

Form of Dietary Methylmercury does not Affect Total Mercury Accumulation in the Tissues of Zebra Finch

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Abstract Exposure to mercury in humans, other mammals, and birds is primarily dietary, with mercury in the methylated form and bound to cysteine in the tissues of prey items. Yet dosing studies are generally carried out using methylmercury chloride. Here we tested whether the accumulation of total mercury in zebra finch blood, egg, muscle, liver, kidney or brain differed depending on whether dietary mercury was complexed with chloride or cysteine. We found no effect of form of mercury on tissue accumulation. Some previous studies have found lower accumulation of mercury in tissues of animals fed complexed mercury. Much remains to be understood about what happens to ingested mercury once it enters the intestines, but our results suggest that dietary studies using methylmercury chloride in birds will produce similar tissue accumulation levels to those using methylmercury cysteine.

Keywords Birds · Dietary dosing · Mercury · Methylmercury chloride · Methylmercury cysteine · *Taeniopygia guttata*

Mercury is a neurotoxin with numerous well-established deleterious effects on wildlife and humans (reviewed in

Scheuhammer et al. 2007; Choi and Grandjean 2008). Anthropogenic sources of mercury pollution have contributed to a threefold increase in the amount of bioavailable mercury in the environment (Mason et al. 1994). Once in the environment, mercury can be methylated by microorganisms (reviewed in Ullrich et al. 2001), whereupon it can bioaccumulate and biomagnify up the food chain (reviewed in Wiener et al. 2003). In order to understand the effects of methylmercury exposure, it is necessary to conduct carefully controlled dosing studies on model organisms in a laboratory setting. However, if the findings of dosing studies are to be applied to natural populations, the methods used should closely replicate natural exposure patterns. Many mercury dosing studies to date do not closely replicate mercury exposure in the field. Mercury is commonly delivered through injection (e.g., Gao et al. 2008; Robinson et al. 2010; Mori et al. 2012) or in a single bolus (e.g., Liu et al. 2009) rather than delivered gradually in the diet as is more likely during environmental exposure. Additionally methylmercury is often delivered in a commercially available form (methylmercury chloride (MeHgCl)) that is not likely to be encountered in natural diets (e.g., Gao et al. 2008; Heinz et al. 2009; Liu et al. 2009).

Methylmercury has a strong affinity to the thiol groups in amino acids such as cysteine and methionine (reviewed in Clarkson 1993). Methylmercury cysteine (MeHgCys) is the predominant form of mercury found in contaminated fish (Harris et al. 2003; Kuwabara et al. 2007) and it is likely that most dietary mercury in wild populations occurs in this form. Further, simulated digestion does not dissociate methylmercury from cysteine (George et al. 2008). MeHgCys appears to serve as a molecular mimic of other amino acids and is transported into cells (reviewed in Bridges and Zalup 2005) and across the blood brain barrier (Kerper et al. 1992; Yin et al. 2008) by large neutral

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amino acid carriers (reviewed in Clarkson et al. 2007). In a natural diet, MeHgCys is likely incorporated into larger proteins (Bourdineaud et al. 2012). Because of the difference in form, mercury ingested in the form of MeHgCys may be taken up and distributed differently than the more commonly used form, MeHgCl.

Several studies have examined differences between MeHgCl and MeHgCys exposure. In general, organisms exposed to methylmercury through non-dietary methods (e.g., injected intravenously or into the abdominal cavity) showed differences between MeHgCl and MeHgCys in the accumulation and distribution of mercury throughout the body (e.g., Leaner and Mason 2001; Roos et al. 2010; Mori et al. 2012). However, natural exposure occurs through the diet and few studies have examined effects of the different forms of methylmercury when introduced through the diet. Several studies have examined the differences between diets containing naturally contaminated fish versus MeHgCl. Rats fed a diet of naturally contaminated fish accumulated less mercury in their tissue and excreted more in their feces than did rats fed a diet of uncontaminated fish spiked with MeHgCl (Berntssen et al. 2004). Despite the lower accumulation, rats exposed to naturally contaminated fish had greater behavioral impairment when compared to rats fed MeHgCl (Bourdineaud et al. 2012). While using fish contaminated naturally with methylmercury may be the most realistic means of dosing in some systems, in many cases it is not possible to dose with fish.

An alternative to dosing with fish is to mix MeHgCys with the food directly. In two studies comparing MeHgCys to MeHgCl exposure in rodents, no difference in total mercury concentration was detected in any tissue measured (Glover et al. 2009; Mori et al. 2012). However, few tissues were examined in either study (liver and blood only). In order to determine whether the form of methylmercury impacts uptake and distribution of mercury, a more comprehensive and long-term study of multiple tissues, particularly the brain, is necessary.

We conducted an environmentally realistic dosing study using zebra finch (*Taeniopygia guttata*). Terrestrial songbirds have recently been shown to be at risk when living near mercury-contaminated sites (Cristol et al. 2008). The well-studied zebra finch is the model laboratory songbird and is only the second bird, after the chicken, to have its genome sequenced (Warren et al. 2010). Furthermore, it is a model system for speech learning (Jarvis 2004) which can be impacted in humans prenatally exposed to methylmercury (Grandjean et al. 1997). We administered environmentally relevant doses of methylmercury to adult zebra finches in the diet. In half of the birds, methylmercury was delivered as MeHgCl, whereas the other half received MeHgCys. Mercury accumulation was measured in the blood, muscle, brain, liver, kidney and eggs. If dietary MeHgCl

has a reduced bioavailability relative to MeHgCys, as is seen in injection studies, we would expect lower accumulation of mercury in birds exposed to MeHgCl, particularly in the brain and egg where additional processes are required for bioaccumulation.

Materials and Methods

Birds were maintained indoors in small (60"×30"×24") wire cages in single sex groups until 10 weeks after the start of dosing, and in pairs thereafter. Animal rooms were maintained on a 14:10 h light:dark cycle at ~20°C. Birds were provided with food and water ad libitum as well as access to digestion-aiding grit and calcium-rich cuttlefish bone. A liquid vitamin supplement (Vita-Sol UltraVite Bird Vitamin Supplement) was added to the water supply to encourage breeding.

Birds were fed a commercially available pelletized finch food (Zupreem FruitBlend Flavor Premium Daily Bird Food XS). Zebra finches are a primarily granivorous bird; the birds in our population prefer a natural seed food, such as millet. However, it is challenging to dose millet based foods as the seeds have a husk the birds remove before eating, which could reduce the exposure to mercury. The pelletized food readily absorbs a mercury solution, however our birds had to be trained to consume this food, and refused several other brands of pelletized food. In natural situations, birds likely consume methylmercury complexed into proteins in their prey. In the lab, this is often duplicated by feeding a diet based on contaminated wild fish. In this case, due to the difficulties with food preference and dietary requirements, it was not practical to feed a fish-based food to the zebra finches. However, fish-based diets may be impractical in many systems, and understanding the differences between methylmercury solutions is still of value to many dosing studies.

MeHgCl solutions were made by dissolving 100 mg of solid MeHgCl in 20 mL of 100% ethanol. This solution was then added to 2 L of distilled water to make a 40 ppm stock solution. To make MeHgCys, 2 L of distilled water was degassed with nitrogen and 100 mg of L-cysteine was added representing 2 times the molarity of methylmercury. 100 mg MeHgCl was dissolved in 20 mL of 100% ethanol and added to the cysteine solution to make a 40 ppm stock solution. These stocks were then diluted with distilled water to the desired concentration.

The ability of cysteine to displace the chloride in the MeHgCl salt was confirmed by using a chloride selective electrode (Vernier, Model CL-BTA) to measure free chloride ion in solution. The electrode was calibrated using a two point calibration. A 5.0 mL volume of each standard, deionized water blanks, and the methylmercury solutions

were spiked with five drops of 5 M NaNO₃ solution to provide sufficient conductivity in all of the solutions as recommended for the electrode when measuring solutions with low ion concentrations. The solution containing 40 ppm Hg with no cysteine added generated a negligible signal when corrected for the blank signal, which was expected since the chloride is bound to the methylmercury. Triplicate measurements of the cysteine-containing solution generated an average signal of 6.9 (+/−0.6) mg/L. The expected chloride concentration for the 40 ppm MeHgCys solution was 7.06 mg/L assuming complete displacement of the chloride by cysteine. Given that the measurement made is close to the limit of detection for the electrode (~1 mg/L), the value is in good agreement with the expected concentration, and a good confirmation that the MeHgCys analog is produced.

Food was dosed by adding an aqueous solution of MeHgCl or MeHgCys equal to 10% of the weight of the food. For both forms of mercury, two dosing levels were used, 0.5 and 1.0 ppm fresh weight. After thorough mixing of the food and aqueous solution, actual mercury concentrations of each batch were measured from ten subsamples of food. The average concentration of these subsamples was calculated to confirm that the concentration of the batch did not differ from the target concentration by more than 10%. All food was stored in freezers until being fed to birds.

Adult birds (~100–300 days old) were randomly assigned to the four treatments (0.5 MeHgCl, 0.5 MeHgCys, 1.0 MeHgCl, 1.0 MeHgCys) with 14 birds (7 males, 7 females) in each treatment. Birds were provided only with dosed food through the end of the study. For the first 10 weeks of dosing, birds were kept in single sex cages with 3–4 birds in each cage. Every 2 weeks during this period small blood samples (~50 µL) were collected from each bird for mercury analysis (see tissue collection below). At 10 weeks the blood mercury levels appeared to have plateaued and the birds were randomly paired within treatment groups. After pairing, blood samples were collected for mercury analysis every 3 weeks. Each pair was kept in a separate cage visually isolated from other birds and provided with a nesting box and nesting material (hay) ad libitum. The first clutch of eggs was collected for mercury analysis. Pairs were then allowed to raise one clutch of offspring until independence (50 days post hatching). After the offspring were independent the parents were humanely sacrificed. Because of differences in the rate of reproduction between pairs, total time of dosing varied from 6 to 9 months.

Blood samples were collected by puncturing the brachial vein with a 30-gauge sterile needle. The resulting droplet of blood was then collected into heparinized microcapillary tubes, and sealed with critocaps (Leica BioSystems). Eggs were collected 2 days after clutch completion and cracked

into small glass vials. All eggs were very early in development (less than approximately stage 15, Murray et al. 2013) reflecting fewer than 3 days of incubation. In the further analysis the entire egg contents, without the shell or shell membranes, were used. Immediately prior to sacrifice, 50 µL of blood was taken from the jugular vein using an insulin syringe with a 30 gauge needle. Birds were then killed by rapid decapitation and the brain, liver, kidneys, and one-half of the pectoral muscle were removed for mercury analysis. All tissues were stored at −20°C until processing for mercury analysis.

All mercury analyses were performed using combustion-amalgamation atomic absorption spectrophotometry on a Milestone DMA-80 direct mercury analyzer (Shelton, CT, USA). As a result, all mercury values reported are total mercury. For some bird tissues (e.g., blood, muscle, and egg content), most mercury exists in the methylated form and total mercury is likely to be a good approximation for methylmercury (Ruelas-Inzunza et al. 2009; Ackerman et al. 2013; Rodríguez Álvarez et al. 2013). Demethylation may occur in the liver and kidneys and the percentage of methylmercury is often lower but can vary widely between species (Thompson and Furness 1989; Kim et al. 1996). In our population of zebra finches, birds fed low dietary concentrations of MeHgCys (0.1–0.4 ppm) have relatively high percentage of methylmercury in the liver (88.9% ± 1.1%) and kidney (83.2% ± 2.5%) when compared to previous studies in seabirds (Kim et al. 1996) suggesting that total mercury may be a fairly good representation of methylmercury concentration in these organs in this species. While there are fewer studies on brain tissue, the percentage of methylmercury appears to be variable between species (Cheng et al. 2013; Kalisinska et al. 2014), however, no data are available for the percentage of methylmercury in the brains of zebra finches. Blood samples were run without drying, immediately after being removed from the freezer and allowed to thaw. Blood mercury concentration is reported based on wet weight (ww) throughout. All other tissues were freeze dried and homogenized before being run. All other tissue concentrations except for blood are reported based on dry weight (dw). Food samples were run fresh. The DMA-80 was calibrated using known standards according to machine specifications approximately every 2 months throughout the study period or more often when necessary to keep standard reference material values within 7.5% of certified values. A sample blank, methods blank, duplicate and two samples of each standard reference material (DORM-3 and DOLT-4, NRC-CNRC) were run with every 20 samples. Duplicates were either two separate capillary tubes of blood from the same collection date of the same bird or two samples of homogenized tissue from the same individual. The mean relative difference in duplicate samples was 10.1% ± 2.5%. Minimum detection limit

was 0.003 ng over the entire period of the study. Recovery of total Hg was $102.5\% \pm 0.2\%$ for DORM-3, and $99.6\% \pm 0.2\%$ for DOLT-4.

All statistics were performed using SPSS 19 (IBM). We used general linear models (GLMs) for all analyses of mercury accumulation. The assumption of normality was tested in each treatment for each tissue type using a Shapiro–Wilk test. Only the 0.5 MeHgCl and 1.0 MeHgCl treatment in blood and the 0.5 MeHgCl in brain deviated statistically from normality and the histograms for these revealed a slight skew to the right. Since GLMs are robust to moderate violations of normality (Mardia 1971), we concluded that this method was appropriate for the data. For all GLMs, the number of days since onset of dosing was also included as a covariate to control for differences in the length of the dosing period between individuals. We used the blood sample taken immediately prior to sacrifice for our analysis of mercury accumulation in the blood. For this analysis mercury type (MeHgCl and MeHgCys) and dietary mercury level (0.5 and 1.0 ppm) and the interaction between the two were fixed effects. Because there was a large amount of variation in the accumulation of mercury in the blood (a close to threefold difference between the highest and lowest individuals in each treatment) and because blood mercury levels correlate strongly with mercury levels in other tissue types, we analyzed the other five tissues in two ways, using dietary mercury level as a fixed effect and using individual blood mercury as a covariate. We did this because much of the variation in tissue levels is explained by individual difference in overall accumulation. The large amount of individual variation could swamp a subtle difference in accumulation to the different tissues. These analyses also included mercury type and the interaction between mercury type and either dietary mercury level or blood mercury. For the analysis of egg mercury levels, the female's blood mercury concentration from the sample prior to the onset of laying was used instead of the terminal blood mercury value and the number of days between the onset of dosing and the day the first egg was laid was used to control for differences in latency to the onset of breeding.

Results and Discussion

The GLM for total blood mercury explained a significant amount of the variation in blood mercury level between individuals (Table 1), and dietary mercury level had a strong positive effect on blood mercury concentration (Table 1). However, type of mercury had no effect on total blood mercury concentration and there was no interaction between dietary level and mercury type (Table 1; Fig. 1a). Similarly, the GLMs using dietary mercury level for all other tissues also explained a significant amount of

variation between individuals (Table 1). In all cases, dietary mercury level had a significant effect on total mercury concentration, but mercury type had no effect and there was no interaction between the two (Table 1; Fig. 1b–f).

Models using blood mercury instead of dietary mercury levels were similar but explained a greater amount of the variation in tissue mercury concentration (Table 2). Mercury concentrations in all tissues showed a strong positive relationship with blood mercury concentration (Table 2). However, in no tissue was the type of mercury in the diet related to mercury accumulation (Fig. 2) and there was no significant interaction between blood mercury and mercury type (Table 2).

The type of methylmercury added to the diet had no effect on mercury accumulation in any of the tissues measured. Mercury concentrations were related to the concentration of mercury present in the diet; however there was a large amount of individual variation in accumulation within each treatment. Blood mercury concentrations were closely associated with concentrations in all other tissues sampled, suggesting no differences between tissues in how the types of mercury were distributed throughout the body. This included egg and brain, for which additional processes, such as passage through the blood–brain barrier or deposition into egg contents, are necessary for accumulation. It is possible that differences in the percentage of mercury that is methylated exists, particularly in the liver, kidney, and brain where demethylation is known to occur, but we are unable to detect this because we only measured total mercury. Rats fed a diet spiked with MeHgCl accumulated mercury to a higher level than those fed naturally contaminated fish in which the methylmercury was presumably bound to cysteine (Berntssen et al. 2004). The discrepancy between our lack of a difference and that study may have resulted from the methylmercury naturally present in fish being bound into whole proteins in contrast to its attachment to independent cysteine molecules in our dosed diet (Bourdineaud et al. 2012). Inefficiency in protein digestion may explain why larger amounts of methylmercury were excreted in the rat feces when they were fed protein-bound mercury. Since methylmercury was not bound to whole proteins in either of the treatments used here, this may help explain the lack of differences in accumulation between the two forms.

Much remains unknown about the fate of methylmercury complexes during digestion. Methylmercury cysteine is stable during simulated digestions (George et al. 2008). It is possible that MeHgCl converts to MeHgCys during the course of digestion due to methylmercury's strong affinity for thiol groups. If this is the case, it would explain the lack of difference between the two forms found here. Previous studies have suggested that MeHgCys may form large hydrophobic complexes that are excreted in the feces,

Table 1 Statistical results from general linear models of tissue total mercury concentration using mercury type and dietary dose as fixed effects and days between onset of dosing and sampling as a covariate

Dependent variable	Model component	F stat.	DF	<i>p</i> value	N	R ²
Blood Hg (ww)	Full model	12.37	4,51	> 0.001	56	0.49
	Hg type	0.45	1,51	0.51		
	Hg dose	35.96	1,51	> 0.001		
	Days dosed	3.36	1,51	0.07		
	Hg type × Hg dose	0.13	1,51	0.73		
Eggs Hg (dw)	Full model	6.085	4,23	0.002	28	0.51
	Hg type	0.38	1,23	0.54		
	Hg dose	23.51	1,23	> 0.001		
	Days dosed	1.24	1,23	0.28		
	Hg type × Hg dose	0.13	1,23	0.72		
Muscle Hg (dw)	Full model	20.28	4,51	> 0.001	56	0.61
	Hg type	0.43	1,51	0.52		
	Hg dose	60.24	1,51	> 0.001		
	Days dosed	4.89	1,51	0.03		
	Hg type × Hg dose	0.15	1,51	0.70		
Brain Hg (dw)	Full model	20.46	4,51	> 0.001	56	0.62
	Hg type	1.08	1,51	0.31		
	Hg dose	65.23	1,51	> 0.001		
	Days dosed	2.67	1,51	0.11		
	Hg type × Hg dose	0.09	1,51	0.77		
Liver Hg (dw)	Full model	17.65	4,51	> 0.001	56	0.58
	Hg type	0.16	1,51	0.69		
	Hg dose	59.42	1,51	> 0.001		
	Days dosed	1.19	1,51	0.28		
	Hg type × Hg dose	0.08	1,51	0.78		
Kidney Hg (dw)	Full model	15.31	4,50	> 0.001	55	0.55
	Hg type	1.19	1,50	0.28		
	Hg dose	49.01	1,50	> 0.001		
	Days dosed	1.43	1,50	0.24		
	Hg type × Hg dose	0.53	1,50	0.47		

whereas MeHgCl is more easily absorbed into the intestinal wall (Berntssen et al. 2004). However, a study where MeHgCl and MeHgCys were injected directly into the intestinal lumen showed no difference in uptake rates (Mori et al. 2012). These results combine with the findings of this study to suggest that there is no difference in intestinal uptake between MeHgCys and MeHgCl, or that chemical conversion of these two forms to a similar form occurs very rapidly in the digestive system. Further research is needed to determine the form of methylmercury that occurs in the intestine after digestion.

Although we found no difference in mercury accumulation between the two forms of mercury in any of the tissues studied, it is possible that there is still a subtle effect of the form of methylmercury that could result in differences in how exposure affects individuals. In mice there is some suggestion that the form of mercury fed in the diet may alter behavioral and gene expression endpoints (Glover et al. 2009; Bourdineaud et al. 2012). However, in studies

run parallel to that described here, no effect of form of dietary methylmercury was found on reproductive success (Maddux et al. 2015), behavior (Bessler 2011), endocrinology (Maddux et al. 2015), or immunocompetence (Lewis et al. 2013). In all, we found no reason to suspect that birds respond any differently to dietary exposure to MeHgCl relative to MeHgCys.

Because there do not appear to be any differences in accumulation or effects between MeHgCl and MeHgCys, the choice of form of dietary methylmercury does not appear to be of major consequence in dosing studies and MeHgCys may not represent a “more realistic” form of the contaminant. On the other hand, much more needs to be known before it can be concluded that off-the-shelf MeHgCl provides a good model for environmental contamination. Results to date suggest that there are differences between dosing with fish contaminated in its environment versus experimental diet with added mercury (Berntssen et al. 2004; Bourdineaud et al. 2012). Further

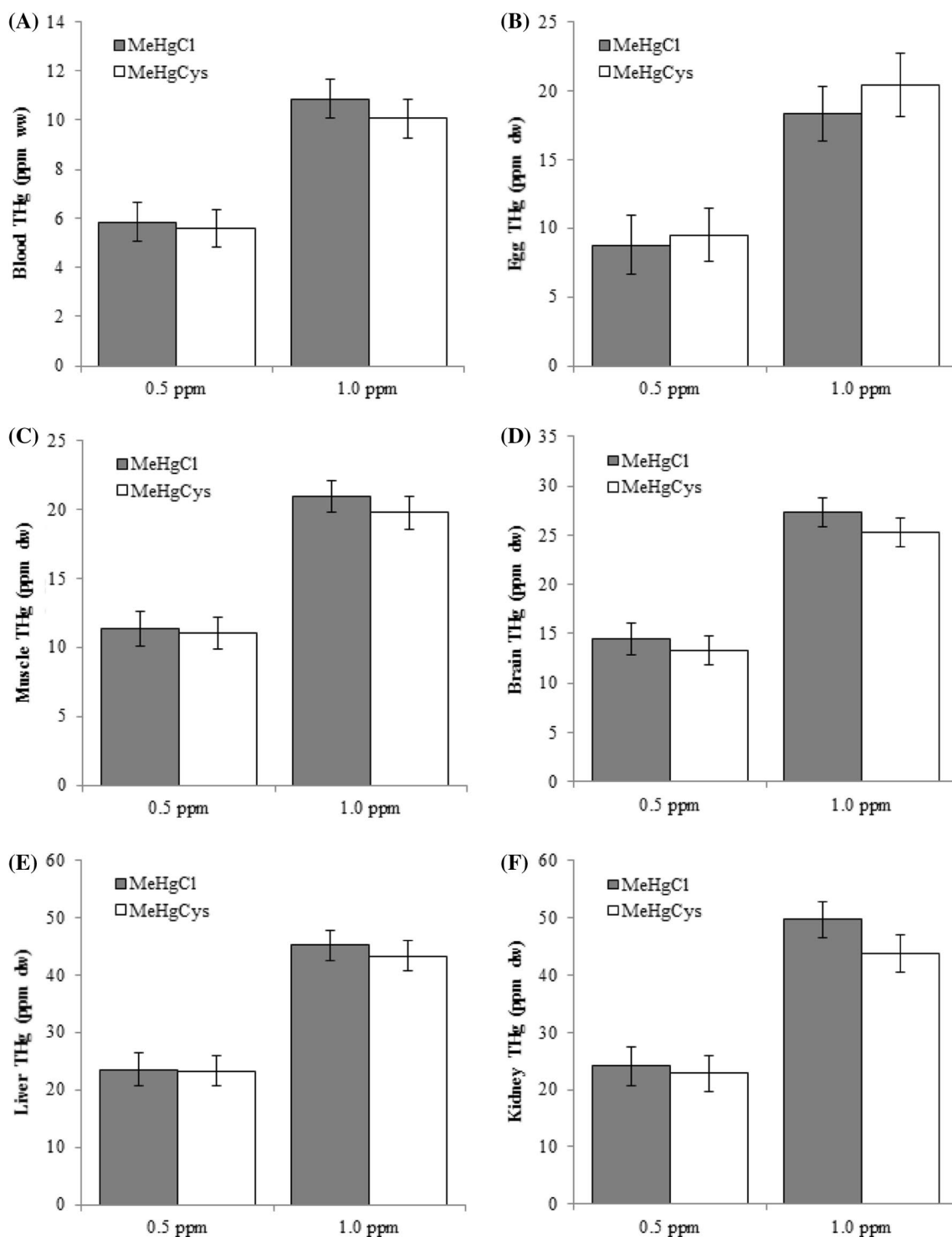
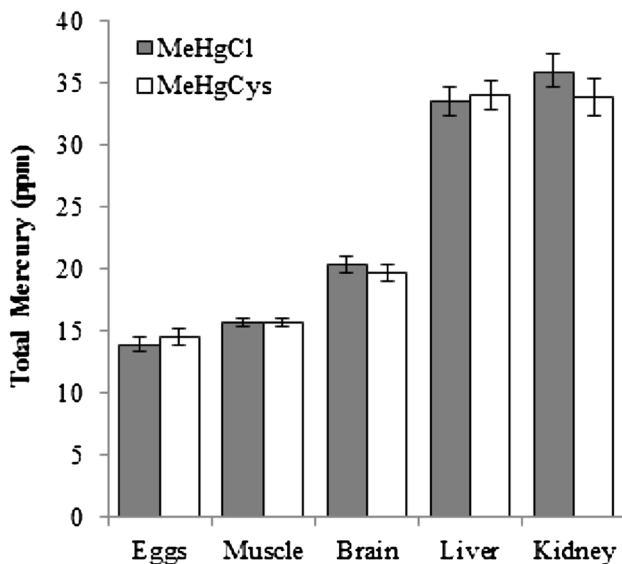


Fig. 1 Least-square mean tissue total mercury concentrations from a general linear models including methylmercury type (MeHgCl *gray*, MeHgCys *white*), dietary mercury level, the interaction between type and level, and the number of days dosed. *Error bars* are one standard error. **a** Blood total mercury concentration (wet weight). **b** Egg con-

tents total mercury concentration (dry weight). **c** Muscle total mercury concentration (dry weight). **d** Brain total mercury concentration (dry weight). **e** Liver total mercury concentration (dry weight). **f** Kidney total mercury concentration (dry weight)

Table 2 Statistical results from general linear models of tissue total mercury concentration using mercury type as a fixed effect and blood mercury concentration and days between onset of dosing and sampling as a covariates

Dependent variable	Model component	F stat.	DF	p value	N	R ²
Eggs Hg (dw)	Full model	60.76	4,23	>0.001	28	0.91
	Hg type	2.11	1,23	0.16		
	Blood Hg	217.55	1,23	>0.001		
	Days dosed	2.25	1,23	0.15		
	Hg type × blood Hg	1.75	1,23	0.20		
Muscle Hg (dw)	Full model	205.88	4,51	>0.001	56	0.94
	Hg type	0.29	1,51	0.59		
	Blood Hg	671.51	1,51	>0.001		
	Days dosed	1.52	1,51	0.22		
	Hg type × blood Hg	0.37	1,51	0.55		
Brain Hg (dw)	Full model	82.87	4,51	>0.001	56	0.87
	Hg type	0.02	1,51	0.87		
	Blood Hg	277.40	1,51	>0.001		
	Days dosed	0.01	1,51	0.92		
	Hg type × blood Hg	0.25	1,51	0.62		
Liver Hg (dw)	Full model	68.81	4,51	>0.001	56	0.84
	Hg type	0.05	1,51	0.83		
	Blood Hg	241.40	1,51	>0.001		
	Days dosed	0.42	1,51	0.52		
	Hg type × blood Hg	0.01	1,51	0.93		
Kidney Hg (dw)	Full model	52.07	4,50	>0.001	55	0.81
	Hg type	0.03	1,50	0.87		
	Blood Hg	176.76	1,50	>0.001		
	Days dosed	0.05	1,50	0.83		
	Hg type × blood Hg	0.07	1,50	0.79		

**Fig. 2** Least-square mean tissue mercury concentrations (dry weight) from general linear models including methylmercury type (MeHgCl gray, MeHgCys white), blood mercury concentration, the interaction between type and blood mercury, and the number of days dosed. Error bars are one standard error

research is needed to determine how uptake and distribution differs between these forms, and the mechanisms underlying these differences. These studies would allow for more realistic dosing studies with broader applications in the future.

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Compliance with Ethical Standards

Conflict of interest DA Cristol received research funding from E.I. DuPont de Nemours and Company.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. 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).

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