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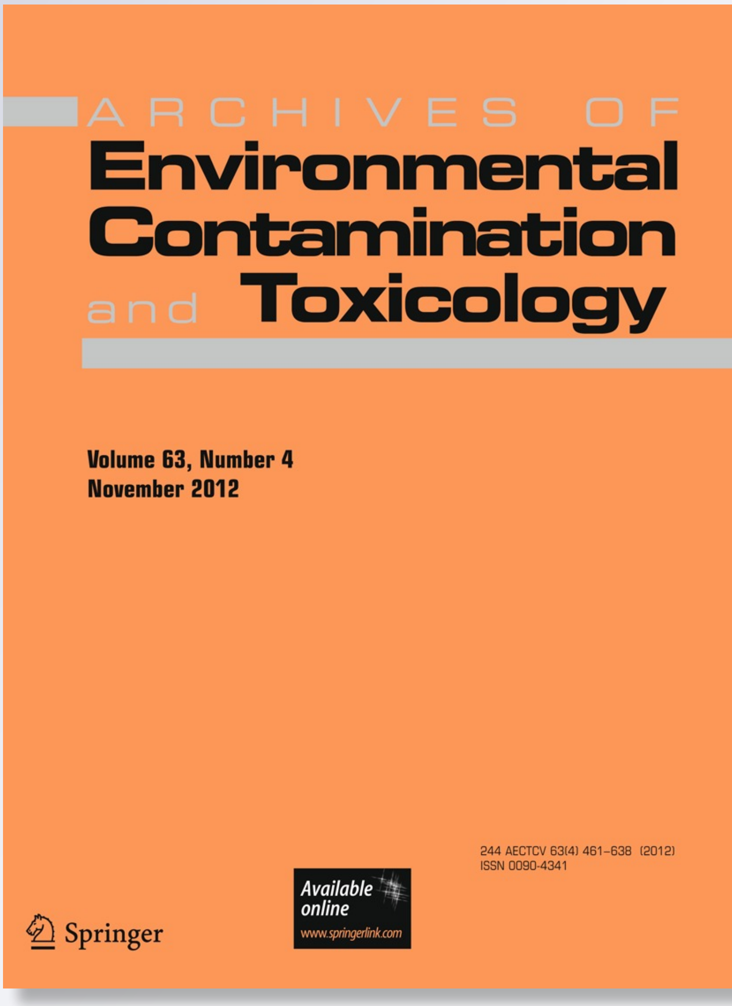

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Increased Mercury and Body Size and Changes in Trophic Structure of *Gambusia puncticulata* (Poeciliidae) Along the Almendares River, Cuba

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Abstract The Almendares River is the largest river draining the area around Havana City, Cuba. The watershed is heavily populated and industrialized, which has had a significant impact on the flow and water quality of the river. The main goal of this study was to analyze the spatial variability in dietary habits, nutrient flow (using stable isotope ratios δ^{15}N and δ^{13}C), and mercury (Hg) levels along the Almendares River upstream and downstream of point-source discharges using localized fish *Gambusia puncticulata*. Stomach contents of *G. puncticulata* were similar among these sites. However, mean δ^{15}N values ranged from 6 to 18 ‰ across sites and were lower in fish from downstream than upstream sites, suggesting localized influences of nutrient inputs along the river. δ^{13}C values were between –22 and –25 ‰, except at a mid-basin site (–26 to –27‰), indicating that fish relied on similar carbon sources at most sites. Total mercury concentrations ranged from 0.04 to 0.49 µg/g wet weight whole body and were unrelated to the among-site differences in δ^{15}N, but Hg exceeded the threshold considered to be protective of fish health (0.2 µg/g ww whole body) in the majority of fish from all sites but one. Results of this study indicate that although the dietary habits of this species do not vary across sites, tissue differences in δ^{15}N, δ^{13}C and Hg show little movement of this species among sites. Localized effects of human activities on nutrients and metals may be affecting the health of this species and posing a risk to other consumers in the ecosystem.

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The city of Havana, Cuba, still lacks many basic sanitation and waste-treatment facilities, and almost half of the city relies on local groundwater for drinking water (Olivares-Rieumont et al. 2005). The watershed of the Almendares River (40,202 km^2) dominates the western part of the city, and >500,000 inhabitants are impacted daily by water from the watershed (Advisory Committee for the Almendares-Vento Basin [ACAVB] 1999). This river is crucial for Havana residents for social, economic, community health, and recreational reasons, and poor water and soil management, overpopulation, intensive deforestation, and unregulated waste discharges have caused major decreases in river water quality.

The Almendares River flows through Havana City, and Cepero (2000) stated that 19,315 m^3 of wastes are deposited in to the river daily; approximately 80 % are of urban origin and the rest are from industrial discharges. More than 70 point sources of pollution have been identified in the watershed (ACAV 1999), many of which are uncontrolled and some of which are potential sources of metal contamination (*e.g.*, smelters, landfills, factories producing paints, electronics, untreated sewage) (Kabata-Pendias and Pendias 1992). There is a gradient of contamination in the Almendares River that starts in the upper river zone, where there are discharges of sewage and other wastes from >30 sources, and progresses through to the lower river where there is a greater number of industrial and residential discharges. Sediments and water hyacinth (*Eichhornia crassipes*) collected along the river have increasing concentrations of lead (Pb), chromium (Cr), copper (Cu), cadmium (Cd), and zinc (Zn) from upstream to downstream, and human activities have increased metal concentrations well above natural levels (Olivares-Rieumont et al. 2005, 2007). River waters are characterized by high fecal coliform ($2*10^3$ to $8*10^4$ Most Probable Number (MPN)/100 mL), nitrite (0.013 to 0.503 mg/L), nitrate (1 to 29 mg/L), and ammonium (0.014 to 5.37 mg/L), and the river becomes increasingly eutrophic from upstream to downstream (Instituto Nacional de Recursos Hidraulicos 2002).

Given the gradient in sediment, water, and aquatic plant contamination in this river and the importance of the watershed for subsistence fishing, it is critical to understand whether fish health and their metal and nutrient concentrations also vary among sites and increase from upstream to downstream with the known gradient of contamination. Stable isotopes of carbon (δ^{13}C) and nitrogen (δ^{15}N), respectively, are commonly used to assess and compare sources of energy and trophic position of biota (Peterson and Fry 1987). However, isotope ratios of these nutrients are also affected by human activities (*e.g.*, sewage inputs, agriculture), and both increase in biota from reaches where anthropogenic nutrient inputs are high (Gray et al. 2004; Anderson and Cabana 2005). As a result, these ratios can also be used to assess the degree and gradient of human impact within a watershed (Anderson and Cabana 2005) as well as the degree of movement of organisms among sites if there are gradients in nutrient inputs and their isotopic composition (Gray et al. 2004).

G. puncticulata is a common Cuban cyprinodont (de Leon et al. 2011), is viviparous with a short generation time, has flexible life-history characteristics (Abney and Rakocinskia 2004) and is well adapted to low-oxygen conditions (Stevens et al. 2010). In a previous, preliminary study, *G. puncticulata* were collected at three sites along the Almendares River, and those collected at the site furthest downstream had the best condition and the lowest stable isotope values (Cabrera et al. 2008). In this study, we collected fish from a greater number of sites to determine the extent of nutrient and mercury (Hg) contamination, to determine whether this species moves within the river and varies in its dietary habits, and to determine any potential risks from Hg. Therefore, the main goal was to analyze the nutrient gradient and contaminants detected in *G. puncticulata* along the Almendares River.

Materials and Methods

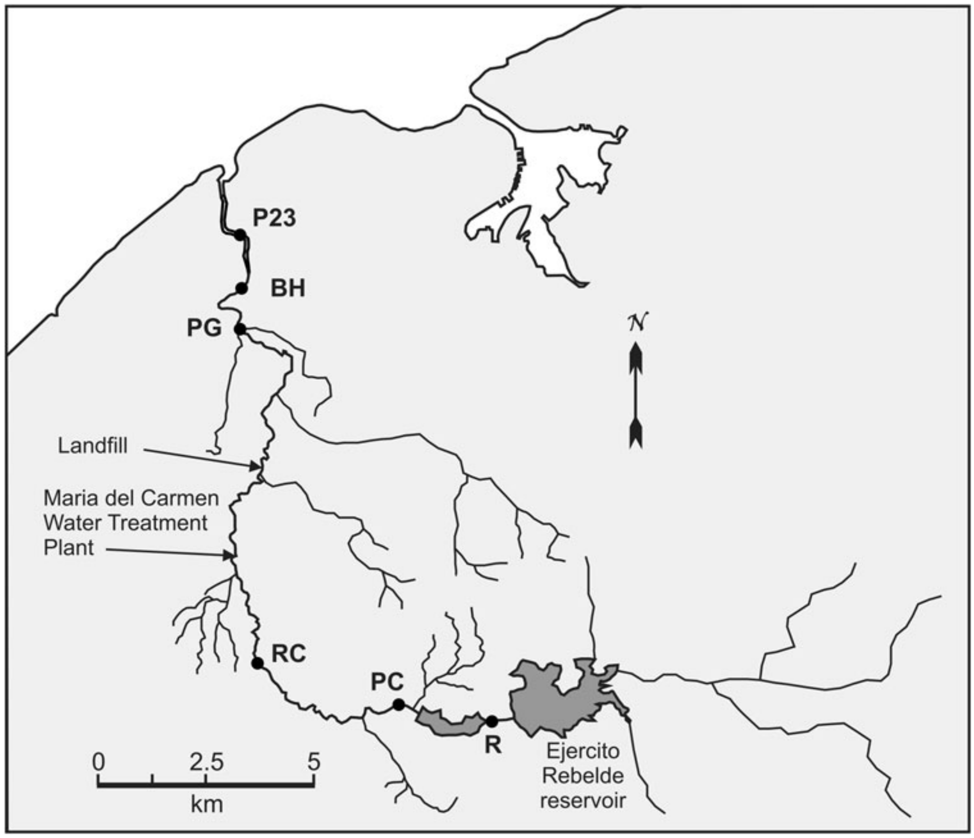
Study Area and Sampling Sites

The research was performed on fish at six sites along the Almendares River (Fig. 1). Sampling sites were as follows: Rodeo (R) (23 01′11″ N, 82 21′03″ W), Calabazar Bridge (PC) (23 01′09″ N, 82 22′19″ W), Crystal River (RC) (23 02′01″ N, 82 24′05″ W), Grandes Bridges (PG) (23 05′55.3″ N, 82 24′27.4″ W), Havana Forest (BH) (23 06′30″ N, 82 24′26″ W), and 23 Bridge (P23) (23 07′07″ N, 82 24′31″ W). Site R is just downstream of the Ejercito Rebelde reservoir, and, according to Olivares-Rieumont et al. (2005), has high organic pollution but the lowest metal concentrations (Cu, Cr, Cd, Zn, and Pb) in sediments collected along the river. Hg was not measured in the Olivares-Rieumont et al. (2005) study. The RC site is on the Almendares River proper; it was named after a tourist center adjacent to the river. At this site the sediment metal concentrations are 1.5- to 5-fold greater than those at site R (Olivares-Rieumont et al. 2005). Between sites RC and PG, there is a large solid waste landfill, and sediment and water hyacinth metal concentrations are ≤10 times greater at the latter than former site (Olivares-Rieumont et al. 2005, 2007). As the river flows downstream, there is an increase in the discharge of nontreated sewage from adjacent towns to the river (Lima-Cazorla et al. 2005). In addition, antibiotic levels were generally low upstream (sites R, PC, and RC) but were increased substantially downstream, probably due to inputs from the pharmaceutical factories (Graham et al. 2011). There is a clear gradient in Pb, Cu, Cd, and Zn concentrations as distance downstream increases with concentrations increasing by 10.8-, 36.2-, 10-, and 8.8-fold, respectively, from sites R to BH (Olivares-Rieumont et al. 2005). Site P23 is furthest downstream (2 km from the river mouth) and has much greater concentrations of nutrients, hydrocarbons, fats, and oils, showing substantial deterioration in the quality of the water in this area (Hernández 2004). Salinity profiles did not show a significant tidal influence at this site (Cabrera Páez [unpublished data]).

Sampling and Methods for Biological Analyses

G. puncticulata were captured using hand-held nylon nets (stretched mesh size 1 mm) every 2 months from July 2008 to December 2009 for stomach content analysis and in July 2008 for stable isotope and Hg analyses. Fish were caught in the shallow waters near the shore, and between 10 and

Fig. 1 Locations of sites sampled on the Almendares River, Cuba. *Sites* Rodeo (R), Calabazar Bridge (PC), Crystal River (RC), Grandes Bridge (PG), Havana Forest (BH), and 23 Bridge (P23)



100 individuals were collected on each date from each site. The fish were transferred alive to the laboratory at the Centre of Marine Research, University of Havana, Cuba (approximately 60 min) where they were killed by spinal severance before taking measurements, stomach contents, and tissue samples. Fish were individually measured for total length and total mass (both ±1 mg); viscera were then removed and carcass mass recorded; and liver and gonad weights (±0.001 g; Table 1) were determined. The stomachs were preserved in 70 % ethanol for later analysis. Calculations were made from raw data for liver somatic index (100 × liver weight/body weight), gonadosomatic index (100 × gonad weight/body weight), condition factor (CF; 100,000 × weight [g])/(length^3 [mm]), and adjusted CF (100,000 × (body weight–gonad weight [g])/(length^3 [mm])).

Stomach contents ($N = 783$ fish; sample size at each site: R $n = 61$, PC $n = 116$, RC $n = 63$, PG $n = 252$, BH $n = 143$, and P23 $n = 148$) were viewed through stereoscopic microscope, and each food item was identified to the lowest possible taxa. To express the diet composition, the frequency of occurrence method was used (% F_i) as defined by (Rosecchi and Nouaze 1987) as follows:

$$F_i = (f_i \times 100)/n,$$

where f_i = the number of stomachs where entity i was found, and n = total number of analyzed stomachs.

The numerical method was also used as a complement for the analysis of the stomach contents. It counts the total number of individuals of each food item and is usually expressed as a percentage of the total number of organisms found in all fish examined at that site (Hyslop 1980). The item ''insect remains'' includes a wide range of organisms (aquatic and terrestrial) and was used because further identification was not possible due to the fragmentation of individuals or high degree of digestion of the samples. In many cases it was impossible to distinguish the habitat (aquatic *vs*. terrestrial) of the insect remains.

For stable isotope analyses, a piece of white muscle was dissected from each fish and kept at –20 °C until tissues were processed. The samples were dried at 60 °C for 48 h and then ground into a fine powder with a mortar and pestle. An aliquot of 200 μg was taken from every sample and packed into a 3 × 5 mm tin cup. The individual fish samples were combusted using a Thermoquest NC 2500 (ThermoQuest Corp. Austin, TX), and gases were submitted by way of a continuous flow of helium to a Finnigan MAT Delta Plus isotope-ratio mass spectrometer for $\delta^{13}C$ and $\delta^{15}N$ analyses at the Stable Isotopes in Nature Laboratory, University of New Brunswick. Precision and accuracy of the method was verified by analyzing replicates of commercial isotopes standards (N_2, sucrose, acetanilide, nicotinamide, SMB-M, BLS; International

Table 1 Sample sizes, lengths, and carcass weights for *G. puncticulata* analyzed for among-site differences regarding condition, THg, and stable isotopes

Sites	Total N	Total length (mm)	Total carcass weight (g)	N THg	Total length (mm) THg (µg/g)	Total carcass weight (g) THg (µg/g)	N $\delta^{13}C/\delta^{15}N$	Total length (mm) $\delta^{13}C/\delta^{15}N$	Total carcass weight (g) $\delta^{13}C/\delta^{15}N$
	140	29.14 ± 0.55^{ab}	0.328 ± 0.020^{ab}	21	33.95 ± 1.25	0.48 ± 0.03	10	29.9 ± 1.47	0.40 ± 0.06
PC	223	28.29 ± 0.37^{b}	0.353 ± 0.017^{ab}	20	32.20 ± 1.37	0.47 ± 0.07	10	36.6 ± 1.62	0.49 ± 0.08
RC	120	29.28 ± 0.59^{ab}	0.375 ± 0.029^{ab}	20	34.80 ± 1.40	0.65 ± 0.09	11	32.00 ± 1.45	0.46 ± 0.05
PG	373	29.39 ± 0.33^{ab}	0.339 ± 0.015^{b}	10	23.30 ± 0.67	0.18 ± 0.01	11	29.00 ± 1.45	0.42 ± 0.07
BH	269	30.31 ± 0.47^{a}	0.438 ± 0.024^{a}	13	31.77 ± 0.45	0.46 ± 0.02	13	33.00 ± 1.11	0.54 ± 0.07
P23	254	31.13 ± 0.44^{a}	0.437 ± 0.023^{a}	30	32.00 ± 1.09	0.43 ± 0.04	10	31.8 ± 1.40	0.42 ± 0.06

Values are means ± SE. Values sharing a superscript letter are not significantly different

Sites Rodeo (R), Calabazar Bridge (PC), Crystal River (RC), Grandes Bridge (PG), Havana Forest (BH), and 23 Bridge (P23)

Atomic Energy Agency). Duplicates of some fish were analyzed every 10th sample, and the relative percent differences were 0.9 and 1.1 % for $\delta^{13}C$ and $\delta^{15}N$, respectively. Stable isotope ratios were reported as follows:

$$\delta X = \left[\left(R_{\text{samples}}/R_{\text{standard}}\right) - 1\right] \times 1000,$$

where $X = {}^{13}C$ or ${}^{15}N$ and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

THg analyses were also performed on muscle tissue of individual fish collected in July 2008 ($N = 114$; R $n = 21$, PC $n = 20$, RC $n = 20$, PG $n = 10$, BH $n = 13$, and P23 $n = 30$). However, fish analyzed for THg were different from the fish used for the isotope analyses; due to their small size, it was impossible to take muscle for both analyses. For THg analyses, approximately 10 mg freeze-dried, homogenized individual fish muscle was weighed into a precleaned quartz boat to the nearest 0.01 mg. Samples were analyzed on a Milestone DMA-80 Direct Mercury Analyzer by thermal decomposition, amalgamation, and atomic absorption spectrophotometry according to United States Environmental Protection Agency (USEPA) Method 7473 (USEPA 2007). To ensure accuracy and precision of the data, standards and certified reference materials (CRMs) (TORT-2 and DORM-2; National Research Council of Canada) were analyzed after every 10th sample. Mean (±SD) recoveries of CRMs were 115.7 ± 8.3 % ($n = 21$) and 95.6 ± 4.6 % ($n = 14$) for TORT-2 and DORM-2, respectively. A 15 ng calibration standard yielded recoveries of 99.0 ± 10.8 % ($n = 21$). Relative percent differences of 14 duplicate samples ranged from 1.5 to 19.0 %. The limit of detection (LOD) for these analyses was 0.0341 µg/g dry weight (dw). For comparisons of THg concentrations with known toxicity thresholds for fish, dry-weight concentrations were converted to whole body wet-weight (ww) concentrations assuming 80 % moisture and an average whole body–to–muscle THg ratio of 0.6 (Wyn et al. 2009).

Statistical Analysis

Before statistical analyses, normality of the data and homogeneity of variances were examined according to the criteria of Zar (1996) and Underwood (1997). Analysis of variance (ANOVA) was used to determine whether stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) and Hg concentrations varied among sites. In cases where ANOVA yielded significant *F*-values, the Student-Newman–Keuls (SNK) post hoc procedure was used to compare all pairs of means. Spearman's correlation coefficients were used to assess the similarity in diets among sampling sites. The significance level for all tests was 0.05. All data are expressed as mean ± SE. STATISTICA version 6.0 software for Windows (Statsoft Inc., Tulsa, OK) was employed for data processing.

Results

More than 1350 *G. puncticulata* were collected among the six sites, and fish at the two sites furthest downstream weighed considerably more (33 %) than fish at all of the four upstream sites (Table 1). Fish were collected during 4 or 5 months at all sites, and the two sites furthest upstream (sites R and PC) showed no significant differences in length of fish between months ($F_{3,136} = 1.210$; $p = 0.37$, $F_{4,218} = 1.073$, $p = 0.37$, respectively). All other more highly contaminated sites showed significant differences between months, although there were no consistent differences. As distance downstream increased, fish at site RC were largest in January and smallest in July ($F_{4,115} = 8.237$; $p < 0.001$); at site PG fish were largest in January and smallest in October ($F_{4,368} = 15.18$; $p < 0.001$); at site BH fish were smallest in April ($F_{3,265} = 7.621$; $p = < 0.001$); and at site P23 they were largest in June ($F_{3,250} = 12.633$; $p < 0.001$).

Table 2 Length, weight, condition, adjusted condition (using carcass weight), LSI, and GSI for *G. puncticulata* from sites sampled on the Almendares River pooled in June and July

Sex	Site	Length (cm)	Weight (g)	K	Kadj	LSI (% body weight)	GSI (% body weight)
Females	R	3.52 ± 0.08 (51)^{bc}	0.72 ± 0.05 (51)^{c}	1.57 ± 0.03 (51)	2.03 ± 0.07 (51)^{b}	2.60 ± 0.31 (18)^{ab}	20.08 ± 1.83 (46)^{a}
	PC	3.37 ± 0.08 (51)^{c}	0.69 ± 0.06 (40)^{bc}	1.83 ± 0.08 (40)	2.41 ± 0.07 (51)^{a}	4.88 ± 0.47 (26)^{a}	16.67 ± 1.48 (40)^{ab}
	RC	3.31 ± 0.10 (43)^{c}	0.34 ± 0.05 (21)^{c}	1.25 ± 0.05 (21)	2.10 ± 0.07 (43)^{b}	5.20 ± 1.37 (13)^{ab}	13.88 ± 1.22 (28)^{ab}
	PG	3.43 ± 0.10 (49)^{bc}	0.67 ± 0.06 (49)^{c}	1.59 ± 0.08 (49)	1.96 ± 0.10 (49)^{bc}	1.64 ± 0.57 (7)^{b}	16.49 ± 1.52 (30)^{ab}
	BH	3.70 ± 0.07 (99)^{ab}	1.04 ± 0.06 (99)^{ab}	1.80 ± 0.05 (99)	2.20 ± 0.06 (99)^{ab}	3.16 ± 0.54 (25)^{b}	18.65 ± 1.13 (70)^{a}
	P23	3.88 ± 0.07 (77)^{a}	1.07 ± 0.09 (57)^{a}	1.54 ± 0.05 (57)	2.06 ± 0.06 (77)^{bc}	1.75 (2)	14.17 ± 1.95 (52)^{b}
Males	R	2.65 ± 0.05 (58)^{a}	0.30 ± 0.01 (52)^{a}	1.63 ± 0.08 (52)^{a}	2.39 ± 0.11 (58)^{a}	2.25 ± 0.83 (4)	10.25 ± 5.33 (17)^{a}
	PC	2.83 ± 0.03 (75)^{a}	0.34 ± 0.01 (75)^{a}	1.46 ± 0.03 (75)^{a}	2.27 ± 0.05 (75)^{a}		11.25 ± 1.72 (22)^{a}
	RC	2.65 ± 0.07 (25)^{a}	0.31 ± 0.03 (25)^{a}	1.75 ± 0.29 (25)^{a}	2.99 ± 0.48 (25)^{a}		16.63 ± 4.67 (6)^{a}
	PG	2.98 ± 0.03 (136)^{a}	0.35 ± 0.01 (136)^{a}	1.28 ± 0.02 (136)^{a}	1.79 ± 0.04 (136)^{a}		
	BH	3.06 ± 0.05 (69)^{a}	0.43 ± 0.02 (69)^{a}	1.47 ± 0.04 (69)^{a}	2.31 ± 0.07(69)^{a}		
	P23	2.98 ± 0.05 (60)^{a}	0.39 ± 0.02 (40)^{a}	1.36 ± 0.04 (40)^{a}	2.21 ± 0.05 (60)^{a}		

Data are shown as mean ± SE (*n*). Values sharing an superscript letter are not significantly different within sex

Sites Rodeo (R), Calabazar Bridge (PC), Crystal River (RC), Grandes Bridge (PG), Havana Forest (BH), and 23 Bridge (P23)

There were no differences between June, July, and October of the relationships between gonad weight and body weight, and pooled values for June and July were used for comparisons between sites. During June and July, female fish at site P23 were significantly longer and heavier than all other sites except site BH ($F_{5,364} = 7.876$, $p < 0.001$; $F_{5,364} = 7.481$, $p < 0.001$; Table 2), although the best-condition fish were seen at sites PC and BH (k_{adj} $F_{5,364} = 4.336$, $p = 0.001$). The largest relative liver sizes (LSI) were seen at two of the three upstream sites (sites PC and RC; $F_{4,84} = 3.315$, $p = 0.014$), and the largest relative gonad sizes (GSI) were seen at site R and BH ($F_{5,262} = 3.44$, $p = 0.005$). During June and July, there were no differences among sites regarding male fish length ($F_{2,40} = 0.946$, $p = 0.40$), carcass weight ($F_{2,40} = 0.95$, $p = 0.40$), k_{adj} ($F_{2,40} = 0.782$, $p = 0.78$), or GSI ($F_{2,48} = 2.57$, $p = 0.089$) (Table 2).

Between months at each site, there were no differences in the length of female fish at the furthest upstream sites, including sites R ($F_{3,44} = 0.43$, $p = 0.68$) and PC ($F_{4,40} = 0.184$, $p = 0.95$), but there were seasonal differences at all downstream sites although they were inconsistent. At site RC ($F_{2,29} = 7.714$; $p = 0.002$) in October, fish were shorter than in other months; at site PG ($F_{3,32} = 8{,}26$, $p < 0.001$) fish became progressively larger throughout the year; and at site BH ($F_{2,67} = 5.228$; $p = 0.014$) fish were largest in June. Similar to site PG, site P23 showed progressive increases in female fish size throughout the year ($F_{3,68} = 13.583$; $p < 0.001$), with January fish being smaller than June fish ($p < 0.001$), June fish smaller than July fish ($p = 0.01$), July fish smaller than October fish ($p = 0.003$), and October fish longer (by 27 %) than January fish.

Mean THg concentrations ranged from 0.08 to 0.36 μg/g ww whole body (0.70 to 2.99 μg/g dw) across all sites and were significantly different between sites in both male ($F_{5,31} = 17.189$; $p < 0.001$) and female fish ($F_{5,46} = 39.858$; $p < 0.001$) (Table 3). Hg concentrations were lowest at site RC and highest at site BH, with all other sites having intermediate concentrations (site BH [0.36] > site R [0.27]; site PG [0.25] > site PC [0.19]; and site P23

Table 3 THg concentrations in muscle (μg/g dw) and whole body (μg/g ww) for *G. puncticulata* collected from sites along the Almendares River (mean ± SE [*n*])

Site	Male fish		Female fish		Juvenile fish	
	Muscle (dw)	Whole body (ww)	Muscle (dw)	Whole body (ww)	Muscle (dw)	Whole body (ww)
R	3.44 ± 0.21 (6)^{a}	0.41 ± 0.03 (6)	1.69 ± 0.21 (12)^{a}	0.20 ± 0.03 (12)		
PC	1.48 ± 0.22 (9)^{c}	0.18 ± 0.03 (9)	1.67 ± 0.21 (9)^{a}	0.20 ± 0.03 (9)		
RC	0.79 ± 0.09 (6)^{d}	0.10 ± 0.01 (6)	0.65 ± 0.06 (12)^{c}	0.08 ± 0.01 (12)		
PG	2.48 ± 0.42 (4)^{b}	0.30 ± 0.05 (4)			1.84 ± 0.38 (6)^{a}	0.22 ± 0.05 (6)
BH	3.23 ± 0.19 (7)^{a}	0.39 ± 0.02 (7)	2.57 ± 0.61 (4)^{a}	0.31 ± 0.07 (4)		
P23	2.39 ± 0.43 (5)^{b}	0.29 ± 0.05 (5)	1.10 ± 0.07 (14)^{b}	0.13 ± 0.01 (14)	1.65 ± 0.22 (9)^{a}	0.20 ± 0.03 (9)

Data are shown as mean ± SE (*n*). Values sharing an superscript letter are not significantly different within sex

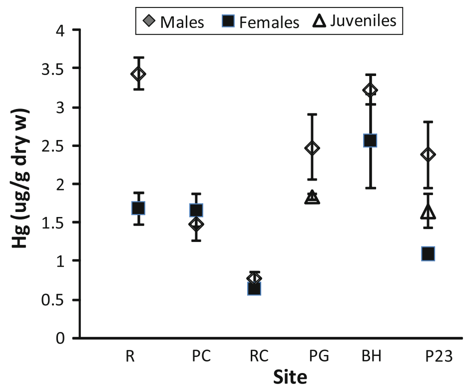


Fig. 2 Hg concentrations (μg/g dw; mean ± SE) in male, female, and juvenile *G. puncticulata* across sites along the Almendares River

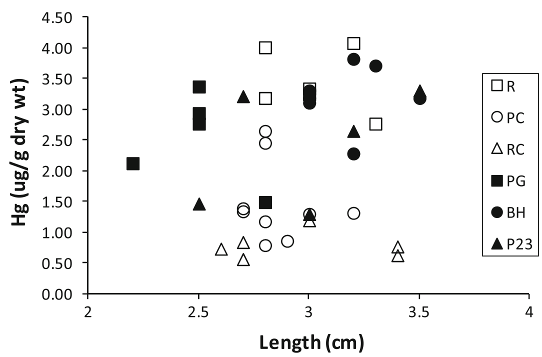


Fig. 3 THg (μg/g dw) and length (cm) of *G. puncticulata* at sites along the Almendares River

[0.18] > site RC 0.08 μg/g ww whole body) (Fig. 2). No consistent upstream-to-downstream gradient in Hg concentrations were found, but Hg concentrations at site BH were on average 4.5-fold greater than at the site with the lowest concentrations. There was no evidence of a relationship between body size and THg for *Gambusia* sp. (Fig. 3).

Some among-sex differences in THg were observed for *Gambusia* sp. from the Almendares River. There was an interaction between sex and site ($F_{54,1,4,74} = 8.044$; $p = 0.008$) because there were no sex differences at the two lowest sites in contamination (site PC and RC [$F_{1,1,1,32} = 0.033$; $p = 0.86$]), but there were differences at all of the more highly contaminated sites ($F_{2,1,2,42} = 36.639$; $p < 0.001$), with male fish having on average 57 % greater Hg concentrations. Across all sites, many fish exceeded a threshold of 0.2 μg THg/g ww whole body considered to be protective (Beckvar et al. 2005) and included 14 of 52 female fish (27 %), 21 of 37 male fish (57 %), and 5 of 15 juvenile fish (33 %).

Stable Isotope Ratios

G. puncticulata collected from the three sites in the upper Almendares River had $\delta^{15}N$ values (means 12–19 ‰) that were 6–11 ‰ greater than fish from the three sites sampled downstream (Fig. 4). Within the three upstream sites, the highest $\delta^{15}N$ values (sexes pooled) were found at site RC (mean = 18 ‰), whereas mean $\delta^{15}N$ was 15.02 ± 0.56 and 15.75 ± 0.47 ‰ at site R and PC, respectively. In contrast, mean $\delta^{15}N$ was lower in fish from the downstream sites (site PG = 6.47 ± 0.25 ‰; site BH = 7.31 ± 0.22 ‰; and site P23 = 7.74 ± 0.31 ‰). Mean $\delta^{15}N$ values were not significantly different between sexes (ANOVA, $F_{1.53} = 0.413$, $p = 0.52$), but among-site differences (pooled across sex) were found ($F_{5.53} = 283.19$, $p < 0.001$). Fish at site RC had the greatest $\delta^{15}N$ values compared with all other sites, and fish from the three downstream sites (sites PG, BH, and P23) had lower $\delta^{15}N$ values (SNK test).

The $\delta^{13}C$ values of *G. puncticulata* also varied but showed fewer differences across sites than $\delta^{15}N$ values (Fig. 4). No among-sex differences were found ($F_{1.53} = 0.20$, $p = 0.65$), and data were pooled within sites for subsequent analyses. Across sites, mean $\delta^{13}C$ values ranged from –22 to –26 ‰ (R = –23.48 ± 0.71 ‰; PC = –23.78 ± 0.27 ‰; RC = –26.22 ± 0.35 ‰; PG = –22.97 ± 0.13 ‰; BH = –23.95 ± 0.17 ‰; and P23 = –22.03 ± 0.20 ‰) and were significantly different for fish from site RC compared with all other sites (ANOVA, $F_{5.53} = 15.52$, $p < 0.001$, SNK test).

Food

The feeding habits of *G. puncticulata* were grouped into 14 categories and were similar among sites (Table 4). This species fed on a mix of organisms with invertebrates (aquatic and terrestrial) and algae being the most common. According to the frequency-of-occurrence method, the most frequent item was "insect remains" across all sites (similar results were also obtained using the numerical method [Tables 4 and 5]). Fish from site P23 most frequently consumed *Oscillatory* spp., which belongs to the cyanobacteria group. This prey group was only observed at the most impacted sites (site P23 = 13.51 %; site BH = 1.40 %; site PG = 1.19 %; and site RC = 1.62 %), and fish remains were only found at downstream sites P23 and BH. Some prey items (Homoptera, Zygoptera, larval individuals, and *Gundlachia radiata*) were only observed in fish from site BH. Fish from site PC had more plant remains than those from all other sites. The mollusk species *Tarebia granifera* was found only in fish at the farthest upstream sites of the river (site R and PC), and a copepod species (family Diaptomidae) was only found in one fish stomach from site R (Table 4). Prenolepini (belonging to a "Caribbean crazy ants" group) was abundant in fish from a less contaminated site (site R = 36.07 %), followed by two other sites with very low values (site RC = 3.17 %; site PG = 0.79 %).

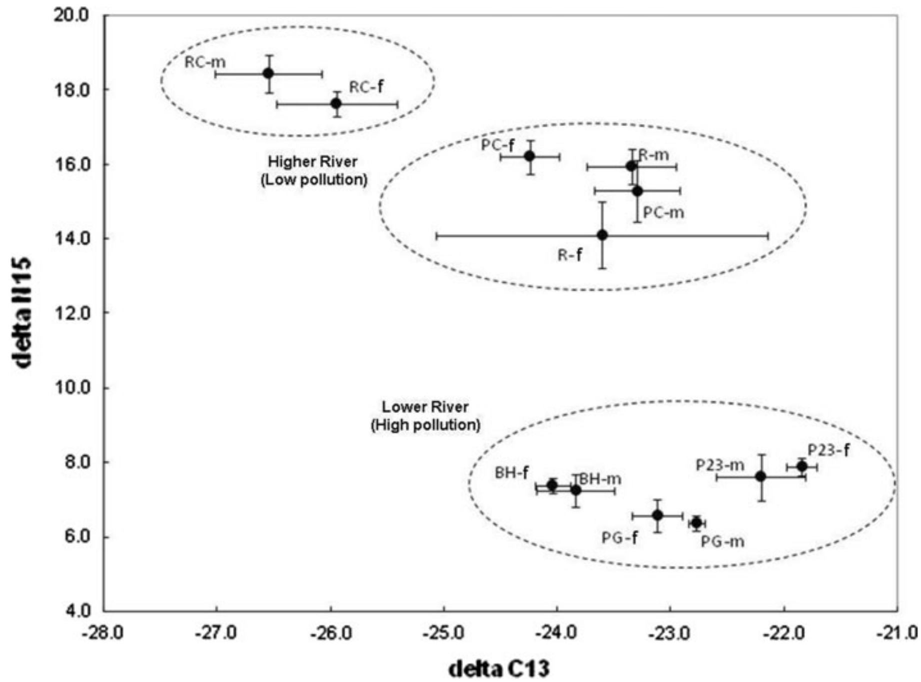


Fig. 4 Mean (±SE) $\delta^{13}C$ *versus* $\delta^{15}N$ (‰) of male fish (m) and female fish (f) *G. puncticulata* across sites along the Almendares River. R-f = Rodeo female and R-m = Rodeo male; PC-f = Calabazar Bridge female and PC-m = Calabazar Bridge male; RC-f = Crystal River female and RC-m = Crystal River male; PG-f = Grandes Bridge female and PG-m = Grandes Bridge male; BH-f = Havana Forest female and BH-m = Havana Forest male; P23-f = 23 Bridge female and P23-m = 23 Bridge male

Table 4 Diet composition comparative analysis using frequency methods (%) for *G. puncticulata* from sites sampled along the Almendares River

Food item	Sites					
	R	PC	RC	PG	BH	P23
Insect remains	47.54	24.14	49.21	37.30	43.36	27.03
Oscilatoria spp.			1.59	1.19	1.40	13.51
Homoptera					0.70	
Diptera				0.40		0.68
Gundlachia radiata					0.70	
Zygoptera					1.40	
Prenolepini	36.07		3.17	0.79		
Solenopsini	1.64		4.76	1.98	2.80	3.38
Diaptomidae	1.64					
Tarebia granifera	1.64	0.86				
Plant remains		7.76		1.19		
Physa acuta			3.17		1.40	
Rock remains		0.86		0.40		
Fish remains					0.70	0.68

Sites Rodeo (R), Calabazar Bridge (PC), Crystal River (RC), Grandes Bridge (PG), Havana Forest (BH), 23 Bridge (P23)

Across most sites, *G. puncticulata* had similar dietary habits. Spearman's rank correlation coefficients for pairs of sites yielded significant correlations for this species studied (RC to P23: $r_s = 0.54$, $p = 0.048$; R to RC: $r_s = 0.56$, $p = 0.036$; BH to P23: $r_s = 0.57$, $p = 0.033$; RC to BH: $r_s = 0.63$, $p = 0.016$; PG to P23: $r_s = 0.63$, $p = 0.015$; and RC to PG $r_s = 0.64$, $p = 0.014$).

Discussion

In the Almendares River, the fish were largest at the two sites furthest downstream as was found in a preliminary study (Cabrera et al. 2008). Unlike the preliminary study, however, in this study there was no consistency in fish from sites with the best condition, LSI, or GSI, and the site furthest downstream did not reflect the largest changes. The earlier study did not separate sites by sex and season, and the inconsistency shown in this study regarding how body sizes and organ sizes change between months and between sexes complicates interpreting the results of the pooled data in the earlier study. The clear separation in isotopic composition of *G. puncticulata* across sites (Fig. 4) shows that this species uses localized habitats within the river and that the among-site differences in condition, liver, and gonad sizes were related to local inputs.

Table 5 Diet composition comparative analysis using numerical methods for *G. puncticulata* from sites sampled along the Almendares River

Food item	Sites					
	R	PC	RC	PG	BH	P23
Insect remains	55.72	77.27	88.96	93.85	86.51	82.29
Oscilatoria spp.			1.84	0.77	0.79	10.42
Homoptera					0.40	
Diptera				0.26		0.52
Gundlachia radiata					4.37	
Zygoptera					0.79	
Prenolepini	40.80		3.68	0.26		
Solenopsini	2.49		2.45	1.79	1.98	5.73
Diaptomidae	0.50					
Tarebia granifera	0.50	0.65				
Plant remains		20.78		2.31		
Physa acuta			3.07		3.97	
Rock remains		1.30		0.77		
Fish remains					1.19	1.04

Sites Rodeo (R), Calabazar Bridge (PC), Crystal River (RC), Grandes Bridge (PG), Havana Forest (BH), and 23 Bridge (P23)

We observed that mean THg concentrations in fish in the Almendares River varied by 4-fold across sites (0.08–0.35 µg/g ww whole body). The proximity to anthropogenic sources has been linked to spatial differences in Hg levels of fish from the Virginia River (Hildebrand et al. 1976) and Florida estuaries (Strom and Graves 2001). Unlike previous studies on the Almendares River of metals in sediments and water hyacinth, which tended to increase from upstream to downstream (Olivares-Rieumont et al. 2005), Hg concentrations decreased in fish from sites R to RC (across the three upstream sites) and was lower in fish from site P23 (furthest downstream) than those from the two sites just upstream. Although the highest Hg concentrations were found in fish at site BH within Havana, no consistent gradient in fish Hg concentrations was observed. The behaviour of Hg in aquatic environments is complex and affected by numerous chemical and physical processes. Even if Hg inputs are less in the Almendares River greater, physical and chemical differences within the system may decrease the bioavailability of Hg to fish (Munn and Short 1997; Paller et al. 2004). Alternatively, if nutrient inputs are greater in the lower Almendares River, faster-growing fish may also have lower Hg levels because of growth dilution.

Mean Hg concentrations in *Gambusia* often exceeded the threshold believed to be protective of fish health. These Hg concentrations were generally greater than the maximum average levels (0.09 µg/g ww whole body [assuming 80 % moisture]) in wild *G. affinis* in permanent wetlands in California (Ackerman and Eagles-Smith 2010),C > but lower than the 3 to 4 µg/g ww whole body reported for mosquitofish from artificial ponds dosed with high concentrations of Hg (*G. holbrooki* [Hopkins et al. 2003]). At five of the sites (sites P23, PC, PG, R, and BH) on the Almendares River, 21, 39, 50, 61, and 91 %, respectively, of fish had THg concentrations greater than the 0.2 µg/g ww threshold believed to be protective of adult and juvenile fish (Beckvar et al. 2005 [no fish exceeded the threshold at site RC]). Fish with greater than this concentration are at risk of Hg intoxication, and the effects can include decreased growth, development, and reproduction as well as changes in behaviour (Beckvar et al. 2005). Mercury hotspots have been found in the Everglades, with G. holbrooki concentrations above 400 ng/g (United States Environmental Protection Agency 1998) and that pose a risk to their predators (Rumbold et al. 2008).

THg concentrations in male *Gambusia* spp. were greater at four of the six sites in the Almendares River compared with those in female fish, thus supporting results of other studies (*e.g.*, Nicoletto and Hendricks 1988). Ovarian tissues are known to contain Hg, although at lower concentrations than in muscle, and Hg levels in ovarian tissues are greater in fish with greater levels of muscle Hg (Donald and Sardella 2010; Johnston et al. 2001). Lower concentrations of Hg in female than male fish in this study may be due to some loss of Hg through egg production and spawning ($\leq$2 % loss; Johnston et al. 2001), although most of the Hg in eggs comes directly from the diet and not from Hg previously stored in body tissues (Hammerschmidt and Sandheinrich 2005).

Because Hg is biomagnified upward through the food web (Newman 1998), larger-bodied fish feeding on *Gambusia* spp. will have greater concentrations and may pose a risk to those fish as well as their human and wildlife consumers (Oken et al. 2005; Onsanit et al. 2012). This risk may spread beyond the river itself to the coastal zone; Aguilar et al. (2008) attributed differences in δ^{13}C values among coastal sites near Havana to carbon from terrestrial sources arriving in the coastal zone from the Havana harbour and Almendares River. They also found that fish were larger in the vicinity of the harbour because of nutrient additions due to sewage, which can increase ecosystem productivity and enhance the growth of fish. Analyses conducted concomitantly with the current study at Playita site 16 showed increased mean THg in male *Holocentrus rufus* of 0.87 (SD; $n = 0.50$; 4, maximum 1.46) and male *Stegastes partitus* of 0.37 (0.18, 5) (data not shown).

Hg accumulation in freshwater fish usually depends on several factors, such as the size of the fish, aqueous pH and dissolved organic carbon concentrations (Haines et al. 1994), ecological and geological characteristics of the basin, type of human activity, algal productivity (Kamman et al. 2004), and zooplankton community structure (Chen et al. 2005). Kamman et al. (2004) found lower average of Hg levels in urbanized areas and attributed this to a dilution of Hg by greater algal production at these sites. In this study, there was no strong relationship between fish body size and Hg concentrations. There were also no consistent trends between Hg and $\delta^{15}N$ values across sites, although fish at site RC had the lowest Hg and highest $\delta^{15}N$ values, and fish at site BH had the highest Hg and lowest $\delta^{15}N$ values; fish from the other sites had intermediate Hg concentrations but variable $\delta^{15}N$ values (Figs. 2 and 4). Among-site differences in nutrient inputs in the Almendares River could explain some of the variability in fish Hg concentrations, although fish Hg did not progressively decrease with increasing eutrophication downstream, and there was no relationship between Hg and $\delta^{15}N$, suggesting that nitrogen and Hg cycling are disconnected in this river. Hg concentrations were lowest at site RC (where fish had the lowest $\delta^{13}C$ values), which suggests that source of the food affects fish Hg concentrations. The among-site differences in $\delta^{13}C$ values are not possible to explain from the results of this current study, and the interactions between $\delta^{13}C$ or $\delta^{15}N$ and Hg cycling, if any, require further study.

The high values of $\delta^{15}N$ at some sites in the Almendares River suggest that there were greater inputs of nitrogen (or nitrogen with increased $\delta^{15}N$) in some reaches. Human activities, such as crop production and ranching, are known to increase the $\delta^{15}N$ values of resident aquatic biota (Anderson and Cabana 2005). However, the $\delta^{15}N$ values of the fish did not increase from upstream to downstream as one would expect in a system with greater human activities in the lower than upper part of the river (Anderson and Cabana 2005). Fish from the upper river sites were greater in $\delta^{15}N$ values than those from the lower river sites, indicating inputs of $\delta^{15}N$-increased nitrogen to the upper reaches that dissipated downstream. This result matches with that of Cabrera et al. (2008) for the same species in three sites at the Almendares River. The effects of human sewage inputs on $\delta^{15}N$ values are conflicting. Some studies have found greater $\delta^{15}N$ values in systems influenced by sewage than in those affected by agriculture, aquaculture, and other industries (Macko and Ostrom 1994; Heikoop et al. 2000; Deudero et al. 2004; Townsend-Small et al. 2007), whereas other studies have shown lower $\delta^{15}N$ values in sewage-impacted coastal regions (Rau et al. 1981). It is possible that the greater sewage inputs from the city of Havana to the Almendares River are reflected in lower, rather than higher, $\delta^{15}N$ values in the fish from these sites. Aguilar (2005) stated the possibility that certain types of sewage may have low relative values of $\delta^{15}N$. This may be associated with the sewage-disposal process (Gaston and Suthers 2004) or by the influence of several nitrogen sources.

In this study, the $\delta^{15}N$ values of *G. puncticulata* varied by 11 ‰ across sites and were clearly affected by nutrient inputs to the river rather than simply among-site differences in trophic position. Previous comparisons of gut contents and $\delta^{15}N$ analyses of fish have shown that the two measures of trophic position are comparable for fish (Vander Zanden et al.1999). The latter is based on the premise that $\delta^{15}N$ is enriched approximately 3–4 ‰ from prey to predator, a difference that indicates a separation of one trophic level between these organisms (Peterson and Fry 1987). However, this comparison requires that the $\delta^{15}N$ signal at the base of the food web is not affected by cultural nutrient inputs or that these among-site differences are removed by standardizing $\delta^{15}N$ data to a common lower trophic–level organism. Ideally, lower trophic–level organisms should be collected to assess whether any differences in the trophic position of *G. puncticulata* exists across sites. However, stomach content data suggest that *G. puncticulata* occupy a similar trophic niche at all sites.

The differences in size and condition between sites were not associated with differences in *Gambusia* sp. diets. Diets ranged from terrestrial arthropods (adults and larvae) as essential food items to aquatic plants and animals as secondary dietary components. Food habits of the species studied were similar to those described for other *Gambusia* spp. in different regions (Meffe 1985; Greenfield et al. 1983; Gragson 1992; Gluckman and Hartney 2000). *G. puncticulata* feed mainly on small invertebrates, although fish and plant matter remains were also found in their stomachs (Fink 1971). According to these results, this species could be considered as a general practitioner/opportunist predator due to the wide variability in their diet. This is consistent with the results found by Specziár (2004) for *G. holbrooki.* Seasonal changes in diets are known for several species of the genus *Gambusia* (Harrington and Harrington 1961; Greenfield et al. 1983). However, dietary habits vary depending on sex or size (Specziár 2004) as well as ontogenic changes (Garcia-Berthov 1999).

The six studied sites were characterized by abundant vegetation on the banks of the river, which provides habitat for terrestrial invertebrates. In this work, the invertebrates were represented, mostly by species of Prenolepini and Solenopsini tribes, which includes "Caribbean crazy ants and fire ants," respectively. These were often found in analyzed fish stomachs and comprised a high percentage of insect remains (Table 5). Fish from site R differed from those at other sites by presenting a diet rich in species of Prenolepini tribe (Caribbean crazy ants), and those from site PC had considerable amount of plant remains. An increase of "*Oscillatory* spp." cyanobacteria was found at greater impact sites, with fish from site P23 having the highest average. Excessive inputs of nutrients at this site could be causing a eutrophic environment favourable for the growth and development of cyanobacteria, which matches with Caquet's 2006 statement.

Conclusion

G. puncticulata in the Almendares River showed increased concentrations of Hg, and the majority of individuals had levels that exceeded the thresholds believed to be protective against health effects. The fish were not mobile between sites and reflected local conditions, and the site fidelity and widespread nature of this species makes it a good candidate for biomonitoring studies. As such, future studies could examine whether discharges into the Almendares River are affecting the health of the fish and, if so, identify sites that require remediation. In addition, this species would be a good choice for assessing the effectiveness of such remediation.

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