NeuroToxicology 59 (2017) 33–39

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Assessment of neuroanatomical and behavioural effects of *in ovo* methylmercury exposure in zebra finches (*Taeniopygia guttata*)

Maria S. Yu[a,*], Margaret L. Eng[a,b], Tony D. Williams[a], Mélanie F. Guigueno[c], John E. Elliott[b,**]

[a] Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada
[b] Environment and Climate Change Canada, Science & Technology Branch, Pacific Wildlife Research Centre, Delta, British Columbia, Canada
[c] Faculty of Agricultural and Environmental Sciences, McGill University, Sainte-Anne-de-Bellevue, Québec, Canada

ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form 3 January 2017
Accepted 4 January 2017
Available online 7 January 2017

Keywords:
Methylmercury
Passerines
In ovo exposure
Developmental neurotoxicity
Courtship behavior

ABSTRACT

Methylmercury (MeHg) readily crosses the blood brain barrier and is a known neuro-toxicant. MeHg accumulation in the brain causes histopathological alterations, neurobehavioral changes, and impairments to cognitive motor functions in mammalian models. However, in birds the neurotoxic effects of MeHg on the developing pre-hatching brain and consequent behavioral alterations in adult birds have not received much attention. Moreover, passerine birds are poorly represented in MeHg neurotoxicology studies in comparison to other avian orders. Hence in this study, we used the egg injection method to investigate the long term effects of *in ovo* MeHg exposure on brain histopathology and courtship behavior in a model songbird species, the zebra finch (*Taeniopygia guttata*). Egg treatment groups included: a low MeHg dose of 0.2 μg Hg g^{-1} egg, a high MeHg dose of 3.2 μg Hg g^{-1} egg, and a vehicle control (water). No adverse effects of *in ovo* MeHg treatment were detected on courtship song quality or on mating behavior in experimental males at sexually maturity which would suggest that observable neurobehavioral effects of MeHg exposure may depend on the timing of exposure during offspring development. However, neuroanatomical analysis indicated an increase in telencephalon volume with increased MeHg concentrations which may suggest a prolonged inflammatory response in this region of the brain.

1. Introduction

The neurotoxic potential of methylmercury (MeHg), the organic form of mercury (Hg) and a ubiquitous anthropogenic contaminant, is well recognized (Burbacher et al., 1990). In birds, research into the neurological impacts of methylmercury exposure has included a variety of endpoints ranging from neuroanatomical and biochemical alterations to behavioral and motor function impairments. Dietary dosing and field studies have demonstrated that MeHg accumulation in the avian brain results in altered structure (i.e., brain lesions, neuronal degeneration, inflammation) (Heinz and Locke, 1976; Wolfe et al., 1998; Spalding et al., 2000); impairments to motor function and coordination (Kenow et al., 2010; Carvalho et al., 2008); cognitive, memory, and learning deficiencies (Heinz, 1975, 1979) and disruptions to complex behaviors such as reproductive and social behavior (Frederick and Jayasena, 2011; Bouton et al., 1999). Studies conducted on humans and experimental animals have demonstrated that early life stages (i.e., prenatal) are more sensitive to developmental stressors including MeHg toxicity (Choi, 1989); further, adverse effects of exposure may not become apparent until later in life (Ceccatelli et al., 2013; Burbacher et al., 2005; Weiss et al., 2005; Beyrouty et al., 2006). This latency in effects has been investigated in some animal models (Ceccatelli et al., 2013; Bornhausen et al., 1980; Weis, 2012; Bergeron et al., 2011), as well as potential mechanisms of action (Monnet-Tschudi et al., 2006) and epigenetic factors (Basu et al., 2014). However, the early *in ovo* exposure and later developmental effects of MeHg have yet to be as extensively elucidated in birds. An improved understanding of MeHg-induced neurotoxicity at the early prenatal stage is important given that the embryonic brain is considered to be particularly sensitive to MeHg during development (Wiener et al., 2003), and because female birds depurate MeHg into their eggs (Kambamanoli-Dimou et al., 1991; Heinz and Hoffman, 2004). Moreover, passerines have been poorly represented in Hg

* Corresponding author.
** Corresponding author at: Environment Canada, Science & Technology Branch, Pacific Wildlife Research Centre, 5421 Robertson Rd., Delta, British Columbia V4K 3N2, Canada.
E-mail addresses: mariay@sfu.ca (M.S. Yu), john.elliott@canada.ca (J.E. Elliott).

http://dx.doi.org/10.1016/j.neuro.2017.01.001
0161-813X/

neurotoxicology studies in comparison to other avian orders, despite their demonstrated ability to accumulate high concentrations of Hg (Rimmer et al., 2005; Jackson et al., 2015).

Recent field studies have reported that environmental exposure to MeHg may be linked to altered male singing behavior in three song-learning passerine species inhabiting areas with elevated environmental Hg concentrations in Northeastern North America (Hallinger et al., 2010; McKay and Maher, 2012). In some songbirds, the acquisition and production of song is a learned behavior that is controlled by a discrete series of brain nuclei and neural pathways often referred to as the song control system. The robust nucleus of the arcopallium (RA) and the HVC (acronym used as proper name, formerly was referred to as hyperstriatum ventral pars caudale or higher vocal center, which are misnomers (Reiner et al., 2004)) are song control nuclei involved in song production via the caudal motor pathway in the song control system (Brenowitz et al., 1997). The anterior forebrain pathway, which indirectly links the HVC and RA via Area X, is involved in song learning and recognition. Exposure to some environmental contaminants, such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and polybrominated biphenyl ethers (PBDEs) can alter the size of these neural structures (Hoogesteijn et al., 2008; Iwaniuk et al., 2006; Eng et al., 2014), which may influence singing behavior (Blocker and Ophir, 2013). Bird song plays a significant role in reproductive success in songbird populations in that variation in courtship singing behavior and song quality is associated with male mating behavior, male quality and attractiveness to females and subsequent breeding and reproduction (McGregor et al., 1981; Baker et al., 1986). As well, the song control system is suggested to be sensitive to early developmental conditions (Nowicki et al., 1998), and the accumulation of MeHg in developing brains of avian neonates may result in observable changes in male song learning and formation [(Varian-Ramos et al., 2011) *unpublished*]. However, few studies have specifically related MeHg exposure to song quality or production in songbird species, and to our knowledge, none have utilized the egg injection method (i.e., minimizing maternal effects) to experimentally test the causal link between early exposure to MeHg and effects on the song control system.

In the present study, we investigated the long-term neurobehavioral effects of early developmental exposure to MeHg, via *in ovo* dosing, on male zebra finches (*Taeniopygia guttata*). These male finches were obtained from an earlier egg injection study designed to investigate embryo mortality following *in ovo* dosing (Yu et al., 2016). In that study, an egg injection method was utilized to experimentally manipulate *in ovo* or embryonic exposure to MeHg at environmentally-relevant concentrations (Yu et al., 2016). The study found that *in ovo* exposure to MeHg significantly reduced hatching success (53% in the high-MeHg dose group vs 94% in vehicle controls). The main objectives of the present study were (a) to assess male courtship behavior and song quality, and (b) to conduct a neuroanatomical analysis of the necropsied brains of the experimental males at sexual maturity (>90 days of age). We hypothesized that *in ovo* exposure to MeHg would impair male song output (i.e. song duration, song phrase rate) and complexity (i.e. number of syllables, repertoire size) and alter the structure of the song control system in the brain.

2. Materials and methods

2.1. Animals and husbandry

The behavioral and neuroanatomical data presented in the present study were collected from the surviving offspring of an egg injection acute study designed to investigate embryo mortality and various physiological aspects of the relationship between *in ovo* MeHg exposure and developmental effects (Yu et al., 2016). To briefly recap, the study was conducted on a captive breeding colony of zebra finches maintained at the Simon Fraser University Animal Care Facility, Burnaby, British Columbia following previously published methods (Eng et al., 2012; Winter et al., 2013). Zebra finches were housed under controlled conditions (temperature 19–23 °C, humidity 35–55%; with a constant 14 h light:10 h dark photoperiod cycle). All birds were provided with mixed seed (panicum and white millet 1:2; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate by dry mass), water, grit, and cuttlefish bone ad libitum. In addition, a multivitamin supplement was provided in the drinking water once per week. Experiments and animal husbandry were conducted under a Simon Fraser University Animal Committee Permit (1070B-08) in accordance with guidelines of the Canadian Committee on Animal Care.

2.2. Experimental protocol

The experimental breeding protocol followed previously described methods (Yu et al., 2016), *in press*], experienced adult male and female birds were randomly paired and housed in individual breeding cages (51 × 39 × 43 cm) each with an external next box (14 × 14.5 × 20 cm). In addition to the *ad libitum* seed diet, breeding pairs were provided with 6 g of an egg-food supplement (20.3% protein, 6.6% lipid on a daily basis from pairing to the end of the chick-rearing stage) (30 days from day of hatch). Breeding pairs that did not produce eggs within 15 days of pairing were separated and re-paired. Following the egg injection methodology outlined by Winter et al. (Winter et al., 2013), doses of methylmercury (II) chloride (MeHgCl, PESTANAL, analytical standard from Sigma-Aldrich) dissolved in nanopure filtered water were injected into the albumen of newly laid eggs (Day 1) on the day the eggs showed signs of viability (onset of blood circulation, at 3 d incubation) using 10-mL Hamilton syringes (Gastight 1700 Series) and sterile 26-gauge beveled needles The injection hole was sealed with cyanoacrylate glue (Loctite Gel Control), and the egg was returned to the nest once the glue was dry. Each viable egg in a clutch was randomly assigned to either the water vehicle control (n = 34), low dose (0.2 μg Hg g^{-1} egg) (n = 36), or high dose (3.2 μg Hg g^{-1} egg) (n = 49) treatments. Following egg injections, dosed eggs were immediately returned to nests and continuously monitored. Surviving chicks were reared by parents until they reached 30 days of age, at which time they were separated and maintained in non-breeding juvenile groups. An adult male tutor was placed in each juvenile cage containing males, and birds were not visually or acoustically isolated from birds in adjacent cages. Once birds could be sexed by the appearance of bill color and sexually dimorphic plumage, they were separated into sex-specific groups and maintained until sexual maturity (90 days of age) at which time courtship trial were conducted.

2.3. Chemical mercury analysis

The vehicle control and MeHg dosing solutions were analyzed for total mercury (THg) at the National Wildlife Research Centre (NWRC) laboratories in Ottawa, Ontario, according to previously described methods (Yu et al., 2016).

2.4. Assessment of male courtship behavior and song analysis

Courtship trials were conducted on all *in ovo* MeHg-exposed males which survived to sexual maturity (90 days) in all treatment groups: water vehicle control (n = 11), low MeHg dose (n = 9), and high MeHg dose (n = 12). For each experimental male, two courtship trials were conducted on two separate days. All courtship trials were conducted between 1000 and 1300 h. Each courtship trial was initiated with the placement of an experienced,

clean wild-type female randomly selected from a pool of 30 females into a cage for 5 min to acclimate alone. A different female was paired with the experimental male for the two successive trials. The cage contained two perches, and grit, but no food or water. A microphone was positioned near the right portion of the cage facing in to the cage. After the 5 min acclimatization period for the female, an experimental male was placed in the cage, and the behaviors of both the male and female were recorded for 10 min by an observer blind to the treatment from behind a cardboard screen. Typical male zebra finch courtship displays, as described in Zann (Zann, 1996), were recorded and scored during each trial. These included mating invitation (Y or N), male singing (Y or N), bill wipe displays (# of wipes made against perch), head or tail twisting (left to right cycle), female followings (# of times male followed female), # of mounting attempts, # of successful mounts (defined as mounts with cloacal contact) and time (in sec) to initiate first mount attempt. The female's response to the male was also scored on a scale of 1–5 (1 = no response, 5 = solicitation of copulation). All male songs were digitally recorded for the duration of a trial using a Sennheiser ME62 microphone connected to a laptop computer running Syrinx-PC software Version 2.6 h. (J. Burt, Seattle, WA).

The audio song recordings and associated sonograms were assessed using Syrinx-PC for four variables of song output and complexity: (1) song duration (2) # of phrases per hour (song rate) (3) # of syllables per song phrase, and (4) # of unique syllables per phrase (repertoire). The structure of a zebra finch song consists of a series of phrases which are composed of repeated sequences of syllables or notes. Song recordings were analyzed based on methods described by Eng et al. (Eng et al., 2012) and supplementary information on zebra finch vocalizations described by Zann (Zann, 1996). Those experimental males that did not sing during either trial were not included in the song analysis. All courtship behaviors and song analyses were recorded blind to the treatment group of the males.

2.5. Tissue collection and neuroanatomy analysis

Following completion of the courtship trials, males were euthanized by decapitation and brains were extracted, dissected, and frozen immediately on dry ice then stored at −80 °C until neuroanatomical analysis. The telencephalon samples were sent to McGill University in Montreal, Quebec for analysis by M. Guigueno. These samples were sectioned into 25 μm sections along the coronal plane using a cryostat. Sections were immediately mounted onto microscope slides (VWR Histobond), fixed in 4% paraformaldehyde for 10 min, Nissl-stained with thionin, serially dehydrated in progressively higher ethanol concentrated solutions, cleared in solvent (NeoClear, EMD chemicals), and coverslipped with Permount (Fisher). Every second section throughout Area X, HVC, and RA and every tenth section throughout telencephalon were collected and used in the volume analyses described below.

Images of the telencephalon were captured using a high-resolution (4800 dpi) flatbed scanner (Epson Perfection 4490 Photo). In addition to the telencephalon, these images were also used to delineate the perimeter of Area X and HVC. Microphotographs of RA were taken with a QICAM Fast 1394 digital camera (QImaging) mounted on a Nikon SMZ1500 stereomicroscope. The perimeter of every tenth telencephalon section and every second section in Area X, HVC, and RA was traced in ImageJ (NIH) to measure cross-sectional surface areas. The same observer, who was blind to the treatment groups, completed all the tracings. Volumes of telencephalon, Area X, HVC, and RA were then estimated using the formula for a frustum. The frusta volumes were summed between each tissue section (250 μm for telencephalon and 50 μm for Area X, HVC, and RA) to estimate the total volume of each region of interest. If tissue was damaged or poorly stained, the next intact and well stained section was used and the sampling interval was adjusted accordingly.

2.6. Statistical analysis

All statistical analyses were conducted using the SAS statistical computing system, Version 9.4 (SAS Institute 2003). Data were found to meet assumptions of normality and homogeneity of variance following the Shapiro-Wilk's test, and by reviewing q-q plots and residual plots. The effect of MeHg dose on continuous variables for male song analysis and courtship behavior were assessed using mixed effects models (proc MIXED), with nest as a random factor. *Post-hoc* tests for differences between means were adjusted for multiple comparisons using the Tukey-Kramer method. Repeatability of male courtship behavior across the two mating trials was tested using nested ANOVA. Proportion measures of experimental male courtship behavior (i.e., invite, sang) were modeled with generalized linear mixed models (proc GLIMMIX) using a binomial distribution and a logit link, and nest as a random factor. For the neuroanatomical analyses, volume measures for Area X, HVC, RA, and telencephalon were modeled with mixed effects models (proc MIXED) with nest as a random factor and Tukey-Kramer post-hoc tests were completed to analyze differences in telencephalon volumes between treatment groups. Correlation between brain and body mass was analyzed. Telencephalon was included as a covariate in the analyses for Area X, HVC, and RA to control for changes in overall telencephalon size (i.e., allometry). All values are presented as mean ± standard error of the mean (SEM), and statistical significance for all tests was set at $p < 0.05$. All values are presented as mean ± standard error of the mean (SEM), and statistical significance for all tests was set at $p < 0.05$.

3. Results

3.1. Male courtship behavior and song quality

Of the multiple courtship displays assessed, whether the experimental male invited the female, whether the male sang, the number of bill wipes, the number of times the male followed the female, the number of mounts, and the number of successful mounts were repeatable within individual males across mating trials. Individual explained 31% of the variation in male invites ($F_{31,32} = 1.90$, $p = 0.038$), 52% of variation in whether the male sang ($F_{31,32} = 3.13$, $p = 0.0009$), 49% of variation in number of bill wipes ($F_{31,32} = 2.93$, $p = 0.0017$), 33% of variation in female followings ($F_{31,32} = 1.98$, $p = 0.029$), 34% of variation in number of mounts ($F_{31,32} = 2.04$, $p = 0.025$), and 35% of variation in number of successful mounts ($F_{31,32} = 2.07$, $p = 0.022$). However, *in ovo* treatment with MeHg did not affect male invitation to the female ($F_{2,29} = 0.25$, $p = 0.78$), or whether the male sang ($F_{2,29} = 0.57$, $p = 0.57$), number of bill wipes ($F_{2,29} = 1.20$, $p = 0.32$), number of female followings ($F_{2,29} = 0.48$, $p = 0.62$), number of mounts ($F_{2,29} = 0.20$, $p = 0.82$), or number of successful mounts ($F_{2,29} = 0.38$, $p = 0.68$). Other displays such as number of head-tail twists and first mount attempt were not repeatable and not included in subsequent analysis. There was no significant difference in female responses to the experimental males among the dose groups ($F_{2,29} = 0.40$, $p = 0.68$).

From the male song analysis, the song characteristics that were repeatable included: average song duration ($F_{31,32} = 1.96$, $p = 0.031$, 32%), number of syllables sang ($F_{31,32} = 2.53$, $p = 0.0054$, 43%), and repertoire size ($F_{31,32} = 2.52$, $p = 0.0055$, 43%) (Fig. 1). However, there was no effect of MeHg treatment on any song traits ($p > 0.80$ in all cases). Song phrase rate was not repeatable ($F_{31,32} = 1.51$, $p = 0.12$, 20%).

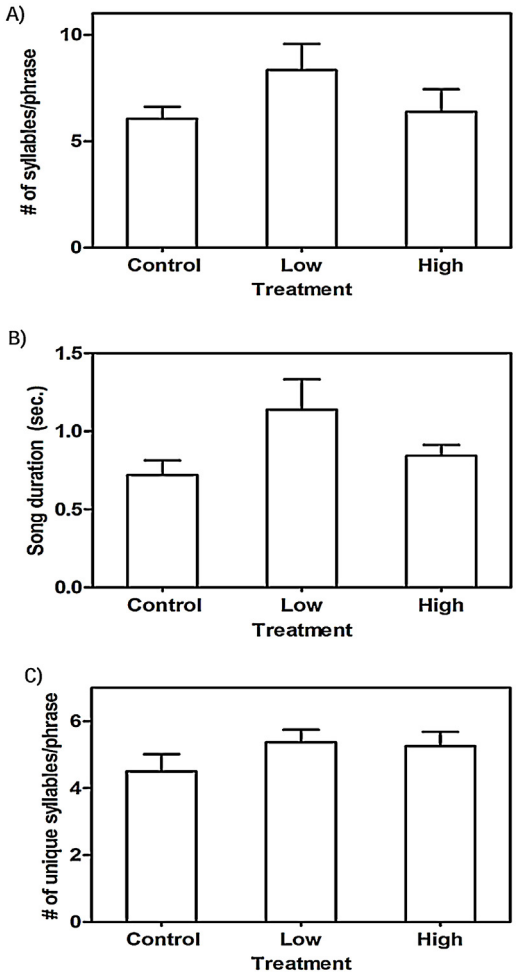

Fig. 1. Effect of *in ovo* MeHg exposure on courtship song quality in reproductively mature experimental males. (A) There was no effect of treatment on the number of syllables per song phrase ($p = 0.89$). (B) There was no effect of treatment on the average song duration ($p = 0.95$). (C) There was no effect of treatment on song repertoire ($p = 0.92$). Bars represent the mean ± standard error.

3.2. Neuroanatomical analyses

There was no effect of MeHg dose on total brain mass ($F_{2,8} = 0.12$, $p = 0.89$) or on volumes of Area X, HVC or RA song nuclei ($p > 0.23$ in all cases). However, there was an effect of MeHg treatment on telencephalon volume ($F_{2,11} = 4.36$, $p = 0.040$) (Fig. 2), with the high-dosed males having the largest telencephalon volumes (telencephalon volume: control < low dose < high dose).

4. Discussion

In the present study, we investigated the long-term behavioral and neuroanatomical effects of early *in ovo* developmental exposure to MeHg in zebra finches. The high dose (3.2 μg Hg g^{-1} egg ww) is typical of egg concentrations at highly contaminated sites, and was previously established as acutely toxic to embryos (53% hatching success in high dose vs 94% hatching success in vehicle controls) (Yu et al., 2016). Based on reported relationships between Hg in zebra finch eggs and adult blood (egg Hg = 0.267* blood Hg on a wet weight basis with 82% moisture in eggs (Ou et al., 2015), we would estimate that eggs with concentrations similar to the low and high doses would be found in populations where adult blood Hg concentrations were ~0.75 μg g^{-1} ww and ~12.0 μg g^{-1} ww, respectively. For comparison, in a survey of 2156 adult passerines in Hg contaminated sites, the average blood Hg was 0.975 ± 0.024SE μg g^{-1} ww, and the highest recorded blood concentration was 14.6 μg g^{-1} ww (Jackson et al., 2015).

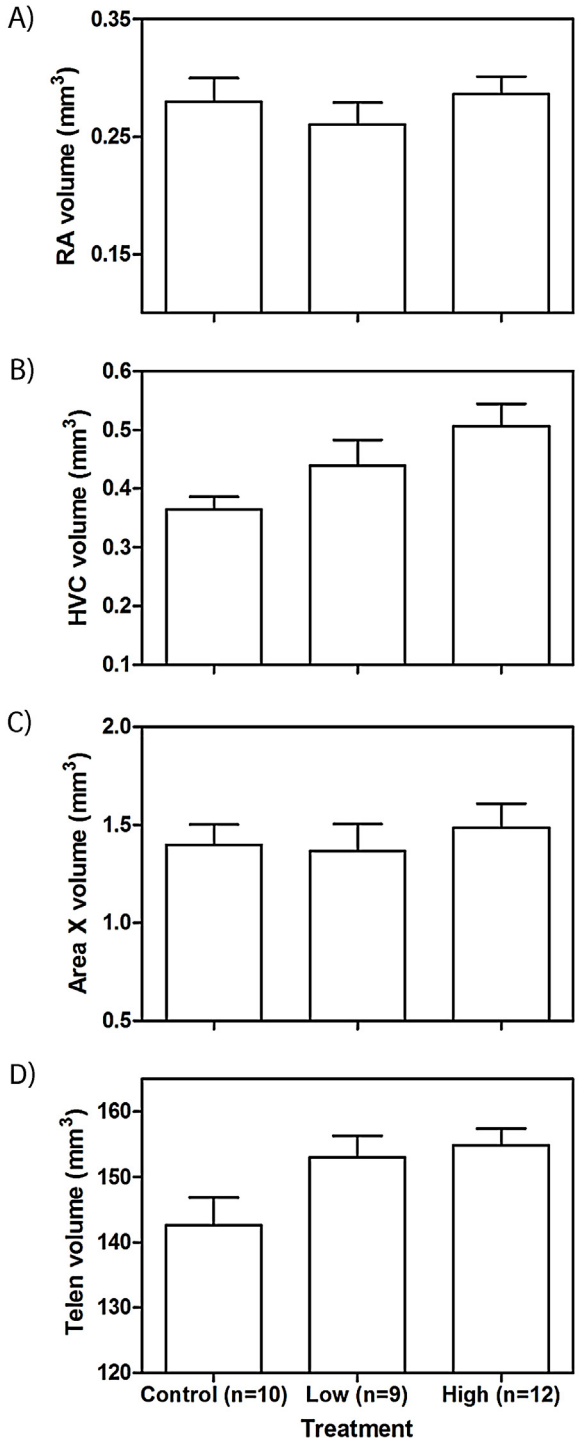

Fig. 2. Effect of *in ovo* MeHg exposure on volumes (mm^3) of select brain regions in experimental males. There was no effect of MeHg treatment on volumes of (A) RA, (B) HVC or (C) Area X song control nuclei ($p > 0.23$ in all cases). (D) There was an effect of MeHg treatment on telencephalon volume ($F_{2,11} = 4.36$, $p = 0.040$). Box plot whiskers represent the minimum and maximum values, central horizontal line represents the median, and box outline represents first and third quartiles.

We found no adverse effect of *in ovo* MeHg treatment on courtship behavior, song quality, or size of different song control nuclei (Area X, HVC, RA) in the experimental males. However,

increased telencephalon volume was observed in the high MeHg dose group which would be consistent with a chronic MeHg-induced inflammatory response. In the mammalian brain, the inflammatory response is triggered by the activation of microglia cells, heterogenous monocyte-derived cells which play a crucial role in brain injury. In the developing central nervous system, these cells will react to neurotoxic insults via neuronal proliferation and differentiation processes (Harry and Kraft, 2012). In mice, embryonic exposure to MeHg has been reported to severely affect proliferating neuroepithelial germinal cells in developing telencephalic vesicles and result in edema and spongiosis (Choi, 1989). Similarly, brain tissue of rat neonates maternally exposed to MeHg exhibited swollen endothelial cells (Chang and Reuhl, 1977). Some avian studies have reported Hg concentrations in the brain causing vacuolar degeneration and inflammation due to oxidative stress (Hoffman et al., 2009), (Hoffman et al., 2011). In our study, the observed causal linkage between MeHg dose and the increase in telencephalon volume may suggest that MeHg induced a neuro-inflammatory response. However, further investigation of this observed effect at the cellular level, specifically focusing on cell types, is warranted.

We hypothesized that *in ovo* exposure to ecologically relevant concentrations of MeHg would impact the quality of male song and courtship behavior; however, our study did not find an effect of *in ovo* MeHg exposure on the song output, song complexity, or behavior of the sexually mature experimental males, even at high concentrations that were acutely toxic to embryos. We selected male song output and complexity as endpoints based on findings from recent field and lab studies in songbirds that reported MeHg exposure was associated with altered male song characteristics and complexity (Hallinger et al., 2010; McKay and Maher, 2012; Varian-Ramos et al., 2011). In a field study conducted on Nelson's sparrows (*Ammodramus nelson*) inhabiting a Hg-contaminated area, adult males with elevated blood Hg (average 2.9 μg/g ww, range 1.25–6.04 $\mu g\,g^{-1}$ ww) produced faster songs than males inhabiting references sites (blood Hg $< 0.9\ \mu g\,g^{-1}$ ww), which the authors suggested was related to Hg exposure at natal sites and accumulation of Hg in developing brains experienced as juveniles that negatively affected the learning and subsequent production of song (McKay and Maher, 2012). In another field study of 4 oscine species, males of Carolina wrens (*Thryothorus ludovicianus*), house wrens (*Troglodytes aedon*), and song sparrows (*Melospiza melodia*) exposed to Hg (average approximate blood Hg = 3.66 $\mu g\,g^{-1}$ ww) on natal sites sang at lower frequencies and produced a lower diversity of notes than birds from reference sites (Hallinger et al., 2010). It should be noted that male songs in a fourth species, the Eastern Phoebe (*Sayornis phoebe*), did not differ between the Hg-contaminated and reference sites. However, unlike oscine songbirds including the zebra finch, wren, and song sparrow, which learn their songs from conspecifics, suboscines like the Eastern phoebe have innate songs and generally do not display vocal learning. The results of the multi-species study may thus suggest that Hg exposure may alter song through effects on learning (Hallinger et al., 2010). While the two correlational birdsong field studies suggest that song output and complexity is sensitive to MeHg exposure, the lack of effects in our *in ovo* study suggests that timing of exposure may influence the outcome of adverse effects to song. In zebra finches, the regions of the song control system show protracted development during the mid to late embryonic stage and neurogenesis continues past the *in ovo* stage into the early post hatch age and adulthood (Zann, 1996; Kirn, 2010). Findings from avian studies conducted on other environmental contaminants lend support to the suggestion that timing of exposure (i.e., age) can affect the degree of consequent neurological damage and alterations to the song control system. For organohalogen contaminants, *in ovo* exposure appears to be important. For example, in zebra finches, exposure to 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) over the nestling period had no effect on the male song-control nuclei or on song quality (Eng et al., 2012), while *in ovo* exposure to BDE-99 via egg injections reduced the volumes of key song control nuclei (Eng et al., 2014). Two other studies of organohalogens that showed significant effects on song control nuclei volumes also included *in ovo* exposure. A correlational study conducted on American robins (*Turdus migratorius*) reported smaller song nuclei regions in adult males naturally exposed to DDT both *in ovo* and during early post-hatching development (Iwaniuk et al., 2006), and Hoogesteijn et al. (Hoogesteijn et al., 2008), reported smaller song control nuclei RA in zebra finches exposed *in ovo* via maternal transfer to PCBs. Further, lab studies on other animal models have reported that factors such as route of exposure and dosing regimen result in significant differences in the distribution of Hg within brain tissue as well as brain Hg accumulation and concentrations (Bellum et al., 2007). It is possible that ongoing post-hatch exposure to MeHg may be required to see effects on the song-control system, particularly considering that chicks rapidly eliminate MeHg during early development and growth (Yu et al., 2016; Ackerman et al., 2011). In our study, the blood Hg of nestlings was significantly elevated in the high dose group relative to the low dose and controls, but between 15 and 30 days of age, nestlings lost 71% and 57% of their total blood Hg in the high and low dose groups, respectively (Yu et al., 2016). While the highest dose in our study was acutely toxic *in ovo* and typical of concentrations in highly contaminated sites (Yu et al., 2016), perhaps the accumulation of MeHg in the developing brains of the experimental males following *in ovo* injections was insufficient to cause post-hatch adverse effects to the developing song control system without continued post-hatch exposure. Additionally, while our study focused on assessing song output and complexity, it is possible that learning local song structure (tutor imitation) could also influence breeding outcomes (Nowicki et al., 2002), and assessment of MeHg effects on song learning could serve as a potential sensitive indicator of effects.

No detectable effects of *in ovo* MeHg exposure on courtship behavior in the experimental males were observed in this study. Such reproductive and sexually selective behaviors are mainly influenced by the endocrine system and the homeostasis of steroidal hormones and their interactions with other systems (Blocker and Ophir, 2013). In White ibises (*Eudocimus albus*), for instance, MeHg was shown to interfere with the proper functioning of sex steroids (Jayasena et al., 2011) and resulted in significantly reduced male-female courtship behavior and reduced likelihood of females to approach males (Frederick and Jayasena, 2011). However, these effects were the result of chronic exposure (3 years) to dietary MeHg levels, which may suggest that altered mating behavior as a result of endocrine disruption is more influenced by chronic exposure to dietary MeHg than *in ovo* exposure alone.

5. Conclusions

In summary, we found no adverse long term effects of *in ovo* MeHg treatment on courtship behavior or singing in male zebra finches, despite high *in ovo* dosing levels that killed a substantial proportion of embryos (Yu et al., 2016). Singing behavior in songbirds is considered an essential component of pairing and reproduction (McGregor et al., 1981; Baker et al., 1986), which is an obvious factor to population growth and survival. It is important to note that often laboratory and field studies proceed in isolation, making interpretations or comparison of data difficult. Because the correlational results from field studies have the potential to be influenced by other co-varying factors, laboratory studies are useful in assessing the direct causal linkage between toxicant

exposure and an adverse effect. Hence, in our study we attempted to investigate the direct link between MeHg exposure and altered male song suggested in recent field studies (Hallinger et al., 2010; McKay and Maher, 2012). Our study did not detect an effect of *in ovo* exposure to environmentally relevant concentrations of MeHg on the quality of male song, which would suggest that exposure during the embryonic stage alone may not be enough to noticeably affect the development of the song control system. Future research on assessing the effects of dietary exposure to MeHg or the combination of *in ovo* exposure and dietary exposure to song production as well as song learning would be useful in further understanding the possible effects of MeHg on male song behavior, and the importance of timing of exposure. As well, because our study was limited to investigating male song quality only, future studies assessing *in ovo* MeHg exposure on song learning in juveniles may be valuable. Interestingly, telencephalon volume appeared to increase with MeHg dose, which may suggest a MeHg-induced neuro-inflammatory response. Further research of the cellular components could potentially elucidate the mechanisms associated with this response.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

Funding was provided by the Canadian Atmospheric Regulatory Agenda program of Environment Canada and National Science and Engineering Research Council Discovery Grants to J Elliott and T Williams. The authors thank D Bond, G Savard, and the laboratory staff at the National Wildlife Research Centre in Ottawa, Ontario for completing the analysis of the dosing solutions and mercury content in egg samples.

References

Ackerman, J.T., Eagles-Smith, C.A., Herzog, M.P., 2011. Bird mercury concentrations change rapidly as chicks age: toxicological risk is highest at hatching and fledging. Environ. Sci. Technol. 45, 5418–5425.
Baker, M.C., Bjerke, T.K., Lamph, H., Espmark, Y., 1986. Sexual response of female great tits to variation in size of males' song repretoires. Am. Nat. 128, 491–498.
Basu, N., Goodrich, J.M., Head, J., 2014. Ecogenetics of mercury: from genetic polymorphisms and epigenetics to risk assessment and decision-making. Environ. Toxicol. Chem. 33 (6), 1248–1258.
Bellum, S., Thuett, K.A., Taylor, R.J., Abbott, L.C., 2007. Assessment of mercury concentrations in mouse brain using different routes of administration and different tissue preparations. Toxicol. Mech. Methods 17, 165–173.
Bergeron, C.M., Hopkins, W.A., Todd, B.D., Hepner, M.J., Unrine, J.M., 2011. Interactive effects of maternal and dietary mercury exposure have latent and lethal consequences for amphibian larvae. Environ. Sci. Technol. 45 (8), 3781–3787.
Beyrouty, P., Stamler, C.J., Liu, J.N., Loua, K.M., Kubow, S., Chan, H.M., 2006. Effects of prenatal methylmercury exposure on brain monoamine oxidase activity and neurobehavior of rats. Neurotoxicol. Teratol. 28 (2), 251–259.
Blocker, T.D., Ophir, A.G., 2013. Cryptic confounding compounds: a brief consideration of the influences of anthropogenic contaminants on courtship and mating behavior. Acta Ethol. 16 (2), 105–125.
Bornhausen, M., Müsch, H.R., Greim, H., 1980. Operant behavior performance changes in rats after prenatal methylmercury exposure. Toxicol. Appl. Pharmacol. 56 (3), 305–310.
Bouton, S.N., Frederick, P.C., Spalding, M.G., McGill, H., 1999. Effects of chronic, low concentrations of dietary methylmercury on the behavior of juvenile Great Egrets. Environ. Toxicol. Chem. 18, 1934–1939.
Brenowitz, E.A., Margoliash, D., Nordeen, K.W., 1997. An introduction to birdsong and the avian song system. J. Neurobiol. 33 (5), 495–500.
Burbacher, T.M., Rodier, P.M., Weiss, B., 1990. Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. Neurotoxicol. Teratol. 12 (3), 191–202.
Burbacher, T.M., Grant, K.S., Mayfield, D.B., Gilbert, S.G., Rice, D.C., 2005. Prenatal methylmercury exposure affects spatial vision in adult monkeys. Toxicol. Appl. Pharmacol. 208 (1), 21–28.
Carvalho, M.C., Nazari, E.M., Farina, M., Muller, Y.M.R., 2008. Behavioral, morphological, and biochemical changes after *in ovo* exposure to methylmercury in chicks. Toxicol. Sci. 106 (1), 180–185.
Ceccatelli, S., Bose, R., Edoff, K., Onishchenko, N., Spulber, S., 2013. Long-lasting neurotoxic effects of exposure to methylmercury during development. J. Intern. Med. 273 (5), 490–497.
Chang, L.W., Reuhl, K.R., 1977. Ultrastructural study of the latent effects of methyl mercury on the nervous system after prenatal exposure. Environ. Res. 13, 171–185.
Choi, B.H., 1989. The effects of methylmercury on the developing brain. Prog. Neurobiol. 32, 447–470.
Eng, M.L., Elliott, J.E., MacDougall-Shackleton, S.A., Letcher, R.J., 2012. Early exposure to 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) affects mating behaviour of zebra finches. Toxicol. Sci. 127 (1), 269–276.
Eng, M.L., Winter, V., MacDougall-Shackleton, S.A., Williams, T.D., Elliott, J.E., 2014. 239 *in ovo* exposure to BDE-99 affects the song control system of a model songbird species, the zebra finch. SETAC Europe 24th Annual Meeting, Basel, Switzerland.
Frederick, P.C., Jayasena, N., 2011. Altered pairing behavior and reproductive success in white ibises exposed to environmentally relevant concentrations of methylmercury. Proc. R. Soc. 278, 1851–1857.
Hallinger, K.K., Zabransky, D.J., Kazmer, K., Cristol, D.A., 2010. Birdsong differs between mercury-polluted and reference sites. Auk 127 (1), 156–161.
Harry, G.J., Kraft, A.D., 2012. Microglia in the developing brain: A potential target with lifetime effects. Neurotoxicology 33, 191–206.
Heinz, G.H., Hoffman, D.J., 2004. Mercury accumulation and loss in mallard eggs. Environ. Toxicol. Chem. 23 (1), 222–224.
Heinz, G.H., Locke, L.N., 1976. Brain lesions in mallard ducklings from parents fed methylmercury. Avian Dis. 20 (1), 9–17.
Heinz, G.H., 1975. Effects of methylmercury on approach and avoidance behavior of mallard ducklings. Bull. Environ. Contam. Toxicol. 13 (1975), 554–564.
Heinz, G.H., 1979. Methylmercury: reproductive and behavioral effects on three generations of mallard ducks. J. Wildl. Manage. 43, 394–401.
Hoffman, D.J., Henny, C.J., Hill, E.F., Grove, R.A., et al., 2009. Mercury and drought along the lower Carson River, Nevada: III. Effects on blood and organ biochemistry and histopathology of Snowy Egrets and Black-crowned Night Herons on Lahontan Reservoir. J. Toxicol. Environ. Health Part A 72, 1223–1241.
Hoffman, D.J., Eagles-Smith, C.A., Ackerman, J.T., Adelsbach, T.L., Stebbins, K.R., 2011. Oxidative stress response of Forster's terns (*Sterna forsteri*) and Caspian terns (*Hydropogne caspia*) to mercury and selenium bioaccumulation in liver, kidney, and brain. Environ. Toxicol. Chem. 30 (4), 920–929.
Hoogesteijn, A.L., Kollias, G.V., Quimby, F.W., De Caprio, A.P., et al., 2008. Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by aroclor 1248. Environ. Toxicol. Chem. 27 (10), 2071–2075.
Iwaniuk, A.N., Koperski, D.T., Cheng, K.M., Elliott, J.E., Smith, L.K., Wilson, L.K., Wylie, D.R.W., 2006. The effects of environmental exposure to DDT on the brain of a songbird: changes in structures associated with mating and song. Behav. Brain Res. 173 (1), 1–10.
Jackson, A.K., Evers, D.C., Adams, E.M., Cristol, D.A., Eagles-Smith, C., Edmonds, S.T., Gray, C.E., Hoskins, B., Lane, O.P., Sauer, A., Tear, T., 2015. Songbirds as sentinels of mercury in terrestrial habitats of eastern North America. Ecotoxicology 24, 453–467.
Jayasena, N., Frederick, P.C., Larkin, I.L.V., 2011. Endocrine disruption in white ibises (*Eudocimus albus*) caused by exposure to environmentally relevant levels of methylmercury. Aquat. Toxicol. 105, 321–327.
Kambamanoli-Dimou, A., Kamarianos, A., Kilikidis, S., 1991. Transfer of methylmercury to hens' eggs after oral administration. Bull. Environ. Contam. Toxicol. 46, 128–133.
Kenow, K.P., Hines, R.K., Meyer, M.W., Suarez, S.A., Gray, B.R., 2010. Effects of methylmercury exposure on the behavior of captive-reared common loon (*Gavia immer*) chicks. Ecotoxicology 19, 933–944.
Kirn, J.R., 2010. The relationship of neurogenesis and growth of brain regions to song learning. Brain Lang. 115 (1), 29–44.
McGregor, P.K., Krebs, J.R., Perrins, C.M., 1981. Song repertoires and lifetime reproductive success in the great tit (*Parus major*). Am. Nat. 118, 149–159.
McKay, J.L., Maher, C.R., 2012. Relationship between blood mercury levels and components of male song in Nelson's sparrows (*Ammodramus nelsoni*). Ecotoxicology 21 (8), 2391–2397.
Monnet-Tschudi, F., Zurich, M.G., Boschat, C., Corbaz, A., Honegger, P., 2006. Involvement of environmental mercury and lead in the etiology of neurodegenerative diseases. Rev. Environ. Health 21 (2), 105–117.
Nowicki, S., Peters, S., Podos, J., 1998. Song learning, early nutrition and sexual selection in song birds. Am. Zool. 38, 19–190.
Nowicki, S., Searcy, W.A., Peters, S., 2002. Brain development, song learning and mate choice in birds: a review and experimental test of the nutritional stress hypothesis. J. Com. Physiol. A 188, 1003–1014.
Ou, L., Varian-Ramos, C.W., Cristol, D.A., 2015. Effect of laying sequence on egg mercury in captive zebra finches: an interpretation considering individual variation. Environ. Toxicol. Chem. 34, 1787–1792.
Reiner, A., Perkel, D.J., Bruce, L.L., Butler, A.B., et al., 2004. Revised nomenclature for avian telencephalon and some related brainstem nuclei. J. Comp. Neurol. 473, 377–414.
Rimmer, C.C., McFarland, K.P., Evers, D.C., Miller, E.K., Aubry, Y., Busby, D., Taylor, R.J., 2005. Mercury concentrations in Bicknell's thrush and other insectivorous passerines in Montane forests of northeastern North America. Ecotoxicology 14, 223–240.
Spalding, M.G., Frederick, P.C., McGill, H.C., Bouton, S.N., Richey, L.J., et al., 2000. Histologic, neurologic, and immunologic effects of methylmercury in captive great egrets. J. Wildl. Dis. 36, 423–435.

Varian-Ramos, C.W., Sheridan, E.R., Cristol, D.A., Swaddle, J.P., 2011. Methylmercury exposure alters pitch and complexity of learned songs in the zebra finch. Presented at Ecotoxicology of Mercury: The 10th International Conference on Mercury as a Global Pollutant, Halifax, Nova Scotia, 2011.
Weis, J., 2012. Delayed behavioral effects of early life toxicant exposures in aquatic biota. Toxics 3, 165–187.
Weiss, B., Stern, S., Cox, C., Balys, M., 2005. Perinatal and lifetime exposure to methylmercury in the mouse: behavioral effects. Neurotoxicology 26, 675–690.
Wiener, J.G., Krabbenhoft, D.P., Heinz, G.H., Scheuhammer, A.M., 2003. Ecotoxicology of mercury, In: Hoffman, D.J., Rattner, B.A., Burton, G.A., Cairns, J. (Eds.), Handbook of Ecotoxicology. 2nd ed. Lewis Publishers, Boca Raton, FL, USA, pp. 409–463.
Winter, V., Elliott, J.E., Letcher, R.J., Williams, T.D., 2013. Validation of an egg-injection method for embryotoxicity studies in a small model songbird, the zebra finch (*Taeniopygia guttata*). Chemosphere 90, 125–131.
Wolfe, M.F., Schwarzbach, S., Sulaiman, R.A., 1998. Effects of mercury on wildlife: a comprehensive review. Environ. Toxicol. Chem. 17, 146–160.
Yu, M.S., Eng, M.L., Williams, T.D., Basu, N., Elliott, J.E., 2016. Acute embryotoxic effects but no long term reproductive effects of *in ovo* methylmercury exposure in zebra finches (Taeniopygia guttatta). Environ. Toxicol. Chem. 35 (6), 1534–1540.
Zann, R.A., 1996. The Zebra Finch: A Synthesis of Field and Laboratory Studies. Oxford University Press, New York, NY, USA.