Validation of an egg-injection method for embryotoxicity studies in a small, model songbird, the zebra finch (*Taeniopygia guttata*)

V. Winter [a], J.E. Elliott [b], R.J. Letcher [c], T.D. Williams [a,*]

[a] Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6
[b] Environment Canada, Pacific Wildlife Research Centre, Delta, British Columbia, Canada V4K 3N2
[c] Environment Canada, National Wildlife Research Centre, Ottawa, Ontario, Canada K1A 0H3

HIGHLIGHTS

► We validated an egg-injection method to study *in ovo* exposure to xenobiotics.
► Zebra finch eggs (1 g) were injected with 5 μL DMSO, safflower oil, or sham-injected.
► Neither embryo fate nor hatchability was affected by vehicle or egg-injection.
► DMSO had an inhibitory effect on post-hatching growth but only in male chicks.
► Egg-injection is a suitable method for embryotoxicity studies in small passerines.

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ABSTRACT

Female birds deposit or 'excrete' lipophilic contaminants to their eggs during egg formation. Concentrations of xenobiotics in bird eggs can therefore accurately indicate levels of contamination in the environment and sampling of bird eggs is commonly used as a bio-monitoring tool. It is widely assumed that maternally transferred contaminants cause adverse effects on embryos but there has been relatively little experimental work confirming direct developmental effects (cf. behaviorally-mediated effects). We validated the use of egg injection for studies of *in ovo* exposure to xenobiotics for a small songbird model species, the zebra finch (*Taeniopygia guttata*), where egg weight averages only 1 g. We investigated a) the effect of puncturing eggs with or without vehicle (DMSO) injection on egg fate (embryo development), chick hatching success and subsequent growth to 90 days (sexual maturity), and b) effects of two vehicle solutions (DMSO and safflower oil) on embryo and chick growth. PBDE-99 and -47 were measured in *in ovo* PBDE-treated eggs, chicks and adults to investigate relationships between putative injection amounts and the time course of metabolism (debromination) of PBDE-99 during early development. We successfully injected a small volume (5 μL) of vehicle into eggs, at incubation day 0, with no effects on egg or embryo fate and with hatchability similar to that for non-manipulated eggs in our captive-breeding colony (43% vs. 48%). We did find some evidence for an inhibitory effect of DMSO vehicle on post-hatching chick growth, in male chicks only. This method can be used to treat eggs in a dose-dependent, and ecologically-relevant, manner with PBDE-99, based on chemical analysis of eggs, hatchling and adults.

1. Introduction

Female birds deposit or 'excrete' contaminants which have accumulated in their lipid-rich tissues to their eggs during yolk formation (Fry and Toone, 1981; Dauwe et al., 2006). As a consequence concentrations of xenobiotics in bird eggs and post-hatching nestlings can accurately indicate levels of contamination in the environment and sampling of bird eggs is commonly used as a bio-monitoring tool for detecting levels, distribution, and long-term trends in environmental contamination such as persistent organic pollutants (Norstrom et al., 2002; Elliott et al., 2005; van den Steen et al., 2010). For example, concentrations of PBDE congeners derived from Penta- and Octa-BDE formulations were reported to have increased exponentially in herring gull (*Larus argentatus*) eggs from the Great Lakes basin between 1981 and 2006 (Norstrom et al., 2002; Gauthier et al., 2008). Similarly, in British Columbia the concentration of PBDE congeners derived from the Penta-BDE

* Corresponding author. Address: Department of Biological Sciences, Simon Fraser University, 8888 University Dr., Burnaby, British Columbia, Canada V5A 1S6. Tel.: +1 604 318 2712; fax: +1 778 782 3496.
E-mail address: tdwillia@sfu.ca (T.D. Williams).

formulation increased 305-fold from 1.3 to 455 µg g^{-1} with a doubling time of 5.4 years in the eggs of great blue herons (*Ardea herodias*) between 1983 and 2002 (Elliott et al., 2005). A major congener in Penta-BDE formulations is 2,2′,4,4′,5-pentabromodiphenyl ether (PBDE-99).

Numerous studies have investigated the processes of maternal transfer of xenobiotics to eggs in birds (Bargar et al., 2001; Drouillard and Norstrom, 2001; Verreault et al., 2006; van den Steen et al., 2009; Gebbink and Letcher, 2012). It is widely assumed that maternally transferred contaminants can cause adverse effects on the embryo, given that early developmental stages are 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 (PCDEs), 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). However, there has been relatively little *experimental* work on the effects of this maternal transfer of contaminants to embryo development. This is important because negative effects on embryos and post-hatching chicks could be related to the direct effects of *in ovo* exposure to contaminants, but they could also be due to indirect effects of contaminants reducing parental (female) quality and parental care, e.g. incubation behavior, affecting egg temperature and embryo development, or chick-provisioning, affecting chick growth. Experimentally the effect of maternally-derived xenobiotics on offspring development can be studied indirectly via treatment of the mother taking advantage of maternal transfer to eggs. However, contaminant exposure of laying females could also generate behavioral and 'non-target' physiological effects and involves other potential sources of 'bias', e.g. among-female variation in food intake, uptake and accumulation of contaminants or transfer to eggs. Direct injection of contaminants into the air cell or yolk sac of the egg avoids many of these problems and allows for embryotoxicity studies utilizing known, specific amounts of xenobiotic, and assessment of direct effects on embryo development (McKernan et al., 2009, 2010). In addition, this approach can help address the issue of whether maternal transfer is a more realistic route of contaminant exposure, e.g. Heinz et al. (2009) showed that injected methylmercury was more toxic than maternally deposited methylmercury.

Some previous 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; McKernan et al., 2009). However, all studies to date have been on relatively large birds, with correspondingly large egg sizes (15–60 g), 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 investigating organic contaminants in small songbirds, perhaps due to technical difficulties associated with injection/incubation of the small eggs (1–5 g). 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* (developmental) exposure to environmentally relevant levels of PBDE-99 flame retardant on embryo development, hatchability, chick development and adult phenotype (Eng et al., 2012; Winter et al., submitted for publication). First, 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). Second, we investigated the effects of two vehicle solutions (DMSO and safflower oil) on embryo and chick growth, as well as repeating the puncture-only treatment, in relation to that of non-manipulated eggs within each clutch. Finally, we measured PBDE-99 and 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47; and a known debrominated metabolite of PBDE-99) in PBDE-treated eggs at day 3 of incubation, in chicks at hatching, and in offspring at sexual-maturity (150 days post-hatch) to investigate relationships between putative injection amounts and the time course of metabolism of xenobiotics during development.

2. Materials and methods

2.1. General animal husbandry and breeding protocol

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

Male and female birds were assigned to breeding pairs at random (first experiment, $n = 24$ pairs; second experiment, $n = 42$ pairs). 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 ($50 \times 41 \times 37$ cm) each with an external nest box ($14.5 \times 14.5 \times 20$ cm). On the day of pairing, body mass (±0.01 g) and tarsus length (±0.01 mm) of all birds were recorded, and females were weighed on the day they laid their first egg and when the clutch was completed (2 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). These data provided information related to laying date and laying sequence of eggs for the egg-injection experiments.

2.2. Egg injection protocol

In the first experiment all eggs were injected or sham-treated within 24 h of being laid (day 0). Eggs were randomly assigned to either the punctured-only treatment ($n = 32$ eggs) or a DMSO-injected treatment ($n = 32$ eggs). Each clutch contained the eggs from both treatments to control for variation among females, and genetic background. Eggs were held in an upright position, with the long axis of the egg vertical, and the rounded end (with the air sac) towards the bottom, and the pointed end of the egg was cleaned with 70% ethyl alcohol, using a swab. A hole was made with a sterile needle (26½ gauge) about 2 mm down the side of the egg, from the pointed end, and a sterile needle was pushed through the shell downwards into the egg approximately 7 mm deep at a 45° angle to the long-axis of the egg to reach the yolk. Eggs in the puncture-only treatment received a sham injection, where the sterile needle was pushed through the shell but nothing was injected. For DMSO-injected eggs, a constant volume (5 µL egg^{-1}) of DMSO was injected into the yolk using a sterile Hamilton syringe. Our injection volume represents approximately 0.5% of egg volume (1 g = 1 mL). 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.

For the second experiment 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), puncture-only group (n = 45 eggs), and non-injected or non-manipulated eggs which were only handled and replaced in the nest (n = 44 eggs). After treatment eggs were sealed with Loctite Ultra Gel Super Glue® (cyanoacrylate), rather than Vetbond®, to achieve better control of the sealed surface (topical application volume was approximately 1 μL see Section 4). 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. Any eggs that were broken during injection (<5) were eliminated from the experiment, and consequently, from further analysis.

Following egg treatment, in both experiments, nest boxes were monitored daily to record "missing" or "broken" eggs. Eggs were considered 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 2 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 h) to determine hatching success of each egg. All newly hatched chicks were then weighed within 24 h 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, when chicks leave the nest box) in both experiments, and additionally mass and tarsus length were measured at day 90 post-hatching (=sexual maturity) in the second experiment.

2.3. In ovo PBDE exposure and metabolism

To evaluate chemical metabolism of PBDE-99 and -47 (as a possible, meta-debrominated metabolite of PBDE-99) following *in ovo* injection we conducted a third experiment with eggs dosed with low (2 ng μL^{-1}, or 10 ng egg^{-1}), medium (20 ng μL^{-1}, or 100 ng egg^{-1}) and high (200 ng μL^{-1}, or 1000 ng egg^{-1}) levels of PBDE-99 (5 μL injection volumes using DMSO vehicle). Eggs from control, low, medium and high groups were randomly collected at two stages of development: the 3rd day of incubation (n = 3 per treatment), and at hatching (n = 3 per treatment) for chemical analysis. In addition we collected liver tissue from birds at 150 days post-hatching that had been dosed using the same protocol in our main study (n = 8 per treatment, 4 of each sex; Winter et al. submitted for publication). All samples were frozen and stored at −80 °C until further analysis.

2.4. PBDE analysis

PBDE (BDE-17, -28/33, -47, -49, -66, -85 and -99) analyses were carried out in the Organic Contaminant Research Laboratory (OCRL; Letcher Lab) at Environment Canada's National Wildlife Research Centre (NWRC) in Ottawa, Canada. All PBDE standards were purchased from Wellington Laboratories (Guelph, ON, Canada). Plasma, egg and dosing solutions were analyzed for BDE-17, -28/33, -47, -49, -66, -85 and -99.

An amount of 0.5–1.0 g of egg homogenate, whole hatchling homogenate or adult liver sample were accurately weighed, and neutral fractions were extracted and cleaned up as recently described elsewhere (Chen et al., 2012; Eng et al., 2012). In brief, samples were ground with ∼25 g of anhydrous sodium sulfate and extracted with 50% dichloromethane (DCM)/hexane using an accelerated solvent extraction system (Dionex ASE 200). A volume of 20 μL internal standard solution, which included BDE-30 and BDE-156, each with concentrations of 2000 pg μL^{-1} in isooctane, was added. The column extraction eluent was concentrated to 10 mL and a 10% portion was removed for gravimetric lipid determination. The remaining extracts were cleaned by gel permeation chromatography (GPC; O.I. Analytical, College Station, TX, USA) and eluted from the GPC column with 50% DCM/hexane.

The GPC fraction was further cleaned up using a LC-Si SPE cartridge (500 mg, 5 mL, J.T. Baker, USA). After conditioning the SPE cartridge with successive washes of 6 mL of Methanol/DCM (10v/90v) and then 8 mL of DCM/hexane (5v/95v) the sample was loaded on the cartridge, and eluted with 8 mL of DCM/hexane (5v/95v). The eluent was concentrated under a gentle stream of nitrogen and reconstituted with isooctane to a final volume of approximately 200 μL for GC/MS determination.

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

PBDE congeners were monitored by ECNI using the bromine anions of m/z 79 and 81. Analytes were identified by comparison of retention times and ECNI mass spectra to those of the authentic standards. Mean internal standard recoveries of BDE-30 and BDE-156 were 27 ± 13% and 25 ± 15%, respectively, and thus were highly reproducible. Despite the systematically lower recovery efficiencies, PBDE-47 and -99 were quantified using an internal standard approach, thus all reported values were inherently recovery-corrected. The method limits of quantification (MLOQs) for PBDEs, based on a signal-to-noise ratio of 10, were generally <0.05 ng g^{-1} wet weight (w.w) for all samples. One method blank sample was analyzed with each batch of samples (n = 12) to check interferences and contamination. Blank subtraction was done for PBDE-47 and -99. For each batch of samples (n = 12), a reference material sample (double-crested cormorant, *Phalacrocorax auritus*, egg homogenate prepared at NWRC) was included in the extraction and analyzed to ensure consistency of data acquisition.

2.5. 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 Section 3). 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.

3. Results

3.1. Effect of puncturing and vehicle injection – experiment 1

There was no difference in mass of eggs subsequently assigned to DMSO ($1.085 ± 0.030$ g) or sham/puncture only treatments ($1.078 ± 0.030$ g; $F_{1,48.9} = 0.07$, $p > 0.7$). Similarly, 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 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 $\chi^2 = 0.006$, $p > 0.80$). However, many cells had expected counts <5 so we pooled data to compare "hatched" vs. all "not hatched" eggs. There was no effect of treatment on overall hatching success: DMSO, 25.8% hatched vs. sham, 24.2% hatched ($\chi^2 = 0.021$, $p > 0.80$). However, overall hatching success was low compared to normal values for this captive population (48%, T.D. Williams, unpub. data; see Section 4).

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 in this first experiment 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), but DMSO-treated chicks tended to be 1–2 g lighter on average at 14, 21, and 30 days of age (Table 1).

3.2. Effect of puncturing, and different vehicle solutions – experiment 2

There was no difference in egg mass for eggs subsequently assigned to DMSO-injected ($1.062 ± 0.020$ g), oil-injected ($1.063 ± 0.021$ g), sham ($1.073 ± 0.020$ g), or control treatments ($1.058 ± 0.021$ g; $F_{3,141} = 0.54$, $p > 0.6$). 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, $3.41 ± 0.26$; $F_{3,176} = 0.23$, $p > 0.80$). In other words, eggs were randomly assigned to 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 ($\chi^2 = 14.0$, $p > 0.25$). Again, 40% of cells had expected counts <5 so we deleted "missing eggs" and pooled fertile eggs and those with more developed, but non-hatching, embryos. There was no effect of treatment on egg fate comparing infertile eggs, hatched eggs and non-hatched eggs (Fisher's Exact $\chi^2 = 9.98$, $p > 0.05$). Similarly, there was no effect of treatment on overall hatching success, comparing "hatched" vs. all "non-hatched" eggs: DMSO, 43.5% hatched, oil, 34.8% hatched, sham, 42.6% hatched, control, 53.3% hatched ($\chi^2 = 3.27$, $p > 0.30$). Overall hatching success was higher for experiment 2 (39%) compared with experiment 1 (25%) although this difference was marginally non-significant ($\chi^2 = 3.56$, $p = 0.06$).

Hatching mass was strongly positively correlated with egg mass in ($F_{1,47.4} = 16.75$, $p < 0.001$). However, there was no effect of treatment on hatching mass, controlling for egg mass or brood size at hatching ($F_{3,55.6} = 0.59$, $p > 0.60$). Analyzing chick growth in a single model with age, sex, and treatment as main effects there was a significant effect of treatment ($F_{3,45.6} = 3.36$, $p = 0.027$), but also a significant effect of sex ($F_{1,52.9} = 6.55$, $p = 0.013$) and a sex * treatment interaction ($F_{3,50} = 3.16$, $p = 0.033$). We therefore analyzed the effect of treatment on chick growth for each sex separately, controlling for effects of egg mass and brood size. In female chicks there

Table 1
Chick mass (g) at hatching (day 0), and at 7, 14 and 21 days of age in relation to egg injection treatment for the first experiment. Values are ls means ± SE.

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	$11.28 ± 1.11$	$12.72 ± 0.83$

was, not surprisingly, a highly significant effect of age of body mass ($F_{4,128} = 2081.3$, $p < 0.001$) but there was no effect of treatment ($p > 0.4$) or a treatment * age interaction ($p > 0.6$), i.e. the pattern of growth did not differ among treatments for females (Table 2). In contrast in male chicks, there was a significant effect of treatment ($F_{3,129} = 7.74$, $p < 0.001$) as well as age ($F_{4,125} = 1881.6$, $p < 0.001$), although the treatment * age interaction was not significant in the overall model ($F_{12,125} = 1.55$, $p = 0.11$). We therefore compared chick mass among treatments for each age separately in males. There was a significant effect of treatment on body mass of male chicks at 21-days ($F_{3,17.8} = 6.90$, $p = 0.003$) and 90-days of age ($F_{3,11.3} = 9.77$, $p = 0.002$), but not at 7 or 14 days ($p > 0.10$ in both cases). DMSO-treated chicks were significantly lighter than all other chicks at both ages (Bonferroni-adjusted $p < 0.05$; Table 2). Furthermore, tarsus length at 90-day of age was affected by treatment in male chicks ($F_{3,24} = 4.62$, $p = 0.011$) but not in females ($p > 0.15$). Again, DMSO-treated chicks had smaller tarsi than control chicks ($16.2 ± 0.2$ mm vs. $17.2 ± 0.2$ mm; Bonferroni-adjusted $p = 0.020$).

3.3. Egg and chick contaminant levels following in ovo PBDE-99 treatment

There was a highly significant dose-dependent relationship between PBDE-99 content and *in ovo* injection treatment in eggs on day 3 of incubation ($F_{3,9} = 96.45$, $p < 0.001$), at hatching on day 12 of incubation ($F_{3,9} = 7.44$, $p = 0.011$), and in livers of adult birds at 150 days post-hatching ($F_{2,21} = 11.27$, $p < 0.001$; Fig. 1 top). Similarly, there was significant variation in PBDE-47 concentrations in relation to *in ovo* PBDE-99 treatment at day 3 ($F_{3,9} = 11.27$, $p = 0.007$) and day 12 of incubation ($F_{3,9} = 5.37$, $p = 0.025$; Fig. 1 bottom). Since there was no detectable PBDE-47 in the dosing solution, all of the PBDE-47 present in samples had to be the result of PBDE-99 debromination to PBDE-47. Regardless, there was a treatment effect on PBDE-47 concentrations in livers of 150 day old adults ($F_{2,21} = 6.73$, $p = 0.006$), although tissue concentrations were much lower compared with egg values (Fig. 1). There was no effect of sex and no treatment * sex interaction for either PBDE-99 or PBDE-47 in livers of 150 day old birds ($p > 0.6$ in all cases).

4. Discussion

Here we successfully 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, where egg weight averages only 1 g. Using sterile technique and a Hamilton syringe we were able to inject a small volume (5 μL) of vehicle into eggs with no effects on egg or embryo fate and with hatchability similar to the norm for non-manipulated eggs in our captive-breeding colony (43% vs. 48%). We confirmed that this small injection volume is sufficient to accurately treat eggs in a dose-dependent manner with PBDE-99, based on chemical analysis of eggs, hatchling and adults. We did find some evidence for an inhibitory effect of DMSO vehicle on post-hatching chick growth, in male chicks only, which we discuss further below.

Table 2
Chick mass (g) at hatching (day 0) and at 7, 14, 21 and 90 days of age in relation to egg injection treatment for the second experiment. Values are ls means ± SE; values in bold are significantly different from other treatments in each row (Bonferroni-adjusted $p < 0.05$).

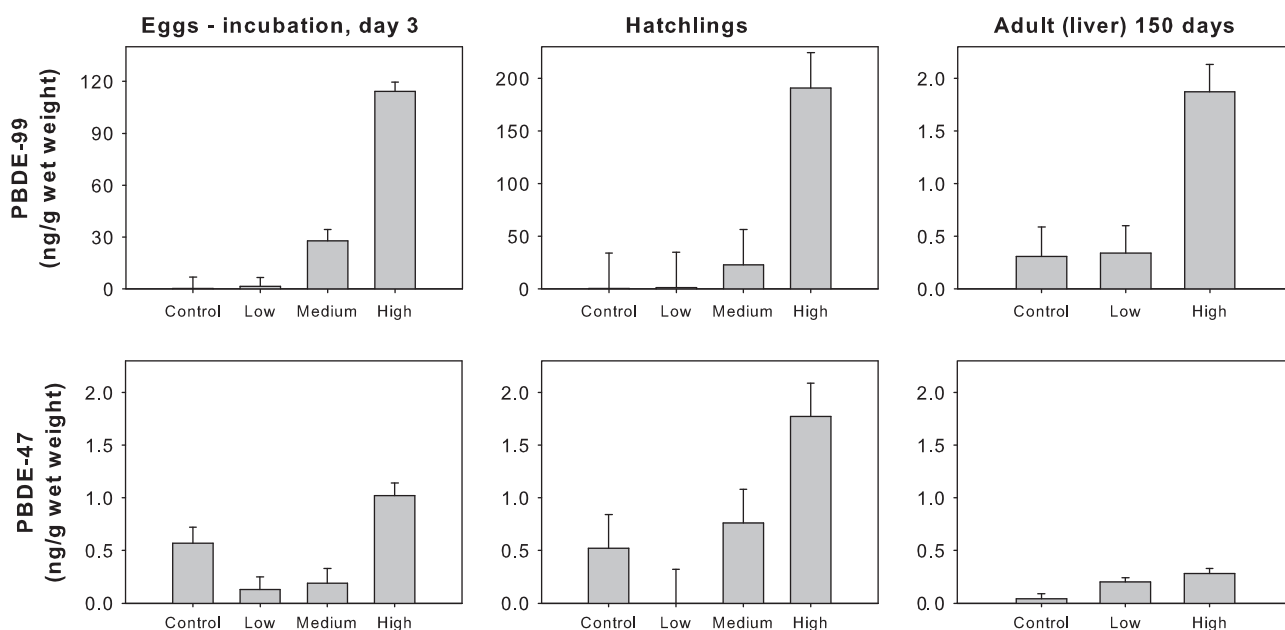| Sex	Age	DMSO	Sham	Oil	Control
Male	0	0.82 ± 0.04	0.84 ± 0.05	0.83 ± 0.06	0.91 ± 0.04
	7	6.1 ± 0.3	6.9 ± 0.4	6.3 ± 0.4	6.9 ± 0.3
	14	11.0 ± 0.3	11.3 ± 0.3	11.4 ± 0.4	11.9 ± 0.3
	21	**11.4 ± 0.2**	11.7 ± 0.2	12.7 ± 0.3	12.2 ± 0.2
	90	**13.3 ± 0.2**	14.3 ± 0.3	14.8 ± 0.3	14.7 ± 0.2
Female	0	0.75 ± 0.06	0.79 ± 0.05	0.80 ± 0.04	0.81 ± 0.05
	7	6.6 ± 0.4	6.9 ± 0.3	6.6 ± 0.3	6.8 ± 0.4
	14	11.8 ± 0.4	12.1 ± 0.3	12.0 ± 0.3	11.7 ± 0.3
	21	12.2 ± 0.3	12.4 ± 0.3	12.5 ± 0.3	12.5 ± 0.3
	90	15.1 ± 0.5	15.7 ± 0.4	15.0 ± 0.4	14.7 ± 0.4

Fig. 1. Dose-dependent variation in tissue PBDE-99 (top panels) and PBDE-47 (bottom panels) levels found in eggs at day 3 of incubation, hatchlings at day 12 of incubation, and in the liver of adult zebra finches at 150 days post-hatching. Values are ls means ± SE (ng g^{-1} wet weight).

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. However, since hatchability of eggs was higher in our second experiment we believe that the abnormally low hatching success was related to technical or methodological problems in the first experiment, i.e.. 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, personal communication) when each needle was used up to five times, we switched to single-use, disposable sterile needles in our second experiment to avoid any potential problem with cross-contamination. Another methodological improvement was that Vetbond® was replaced with Loctite Ultra Gel Super Glue® for sealing the injection site. Vetbond® was found to be less viscous which meant that it was easy to accidentally cover a large surface area of the egg, potentially inhibiting gas exchange for the developing embryo and increasing pre-hatching mortality. Use of Loctite also minimized the possibility of eggs sticking to nest material or

other eggs (O. Love, personal communication). However, Loctite® required more time to dry out completely, so that we increased the drying time to 10 min before replacing eggs in the nest. 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%; and similar to hatching success reported for some other zebra finch colonies, e.g. von Engelhardt et al., 2004, 2006; Criscuolo et al., 2011).

We found that DMSO-treated male chicks had significantly lower body mass at 21 and 90 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, although there was no effect of DMSO on hatching mass. Thus, DMSO appeared to have a negative effect on post-hatching 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. DMSO (CAS 67-68-5) is a widely-used vehicle in toxicological studies due to its excellent solvent properties and is readily absorbed via dermal and oral routes, persisting in serum for more than 2 weeks after a single exposure (Gad, 2005). Several studies have investigated toxicity of DMSO in non-avian species and acute toxicity has been reported to be low

(Dresser and Finch, 1992; Guerre and Casali, 1999; Gad, 2005). Hallare et al. (2004) did report increased heat shock protein 70 expression following 96-h DMSO exposure in zebrafish (*Danio rerio*) and warned against use of DMSO as a solvent in toxicity assays. Fewer studies have directly tested for DMSO toxicity in avian species. Scialli et al. (1994) found DMSO to be toxic for chicken embryo, when injecting 50 μL egg^{-1} into the air sac (although this study did not include a non-manipulated or sham-control). In contrast DMSO has been used successfully in chicken egg injection studies at volumes of 1 μL g^{-1} or 50 μL egg^{-1} (O'Brien et al., 2009), which is relatively less than the amount used in our experiments (5 μL g^{-1}, or 5 μL egg^{-1}). Similarly, Heinz et al. (2006) evaluated DMSO as a solvent for methylmercury injection into chicken eggs and found that DMSO itself had little if any toxicity when injected into the air sac just before the start of incubation at 0.5 μL g^{-1}, or approximately 25 μL egg^{-1}: at the end of incubation, 8/10 DMSO-injected eggs survived compared with 10/10 un-injected control eggs. It is possible that the delayed effect of DMSO on chick growth in our study was 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) and has been evaluated as a more potent cytotoxic drug than DMSO, with less reversible effect. DMSO$_2$ has also been shown to be a potent growth inhibitor in smooth muscle cells and endothelial cells (Layman, 1987). However, it is not clear why any effect of DMSO or its metabolite was sex-specific in our study, only occurring in males but not in female chicks. If DMSO is inhibiting growth one would predict that the larger, more rapidly-growing sex would be most affected. In many avian species this would be the male, but in our species female chicks are slightly but significantly larger. However, there are obviously many other sex-specific developmental and hormonal differences providing a myriad of targets for the effect of DMSO.

After background correction, in the control samples of egg, hatching or adult liver dose group, PBDE-47 or PBDE-99 were either not detectable of at very low levels <0.5 ng g^{-1} ww (and just above the MLOQ). However, PBDE-99 was quantifiable in all egg, chick and adult samples from *in ovo* PBDE-99 treated birds. Concentrations of PBDE-99 in eggs at day 3 of incubation and in nestlings at day 12 of incubation (around hatching) showed a strong dose-response relation to treatment dose but only represented about 20–30% of the nominal amount of PBDE-99 injected at each dose. This suggests that there can be rapid metabolism of xenobiotics by the developing embryo, as has been documented for maternally-derived steroid hormones (Paitz and Bowden, 2009; Paitz et al., 2010). Furthermore, regardless of the egg, hatching or adult liver from the PBDE-99 dosed groups, concentrations of PBDE-47 was consistently about 1.5 ng g^{-1} ww or lower and essentially barely quantifiable above the MLOQ. Again, this indicates that some PBDE-47 background contamination was present, which most likely reflects chemicals accumulated from the diet (either seed or the supplemental egg food all birds received during breeding). Fernie et al. (2006) also reported small quantities of PBDE-99 (0.97 ng g^{-1}) but no PBDE-47 in a control group of American kestrels after exposure *in ovo* to safflower oil and after being fed with small chicks for 29 days after hatching. In liver PBDE-99 like PBDE-47 was barely quantifiable, which suggests that PBDE-99 were eliminated from the adult birds. These results are consistent with that of Eng et al. (2012), where PBDE-47 was below the MLOQ or at sub ng g^{-1} w.w. levels, and thus essentially not present in the plasma or adipose of PBDE-99 dosed zebra finches.

The results of our chemical analysis therefore confirm that our egg injection method is suitable to allow for experimental exposure to specific, environmentally relevant, chemical concentrations during the early, critical stages of embryo development. Although liver PBDE-99 concentrations were very low in adult birds

4 months after *in ovo* dosing, representing <1% of the injected dose, we still found significantly elevated PBDE-99 levels in birds dosed at the highest treatment level (200 ng μL^{-1}, or 1000 ng egg^{-1}). Given the small size of zebra finch eggs we were unable to confirm that the vehicle and/or PBDE were injected directly into the yolk, and we did not investigate how the vehicle partitioned within the egg. von Engelhardt et al. (2009) injected radio-labelled steroid hormones in oil vehicle into chicken eggs and showed that although oil initially concentrated in a small area at the top of the yolk during incubation radioactivity spread within the egg with highest concentrations in yolk and yolk sac and lower concentrations in albumen, embryo, allantois, and amnion. In conclusion, the egg injection method validated in our study, could be used to test a wide variety of organic pollutants in small songbird species. Even with eggs of only 1 g mass, on average, and with injection volumes of only 5 μL this method allows for dosing up to quite high, ecologically-relevant, levels (200 ng μL^{-1}, or 1000 ng egg^{-1} in this study).

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