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Compromised immune competence in free-living tree swallows exposed to mercury

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Abstract Mercury is a pervasive environmental contaminant and a well-documented immunosuppressor. However, little is known about the effects of mercury contamination on health of free-living vertebrate populations. The South River in Virginia, USA was heavily contaminated with industrial mercury from 1929 to 1950, and recent studies have documented high levels of circulating mercury in riparian songbirds breeding below the site of contamination. Here we used two standardized immune assays, mitogen-induced swelling in response to phytohaemagglutinin (PHA) and antibody response to sheep red blood cells (SRBCs), to test for effects of mercury toxicity on the immune system of female tree swallows (*Tachycineta bicolor*) which feed on terrestrial and aquatic insects along the contaminated waterway. We found that females breeding at mercury-contaminated sites mounted significantly weaker PHA-induced swelling responses than those at reference sites in both years of study. However, among females on the contaminated sites, individual bloodstream mercury concentration did not predict the extent of mitogen-induced swelling. We did not detect any differences between reference and contaminated females in the strength of antibody responses to SRBCs, but sample sizes for this assay were significantly smaller. Overall, our results suggest that mercury toxicity can exert sub-lethal immunosuppression in free-living, insectivorous songbirds. The potential fitness consequences of the detected differences in immunocompetence caused by mercury toxicity warrant further study.

Keywords Mercury · Songbird · Immune competence · Tree swallow · *Tachycineta bicolor*

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Introduction

The health implications of mercury toxicity in wildlife are widely documented (Wiener et al. 2003; Scheuhammer et al. 2007). In vivo and in vitro, mercury is a potent immune suppressor and/or modulator across a range of vertebrate taxa (Zelikoff et al. 1994; Day et al. 2007). The effects of mercury on avian health have been particularly well studied in poultry (Kumar et al. 1999) and a range of piscivorous, or fish-eating, bird species due to their susceptibility to bioaccumulation. In studies with captive birds, both low (0.5 mg/kg) and high (5 mg/kg) doses of methylmercury chloride caused severe immune lesions in captive great egrets (*Ardea alba*; Spalding et al. 2000). Similarly, in juvenile common loons (*Gavia immer*), Kenow et al. (2007) documented significant effects of experimental methylmercury doses on antibody responses to sheep red blood cells, but no effects on swelling response to phytohaemagglutinin (PHA). However, the effect of methylmercury on antibody responses was only present at low dose levels, making the results difficult to interpret (Kenow et al. 2007). Finally, chickens fed mercury at low doses showed significantly suppressed humoral immune responses (Kumar et al. 1999). Overall, immune suppression from mercury toxicity appears to be universal in the avian species studied to date, but the effect of mercury contamination on songbird immunity remains unknown, as does the likelihood of effects in free-living birds.

Understanding the effects of mercury toxicity on songbird health, including immune competence, is of increasing conservation value and concern. Songbirds comprise the majority of all bird species, but they have been largely overlooked by toxicologists to date because few are piscivorous (Scheuhammer et al. 2007). However, recent studies indicate that a diversity of songbirds bioaccumulate mercury in montane (Rimmer et al. 2005), freshwater (Evers et al. 2005), estuarine (Shriver et al. 2006) and riparian systems (Cristol et al. 2008). Insectivorous songbirds appear to be particularly susceptible to mercury bioaccumulation (terrestrial: Rimmer et al. 2005; aquatic: O'Halloran et al. 2003; Henny et al. 2005), in some cases harboring blood mercury levels at or above those of sympatric piscivorous birds (Cristol et al. 2008). Therefore, studies of the health impacts of mercury toxicity are relevant and sorely needed in non-piscivorous taxa subject to high levels of mercury contamination. Furthermore, studies of mercury effects on health in free-living populations are critical (Day et al. 2007) in order to understand the effects of mercury toxicity in an ecological context.

Here we examine the effects of mercury toxicity on immune competence in free-living insectivorous songbirds (tree swallows, *Tachycineta bicolor*) breeding along the South River in Virginia, USA, where bloodstream mercury levels are among the highest ever documented in terrestrial birds (Cristol et al. 2008). The South River was heavily contaminated with industrial mercury from 1929 to 1950 (Carter 1977), and more than a half-century later, mercury levels remain elevated in both fish and birds compared with sympatric reference rivers (Cristol et al. 2008). Tree swallows breeding along the contaminated South River eat a variety of mercury-containing terrestrial and aquatic insects, resulting in significantly elevated blood-stream mercury levels (Cristol et al. 2008) and reduced fledging success compared to conspecifics breeding at nearby reference sites (Brasso and Cristol 2008). The effects of mercury toxicity in tree swallows on other components of fitness, such as immune competence, remain unknown. Here we use two standardized immune assays that broadly target both arms (e.g. cell-mediated and humoral) of the vertebrate immune response (but see Martin et al. 2006). Our study was designed to investigate whether mercury toxicity causes detectable immune suppression in a free-living songbird, the tree swallow.

Methods

Study area

The tree swallow is an insectivorous, migratory songbird that breeds in the northern half of North America (Robertson et al. 1992). In 2005, nest boxes were erected at 36 sites along the South River, VA, USA and two nearby rivers with no history of mercury contamination, the North and Middle Rivers (described in Cristol et al. 2008). Nest boxes were placed in hayfield or pasture approximately 25 m apart and within 300 m of river shoreline. In 2005, 233 nest boxes were provided. This number was increased prior to the breeding season to 286 in 2006 and 347 in 2007. There is no natural wetland habitat suitable for tree swallow nesting in the study area and prior to the establishment of our nest box trail, few, if any, tree swallows were nesting on or near the study sites.

Capture and sampling

Adult female tree swallows were captured in their nest boxes during incubation or the nestling period using one of two trapping methods (Stutchbury and Robertson 1986; Friedman et al. 2008) or by hand. Upon capture, each female was uniquely banded with a USGS metal band and aged via plumage. In addition, a small blood sample (<100 μl) was collected via brachial venipuncture for mercury analysis (as described in Brasso and Cristol 2008). All procedures for wild bird handling were approved by the College of William and Mary's Institutional Animal Care and Use and Biosafety Committees and performed under US Geological Survey Bird Banding permit 22792 to Daniel Cristol.

Mercury analysis

Following collection, blood was kept on ice for approximately 3–6 h, after which time it was stored at −25°C. Samples of whole blood were analyzed for total mercury on a Milestone® DMA 80 using cold vapor atomic absorption spectroscopy at the College of William and Mary (W&M, $n = 51$ blood samples) or at the Trace Elements Research Laboratory at Texas A&M University (TERL, $n = 40$ blood samples). Method detection limit was 0.003–0.006 μg at W&M and 0.001–0.018 μg at TERL. A sample blank, methods blank, duplicate sample and two of three standard reference materials (DORM-2, DORM-3 or DOLT-3) were included with every 20 samples. Recovery of total Hg was 97.7–98.3% at W&M and 98.4–103.8% at TERL. Duplicate samples were obtained at W&M by comparing two capillary tubes of blood from the same collection of the same bird run in the same batch. At TERL, blood samples with larger volumes, not collected for this study, were split and run in the same batch as samples from this study. Relative percent differences were 10.26 ± 18.20% for W&M ($n = 10$) and 6.19 ± 5.99% for TERL ($n = 37$).

In both 2006 and 2007, a portion of blood samples from the current study were analyzed by each facility. Inter-laboratory duplicates were run to ensure comparability

between the two labs. The relative percent difference between inter-laboratory duplicates for the period when these samples were run was 15.73 ± 27.53%, less than the generally accepted 20%, for samples greater than 10 times the MDL. All results are presented as wet/fresh weight (μg/g) concentrations. We did not obtain mercury blood concentrations for two of the 92 individuals for which we measured PHA response.

PHA assay

We performed all immune assays when females were feeding 2–5 day old nestlings due to the ease of capture during this high provisioning period. In 2006 and 2007, we quantified T-cell proliferation in response to phytohaemagglutinin (PHA) for 92 female tree swallows using a protocol described by Smits et al. (1999). We injected the right patagium ("wing web") of each individual with 0.15 mg of PHA (Sigma–Aldrich, St. Louis, MO, USA) suspended in 30 μl of phosphate-buffered saline (PBS). We captured each subject and measured patagial width at the site of injection to 0.01 mm using a micrometer, 5–10 min prior to and 24–26 h after injection. In order to control for variation inherent in this small measurement (wing web width is ~1 mm), we made five independent measurements each time, discarded the smallest and largest measurement and averaged the remaining three measurements to obtain a single pre- and post-injection value. Measurements were made blind to previous measurements on the same bird by having a second observer record each value measured by Dana M. Hawley, who could not see the instrument dial while measuring. In 2006, measurements were made blind to female status (reference or contaminated), because Dana M. Hawley was unfamiliar with layout of the study site with respect to source of contamination. We divided change in width (mm) by pre-injection width (mm) to obtain a measure of PHA response, thus standardizing for initial patagial size.

SRBC assay

In 2006 only, we injected a subset of females ($n = 25$) with sheep red blood cells (SRBCs) as an assay of humoral immune function. Following the final PHA measurement, we intra-abdominally injected individuals with 5×10^7 SRBCs (MP Biomedicals, Irvine, CA, USA) suspended in 50 μl PBS (as per Deerenberg et al. 1997). We took blood samples 10–15 min prior to and 8 days following injection and kept the blood at 4°C for 2–4 h until centrifugation. We quantified antibody titer as the reciprocal of the highest $\log_2$ dilution at which an individual's plasma showed positive haemagglutination, subtracting pre-injection titers from post-injection titers.

Statistics

We performed all statistical analyses in JMP 5.0 (SAS Institute, Cary, NC). In order to test for the effects of mercury contamination on immune response, we used mixed linear models including fixed effects of year, female age, status (contaminated vs. reference), and their two-way interactions. We included site (within contaminated or reference status) as a random effect for all models. For the SRBC assay, which was only conducted in 2006, we did not include year in our analyses. We removed all non-significant interactions from the final model.

Results

Mercury levels

As detected in previous studies (e.g. Brasso and Cristol 2008), female tree swallows breeding at contaminated sites had significantly higher blood mercury levels than females breeding at reference sites (Table 1; Fig. 1a; $n = 90$, $F_{1,20.5} = 42.8$, $P < 0.0001$). Year of study ($F_{1,75.0} = 31.1$, $P < 0.0001$) but not female age ($F_{3,65.98} = 1.75$, $P = 0.16$) significantly predicted mercury concentrations (Table 1).

PHA assay

Tree swallows breeding at contaminated sites mounted significantly reduced swelling responses to PHA injection in both years (Fig. 1b; $n = 92$; $F_{1,13.6} = 15.5$, $P = 0.002$). Year of study also significantly influenced PHA response: swelling responses were significantly higher in 2007 ($F_{1,53.2} = 12.8$, $P = 0.0007$). Female age did not influence PHA response ($F_{3,82.7} = 0.35$, $P = 0.79$) and none of the two-way interactions were significant. Within contaminated sites, variation in blood mercury levels of females

Table 1 Bloodstream mercury concentrations (μg/g) of female tree swallows (*Tachycineta bicolor*) varied across the 2 years of study at contaminated sites, but were significantly higher than reference sites in both years

Study year	Bloodstream mercury concentration	
	Reference	Contaminated
2006 ($n = 40$)	Range 0.09–0.40	Range 0.80–7.36
	Mean 0.16 ± 0.02	Mean 3.25 ± 0.37
2007 ($n = 50$)	Range 0.11–0.33	Range 1.12–4.52
	Mean 0.16 ± 0.01	Mean 2.51 ± 0.16

Error values represent one standard error around the mean

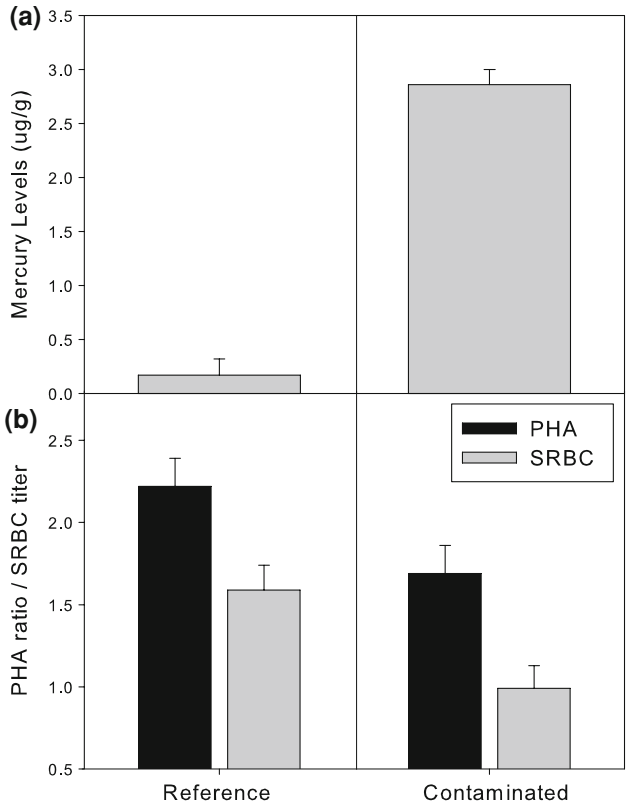

Fig. 1 **a** Blood mercury concentrations **b** the strength of response to two standardized immune assays in female tree swallows (*Tachycineta bicolor*) breeding along contaminated and reference waterways. Values are averaged across the 2 years of study. *Error bars* represent one standard error around the mean

did not predict the extent of swelling in response to PHA injection ($F_{1,30.0} = 0.28$, $P = 0.60$).

SRBC assay

Antibody titer in response to SRBC injection did not vary with contamination status (Fig. 1b; $n = 25$; $F_{1,12.34} = 0.43$, $P = 0.55$) or female age ($F_{3,20.47} = 0.35$, $P = 0.71$).

Discussion

Our results indicate that tree swallows breeding at mercury-contaminated sites incur sub-lethal fitness effects in the form of reduced immune competence: in both years, females breeding at contaminated sites showed significantly lower mitogen-induced swelling in response to PHA injection. Mercury levels were significantly higher in 2006 (Table 1), likely as the result of a severe drought that affected dietary mercury exposure, and this corresponded with reduced responses to PHA injection in that year. However, although blood mercury concentrations among females breeding at contaminated sites varied by almost ten fold (Table 1), this variation did not directly predict female immune response to PHA. This lack of direct correlation between individual blood mercury concentration and immune response has several possible interpretations: first, blood-level of mercury may not have a direct effect on immunity in breeding tree swallows, but may act indirectly on female immunity at contaminated sites via changes in individual condition or health. Second, nearly all mercury levels at these highly contaminated sites may be above the minimum threshold necessary for immune effects to be detected. We cannot distinguish between these possibilities with our current data set, but future studies in captivity may reveal the extent to which mercury effects on immunity are dose-dependent in tree swallows.

Our sample sizes for the humoral immune assay used in this study were insufficient to reject the null hypothesis that mercury had no effect on the strength of antibody responses to sheep red blood cells. Although mercury is known to affect both arms of the vertebrate immune response (Wiener et al. 2003; Scheuhammer et al. 2007), studies of mammals indicate that B-cells (the primary cell type for the humoral immune response) may be more sensitive to mercury toxicity than T-cells (the primary cell type for cell-mediated immunity). In humans, B-cells were suppressed at significantly lower doses of mercury than mitogen-induced T-cell proliferation (Shenker et al. 1992). Furthermore, in mice, equivalent mercury doses caused a significantly larger reduction in B-cells (47%) than T-cells (9%) (Haggqvist et al. 2005). Taken together, these studies suggest that mercury has the potential to negatively impact both arms of vertebrate immunity, but differential responses may exist for humoral versus cell-mediated components. Although the patterns for PHA and SRBC response appeared broadly similar in our study (Fig. 1b), our sample sizes for the humoral immune assay were not sufficiently large to detect significant effects. Furthermore, the PHA assay used in this study is likely an integrated measure of both innate and cell-mediated immune components (Martin et al. 2006), rendering it difficult to interpret as an assay of cell-mediated immunity per se (Kennedy and Nager 2006). Further study is needed on potential differences between vertebrate immune arms in the effects of mercury toxicity.

To our knowledge, this is the first study to demonstrate effects of mercury toxicity on immune competence in a free-living bird species, and more specifically, a songbird. These results add to an accumulating set of evidence that heavy metal toxicity causes detectable sub-lethal effects in free-living songbirds. Nestling great tits (*Parus major*) at sites heavily contaminated with a suite of heavy metals, including mercury, had significantly lower body mass and condition than those at uncontaminated sites (Janssens et al. 2003). Furthermore, adult male great tits breeding at

contaminated sites showed significantly smaller song repertoire size (Gorissen et al. 2005). The additive consequences of these sub-lethal effects on songbird populations remain unknown. In particular, understanding how the detected effects of mercury toxicity on tree swallow immune competence translate into differences in pathogen susceptibility and/or survival is critical. An experimental link between mercury toxicity and resistance to *Salmonella* infection has been made in poultry (Hill 1979), but studies on free-living birds are lacking. The large suite of insectivorous songbird species now known to be impacted by mercury contamination (Rimmer et al. 2005; Cristol et al. 2008) underscore the critical need to understand the impacts of mercury toxicity on songbird health more broadly.

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