Regression of dark color in subterranean fishes involves multiple mechanisms: response to hormones and neurotransmitters

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Organisms with source-populations restricted to the subterranean biotope (troglobites) are excellent models for comparative evolutionary studies, due to their specialization to permanent absence of light. Eye and dark pigment regression are characteristics of most troglobites. In spite of the advance in knowledge on the mechanisms behind eye regression in cave fishes, very little is known about pigmentation changes. Studies were focused on three species of the genus *Pimelodella*. Exemplars of the troglobitic *P. spelaea* and *P. kronei* were compared with the epigean (surface) *P. transitoria*, putative sister-species of the latter. Melanophore areas and densities are significantly lower in the troglobitic species. Evaluating the *in vitro* response of these cells to adrenaline, acetylcholine and MCH, we observed a reduced response in both troglobites to adrenaline. The same trend was observed with MCH, but not statistically significant. No response to acetilcholine was detected in all the three. Contrary to expectations, even though eye-regression in *P. spelaea* was much lower than in *P. kronei*, pigmentation regression was more advanced. Multiple mechanisms of loss showing a mosaic of traits in troglobitic fishes are discussed here.

Keywords: Adrenaline, Melanin Concentrating Hormone, Melanophores, Physiology of Pigmentation, Troglobitic Fish.

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Organismos com populações-fonte restritas ao biótopo subterrâneo (troglóbios) são excelentes modelos para estudos evolutivos comparativos, devido à especialização resultante do isolamento sob um regime seletivo particular, com ênfase na permanente falta de luz. A regressão do olho e da pigmentação são características dos troglobites. Apesar do avanço do conhecimento sobre os mecanismos subjacentes à regressão ocular em peixes de caverna, pouco se sabe sobre mudanças de pigmentação. Os estudos foram focados em três espécies do gênero *Pimelodella*. Exemplares das espécies troglóbias *P. spelaea* e *P. kronei* foram comparados com a epígea *P. transitoria*, provável espécie-irmã dessa última. As áreas e densidades dos melanóforos são significativamente menores nas espécies troglóbias. Avaliando a resposta *in vitro* dessas células à adrenalina, acetilcolina e MCH, observamos uma resposta reduzida em ambos os troglóbios à adrenalina. A mesma tendência foi observada com o MCH, mas não estatisticamente. Nenhuma resposta à acetilcolina foi detectada três. Contrariamente às expectativas, embora a regressão ocular em *P. spelaea* seja bem menor do que em *P. kronei*, a regressão na pigmentação foi mais acentuada. Múltiplos mecanismos de regressão, mostrando um mosaico de características em peixes troglóbios, são discutidos aqui.

Palavras-chave: Adrenalina, Fisiologia da pigmentação, Hormônio Concentrador de Melanina, Melanóforos, Peixes troglóbios.

INTRODUCTION

Troglobites, source-populations restricted to subterranean habitats, usually present autapomorphies expressed as morphological, physiological, behavioral and ecological characteristics that can be directly or indirectly related to the underground environment, and are known as troglomorphisms (Trajano, 2001).
Subterranean habitats are distinguished by strong ecological filters representing selective regimes highly contrasting to epigean. In fact, caves may be considered as a natural experiment in the exclusion of certain ecological factors, such as photoperiods and extreme fluctuations in temperature (Trajano, Bockmann, 1999).

Thus, in evolutionary studies, troglobitic organisms are an excellent source of data on both constructive and regressive mechanisms, since the selective pressures they undergo are relatively easy to identify (Trajano, Bockmann, 1999; Trajano, 2001). The large Brazilian diversity in subterranean fishes, one of the richest in the world and which includes species at different stages of eye and pigmentation regression (Trajano, Bichuette, 2010), offers an excellent opportunity for comparative studies on these specializations (Felice *et al.*, 2008). Between the two most recognized and common troglomorphisms, *i.e.*, regression of visual structures and cutaneous dark pigmentation, the former has received greater attention, with numerous publications dedicated to light-receptor structures in vertebrate troglobites. Studies range from morphology, ontogeny and classical genetics to ultra-structure and molecular biology, mostly focusing on the Mexican tetra characins, genus *Astyanax* Baird, Girard, 1854 (Jeffery, 2006; Protas *et al.*, 2006, 2007; Wilkens, Strecker, 2017).

Concerning cutaneous pigmentation, the literature is scarce on Brazilian fishes (Pavan, 1945; Trajano, de Pinna, 1996; Felice *et al.*, 2007, 2008), and also on the Mexican *Astyanax* when compared to studies on photorreceptors (Wilkens, 1988; McCauley *et al.*, 2004, Bilandzija *et al.*, 2013; Wilkens, Strecker, 2017).

It has been assumed that the degree of troglomorphisms, *i.e.*, the number of troglomorphisms and their modifications in relation to close epigean relatives is correlated with the time of isolation under conditions of permanent darkness (Wilkens, Meyer, 1992). Nevertheless, as was pointed out by Trajano (2007), other factors may have also exerted an influence, such as variable rates of differentiation, population sizes and life-cycle strategies.

Felice *et al.* (2008) distinguished four types of modification in pigmentation observed in Brazilian troglobitic fishes: decrease in the number/density of melanophores; decrease in the size of melanophores usually associated to the former; DOPA (+) and DOPA (-) albinism; and lower capacity to respond to the hormones and neurotransmitters involved in pigmentation control, such as adrenaline, the melanin concentrator hormone (MCH) and acetylcholine. The first three mechanisms have been described for the Mexican troglobitic *Astyanax*, viz., the decrease in density of melanophores, and of melanin content in "phylogenetically old" populations (Wilkens, 1988), and depigmented fish are DOPA (+) (McCauley *et al.*, 2004). So far, no cave fish other than the Brazilian *Trichomycterus itacarambiensis* Trajano, de Pinna, 1996 and the presently studied *Pimelodella* spp., have been investigated with focus on the response of melanophores to hormones and neurotransmitters.

In teleosts, body pigmentation basically depends on four types of pigment cells or chromatophores, dark-colored melanophores containing melanin, white or silver-colored iridophores with grain or plate-shaped purine, yellow-colored xanthophores and red-colored erythrophores, both containing pteridines and carotenoids (Bagnara, Hadley, 1973). These cells synthesize and/or store pigments, and possess numerous dendrites (cytoplasmic projections) that give them a star-like appearance (Sherbrooke *et al.*, 1988; Fujii, 2000).

Some hormones and neurotransmitters modulate movements of pigment-granules inside chromatophores. This can occur in various processes, such as endocrinal, neural or both, as is the case in most teleost pigment cells (Fujii, Oshima, 1986; Fujii, 2000). To a large extent, environmental stimuli are perceived through the senses, whence this information is transferred through the optic nerve to the central nervous system. Here, processing occurs with the posterior conversion into the appropriate chromatic reactions, thereby giving rise to a "secondary" chromatic response. However, other factors, such as light, temperature and osmosis, exert a direct influence on chromatophores, thence provoking a "primary chromatic response" (Fujii, 2000).

The hormone MCH is a neurohypophysial peptide that is synthesized by hypothalamic neurons, and released through axonal terminals in the neurointermediate lobe (Bittencourt *et al.*, 1992). The role of MCH in color-change in heterothermic vertebrates, more specifically teleosts, has been well-reported (Baker, 1991). MCH has been widely shown to be efficient in aggregating teleost melanosomes (Nagai *et al.*, 1986; Logan *et al.*, 2006; Kawauchi, 2006). Concomitantly, in *Synbranchus marmoratus* Bloch, 1795, MCH at high concentrations was active in dispersing pigment granules, the opposite being the case at lower (Castrucci *et al.*, 1988).

Catecholamines, synthesized and released by the sympathetic nervous system and the adrenal gland, are aggregating pigment granules agents (Fujii, Oshima, 1986; Visconti, Castrucci, 1993; de Oliveira *et al.*, 1996; Fujii, 2000). Furthermore, the chromatophores of several fishes are primarily regulated by the sympathetic nervous system (Fujii, 2000). In synthesis, the aggregation of pigment cells is induced by the action of α-adrenoceptors present in the cell membrane, with adrenaline, one of the most common catecholamines, playing an important role in color-change. There are also β-adrenoceptors identified in some species, and these adrenoceptors mediate the dispersion of pigment granules, but as the resting state in fishes is with pigment granule dispersed, the aggregation mediated by α-adrenoceptors prevails (Fujii, 2000; Burton, Burton, 2018; Grempel, Visconti, 2020). In some fishes, acetylcholine (released form the parasympathetic nervous system) has proved to be efficient in pigment granules translocations (Fujii, Miyashita, 1976; Hayashi, Fujii, 1993).

Pavan (1945), when studying the morphology, biology and behavior of the blind catfish, *Pimelodella kronei* (Miranda Ribeiro, 1907), the first-described Brazilian troglobite, compared it with the epigean *P. transitoria* (Miranda Ribeiro, 1907), the generator of subterranean troglophile source-populations in SE Brazil, that are genetically connected with the epigean by individuals that commute between the two habitats (Dazzani *et al.*, 2012), some of which syntopic with *P. kronei*. The author noted that blind catfish kept in the laboratory and exposed to natural light became progressively darker and lost the ability to change color in response to background changes. This was the first evidence that pigmentation regression in subterranean fishes involved more than a simple decrease in skin melanin.

Herein we evaluate the degree of regression in pigment cells, as well as eventual differences in cell-response to hormones and neurotransmitters, through comparison of three catfish species belonging to the monophyletic genus *Pimelodella* Eigenmann, Eigenmann, 1888 (Siluriformes: Heptapteridae), viz., the troglobitic *P. spelaea* from central Brazil, and the sympatric troglobitic *P. kronei* and epigean *P. transitoria* from the southeast. The latter are considered sister-species, due to geographic distribution and great morphological similarity – they differ basically in the troglomorphisms of *P. kronei* (Trajano, 1997; Trajano, Bockmann, 1999).

MATERIAL AND METHODS

The catfish. *Pimelodella transitoria* and *P. kronei* are sympatric in the Alto Ribeira karst area of the Upper Rio Ribeira basin, São Paulo State. The nominal species *P. kronei* occurs in six caves. These populations are characterized, besides others, by individual variation in the degree of melanic pigmentation, with bell-shaped distribution in classes. According to Trajano, Britski (1988), while the eyes in most individuals were invisible externally, in about 9.4% these were vestigial, and appeared as often asymmetric black dots. In rare cases, the structure was apparently normal, with iris and lens, and almost the same size as in *P. transitoria*. While other morphological and behavioral characters are also subject to regression, the mosaic distributions of character-states, including eyes and pigmentation, indicated independent regression.

Pimelodella spelaea Trajano, Reis, Bichuette, 2004 is endemic to the São Bernardo

cave, in the São Domingos karst area of the Upper Tocantins, a tributary in the Amazon basin. The population is relatively homogeneous when compared to *P. kronei*: in general, cave catfishes are paler than epigean congeners and have eyes with an apparently normal structure, but significantly smaller than those of epigean catfish (Trajano *et al.*, 2004). Representative specimens can be found at the Museu de Zoologia of Universidade de São Paulo (MZUSP), as follows: *P. transitoria* – 58531, 58536, 37540, 37591; *P. kronei* – 36499, 36508, 36510; *P. spelaea* – 81726 to 81729.

Collection and maintenance of specimens. Samples of *P. spelaea* were collected in an upper tributary of the São Bernardo cave stream, in the São Domingos karst area, Goiás State, Central Brazil. For a description of the area, see Bichuette, Trajano (2003). *Pimelodella kronei* and *P. transitoria* were collected in the Alto Ribeira karst area, São Paulo State, southeast Brazil (see Trajano, 1991). *Pimelodella transitoria* specimens were from the Betari River, and *P. kronei* from the Areias de Cima cave, the type-locality with the largest of the six known populations (Areias de Cima, Areias de Baixo, Bombas Resurgence, Águas Quente Resurgence, Córrego Seco and Gurutuva, Trajano, *op. cit.*).

Pimelodella spelaea and *P. kronei* specimens were maintained in a laboratory at the Department of Zoology, Biosciences Institute, University of São Paulo – IBUSP, under constant darkness and aeration, except during maintenance activities, at a temperature of around 20–22 °C. They were fed live *Artemia* Leach, 1819 crustaceans, earthworms or commercial food for fish twice a week. *Pimelodella transitoria* specimens were kept at the Laboratory of Comparative Physiology of Pigmentation, Department of Physiology – IBUSP, under 12:12 light/dark cycles, at a temperature of around 25 °C.

Methodology. Caudal-fin clippings from adult fish (*P. spelaea*, N = 7; *P. kronei*, N = 8; *P. transitoria*, N = 6) were used. The partly pigmented *P. kronei* specimens were from the most frequent pigmentation class in the Areias de Cima population (Trajano, Britski, 1988). Although fish-fins regenerate after lesions, excision was only carried out once per individual, to so minimize stress.

Immediately after excision, the fin clippings were immersed in a physiological solution (NaCl – 7.48 g / KCl – 0.2 g / $CaCl_2$ – 0.2 g / $NaHCO_3$ – 0.2 g / Glucose – 1.0 g) for 30 minutes prior to microscopic analysis. This was our control, since the melasomes were fully dispersed in this experimental condition.

Hormone and neurotransmitters were tested at the following concentrations (in mM): Adrenaline, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}; Acetylcholine, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}; and MCH, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}. In order to evaluate responses, caudal fin fragments were cut into three smaller pieces, whereupon each was exposed to only one hormone or neurotransmitter administered in a sequence of increasing concentrations at intervals of 5 minutes (adrenaline), 10 minutes (acetylcholine) or 15 minutes (MCH). The percentage of response was calculated regarding the melanophores with fully aggregated melanosomes, as 100%. This response was obtained with the higher doses of the hormones. The control were the fragments immersed in physiological salt solution before apply the hormones, when the melanosomes were fully dispersed.

Observation was with an inverted optical microscope (*Zeiss – Axiovert 100*), with attached perfusion system for fixing fragments. Responses were registered by a digital camera *Canon – A64* attached to the microscope.

Fin fragments were exposed to adrenaline in a concentration of 10^{-4} mM for five minutes, to so obtain data on chromatophore relative size and density. Since adrenaline promotes pigment aggregation, this procedure facilitated counting and measuring the cells. Photos of homologous areas of the fragments were taken for measurements with *ImageJ 1.41N*. Density was evaluated as the number of melanophores per 25 mm². Due to the morphology of the melanophores, with dendritic shape, we measured the area occupied by the melanosome after the aggregation, which is an indirect estimate of the size of melanophores. The software uses a unit defined as arbitrary, because there is not a correspondence to conventional unit of the metric system. However, as the aim of these measurement was comparing the three species, it fills this objective.

The three species were compared using Hogben-and-Slome's chromatophorotropic index (Bagnara, Hadley, 1973). This method has been widely used in studies on the response of the melanophores of teleosts, amphibians and reptiles to various chemical stimuli, including drugs and melanotropic peptides. The percentage change in melanophore index scale to the tested hormones was plotted in graphs.

Data analysis was with *ImageJ 1.41N*, *Microsoft®* and *GraphPad.* The data were analyzed with Variance Analysis test (ANOVA) with multiple comparisons, and statiscally significant for p ≤ 0.05.

RESULTS

Area and density of melanophores. Tab. 1 presents the data for density and relative size. There were significant differences (p ≤ 0.05) between average aggregated melanosome areas in *P. kronei* and *P. spelaea* caudal fins compared with *P. transitoria*. In the two troglobites these were, on an average, less than half the size of those of the epigean *P. transitoria*. No significant differences were observed between *P. kronei* and *P. spelaea*.

As regards density (number of melanophores per 25 mm²), there were significant differences (p ≤ 0.05) between *P. kronei* and *P. spelaea* on the one hand, and *P. transitoria* on the other. Analysis revealed no differences between *P. kronei* and *P. spelaea*.

Chromatophore response to hormones and neurotransmitters. Tab. 2 presents the number of cells observed in 25 mm² (in percentages) containing melanosomes aggregated in response to hormones. In the troglobites, there is a clear reduction in the number of responsive cells compared to *P. transitoria*, and this reduction is more pronounced in *P. spelaea*, that also differs significantly from *P. kronei*, in response to adrenaline. On the other hand, although there is apparently the same tendency for MCH, they did not differ significantly.

TABLE 1 | Density and relative size of the studied species (* p ≤ 0.05 in comparisons with *P. transitoria*). (a.u. arbitrary unit of *Image J* software).

	Size (a.u.)	Density Per 25 mm²
P. transitoria	3,375 ± 709.4	116.5 ± 28.8
P. kronei	1,067 ± 441.5 *	53.1 ± 22.6 *
P. spelaea	1,212.5 ± 25.5 *	67.4 ± 24.2 *

TABLE 2 | Percentages of cells with fully aggregated melanosomes in response to hormones in the studied species (* p ≤ 0.05 in comparisons with *P. transitoria*, *a different also from *P. kronei*) (n.r. without response, the melanosomes remain disperse).

	Adrenaline 10^{-5} mM	MCH 10^{-6} mM	Acetylcholine 10^{-5} mM
P. transitoria	92.7 ± 8.20	85.5 ± 9.90	n.r.
P. kronei	42.8 ± 7.20 *	52 ± 7.8 *	n.r.
P. spelaea	18.7 ± 8.30 *a	36.2 ± 14.7 *	n.r.
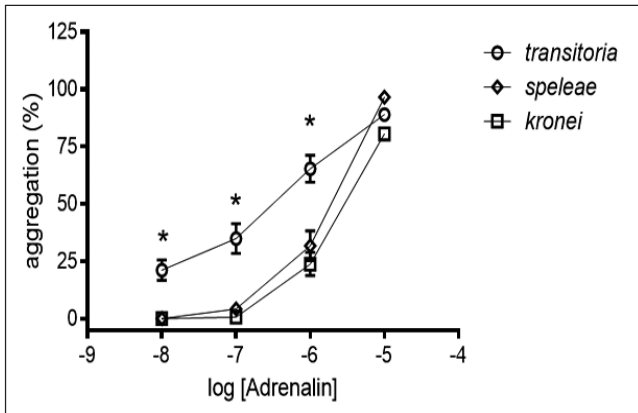
FIGURE 1 | Dose-response curves for Adrenalin in melanophores of the species of *Pimelodella*. Each point is the mean ± SEM (*P. transitoria*, n = 6, *P. speleae*, n = 7, and *P. kronei*, n = 8). * statistically different (p≤ 0.05). Control – melanosomes fully dispersed.
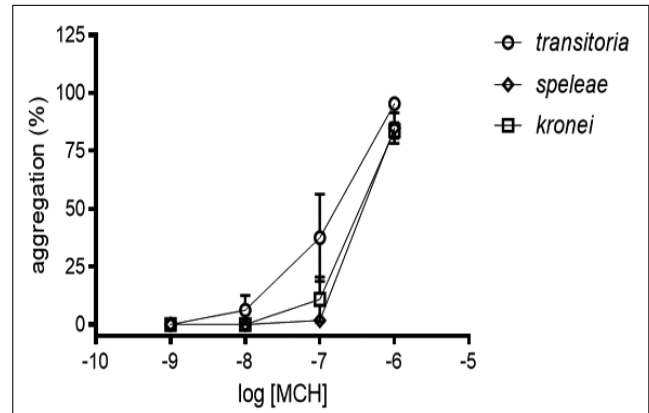

FIGURE 2 | Dose-response curves for MCH in melanophores of the species of *Pimelodella*. Each point is the mean ± SEM (*P. transitoria*, n = 6, *P. speleae*, n = 7, and *P. kronei*, n = 8). Control – melanosomes fully dispersed.

Figs. 1 and 2 show the dose–response curves to adrenaline and MCH, in the three species. There was no response to acetylcholine. The responses to adrenaline differed significantly (p ≤ 0.05) and were smaller in *P. kronei* and *P. spelaea* (that did not differ from each other) compared to *P. transitoria*. The same tendency was observed for MCH, although with no statistical differences. The cells of the three spp. have their melanosomes completely dispersed prior the application of hormones, as this is the resting state in physiological salt solution.

DISCUSSION

According to Felice *et al.* (2007, 2008), at least in fish, and probably in amphibians, the reduction of dark color in troglobites may involve multiple mechanisms, both morphological and physiological, sometimes simultaneously and beyond a simple decrease in the amount of dark pigment (melanin, in the case of vertebrates). Regarding pigmentation, Trajano, de Pinna (1996) were the first to report a case of DOPA (–) albinism among cave fishes, when describing a polymorphous population of the trichomycterid catfish, *Trichomycterus itacarambiensis*. Among all the other Brazilian

homogeneously depigmented species tested, only the heptapterid catfish *Rhamdiopsis* undescribed sp. from the Campo Formoso karst area, NE Brazil (cited as *Rhamdiopsis* sp. 2) responded as DOPA (-). The others, the catfish *Rhamdiopsis krugi* Bockmann, Castro, 2010 (cited as *Rhamdiopsis* sp. 1), the armored catfish *Ancistrus formoso* Sabino, Trajano, 1997, and the caraciform *Stygichthys typhlops* Brittan, Böhlke, 1965, are DOPA (+), as is also the case with the troglobitic *Astyanax* fish from the Pachón, Yerbaniz/Japonés and Molinos caves, due to independent mutations (McCauley *et al.*, 2004; Wilkens, Strecker, 2017).

Reduction in the number and size of melanophores is one of the mechanisms underlying the paler skin of *P. spelaea* and *P. kronei*. The number of melanophores in the caudal fin of these troglobites was about half that observed in the epigean *P. transitoria*, a putative sister-species of *P. kronei*. When compared to an epigean *Pimelodella* species from the same river basin, the Upper Tocantins, the density of chromatophores in equivalent dorsal areas of preserved specimens of *P. spelaea* was also about a half (Trajano *et al.*, 2004). Worthy of note: the percentage of reduction in melanophore density observed in cave *Astyanax*, with variable eyes and pigmentation, is the same as in the species studied here (Wilkens, 1988; Wilkens, Strecker, 2017).

Comparative morphological, histological and genetical studies of the Mexican *Astyanax* revealed that differences in complex traits, such as eye-size, melanophore density, number of free neuromasts and taste organs, feeding posture, sleep duration, amount of yolk content, ability to store fat, degree of darkness-preference etc., between cave and surface fish have a quantitative character (Jeffery *et al.*, 2000; Jeffery, 2001). The method of inheritance in melanophore number in classical crossing experiments indicated that this depends on either eight quantitative trait loci or two genes: quantitative trait-loci analysis revealed at least 18 affecting pigmentation (Wilkens, Strecker, *op. cit.*).

Pimelodella kronei catfish are aggressive, and when two or more individuals are kept together, a dominance relationship is established, which is very stressful for subordinate fishes (Trajano, 1992). Probably due to confinement, this happens even with individuals of different sexes and fully mature gonads. Therefore, intraspecific crosses and those with the less aggressive *P. transitoria*, were not feasible. However, the bell-shaped distribution of pigmentation classes in natural populations (Pavan, 1945; Trajano, Britski, 1988) is evidence in support of the additive polygenic-system hypothesis.

Preliminary tests by Felice *et al.* (2007) indicated a decrease in melanophore response to adrenaline in the trichomycterid *Trichomycterus itacarambiensis*, with high individual variation among nine of the tested individuals. In four there was no response whatsoever, even after 18 minutes of observation. The maximum number of responsive melanophores also varied appreciably. The heptapterid catfish *Rhamdia enfurnada* Bichuette, Trajano, 2005 from northeast Brazil differed from *P. spelaea* and *P. kronei* (present data) in its positive response to acetylcholine 10^{-7} M. However, in these preliminary tests, no response to MCH was observed.

According to Wilkens, Strecker (2017), and apparently based on *in vivo* observations, pigment cells of the troglobitic *Astyanax* are still capable of performing physiological color changes. This could indicate an evolutionary step regarding regression in hormonal control of dark color, beyond that observed in Brazilian catfish.

In fish, various hormones/neurotransmitters, such as MCH, adrenaline and acetylcholine, may moderate the movements of certain pigment granules inside

chromatophores. Our studies on troglobitic species have shown that, parallel with the reduction in melanophore density and loss in the capacity to synthesize melanin, the regressive evolution of characters may also affect the response of melanophores to these modulators. This reduction affects different hormones and neurotransmitters independently, thereby forming a mosaic of character states together with other traits, such as melanophore density, eye development, and many other morphological, physiological and behavioural constructive and regressive characters (*e.g.*, Bessa, Trajano, 2001; Parzefall, Trajano, 2010; Trajano, Bichuette, 2010).

When considering the positive response to acetylcholine in *Rhamdia enfurnada* (Felice *et al.*, 2007), the most parsimonious explanation for the absence of such a response in the epigean–troglobite pair *P. transitoria* – *P. kronei*, and the distantly related *P. spelaea* that lives in a discontinuous karst area, is that the loss originated from some common epigean ancestor of these species, but not of *Rhamdia* catfishes, and was possibly an inherited plesiomorphic trait.

Our results point to more advanced pigmentation regression in *P. spelaea* than in *P. kronei*, regarding physiological response to adrenaline (maximum percentage of cells responding the hormone). We also confirm Pavan's (1945) observations about darkening in *P. kronei* specimens kept in the laboratory and exposed to light from time to time. However, this was not observed for *P. spelaea* when kept under similar conditions, which indicates loss of response to light as a stimulus of melanin synthesis. The higher degree of regression affecting, apparently independent mechanisms related to the development of dark pigmentation in *P. spelaea*, when compared to *P. kronei*, is contrary to expectancy, when considering the eyes to be much more reduced in the latter.

Likewise, when exposed to light, even artificial, for some minutes once or twice a week, *Trichomycterus rubbioli* Bichuette, Rizzato, 2012, catfish, from Bahia State, NE Brazil, which are normally pale yellow in the natural habitat, become progressively darker, and after several months become dark grey, almost black. There is no reversion in color change, even when individuals are kept in the dark for several days. In contrast, congeneric *T. dali* Rizzato, Costa, Trajano, Bichuette, 2011 catfishes from Mato Grosso do Sul State, SW Brazil, do not become dark when exposed to light. The same is true for *Rhamdiopsis krugi* from Campo Formoso, as well as *Ancistrus formoso* and *Stygichthys typhlops* (E. Trajano, pers. obs.), thereby indicating the loss of light-induced melanogenesis in these highly specialized troglobites. Among troglobitic *Astyanax*, Micos fishes generally become darker in the light, due to an increase in the number of melanophores and melanin content, but "strongly eyed and pigmented – SEP" individuals in reduced populations do not react to light, although they do possess melanophores (Wilkens, Strecker, 2017).

In conclusion, *P. kronei* and *P. spelaea* have melanophores of smaller size and lower density than their congeneric epigeous, *P. transitoria*. Our results did not indicate a positive correlation between the degree of troglomorphisms, as measured by macroscopic characteristics, and responsiveness to hormones and neurotransmitters. There was no response to acetylcholine. The response to adrenaline was significantly lower in *P. kronei* and *P. spelaea* and although the same trend was observed in relation to MCH, there was no statistical significance. This was possibly due to the larger standard error, thereby indicating greater individual variation in response. Contrary to the expected with basis

on eye reduction, more advanced in *P. kronei*, regression of physiological mechanisms of pigmentation control regarding responses to adrenaline is more advanced in *P. spelaea*.

ACKNOWLEDGEMENTS

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AUTHOR'S CONTRIBUTION

Renato Grotta Grempel: Formal analysis, Investigation, Methodology, Writing–original draft.

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Maria Aparecida Visconti: Conceptualization, Formal analysis, Investigation, Project administration, Writing–original draft, Writing–review & editing.

ETHICAL STATEMENT

Permission for collect the fishes was given by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA).

COMPETING INTERESTS

The authors declare no competing interests.

HOW TO CITE THIS ARTICLE

Neotropical Ichthyology

OPEN ACCESS

Official Journal of the
Sociedade Brasileira de Ictiologia

SBI