Chemosphere 90 (2013) 125…131

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier .com/locate/chemosphere

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

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h i g h l i g h t s

- We validated an egg-injection method to study in ovo exposure to xenobiotics.
- Zebra “nch eggs (1 g) were injected with 5 l L DMSO, saf”ower 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.

a r t i c l e i n f o

Article history:
Received 10 May 2012
Received in revised form 2 August 2012
Accepted 7 August 2012
Available online 6 September 2012

Keywords:
In ovo exposure
Embryotoxicity
Developmental effects
Zebra “nch
Flame retardant
PBDE-99

a b s t r a c t

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 con“rming 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 “nch (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 saf”ower oil) on embryo and chick growth in PBDE-99 and -47 (Stapleton et al., 2002; Gauthier et al., 2007). Similar sured in ovo PBDE-treated eggs, chicks and adults to investigate relationships between tissue PBDE concentration amounts and the time course of metabolism (debromination) of PBDE-99 during early development. We successfully injected a small volume (5 l) into Zebra finch eggs (Taeniopygia guttata) without significantly affecting hatching success, and accurately indicate levels of PBDE congeners derived from the commercial mixture DE-71 (La Guardia et al., 2006). As a consequence concentrations of xenobiotics in bird eggs and post-

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0045-6535/$ - see front matter
http://dx.doi.org/10.1016/j.chemosphere.2012.08.017

formulation increased 305-fold from 1.3 to 455 l g 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

For the second experiment eggs were randomly assigned to four treatments within each clutch. The treatments included a DMSO-injected group (n = 45 eggs), saf"ower 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 l L 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 classi"ed 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 speci"c 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 (="edging, 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 l L ^{1}, or 10 ng egg ^{1}), medium (20 ng l L ^{1}, or 100 ng egg ^{1}) and high (200 ng l L ^{1}, or 1000 ng egg ^{1}) levels of PBDE-99 (5 l L 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 l L internal standard solution, which included BDE-30 and BDE-156, each with concentrations of 2000 pg l L ^{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 "nal volume of approximately 200 l L 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 quanti"ed 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 l m DB-5HT fused-silica column (J & W Scienti"c, Brockville, ON, Canada). Helium and methane were used as the carrier and reagent gases, respectively. A sample .8(the)-316.p02.5Sc.4(Ba-455.6(5973)]T000)-and70ime

Values are presented as least square means ± SEM unless otherwise stated.

3. Results

3.1. Effect of puncturing and vehicle injection … experiment 1

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