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Effects of Maternally-Transferred Methylmercury on Stress Physiology in Northern Water Snake (*Nerodia sipedon*) Neonates

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Abstract Biomagnification of methylmercury in aquatic systems can cause elevated tissue mercury (Hg) and physiological stress in top predators. Mercury is known to affect stress hormone levels in mammals, birds and fish. In this study, the effects of maternally-transferred methylmercury on the stress physiology of Northern Water Snake (*Nerodia sipedon*) neonates were tested. Gravid females were dosed via force-fed capsules during late gestation with 0, 0.01, or 10 µg methylmercury per gram of body mass. Plasma corticosterone levels and leukocyte differentials were analyzed in baseline and confinement-stressed neonates from all dose levels. Neither Hg nor confinement stress had a significant effect on leukocyte differentials nor was Hg related to corticosterone levels. However, stress group neonates showed lower heterophil/lymphocyte ratios and this study was the first to show that neonate *N. sipedon* can upregulate CORT in response to stress. These results indicate that *N. sipedon* may be somewhat tolerant to Hg contamination.

Keywords Methylmercury · Corticosterone · Maternal transfer · Leukocytes · Neonate · Reptile

Mercury (Hg) is a common contaminant that can be found in various forms in the environment (Selin 2009). Methylmercury (MeHg) is particularly toxic to wildlife, and is most common in aquatic systems where inorganic mercury is converted to MeHg by anaerobic sulfur-reducing bacteria (Wolfe et al. 1998; Schaefer et al. 2011). MeHg is easily absorbed by lower trophic level organisms, and can be transferred up the food chain leading to biomagnification in aquatic systems (Chumchal et al. 2011). The toxic effects of MeHg are varied, but neurological damage, deficiencies in motor and sensory systems, and effects on reproduction have been reported (Wolfe et al. 1998).

Maternal transfer of Hg has been demonstrated in many vertebrate taxa, including fish, amphibians, reptiles and mammals (Hammerschmidtt and Sandheinrich 2005; Bergeron et al. 2010; Chin et al. 2013a, b, Kajiwara et al. 1996). Mammals are able to transfer MeHg directly from mother to offspring across the placenta (Kajiwara et al. 1996) and although most squamate reptiles are oviparous, some exhibit viviparity and possess a primitive chorioallantoic placenta (Conway and Fleming 1960). These viviparous reptiles still undergo yolk formation (vitellogenesis) as a nutritional source for prenatal offspring, but do not produce a definitive egg. The majority of nutrients for development are in the yolk, but it has been shown that extra-vitellogenic ions and amino acids in *Nerodia sp.* (Stewart and Castillo 1984; Van Dyke and Beaupre 2012) can be transferred to offspring during gestation, possibly via the placenta.

Studies on maternal transfer of contaminants in squamate reptiles have largely been limited to oviparous species (e.g. Hopkins et al. 2004). However, maternal transfer of Hg has been demonstrated in the viviparous snake *Nerodia sipedon* (Chin et al. 2013a, b) with subsequent negative effects on strike efficiency in neonates (Chin et al. 2013b).

Levels of glucocorticoid hormones, such as corticosterone (CORT), can be monitored as indicators of acute stress in individuals because they are quickly elevated following a stressor, such as capture and/or confinement

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(Bailey et al. 2009). This has been established in adults of a variety of species, but there can be key differences in physiology between adults and juveniles of the same species (e.g., heat tolerance, Winne and Keck 2005). Mercury has been shown to affect the glucocorticoid stress response in mammals and birds in various ways, including reducing the glucocorticoid response to stress (Burton and Meikle 1980; Thaxton et al. 1982) and researchers have documented the effects of metals on the stress response in fish (e.g. Norris et al. 1999) and possibly amphibians (Ward and Mendonça 2006). Elevation of glucocorticoids in vertebrates has also been shown to initiate changes in total and differential leukocyte numbers in the blood stream (Davis et al. 2008). In reptiles, the most commonly observed of these changes is an increase in the heterophil/lymphocyte (H/L) ratio, as heterophils are released into the blood stream, and lymphocytes migrate from the bloodstream into tissues, organs, and lymph nodes. The result of this is a net decrease in leukocytes in the blood stream, another observation commonly used to infer an elevation of stress hormones (Davis et al. 2008).

Northern Water Snakes (*N. sipedon*) are viviparous semi-aquatic natricine snakes with a wide range, inhabiting most of the eastern United States. They are upper-trophic level consumers, with a generalist diet comprised mostly of fish (Gibbons and Dorcas 2004), making their potential for exposure to MeHg high at contaminated sites. Breeding in most populations occurs in the spring (late April-early/mid May), and birth occurs in late August-early/mid-September, with litters ranging from 10 to 30 neonates averaging approximately 5 g each (Gibbons and Dorcas 2004). The purpose of this study was to examine maternal transfer of MeHg and its effects on certain physiological endpoints of neonate *N. sipedon*. The specific objectives were to: (1) orally dose adult female post-vitellogenic *N. sipedon* with MeHg via force-fed capsules in a laboratory setting; (2) determine the fate of ingested Hg in adults and maternally-transferred Hg in neonates; (3) determine if CORT and leukocyte differences exist in neonates exposed to different acute confinement stress conditions and Hg concentrations.

Methods and Materials

Twenty-four adult female *N. sipedon* were hand collected 19–23 June 2010 from northern Ohio (Ottowa County) along the shoreline of Lake Erie (41°28.503′N, 82°49.587′W). Sex was verified using sexing probes and since it was often not possible to feel developing neonates by palpation at the time, large females were assumed to be gravid (King 1986). The individuals were transported to Middle Tennessee State University (MTSU) where they were measured for mass and snout-vent length, then housed in individual plastic containers (~61:91:20 cm, L:W:H) with dry newspaper bedding, a water bowl, and a hide box. Snakes were maintained at approximately 28.5°C with a 14:10 h light:dark cycle and each female was fed live fish (*Notropis sp.*) totaling approximately 15 % of her body mass once per week.

Seventeen of the 24 snakes originally collected were ultimately determined to be gravid by palpation. After a 1 month acclimation period, individuals were randomly assigned to treatment groups (control, low and high dose) and methylmercury(II) chloride (Sigma-Aldrich) stock solutions were prepared at 25 (for the high dose) and 0.25 (for the low dose) mg/mL in corn oil. Appropriate amounts of stock solution and corn oil were added to size 0 cellulose capsules such that each capsule contained 0.25 mL of one of the following: corn oil only (control, $n = 6$), 0.01 (low, $n = 6$) or 10 (high, $n = 5$) μg MeHg/g of snake dissolved in corn oil. The capsules were then administered to the snakes by force-feeding and the snakes were observed for a period of several hours to ensure capsules were not regurgitated. These mercury doses correspond to a snake eating 10 % of its body weight in fish that contain 0.1 and 100 μg MeHg/g of fish respectively. The 0.1 μg/g dose represents a typical fish from a MeHg-contaminated site (Hogan et al. 2007) and the 100 μg/g dose represents a dose not typically found in nature. Snakes were dosed once per week for 2 weeks (total of two doses per snake). Following dosing, snakes were maintained in similar conditions to those prior to dosing, during which time fecal and shed samples were collected as possible, stored in plastic centrifuge tubes, and frozen at −20°C until analysis. Within 24 h after giving birth, adult snakes were euthanized by CO_2 asphyxiation, then liver and muscle tissues were removed, stored in plastic centrifuge tubes, and frozen at −20°C until mercury analysis was performed.

Within 24 h after each female gave birth, individual neonate snakes were weighed, housed similarly to the adults, and 10 neonates from each litter were randomly assigned into treatment groups for CORT and leukocyte analysis. Two treatment groups were created: a baseline treatment group ($n = 5$ per litter) and a stress treatment group ($n = 5$ per litter). Neonates in the baseline treatment group had blood samples collected from the neck in less than 3 min using heparinized capillary tubes (1.1 mm diameter; 75 mm length) immediately following euthanasia by decapitation and pithing, thus avoiding an elevated baseline CORT concentration due to the acute stress of handling (Romero and Reed 2005). Neonates in the stress treatment group were exposed to confinement stress in a bag for 1 h and were immediately blood sampled as above. Confinement is a standard stressor used in reptile research known to elicit an increase in plasma CORT (Moore et al. 2000; Bailey et al. 2009). A drop of blood from each

neonate was used to make a blood smear for leukocyte analysis (see below) and the remaining blood was then centrifuged at 12,000 rpm for 15 min and plasma was stored at −20°C until CORT analysis was performed. After euthanasia, samples of liver tissue and tail clip samples (0.2–0.5 g) were taken from each individual neonate and stored at −20°C until mercury analysis was performed.

Analysis of total mercury in liver, muscle, sheds, and fecal samples of adult snakes and liver and tail clip samples in neonates were performed following digestions in 1:1 (v:v) sulfuric acid:nitric acid and 30 % H_2O_2. All digested samples were then filtered through glass fiber filters and analyzed by cold vapor atomic absorption spectroscopy (Adair and Cobb 1999).

Analysis for CORT was performed on neonate blood plasma via an enzyme-linked immunosorbent assay (Enzo Life Sciences, ADI-901-097), which has been validated for use with *N. sipedon* (Sykes and Klukowski 2009). Plasma was diluted 30-fold in the assay buffer and plated in duplicate.

Blood smears for leukocyte analysis were made on 7.62 × 2.54 cm glass slides for each baseline and stress group neonate and allowed to air dry before fixing in 95 % ethanol, and staining with giemsa. The smears were examined at 600× magnification to count both leukocytes and erythrocytes. No fewer than 100 leukocytes were counted per slide and classified as lymphocytes, heterophils, basophils or monocytes (azurophils and monocytes were pooled) and reported as percentage of total leukocytes contributed by each leukocyte class and as the number of each leukocyte class per 1000 erythrocytes. For each smear, heterophil to lymphocyte ratio (H/L) was calculated by dividing the total number of heterophils by the total number of lymphocytes.

Measurements of neonates from the same litter were averaged prior to analyses because of potential maternal effects. A Chi square test of independence of factors was used to analyze for differences in adult female mortality before birth among the doses. Independent one-way analyses of variance (ANOVA) were used to determine if dosing treatment of the adults was related to (1) total mercury in tissues from neonates and adults and (2) neonate birth weights. The effects of stress treatment and mercury dose on CORT levels in neonates were determined by two-way ANOVA followed by a Student's *t* post hoc analysis. Because females were wild caught, timing of reproductive events (e.g., mating and fertilization) were not known. Thus, time between dosing and birth varied among individuals. To test for this, the analyses of differences by dosing treatment in baseline CORT and mercury in neonate tissues were repeated using one-way ANCOVAs with days until birth following dosing as the covariate. A one-way repeated-measures ANOVA was used to determine if dosing treatment was related to stress-induced differences between stress and baseline treatment CORT levels. The effects of stress treatment and mercury dose on H/L ratio, as well as on the percentage and absolute numbers of each leukocyte class were determined by two-way ANOVA. Correlation analyses were conducted to determine relationships between CORT, H/L ratio, percent of lymphocytes and percent of heterophils. For all analyses significance was defined as having a $p < 0.05$. All analyses were performed using JMP 12 (SAS Institute).

Results and Discussion

Average adult snake mass (mean ± SE = 359.89 g ± 23.5) and average snout-vent length (mean ± SE = 74.15 cm ± 1.83) were not significantly different by mercury treatment group ($p = 0.536$; $p = 0.776$, respectively). Of the 17 originally dosed, 14 females survived to birthing (6 – control, 5 – low dose, 3 – high dose) following dosing. Adult female mortality did not differ significantly among doses (Chi square = 3.002, critical value = 5.991). Although mortality was not found to be significantly related to dose, the individuals who did perish at the high dose level exhibited some symptoms of acute mercury toxicity, such as lethargy and lack of coordination.

Average adult mercury concentrations in muscle ($F_{(2,11)} = 43.1$, $p < 0.0001$), liver ($F_{(2,12)} = 5.12$, $p = 0.0029$), shed skins ($F_{(2,13)} = 43.6$, $p < 0.0001$) and fecal samples ($F_{(2,34)} = 7.45$, $p = 0.0022$) were significantly greater in the high dose treatment group compared to tissues from both control and the low dose treatment group, which did not differ from each other (Table 1). In the high dose treatment group, mean liver (13.5 μg/g) and muscle (13.2 μg/g) concentrations were similar, and in both dose treatment groups the shed skins exhibited the highest concentrations, with the high dose averaging 95.4 μg/g. The percentage of the total body mass represented by shed skins, however, is relatively low and that of muscle is relatively high indicating that of the tissues tested, the vast majority of mercury is being stored in muscle. This is consistent with other studies that show that MeHg tends to partition to muscle (Chumchal et al. 2011). However, because snakes shed skins throughout their lives, the present study is in agreement with others that shed skins may be used as a non-lethal indicator of exposure to metals (e.g. Jones and Holladay 2006).

Neonates were born from late August to mid-September with litter sizes ranging from 15 to 25 neonates. Neonate weights ranged from 4.17 to 5.86 g (mean = 5.00 g) and did not differ significantly by Hg treatment ($F_{(2,12)} = 0.5798$, $p = 0.5778$). As with adult tissues, total mercury concentrations in neonates were significantly

Table 1 Mean (±SE) mercury concentrations in tissues and fecal samples (μg Hg/g, wet weight) from adult female *N. sipedon*

Dose	Muscle	Liver	Fecal	Shed
0	0.112 (0.019)[a]	0.116 (0.023)[a]	0.00377 (0.00078)[a]	0.286 (0.059)[a]
	5	*6*	*11*	*6*
Low	0.199 (0.047)[a]	0.0935 (0.021)[a]	0.00704 (0.0017)[a]	0.375 (0.032)[a]
	4	*4*	*17*	*5*
High	13.2 (2.58)[b]	13.5 (8.16)[b]	2.87 (1.53)[b]	95.4 (21.1)[b]
	3	*3*	*7*	*3*

Shared letters within a tissue type denote treatment groups that did not differ significantly

Values in italics represent sample sizes

higher in the high dose treatment group compared to the control and low dose treatment groups for both liver and tail tissues ($F_{(2,8)} = 15.590$, $p = 0.0042$ and $F_{(2,8)} = 34.537$, $p < 0.0002$, respectively; Fig. 1) while the control and low dose treatment group were not found to differ from each other. ANCOVA revealed that days until birth following dosing had no significant effect on Hg uptake in neonate tissues.

Overall, mercury results from the present study indicate that maternal transfer of mercury did occur, even in the controls. Presumably, the Hg that was transferred to neonates in the present study's control group came from maternally accumulated Hg or dietary sources of the mother at Lake Erie early in pregnancy, and thus the majority of the Hg in the 10 μg/g dose neonates came from the doses administered to the mother after gestation had begun. Although the lack of significant differences in Hg concentration in neonate tissues between the control and 0.01 μg/g dose was not expected, it was also not surprising because all of the females used in the study, including the controls, were found to contain at least low levels of Hg, most likely from Hg-contaminated food items (i.e. round goby, *Neogobius melanastomus*) at Lake Erie (Hogan et al. 2007). Apparently at the 0.01 μg/g dose, the females did not transfer enough additional Hg to embryos to gain statistical significance over the control group.

There was no significant difference found in the average CORT levels in either baseline or stress group neonates across Hg doses ($F_{(2,16)} = 2.7535$, $p = 0.0938$; Fig. 2) and time between dosing and birth was not found to have an effect on dosing treatment as a predictor of baseline CORT levels ($F_{(2,7)} = 2.805$, $p = 0.127$). However, stress level was significantly related to CORT levels ($F_{(1,16)} = 6.3635$, $p = 0.0004$) with stressed individuals having significantly higher concentrations of CORT than baseline individuals in all dosing treatments (Fig. 2). Baseline CORT levels ranged from 16.9 to 103 ng/mL, and mean levels for the control, low, and high dose Hg treatments were 30.8, 48.7, and 75.2 ng/mL, respectively. Stress treatment CORT levels ranged from 82.6 to 270 ng/mL, and mean levels for the control, low, and high dose Hg treatments were 117, 126, and 192 ng/mL, respectively. A comparison of the differences in CORT levels between the baseline and stress treatment groups revealed that mercury dose was not a predictor of the amount CORT level increase due to stress ($F_{(2,8)} = 1.046$, H–F $p = 0.395$).

The baseline plasma CORT concentrations reported in the present study for *N. sipedon* neonates are typical of

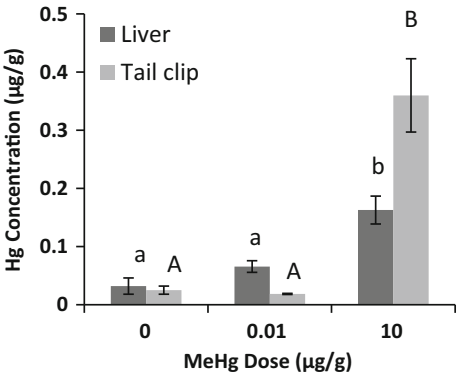

Fig. 1 Mean (±SE) mercury concentration by dose in neonate *N. sipedon* measured as μg Hg/g tissue. *Shared letters* within a tissue type denote treatment groups that did not differ significantly (N = 3 for both treatments at each dose level). Different tissue types were not compared statistically

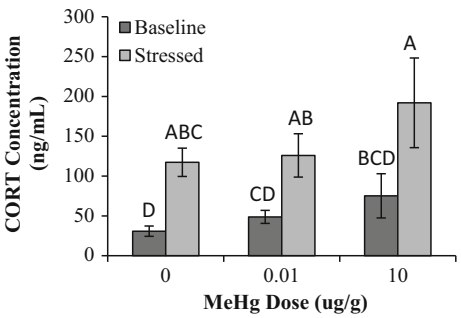

Fig. 2 Mean (±SE) baseline and stressed corticosterone levels in neonate *N. sipedon* by MeHg dose. *Shared letters* denote groups that did not differ significantly. N = 4 for 0 and 0.01 μg/g and 3 for 10 μg/g baseline and stress treatments

what has been found in juveniles of most species that exhibit the ability to upregulate glucocorticoids in response to stress, with mean juvenile CORT levels (51.6 ng/mL) being similar to that of adults of *N. sipedon* (mean = 45.8 and 45.7 ng/mL for males and females, respectively; Sykes and Klukowski 2009). One possible contributor to the high variation in neonate baseline CORT seen in the present study is maternal stress effects on neonatal glucocorticoids (Robert et al. 2009). Any stress (including possible body Hg burden) the adult females experienced in the present study may have affected the prenatal juveniles.

The present study is the first to show that neonates of *N. sipedon* are able to upregulate glucocorticoid hormones in response to confinement stress. These results are similar to those found by Jessop and Hamann (2005) who examined the elevation of CORT in green turtles across age classes. They found that newly hatched juveniles exhibited a strong elevation of CORT following capture. This upregulation can facilitate an effective flight or fight response when in physiologically suboptimal conditions, such as at low ambient temperatures or during times of starvation (Jessop and Hamann 2005).

Amount of post-natal care by parents may be linked to the response of neonates to stressors. For example, nest-bound (i.e., altricial) hatchling birds often receive ample parental care. Elevated stress hormones prior to fledge in altricial birds may lead to inappropriate and unnecessary utilization of energy resources and thus be detrimental to development, and would be suppressed until fledge (Blas et al. 2006). In contrast, some species have precocial young that leave the nest shortly after hatching and receive little parental care. These young can exhibit a hormonal stress response equal to or higher than adults (e.g., chuckar, Dickens and Romero 2010). Juvenile snakes are typically precocial and thus may have evolved the ability to upregulate glucocorticoids immediately following birth, allowing for rapid stress responses as observed in precocial bird species.

The H/L ratio was found to be lower in neonates from the stress treatment group (mean ± SE = 0.013 ± 0.0026) than in the baseline group (mean ± SE = 0.026 ± 0.0043) ($F_{(1,24)} = 5.6284$, $p = 0.0260$) but did not differ significantly by Hg dose. Correlation analyses revealed that the percentage of heterophils ($r^2 = 0.9912$, $p < 0.0001$; as a percent of total leukocytes) but not the percentage of lymphocytes ($r^2 = 0.100$, $p = 0.0888$) were directly related to the H/L ratio. This indicates that the lower H/L ratio in the stress group was related to a decrease in heterophils, whereas an increase in heterophils and a decrease in lymphocytes were expected in response to stress (Davis et al. 2008). The proportion and absolute numbers of each leukocyte class did not differ by stress or Hg treatment, so are reported with treatment groups combined in Table 2. To our knowledge, this is the first reporting of leukocyte counts in neonate *N. sipedon*.

The unexpected reduction in the H/L ratio in response to acute stress and lack of correlation between CORT and H/L ratio ($r^2 = 0.003$, $p = 0.7735$) in *N. sipedon* neonates could be attributed to a variety of things. Most of the literature regarding the effects of stress on leukocytes has been limited to the effects of acute/short-term stress; however it's been shown that short-term and long-term stress interactions can yield unpredictable results (Dhabhar and McEwen, 1997). In the present study, neonates were subjected to a variety of stressors, including short-term (confinement), long–term (Hg exposure), and prenatal stress, which could potentially explain discrepancies between the expected leukocyte response and our observations. It is also possible that stress induced leukocyte changes are delayed in reptiles and amphibians, with one study reporting that the leukocyte response in newts was not measurable until 3-days after exogenous stress-hormone administration (Bennett et al. 1972).

The present study was the first to show that neonate *N. sipedon* can upregulate CORT in response to stress in a similar manner to precocial birds and adult snakes. The lack of a CORT increase with Hg dose indicates that these dose levels of MeHg have no effect on some hypothalamic–pituitary–adrenal (HPA) axis functions. However, because Hg was also maternally transferred to control neonates, an absolute baseline for CORT could not be determined. It is also possible that the duration of in utero exposure to mercury in this study was not long enough or was not at the appropriate period during embryonic development to cause significant toxic effects on CORT levels or leukocyte differentials. If dosing had occurred during vitellogenesis, there may have been a greater transfer of mercury to the neonates. Mercury levels in adult snakes in other studies (e.g. Rainwater et al. 2005) suggest that snakes exhibit a high tolerance

Table 2 Leukocyte counts in neonate *N. sipedon* with all treatment groups combined

Leukocyte class	N	Number/1000 erythrocytes (±SE)	Percent of total leukocytes (±SE)
Lymphocyte	30	30.4 (2.9)	75.8 (1.4)
Heterophil	30	0.590 (0.09)	1.47 (0.20)
Monocyte	30	4.70 (0.46)	12.5 (1.0)
Basophil	30	2.60 (0.27)	7.47 (0.88)

for accumulated Hg burden with little apparent toxic effects (although none were assessed in the aforementioned study). Wolfe et al. (1998) cite unpublished data by Wolfe in which MeHg was fed to garter snakes (*Thamnophis sirtalis*) at high concentrations (highest was 200 μg/g food) with no apparent toxic effects or decrease in food consumption by adults or subsequent offspring. Similarly, Chin et al. (2013a, b) observed no adverse effects on reproductive success or offspring viability in *N. sipedon* from a historically Hg contaminated site when compared those from a reference site. However, effects on strike efficiency in neonates of females from the Hg-contaminated sites were seen (Chin et al. 2013b). Their results, in conjunction with our own, support that *N. sipedon* may be somewhat tolerant to Hg contamination.

All procedures involving *N. sipedon* adults and neonates were carried out according to MTSU IACUC protocol 10-011. *Nerodia sipedon* adults were collected in accordance with Ohio Division of Wildlife scientific collection permit #11-305 issued to Frank C. Bailey.

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