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Intraspecific discrimination of fish populations by fluorescence spectroscopy

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ABSTRACT. This study used the visible fluorescence signal of scales from *Astyanax lacustris* fishes to differentiate ten populations of streams in Ivinhema River Basin, Upper Paraná Basin, Brazil. Scales were removed from the humeral region of each fish and the fluorescence spectroscopy was carried out with two excitation wavelengths: at 360 nm (UV-A) and 405 nm. The broad emission covers all visible regions and it is related to the organic fraction of scale, basically composed from type I collagen. By interpreting the experimental fluorescence spectra with multivariate statistical analysis, it was possible to discriminate the investigated populations. By exciting the inner face of scales at 405 nm, for instance, the obtained Wilk's lambda was 0.143, and the ten sampled streams could be statistically differentiated with 85.2% of explanation. This fluorescence interpretation exhibits very good correlation with the diet composition, which was also investigated for the same fishes from which the scales were removed. The applied methodology was capable to analyze the scales of *A. lacustris,* and to provide meaningful and enlightening results for the differentiation of populations. This methodology is very important for aquatic environmental study, mainly because small fishes, non-migratory or with small migration rate, can exhibit differences among habitats, as response to genetic isolation and adjustment to local conditions.

Keywords: fluorescence spectroscopy; collagen fluorescence; *Astyanax lacustris* diet composition; fish scales; bioindicators.

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Introduction

In aquatic environmental studies using fishes as bioindicators, the most valuable aspects for the analysis are the occurrence (or not) and abundance of some species, the frequency of malformations, parasitic fauna and hematological damage in response to environmental quality (Li, Zheng, & Liu, 2010). Some structures of fish, such as scales and/or otolith, are also used in this approach, in studies of fish growth and to differentiate populations (Hägerstrand et al., 2016).

Recent studies have shown that fish scales are able to incorporate pollutants, and they can act as a chemical fingerprint of the events that are occurring in the environment (Moura, Vieira & Cestari, 2012; Santana et al., 2016; Santos, Vieira, Cestari, & Barreto, 2009). Fish scales are bony elements that assist the fish body protection and have significant function in their hydrodynamics. They are considered good bioindicators and can be distinguished basically in two faces, which depends on its chemical composition: the outside (inorganic phase) and inside (organic phase). The outer face has direct contact with the environment, and it is mainly composed by hydroxyapatite ($Ca_{10}(O_4)_6(OH)_2$) with calcium deficiency. The inner face exhibits highest concentration of type I collagen (Huang, Hsiao, & Chai, 2011; Mori et al., 2012; Pati, Adhikari, & Dhara, 2010), which is directly related to the chemical composition of the diet of the fishes.

The *Astyanax* genus is one the most diversified of Characidae family, with 171 valid species (Fricke, Eschmeyer, & Van Der Laan, 2019). *Astyanax lacustris* species is commonly found in the Upper Paraná and Paraguay River Basins. It is one of the most abundant and widely distributed fish species in these Basins (Súarez et al., 2011; Ferreira, Duarte, Severo-Neto, Froehlich, & Súarez, 2017). In a recent study, the chemical composition of scales from *A. lacustris* was monitored by infrared spectroscopy to distinguish environments according to their integrity and populations (Santana et al., 2015; Almeida et al., 2016). More recently, the

fluorescence signal of fish scales from *A. lacustris* was used to distinguish environmental integrity (Santana et al., 2015). In all these works, the results indicated a high correlation between the chemical signature of the fish scales and habitat characteristics, showing that the fish scales of *A. lacustris* are great bioindicators.

Regarding the efficiency and versatility of fluorescence spectroscopy and the elevated plasticity of *A. lacustris* fish species in response to resource availability, the purpose of this study was to test the hypothesis that a population of *A. lacustris* submitted to environmental differences and isolation, in a neotropical basin, can be distinguished by the fluorescence signal of scales. The multivariate statistical analyzes was carried out considering the emission intensities for some specific types of collagen.

Material and methods

For this study, the *A. lacustris* fish were sampled in ten streams of Ivinhema River Basin, Upper Paraná River. Figure 1 is a local map with the indication of the streams. A multi-probe field instrument (Model 556, YSI) was used to measure some physico-chemical variables of each stream, such as pH, water electrical conductivity ($\mu S.cm^{-1}$), water temperature (°C), turbidity (NTU), water velocity ($m.s^{-1}$), and concentration of dissolved oxygen ($mg.L^{-1}$).

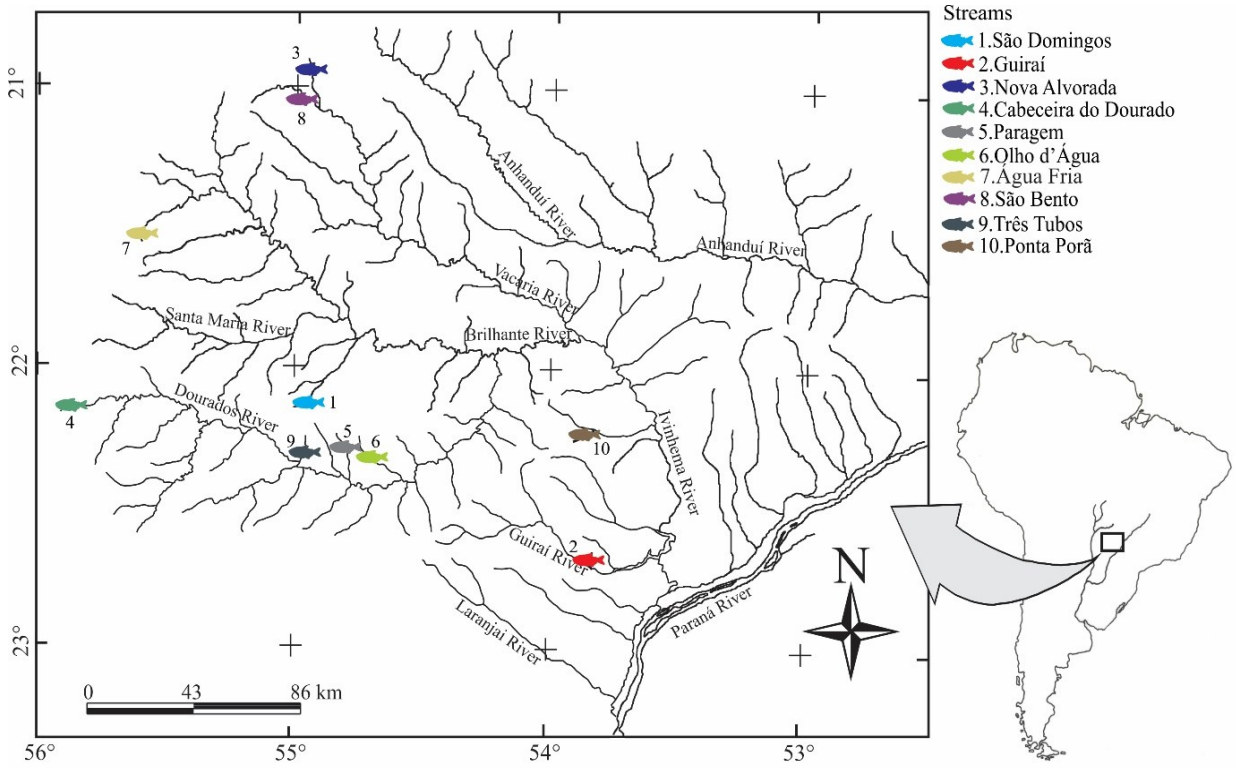

Figure 1. Local map of Ivinhema River Basin with the indication of the stream's names where the fish were sampled.

In each stream, five to ten fish individuals were analyzed. They were fixed in 10% formaldehyde to be transported to the laboratory, where they were transferred to bottles containing 70% alcohol. Five scales from each fish were removed and washed in distilled water, and then placed between two microscope slides in order to keep them flat. The humeral region was the one chosen to sample the scales, because it has the environmental characteristics fingerprint of the sampled habitat (Santana et al., 2015).

The better excitation wavelengths to perform the photoluminescence study were chose by the excitation photoluminescence spectrum. In this case, a Xe pump light was collimated to a monochromator, and the selected wavelength was directed to excite the scale. The photoluminescence signal was registered for each excitation wavelength, from 250 to 500 nm with variation of 5 nm. Two excitation lasers were employed in the fluorescence spectroscopy: a diode laser at 405 nm, and an Argon laser at 360 nm (Innova 308C, Coherent). A bifurcated optical fiber was used to conduct the excitation light to the scale and to transport the captured fluorescence signal to a portable spectrometer (Model HR4000, Ocean Optics). For each scale, spectra were obtained with excitation in both external and internal faces of the scale.

The average fluorescence spectra of the scales were deconvoluted with appropriate Gaussian functions. The relative fluorescence intensities for the Gaussians in each experimental spectrum were used to perform multivariate discriminant statistical analysis.

The geographical distance was obtained by the cartographic base (1:1000000) and the distance between the sites was measured using the stream flow. Environmental physicochemical variables were standardized (mean = 0 and standard deviation = 1) to produce a matrix of environmental distance between environments, by using the Euclidean distance method. The partial Mantel test was used to quantify the influence of limnology and the geographical distance on the differentiation of populations. These procedures were performed in order to correlate with the fluorescence information.

Results and discussion

From a scale of *A. lacustris*, the photoluminescence excitation (PLE) spectrum was obtained by observing the emission at 500 nm, and the photoluminescence (PL) spectra were also determined with excitation at 360 and 405 nm. The spectra are plotted in Figure 2. The PLE indicates that the fish scale exhibits a broad absorption band, ranging between 250 and 500 nm, which makes it possible to investigate the emission shape with different excitation wavelengths. The PL spectra of the scale exhibit maximum intensity around 490-500 nm, with a small difference in the region around 450 nm, mainly due to the excitation wavelengths used to excite the scale.

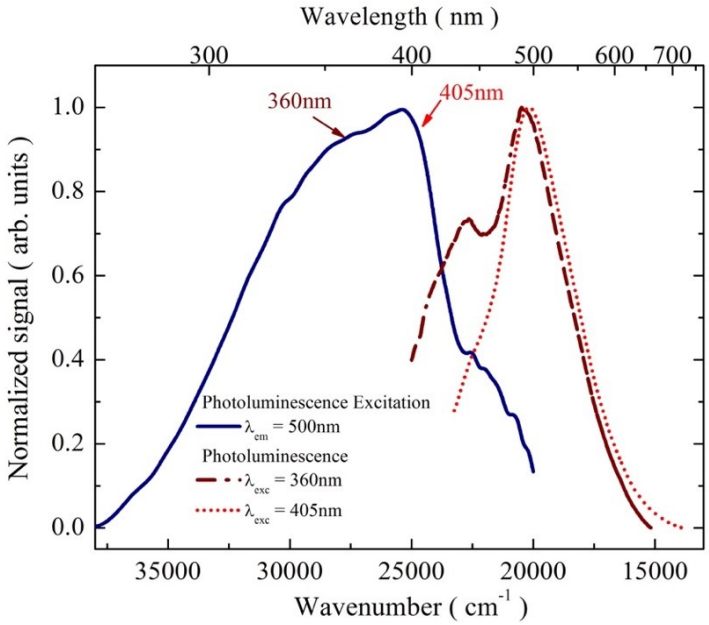

Figure 2. Photoluminescence excitation (PLE) and photoluminescence (PL) spectra obtained in a scale of *A. lacustris* by exciting the outside face of the scale.

Figure 3 shows the plotted average PL spectra (solid thick black lines) for the scales of *A. lacustris* excited in the outside face at 360 nm (A) and 405 nm (B). The correspondent spectra for the internal face are similar, so they are not shown. The spectra were fitted by Gaussians functions, and the obtained fit spectra (solid thin red lines) in both (A) and (B) are the cumulative curves determined by using four or three Gaussians, depending if the used excitation wavelength was 360 nm or 405 nm, respectively.

The Gaussians centered at 440 and 490 nm are mainly related to collagen (Santana et al., 2015), while those centered at 424 and 576 nm are due to HAp (Roman-Lopez, Correcher, Garcia-Guinea, Rivera, & Lozano, 2014). Wu, Hsiao, Chu, Hu and Chen (2012) also attributed the emission peaks between 440 and 460 nm for collagens type I and V, which are also close to those observed by Andersen and Wold (2003). These same fluorescence peak intensities were recently used by Santana et al. (2015) to assess the environmental integrity of ecosystems based in the emission of fish scales.

As it is known, each face of the fish scale has a different chemical composition, however, a distinction in the spectra was not observed when the scales were excited in the internal or external faces. Through the

analyzes of the emission curves for each stream, the broad band emission and the wavelength positions of the Gaussians were always the same, which turns difficult to distinguish the streams by direct spectra inspection. Therefore, in order to make the experimental interpretation easier, an alternative way is to use a multivariate statistical analysis with the fluorescence intensities correspondent to the Gaussian wavelength positions for each spectrum. Thus, the relative intensities of the identified peaks (correspondent to the Gaussians indicated in Figure 3) were used to construct a matrix with the wavelengths as variables. The obtained statistical results (Wilk's lambda, F, p and the first canonical root percentage) are listed in Table 1.

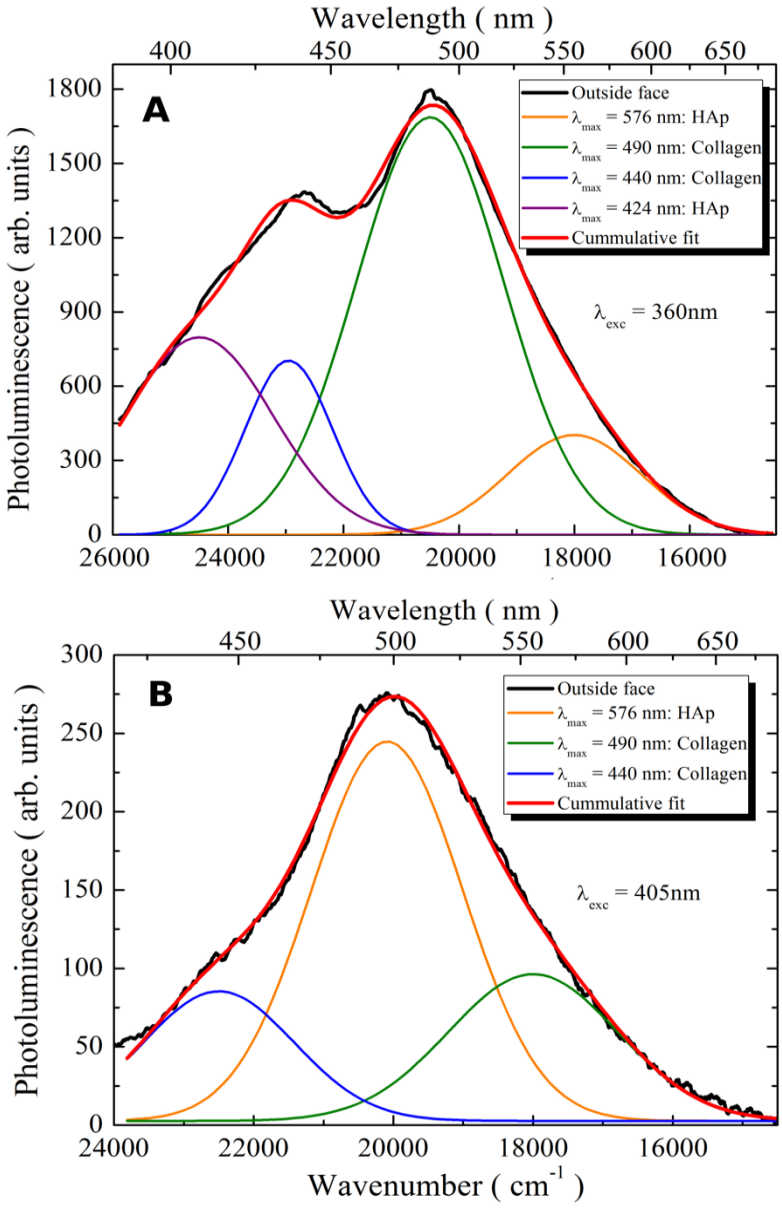

Figure 3. Average fluorescence spectra obtained in fish scales exciting the outside face at 360 nm (A) and 405 nm (B).

The discriminant function analysis performed with the fluorescence intensity of the scales showed a significant difference between the ten streams analyzed, for both scale's faces and excitation wavelengths. The highest first canonical root percentage (85.2%) corresponds to the excitation with 405 nm in the internal face (see Table 1). This distinction among the streams was not observed by analyzing the environmental characteristics; the correlation with the fluorescence spectra and the physico-chemical variables of each stream was not found (Mantel $r = 0.05$; $p > 0.05$). By comparing the geographic data (distance between the collected points) aiming to investigate a possible genetic influence, as the streams are communicated and, in some cases, they are near each other, we found that there is no correlation with the fluorescence (Mantel $r = 0.37$; $p > 0.05$) for the differentiation of populations.

Table 1. Statistical results for the excitation of the internal and external faces of the scales at 360 and 405 nm: first canonical root percentages (1ª C.R.), and Wilks' lambda, *F*, and *p* values.

Scale face	λ_{exc} (nm)	Wilk's lambda	*F*	*p*	1ª C.R. (%)
Internal	360	0.145	10.66	< 0.05	84.3
	405	0.143	10.77	< 0.05	85.2
External	360	0.106	13.54	< 0.05	80.3
	405	0.073	15.35	< 0.05	76.1

In order to investigate the influence of fish diet on the fluorescence signal and if it is correlated with the fish populations, the food components in the stomachs of six populations were verified, according to Almeida et al. (2016). A new multivariate statistical analysis was performed to correlate the fluorescence spectroscopic data with those of the composition of food findings in the stomachs. The same Gaussians described above were used in this statistical analysis, and the interpretation for the excitation wavelength at 405 nm in the outside face resulted in Wilk's Lambda = 0.012; $F = 60.99$; and $p < 0.05$. The discriminant function analysis showed a significant difference between the six analyzed streams, with the first canonical root explaining 90.9% of the variation of the data. Figure 4 shows the dispersion diagram obtained with the fluorescence data for the outside scale face excited at 405 nm. The positions of the ellipses along the first canonical root axis reflect the differentiation on the populations of streams. This arrangement of the ellipses corroborates the findings by Almeida et al. (2016), who used statistical analysis to evaluate the infrared absorption peaks related to collagen and suggested a correlation between the fish diet and the chemical functional groups present in the scales.

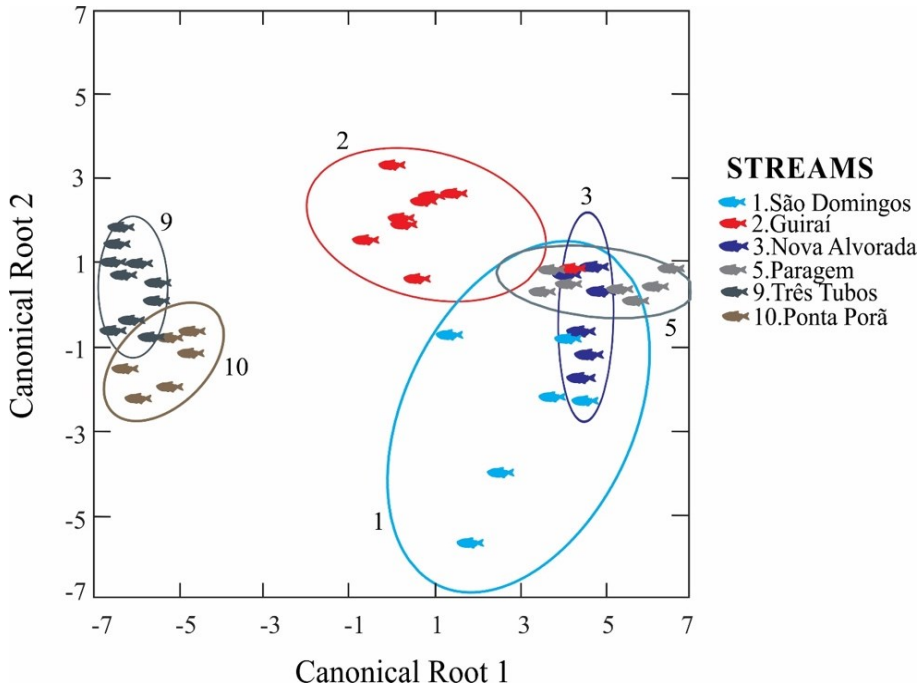

Figure 4. Dispersion diagram obtained from statistical analysis of the fluorescence data for the outside scale faces excited at 405 nm.

The position of the ellipses can be explained by the diet of the fishes sampled in the six streams, as can be noted by the percentage of food concentration found in stomachs of the studied fishes listed in Table 2. According to the stomach composition, the population of Nova Alvorada stream has a diet based exclusively of terrestrial insects (TI) and plants (TP) and its ellipse in Figure 4 is positioned on the right (positive side) of the first canonical root. In the opposite side, the population of Três Tubos stream has a diet based on terrestrial food (TI + TP = 90.7%) with a small concentration of aquatic insects (AI = 3.6%), nematodes (NE = 3.6%) and sediments (SE = 2.1%), so that its ellipse is located on the left in Figure 4.

Table 2. Percentage of food concentration found in the stomachs of the studied fishes. TI are terrestrial insects, AI are aquatic insects, TP are terrestrial plants, AP are aquatic plants, AL are algae, PF are debris and/or parts of fishes, AR are arachnids, NE are nematodes, S are sediments and UM are unidentified material.

Streams	TI (%)	AI (%)	TP (%)	AP (%)	AL (%)	PF (%)	AR (%)	NE (%)	S (%)	UM (%)
Nova Alvorada	17.0	-	83.0	-	-	-	-	-	-	-
São Domingos	44.5	-	53.0	-	-	-	-	-	2.5	-
Paragem	20.5	19.5	35.5	0.2	1.7	7.1	-	2.2	7.7	5.6
Guiraí	38.0	7.0	29.0	-	-	8.0	0.3	-	10.7	7.0
Três Tubos	42.2	3.6	48.5	-	-	-	-	3.6	2.1	-
Ponta Porã	36.4	27.1	20.7	2.0	0.4	0.4	-	-	7.9	5.0

An additional point to be considered is based on the *F* values obtained for each variable (wavelength) in the statistical analysis shown in Figure 4: the most and least relevant fluorescence intensities for discrimination of streams are those corresponding to 440 and 576 nm, respectively, which are the same observed by Santana et al. (2015). The least important peak is related to hydroxyapatite and therefore presents low sensitivity to environmental changes, while the most important peak is due to collagen concentration and it is highly sensitive.

Conclusion

The results show that the fluorescence spectroscopy is a potential tool to differentiate scales of the *Astyanax lacustris* fishes from different habitats based on the interpretation of multivariate statistical analysis. Both excitation wavelengths (360 nm and 405 nm) are useful for conducting this type of research. A strong correlation was verified between the fluorescence results and the type of food found in the stomachs of the fishes, which indicates that the habitat has a strong influence in the scale composition and consequently in the fluorescence signal. Therefore, the methodology used in this study is a promising tool for aquatic environmental studies.

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