PHA-Stimulated Immune-Responsiveness in Mercury-Dosed Zebra Finches Does Not Match Results from Environmentally Exposed Songbirds

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Abstract Dietary mercury exposure is associated with suppressed immune responsiveness in birds. This study examined the immune-responsiveness of domestic zebra finches (*Taeniopygia guttata*) experimentally exposed to mercury through their diet. We used the phytohemagglutinin (PHA) skin-swelling test to assay the effect of two modes of mercury exposure. Some finches received exposure to mercury only after reaching sexual maturity, while others were maintained on a mercury-dosed diet throughout life, including development. Each bird received one of five dietary concentrations of methylmercury cysteine (0.0, 0.3, 0.6, 1.2 or 2.4 ppm). In contrast to a study on wild songbirds at a mercury-contaminated site, we detected no relationship between mercury level and immunological response to PHA, regardless of mode of exposure. This result represents the first major difference found by our laboratory between wild birds exposed to environmental mercury and captive birds experimentally exposed to mercury.

Mercury is a persistent environmental toxin that current global regulations are unlikely to reduce (Selin 2013). In its methylated form, mercury has demonstrable impacts on organisms in aquatic ecosystems (Wolfe et al. 1998; Scheuhammer et al. 2007) and also enters terrestrial food webs, where it negatively impacts wildlife, including songbirds (Cristol et al. 2008). Studies on wild songbirds have revealed correlations between mercury exposure in the diet and many fitness-related endpoints such as lower nestling survival (Hallinger and Cristol 2011), reduced corticosterone response to stress (Wada et al. 2009; Franceschini et al. 2009), and damage to internal organs (Nicholson and Osborn 1984). Because field studies typically demonstrate only correlations, lab studies using experimental manipulations of dietary mercury are necessary to determine if mercury is, indeed, the causal agent. Several such laboratory studies, using environmentally-relevant doses to examine sub-lethal effects of mercury, have reproduced findings from the field. Using the examples cited above, nestling survival was reduced in a dose-dependent manner in captive zebra finches (*Taeniopygia guttata*) (Varian-Ramos et al. 2014), much as it was in the field. Also, laboratory studies of the corticosterone response to stress using dosed zebra finches have found results consistent with those reported for wild songbirds (Moore et al. 2014). Finally, oxidative damage to an internal organ (liver) has recently been demonstrated in a dosing study with zebra finches (Henry et al. 2014).

Another observed effect of long-term exposure to mercury is altered immune function (Moszczynski 1997; Siebert et al. 1999; Das et al. 2008; Frouin et al. 2012; Singaram et al. 2013), which has high potential for reducing an organism's fitness. The immune system is critical in preventing endogenous disease (Takeda et al. 2002; Reiche et al. 2004; de Visser et al. 2006) as well as defending organisms against parasites and infection (Andre et al. 2003; Kawai and Akira 2006; Schmid-Hempel 2008). In birds, previous studies have found that exposure to mercury may impair white blood cell phagocytosis (Holloway et al. 2003; Finkelstein et al. 2007) and reduce B cell

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proliferation (Lewis et al. 2013). Understanding mercury's impact on avian immune systems, then, is critical for understanding the potential environmental impact of future mercury emissions on birds.

Subcutaneous injection of phytohemagglutinin (hereafter PHA) has proven to be a popular assay of immune-responsiveness in birds (e.g., Smits et al. 2002; Navarro et al. 2003). PHA is a protein derived from red kidney beans, and while the exact mechanism of assay response is unknown, PHA is thought to act as a mitogen inducing a T-cell mediated response (Goto et al. 1978; Tella et al. 2008). Histological evidence, however, indicates that PHA may induce a more complex immune response (Martin et al. 2006). A previous study on wild breeding female tree swallows (*Tachycineta bicolor*) living at a mercury-contaminated site found that birds with elevated blood mercury had reduced swelling after PHA injection, compared to birds from reference sites, suggesting a weakened immune response (Hawley et al. 2009).

Our objective was to use an experimental manipulation of mercury exposure to determine whether mercury causes suppression of immune responsiveness in captive-bred zebra finches exposed to mercury either solely during adulthood, or throughout their lifetime. The PHA skin-swelling assay was used to evaluate immune responsiveness.

Materials and Methods

This study was carried out in accordance with the recommendations in the Guide of the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures and protocols were approved and overseen by The College of William and Mary's Institutional Animal Care and Use Committee (IACUC 2012-05-23-7982). Finches were randomly assigned to five treatment groups and thereafter fed a complete artificial diet (Zupreem Fruitblend, Shawnee, KS, USA) containing one of five concentrations of aqueous methylmercury cysteine: 0.0 µg/g (parts per million, hereafter ppm), 0.3, 0.6, 1.2 and 2.4 ppm. The treatment doses were calculated on a wet weight basis and would be the equivalent of dry weight concentrations of 0.0, 0.35, 0.70, 1.39 and 2.78 ppm, respectively. Every batch of artificial diet was tested on a direct mercury analyzer to ensure that it was within 10 % of the nominal concentration. A complete description of the mercury analysis methods and quality assurance data for these samples can be found in Varian-Ramos et al. (2014). The average calculated minimum detection limit was 0.008 ± 0.001 ppm.

The 130 adult-exposed zebra finches used in this study were housed in pairs in an indoor aviary with ad libitum access to food, grit, nesting materials, and water. Birds were fed a mercury-dosed diet starting from the age of 150–400 days old. The lifetime-exposed birds were raised by their dosed parents until 50 days old, and then released into a group outdoor aviary, where they continued to be fed their treated diets. Lifetime-exposed birds, therefore, received the same dose of mercury from the point of conception to the completion of the study. Before testing, 109 lifetime-exposed birds were transferred to an indoor aviary and housed in pairs using the same conditions described for adult-exposed birds.

We conducted PHA assays on adult-exposed birds from June to July, 2011 and on lifetime-exposed birds from October, 2012–February, 2013. A few small feathers from each wing were plucked to allow for measurement of the patagium ("wing web") thickness with a micrometer. Both patagia were measured to 0.01 mm five times in rapid sequence, with the highest and lowest measurements discarded, and the remaining three averaged. To ensure that measurements were taken independently, a second observer watched the micrometer dial and recorded the results while the measurer could not see the micrometer dial. Following this, we injected the right patagium with 0.15 mg PHA (Sigma-Aldrich, St. Louis, MO, USA) suspended in 30 µl phosphate-buffered saline (PBS), replicating the dosages used in Hawley et al. (2009). The left wing was injected with 30 µl PBS as a control. After 23–25 h, we re-measured both patagia using the same procedures with the measurer blind to previous measurements or treatment. The change in width (mm) in each patagia was divided by the pre-injection width to standardize swelling. We then subtracted the standardized control (PBS) swelling measurement from the standardized PHA swelling to give a stimulation index.

All statistics were completed using SPSS (version 20, IBM Corp., Armonk, NY, USA). A general linear model was constructed with mercury dose (0.0–2.4 ppm) and life-stage of exposure (adult vs. lifetime), and the interaction between the two, as independent variables and stimulation index as the dependent variable. Means are presented with standard errors throughout.

Results and Discussion

Neither dose ($F = 0.99$, $p = 0.41$), nor life-stage of exposure ($F = 1.29$, $p = 0.26$) had a significant effect on stimulation index, and there was no significant interaction term ($F = 0.42$, $p = 0.80$) (Table 1; Fig. 1). Because we wished to compare our results to those from wild birds exposed to environmental mercury, we also analyzed the data as in Hawley et al. (2009), omitting the correction for swelling of the saline-injected wing. The alternate analysis

Table 1 Estimated marginal means of PHA stimulation index as a function of mercury dose and mode of exposure

Dose (ppm)	Mode of exposure	Sample size	Mean	SE	95 % Confidence interval	
					Lower	Upper
0.0	Adult	23	0.807	0.104	0.601	1.012
	Lifetime	24	0.736	0.091	0.556	0.916
0.3	Adult	26	0.920	0.098	0.727	1.112
	Lifetime	21	0.779	0.100	0.582	0.976
0.6	Adult	27	0.771	0.096	0.581	0.960
	Lifetime	19	0.836	0.091	0.656	1.016
1.2	Adult	27	0.956	0.096	0.767	1.145
	Lifetime	21	0.908	0.091	0.729	1.088
2.4	Adult	26	0.980	0.098	0.787	1.173
	Lifetime	22	0.830	0.093	0.647	1.012
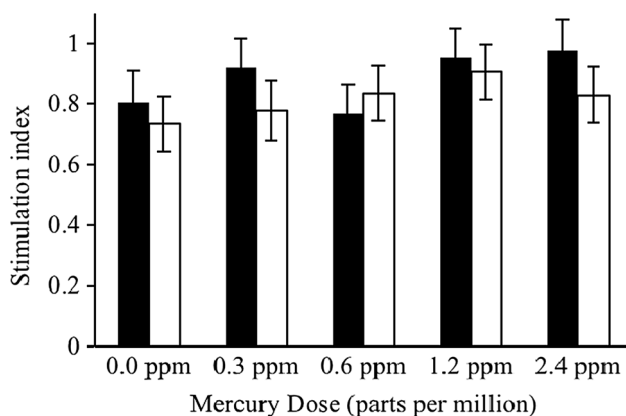
Fig. 1 Estimated marginal means of PHA stimulation index as a function of mercury dose and mode of exposure (*black* adult exposure only, *white* lifetime exposure). *Error bars* are 1 standard error

also revealed no effect of mercury dose on PHA skin-swelling response ($F = 0.85$, $p = 0.49$). On average the blood mercury level of the finches was 13.2 ± 0.2 times the dietary dose (e.g., birds consuming 0.6 ppm had blood mercury levels of ~ 8 ppm), and the lifetime-exposed birds had slightly, but not significantly, higher blood mercury concentrations than the adult-exposed birds (see Fig. 1 in Varian-Ramos et al. 2014).

Thus, we detected no relationship between dose of dietary mercury exposure and responsiveness of the immune system to an injection of the PHA antigen, despite dietary mercury levels that matched or exceeded those found in the field. Our objective was to experimentally manipulate mercury exposure so as to assess the evidence for causality in the relationship between mercury exposure and PHA response observed in the field at a mercury-contaminated site (Hawley et al. 2009). In contrast to the results from the field, we did not find evidence for a weakened swelling response to in vivo injection of PHA antigen in zebra finches dosed either throughout life or only

as adults. There are several possible explanations for this discrepancy between field and laboratory findings.

First, it is possible that the correlation between mercury exposure and reduced PHA response found in the field with tree swallows was not due to the difference in their contaminant exposure, but to a third, unmeasured factor that differed between mercury-contaminated and reference sites, for example prey availability. This cannot be ruled out because field studies are inherently reliant on inference from correlations. It is also possible that mercury was the cause of the difference in the field-tested swallows, but that under captive conditions the immune system did not respond in the same way to mercury exposure. Captive finches had never experienced food shortage, were raised without threat of predation, and likely faced reduced exposure to diverse diseases and parasites than free-living swallows. Immunological response to PHA injection may be condition-dependent, and thus sensitive to environmental conditions of field or lab (Thompson et al. 2014). There is also a third possibility, that the differential relationship between mercury and immune responsiveness of zebra finches and tree swallows is the result of evolutionary differences between the two species. Distinguishing between these explanations for the discrepancy between the two results is not possible without much further study, but it is clear that the relationship between mercury and immune suppression assayed via PHA injection should be considered tentative and correlational until further dosing studies are completed. The only relevant avian dosing study of which we are aware, on chicks of common loons (*Gavia immer*) dosed with 0.4 or 1.2 ppm methylmercury chloride, also detected no effect of mercury exposure on PHA-induced immune response (Kenow et al. 2007).

In general, though, physiological and behavioral effects of mercury identified with wild birds have been reproduced with recent dosing studies on captive-bred zebra finches. For example, similar levels of reproductive inhibition have been reported from the field and aviary (Hallinger and

Cristol 2011; Varian-Ramos et al. 2014) and the corticosterone stress response is inhibited in both as well (Wada et al. 2009; Moore et al. 2014). In addition, captive zebra finches dosed with mercury show modifications of singing behavior similar to wild sparrows and wrens at mercury-contaminated sites (Hallinger et al. 2010; CW Varian-Ramos, unpublished data) and there is evidence of disruption of coloration in free-living, mercury-exposed belted kingfishers (*Megaceryle alcyon*) and captive zebra finches (White and Cristol 2014; Spickler 2014). Thus, the discrepancy between field and lab results found in this study cannot be easily dismissed as being due to differences in the evolutionary histories of species used or the lack of environmental challenges in the laboratory.

Aligning the results of experimental contaminant dosing studies with the tissue levels and physiological effects of the same contaminants in similar free-living species is of paramount importance for risk assessment (Burger and Gochfeld 1997). In the case of songbirds and mercury, it is only in the last decade that there has been concern about the effects of mercury contamination on population health. Tissue mercury residues have recently been reported from many species of songbirds (e.g., Jackson et al. 2011; Keller et al. 2014), but the picture is still incomplete. Effects studies have focused on only a few songbird species, either dosed in captivity or living free at contaminated sites (e.g., Varian-Ramos et al. 2014; Brasso and Cristol 2008). Progress has been slow at matching laboratory and field results, and even when studies have examined the same endpoints, results sometimes vary. For example, the relationship between baseline corticosterone and mercury has been reported as positive or negative, depending on species and age (Franceschini et al. 2009; Wada et al. 2009; Herring et al. 2012). The results of the present study suggest that further attempts to establish causality for reported correlational effects of mercury and other contaminants are worthwhile and important for improving risk assessment and our ability to predict the effects of environmental contamination and remediation on the health of animal populations.

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