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NIVELES DE MERCURIO EN HUEVOS, EMBRIONES Y NEONATOS DE *Trachemys callirostris* (TESTUDINES, EMYDIDAE)

Mercury Levels in Eggs, Embryos, and Neonates of *Trachemys callirostris* (Testudines, Emydidae)

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RESUMEN

Cuantificamos la concentración total de mercurio en cáscaras, yemas y embriones de 16 nidos de hicotea (*Trachemys callirostris*). Los nidos fueron colectados en diferentes estadios de desarrollo embrionario. No hubo una correlación entre el tiempo estimado desde el desove y los niveles de mercurio en los huevos, sugiriendo que el metal no fue absorbido del substrato, sino que probablemente éste fue transferido a los huevos durante el proceso de foliculogénesis en las hembras reproductivas, las cuales bioacumularon el mercurio de fuentes ambientales. La concentración promedio de mercurio fue mayor en los embriones que en las cáscaras o yemas, indicando que los embriones también bioacumulan el metal presente en otros tejidos del huevo. La variación de la concentración de mercurio dentro de una misma nidada fue relativamente alta. Las concentraciones de mercurio en las yemas no estuvieron asociadas con ninguna de las medidas de *fitness* que fueron evaluadas (éxito de eclosión, tamaño inicial de los neonatos y tasas de crecimiento de los juveniles en el primer mes). Después de cinco meses de mantenimiento en cautiverio, en un ambiente libre de mercurio, 86 % de los juveniles había eliminado completamente este metal de sus tejidos.

Palabras clave: bioacumulación, hicotea, mercurio, metales pesados, yema.

ABSTRACT

We quantified total mercury concentrations in eggshells, egg yolks, and embryos from 16 nests of the Colombian Slider (*Trachemys callirostris*). Nests were collected in different stages of development, but estimated time of incubation in natural substrates was not correlated with mercury levels in the eggs, suggesting that mercury was not absorbed from the substrate, but more likely passed on to the embryos during folliculogenesis by the reproductive females who had bioaccumulated the mercury from environmental sources. Mean mercury concentrations were higher in embryos than in eggshells or egg yolks, indicating that embryos also bioaccumulate mercury present in other egg tissues. Intra-clutch variation in egg yolk mercury concentrations was relatively high. Egg yolk mercury concentrations were not associated with any of the fitness proxies we quantified for the nests (hatching success rates, initial neonate sizes and first-month juvenile growth rates). After five months of captive rearing in a mercury-free laboratory environment, 86 % of the juveniles had eliminated the mercury from their tissues.

Keywords: bioaccumulation, Colombian slider, heavy metals, mercury, yolk.

INTRODUCTION

Elemental and inorganic mercury enters natural ecosystems through its conversion by microorganisms into methylmercury, which readily bioaccumulates and biomagnifies as it passes through the food web. Most studies of mercury bioaccumulation focus on species consumed by humans and simply document whether mercury levels in different tissues pose threats to the humans who consume them (e.g., Holden, 1973; Hall *et al*., 1978; Eisler, 1981; Goldstein *et al.,* 1996; Sunderland, 2007). However, many wildlife species consumed by humans are over-exploited, and contamination of their habitats with mercury released by human activities represents an additional threat to these populations (Eisler, 2006; Schneider *et al.,* 2013). Mercury bioaccumulation by wildlife species has been associated with changes in patterns of genetic diversity, reduced reproductive success rates, slower growth rates, impaired behavior, infertility, and suppressed immune functions (Hall *et al*., 1980; Wolfe *et al*., 1998; Hopkins, 2000; Theodorakis *et al*., 2000; Day *et al*., 2007). Therefore, it is important to study the rates and mechanisms of mercury elimination by individuals of threatened wildlife species that occur in contaminated habitats and how mercury bioaccumulation affects the demography of their populations.

Wildlife species may purge their bodies of bioaccumulated mercury via urine, feces, and by sequestering it in inert body components such as hair, feathers, claws, or shells (USNAS, 1978; EPA, 2000). Females have an additional mechanism that is unavailable to males, because they also may eliminate mercury when they produce offspring (Becker, 1992; Lewis and Furness, 1993; Burger, 1994). However, this strategy may affect the fitness of their offspring, especially given that the adverse effects of methylmercury are often strongest in the earliest stages of development of an organism (Rodier, 1995; Boening, 2000).

In turtles, mercury has been found in liver, kidney, carapace, muscle, and egg tissues (in order of usual decreasing concentrations); levels of mercury in blood are usually lower than in the previously mentioned tissues except immediately following exposure (Schneider *et al.,* 2013). Given the relatively high concentrations of mercury in liver tissues of contaminated turtles and the role the liver plays in the production of egg yolk components, it appears that mercury present in egg tissues is deposited during vitellogenesis (Schjeide *et al.,* 1963; Heck *et al.,* 1997), although the alternate possibility that mercury in eggs is absorbed directly from the incubation substrate is usually not evaluated (Schneider *et al*., 2013). Mercury levels in tissues in turtles sometimes exceed maximum recommended levels for human consumption (0.2 µg/g tissue in sensitive populations, IPCS, 1990; 0.5 µg/g tissue in general populations, EPA, 2000), but the effects of sub-lethal levels of mercury bioaccumulation on the fitness of contaminated turtles, or the levels of bioaccumulation necessary to evoke them, have not been widely studied (Schwartz and Flamenbaum, 1976; Day *et al.,* 2007).

The Colombian Slider (*Trachemys callirostris*, or *Trachemys ornata callirostris*, *sensu* Fritz *et al.,* 2012) is the most heavily exploited wildlife species in northern Colombia and is classified as Near Threatened (Castaño-Mora, 2002; Bock *et al.,* 2010; Bock *et al.,* 2012). Northern Colombia suffers from high levels of mercury contamination because the use of agricultural fungicides and pesticides that contain mercury is common there, and because local artisanal gold miners employ amalgamation methods that produce large amounts of mercury wastes as a by-product (UNEP, 2002). In this study, we show that female *T. callirostris* from the middle Magdalena River drainage in northern Colombia lay eggs containing mercury, investigate possible effects of this presumed trans-generational transfer of contamination on the offspring produced, and examine how juveniles eliminate the mercury passed on to them by their mothers.

MATERIALS AND METHODS

In March 2011 we collected 16 *T. callirostris* nests in different stages of development along the margins of the Chicagua River (9° 07' N, 74° 37' W), a branch of the Magdalena River located downstream from a major artisanal mining focus of mercury contamination in northern Colombia. It was not possible to obtain samples from a reference site, because Hg contamination occurs throughout the Magdalena River drainage (Olivero-Verbel *et al*., 2004; Alvarez *et al*., 2012; Rúa *et al*., 2013). Mean clutch size was 8.4 eggs, comparable to reports of 9.5 eggs (Cortés-Duque, 2009) and 11 eggs (Bernal *et al.,* 2004; Restrepo *et al.,* 2007) per clutch on average in other locations.

Eggs were packed in soil inside of Styrofoam coolers and flown to the Laboratory of Herpetology of the Universidad de Antioquia in Medellin. All eggs were cleaned, weighed (± 0.1 g), and measured for maximum length and width (± 0.01 mm). Egg volumes were calculated using the ellipsoid formula of Iverson and Ewert (1991),

$$Vol = \frac{4\pi}{3000}\left(\frac{EL}{2}\right)\left(\frac{EW}{2}\right)^2$$

where Vol = egg volume (cm^3), EL = egg length (mm), and EW = egg width (mm). Two small clutches of two and five eggs were frozen for later analysis of total mercury content (THg, see below), as was one egg from each of the remaining 14 clutches. When a clutch contains eggs that seem inviable, they are usually the smallest, so we selected the smallest egg from each nest, to help maximize the number of hatchlings produced from the incubated eggs.

The remaining eggs were half-buried in a 1:2 weight ratio of vermiculite:H_20 in containers that were covered with plastic wrap to slow dehydration (see Páez *et al.,* 2009 for

the details of the incubation protocols) and assigned to one of three incubators (Binder, Inc., Bohemia, New York, USA). An approximately equal number of eggs from each nest were assigned to the three incubators, each set to maintain constant temperatures of 28.5, 30.0, or 31.5 °C (± 0.4 °C). These temperatures were selected to help insure that both male and female hatchlings were obtained from the nests, given sex ratios of nests in the field that suggest this species has temperature-dependent sex determination, with nests incubating at higher temperatures producing females and nests incubating at lower temperatures producing males (TSD pattern Ia; Restrepo *et al.,* 2007). Each incubator held seven containers, each with five or six eggs from different nests, and containers were rotated weekly among shelves to minimize effects of temperature gradients within the incubators. The containers were weighed before they were placed into the incubators, and were re-weighed weekly and water lost to evaporation was replaced.

When hatching began, all eggs were inspected daily and pipped eggs were isolated in their incubators until the neonates emerged and absorbed the majority of their yolk sacs. Neonates were then individually marked by notching their marginal scutes, measured (straight-line carapace length, SCL ± 0.01 mm), weighed (± 0.1 g) and placed into one of two aquaria that contained 80 x 60 x 10 cm of tap water maintained at 28 °C. The aquaria contained rocks to provide hiding and basking sites and were illuminated with UVB light bulbs on a 12:12 h on:off schedule. Food was provided *ad libitum* and consisted of carrot (*Daucus carota*) shavings and water hyacinth (*Eichornia crassipes*).

Because the nests were in different stages of development when collected and the eggs were incubated at different temperatures, growth rate analyses were only conducted on nests that incubated a minimum of 25 days in the artificial conditions, with each neonate being re-measured and re-weighed upon attaining 30 and 60 days of age. In the field, growth rates of juvenile slider turtle species are essentially linear during the first year (Dunham and Gibbons, 1990; Tucker *et al.,* 1999). However, our laboratory growth rates were initially high, but slowed markedly after the first month of life. We interpret this change as being related to a switch from reliance primarily on internalized yolk reserves for growth to reliance on possibly sub-optimal diet items. For this reason, in the following analyses, we only consider the initial growth rate data obtained during the first month.

Hatchlings from the two nests that exhibited the fastest initial growth rates and the two nests that exhibited the slowest initial growth rates were sacrificed after five months of captive rearing with injections of sodium pentobarbital and samples of skeletal muscle and carapace were frozen for later analysis of THg levels. The remaining turtles were incorporated into another study, but eventually also were sacrificed at approximately 15 months of age. All of the sacrificed turtles were sexed by one person (VPP) via inspection of the gross anatomy of the gonads and oviducts (Malvasio *et al.,* 1999).

We quantified THg levels in samples of eggshell and egg yolk obtained from the 21 eggs that were refrigerated, as well as in samples of total tissue from embryos in later stages we encountered in 11 of these eggs. We also quantified THg levels in the skeletal muscle and carapace samples from the 22 individuals sacrificed at five months of age, to see whether they had been able to purge the mercury from a typical and a relatively inert tissue type, respectively. All samples were digested using sulfuric acid, nitric acid and potassium permanganate (EPA, 2000) and THg concentrations were determined by cold vapor atomic absorption spectrometry, using a BUCK Scientific 410 cold vapor mercury analyzer (Buck Scientific Inc., Norwalk, Connecticut, USA). THg concentrations are reported as µg/g wet weight. Quality assurance was determined using 10 yolk sample duplicates, calibration standards, blank samples, and certified samples (DORM-2 dogfish muscle from the National Research Council of Canada). The DORM-2 certified value for THg was 4.64 ± 0.26 and our analyses yielded a value of 4.31 ± 0.18 µg/g (n = 3). Recoveries were reported at 92.7 % for THg for the DORM-2 reference material. Coefficients of variation for the yolk sample duplicates were estimated to be less than 0.7. The reliable quantification limit for THg of the method was 0.1 µg/g in 0.5 g of sample. Because some of the values we obtained in this study were below this level, we employed non-parametric analyses for those data.

In two nests, THg data were obtained from more than one egg, and in one of these nests, we also quantified THg levels in both embryos the eggs contained. Given the lack of independence of these data, we used mean values for these nests to compare nests for THg levels in eggshells, egg yolks and embryos using a Kruskal-Wallis test and to inspect for associations among these tissue types using Spearman correlations.

Hatching success rates and proportions of embryos vs. neonates with mercury concentrations that exceeded the mercury detection limit of the method (0.02 µg/g) were compared using X^2 tests. ANOVAs were employed to inspect for sex, incubator, or nest effects on neonate dependent variables that were all normally distributed, except for neonate weight in one nest (Shapiro-Wilk tests). Spearman correlations were used to examine for effects of THg egg yolk concentrations on neonate dependent variables. All analyses were conducted using the JMP software package with a critical value of $\alpha < 0.05$.

Juvenile turtles were reared and sacrificed according to the animal welfare guidelines of the American Society of Ichthyologists and Herpetologists (HACC, 2004) and voucher specimens (MHUA-R 18328-18349) were deposited in the Museo de Herpetología de la Universidad de Antioquia, an officially recognized depository of biodiversity in Colombia (Instituto von Humboldt, Collection 080).

RESULTS

The *T. callirostris* eggs obtained from the Chicagua River contained mercury, with embryos exhibiting the highest levels (mean = 0.079 µg/g, range = 0.013–0.389), egg yolks having intermediate levels (mean = 0.031 µg/g, range = 0.011–0.145), and eggshells the lowest levels of THg (mean = 0.010 µg/g, range = 0.001–0.023; Kruskal-Wallis, df = 2, $p < 0.001$; Table 1, Fig. 1). THg levels were higher in embryos than in egg yolks in nine of the eleven eggs found to contain embryos. Egg yolk THg levels were not related to the time the eggs incubated artificially (Spearman Rho = 0.07, $p > 0.10$) and were comparable in the two eggs examined from one nest, but in the nest where all five eggs were analyzed, variance in THg was almost 50 % of the total variance documented among the 16 nests inspected (Fig. 2). Mercury levels in eggshells were positively correlated with levels quantified in yolks from the same eggs (Spearman Rho = 0.56, $p < 0.05$) and with levels in embryos (Spearman Rho = 0.81, $p < 0.05$), but the relationship between THg levels in egg yolks and embryos was not significant (Spearman Rho = 0.47, $p > 0.10$). THg levels in egg yolks were not related to egg size (egg volume, Spearman Rho = -0.39, $p > 0.10$; egg weight, Spearman Rho = -0.48, $p = 0.06$).

The hatching success rate in this study was 93 % and did not vary among incubators ($X^2 = 2.96$, df = 2, $p > 0.10$), but differed among nests ($X^2 = 25.70$, df = 9, $p < 0.005$) and was not associated with the time the nests incubated artificially (Spearman Rho = -0.30, $p > 0.10$), or with mean egg yolk THg concentrations (Spearman Rho = 0.39, $p > 0.10$). Although this study was not designed to corroborate the existence of TSD, we documented significant sex ratio differences among the incubators ($X^2 = 17.09$, df = 2, $p < 0.001$), with approximately equal numbers of males and females obtained from the lowest incubation temperature and an excess of females produced in the other two incubators. Seven nests produced only females or just one male, but in the remaining five nests, sex did not influence initial hatchling size (SCL, $F_{1,39} = 0.01$, $p > 0.10$; weight, $F_{1,39} = 0.04$, $p > 0.10$), so males and females were pooled for subsequent analyses.

Initial hatchling sizes were not affected by laboratory incubation temperature (neonate SCL, $F_{2,89} = 0.34$, $p > 0.10$; neonate weight, $F_{2,89} = 0.32$, $p > 0.10$) or time the eggs incubated artificially (neonate SCL, Pearson $r = -0.24$, $p > 0.10$; neonate weight, Pearson r = -0.33, $p > 0.10$). However, there were significant maternal (nest) effects on initial

Table 1. Total mercury concentration (µg/g) in samples of egg yolk, eggshell, and embryos for each nest, with mean times the eggs incubated in the laboratory and nest hatching success rates.

Nest	[Hg] yolk	[Hg] eggshell	[Hg] embryo	Mean days in artificial incubation	% Hatching success
1	0,011	0,006	0,02	19	100
2	0,012	0,001	0,013	27	100
3	0,013	0,007	0,051	38	100
4	0,013	0,008	0,026	12	100
5	0,013	0,002	NA	40	100
6	0,016	0,007	NA	34	60
7	0,016	.	0,119	35	88
8*	0,014	0,05	0,08	NA	NA
8*	0,024	.	0,012	NA	NA
9	0,020	0,022	0,043	36	100
10	0,020	0,01	0,064	25	100
11*	0,009	0,006	NA	NA	NA
11*	0,012	0,047	NA	NA	NA
11*	0,013	0,005	NA	NA	NA
11*	0,013	0,018	NA	NA	NA
11*	0,061	0,039	NA	NA	NA
12	0,023	0,002	0,016	14	100
13	0,033	0,013	0,389	28	100
14	0,035	.	NA	NA	NA
15	0,084	0,008	NA	41	88
16	0,145	0,019	NA	33	67

*Clutches in which all eggs were used for analysis of total mercury content (THg), NA: Not applicable

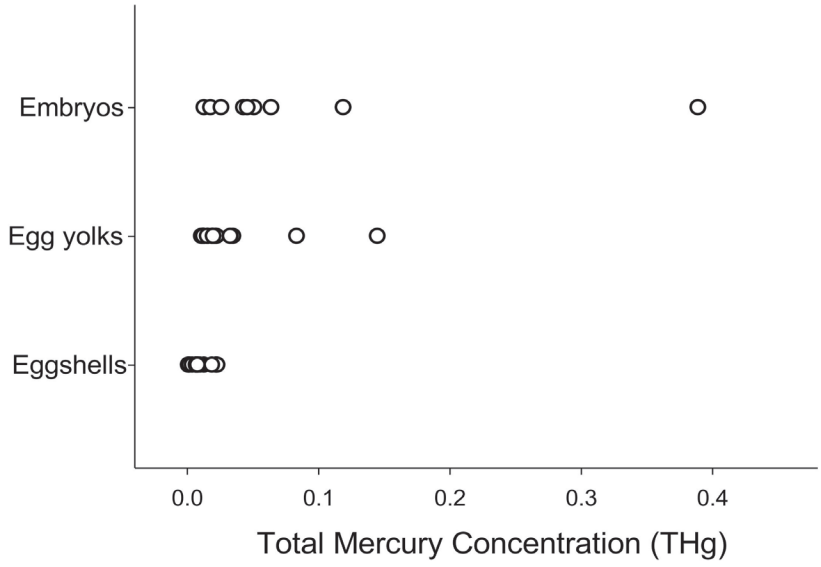

Figure 1. Total mercury concentration (µg/g) in samples of eggshell, egg yolk, and embryos of *Trachemys callirostris* from the Chicauga River, northern Colombia.

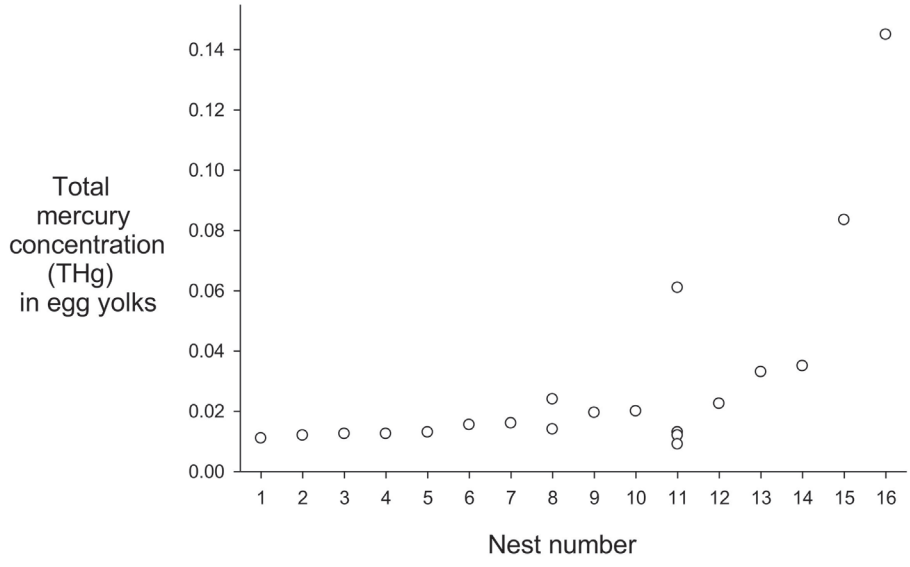

Figure 2. Levels of intra and inter-clutch variation in total mercury concentration (µg/g) of egg yolks of *Trachemys callirostris* from the Chicauga River, northern Colombia.

neonate sizes (neonate SCL, $F_{11,80} = 13.71$, $p < 0.001$; neonate weight, $F_{11,80} = 14.06$, $p < 0.001$). Finally, nest mercury contents were not related to mean nest initial hatchling sizes (neonate SCL, Spearman Rho = -0.29, $p > 0.10$; neonate weight, Spearman Rho = -0.23, $p > 0.10$). The maternal effect persisted during the first month of growth (SCL growth, $F_{11,80} = 3.15$, $p < 0.005$; weight growth, $F_{11,80} = 9.92$, $p < 0.001$), but again nest mercury contents were not related to mean nest initial growth rates (SCL growth, Spearman Rho = -0.20, $p > 0.10$; weight growth, Spearman Rho = 0.07, $p > 0.10$).

After five months of captive rearing in the laboratory in mercury-free conditions, only three of the 22 individuals examined contained levels of mercury above the quantification level (muscle samples for two individuals and a carapace sample for another), indicating a significant reduction in the proportion of individuals showing signs of substantial mercury bioaccumulation, as compared to the proportion of embryos with substantial mercury concentrations in their muscle tissues ($X^2 = 12.6$, df = 1, $p < 0.001$).

DISCUSSION

The time the eggs spent incubating in natural substrates in this study before being transferred to vermiculite was not related to their THg levels, and it has been shown that female *T. callirostris* from this region bioaccumulate THg into their tissues (Zapata *et al.,* 2014), so it seems likely the mercury present in the eggs in this study was maternally derived. The fact that the embryos contained higher THg concentrations than the shell or yolk samples also suggests that the embryos bioaccumulate the mercury present in these egg tissues during development. But the larger (presumably more developed) embryos did not contain higher concentrations of mercury in their tissues than smaller embryos. Perhaps the flux of mercury during development is initially into the embryos, but after the allantois develops (approximate Stage 12 of Yntema, 1968) and the excretory system of the embryos begins to function, they may begin to purge their tissues of mercury to some extent.

In birds, THg levels in egg yolks are often highest in the first eggs in a clutch, and decrease gradually as subsequent eggs are laid, presumably because the THg load of the female decreases with each egg produced (Becker, 1992; Sanpera *et al.,* 2000; Akearok *et al.,* 2010). However, turtles deposit the yolk in all eggs in a clutch simultaneously, leading some studies to argue that it is sufficient to examine just one egg per clutch, both when assessing levels of contaminants in egg yolks (Godley *et al.,* 1999; Tryfonas *et al.,* 2006) and when quantifying levels of endogenous hormones that females invest in egg yolks (Bowden *et al.,* 2001; Elf *et al.,* 2002). Some studies have explicitly examined this assumption and corroborated low levels of intra-clutch variation in yolk component concentrations in turtle clutches (i.e., Sakai *et al.,* 1995). Thus, it was surprising in this study to show that one egg (from Nest 12, Fig. 2) contained five times more THg than the other four eggs from this nest that were examined. It might actually be adaptive for female turtles to concentrate most of the Hg that they purge when reproducing into one or a few eggs, thereby minimizing the deleterious impacts of the toxin on the remainder of the clutch. We recognize our data are limited, because our study was not designed to rigorously document such a possibility, but our pilot data argue that more studies should test the assumption that there is minimal intra-clutch variation in yolk composition in turtle clutches, not only to better justify the argument that one egg per clutch is sufficient in yolk content studies, but also to explore the intriguing possibility that females are more sophisticated in their capabilities to adjust investments in individual egg yolks than is currently believed.

In this study, egg yolk THg concentrations were not associated with hatching success rates, initial neonate sizes, or initial growth rates. Our failure to demonstrate significant effects of the levels of mercury in egg yolks on these different fitness proxies could be because the negative effects of mercury are more subtle and difficult to demonstrate, or that all clutches were affected negatively regardless of the magnitude of contamination in the yolks, or because assessing only one egg from a clutch is not a valid method for estimating mercury contents in all the eggs from a given clutch that were incubated. Presumably, maternally-derived mercury intoxication of the neonates ended approximately one month after hatching, when they finished utilizing their internalized yolk reserves. At five months of age, most of the laboratory-reared individuals in this study had purged their bodies of most of the mercury they had accumulated from their egg yolks. Unfortunately, juvenile *T. callirostris* inhabiting the Chicagua River do not experience mercury-free conditions like those we provided in the laboratory, so one cannot conclude from these results that mercury contamination does not represent a problem for juveniles living in the environment.

CONCLUSIONS

This study produced valuable baseline information on Hg bioaccumulation in *T. callirostris* and its maternal transfer to eggs. While the results documented relatively low levels of this toxic metal in the eggs and no evidence that it had adverse effects on the neonates produced, it highlights the need for more studies to attempt to assess the ecological and demographic effects of Hg persistence, bioaccumulation, and biomagnification in aquatic ecosystems on wildlife species. The levels of mercury we documented in the egg yolks were below levels normally deemed dangerous for daily human consumption (IPCS, 1990; EPA, 2000), but additional data are needed before firm recommendations on consumption may be made. Most local people in the middle Magdalena River drainage subsist primarily on fish, and many of the species they harvest for consumption have been shown to contain dangerous levels of mercury in their muscle tissues (Alvarez *et al.,* 2012). Adult *T. callirostris* also have been shown recently to contain comparably dangerous levels of mercury in their muscle tissues (Zapata *et al*., 2014). Thus, it would seem appropriate for educational campaigns to educate local people about the risks of consuming *T. callirostris* adults and eggs, in terms of both human health concerns and the impact such consumption has on the demography of this over-exploited species.

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