Male nuptial ornamentation of invasive guppies (*Poecilia reticulata*) responds to water pollution via phenotypic plasticity and microevolutionary change

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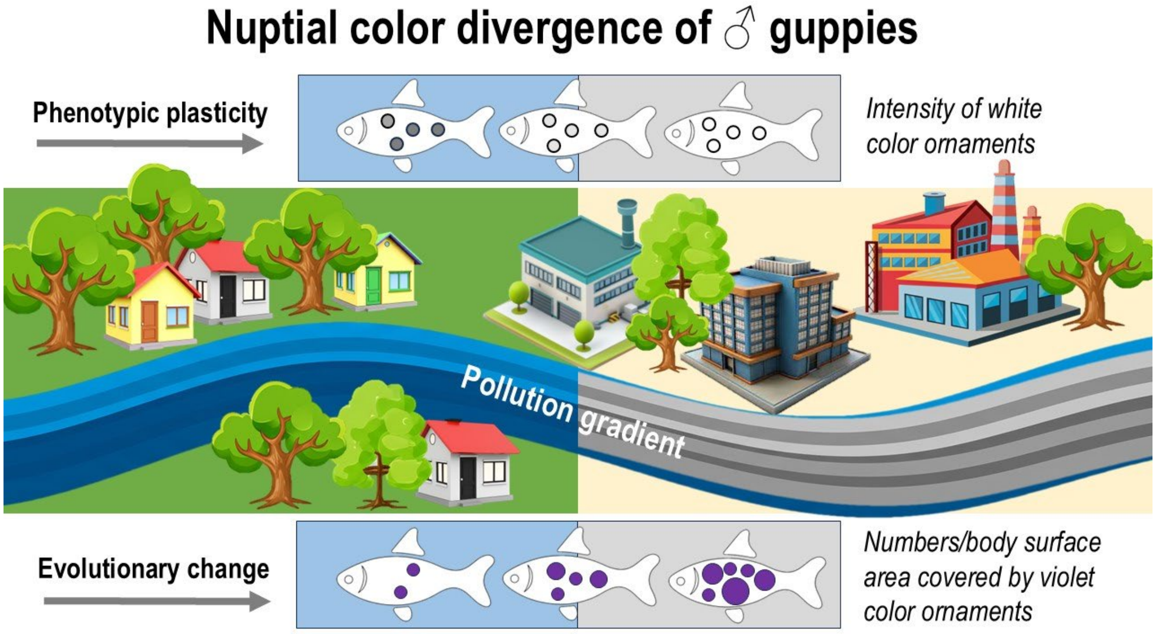
Graphical abstract



Highlights

- Water pollution affects male nuptial ornamentation of invasive guppies
- Population differences may involve plasticity (P) and evolutionary change (E)
- P: Color intensity of white ornaments increased in polluted waters
- E: Numbers and % body cover of violet ornaments increased in polluted waters
- Water pollution appears to alter multiple components of sexual selection

Abstract

Invasive alien species are increasingly replacing native species, with human habitat alterations often favoring invasive taxa. The Brazilian Rio Uberabinha, for instance, was found to harbor more invasive guppies (*Poecilia reticulata*) at polluted than non-polluted sites. Studies in other regions reported numeric frequencies of certain nuptial color ornaments of guppy males to either in- or decrease along pollution gradients, which has been interpreted to largely reflect population differences of (heritable) male ornamentation patterns. But could plasticity play an additional, or possibly even greater role in creating phenotypic variation of nuptial ornaments? Here, we examined male guppies along a gradient of diffuse water pollution of domestic and industrial origins in the Rio Uberabinha and quantified not only the number of ornaments (and percentage body cover), but also population differences in color intensity (total coloration, ΔE). The latter metric likely reflects plasticity during ornament development, *e.g.*, through general challenges to physiological homeostasis or suppression of male ornamentation following xenestrogen exposure. We found numbers and % body cover of violet ornaments to increase as water pollution intensified. Black, orange, blue and violet ornaments responded to additional components of environmental variation that were not the focus of our present study (*e.g.*, stream velocity, dissolved oxygen). Moreover, we found systematic variation of ΔE along the examined pollution gradient in the case of white/iridescent ornaments, with fish becoming more brightly colored at more polluted sites, possibly related to an alteration of uric acid and guanine biosynthesis and/or accumulation. White/iridescent and green ornaments also responded to additional components of environmental variation. Hence, it appears that both, plasticity and evolutionary changes, jointly create phenotypic diversification of different ornament types. Our study provides novel insights into the manifold ways by which man-made habitat alterations can alter evolutionary trajectories (here: components of sexual selection) of the populations exposed to them.

Key words: biological invasion, color polymorphism, contemporary evolution, sexual selection, urban ecology

1. Introduction

Invasive alien species (IAS), *i.e.*, species that established populations outside their natural range and cause economic and/or ecological harm in newly colonized habitats (Bartz et al. 2010; Schirmel et al. 2016; Booy et al. 2017; Lockwood et al. 2013), are virtually omnipresent and represent the third biggest threat to biodiversity behind only habitat loss and fragmentation in a world undergoing ever-accelerating rates of Global Change (United Nations Environment Programme 2007). Freshwater ecosystems are simultaneously amongst the most diverse but also the most threatened ones (Dudgeon et al. 2006; Gallardo et al. 2016; Dayton 2019). Neotropical livebearing fishes of the family Poeciliidae—especially mosquitofish (*Gambusia affinis* and *G. holbrooki*) and guppies (*Poecilia reticulata*)—rank among the most invasive freshwater organisms as they have been introduced to numerous countries worldwide for malaria and dengue prophylaxis (Pyke 2008; Deacon et al. 2011; Stockwell and Henkanaththegedara 2011). Extensive work has addressed whether and how IAS alter ecosystems functions and pose risks to ecological communities because they exert predation, act as (or introduce) novel parasites, or compete with local faunal elements, potentially driving the local extinction of native taxa (Clavero and Garcia-Berthou 2005). At the same time, several species appear to continuously invade new habitat types at the edge of their distribution or simply by filling previously unoccupied habitats within their natural range (Hickling et al. 2006; Thomas et al. 2012). As a particularly well-researched example, guppies on Northern Trinidad repeatedly invaded stream headwaters with relaxed piscine predation (Haskins and Haskins 1951; Haskins et al. 1961; Endler 1980). Guppies occurring in both lowland (downstream) and headwater stream sections experience dissimilar selective regimes and have emerged as a model system for various questions in evolutionary ecology, asking, *e.g.*, how relaxed predation might affect the evolution of male nuptial color ornaments (Endler 1980; Millar et al. 2006; Kemp et al. 2009) or certain reproductive life-history traits (Rodd and Reznick 1991; Reznick et al. 1990, 2001, 2012).

Some IAS benefit from man-made habitat alterations at least during some stage(s) of the invasion process (Light and Marchetti 2007; Wang et al. 2021). In fact, adaptation to human-altered habitats in the native range (the 'anthropogenically-induced adaptation-to-invade' scenario), has been proposed as another mechanism facilitating biological invasions (Hufbauer et al. 2012). Even pollution often tends to have less of a negative effect on IAS compared to native species. Invasive gammarid crustaceans, *e.g.*, became dominant in the river Rhine only after a major industrial chemical spillover in the 1980ies, with different tolerances to certain toxicants largely explaining this pattern (Kuhn and Streit 1994), and in San Francisco Bay, copper exposure significantly decreased species richness of native species but not of IAS (Crooks et al. 2011). Likewise, a previous study comparing fish communities in the Brazilian Rio Uberabinha found the native poecilid *Phalloceros* sp. to primarily occur in unpolluted waters, whereas guppies—invasive to southern Brazil (Dias et al. 2020; Bueno et al. 2023)—dominated in more polluted stream sections (Gomes-Silva et al. 2020b). While the exact mechanism is not currently known, species differences in their tolerances to certain toxicants and an alteration of competitive interactions likely explain this pattern. Comparing 15 sampling sites in the Rio Uberabinha, Gomes-Silva et al. (2020b) further reported that pollution-related ecological predictor variables (condensed via Principal Component Analysis) were a strong predictor of numerical frequencies of cytonuclear abnormalities (such as the formation of micronuclei) of poeciliids across sites. A sub-set of sampling sites from that study (*i.e.*, those slightly to heavily polluted sites harboring guppies) served as the basis for our present study on the divergence of male nuptial coloration along the well-characterized pollution gradient.

Independent of whether fish populations encounter polluted water conditions because of an anthropogenic introduction, or natural invasion/range expansion of hitherto uninhabited habitats, the altered environmental conditions are likely to create starkly divergent selective regimes (Oziolor and Matson 2015;

Candolin 2019). However, to better understand the impact of anthropogenic pollutants on animal phenotypes, including communication and signaling, broad-scale monitoring of animal pigmentation in general, and nuptial ornamentation in particular, is of utmost importance (Lifshitz and St Clair 2016). The role played by pollution-gradients in generating phenotypic variation of male nuptial ornaments has been studied in a comparison of invasive guppy populations in rivers draining the degraded and heavily overstocked Mutara Rangelands of north-eastern Rwanda, which receive pollution from domestic wastewater and cattle dung (Gomes-Silva et al. 2020a). The study was based on the key assumption that, since guppies are color-polymorphic, and color patterns have a heritable basis (Winge 1922; Khoo et al. 1999; Sato and Kawata 2020), population differences in numerical frequencies of nuptial ornaments likely reflect microevolutionary (*i.e.*, genetic) population divergence. Indeed, statistical analyses of numbers and percent body surface covered by different color ornaments uncovered several pollution-effects and interactions with other environmental parameters. The direction of interaction effects was similar for blue/black and red/orange ornaments, while white/iridescent ornaments responded dissimilarly. The finding that responses differed between ornament types adds to our interpretation of microevolutionary divergence due to site-specific alterations of selective regimes.

Could plasticity play an additional, or possibly even greater role in creating phenotypic variation of nuptial ornaments? Here, using the same analytical approach as employed by Gomes-Silva et al. (2020a), we investigate patterns in male guppy nuptial ornamentation along nine sites in the Rio Uberabinha differing in pollution levels. To capture potential signatures of both microevolutionary change (predictions 1, 2) and plasticity (predictions 3, 4), we assessed two different metrics related to male nuptial coloration. Potential differences in ornament distribution patterns (assessed as ornament numbers and areas covered by the respective ornament type on males' body surface) among populations should reflect microevolutionary divergence along the examined pollution gradient (Gomes-Silva et al. 2020a). Prediction 1a, therefore, presumed significant effects of pollution-related environmental principal component 1 (and likely, other environmental PCs adopted from Gomes-Silva et al. 2020b) on ornament distribution patterns. Alternatively, if evolutionary change is too slow to have left its footprints on population differences in our study system, or if evolutionary change is impeded by antagonistic selection (*e.g.*, Keith and Mitchell-Olds 2019), no such effect would be expected (prediction 1b). Moreover, adult male guppies show a much weaker body size polymorphism compared to numerous other poeciliids, in which males may adopt alternative mating tactics depending on their body size (Evans and Pilastro 2011). Even though body size-effects were not the main focus of our present study, we included male size as a covariate into our analyses. We predicted that large-bodied males, which likely represent those individuals whose 'genomic makeup' renders them more able to withstand (and thus, grow to a larger size under) the adverse effects of water pollution, would sport more ornaments than smaller ones (prediction 2).

Whether or not phenotypically plastic responses to pollution contribute to population differentiation should become apparent when analyzing color properties of nuptial ornaments, assessed as total color differences (ΔE; *e.g.*, Mokrzycki and Tatol 2011). More detail on information conveyed by ΔE can be found in the Methods section. Prediction 3a assumed significant effects of our pollution-related covariate (PC1, and likely other environmental effects) in statistical analyses treating ΔE as the dependent variable. By contrast, no such effect would be expected, *e.g.*, when the availability of certain nutrients is more important during ornament development than overall pollution levels (*e.g.*, carotenoids: Hudon et al. 2003; prediction 3b). Finally, assuming that *e.g.*, challenges to physiological homeostasis or endocrine disruption play a role during ornament development, large males could be more able to defy adverse environmental conditions if tolerance depended on more beneficial surface-to-volume ratios (*e.g.*, Riesch et al. 2014). Prediction 4, therefore, assumed large-bodied males to be more brightly colored than smaller ones.

2. Methods

2.1 Data collection and availability

2.1.1 *Sampling sites, environmental and pollution-related parameters*

All data presented here were collected between 23rd January and 9th February 2018 and as part of a previous study that focused on changes in fish communities and nuclear abnormalities of guppies exposed to water pollution in the upper Rio Uberabinha (Gomes-Silva et al. 2020b). Here, we present unpublished data on male nuptial coloration from nine sampling sites, excluding sites at which no (or not enough) guppies were encountered as well as an artificial fish pond ('Pesque e Pague') included in Gomes-Silva et al. (2020b) that is regularly stocked with several fish species, precluding meaningful conclusions about the evolutionary trajectory of guppies encountered at that site.

Gomes-Silva et al. (2020b) collected 23 geo-physical, physico-chemical, and water pollution-related parameters at 15 sites and condensed those variables by means of Principal Component Analysis into four PCs (Table 1 in Gomes-Silva et al. 2020b). Of particular interest in the context of our current study is environmental PC1, which received high axis loadings from all pollution-related parameters included; namely the Biological Monitoring Work Party (BMWP) index (Junqueira and Campos 1998), biochemical oxygen demand (BOD), concentrations of fecal coliforms, chlorides, nitrates, nitrites, ammonia, total phosphorus, various measures of heavy metal contamination in the sediment (Cd, Pb, Cu and Cr) and water (Cd, Pb and Cr), as well as the pH, concentrations of dissolved solids and water turbidity. Environmental PC2 captured variation between fast- and slow-flowing stream sections and received high axis loadings from stream velocity, dissolved oxygen concentrations, as well as local water temperatures. Environmental PC3 received a high axis loading from variation in water depth across sampling sites, while environmental PC4 received high axis loadings from altitudinal variation, as well as dissolved oxygen concentrations. Our current study makes use of those four PCs, treating them as predictor variables in our statistical analyses.

A potential point of criticism could be that our analyses utilized environmental PCs that were obtained from a total of $n = 15$ sampling sites but compare a sub-set of $n = 9$ sites at which guppies were present. However, running the aforementioned PCA with only nine sites led to a much less clear-cut separation of sites by water quality parameters, with pollution-related parameters being split over two PC axes (Online Supplementary Table 1), largely owing to the fact that the most pristine, unpolluted sites in our previous sampling scheme were excluded here as they did not harbor guppies (Gomes-Silva et al. 2020b). Alternative PCAs are shown in Online Supplementary Table 1, while analyses of covariance (ANCOVA) that we ran with those alternative PCs as predictor variables nevertheless qualitatively confirm our main results (not shown in detail).

2.1.2 *Collection and general processing of color photographs*

We collected 171 male guppies for the analysis of body coloration (12–21 individuals per site; for details see Gomes-Silva et al. 2020b). To take photographs, we anesthetized males by adding few drops of clove oil to the water in the buckets in which they were collected. This would relax (extend) chromatophores such that close to full coloration was measured (see Javahery et al. 2012 for discussion). We then laid each male flat on a small piece of laminated millimeter paper (IT8.7/2 LaserSoft Imaging, ID no. R051025) placed on a laminated color calibration plate (IT8.7/2 LaserSoft Imaging, ID No. R051025; Fig. 1a). We took digital photos of the left body side from centrally above (at approximately 30 cm distance) using a digital Nikon 5200 camera equipped with a 55 mm lens and saved them in *jpg format. Photos were taken in the shade to avoid glare and reflection effects. Prior to all subsequent measurements, we used the gradation curve modulation in Adobe Photoshop CS5 and calibrated each image according to the manufacturer's instructions (LaserSoft Imaging, Kiel, Germany). We adjusted the black-and-white squares as well as squares L13 to L19 of the calibration plate to the provided (standardized) L*a*b*-values. This procedure ensured that each photograph had the same standard coloration and thus allowed for a quantitative comparison of individual color differences (Zimmer et al. 2018).

2.1.3 *Ornament numbers and percent cover on males' body surface*

We processed the photos using Adobe Photoshop CS5 (version 12, 2010). First, we assessed each male's body size (standard length, SL) and body surface area (excluding fins). We largely followed the classification of color ornaments described by Endler (1978) and considered black (including 'fuzzy black': Ruell et al. 2013; Martínez et al. 2016), orange, green, yellow, blue, violet, and white/iridescent ornaments. For each color category, we counted the total number of spots on the left body side and measured the surface area covered by each spot. We then calculated the percentage of body surface covered by the respective color type by dividing the cumulative surface area for that color category by the total body surface area (Martínez et al. 2016). A more detailed description of the employed methods can be found in Gomes-Silva et al. (2020a).

2.1.4 *Total color difference of nuptial ornaments*

While it is possible that some of the changes in color intensity (ΔE) could also result from microevolutionary change, our analysis of potential phenotypic plasticity was based on the consideration that color intensity should largely reflect males' ability to deposit pigments into existing ornaments (for pigment-based ornaments) and to build and arrange the crystals and compounds required for structural ornaments. These processes depend on several ecological factors; for instance, high carotenoid availability results in males developing brighter orange and red ornaments in laboratory experiments and in the wild (Endler 1983; Kodric-Brown 1989; Grether et al. 2005). To analyze color differences of male ornaments, we used the relative values of L*a*b* in CIELAB color space. L*-values describe relative lightness, ranging from black (L* = 0) to white (L* = 100), a*-values describe relative variation from green (a* = −150) to red/magenta (a* = +100), and b*-values describe relative variation from blue (b* = −100) to yellow (b* = +150). Adobe Photoshop CS5 provides L*-, a*- and b*-values automatically for areas delimited using the program's toolbox. This procedure was done for each ornament of a given color category, after which we calculated mean values for each category.

2.2 Statistical analyses

We excluded the category of yellow ornaments from our analyses, as 40.35% of individuals did not sport this type of ornament. For the remaining color categories, the percentage of individuals that were missing a given ornament type did not exceed 22.80%. In those cases, we replaced missing ΔE-values by the respective population mean. All statistical analyses were conducted using SPSS v. 19 (IBM 2010, Chicago, USA). If necessary, we transformed data to meet the assumptions of normality and homoscedasticity of model residuals, which we tested using Shapiro-Wilks' and Levene's tests, respectively.

2.2.1 *Ornament numbers and percent body cover*

We decided to condense information on numbers (square root-transformed for all statistical analyses) and percentages of males' body surface covered by the six color ornament types [arcsine (square root)-transformed], as both metrics tend to be highly correlated (Gomes-Silva et al. 2020a). We confirmed significant correlations between both metrics across all males in our empirical data set (black: Pearson's $r = 0.546$; orange: $r = 0.728$; green: $r = 0.793$; blue: $r = 0.792$; violet: $r = 0.781$; white/iridescent: $r = 0.784$; all $P < 0.001$, $N = 171$) and then ran separate principal component analyses (PCAs), based on a covariance matrix, to condense data into a single PC for each ornament type, each of which obtained Eigenvalues > 1.0 (Eigenvalues, black: 1.568, 78.4.% variance explained; orange: 1.728, 86.4%; green: 1.793, 89.7%; blue: 1.792, 89.6%; violet: 1.781, 89.1%; white/iridescent: 1.784, 89.2%).

To answer the question of whether ornaments show microevolutionary divergence in response to the four components of environmental variation considered here (prediction 1), we subjected the six PCs to MANCOVA (operated as a multivariate general linear model, GLM). We conducted a preliminary analysis to confirm general variation among populations and thus, included 'sampling site' as a fixed factor and body size (log-transformed standard length, SL) as a covariate. We discovered significant differences among populations ($F_{56.000,\ 840.012} = 2.687$, $P < 0.001$, Wilk's partial $\eta_p^2 = 0.119$) and as a function of male body size ($F_{7.000,\ 155.000} = 2.522$, $P = 0.018$, Wilk's partial $\eta_p^2 = 0.102$), while the interaction effect was non-significant and hence, excluded from the final model ($F_{56.000,\ 796.930} = 1.112$, $P = 0.271$, Wilk's partial $\eta_p^2 = 0.056$). Therefore, we proceeded with our main analyses, this time using the four previously published environmental PCs (Gomes-Silva et al. 2020b)—with pollution-related environmental PC1 being of prime interest (prediction 1)—as well as body size (prediction 2) as predictor variables, coded as covariates. This was followed by separate ANCOVAs of a similar model structure on single color PCs to identify the source(s) of variation. In each case, we first ran a model with all possible two-way interactions, but removed non-significant interaction terms from the final model. Throughout, effect strengths are provided as Wilk's partial η_p^2.

2.2.2 *Total color difference of nuptial ornaments*

We screened the obtained data (*i.e.*, L*-, a*- and b*-values) for a correlation structure, which was detected for 11 out of 18 binary comparisons (61.1%) at the level of $P < 0.05$ and two more comparisons (11.1%) at $0.05 < P < 0.1$ (Pearson correlations, black: L* vs a*, $r = 0.072$, $P = 0.352$; L* vs b*, $r = 0.150$, $P = 0.050$; a* vs b*, $r = 0.140$, $P = 0.068$; orange: L* vs a*, $r = 0.235$, $P = 0.002$; L* vs b*, $r = 0.211$, $P = 0.006$; a* vs b*, $r = 0.307$, $P < 0.001$; green: L* vs a*, $r = -0.555$, $P < 0.001$; L* vs b*, $r = -0.043$, $P = 0.577$; a* vs b*, $r = 0.094$, $P = 0.223$; blue: L* vs a*, $r = -0.189$, $P = 0.013$; L* vs b*, $r = -0.434$, $P < 0.001$; a* vs b*, $r = -0.330$, $P < 0.001$; violet: L* vs a*, $r = 0.100$, $P = 0.192$; L* vs b*, $r = -0.274$, $P < 0.001$; a* vs b*, $r = -0.517$, $P < 0.001$; white/iridescent: L* vs a*, $r = -0.293$, $P < 0.001$; L* vs b*, $r = -0.014$, $P = 0.845$; a* vs b*, $r = -0.233$, $P = 0.002$; all $N = 171$). To obtain a single metric that expresses total color differences, we this time refrained from using a factor reduction procedure (*i.e.*, PCA) but rather decided to calculate the more commonly used ΔE-values (Mokrzycki and Tatol 2011). One possible approach to express differences in total coloration (ΔE) would have been to use a standard color template for comparison. This, however, raises the question as to what the expected L*a*b*-values of a 'standard' ornament for any given ornament type would actually look like. Hence, we used the following approach: for each ornament type and color space (*i.e.*, L*-, a*-, and b*-values, separately), we first determined the general distribution pattern in the empirical data set by visual inspection. As one example, black ornaments showed the expected distribution pattern of positive L*-values, with a strong skew towards zero. We then determined the maximum (if data were skewed towards large and positive values, as was the case, *e.g.*, for a*-values in the case of orange ornaments) or minimum value (if the skew was in the direction of zero or negative values) and used the respective extreme value to express ΔE for each individual and ornament type as:

$$\Delta E = \text{square root}\ [(L^*_{\text{observed}} - L^*_{\text{maximum}})^2 + (a^*_{\text{observed}} - a^*_{\text{maximum}})^2 + (b^*_{\text{observed}} - b^*_{\text{maximum}})^2]$$

Hence, the obtained data expressed differences in total coloration of each ornament type between each individual male in our data set and the most extremely colored ornament that was observed, with large ΔE-values representing less colorful males.

Before we conducted our main analyses, we again confirmed general variation among populations and thus, ran a preliminary MANCOVA that detected significant population differences ($F_{48.000,\ 771.648}$ = 3.238, $P < 0.001$, Wilk's partial η_p^2 = 0.140), but no statistically significant effect of male body size ($F_{6.000,\ 156.000}$ = 2.064, P = 0.060, Wilk's partial η_p^2 = 0.074). Again, the interaction term was excluded as it was not significant ($F_{48.000,\ 732.284}$ = 0.847, P = 0.760, Wilk's partial η_p^2 = 0.043). To answer the question of whether nuptial ornaments show plasticity in response to environmental variation (predictions 3, 4), we followed the same approach as described for ornament numbers, with an initial MANCOVA confirming variation along the four PCs representing environmental/pollution-related variation (Gomes-Silva et al. 2020b). This was again followed by separate ANCOVAs for each ornament type to identify the source(s) of variation. We depict significant model effects by plotting unstandardized residuals, corrected for all other model terms, against the respective covariate of interest.

3. Results

3.1 Evolutionary divergence: ornament numbers and percent body cover

3.1.1 Overall variation along environmental PCs

When we subjected our data on ornament numbers and percent thusly colored body surface (condensed into one PC per ornament type) to MANCOVA, no significant effect of environmental PC4 nor any significant interaction effects were uncovered (Table 1a). By contrast, and in agreement with predictions 1a and 2, the remaining three environmental PCs (PC1–PC3) as well as male body size (SL) all had significant effects, with estimated effect strengths (Wilks' partial η_p^2) ranging from 0.098 to 0.132 (Table 1a). Based on this result, we proceeded with a set of single-trait ANCOVAs to identify the source(s) of variation.

3.1.2 Pollution-related effects

In our *post-hoc* ANCOVAs for each ornament type separately, no significant interaction terms were uncovered (Table 1b-g). Focusing on the main effect of environmental PC1—our explanatory variable related to environmental pollution (Gomes-Silva et al. 2020b)—we detected a significant effect in the case of violet ornaments (Table 1f). A tendency towards such a pattern (*i.e.*, $0.05 < P < 0.1$) was also detected for black, green, blue, and white/iridescent ornaments, even though the results were not statistically significant (Table 1b,d,e,g). When we plotted residuals (corrected for other model terms) against environmental PC1, it became evident that violet ornamentation increased as pollution levels intensified (Fig. 1b). Note, however, that the degree of determination (R^2) of all *post-hoc* partial linear regressions—here and in subsequent single-trait analyses—was low (Fig. 1b).

3.1.3 Additional environmental effects

Environmental PC2, which received high axis loadings from dissolved oxygen concentrations and water velocity, as well as (in opposing direction) water temperature (Gomes-Silva et al. 2020b), had significant effects on black, orange, and blue ornaments (Table 1b,c,e). We visualized those effects by plotting residuals against environmental PC2 and in all cases, found residual values to decrease with increasing values of environmental PC2 (Fig. 2a-c; here and throughout, please note again the low R^2-values of *post-hoc* linear regressions). Including information on axis loadings (Gomes-Silva et al. 2020b), this result can be interpreted as males sporting fewer black, orange and blue ornaments in populations exposed to a more stagnant flow regime, with low levels of oxygen and elevated water temperatures.

Environmental PC3—a correlate mainly of water depth (Gomes-Silva et al. 2020b)—had significant effects on black, orange and violet ornaments (Table 1b,c,f). Residual analysis suggests that males developed more ornaments of those three types in deeper stream sections (Fig. 2d-f).

Finally, environmental PC4 had a significant effect on violet ornaments (Table 1f). When we visualized the relationship using residuals, a decrease with increasing values of environmental PC4 (*i.e.*, towards higher altitudes but less oxygenated conditions; Gomes-Silva et al. 2020b) was observed (Figure 2g).

3.1.4 Effect of male body size

We detected an effect of male body size in the case of green, blue and violet ornaments (Table 1d,e,f); a tendency (*i.e.*, $0.05 < P < 0.1$) was also detected for black ornaments (Table 1b). In agreement with prediction 2, residual analyses confirmed that larger males sported more ornaments in all three cases (Fig. 3a-c).

3.2 Phenotypic plasticity: total color differences of different ornaments

3.2.1 Overall variation along environmental PCs

In our MANCOVA using total color differences (ΔE) of all six ornament types as the dependent variables, environmental PC2, male body size, and all interaction terms were non-significant (Table 2a). By contrast, and conforming to prediction 3a, we discovered significant effects of environmental PCs1, 3, and 4, with Wilk's partial η_p^2-values ranging from 0.112 to 0.171 (Table 2a). In partial disagreement with prediction 4, we found body size to have no significant effect in our MANCOVA (Table 2a; but see below for effects in single-trait analyses). Again, we proceeded with single-trait ANCOVAs to identify the source(s) of variation.

3.2.2 Pollution-related effects

We detected no statistically significant effects along pollution-related environmental PC1 for most ornament types (Table 2). However, the strongest effect we detected in our entire set of single-trait analyses (compare *F*-ratios and partial η_p^2 in Tables 1b-g and 2b-g) was indeed that of PC1 on the ΔE-values of white/iridescent color patterns (Table 2a). Visualization of the effect suggests that ΔE-values decreased with increasing values of environmental PC1—in other words: as pollution levels intensified, males tended to be increasingly similar to the most extreme white/iridescent ornament we used as a reference value to calculate ΔE (Fig. 4a,b). Just as in our previous analyses, however, the degree of determination (R^2) of *post-hoc* partial linear regressions was low (Figs 4-6).

In the case of white/iridescent ornaments, we also detected a weak, albeit statistically significant effect of the interaction of PC1 × SL (Table 1g). When we split the data (residuals) by the empirical mean value of male body size, we found the aforementioned effect of males sporting whiter/more iridescent ornaments with increasing pollution levels to become evident in males of a body size ≤ the empirical mean (Pearson correlation: $r_P = -0.222$, $P = 0.026$, $N = 100$), but not in males larger than the mean ($r_P = -0.024$, $P = 0.844$, $N = 71$).

3.2.3 Additional environmental effects

We found ΔE-values of green ornaments to be affected by environmental PC2 (Table 2d). Increasing ΔE-values with increasing values of environmental PC2 suggests that green coloration became weaker in river sections with higher temperatures, lower dissolved oxygen and reduced stream velocities (Fig. 5a).

Finally, ΔE-values of white/iridescent ornaments decreased along environmental PC3 (Table 2g; Fig. 5b) but increased along environmental PC4 (Fig. 5c). Including information on axis loadings (Gomes-Silva et al. 2020b), and remembering that small ΔE-values characterize more brightly colored individuals, these results can jointly be interpreted as males becoming whiter/more iridescent in lowland river sections with a deeper water column and higher dissolved oxygen.

3.2.4 Effects of male body size

We found ΔE-values of violet ornaments to vary as a function of male body size (Table 2d). A non-significant tendency towards such a pattern (*i.e.*, $0.05 < P < 0.1$) was also detected for black and blue ornaments (Table 2b,e). Congruent with our prediction 4, residual analysis found ΔE-values for violet ornaments to decrease with increasing body size, suggesting that large males were more colorful (Fig. 6).

4. Discussion

4.1 Pollution affects nuptial ornaments of male guppies

Our present study asked whether not only microevolutionary change (Gomes-Silva et al. 2020a; Zhou et al. 2022), but also phenotypic plasticity (Richards et al. 2006; Smith 2009; Engel et al. 2011; Hendry 2016) contributes to the diversification of male nuptial ornaments of invasive guppies exposed to water pollution, exemplified by populations inhabiting the Brazilian Rio Uberabinha. Focusing on a model organism in sexual selection (Houde 1997; Magurran 2005) with a pronounced polymorphism of male nuptial ornaments (Endler 1980; Kemp et al. 2009) and numerous natural or invasive populations occurring in polluted stream sections (Widianarko et al. 2000; Rolshausen et al. 2015; de Carvalho et al. 2019; Santi et al. 2019, 2021), we first considered a metric that largely reflects microevolutionary (*i.e.*, genetic) population divergence: numeric frequencies of violet ornaments increased as water pollution intensified, while black, orange, blue and violet ornaments responded to additional components of environmental variation. Next, we considered a metric that likely captures signatures of plasticity. We detected variation of ΔE along the examined pollution gradient in the case of white/iridescent ornaments, with fish becoming more brightly colored at more polluted sites. White/iridescent and green ornaments also responded to additional components of environmental variation.

Even though our present study was based on relatively few (N = 9) population samples, our sample size was sufficient to confirm statistically significant effects of pollution-related predictor variables on population differentiation previously detected in a comparison of more (N = 26) Rwandan sampling sites (Gomes-Silva et al. 2020a). This could indicate that pollution gradients are repeatedly accompanied by concurrent gradients in male nuptial color ornaments of guppy populations exposed to them, even though the exact nature of the respective change(s) will likely differ geographically, as the kind of pollution and co-occurring selection pressures will be different (see our discussion of not pollution-related effects below and in Gomes-Silva et al. 2020a). We encourage future studies that quantify the patterns detected here and in Gomes-Silva et al. (2020a) across a broader geographic scale and across different types of pollution gradients to address these questions. Moreover, our study is correlational in nature, *i.e.*, it reports associations between environmental parameters and ornament metrics *in situ*. This prevents us from drawing conclusions regarding the causation of any of the observed effects, for which controlled field or laboratory exposure experiments would be necessary. With this in mind, the following discussion provides but an attempt to *post hoc* explain the nature of some pollution-related (and other) ecological factors acting as agents of microevolutionary change through selection (prediction 1) and/or by inducing variation through plasticity (prediction 3). Notably, we found the strongest of all statistically significant effects in analyses of plastic traits.

Male size (*i.e.*, SL) had a significant effect in several of our analyses but always revealed the same pattern, namely that larger males were overall more colorful than smaller ones, thus matching our predictions 2 and 4. Since male guppies have only limited growth after reaching maturity (Snelson 1989), larger size is usually a result of juvenile rather than adult growth. However, previous studies did not report increasing ornamentation in larger males. While the pattern uncovered here is thus somewhat surprising, it could be directly related to pollution: maintenance of homeostasis while dealing with the adverse effects of pollution will be costly (Riesch et al. 2015), and not surprisingly, fish body size is often reduced in polluted waters because of impaired growth (Santi et al. 2019; Gomes-Silva et al. 2020a; but see Santi et al. 2021). In this context, our findings might provide a basis to address a key assumption of sexual selection theory, namely that males with certain beneficial allele combinations ('good genes'-theory; Krebs and Davies 2009) should be overall healthier and thus able to invest more into both, growth and reproduction, including mate attraction. This interpretation would also align with the observed interaction effect of 'PC1 × SL' in the case of white/iridescent ornament plasticity (ΔE-values), where pollution-effects became evident in small- but not in large-bodied males.

The fact that body-size effects were uncovered here but not in other studies may allude towards 'good genes'-mechanisms attaining a greater importance during the early stages of the colonization of (and adaptation to) polluted waters. Could female mate choice for the respective carriers of 'good genes' explain why violet ornaments increased evolutionarily in more polluted waters? As a starting point for future research on female mate choice in the context of water pollution, we envision studies that cross-rear guppy males (ideally sorted by color-phenotype) under polluted vs. non-polluted conditions and conduct binary mate choice (*e.g.*, association preference) tests using both types of males as stimuli (Houde 1997). This might answer the question of whether females generally base their mate choices on condition-dependent traits (Grether 2000; Grether et al. 2005; Fisher and Rosenthal 2006)—potential indicators of males' ability to withstand pollution (*i.e.*, 'good genes'). Studies comparing female preferences across different populations could then ask whether female preferences for condition-dependent traits increased in strength in populations inhabiting polluted waters (*Poecilia mexicana* in naturally H_2S-toxic waters: Plath et al. 2005). Comparative studies could also ask if the observed microevolutionary changes towards more violet ornamentation under more polluted conditions coincide with an alteration of female preferences for this trait (as shown for other other ornamental traits, *e.g.*, in *Haplochromis* cichlids: Seehausen and van Alphen 1998; *Poecilia mexicana*: Bierbach et al. 2013). Those studies could be followed by digital manipulations of male nuptial ornaments on virtual stimulus males to identify the traits affecting female mating decisions (*e.g.*, Greenway et al. 2016).

4.2 Phenotypic plasticity and contemporary (rapid) evolution of color ornaments

The visible color pattern of an organism is usually the result of a complex interplay of multiple different color components (*e.g.*, Kottler et al. 2014) and in fishes, these color components are provided by five different pigment cell types, or chromatophores. They consist of the black melanophores (containing eumelanin), the yellow to orange xanthophores (containing carotenoids and pteridines), the red erythrophores (also containing carotenoids and pteridines), the blue- to green-iridescent iridophores (containing reflective guanine crystals) and the white leucophores (containing uric acid; Braasch et al. 2008; Kottler et al. 2014; Luo et al. 2021; Parichy 2021). In Trinidadian guppies, natural and sexual selection act antagonistically, whereby more colorful males are favored by sexual selection via mate attraction but less colorful males by natural selection through predation (Endler 1983) and this antagonistic interaction is largely responsible for the observed color variation between populations (Endler 1980). Moreover, female mate choice has been shown to stabilize the observed male color polymorphism through negative frequency-dependent selection, in a way that females prefer to mate with rare color phenotypes (Hughes et al. 2013; Potter et al. 2023). We argue that this might partly explain the low degree of determination (R^2) in *post-hoc* regression models in our present study. For example, while water pollution (or an altered female preference for males sporting violet ornaments, please see 4.1) appears to drive violet ornaments towards different population means, female mate choice for rare male phenotypes will counter this effect, thereby weakening the observed effect strength in our analyses.

While extensive research has investigated the effects of different kinds of pollution on the melanophores, xanthophores and erythrophores in guppies (*e.g.*, Berkowitz 1941; Toft and Baatrup 2001; Zhang et al. 2019), data are scarce as to how it might affect iridophores and leucophores. Moreover, most previous research has outlined an overall decrease in coloration in polluted environments (*e.g.*, Toft and Baatrup 2001; Zhang et al. 2019), while we report that certain components of pigmentation actually increase in our study system. Specifically, we found an increase in the number of violet ornaments and the proportion of violet body cover as well as in the strength of white/iridescent ornamentation with increasing levels of pollution. The patterning of ornamentation on the body as well as the size of different ornaments have previously been shown to be highly heritable (*e.g.*, Winge 1922; Haskins and Haskins 1951; Kodric-Brown 1989; Houde 1992). Thus, the differences in coverage of violet body ornaments we discovered here suggests heritable changes via rapid contemporary evolution (Reznick et al. 2019) in these invasive populations exposed to aquatic pollution, likely within few decades. Increased somatic (*i.e.,* mitotic) mutation rates in populations exposed to high pollution levels have been demonstrated using cytonuclear methods (Gomes-Silva et al. 2020b) and assuming that this also applies to the germline, it should arguably increase their evolvability.

Violet coloration is likely the result of a combination of reds/oranges produced by xanthophores and erythrophores and the blues produced by iridophores (Sköld et al. 2016). However, since we did not uncover changes in orange or red coloration, the increases in violet are likely to be the result of increases in the blue components, *i.e.*, changes in iridophores, and thus, guanine platelet crystals and other purines (Braasch et al. 2008; Kottler et al. 2014; Sköld et al. 2016; Luo et al. 2021; Parichy 2021). A similar effect of increasing blueish coloration in more polluted sites was also found by Gomes-Silva et al. (2020a) who investigated pigmentation patterns of guppies exposed to domestic waste-water and cattle-dung pollution in Rwanda. We refrain from more detailed *post-hoc* explanations for both, the mechanisms at play and the selective forces behind the observed microevolutionary change, as the nature of our study (*i.e.*, exposure to diffuse pollution by a variety of chemical pollutants alongside a potential magnitude of further unquantified ecological variation) precludes ruling out even epigenetic effects (*e.g.*, Hou et al. 2022) causing parts of the observed variation. Examining the visual ecology of signaler-receiver interactions under polluted versus non-polluted conditions (*e.g.*, signal transmission properties and potential receiver biases in murky waters) emerges as a promising field of future research (see Endler 1991,1992; Seehausen et al. 1997; Candolin et al. 2016).

Increases in the strength of pigmentation, by contrast, are usually associated with plastic changes (Toft and Baatrup 2001; Grether 2000; Zhang et al. 2019), although we cannot discount that some aspects of color intensity might also be linked to microevolutionary change, *e.g.*, via directional selection for a specific color phenotype in more polluted environments. White/iridescent ornamentation, which we interpret to show plastic responses along the pollution gradient, is derived from leucophores and iridophores, and thus ultimately from guanine platelet crystals and uric acid (Braasch et al. 2008; Kottler et al. 2014; Sköld et al. 2016; Luo et al. 2021; Parichy 2021). But how could pollution result in an increased whiteness when other studies confirm, *e.g.*, the expected developmental inhibition of male secondary sexual characters as a result of xenestrogen exposure (Toft and Baatrup 2001; Ward and Blum 2012). Likewise, general challenges to physiological homeostasis should result in reduced (not increased) expression of male nuptial ornamentation (Johnstone 1995; Cotton et al. 2006; Svensson and Wong 2011; Ward and Blum 2012). As outlined before, our present study represents a comparative approach using wild populations, as opposed to controlled exposure studies under laboratory conditions. This precludes an in-depth discussion as to how exactly differences in nutrient availability across sampling sites, or alterations of the biosynthesis, catabolism, or forms of intracellular deposition of the involved pigments, to mention but some examples, might be involved in creating the observed variation. It is tempting to speculate whether a reduction of other, co-occurring (darker) pigments holds responsible for the increased whiteness of guppy ornaments under more polluted conditions. Moreover, de Carvalho et al. (2019) found guppies from another part of

Brazil to assimilate carbon directly from industrial and domestic sewage, alluding to potential diet-related pathways for the ornamentation patterns we found here. This could be addressed in detailed analyses of the chemical compositions of ornaments across populations.

Research into the manifold ways by which water pollution could affect fish populations exposed to it is still a relatively new, emerging field (Oziolo and Matson 2015; Candolin 2019), and several of the interpretations we outline above remain speculative until more detailed information on the involved populations' evolutionary ecology becomes available. For instance, a follow-up study is being conducted in our team to provide information on behavioral differences between these populations (*i.e.*, boldness, activity and sociability/shoaling tendencies: Gomes-Silva et al. unpublished data). Likewise, another study from our team will shed light on male and female life histories, including investment into reproduction (*i.e.*, reproductive allocation for females and gonosomatic index for males; Riesch et al. unpublished data).

4.3 Effects of not pollution-related ecological variables

Finally, while our results meet *a priori* predictions with respect to their dependencies on water pollution—the focus of our present study—an interpretation of statistically significant effects attributable to additional components of environmental variation (PCs 2-4; Gomes-Silva et al. 2020b) necessarily remains less straightforward. Based on our repeated argument of the correlational nature of our study, we refrain from possible speculation with respect to plastic responses (ΔE). Regarding microevolutionary divergence, we found black, orange and blue ornaments to be reduced in stagnant river sections, while blue, orange and violet ornaments were reduced in shallow river sections. Considering the effects of water depth, previous studies found the cichlid *Pundamilia pundamilia* in Lake Victoria to prefer shallow-water breeding grounds, where blue light dominates, while the more reddish sister species *P. nyererei* adapted to deeper parts of the lake with more red light (Seehausen et al. 2008). However, the same effect is unlikely to explain the microevolutionary divergence of ornamentation reported here, as guppies do not penetrate the deep water column to the extent seen in those cichlids.

Could our findings, therefore, be a result of spatial variation in the type(s) and intensity of predation? Gomes-Silva et al. (2020b) confirmed spatial variation of fish communities along the examined pollution gradient but certainly did not provide enough information from which to draw conclusions regarding predation pressure and intensity. Studies on the evolution of Trinidadian guppies demonstrate the importance of information regarding what species of piscine predators actually consume guppies and how their sensory biases and prey preferences (Godin and McDonough 2003) translate into differential survival probabilities of guppy color phenotypes (Olendorf et al. 2006; Gordon et al 2015). However, also crustacean predation has been suggested as another vital, albeit largely overlooked factor (Magurran and Seghers 1990; Klaus and Plath 2011). Other poeciliids are also under intense avian predation (Riesch et al. 2010; Lukas et al. 2021), or even predation by large-bodied spiders (Horstkotte et al. 2010). In our present study, violet ornamentation actually increased from headwaters towards lowland sections, which likely harbor more piscine predators (Haskins et al. 1961; Endler 1980, 1983, 1995; Winemiller et al. 1990; Houde 1997; Kemp et al. 2008), yet again highlighting the need for an in-depth examination and quantification of (ideally) all co-occurring predators, including often overlooked predators such as the very common chelid water turtle *Phrynops geoffroanus*.

5. Conclusions

Our present study adds to a growing body of literature suggesting that pollution (and other forms of man-made habitat alterations) has the potential to alter the selective landscape under which populations evolve, far beyond the level of mere adaptation to cope with the pollutants (*i.e.*, potential evolution of tolerance or detoxification mechanisms). Specifically, studying components of sexual selection via mate attraction (nuptial ornaments), we find signatures of microevolutionary divergence, directly alluding to altered evolutionary trajectories, but we highlight that also those effects we interpret as being largely plastic will likely leave their future evolutionary footprint in the populations in which they occur.

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Authors' contributions

TW, BBP, RO, SISM, RR and MP conceived the project outline (conceptual and analytical framework). GG-S, KL, BC, VSVS, BBP, RO and MP collected samples. GG-S and EC analyzed color photos and conducted statistical analyses under the supervision of RR and MP. GG-S, RR, EC and MP drafted the first manuscript version, which received critical input from all authors.

Ethical statement

All experiments were performed in accordance with relevant regulations of the Federal Republic of Brazil (CEUA protocol number 106/1).

Data availability

The datasets generated and/or analyzed for the current study are available online at https://doi.org/10.17637/rh.29376452.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1

Results from (*a*) MANCOVA using principal component (PC-)scores obtained from numbers and percent body cover of nuptial ornaments of *P. reticulata* males as dependent variables while including information from all six ornament types. Predictor variables (covariates) were four environmental PCs condensing information on geo-physical, physico-chemical, and pollution-related characteristics of the sampling sites—with PC1 capturing pollution-related effects (Gomes-Silva et al. 2020b)—as well as male body size (standard length, SL). All interaction effects were non-significant ($F < 1.029$, $P > 0.413$) and thus, removed from the final model. (*b*-*g*) Separate *post-hoc* ANCOVAs for each color ornament type. All interaction effects were again removed as they were non-significant ($F < 2.123$, $P > 0.147$). Effect strengths are provided as Wilk's partial η_p^2 and statistically significant effects are highlighted in bold font.

Explanatory variable	*df*	*F*	*P*	Wilks' partial η_p^2
(*a*) All ornament types				
Environmental PC1	**6**	**3.134**	**0.004**	**0.121**
Environmental PC2	**6**	**3.463**	**0.002**	**0.132**
Environmental PC3	**6**	**2.455**	**0.020**	**0.098**
Environmental PC4	6	1.216	0.297	0.051
SL	**6**	**3.424**	**0.002**	**0.131**
Error	160			
(*b*) Black ornaments				
Environmental PC1	1	3.477	0.064	0.021
Environmental PC2	**1**	**14.614**	**< 0.001**	**0.081**
Environmental PC3	**1**	**12.238**	**0.001**	**0.069**
Environmental PC4	1	0.701	0.404	0.004
SL	1	3.328	0.070	0.020
Error	165			
(*c*) Orange ornaments				
Environmental PC1	1	0.326	0.569	0.002
Environmental PC2	**1**	**4.917**	**0.028**	**0.029**
Environmental PC3	**1**	**10.260**	**0.002**	**0.059**
Environmental PC4	1	1.871	0.173	0.011
SL	1	0.019	0.892	0.000
Error	165			
(*d*) Green ornaments				
Environmental PC1	1	3.746	0.055	0.022
Environmental PC2	1	3.487	0.064	0.021
Environmental PC3	1	0.119	0.731	0.001
Environmental PC4	1	1.128	0.290	0.007
SL	**1**	**4.438**	**0.037**	**0.026**
Error	165			
(*e*) Blue ornaments				
Environmental PC1	1	2.769	0.098	0.017
Environmental PC2	**1**	**4.079**	**0.045**	**0.024**
Environmental PC3	1	0.090	0.765	0.001
Environmental PC4	1	0.936	0.335	0.006
SL	**1**	**9.082**	**0.003**	**0.052**
Error	165			

(*f*) Violet ornaments				
Environmental PC1	**1**	**9.495**	**0.002**	**0.054**
Environmental PC2	1	0.411	0.523	0.002
Environmental PC3	**1**	**4.698**	**0.032**	**0.028**
Environmental PC4	**1**	**6.395**	**0.012**	**0.037**
SL	**1**	**10.329**	**0.002**	**0.059**
Error	165			
(*g*) White/iridescent ornaments				
Environmental PC1	1	3.526	0.062	0.021
Environmental PC2	1	0.129	0.720	0.001
Environmental PC3	1	0.218	0.641	0.001
Environmental PC4	1	0.040	0.842	0.000
SL	1	1.130	0.289	0.007
Error	165			

Table 2

Results from (*a*) MANCOVA using differences in total coloration (ΔE) of nuptial ornaments of guppy males as dependent variables. We coded four environmental PCs condensing information on geo-physical, physico-chemical, and pollution-related characteristics of the sampling sites and male standard length (SL) as covariates, whereby PC1 captures pollution-related effects (Gomes-Silva et al. 2020b). All interaction effects were non-significant ($F < 1.668$, $P > 0.133$) and therefore, removed from the final model. (*b*-*g*) Separate *post-hoc* ANCOVAs for the six different ornament types. Non-significant interaction effects were removed from the final models ($F < 2.802$, $P > 0.096$). Effect strengths are given as Wilk's partial η_p^2 and statistically significant effects are highlighted in bold font.

Explanatory variable	*df*	*F*	*P*	Wilks' partial η_p^2
(*a*) All ornament types				
Environmental PC1	**6**	**5.482**	**< 0.001**	**0.171**
Environmental PC2	6	1.544	0.167	0.055
Environmental PC3	**6**	**3.347**	**0.004**	**0.112**
Environmental PC4	**6**	**3.492**	**0.003**	**0.116**
SL	6	1.916	0.081	0.067
Error	160			
(*b*) Black ornaments				
Environmental PC1	1	0.163	0.687	0.001
Environmental PC2	1	0.016	0.899	< 0.001
Environmental PC3	1	1.957	0.164	0.012
Environmental PC4	1	1.892	0.171	0.011
SL	1	3.370	0.068	0.020
Error	165			
(*c*) Orange ornaments				
Environmental PC1	1	1.919	0.168	0.011
Environmental PC2	1	1.645	0.201	0.010
Environmental PC3	1	1.789	0.183	0.011
Environmental PC4	1	0.751	0.388	0.005
SL	1	2.296	0.132	0.014
Error	165			
(*d*) Green ornaments				
Environmental PC1	1	2.641	0.106	0.016
Environmental PC2	**1**	**4.959**	**0.027**	**0.029**
Environmental PC3	1	0.110	0.741	0.001
Environmental PC4	1	0.986	0.322	0.006
SL	1	1.569	0.212	0.009
Error	165			
(*e*) Blue ornaments				
Environmental PC1	1	0.025	0.876	< 0.001
Environmental PC2	1	0.025	0.875	< 0.001
Environmental PC3	1	3.220	0.075	0.019
Environmental PC4	1	0.688	0.408	0.004
SL	1	2.956	0.087	0.018
Error	165			
(*f*) Violet ornaments				
Environmental PC1	1	0.726	0.395	0.004

Environmental PC2	1	0.774	0.380	0.005
Environmental PC3	1	0.851	0.358	0.005
Environmental PC4	1	0.008	0.930	< 0.001
SL	**1**	**6.278**	**0.013**	**0.037**
Error	165			
(*g*) White/iridescent ornaments				
Environmental PC1	**1**	**28.026**	**< 0.001**	**0.145**
Environmental PC2	1	0.543	0.462	0.003
Environmental PC3	**1**	**7.768**	**0.006**	**0.045**
Environmental PC4	**1**	**16.786**	**< 0.001**	**0.092**
SL	1	0.215	0.643	0.001
Environmental PC1 × SL	**1**	**4.073**	**0.045**	**0.024**
Error	164			

Figures

Figure 1

(*a*) Examplary photographs of male guppies sporting colorful nuptial ornaments and steps taken to quantify numbers/percent body cover of different ornament types. In this example, the male shown above sported six black ornaments (covering 14.7% of its body area), as well as three orange (7.1%), three green (8.0%), six blue (10.0%), and one violet ornament (2.0%), while the male shown below had seven black (7.1%), three orange (16.8%), two blue (2.7%) and one white/iridescent ornament (2.7%). Color ornaments are encircled by red lines. (*b*) Scattergram visualizing the relationship between (pollution-related) environmental PC1 and variation in numbers/percent body cover of violet nuptial ornaments. Shown are residuals, corrected for other model terms, the corresponding partial linear regression [solid line, shown with 95% confidence interval (CI), indicated by dashed lines], and the associated degree of determination (R^2). Increasing values of environmental PC1 represent elevated levels of water pollution (Gomes-Silva et al. 2020b), suggesting that males exhibited more violet ornaments in heavily polluted waters.

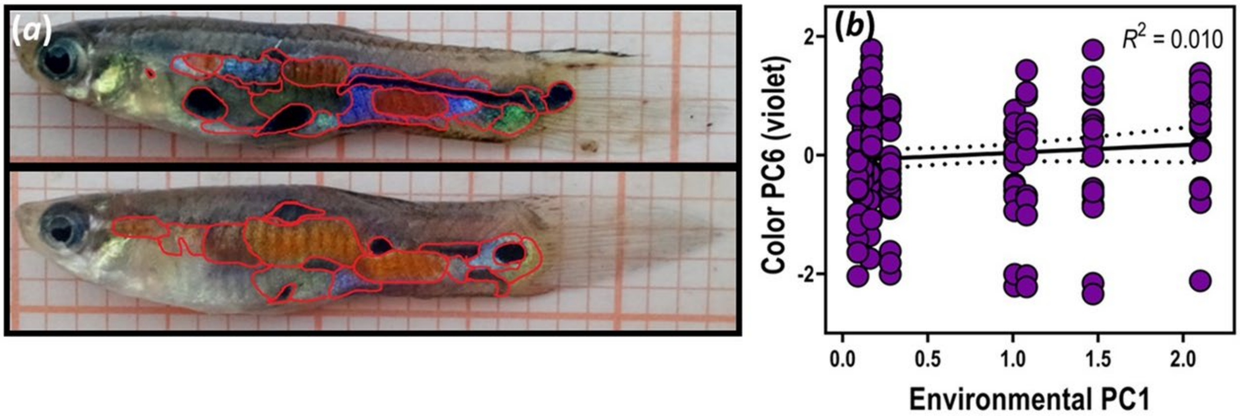


Figure 2

Effects of environmental variation other than water pollution (environmental PCs2–4 in Gomes-Silva et al. 2020b) on residual numbers/percent body cover of different ornament types. To ease the interpretation of our results, main axis loadings (> 0.6) are provided, with arrows indicating axis loadings in negative or positive direction, respectively. Scattergrams include partial linear regression lines (with 95% CI) and the associated degree of determination (R^2). (*a*) Black, (*b*) orange, and (*c*) blue ornaments decreased with increasing values of environmental PC2 (*i.e*., towards slow-flowing, less oxygenated and warmer conditions). (*d*) Black, (*e*) orange, and (*f*) violet ornaments increased as environmental PC3 increased (towards deeper water bodies). (*g*) Finally, violet ornaments decreased as PC4 increased (towards higher altitudes but less oxygenated conditions).

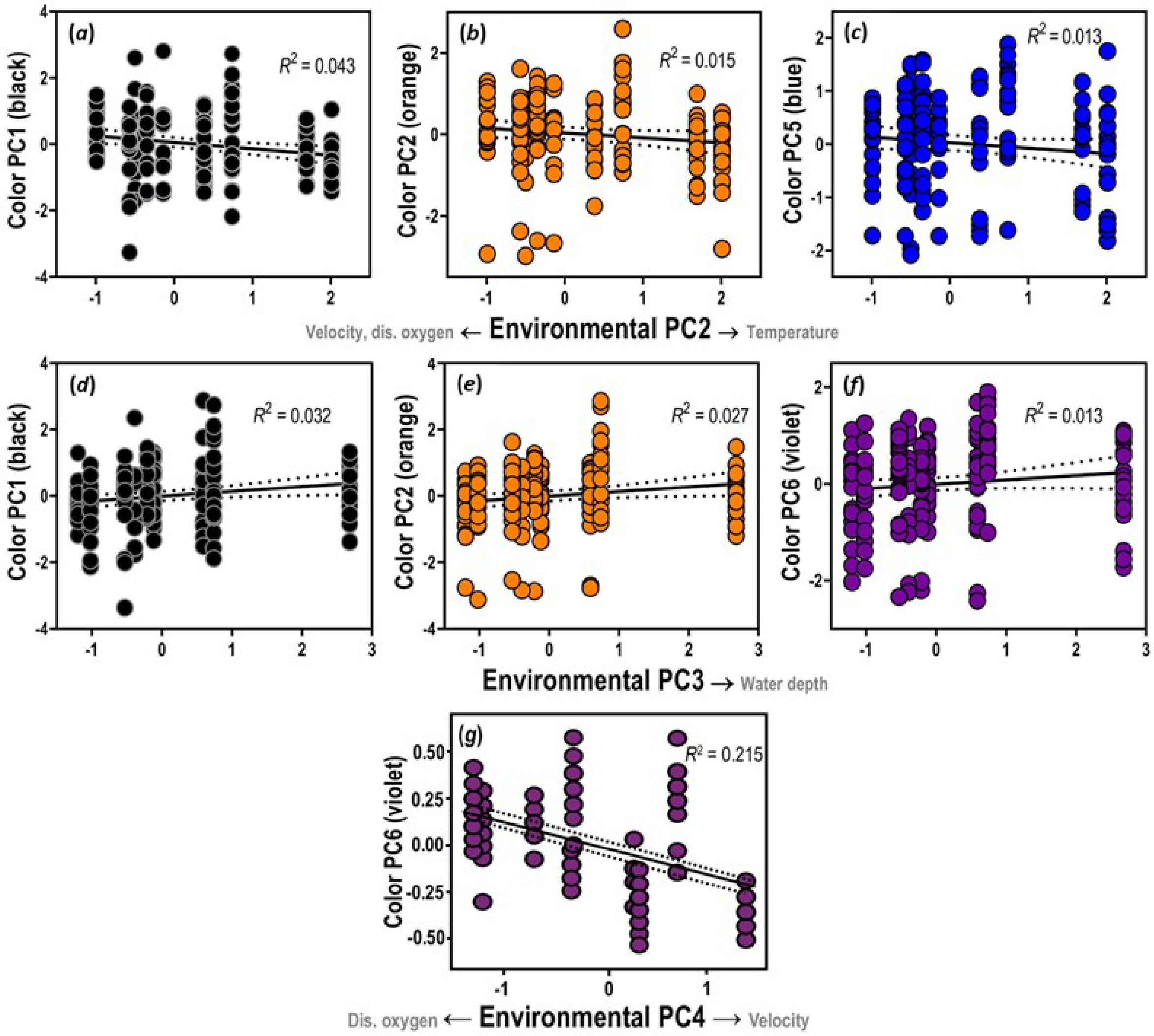


Figure 3

Effects of male body size on residual numbers/percent body cover of (*a*) green, (*b*) blue, and (*c*) violet nuptial ornaments of male guppies. Standard length [mm] was log-transformed for the statistical analyses. Shown are partial linear regression lines (with 95% CI) and the associated degree of determination (R^2). Note that larger males sported more ornaments in all three cases.

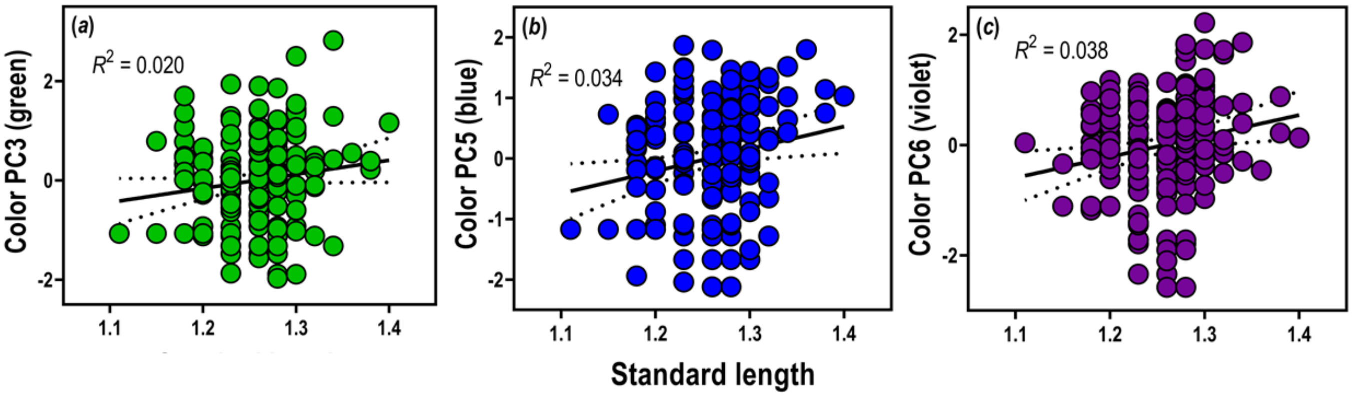


Figure 4

(*a*) Exemplary photographs of male guppies sporting two (above), three (middle) and three (below) white/iridescent color ornaments and illustration of steps taken to quantify total color differences (ΔE). White/iridescent ornaments are encircled by red lines and the highlighted ornaments yielded the following measurements (mean ± SD) in CIELAB color space: above, L* = 78.33 ± 3.06, a* = -5.33 ± 2.52, b* = -6.00 ± 5.00; middle, L* = 87.33 ± 4.31, a* = -7.00 ± 1.00, b* = -10.67 ± 4.16; below, L* = 70.00 ± 1.41, a* = -10.50 ± 7.07, b* = -1.00 ± 0.00. (*b*) Scattergram depicting the relationship between residuals of total color differences (ΔE) of white/iridescent ornaments (with the extremes of L*-, a*- and b*-values observed in our empirical data set serving as a reference point to calculate ΔE) and pollution-related environmental PC1 (see Gomes-Silva et al. 2020b). Depicted are residuals, corrected for other model terms, the corresponding partial linear regression line (shown with 95% CIs), and the associated degree of determination (R^2). Note that small values of ΔE are indicative of males sporting more brightly colored ornaments; hence, ornaments became whiter/more iridescent as pollution levels increased.

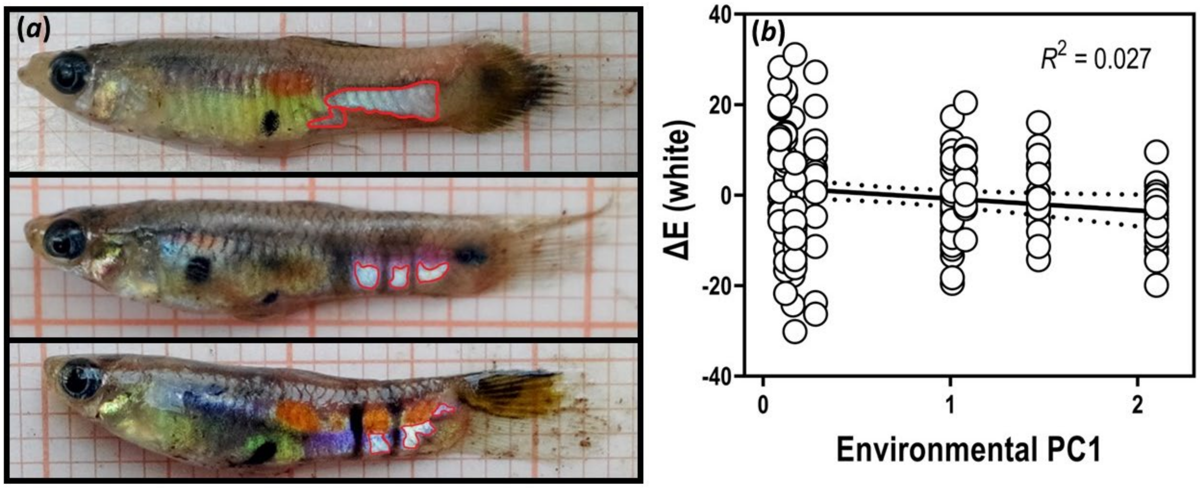


Figure 5

Effects of environmental variation other than pollution-effects on residuals of total color differences (ΔE). We show partial linear regression lines (with 95% CIs) and the associated degree of determination (R^2). For better interpretation, main negative and/or positive axis loadings (> 0.6) are provided (compare Gomes-Silva et al. 2020b). (*a*) Green ornaments responded by increasing ΔE (suggesting that males became less colorful) as environmental PC2 increased (*i.e.*, towards slow-flowing, less oxygenated, and warmer sites). ΔE-values of white ornaments (*b*) decreased (*i.e.*, males became more colorful) as environmental PC3 increased (in deeper water bodies) and (*c*) increased (*i.e.*, males became less colorful) with increasing values of environmental PC4 (towards higher altitudes but lower oxygen concentrations).

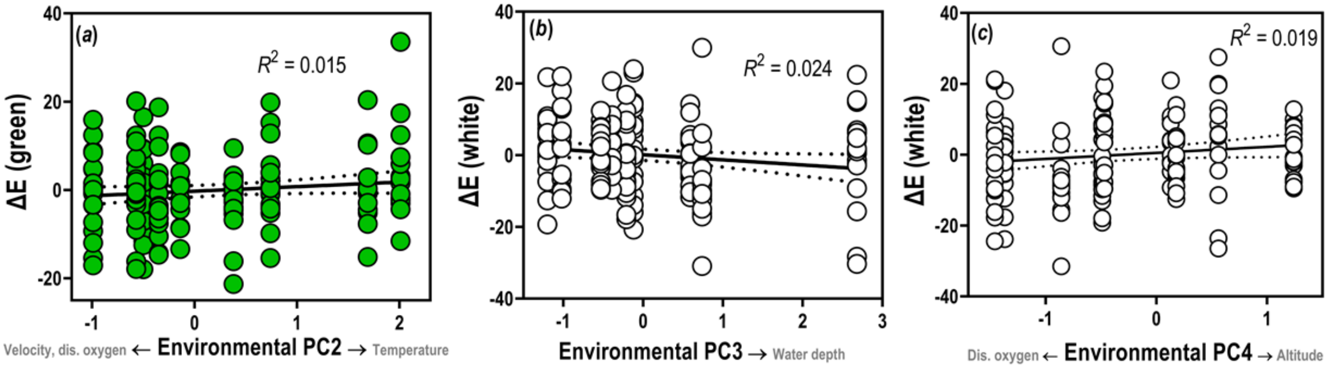
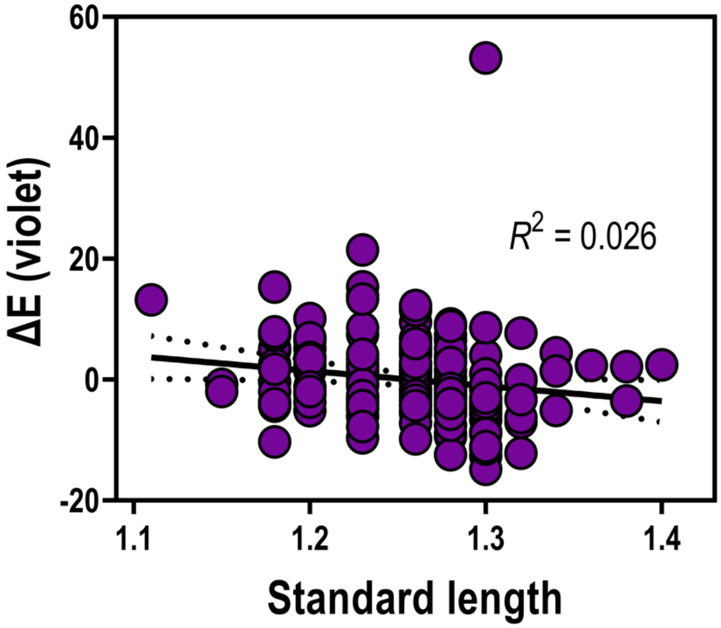


Figure 6

Effect of log-transformed male body size (standard length [mm]) on residual ΔE-values of violet ornaments. Included are the corresponding partial linear regression line (with 95% CIs) and the associated degree of determination (R^2). Note that larger males were characterized by smaller ΔE-values, suggesting that they sported more colorful violet nuptial ornaments than smaller males.



Online Supplementary Material

We present results from principal component analyses (PCA) using environmental information from only those *n* = 9 sampling sites included in our present study. We thus enable a comparison with the results of another PCA that we had previously conducted (Gomes-Silva et al. 2020b) and that included *n* = 15 sampling sites: those included here, which were slightly to heavily polluted, as well as an additional *n* = 6 sites with unpolluted water conditions. Even though the latter were excluded from our current analyses as they did not harbor guppies, our main analyses nevertheless utilize environmental principal components that were calculated from all *n* = 15 sites. This decision was made as the results below suggest that PCA based on only nine sites show a less clear-cut separation of the obtained environmental and pollution-related information. Indeed, all pollution-related variables loaded with high axis loadings on the same PC in Gomes-Silva et al. (2020b). We attribute this to the inclusion of sites with pristine water “spanning” the separation of ecological and pollution-related variables as well as major indicators of water quality onto different PC axes in a more ecogeographically meaningful way.

Supplementary Table 1

Principal component analyses (PCA) condensed 23 geo-physical, ecological and pollution-related predictor variables (please see Table 1 in Gomes-Silva et al. 2020b), based on a correlation matrix, and (*a*) using the Varimax option for axis rotation or (*b*) without axis rotation. We included (*b*) to demonstrate that, based on the set of *n* = 9 sampling sites included here, the distribution pattern of key indicators of pollution—such as the Biological Monitoring Working Party (BMWP) index and the Biochemical Oxygen Demand (BOD)—did not exhibit the distinct association with other components of pollution as reported in Gomes-Silva et al. (2020b) even when we suppressed maximization of variation onto different PC axes.

(*a*) Rotated axis solution	**PC 1**	**PC 2**	**PC 3**	**PC 4**
Eigenvalue	10.39	6.12	3.97	1.21
% variance explained	45.16	26.59	17.26	5.30
Altitude [m]	-0.223	**0.857**	0.002	0.341
Water depth [m]	0.314	**0.640**	-0.598	0.052
Stream velocity [m s^{-1}]	0.209	**-0.605**	-0.126	0.728
pH	**-0.902**	0.270	0.212	0.023
Dissolved oxygen (DO) [mg L^{-1}]	0.329	**-0.904**	0.152	0.063
Temperature [°C]	-0.236	**0.813**	0.454	-0.164
Turbidity [NTU]	**0.713**	0.521	0.353	0.281
Total dissolved solids [mg L^{-1}]	**0.698**	0.505	0.380	0.227
Biological Monitoring Working Party (BMWP) index	-0.451	**-0.691**	-0.446	0.212
Biochemical oxygen demand (BOD) [mg L^{-1}]	0.299	**0.864**	-0.238	0.187
Chlorides [mg L^{-1}]	-0.259	0.497	**0.781**	-0.164
Fecal coliforms [mg L^{-1}]	**0.792**	0.060	0.576	0.142
Nitrates [mg L^{-1}]	**0.875**	0.072	-0.335	-0.221
Nitrites [mg L^{-1}]	**0.759**	0.194	-0.570	0.146
Ammonia [mg L^{-1}]	**0.958**	-0.045	-0.238	-0.079
Total phosphorus [mg L^{-1}]	0.062	**0.890**	-0.418	0.116
Cd (sediment) [mg Kg^{-1}]	**0.978**	-0.038	0.019	0.095
Pb (sediment) [mg Kg^{-1}]	0.597	-0.249	**0.723**	0.140

Cu (sediment) [mg Kg^{-1}]	**0.906**	0.109	-0.013	-0.197
Cr (sediment) [mg Kg^{-1}]	0.460	-0.300	**0.797**	0.083
Cd (water) [mg L^{-1}]	**0.946**	-0.157	-0.083	-0.081
Pb (water) [mg L^{-1}]	**0.911**	-0.101	-0.149	-0.290
Cr (water) [mg L^{-1}]	**0.949**	-0.126	-0.012	-0.157

(*b*) Unrotated axis solution	**PC 1**	**PC 2**	**PC 3**	**PC 4**
Eigenvalue	10.39	6.12	3.97	1.21
% variance explained	45.16	26.59	17.26	5.30
Altitude [m]	-0.371	**0.845**	0.167	-0.146
Water depth [m]	0.410	**0.806**	-0.223	-0.022
Stream velocity [m s^{-1}]	0.063	**-0.219**	0.164	0.937
pH	**-0.904**	0.113	-0.171	-0.269
Dissolved oxygen (DO) [mg L^{-1}]	0.299	**-0.798**	0.145	0.454
Temperature [°C]	-0.379	**0.472**	0.330	-0.688
Turbidity [NTU]	0.394	0.461	0.782	-0.062
Total dissolved solids [mg L^{-1}]	**0.389**	0419	0.774	-0.109
Biological Monitoring Working Party (BMWP) index	-0.261	**-0.393**	-0.586	0.599
Biochemical oxygen demand (BOD) [mg L^{-1}]	0.219	**0.916**	0.145	-0.140
Chlorides [mg L^{-1}]	-0.483	0.078	0.533	-0.654
Fecal coliforms [mg L^{-1}]	0.469	-0.060	**0.871**	-0.027
Nitrates [mg L^{-1}]	**0.951**	0.159	0.041	-0.012
Nitrites [mg L^{-1}]	**0.808**	0.467	-0.042	0.297
Ammonia [mg L^{-1}]	**0.961**	0.076	0.189	0.133
Total phosphorus [mg L^{-1}]	0.087	**0.965**	-0.124	-0.173
Cd (sediment) [mg Kg^{-1}]	**0.854**	0.064	0.440	0.199
Pb (sediment) [mg Kg^{-1}]	0.272	-0.393	**0.854**	0.046
Cu (sediment) [mg Kg^{-1}]	**0.862**	0.085	0.329	-0.115
Cr (sediment) [mg Kg^{-1}]	0.147	-0.491	**0.825**	-0.016
Cd (water) [mg L^{-1}]	**0.909**	-0.079	0.291	0.130
Pb (water) [mg L^{-1}]	**0.957**	-0.080	0.153	-0.047
Cr (water) [mg L^{-1}]	**0.908**	-0.103	0.326	0.030

Altogether, 94.31% of the cumulative variance was explained. Principal components with an Eigenvalue > 1.0 were retained; variables with |axis loading| > 0.6 are highlighted in bold typeface.