutch size effect was robust when we controlled for laying interval. At the same time, there was a trend for delayed laying interval in all exposed groups. We believe that the reduced clutch size in our experiment may be related to delay in laying eggs, and this shorter laying interval may be also considered as a treatment effect. It is possible, that PBDE-exposed birds were not able to recognize courting behaviour timely, or needed longer time to properly response for it. Thus, a response delay in several days might have been cost the breeding pair one or two eggs.

We did not observe any dose-dependent effect on a clutch size. All groups received PBDE-99 injection, had smaller clutch than DMSO-treated birds, with no difference from low (10 ng/g) to high (1000 ng/egg) exposure. Taking into account, that

the PBDE-99 concentration range found in great tit (*Parus major*) eggs was from 0.96 to 2.55 ng/g wet weight (Dauwe et al. 2009), we would recommend closer monitoring of population dynamics within small songbird species.

Another significant difference occurred within the second generation. Chicks from the parents exposed to the highest dose of PBDE-99, had lower body mass than control and low-dosed birds, at the rapid growth stage, day 7. It is an interesting to note, that thise low numbers within generation 2, which are also the lowest compared to generation 3, fell within normal range in rapid growth stage of generation 1 (Table 2.3). Comparing control groups from all generations, we can see that exposed birds are 1 g smaller than their offspring and grand-offspring at the rapid growth stage. We think that this difference may be caused by several reasons, such as embryo stress of injection itself, or DMSO as a vehicle, or even selection pressure on the following generations. Indeed, only good female breeders from exposed groups could produce offspring, and only good female breeders from the second generation gave rise to the third one. The body mass difference within the same generation may be attributed to a treatment effect. By age of sexual maturity, that effect disappeared.

We think that future studies could investigate the long-term effect more thoroughly, to study the possible interactions between PBDE-99 and bird reproductive hormones, such as estradiol and prolactin, because many of thyroid-active environmental chemicals do not solely target the thyroid system. For example, many PCB mixtures contain PCB molecules that are both estrogen- and thyroid-active (Ottinger et al. 2011). Lilienthal et al. (2006) found that in rats, PBDE exposure resulted in decreases in circulating testosterone and estradiol concentrations in male offspring at weaning and in adulthood, demonstrating a long-lasting effect. Prolactin studies in exposed birds may be important, because a potential endocrine mechanism may mediate clutch reduction via the inhibitory effect of prolactin on follicular development (Sockman et al. 2006).

Multigenerational studies are important as evidence of treatment effect on reproductive phenotype, as contaminant exposure effects may not elicit themselves until future generations. The smaller body mass within second generation offspring may be attributed to the altered behaviour of their affected parents. It is conceivable that highly

exposed females did not participate in feeding or other type of parental care at the same rate as other females. To test this hypothesis, we would recommend either cross-fostering experiment, or monitoring of feeding rates. The short period of zebra finch maturation gives us an opportunity to complete the multigenerational studies relatively fast, at least for two generations. Third generation analyses could be more difficult, because of increased selective pressure and reduced genetic variability. Thus, only successful female breeders could lay eggs for further reproductive testing. In addition, while comparing chicks from different broods in offspring and grand-offspring, we have to take into account interbrood variations.

We would also recommend thyroid hormone analyses on the preliminary reproductive stage. In birds, thyroid hormones are essential for reproduction with regard to stimulating and maintaining egg laying in females (McNabb 2007). Therefore, egg laying may be a confounding factor when investigating the effects of PBDEs on thyroid and other hormones which are important for laying eggs.

Further experiments may involve evaluation of male reproductive performance through their courting behaviour and singing ability, because male sexual behaviour has proven to be a sensitive and reliable index of embryonic exposure to endocrine disrupting chemicals (Ottinger et al. 2008).

In summary, we suggest that PBDE-99 may have a significant impact on reproduction, where wet weight concentration in the egg equal or higher than 10 ng/g can cost an adult breeding pair one or two eggs per clutch. Although exposure to PBDE-99 *in ovo* may not influence chick survival and growth directly, the population dynamic may be changed due to smaller clutch, and therefore, lower brood size in following generations. Furthermore, PBDE-99 single exposure *in ovo* can have multigenerational effects. Unlike the parental group, the chicks from high dosed females showed smaller body mass at hatching and early stage of bird life. An additional study is required to investigate possible mechanism of PBDE-99 interaction with bird reproductive hormones and neurodevelopment.

2.5. References

Alaee, M., & Wenning, R. (2002). The significance of brominated flame retardants in the environment: Current understanding, issues and challenges. *Chemosphere, 46*(5), 579-582. doi:10.1016/S0045-6535(01)00224-7

Alm, H., Scholz, B., Fischer, C., Kultima, K., Viberg, H., Eriksson, P., . . . Stigson, M. (2006). Proteornic evaluation of neonatal exposure to 2,2 ',4,4 ',5-pentabromodiphenyl ether. *Environmental Health Perspectives, 114*(2), 254-259. doi:10.1289/ehp.8419

Birnbaum, L., & Staskal, D. (2004). Brominated flame retardants: Cause for concern? *Environmental Health Perspectives, 112*(1), 9-17. doi:10.1289/ehp.6559

Boon, J., Lewis, W., Tjoen-A-Choy, M., Allchin, C., Law, R., de Boer, J., . . . Zegers, B. (2002). Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web. *Environmental Science & Technology, 36*(19), 4025-4032. doi:10.1021/es0158298

Branchi, I., Alleva, E., & Costa, L. (2002). Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE 99) on mouse neurobehavioural development. *Neurotoxicology, 23*(3), 375-384. doi:10.1016/S0161-813X(02)00078-5

Braune, B. M., Mallory, M. L., Gilchrist, H. G., Letcher, R. J., & Drouillard, K. G. (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*(3), 403-417. doi:10.1016/j.scitotenv.2007.03.003

Chen, D., & Hale, R. C. (2010). A global review of polybrominated diphenyl ether flame retardant contamination in birds. *Environment International, 36*(7), 800-811. doi:10.1016/j.envint.2010.05.013

Chen, D., Hale, R. C., Watts, B. D., La Guardia, M. J., Harvey, E., & Mojica, E. K. (2010). Species-specific accumulation of polybrominated diphenyl ether flame retardants in birds of prey from the Chesapeake Bay region, USA. *Environmental Pollution, 158*(5), 1883-1889. doi:10.1016/j.envpol.2009.10.042

Custer, T. W., Kannan, K., Tao, L., Saxena, A. R., & Route, B. (2009). Perfluorinated compounds and polybrominated diphenyl ethers in great blue heron eggs from Indiana Dunes National Lakeshore, Indiana. *Journal of Great Lakes Research, 35*(3), 401-405. doi:10.1016/j.jglr.2009.02.003

Darnerud, P. O. (2008). Brominated flame retardants as possible endocrine disrupters. *International Journal of Andrology, 31*(2), 152-160. doi:10.1111/j.1365-2605.2008.00869.x

Darnerud, P. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environment International, 29*(6), 841-853. doi:10.1016/S0160-4120(03)00107-7

Darnerud, P., Eriksen, G., Johannesson, T., Larsen, P., & Viluksela, M. (2001). Polybrominated diphenyl ethers: Occurrence, dietary exposure, and toxicology. *Environmental Health Perspectives, 109*, 49-68. doi:10.2307/3434846

Dauwe, T., Janssens, E., & Eens, M. (2006). Effects of heavy metal exposure on the condition and health of adult great tits *(Parus major). Environmental Pollution, 140*(1), 71-78. doi:10.1016/j.envpol.2005.06.024

Dauwe, T., Van den Steen, E., Jaspers, V. L. B., Maes, K., Covaci, A., & Eens, M. (2009). Interspecific differences in concentrations and congener profiles of chlorinated and brominated organic pollutants in three insectivorous bird species RID E-1379-2011. *Environment International, 35*(2), 369-375. doi:10.1016/j.envint.2008.09.006

Dorneles, P. R., Lailson-Brito, J., Dirtu, A. C., Weijs, L., Azevedo, A. F., Torres, J. P. M., . . . Covaci, A. (2010). Anthropogenic and naturally-produced organobrominated compounds in marine mammals from Brazil. *Environment International, 36*(1), 60-67. doi:10.1016/j.envint.2009.10.001

Drabkin, D., & Austin, J. (1932). Spectrophotometric studies I. spectrophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood. *Journal of Biological Chemistry, 98*(2), 719-733.

Elliott, J., Wilson, L., & Wakeford, B. (2005). Polyhrominated diphenyl ether trends in eggs of marine and freshwater birds from British Columbia, Canada, 1979-2002. *Environmental Science & Technology, 39*(15), 5584-5591. doi:10.1021/es050496q

Eriksson, P., Jakobsson, E., & Fredriksson, A. (2001). Brominated flame retardants: A novel class of developmental neurotoxicants in our environment? *Environmental Health Perspectives, 109*(9), 903-908. doi:10.2307/3454990

Fernie, K. J., Shutt, J. L., Ritchie, I. J., Letcher, R. J., Drouillard, K., & Bird, D. M. (2006a). 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*(16), 1541-1554. doi:10.1080/15287390500468753

Fernie, K., & Smits, J. (2006b). The effects of exposure to polybrominated diphenyl ethers on reproductive performance and immune function in captive American kestrels. *Journal of Ornitology, 147*(5), 92-92.

Fernie, K., Shutt, J., Mayne, G., Hoffman, D., Letcher, R., Drouillard, K., & Ritchie, I. (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*(2), 375-383. doi:10.1093/toxsci/kfi295

Frouin, H., & Fournier, M. (2011). PBDEs in serum and blubber of harbour, grey and harp seal pups from Eastern Canada. Chemosphere, 82(5), 663-669.

Gauthier, L. T., Hebert, C. E., Weseloh, D. V. C., & Letcher, R. J. (2007). Current-use flaime retardants in the eggs of herring gulls (*Larus argentatus*) from the Laurentian Great Lakes. *Environmental Science & Technology, 41*(13), 4561-4567. doi:10.1021/es0630487

Gauthier, L. T., Hebert, C. E., Weseloh, D. V. C., & Letcher, R. J. (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*(5), 1524-1530. doi:10.1021/es702382k

Hale, R., La Guardia, M., Harvey, E., Mainor, T., Duff, W., & Gaylor, M. (2001). Polybrominated diphenyl ether flame retardants in Virginia freshwater fishes (USA). *Environmental Science & Technology, 35*(23), 4585-4591. doi:10.1021/es010845q

Harrad, S., Hazrati, S., & Ibarra, C. (2006). Concentrations of polychlorinated biphenyls in indoor air and polybrominated diphenyl ethers in indoor air and dust in birmingham, United Kingdom: Implications for human exposure. *Environmental Science & Technology, 40*(15), 4633-4638. doi:10.1021/es0609147

Hayes, A.W. (Ed.). (2001). *Principles and Methods of Toxicology*. (4th Ed.). Philadelphia: Taylor & Francis.

Henny, C. J., Kaiser, J. L., & Grove, R. A. (2009). PCDDs, PCDFs, PCBs, OC pesticides and mercury in fish and osprey eggs from Willamette River, Oregon (1993, 2001 and 2006) with calculated biomagnification factors. *Ecotoxicology, 18*(2), 151-173. doi:10.1007/s10646-008-0268-z

Henny, C. J., Kaiser, J. L., Grove, R. A., Johnson, B. L., & Letcher, R. J. (2009). Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology, 18*(7), 802-813. doi:10.1007/s10646-009-0323-4

Herzke, D., Berger, U., Kallenborn, R., Nygard, T., & Vetter, W. (2005). Brominated flame retardants and other organobromines in Norwegian predatory bird eggs. *Chemosphere, 61*(3), 441-449. doi:10.1016/j.chemosphere.2005.01.066

Ikonomou, M., Rayne, S., & Addison, R. (2002). Exponential increases of the brominated flame retardants, polybrominated diphenyl ethers, in the Canadian Arctic from 1981 to 2000. *Environmental Science & Technology, 36*(9), 1886-1892. doi:10.1021/es011401x

Jaspers, V., Covaci, A., Maervoet, J., Dauwe, T., Voorspoels, S., Schepens, P., & Eens, M. (2005). Brominated flame retardants and organochlorine pollutants in eggs of little owls (*Athene noctua*) from Belgium. *Environmental Pollution, 136*(1), 81-88. doi:10.1016/j.envpol.2004.12.003

Johansson, A., Sellstrom, U., Lindberg, P., Bignert, A., & de Wit, C. A. (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*(1), 9-17. doi:10.1897/08-142.1

Kuriyama, S. N., Wanner, A., Fidalgo-Neto, A. A., Talsness, C. E., Koerner, W., & Chahoud, I. (2007). Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology, 242*(1-3), 80-90. doi:10.1016/j.tox.2007.09.011

Lebeuf, M., Gouteux, B., Measures, L., & Trottier, S. (2004). Levels and temporal trends (1988-1999) of polybrominated diphenyl ethers in beluga whales (*Delphinapterus leucas*) from the St. Lawrence Estuary, Canada. *Environmental Science & Technology, 38*(11), 2971-2977. doi:10.1021/es035187j

Lilienthal, H., Hack, A., Roth-Harer, A., Grande, S., & Talsness, C. (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*(2), 194-201. doi:10.1289/ehp.8391

Lindberg, P., Sellstrom, U., Haggberg, L., & de Wit, C. (2004). Higher brominated diphenyl ethers and hexabromocyclododecane found in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Environmental Science & Technology, 38*(1), 93-96. doi:10.1021/es034614q

Marteinson, S. C., Bird, D. M., Shutt, J. L., Letcher, R. J., Ritchie, I. J., & Fernie, K. J. (2010). Multi-generational 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*(8), 1740-1747. doi:10.1002/etc.200

Martin, P. A., Mayne, G. J., Bursian, S. J., Tomy, G., Palace, V., Pekarik, C., & Smits, J. (2007). Immunotoxicity of the commercial polybrominated diphenyl ether mixture DE-71 in ranch mink (*Mustela vison*). *Environmental Toxicology and Chemistry, 26*(5), 988-997. doi:10.1897/06-246R.1

McKernan, M. A., Rattner, B. A., Hale, R. C., & Ottinger, M. A. (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*(5), 1007-1017. doi:10.1897/08-318.1

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*(1-2), 163-193. doi:10.1080/10408440601123552

Neale, J., Gulland, F., Schmelzer, K., Harvey, J., Berg, E., Allen, S., . . . Tjeerdema, R. (2005). Contaminant loads and hematological correlates in the harbour seal (*Phoca vitulina*) of San Francisco Bay, California. *Journal of Toxicology and*

Environmental Health-Part A-Current Issues, 68(8), 617-633. doi:10.1080/15287390590921748

Norstrom, R., Simon, M., Moisey, J., Wakeford, B., & Weseloh, D. (2002). Geographical distribution (2000) and temporal trends (1981-2000) of brominated diphenyl ethers in Great Lakes herring gull eggs. *Environmental Science & Technology, 36*(22), 4783-4789. doi:10.1021/es025831e

Ottinger, M. A., Dean, K., McKernan, M., & Quinn, M. J. (2011). Endocrine Disruption of Reproduction in Birds. In D. O. Norris & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrate* (Vol. 4, 1st ed., pp. 239-260). New York: Elsevier.

Ottinger, M. A., Lauoie, E., Thompson, N., Barton, A., Whitehouse, K., Barton, M., . . . Viglietti-Panzica, C. (2008). Neuroendocrine and behavioral effects of embryonic exposure to endocrine disrupting chemicals in birds. *Brain Research Reviews, 57*(2), 376-385. doi:10.1016/j.brainresrev.2007.08.011

Polder, A., Venter, B., Skaare, J. U., & Bouwman, H. (2008). Polybrominated diphenyl ethers and HBCD in bird eggs of South Africa. *Chemosphere, 73*(2), 148-154. doi:10.1016/j.chemosphere.2008.03.021

Sockman, K., Schwabl, H., & Sharp, P. (2000). The role of prolactin in the regulation of clutch size and onset of incubation behavior in the American kestrel. *Hormones and Behavior, 38*(3), 168-176. doi:10.1006/hbeh.2000.1616

van den Steen, E., Eens, M., Geens, A., Covaci, A., Darras, V. M., & Pinxten, R. (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*(24), 6142-6147. doi:10.1016/j.scitotenv.2010.09.003

van den Steen, E., & Jaspers, V. L. B. (2009). An exposure study with polybrominated diphenyl ethers (PBDEs) in female European starlings (*Sturnus vulgaris*): Toxicokinetics and reproductive effects. *Environmental Pollution, 157*(2), 430-436.

Stoker, T., Ferrell, J., Hedge, J., Crofton, K., Cooper, R., & Laws, S. (2003). Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male pubertal protocol. *Toxicological Sciences, 72*, 135-136.

Verreault, J., Skaare, J., Jenssen, B., & Gabrielsen, G. (2004). Effects of organochlorine contaminants on thyroid hormone levels in arctic breeding glaucous gulls, *Larus hyperboreus*. *Environmental Health Perspectives, 112*(5), 532-537.

Viberg, H., Fredriksson, A., & Eriksson, P. (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*(2), 283-288. doi:10.1016/j.etap.2005.02.004

Voorspoels, S., Covaci, A., Neels, H., & Schepens, P. (2007). Dietary PBDE intake: A market-basket study in Belgium. *Environment International, 33*(1), 93-97. doi:10.1016/j.envint.2006.08.003

Watanabe, I., & Sakai, S. (2003). Environmental release and behavior of brominated flame retardants. *Environment International, 29*(6), 665-682. doi:10.1016/S0160-4120(03)00123-5

Williams, T. (1996). Variation in reproductive effort in female zebra finches (*Taeniopygia guttata*) in relation to nutrient-specific dietary supplements during egg laying. *Physiological Zoology, 69*(5), 1255-1275.

Williams, T., & Christians, J. (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*(4), 379-386. doi:10.1111/j.0908-8857.2003.03080.x

de Wit, C. (2002). An overview of brominated flame retardants in the environment. *Chemosphere, 46*(5), 583-624. doi:10.1016/S0045-6535(01)00225-9

Wollenberger, L., & Breitholtz, M. (2005). Brominated flame retardants: Activities in a crustacean development test and in an ecdysteroid screening assay. *Environmental Toxicology and Chemistry, 24*(2), 400-407.

Yogui, G. T., & Sericano, J. L. (2009). Levels and pattern of polybrominated diphenyl ethers in eggs of Antarctic seabirds: Endemic versus migratory species. *Environmental Pollution, 157*(3), 975-980. doi:10.1016/j.envpol.2008.10.016

2.6. Tables

Table 2.1. Egg fate by treatment from Generation 1, Generation 2, and Generation 3 in number and percentage (in parentheses). The egg is considered hatched if there was a chick found in the nest (dead or alive). Other fate is determined when the eggs were opened artificially, in 21 days after incubation started. We consider the egg fate to be embryo, if there was a distinct embryo (wt>0.1g) inside. Fertile means some visible sign of fertilization, or indistinct embryo (wt<0.1g). Infertile means no visible sign of fertilization. Missing eggs are those being disappeared (probably, being eaten or destroyed by parents) during incubation time.

Generation	Treatment	Egg fate					
		Hatched	Embryo	Fertile	Infertile	Missing	Total
Parental (1^{st})	DMSO	20 (37.0)	7 (13.0)	8 (14.8)	13 (24.1)	6 (11.1)	54
	Low	25 (47.2)	9 (17.0)	4 (7.6)	11 (20.8)	4 (7.6)	54
	Medium	26 (48.2)	7 (13.0)	1 (1.9)	14 (26.0)	6 (11.1)	54
	High	28 (47.5)	6 (10.2)	4 (6.8)	15 (25.4)	6 (10.2)	59
	Total	99 (44.6)	29 (13.1)	17 (7.66)	55 (24.8)	22 (9.91)	222
Offspring (2^{nd})	DMSO	26 (46.4)	0 (0.0)	2 (3.57)	25 (44.6)	3 (5.36)	56
	Low	20 (33.3)	3 (5.0)	0 (0.0)	35 (58.3)	2 (3.3)	60
	Medium	29 (70.7)	3 (7.32)	1 (2.44)	8 (19.5)	0 (0.0)	41
	High	30	2	1	12	1	46

		(65.2)	(4.4)	(2.17)	(26.1)	(2.17)	
	Total	105 (51.7)	8 (3.9)	4 (2.0)	80 (39.4)	6 (3.0)	203
Grand-offspring (3^{rd})	DMSO	30 (49.1)	1 (1.64)	2 (3.28)	26 (42.6)	2 (3.28_	61
	Low	17 (60.7)	1 (3.57)	0 (0.0)	10 (35.7)	0 (0.0)	28
	Medium	28 (65.1)	1 (2.3)	0 (0.0)	11 (25.6)	3 (7.0)	43
	High	25 (51.0)	1 (2.0)	0 (0.0)	23 (46.9)	0 (0.0)	49
	Total	100 (55.3)	4 (2.2)	2 (1.1)	70 (38.7)	5 (2.8)	181

Table 2.2. Thyroid hormone level by treatment from Generation 1 (parental). Ratio is given as pmol/L to nmol/L for better convenience. To calculate an actual mole ratio we should divide numbers in columns by 1000.

Treatment	total T4 (nmol/L)	total T3 (nmol/L)	free T4 (pmol/L)	free T3 (pmol/L)	fT4/T4 ratio*	fT3/(T3 ratio*
DMSO	13.72 ± 2.14	1.52 ± 0.18	7.43 ± 1.08	4.28 ± 0.84	0.63 ± 0.74	2.31±0.81
Low	7.04 ± 1.55	1.23 ± 0.17	7.73 ± 0.86	2.94 ± 0.94	1.54 ± 0.56	2.19±0.85
Medium	9.71 ± 1.42	1.28 ± 0.13	8.52 ± 1.38	5.56 ± 0.73	1.20 ± 0.74	4.31±0.66
High	9.66 ± 1.33	1.52 ± 0.14	7.42 ± 0.75	3.99 ± 0.73	1.19 ± 0.42	3.20±0.68

Table 2.3. Chick mass (g) at hatching, and at 7, 30, and 90 days of age in relation to egg injection treatment. Values with different superscript letters are significantly different ($P < 0.005$).

(a) *Generation 1 (parental, exposed in ovo)*

Chick age (days)	DMSO	Low	Medium	High
0 (hatching)	0.78 ± 0.03	0.80 ± 0.02	0.81 ± 0.02	0.79 ± 0.02
7	5.98 ±0.37	5.99 ± 0.35	6.51 ± 0.35	6.34 ± 0.37
30	13.27 ± 0.27	13.39 ± 0.25	13.17 ± 0.24	13.26 ± 0.23
90	15.21 ± 0.37	15.45 ± 0.32	15.34 ± 0.32	15.04 ± 0.30

(b) *Generation 2 (offspring)*

Chick age (days)	DMSO	Low	Medium	High
0 (hatching)	0.85 ± 0.03	0.86 ± 0.03	0.89 ± 0.03	0.77 ± 0.03
7	7.96 ± 0.30 [a]	7.59 ± 0.28 [a]	7.09 ± 0.28 [a]	6.51 ± 0.28 [b]
30	14.44 ± 0.37	14.38 ± 0.37	13.31 ± 0.36	13.65 ± 0.37
90	15.96 ± 0.60	16.25 ± 0.65	15.35 ± 0.57	15.57 ± 0.60

(c) *Generation 3 (grand-offspring)*

Chick age (days)	DMSO	Low	Medium	High
0 (hatching)	0.86 ± 0.03	0.90 ± 0.04	0.83 ± 0.03	0.86 ± 0.03
7	7.42 ± 0.22	7.25 ± 0.30	7.04 ± 0.25	6.90 ± 0.28
30	13.72 ± 0.25	13.45 ± 0.35	13.10 ± 0.28	12.69 ± 039
90	16.24 ± 0.47	16.73 ± 0.67	15.20 ± 0.53	16.43 ± 0.61

Table 2.4. Adult reproductive phenotype: an average laying interval per treatment by generation 1 (parental) and generation 2 (offspring).

Generation	DMSO	Low	Medium	High
Parental	8.67 ± 2.60	14.00 ± 2.40	14.10 ± 2.01	13.10 ± 2.01
Offspring	8.00 ± 1.16	7.33 ± 2.32	8.00 ± 1.34	10.33 ± 1.34

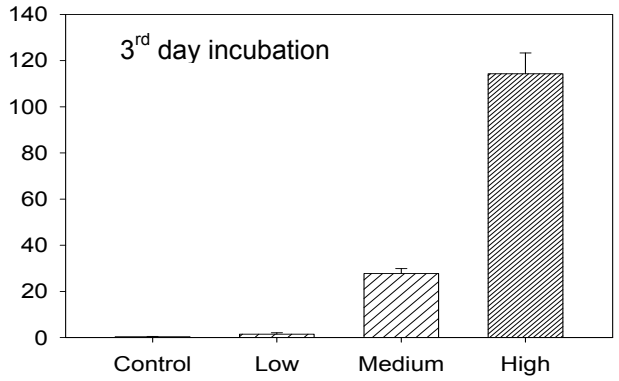
Table 2.5. Adult reproductive phenotype: an average clutch size per treatment by generation 1 (parental) and generation 2 (offspring). Values with different superscript letters are significantly different ($P < 0.05$).

Generation	DMSO	Low	Medium	High
Parental	6.08 ± 0.60^{a}	4.59 ± 0.54^{b}	4.27 ± 0.45^{b}	4.87 ± 0.45^{b}
Offspring	5.07 ± 0.43	5.82 ± 0.86	5.07 ± 0.50	5.23 ± 0.51

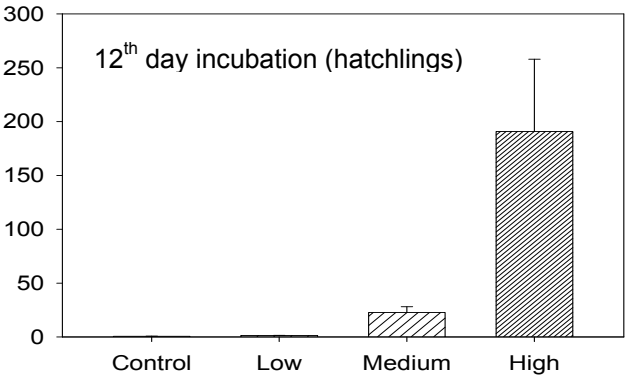
2.7. Figures

Figure 2.1. PBDE-99 found in the (a) eggs, (b) hatchlings, and (c) in the liver of adult zebra finches at three different stages, ng/g wet weight. Up to 90% of the dose was metabolised during first 3 days. By the end of 5 months, PBDE-99 concentration found within the liver of highly exposed group was approximately 100 times less than within the whole body at the hatching stage.

(a)



(b)



(c)

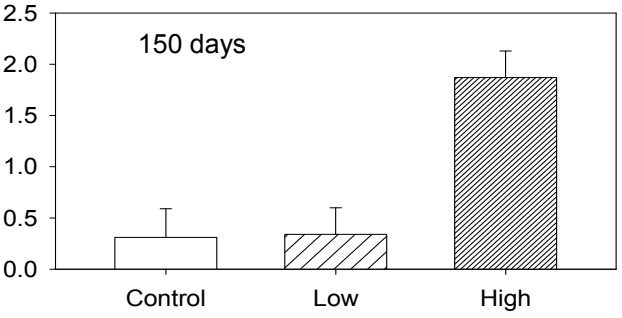
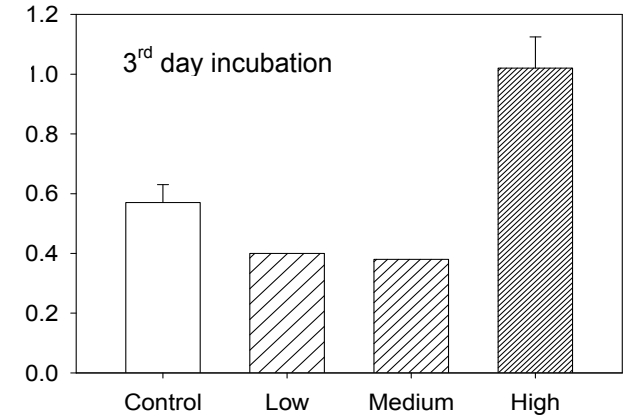


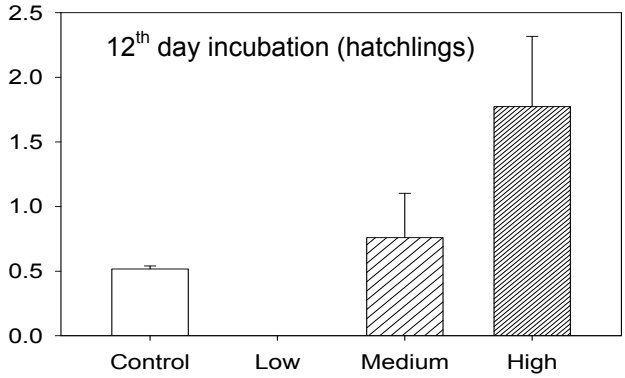
Figure 2.2. PBDE-47, a primary metabolite of PBDE-99, found in the (a) eggs, (b) hatchlings, and (c) in the liver of adult zebra finches at three different stages, ng/g wet weight.

(a)



.

(b)



(c)

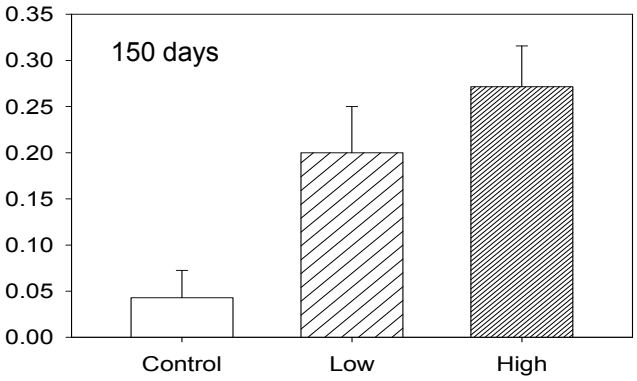
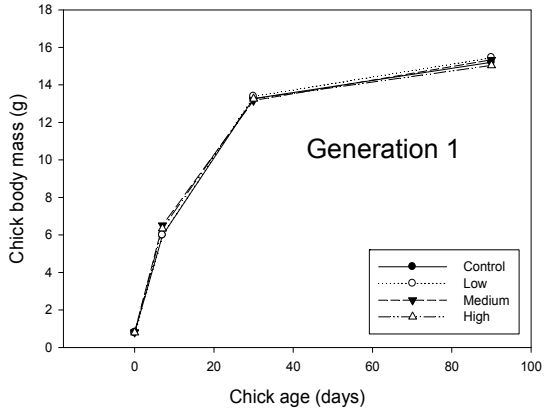
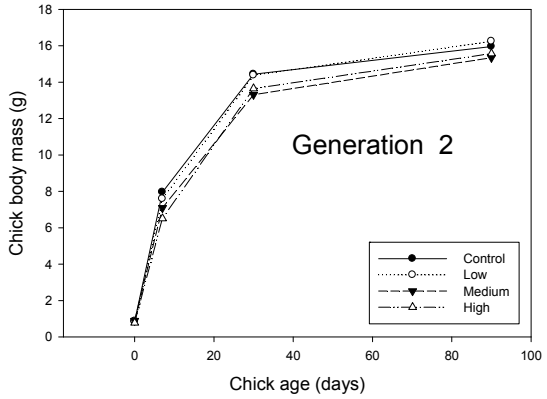


Figure 2.3. Growth curves for a) chicks exposed *in ovo* to PBDE-99 in low (10 ng/egg), medium (100 ng/egg), high (1000 ng/egg), and control (DMSO) doses; b) offspring of females from gen. 1; c) offspring of females from gen.2. In offspring generation body mass was significantly lower ($P < 0.005$) than controls in high-treated chicks at day 7, and marginally significant ($P = 0.07$) at day 30. No differences were significant within generations 1 and 3.

(a)



(b)



(c)

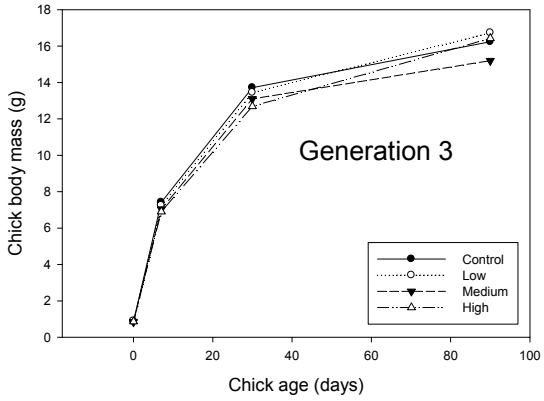
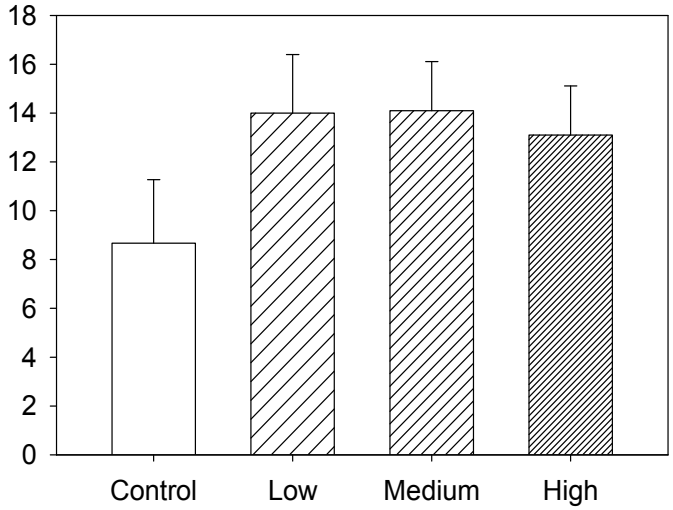


Figure 2.4. Laying interval (days between pairing and laying first egg) in days in (a) females exposed *in ovo* to PBDE-99 in low (10 ng/egg), medium (100 ng/egg), high (1000 ng/egg), and control (DMSO) doses; b) female offspring of exposed *in ovo* females. Statistically non-significant (P > 0.35) in parental generation, it tends to be longer in all PBDE-treated groups, whereas in control females and in offspring it corresponds to the typical laying interval for a 3-month inexperienced bird (Williams and Christians 2003).

(a) Parental (exposed *in ovo*) generation



(b) Offspring generation

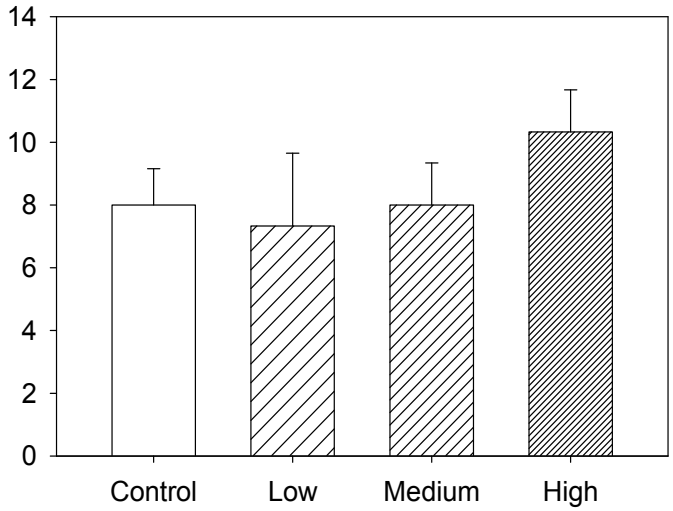
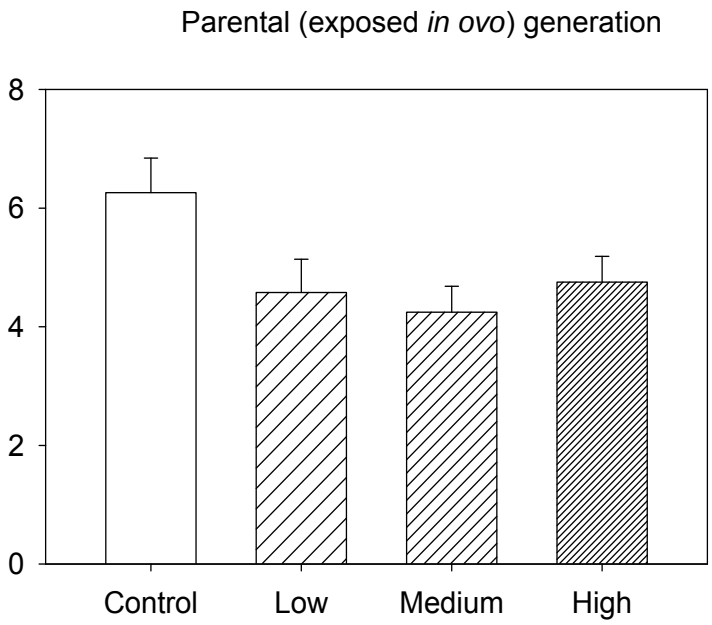
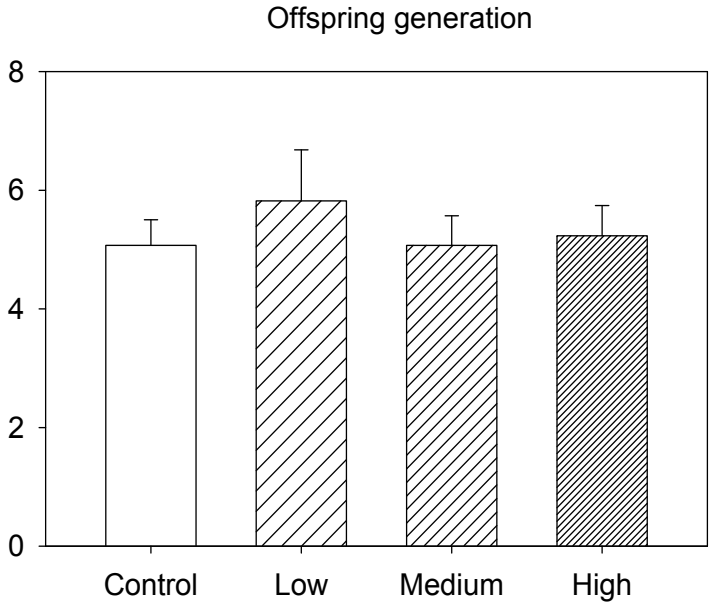


Figure 2.5. Clutch size in (a) females exposed *in ovo* to PBDE-99 in low (10 ng/egg), medium (100 ng/egg), high (1000 ng/egg), and control (DMSO) doses; b) female offspring of exposed *in ovo* females. In exposed generation, clutch size was significantly lower ($P < 0.05$) in all PBDE-treated groups, comparing to control.

(a)



(b)



2.8. Appendix

Concentration of PBDE-99 in avian eggs worldwide, ng/g wet weight

Species	Concentration, ng/g		Year	Place	Source
	Range	Median or mean			
Chinstrap penguin		2.55	2005-2006	King George Island, Antarctic	Yogui & Sericano 2009
Crowned plover		1.60	2004-2006	South Africa	Polder et al. 2008
Double-crested cormorant	0.06 - 44.0		1979-2002	British Columbia	Elliott et al. 2005
Gentoo penguin		3.42	2005-2007	King George Island, Antarctic	Yogui & Sericano 2009
Golden eagle	nd-7	1.00	1992-2002	Norway	Herzke et al. 2005
Goshawk	1.0 - 92	9.20	1991-2002	Norway	Herzke et al. 2005
Great blue heron	0.2 - 174		1983-2002	British Columbia	Elliott et al. 2005
Great tit	0.09 – 0.31	0.15	2005	Belgium	Dauwe et al. 2006
Great tit	0.69 – 2.55	1.55	2006	Belgium	Dauwe et al. 2009
Herring gull	26.2 - 31.0	28.00	1983-2000	Great Lakes	Elliott et al. 2005
Herring gull	85.3 - 89.5	87.40	1983-2000	Great Lakes	Elliott et al. 2005
Herring gull	108 - 302	156.00	2004	Great Lakes	Gauthier et al. 2007
Herring gull	107 - 542	260.00	2006	Great Lakes	Gauthier et al. 2008

Herring gull		12.37	1999	Lake Ontario	Norstrom et al. 2002
Herring gull		10.96	2000	Lake Ontario	Norstrom et al. 2002
Herring gull		18.26	1999	Lake Michigan	Norstrom et al. 2002
Herring gull		32.95	2000	Lake Michigan	Norstrom et al. 2002
Herring gull		24.30	1999	Lake Huron	Norstrom et al. 2002
Herring gull		15.30	2000	Lake Huron	Norstrom et al. 2002
Herring gull	5.08 – 44.84		2000	Great Lakes	Norstrom et al. 2002
Ivory gull		019	1976	Canadian Arctic	Braune et al. 2007
Ivory gull		0.31	1987	Canadian Arctic	Braune et al. 2007
Ivory gull		0.44	2004	Canadian Arctic	Braune et al. 2007
Kepl gull		0.13	2004-2009	South Africa	Polder et al. 2008
Lapwing	0.90 – 2.22	4.76	2006	Belgium	Dauwe et al. 2009
Leach's Storm-Petrel		0.76	1979-2002	British Columbia	Elliott et al. 2005
Little grebe		7.20	2004-2007	South Africa	Polder et al. 2008
Mediterranean gull	0.9 - 37	5.50	2006	Belgium	Dauwe et al. 2009
Merlin	5.0 - 19	9.60	1995-2000	Norway	Herzke et al. 2005
Osprey	2.37 - 17.3		1991-2000	British Columbia	Elliott et al. 2005
Osprey	4.0 - 77.0	17.30	1993-2000	Norway	Herzke et al. 2005
Peregrine falcon	9 - 1017	64.70	1996-2006	Northeastern US	Chen et al. 2008

Peregrine falcon	9.0 - 81	24.90	1993-2000	Norway	Herzke et al. 2005
Sacred ibis		3.04	2004-2005	South Africa	Polder et al. 2008
South polar skua		0.90	2005-2008	King George Island, Antarctic	Yogui & Sericano 2009
White-fronted plover		0.25	2004-2008	South Africa	Polder et al. 2008
White-tailed sea eagle	6 - 184	26.70	1992-2000	Norway	Herzke et al. 2005
Great Blue heron	24.9 - 293.1	100.90	1993	Indiana Dunes national lakeshore	Custer et al. 2009
Osprey	15.9 - 77.7	40.2	2002-2003	Everett	Henny et al. 2009
Osprey	7.07 - 55.3	26.8	2002-2004	Seattle	Henny et al. 2009
Osprey	34.1 - 178	73.9	2002	Yakima River	Henny et al. 2009
Osprey	1.39 - 42.1	14.4	2002-2004	Columbia River	Henny et al. 2009
Osprey	4.40 - 43.7	11.7	2004	Columbia River	Henny et al. 2009
Osprey	4.87 - 53.1	14	2002-2004	Columbia River	Henny et al. 2009
Osprey	5.40 - 25.0	12.8	2004	Columbia River	Henny et al. 2009
Osprey	8.01 - 35.3	18.4	2002-2004	Willamette reservoir	Henny et al. 2009
Osprey	11.2 - 498	52.1	2002-2003	Willamette reservoir	Henny et al. 2009