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Evidence of mercury biomagnification in the food chain of the cardinal tetra *Paracheirodon axelrodi* (Osteichthyes: Characidae) in the Rio Negro, central Amazon, Brazil

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In this study, nitrogen stable isotope ($\delta^{15}\text{N}$) and total mercury (THg) analyses were conducted on algae, submersed and emergent macrophytes, shrubs and trees, *Macrobrachium* sp. and *Paracheirodon axelrodi* collected in three streams that drain a large interfluvial region in the middle Rio Negro, Amazonas State, Brazil. Samples were collected during different hydrological periods over 12 months in lower stream reaches and their headwaters; the latter being characterized by shallow, open-canopy swamps. Additionally, $\delta^{15}\text{N}$ values and mercury concentrations of *Paracheirodon simulans* and *Cichla* spp. from the middle Rio Negro were analysed to demonstrate THg biomagnification in the food web. The highest mercury levels of *P. axelrodi* were found in small individuals, which were collected principally in the low water period. The $\log_{10}$ THg– $\delta^{15}\text{N}$ relationship of vascular plants and algae, *Macrobrachium* sp., *Paracheirodon* spp. and *Cichla* spp. showed significant mercury biomagnification among trophic levels, with regression slopes of 0.15 and 0.25 for the entire food web and heterotrophs-only food web, respectively. The mean $\pm$ s.d. THg concentrations for *Macrobrachium* sp., *P. axelrodi*, *P. simulans* and *Cichla* spp. were 63.6 ± 23.7 , 104.5 ± 40.0 , 112.3 ± 31.4 and 418.5 ± 188.1 ng g⁻¹ wet mass, respectively. Elevated levels of mercury found in *Paracheirodon* spp. and top predators such as *Cichla* spp. in a remote area far from anthropogenic inputs provide evidence that high mercury concentrations occur naturally in Rio Negro aquatic food webs.

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Key words: blackwater; interfluvial swamps; nitrogen stable isotopes; THg.

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INTRODUCTION

Nitrogen stable isotopes ($\delta^{15}\text{N}$) have been used for over 25 years in dietary studies to delineate trophic level (Minigawa & Wada, 1984; Reuss *et al.*, 2013), due to an average enrichment in ^{15}N of *c.* 3‰ between trophic levels (DeNiro & Epstein, 1981). Mercury has been used in many studies in conjunction with $\delta^{15}\text{N}$ as an indicator of trophic level, due to its biomagnification within a food web (Yoshinaga *et al.*, 1992; Endo *et al.*, 2010). Biomagnification occurs when each level in a food chain, from bacteria to plankton to invertebrates, small fishes, larger fishes and fish consumers take in more mercury than is excreted, causing excess accumulation. This results in an organism having a higher mercury concentration than the Hg in its food.

Typically, the regression slopes of $\log_{10}$ -linear plots of total mercury (THg) and $\delta^{15}\text{N}$ are used to estimate the rate of mercury biomagnification through an aquatic food chain (Kidd *et al.*, 1995; Atwell *et al.*, 1998). For this type of model, the slopes are normally positive and significant (with an average value of *c.* 0.2) for fish communities or their food chains and are consistent among different ecosystems including marine, temperate, Arctic and freshwater tropical (Campbell *et al.*, 2008; Swanson & Kidd, 2010; Zhang *et al.*, 2012).

In contrast to other heavy metals that accumulate in bacteria and microalgae but do not biomagnify, mercury is absorbed by microorganisms at the base of a food chain and increases in concentration from one trophic level to the next (Clarckson, 1997). There are two forms of mercury that bioaccumulate in organisms: Hg (II) in the inorganic form and methylmercury (MeHg) in an organic form. It is considered that conversion of inorganic Hg to MeHg is the most important step for Hg entry into the aquatic food chain, resulting in high mercury concentrations in fishes and humans as Hg biomagnifies through each subsequent trophic level (Barbosa *et al.*, 2003; Chumchal *et al.*, 2008; Hall *et al.*, 2008). On average, the proportion of MeHg to THg increases with trophic level in aquatic food webs, from <10% in water (Malm *et al.*, 1995), <1–12% in phytoplankton and other lacustrine primary producers (Watras & Bloom, 1992; Gantner *et al.*, 2010), *c.* 10–80% in lacustrine zooplankton (Back *et al.*, 2003; Loseto *et al.*, 2008; Swanson *et al.*, 2011), *c.* 1 to over 100% in freshwater invertebrates (Mason *et al.*, 2000; Cremona *et al.*, 2008; Bates & Hall, 2012) and *c.* 80–100% (often >90%) in the muscle tissue of most marine and freshwater fishes (Bowles *et al.*, 2001; Wyn *et al.*, 2009; Kasper *et al.*, 2012; van der Velden *et al.*, 2013). Due to the normally high MeHg:THg ratio in fishes, THg concentrations for this group can therefore be a good indicator of MeHg levels (Bloom, 1992; Cleckner *et al.*, 1998; Chumchal *et al.*, 2008).

In Amazonian black-water systems which are rich in dissolved humic and fulvic acids and generally low in pH (Sioli, 1953, 1984), high mercury concentrations have been found in the soils and fishes (Guimarães *et al.*, 1999; Barbosa *et al.*, 2003) and subsequently in the hair of riverine peoples who derive a high percentage of their daily protein from fishes (Silva-Forsberg *et al.*, 1999; Santos *et al.*, 2005). Although some gold mining still occurs in the upper Rio Negro, recent biogeochemical studies have shown the existence of naturally high background Hg levels in the basin (Fadini & Jardim, 2001; Valle *et al.*, 2005, 2006), occurring mainly *via* atmospheric deposition over millions of years. These studies conclude that Hg leaching from soils constitutes the largest regional reservoir of this metal, which is then made available for uptake by plants and other organisms. In Amazonian fishes, MeHg transmission through the aquatic food chain dominates over water-borne Hg (Barbosa *et al.*, 2003).

All previous studies examining mercury bioaccumulation in Rio Negro fishes have focused on species captured for human consumption (Barbosa *et al.*, 2003; Belger & Forsberg, 2006; Dorea *et al.*, 2006). In these studies, many top predators were found to have mercury levels $>1000 \text{ ng g}^{-1}$, which is the maximum concentration recommended for human consumption by the World Health Organization (WHO, 2008) and the Brazilian Health Ministry (ANVISA, 2013). Although concentrations varied widely, some studies showed a consistent pattern of biomagnification among herbivorous, detritivorous, omnivorous and piscivorous fishes, with the highest concentrations being found in piscivorous fishes (Barbosa *et al.*, 2003; Dorea *et al.*, 2006).

The influence of different variables on mercury concentrations in Rio Negro fishes, including standard length (L_S), mass and seasonal water level fluctuations, have yielded varying results. Barbosa *et al.* (2003) found a positive correlation between THg and mass of Rio Negro piscivores (including *Cichla* spp.), but a negative correlation was found between mass and THg for non-piscivores. Belger & Forsberg (2006) found strong positive correlations between Hg levels for the piscivorous species *Cichla* spp. and *Hoplias malabaricus* (Bloch 1794) for both standard length and the percentage of seasonally flooded area upstream of the sampling sites. During flooding, water generally becomes anoxic, due to high concentrations of decomposing organic matter (Meili, 1997), which in turn favours sulphate-reducing bacteria (Bodaly *et al.*, 1984; Watras *et al.*, 1998). It appears that mercury methylation is facilitated by bacteria, which thrive in organic-rich, anaerobic sediments of many aquatic systems, including wetland soils (Compeau & Bartha, 1984; Branfireun *et al.*, 1999).

For the omnivorous cardinal tetra *Paracheirodon axelrodi* (Schultz 1956), a small, ornamental fish preyed on by *Cichla* spp. juveniles (B. G. Marshall, pers. obs.), Marshall *et al.* (2008) showed that *P. axelrodi*'s food strategies and autotrophic energy sources vary depending on habitat and water level. During high water, when *P. axelrodi* is found in flooded forest streams in the middle Rio Negro, stomach content analyses showed a high predominance of insect larva in the diet, while carbon stable isotope analyses indicated allochthonous carbon as the principal autotrophic energy source. During the dry season, however, the contribution of periphytic algae to *P. axelrodi*'s diet and as a carbon source to its food chain increased significantly. It is important to note that few collections in that study were made in interfluvial swamps, where high production of periphytic algae, especially during the dry season (Marshall, 2010), can influence energy flow in the *P. axelrodi* food chain.

Interfluvial swamps occur in headwater regions of many small tributaries in the middle Rio Negro and are characterized by open-canopy vegetation and shallow, slow-moving waters, which along with abundant growth of aquatic herbaceous plants, create an ideal environment for epiphytic and metaphytic algal production (Marshall, 2010). Epiphytic algae grow in the water column attached to both emergent and submerged herbaceous plants, while metaphytic algae float on top of the water in thick, bulbous masses, due to trapped air bubbles (Hillebrand, 1983; Marshall, 2010). Epiphytic algae have been identified as an important site of mercury methylation (Cleckner *et al.*, 1999; Guimarães *et al.*, 2000a), and thus represent an important potential link for mercury transferral to aquatic invertebrates and small fishes like *P. axelrodi* in these environments.

The principal objective of this study was to assess mercury biomagnification in *P. axelrodi*'s food chain. Additionally, natural variations in THg in plants (emergent and submersed aquatic herbaceous plants, tree and shrub leaves, and epiphytic and

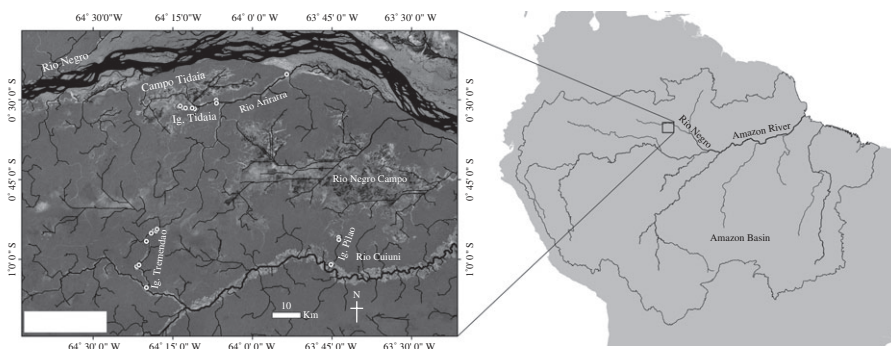


FIG. 1. Map showing collection points (⊙) in the study area, which is located in the middle Rio Negro, Amazonas State, Brazil. Grey areas represent non-flooded forest, black areas indicate streams, rivers and areas of open water with flooded herbaceous plants in the interfluvial region and whitish areas are characterized by mainly flooded forest along main river channels and a mix of flooded palms, shrubs and trees in the interfluvial wetlands.

metaphytic algae), juvenile shrimps *Macrobrachium* sp. and *P. axelrodi* collected in an interfluvial region of the middle Rio Negro were examined. Environmental variables were investigated in relation to THg concentrations of *P. axelrodi* to determine seasonal differences in mercury assimilation. Similarly, the influence of L_S on the THg concentrations of *P. axelrodi* was also investigated to determine whether mercury bioaccumulated during ontogeny.

MATERIALS AND METHODS

The interfluvial region investigated in this study covers an area of 1800 km² and is situated among the following geographical co-ordinates: 0° 25'–1° 05' S and 63° 23'–64° 05' W, in the middle Rio Negro, Amazonas State, Brazil (Fig. 1). This region consists of the Cuiuni and Arirarrá drainage basins, which are seventh-order tributaries of the Rio Negro. The interfluvial region is drained by small streams with seasonally flooded forest in their lower reaches and extensive palm swamps (known locally as *campos*) in their headwaters. The swamps flood principally as a result of local precipitation, with little influence from the yearly monomodal flood pulse that drives the hydrological cycle of large tributaries and main channels of the Rio Negro (Rosenqvist *et al.*, 2002; Marshall *et al.*, 2011). The precipitation and flooding patterns in the interfluvial regions are seasonal, with many wetlands drying out completely during the dry periods while others remain at least partially flooded throughout the year (Belger *et al.*, 2011; Marshall *et al.*, 2011).

Although vegetation in interfluvial swamps can vary greatly, *P. axelrodi* habitat in the palm swamps is generally characterized by: a predominance of buriti palms *Mauritia flexuosa* and buritirana *Mauritiella armata*, shrubs and trees such as *Machaerium floribundum* and *Vitex calothyrsa*, and an abundance of both emergent (e.g. *Becquerelia cymosa*, *Eleocharis* spp. and *Montrichardia arborescens*) and submersed herbaceous plants [e.g. *Utricularia*, *Potamogeton*, *Paepalanthus* and *Nymphaea* (Marshall *et al.*, 2011)]. The palm *Leopoldinia pulchra* and herbaceous plants *Ischnosiphon polyphyllus* and *Cyperus ligularis*, which are indicator plants for occurrence of *P. axelrodi*, are also found in the headwater swamps (Geisler & Annibal, 1986; Marshall *et al.*, 2008).

Samples of *P. axelrodi* were collected in three streams (locally known as *igarapés*): Ig. Cachorro-Tremendão and Ig. Pilão, which pertain to the Rio Negro Campo interfluvial, and Ig. Tidaia, which pertains to Campo Tidaia (Fig. 1). Collections were made on five different excursions over a period of 12 months, occurring in February 2009, May 2009, August 2009,

November to December 2009 and January to February 2010. Due to heavy precipitation over the course of these field campaigns, the first four excursions occurred during moderate to heavy flooding of the interfluvial region, while the last excursion occurred during extreme low water, when the Rio Negro was in the midst of an El Niño dry spell. Water level in the interfluvial region was measured daily between February 2009 and January 2010 using Leveloggers (Solinst Inc., model 3001; www.solinst.com) installed in two areas of open water in both the Rio Negro Campo and Campo Tidaia. Leveloggers are basically hydrostatic sensors which are able to provide accurate water level readings by compensating for barometric pressure. Concurrently, the leveloggers also recorded the water temperature.

Water samples (250 ml) were collected at *P. axelrodi* sampling sites using polyethylene terephthalate bottles and were immediately filtered using 47 mm Whatman (www.whatman.com) GF/F with 0.7 µm porosity for chlorophyll *a* and 47 mm Millipore (www.merckmillipore.com) cellulose membrane filters with 0.45 µm porosity (pre-oven dried at 60° C for 2 h) for total suspended sediments (TSS). A total of 28 water samples were collected in the swamps and 13 in the streams over the course of the campaigns. The filters were dry preserved in a desiccator covered in aluminium foil to keep the light out and then transported to the laboratory at the Instituto Nacional de Pesquisas da Amazônia (INPA).

Some physico-chemical variables were also measured at the sampling sites, including dissolved oxygen (mg l⁻¹) and water temperature (° C), using a portable 110 model Oakton DO meter, and pH (Oakton pH/CON, model 10; www.4oakton.com), although pH was not measured during all of the excursions due to technical problems with the meter.

For isotopic analyses and THg concentrations, 36 samples of *P. axelrodi* (each sample included a minimum of 10 individuals) were collected in the headwater swamps and 16 in the streams (including the flooded forests). The fish were collected with a hand-held net made of nylon mesh and woody vines, locally called a rapiché. Additionally, for δ¹⁵N and mercury analyses, seven samples of green neon tetras *Paracheirodon simulans* (Géry 1963) were also collected in the interfluvial region. *Paracheirodon simulans* is also found in palm swamps in the middle Rio Negro, but in aquatic habitats distinct from those of *P. axelrodi* (Géry, 1963; Marshall *et al.*, 2011). Unlike *P. axelrodi*, *P. simulans* does not move or migrate between habitats, apparently only occurring in very shallow swamps, which can be found exclusively at the headwaters of some small streams that drain into tributaries of the Rio Negro.

Twenty-two samples of epiphytic algae, 11 metaphytic algae, 28 vascular plants (10 emergent, eight submersed and 10 samples of tree and shrub leaves), and four samples of juvenile shrimps (*Macrobrachium* sp.), which were composite samples of at least three specimens, were also collected. Samples of chironomid larvae and oligochaete and ceratopogonide larvae were also collected at the same time as the shrimps, as these prey items are commonly found in stomach contents of *P. axelrodi*. The larvae samples, however, only yielded enough material to perform the isotopic analyses; therefore, it was not possible to determine mercury concentrations for these invertebrate groups.

Finally, in order to test further mercury biomagnification in the *P. axelrodi* food web, δ¹⁵N values and THg concentrations of *Cichla* spp. collected in the middle Rio Negro (62 samples of *Cichla temensis* Humboldt 1821, 43 *Cichla orinocensis* Humboldt 1821 and 18 *Cichla monoculus* Agassiz 1831) were included in the final analyses. These fishes were collected in different aquatic habitats during a period of 12 months in 2005.

The algae were collected manually using first 800 µm and then 275 µm nets, then large detritus particles and invertebrates were removed manually using tweezers. A small portion of each algal sample was preserved in alcohol for identification at INPA, while the rest was frozen in liquid nitrogen for posterior isotopic and mercury analysis. The shrimp samples were collected at the same time as the epiphytic algae and were separated manually while still alive. One individual of each sample was preserved in alcohol for later identification, while the rest were maintained in filtered water for 1 h to clear out the intestine before being frozen. All samples were frozen in liquid nitrogen and transported to INPA for pre-processing prior to isotopic and mercury analysis.

Plant samples, including algae, were washed with tap water followed by a 1 M HCl solution, rinsed with deionized water and then dried at 60° C. After drying, samples were ground to a fine powder with mortar and pestle. Juvenile shrimp samples were dried whole at 60° C and then processed as described above. For *P. axelrodi*, *L*₅ of each fish was measured prior to removing the heads and stomachs, and then dried and ground as above. As each fish sample was a composite of at least 10 individuals, a mean *L*₅ for each sample was determined for use

in the statistical analyses. The samples were preserved in glass vials which had been washed beforehand with a 1 M HCl solution, rinsed with deionized water and then dried at 60° C. Fine particulate organic matter (FPOM) samples on the fibreglass filters were dried at 60° C and then processed as described above.

For $\delta^{15}\text{N}$ analyses, samples were sent to the Centre of Nuclear Energy in Agriculture (CENA) in Piracicaba, SP, Brazil. Briefly, 1 mg of sample was converted by combustion (1020° C) into N_2 gas, separated in a carrier gas by a chromatographic column, before being introduced in a continuous-flow isotope ratio mass spectrometer (ThermoScientific, model Delta Plus; www.thermoscientific.com) coupled to an Elemental Analyser (CarloErba, model CHN-1110; www.carloerbareagents.com). Stable isotope ratios were expressed in delta (δ) notation as parts per thousand (‰) according to the following equation: $\delta^{15}\text{N} = 1000[(R_{\text{sample}}/R_{\text{standard}}) - 1]$, where R is the corresponding ratio of $^{15}\text{N}:^{14}\text{N}$. Atmospheric N_2 was used as a primary international standard to calibrate the nitrogen stable isotope analyses. All of the samples were analysed in triplicate with a precision of 0.5%.

Total mercury analyses were conducted at the Environmental Biology Laboratory at the Federal University of Western Pará (UFOPA) in Santarém, Pará State, Brazil. The mercury analyses were performed following Pichet *et al.* (1999). The accuracy of the method was determined by analysing a Canadian First Nations and Inuit Health Branch Laboratory standard of dried fish tissue, applied during several analytical sample runs. To facilitate comparison with THg concentrations of plants and consumers from other studies, all of the THg values in dry mass (dried oven mass) were converted to wet mass in accordance with conversion ratios (humid wet mass:dry mass) established in the literature. The following ratios were applied: emergent and submerged herbaceous plants: 5.5 (Sarkka *et al.*, 1978); epiphytic and metaphytic algae: 12.5 (Sladeczek & Sladeczkova, 1964); *Macrobrachium* sp.: 6.99 (Castell *et al.*, 1989); *Paracheirodon* spp.: 4.8 (B. G. Marshall, unpubl. data); *Cichla* spp.: 5.0 (M. Thomé-Souza, unpubl. data).

All statistical analyses were performed using STATISTICA 9.0 (Statsoft; www.statsoft.com) and R Software (R Development Core Team; www.r-project.org), with all significant P values set at 0.05. Any data that did not satisfy homoscedasticity conditions (Levene test; Zar, 2009) was $\log_{10}$ transformed. An ANOVA was used to determine whether the THg values of vascular plants, algae, *Macrobrachium* sp. and *P. axelrodi* were statistically different. A linear discriminant function analysis (LDFA) was performed to test the hypothesis that there are no environmental differences between the hydrological seasons (high and low water). The grouping variable was season and the independent variables were total suspended sediments, chlorophyll a , water level, dissolved oxygen and water temperature. Due to a low number of replicates from some of the sites and habitats (stream and swamp), the samples were all pooled together for the analysis. Multiple regression using the CAR Package, version 2.0-19 in R software (R Development Core Team), was then applied with the significant variables detected by LDFA, along with L_S of *P. axelrodi* as an independent variable and $\ln$ THg concentrations. Previous to running the regression model, the Bonferroni test was applied to detect outliers and collinearity was graphically checked. All statistical tests were done using a P level of 0.05. Finally, the slope of $\ln$ -linear plots of THg and $\delta^{15}\text{N}$ was used to estimate the rates of mercury biomagnification through the aquatic food chain.

RESULTS

NATURAL VARIATIONS IN THG CONCENTRATIONS OF PLANTS AND CONSUMERS

Vascular plants, which were composed of emergent and submersed aquatic plants and tree and shrub leaves, presented mean $\pm$ s.d. THg concentrations of 18.1 ± 6.9 , 16.3 ± 6.2 and $12.5 \pm 4.2 \text{ ng g}^{-1}$ wet mass, respectively (Table I). There was no significant statistical difference found between these plants and the average THg concentrations of epiphytic and metaphytic algae: 11.8 ± 5.1 and $14.1 \pm 3.9 \text{ ng g}^{-1}$ wet mass, respectively (Table I). Although there was only small variation among the plant groups,

TABLE I. Mean $\pm$ S.D., minimum and maximum of total mercury concentrations (THg) and nitrogen stable isotope ($\delta^{15}\text{N}$) values of emergent aquatic herbaceous and submerged aquatic herbaceous plants, tree and shrub leaves, epiphytic algae, metaphytic algae, juvenile shrimp (*Macrobrachium* sp.) and *Paracheirodon axelrodi* collected in stream and swamps in the inter-fluvial region

	<i>n</i>	Mean $\pm$ S.D. THg (ng g ⁻¹ wet mass)	Minimum	Maximum	Mean $\pm$ S.D. $\delta^{15}\text{N}$ (‰)	Minimum	Maximum
Plants							
Emergent aquatic herbaceous	8	18.1 $\pm$ 6.9	6.8	28.9	3.6 $\pm$ 2.7	-2.1	6.5
Submerged aquatic herbaceous	10	16.3 $\pm$ 6.2	6.8	27.8	2.8 $\pm$ 1.9	0.6	5.7
Tree and shrub leaves	9	12.5 $\pm$ 4.2	6.4	17.4	0.9 $\pm$ 3.1	-2.2	6.9
Epiphytic algae	21	11.8 $\pm$ 5.1	4.6	25.7	2.5 $\pm$ 1.7	0.3	6.7
Metaphytic algae	11	14.1 $\pm$ 3.9	7.1	19.8	1.7 $\pm$ 1.3	0.3	4.4
Aquatic invertebrates							
<i>Macrobrachium</i> sp.	4	63.6 $\pm$ 23.7	29.1	82.9	7.4 $\pm$ 0.3	7.2	7.9
<i>Paracheirodon axelrodi</i>							
Swamps	36	97.0 $\pm$ 34.1	49.4	193.9	9.2 $\pm$ 0.5	8.0	10.1
Streams	16	121.2 $\pm$ 46.4	51.2	220.1	9.3 $\pm$ 0.6	8.7	10.7

n, number of samples.

there was a significant difference found between the plants, *Macrobrachium* sp. and *P. axelrodi* ($F_{1,50} = 4.318$, $P < 0.05$).

Large variations in THg concentrations were found in the samples of *Macrobrachium* sp., showing a minimum of 29.1 ng g⁻¹ wet mass and a maximum of 82.9 ng g⁻¹ wet mass (Table I). Shrimp were only collected in the swamps, so it was not possible to test for variations in concentrations based on habitat differences. In comparison, samples of *P. axelrodi* in the streams and swamps presented mean $\pm$ S.D. THg concentrations of 97.5 $\pm$ 34.1 ($n = 16$; L_S range = 1.3–2.3 cm) and 121.2 $\pm$ 46.4 ($n = 36$; L_S range = 1.3–2.2 cm) ng g⁻¹ wet mass, respectively (Table I). A significant difference in THg concentrations of *P. axelrodi* was found in relation to habitat (stream and swamp habitats) ($F_{2,113} = 147.2$, $P < 0.001$). The mean $\pm$ S.D. THg concentrations of *P. simulans* (112.3 $\pm$ 31.4 ng g⁻¹ wet mass; $n = 7$; $L_S = 1.3$ –1.5 cm) were within the range of the *Paracheirodon* spp. mercury concentrations, presenting no statistical difference between the two species [see Tables SI and SII (Supporting Information) for THg and L_S data spreadsheets of *P. axelrodi* and *P. simulans*, respectively].

In comparison, the THg concentrations of *Cichla* spp. were 1.5–10 fold higher than the *Paracheirodon* spp., with mean $\pm$ S.D. as following: *C. monoculus* (429.9 $\pm$ 222.1 ng g⁻¹ wet mass; $n = 18$; $L_S = 23.5$ –34.2 cm); *C. orinocensis* (395.1 $\pm$ 150.88 ng g⁻¹ wet mass; $n = 43$; $L_S = 23$ –40.6 cm); *C. temensis* (431.5 $\pm$ 202 ng g⁻¹ wet mass; $n = 62$; $L_S = 18.8$ –74.1 cm) [see Table SIII (Supporting Information) for THg and L_S data spreadsheet of *Cichla* spp.]. Although *C. temensis* had individuals

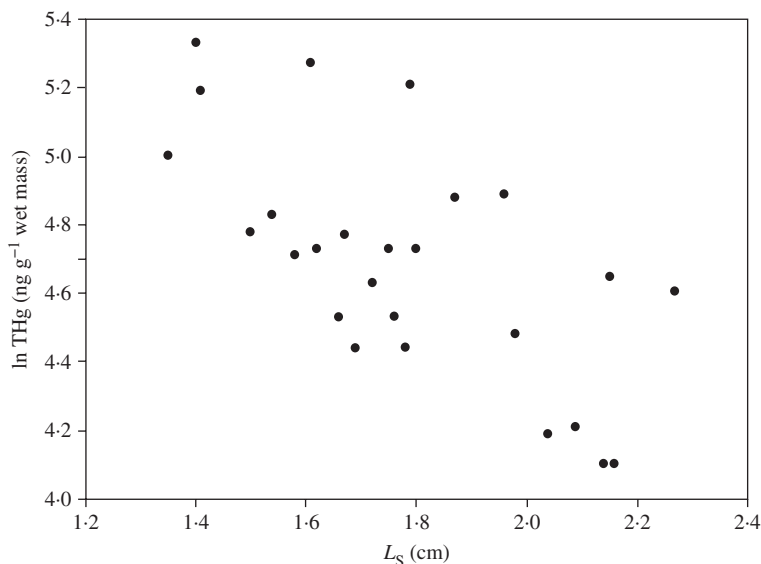


FIG. 2. Relationship between $\ln$ total mercury (THg) concentrations and standard length (L_S) of *Paracheirodon axelrodi* in the interfluvial region.

with greater L_S than the other two species, no statistical difference was found in relation to mercury concentrations.

VARIATIONS IN THg CONCENTRATIONS OF *P. AXELRODI* IN RELATION TO ENVIRONMENTAL VARIABLES AND L_S

Discriminant analysis revealed that there are environmental differences between high and low water seasons (Wilks' $\lambda = 0.095$; d.f. = 5, 26; $P < 0.001$). The variables responsible for the difference were water level ($P < 0.001$) and chlorophyll *a* ($P < 0.01$). Before applying the stepwise multiple regression, however, chlorophyll *a* was removed due to strong collinearity with water level.

Before applying the stepwise regression, THg concentrations of *P. axelrodi* (y) were $\ln$ transformed as they did not satisfy the criteria for a linear model. The final regression equation was: $\ln y = 6.719 - 0.697 L_S - 0.006 D$ ($r^2 = 0.487$; d.f. = 2, 23; $P < 0.001$), where D is water level. L_S explained most of the variation in THg concentrations of *P. axelrodi* ($P < 0.01$), showing a strong negative relationship ($r^2 = 0.442$) (Fig. 2). Although the multiple regression results showed that water level in relation to mercury was not statistically significant ($P > 0.05$), Fig. 3 shows a clear tendency of higher THg concentrations of *P. axelrodi* at low water, but due to two defined groups, one at high water and one at low water, a linear relationship could not be well adjusted.

Hg BIOMAGNIFICATION IN THE *P. AXELRODI* FOOD CHAIN

The THg concentrations of flora and fauna in the Rio Negro food web were significantly correlated to trophic position as indicated by $\delta^{15}\text{N}$ signatures of vascular plants

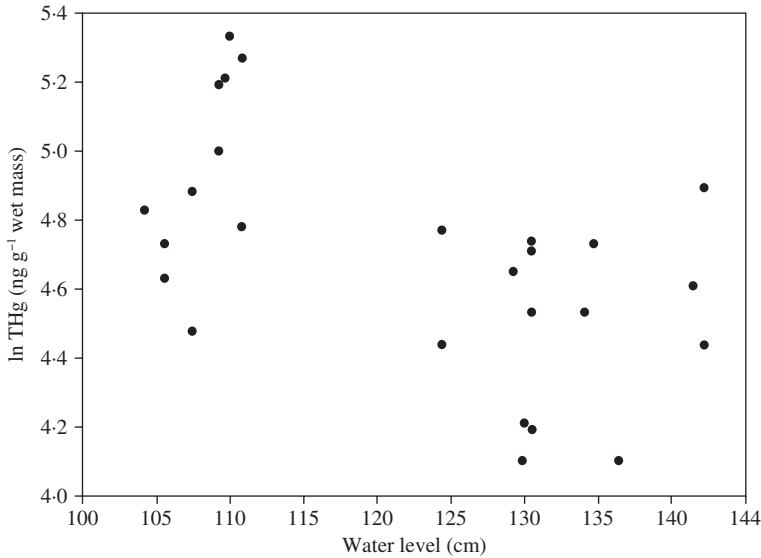


FIG. 3. Relationship between ln total mercury (THg) concentrations and water level of *Paracheirodon axelrodi* in the interfluvial region.

and algae, *Macrobrachium* sp., *Paracheirodon* spp. and *Cichla* spp. (Fig. 4 shows $\log_{10}$ THg concentrations in relation to $\delta^{15}\text{N}$ values, which generated a slope = 0.15).

When the plants were removed to determine Hg biomagnification solely among consumers in the food web, the relationship was also significant (slope = 0.246; Fig. 5). A slope value >0.2 indicates evidence of strong mercury biomagnification in the food chain.

The mean $\pm$ S.D. $\delta^{15}\text{N}$ values of the plants, shrimp and fishes in relation to non- $\log_{10}$ adjusted THg concentrations, demonstrated a pronounced exponential curve (Fig. 6). Relatively large variations in mercury concentrations were found in the shrimps and fishes, with *Cichla* spp. concentrations presenting the largest differences between minimum and maximum values. There was little overlapping of THg concentrations among *Macrobrachium* sp., *Paracheirodon* spp. and *Cichla* spp., however, which facilitated trophic level distinction. In comparison, although THg concentrations of different plant groups overlapped substantially, there was little variation observed, which helped to clearly identify the base of the food chain.

On the other hand, $\delta^{15}\text{N}$ values of the plants presented large variation, probably due to a mix of nitrogen-fixing and non-nitrogen-fixing species (Martinelli *et al.*, 1992). This typically creates uncertainty for determining a reliable $\delta^{15}\text{N}$ average, as it is above this baseline that the higher trophic levels are positioned. The $\delta^{15}\text{N}$ values of *Macrobrachium* sp., *Paracheirodon* spp. and *Cichla* spp., however, varied little within their respective groups, showing distinct separation of trophic levels. The only exception was *P. simulans*, which possesses food strategies very similar to that of *P. axelrodi* (Marshall *et al.*, 2011). Therefore, it is understandable that these two species would present $\delta^{15}\text{N}$ values almost identical to each other. Considering trophic fractionation of 2.1‰ per trophic level for organisms analysed whole (McCutchan *et al.*, 2003),

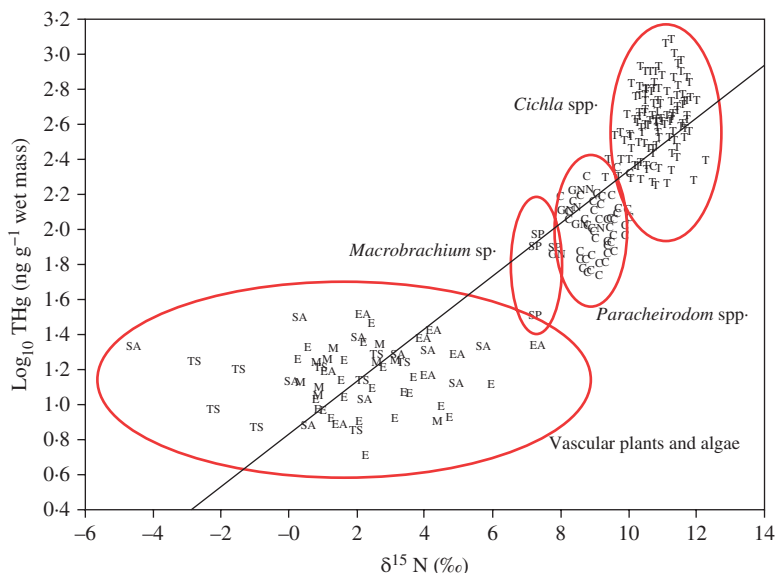


FIG. 4. Log_{10} total mercury (THg) concentrations and nitrogen stable isotope ($\delta^{15}\text{N}$) values of vascular plants and algae [emergent aquatic herbaceous (EA), submerged aquatic herbaceous (SA), metaphytic algae (M), epiphytic algae (E) and tree and shrub leaves (TS)], juvenile *Macrobrachium* sp. (SP), *Paracheiroidon simulans* (GN), *Paracheiroidon axelrodi* (C) and *Cichla* spp. (T). The curve was fitted by $y = 0.15x + 0.832$ ($r^2 = 0.8174$, $P < 0.01$).

Paracheiroidon spp. would be approximately one trophic level above *Macrobrachium* sp. and *Cichla* spp. a little less than one trophic level above *Paracheiroidon* spp.

DISCUSSION

Although relatively low THg concentrations were found in all of the different plant groups analysed in this study, similar results have been found in other Amazonian studies. Aula *et al.* (1995), studying catchment flux of mercury during the dry season in the Tucuruí Reservoir in Pará, Brazil, found THg concentrations of $5.5\text{--}21.8 \text{ ng g}^{-1}$ wet mass in floating aquatic plants, with higher concentrations in the roots and submerged leaves than in the shoots and floating leaves. Roulet *et al.* (2000) reported THg concentrations of $9.7\text{--}19.1 \text{ ng g}^{-1}$ wet mass in epiphyton attached to aquatic macrophytes in floodplain lakes along the clear water Tapajós River in eastern Amazonia. It is important to note, however, that the proportion of MeHg to THg concentrations encountered in the epiphyton ($1.5\text{--}8.3\%$) correlated with its C and N contents, probably as a result of methylation *in situ*, which was influenced by biogeochemical conditions within the macrophyte stands. Bowles *et al.* (2001), investigating Hg biomagnification in a freshwater food web in Papua New Guinea, found significantly higher MeHg concentrations in seston (a mixture of phytoplankton, zooplankton and detritus) than in other plants. It is understood that enrichment of methylmercury occurs at the bacterial and primary producer level, which is then passed on to invertebrates and small fishes (Cleckner *et al.*, 1999).

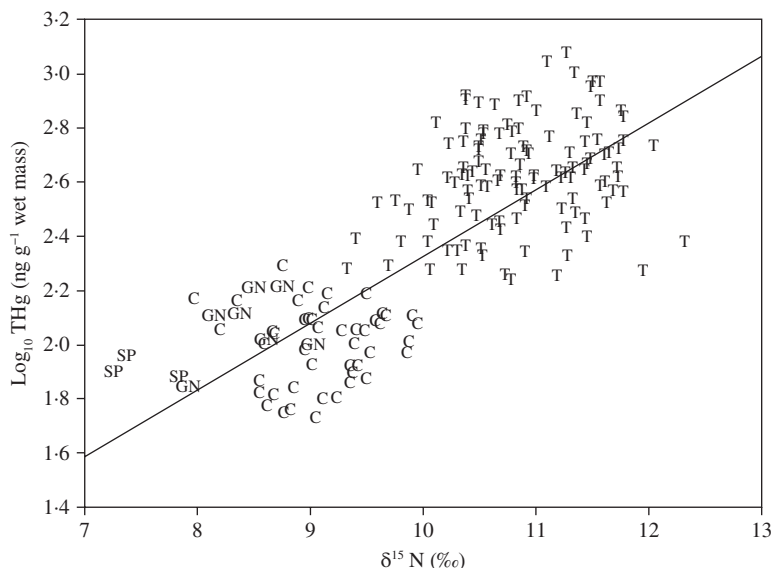


FIG. 5. Log_{10} total mercury (THg) concentrations and nitrogen stable isotope ($\delta^{15}\text{N}$) values of juvenile *Macrobrachium* sp. (SP), *Paracheirodon simulans* (GN), *Paracheirodon axelrodi* (C) and *Cichla* spp. (T). The curve was fitted by $y = 0.246x - 0.133$ ($r^2 = 0.62$, $P < 0.01$).

The THg concentrations of the juvenile shrimp samples in this study were larger than those found by Pereira *et al.* (2010) for *Macrobrachium* sp. ($20\text{--}40\text{ ng g}^{-1}$ dry mass) in a freshwater lake contaminated with transition metals and sewage in southern Brazil. In comparison, the mercury concentrations of *P. axelrodi* and *P. simulans* were similar to low-end THg values of *Hemigrammus* spp. ($124\text{--}368\text{ ng g}^{-1}$ wet mass) found by Barbosa *et al.* (2003) in a study of mercury biomagnification in the Rio Negro. *Hemigrammus* spp. are ornamental fishes that possess omnivorous feeding habits and have approximately the same size as *Paracheirodon* spp. The upper limit of THg concentrations of *Hemigrammus* spp., however, was more than double that encountered for *P. simulans* and 40% more than the highest concentration found for *P. axelrodi* during the low water period.

For the same size organisms, THg concentrations of consumers can vary widely, due to differences related to mercury assimilation in the tissues. This, in turn, is influenced by variation in mercury and methylmercury production in the environment, temporal and spatial changes in food strategies for a given consumer and biodilution associated with an individual's growth rate (Swanson *et al.*, 2006). The similarity in THg concentrations between *P. axelrodi* and *P. simulans* in this study, however, was probably due to the nearly identical food strategies of the two omnivorous species, both with a preference for micro-crustaceans and small insect larva (Marshall, 2010; Marshall *et al.*, 2011).

Mercury concentrations can also vary in relation to fish size (length and mass), due to a number of reasons, including fluctuations in mercury levels in prey species or in superficial water layers, differences in fish physiology, which affects the rate of mercury depuration (Barbosa *et al.*, 2003), or changes in diet, due to ontogeny. While no shift in diet has been observed for *P. axelrodi* during ontogeny, a negatively

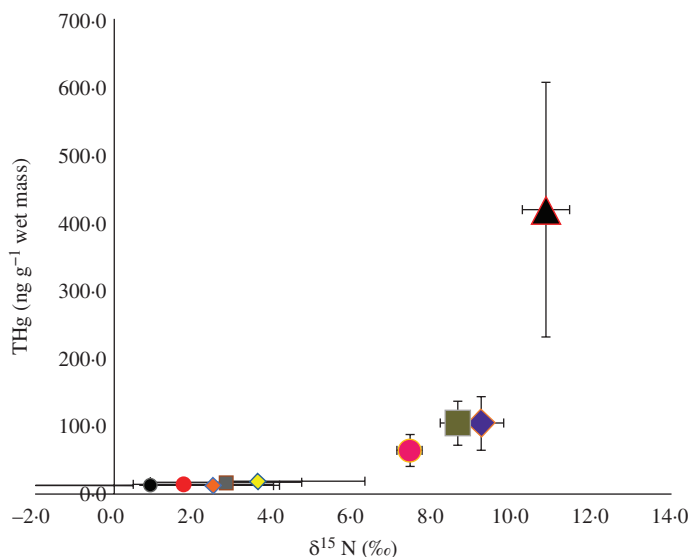


FIG. 6. Mean $\pm$ S.D. total mercury (THg) concentrations and nitrogen stable isotope ($\delta^{15}\text{N}$) values of *Cichla* spp. ($\blacktriangle$), *Paracheirodon axelrodi* ($\blacklozenge$), *Paracheirodon simulans* ($\blacksquare$), juvenile *Macrobrachium* sp. ($\bullet$), emergent aquatic herbaceous plants ($\blacklozenge$), submerged aquatic herbaceous ($\blacksquare$), epiphytic algae ($\blacklozenge$), metaphytic algae ($\bullet$) and tree and shrub leaves ($\bullet$).

significant correlation between L_S and THg concentrations in this study shows that mercury became more concentrated in the tissues of smaller individuals, which were caught principally at low water in streams that drain the interfluvial swamps, when food resources are generally less plentiful.

In floodplain lakes along the margins of the Tapajós River in eastern Amazonia, Silva *et al.* (2005) also found a negative correlation between THg and L_S for *Schizodon fasciatus* (Spix & Agassiz 1829), a herbivorous species. Lima *et al.* (2000), also in the Tapajós River, encountered negative relationships for THg and mass in two carnivorous fish species. In the middle Negro River, Barbosa *et al.* (2003) also found negative correlations between THg and mass for *Potamorhina latior* (Spix & Agassiz 1829), a detritivorous species, and *Leporinus* sp., an omnivorous species. Many omnivorous fish species experience lower growth during the low water period, due to a high density of organisms competing for fewer food resources (Winemiller, 1993, 1998), which can result in higher Hg concentrations being incorporated into the tissues.

According to Junk (1985), the growth rates of some species are 60% higher during the annual flood season, due to a greater availability of food resources. A faster growth rate can result in lower mercury concentrations being incorporated in the tissues, as dilution occurs when nutrients and/or toxins (in this case, transition metals) are being absorbed in the stomach at the same time that the organism is undergoing rapid growth (Essington & Houser, 2003; Swanson *et al.*, 2006).

Hylander *et al.* (2000), studying four carnivorous species in various rivers and lakes in the upper Pantanal in central-western Brazil, found THg concentrations to be 50% higher during the dry period than at high water. They attributed higher concentrations during the dry period to two sources: (1) the decomposition of submerged aquatic

herbaceous plants and the subsequent liberation of organic acids, which increase the release of adsorbed mercury in the soils and particulate organic matter to be transformed in bioavailable MeHg (Meili, 1991, 1997; Forsberg *et al.*, 1999) and (2) atmospheric mercury deposited during the rainy season, which then became more concentrated in shallower lakes and streams during the dry season, when higher water temperatures were also encountered.

Several studies have shown that methylmercury production normally increases in relation to higher surface water temperatures (Weis *et al.*, 1986; Bodaly *et al.*, 1993). This only occurs, however, if the bottom sediments are anoxic and the organic carbon is adequately labile (Lambertsson & Nilsson, 2006; Hall *et al.*, 2008). Weis *et al.* (1986), studying the effects of environmental factors on mercury release from a small temperate stream and its uptake by killifish *Fundulus heteroclitus* (L. 1766), suggested that their observations of elevated Hg concentrations during late spring and summer may be related to warming water temperatures and decreased dissolved oxygen levels, that may enhance microbial production of MeHg.

Marshall *et al.* (2011), using leveloggers which registered changes in water temperature in the interfluvial region every 15 min for 5 months between September 2009 and February 2010, showed that temperatures can reach 35° C in some areas during the dry season. When local rainfall decreases and water levels drop, suspended sediments descend to the bottom and water transparency increases. Consequently, more light entering the water column during this low water period generates higher production of phytoplankton and epiphytic algae than during the high water season (Marshall, 2010). Epiphytic algae utilize submersed and emergent herbaceous plants as substrata, as well as benefiting from nutrients liberated from plants in decomposition. As mentioned previously, Roulet *et al.* (2000) found a higher percentage of MeHg production in epiphyton relative to nitrogen and carbon concentrations than in organic material of allochthonous origins, due to methylation occurring within macrophyte stands.

Although algal productivity in the system studied here was lower than the vascular plant production (Marshall, 2010), the carbon derived from algae is more labile, contains more protein per unit volume and is typically preferred by metazoan consumers (Forsberg *et al.*, 1993; Thorp & Delong, 2002). Stomach content analyses have shown that *Paracheirodon* spp. consume little algae directly (Walker, 2004). They appear, however, to benefit indirectly through consumption of micro-crustaceans and aquatic invertebrates, which are mainly herbivores (Goulding *et al.*, 1988; Walker, 2004). Considering that submersed herbaceous plants and attached epiphytic algae create an environment especially conducive to mercury methylation (Cleckner *et al.*, 1999; Guimarães *et al.*, 2000b), it is hypothesized that the selective herbivory practiced by aquatic invertebrates could be one of the principal links for Hg biomagnification in the food chain. In this study, it appears that mercury found in *Macrobrachium* sp. was successively passed on to *Paracheirodon* spp. and *Cichla* spp. via predation.

Studies have shown that *Macrobrachium* sp. feeds primarily on detritus, algae and fungus (Kensely & Walker, 1982; March & Pringle, 2003), with the algal component becoming more important when available. Chasar *et al.* (2009), in a study analysing trophic dynamics and mercury biomagnification in stream ecosystems with a broad range of environmental conditions across the U.S.A., found that the supply of MeHg to the base of the food web was the strongest determinant of Hg in top-level predators, rather than differences in trophic transfer efficiency or trophic position. Although methylmercury was not analysed in this study, studies in tropical regions have shown

that MeHg increases up the food chain, with fishes presenting ratios between 80 and 100% (Bowles *et al.*, 2001; Campbell *et al.*, 2008). Bowles *et al.* (2001), investigating mercury biomagnification in Lake Murray in Papua New Guinea, found that the proportion of THg present as methylmercury increased from <1% in plants to 94% in piscivorous fishes. Hylander *et al.* (2000) found an average MeHg:THg ratio of 92% in four carnivorous species collected from various rivers and lakes in the upper Pantanal in central-western Brazil. Kasper *et al.* (2012), studying mercury levels in fishes upstream and downstream of an Amazon hydroelectric reservoir, found MeHg:THg ratios that ranged from 75 to 100%, with the highest ratios in carnivorous fishes, such as *C. monoculus*.

Goulding *et al.* (1988) reported that *Cichla* spp. feed on small fishes, including *Paracheirodon* spp. and other species of the Characidae family, as well as larger fishes from Anostomidae and Cichlidae. These prey belong to various trophic levels, comprised of algivores, herbivores, detritivores, omnivores and even piscivores. This could probably explain, in part, the large variation in THg concentrations found in the muscle tissue of *Cichla* spp. individuals analysed in this study, varying between a minimum of 170 ng g⁻¹ wet mass to a maximum of 1093 ng g⁻¹ wet mass, which is over the acceptable concentration allowed by the Brazilian Health Ministry (ANVISA, 2013) and the World Health Organization (WHO, 2008) for human consumption of predator fishes. Two *C. temensis* individuals of similar size, which were collected at the same time of year and presented almost identical $\delta^{15}\text{N}$ values, had THg concentrations different from each other by a factor of 5.

Although variations in THg concentrations were also found for *Macrobrachium* sp. and *Paracheirodon* spp., there was little overlap between these two groups, which helped to identify distinct trophic levels and determine mercury biomagnification in the food chain. The slope of the log THg and $\delta^{15}\text{N}$ regression, which is usually interpreted as indication of the biomagnification rate, for both the entire food web (0.15) and heterotrophs-only food web (0.25) were similar to what has been observed in other studies. Campbell *et al.* (2008), studying mercury fluxes in aquatic biota in Lake Tanganyika in east Africa, found slopes of 0.13 and 0.22 for the whole food web and fish-only food web, respectively. These results were comparable to that seen in other tropical lakes, including Lake Victoria (0.16; Campbell *et al.*, 2003), Lake Malawi (0.20; Kidd *et al.*, 2003) and Lake Murray in Papua New Guinea (0.28; Bowles *et al.*, 2001). Similar results have also been found in temperate freshwater food webs (0.17–0.29 for five to eight fish species; Kidd *et al.*, 1995, and 0.17–0.24 over three seasons for invertebrates and fishes in Lake Ontario, Canada; Zhang *et al.*, 2012) and in Arctic lacustrine food webs (0.14–0.22; van der Velden *et al.*, 2013). These results suggest that trophic level accumulation of Hg is comparable across systems and mercury biomagnification occurs independently of climatic factors.

The results presented here show additional supporting evidence for large, naturally occurring sources of mercury in the Rio Negro and the potential for Hg to be readily transferred up through an aquatic food web. The high total mercury concentrations found in *Cichla* spp. are cause for concern, as fish consumption is the most important predictor of human Hg exposure in the Amazon, explaining a substantial portion of the variation of total and methyl-Hg concentrations found in both blood and hair (Passos & Mergler, 2008). Overall, urban Amazonian fish-consumers are the least exposed, presenting mean hair-Hg levels of 2400 (Santos *et al.*, 2002), 2000 (Passos *et al.*, 2003) and 1900 ng g⁻¹ (Webb *et al.*, 2004). A pilot study in two

municipalities of the Rio Negro (São Gabriel da Cachoeira and Barcelos) showed mean hair-Hg levels of 13 000 and 9700 ng g⁻¹, with upper values as high as 81 300 ng g⁻¹ (Santos *et al.*, 2005). Considering the potential for Hg-induced harmful health effects, notably in women of reproductive age, dietary Hg exposures may be reduced through changes in fish-eating practices. The investigators of one study in a village on the Rio Tapajós in eastern Amazônia used a participatory strategy that aimed at reducing the consumption of contaminated species while maintaining fish consumption (Mertens *et al.*, 2005). Through community involvement and education, villagers continued to eat the same quantity of fish, but decreased the consumption of carnivores at the top of the food chain, which resulted in a reduction of hair-Hg levels of close to 40%.

In the future, more studies should investigate mercury methylation dynamics in wetland areas of the Rio Negro and determine the role of invertebrates in Hg transfer from basal energy sources to higher trophic levels, while at the same time promoting environmental education about the hazards of Hg exposure due to fish consumption of top predators.

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Supporting Information

Supporting Information may be found in the online version of this paper:

TABLE SI. *Paracheirodon axelrodi* sampling data, including dates, sample numbers, nitrogen stable isotope signatures ($\delta^{15}\text{N}$), total mercury concentrations (THg), log₁₀ THg concentrations, ln THg concentrations and mean $\pm$ s.d. standard length (L_S)

TABLE SII. *Paracheirodon simulans* sampling data, including dates, sample numbers, nitrogen stable isotope signatures ($\delta^{15}\text{N}$), total mercury concentrations (THg), log₁₀ THg concentrations and mean $\pm$ s.d. standard length (L_S)

TABLE SIII. *Cichla* spp. sampling data, including dates, species (M, *Cichla monoculus*; O, *Cichla orinocensis*; T, *Cichla temensis*), nitrogen stable isotope signatures ($\delta^{15}\text{N}$), total mercury concentrations (THg), log₁₀ THg concentrations and mean $\pm$ s.d. standard length (L_S)

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