RESEARCH ARTICLE

# Reproduction, development, asymmetry and late eye regression in the Brazilian cave catfish *Ituglanis passensis* (Siluriformes, Trichomycteridae): evidence contributing to the neutral mutation theory

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## Abstract

The troglobitic (exclusively subterranean source population) catfish *Ituglanis passensis* (Siluriformes, Trichomycteridae) is endemic to the Passa Três Cave, São Domingos karst area, Rio Tocantins basin, Central Brazil. This unique population presents variably reduced eyes and melanic pigmentation. We describe reproduction and early development in this species based on a spontaneous (non-induced) reproductive-event that occurred in the laboratory in January–February, 2009, while simultaneously comparing with data from the cave-habitat and a previous reproductive event. Egg laying was parceled. Egg-size and number were within variations observed in epigean congeners. Larvae behavior and growth is described. A single surviving specimen was monitored over two years. Eye-regression started late, one year after birth, and followed a pattern of stasis phases intercalated with slow growth and fluctuating asymmetric rates. Late eye regression, associated with asymmetry in eye development and intra-population variability of troglomorphic traits, as shown by several Brazilian subterranean fishes, provide support for the Neutral Mutation Theory.

## Keywords

central Brazil, eye-regression, *Ituglanis passensis*, life-cycle, reproduction, Troglobitic fish

## Introduction

Character regression, observed in most troglobites (exclusively subterranean source populations – Trajano and Carvalho 2017), is, not surprisingly, a central theme in subterranean biology, with emphasis on visual structures and dark pigmentation. Over the last seven decades and among fishes, Mexican tetra characins of the genus *Astyanax* have been the favored source of material in genetic and developmental studies focused on the regression of eyes and pigmentation in cave derivatives (e.g., Thinès 1960; Wilkens 1971, 1987, 1988, 2007, 2010, 2011; Peters and Peters 1973; Durand 1979; Borowsky and Wilkens 2002; Jeffery 2005; Gross et al. 2009; Yamamoto et al. 2009; Bilandzija et al. 2013; Hinaux et al. 2013; McGaugh et al. 2014; Wilkens and Strecker 2017; among many others). The other troglobitic fishes studied include the cyprinids *Caecobarbus geertsi* (Thinès 1960), *Phreatichthys andruzzi* (Berti et al. 2001; Aden 2008) and *Garra barreimiae* (Aden 2010), besides the Brazilian loricariid, *Ancistrus cryptophthalmus* (Secutti and Trajano 2009). In every case, juveniles were born with apparently normally structured eyes, that degenerated throughout ontogeny. Thus, subterranean fishes provide excellent material for testing hypotheses on the evolutionary causes of character regression.

Two hypotheses have been proposed to explain regressive traits in the Mexican cave characins *Astyanax*, the so-called "cavefish", 1) the accumulation of neutral mutations, due to relaxation of selective pressure on the maintenance of eyes, dark pigmentation and other light-related structures and functions, and 2) the negative pleiotropic effect on the selection of constructive traits in these structures and functions (e.g., Wilkens 1971, 2011; Protas et al. 2007; Jeffery 2010; Yoshizawa et al. 2012; for review see Wilkens and Strecker 2017). They are not mutually exclusive. As the neutralistic model can be applied to any evolutionary regression arising from the loss of function (Wilkens 1971, among others), application is more widespread. When considering specific traits, evidence of pleiotropic effects is only available from these studies of the *Astyanax* cavefish. To date, this knowledge has not been applied to studies of other troglobitic fishes.

When compared to other countries, taxonomically and phylogenetically, Brazil harbors a high diversity of subterranean fishes, comprising troglobites (30+ differentiated lineages in seven families and three orders – Trajano 2021), and troglophiles (source populations, dwelling in epigean and subterranean habitats, genetically connected by mutually commuting individuals (Trajano and Carvalho 2017). Most troglobitic lineages are characterized by troglomophisms, i.e., autapomorphies related to the particular selective regime found in subterranean habitats, the commonest of which regression of both eyes and melanic pigmentation (Trajano et al. 2016). The degree of troglomorphism may present inter- and intrapopulation variability, passing from slightly yet significantly reduced eyes and pigmentation compared to their surface congeners, to highly variable populations, and homogeneously depigmented and anophthalmic species, without any corresponding taxonomic correlations (Trajano and Bichuete 2010). There is also high diversity in habitat type, from the epikarst and vadose tributaries to base-level streams and the phreatic zone (Trajano and Bichuette 2010).

The Trichomycteridae family contains the largest number of troglobitic derivatives in South America, with 10 described species in Brazil included in three genera, viz.,

*Trichomycterus*, 3 spp., *Ituglanis*, 6 spp. and *Glaphyropoma*, 1 sp., besides a few undescribed species (Gallão and Bichuette 2018)

Data on the complete reproductive cycle, from courtship (if present), egg laying, larvae growth, and development until the juvenile phase, are lacking for most of these species. Sparse information is available on *T. chaberti* from Bolivia (Pouilly and Miranda 2003), and the Brazilian *T. itacarambiensis* (Trajano 1997a) and *I. passensis*, cited as *Trichomycterus* sp. (Trajano and Souza 1994).

The reproductive cycles of relatively few among the 200+ troglobitic fishes worldwide have been described. Among these, the Mexican cave-fishes *Astyanax* (*e.g.*, Wilkens 1987, 1988; Parzefall 1993; Jeffery 2005), the USAn amblyopsids (Poulson 1963), and the Brazilian armored catfish *Ancistrus cryptophthalmus* (Secutti and Trajano 2009) based on spontaneous (non-induced) reproduction in the laboratory, the Mexican heptapterids *Rhamdia zongolicensis* and *R. reddelli* (Wilkens 2001), and *Phreatichthys andruzzi* from Somalia (Berti et al. 2001; Aden 2008) based on induced reproduction, and the Cuban bythitid *Lucifuga subterranea* (Durand 1998) based on data from the natural habitat.

The main difficulties when studying reproduction, development and eye regression in troglobitic vertebrates arise from their life-cycle tendency towards K in the r – K continuum (precocial life-cycle *sensu* Balon 1990), as an adaptation to counteract food scarcity. Infrequent reproduction, low fertility and slow development (Culver 1982; Trajano 2001a), allied to generally low population densities, the small proportion of mature individuals at any time and the secretive habit of larvae, hinders encountering juveniles in the natural habitat. Moreover, most subterranean fishes with well-defined spawning cycles reproduce during or just after the rainy season, when many caves are flooded and access is impossible or quite dangerous (Trajano op. cit.). Few species, e.g., the Mexican troglobitic *Astyanax*, reproduce regularly in the lab.

Bichuette (2003), when investigating the distribution, ecology and behavior of the four cave *Ituglanis* from the São Domingos karst area, Central Brazil, transported live specimens to a lab of the Department of Zoology, University of São Paulo, São Paulo, for observations on behavior. In the beginning of 2009, a small group of *Ituglanis passensis* reproduced spontaneously, thereby offering an excellent opportunity to describe egg laying and larval development, with a focus on growth rates and eye regression. Additional evidence bolstering the neutral mutation theory, from data on Brazilian troglobitic fishes, are herein discussed.

## Materials and methods

### Study area and species studied

*Ituglanis passensis* is endemic to the type-locality, the Passa Três Cave (13°25'S, 46°22'W), a part of the São Vicente system, São Domingos karst area, Goiás State. This cave is basically a 2,000 m long stream-conduit, part of an upper vadose tributary of the São Vicente I cave, opening onto the surface through a sinkhole. Large amounts

of organic matter are carried into the cave by flash-floods, the accumulation in the first hundreds of meters sustaining rich communities of invertebrates. Particulate and dissolved organic matter is carried on throughout the entire cave stream, thereby characterizing a mesotrophic environment that supports dense populations of *I. passensis*, as well as the syntopic armored catfish *Ancistrus cryptophthlmus*. Non-troglomorphic jejus, *Hoplerythrinus unitaeniatus* (Erythrinidae), (Bichuette and Trajano 2003) represent potential prey for troglobitic fishes.

This cave area is located in the PETER- Parque Estadual de Terra Ronca (State Park of Terra Ronca), within the Cerrado (savannah-like) domain characterized by a tropical semi-humid climate, with 4–5 dry months (May to September). The water temperature in the Passa Três Cave, measured in the dry seasons of 2000 and 2001, varied from 19 to 22 °C at 100 m from the sinkhole, thereafter stabilizing to around 21 °C at 300 m. For maps and further details on the area, as well as descriptions of the caves, see Bichuette and Trajano (2003) and Reis et al. (2006).

The São Domingos karst area presents high diversity in subterranean fishes, with seven nominal troglobitic species (three families, two orders – Bichuette and Trajano 2003). The four *Ituglanis* species inhabit separate, but parallel, micro-basins fed exogenously by high-flow rivers crossing the limestone outcrop from east to west towards the Tocantins river, in the Amazon basin. As a consequence of parallel evolution, they can be mutually distinguished by different mosaics of troglomorphic traits.

*Ituglanis passensis* is characterized by very reduced eyes and scarce pigmentation, with some individual variations. Color varies from yellowish-pink (most individuals) to light grey. Melanophores are scarce and limited to the dorsal region of both head and body. Eyes vary from vestigial black spots, symmetrical or not, to none visible externally (Trajano and Souza 1994; Fernandez and Bichuette 2002). In the laboratory, the largest sizes observed in females were 90 mm TL (total length) (Trajano and Souza 1994) and 73.1 mm SL (standard length) (present study), whereas in males these were, respectively, 63.0 mm SL and 64.7 SL. *I. passensis* fishes are significantly larger than individuals of the other three species from the São Domingos karst area (Bichuette and Trajano 2004).

## Methods

Four adult *I. passensis* catfish were hand-netted in May, 2000, and afterwards transported to the laboratory in the basement of the Departamento de Zoologia, Instituto de Biociências da USP. They were kept in permanent darkness (except during maintenance activities) in a 55-l aquarium (1650 cm² bottom area), with biological filtering and a dolomite pebble substrate. Limestone rocks from the previous habitat provided shelter. Air and water temperatures were controlled by air conditioning at around 21 to 24 °C, thus close to the normal range in the cave-habitat.

For no apparent reason, one died in 2007. The remainder consisted of a female of 73.1 mm SL, without externally visible eyes (Fig. 1), and two individuals of undefined sex, measuring, respectively 64.7 and 63.0 mm SL, with eyes reduced to externally visible spots and diameters of approximately 0.85% of SL (Fig. 1). At least one of the latter two was a male, proven by spontaneous reproduction in January-February, 2009.

To avoid predation by the parents, free-swimming larvae were individually transferred to small (2.5-l, bottom area 200 cm²) aquaria, with aeration but devoid of a substrate, so as to facilitate observation. When collecting eggs from the substrate of the home aquarium, the female was placed in a clean aquarium without substrate for one hour. Those encountered were measured (total diameter, including the turgid hyaline membrane) and subsequently preserved in diluted formalin.

The surviving larvae were fed *ad libitum* once a week with nauplii and young *Artemia* crustaceans. When reaching 14.0–15.0 mm SL, juveniles were transferred to larger aquaria (7-l, bottom area 375 cm²), with small limestone blocks and a "mop" (a bundle of acrylic wool-yarns hanging from a floating styrofoam piece) for shelter. They were fed with young to adult *Artemia* crustaceans.

Eggs and larvae were measured and photographed under a stereomicroscope with ocular lens (millimeter scale – precision 0.1 mm) coupled to a digital camera. Once a week, living fry were placed in a Petri dish with water from their maintenance aquaria. After settling down, standard length (SL), head length (HL) measured dorsally from the tip of the snout to the beginning of spinal cord, and horizontal diameter of both eyes (40 × magnification), were measured. The study started with 12 larvae. However, in spite of every care, most died in the first few months: one individual survived for more than two years.

## Results

### Reproductive behavior and eggs

External sexual dimorphism was not apparent. Gender could only be distinguished in mature individuals with fully developed gonads, visible through transparency (facilitated by light coloration), as observed in the case of the female in January, 2009 (Fig. 1). These fish presented cryptobiotic habits and avoidance of light. Hence, mating could not be directly observed. Maybe it occurred inside dens and/or in the dark.

Spawning is parceled, i.e., spaced out – during the reproductive season, eggs are released several times intercalated with resting phases. Gonad maturation and egg laying in the laboratory occurred in January-February, thus corresponding to the middle of the rainy season in the natural habitat, when the minimum air temperatures are the highest.

Non-adhesive translucent eggs (Fig. 2A) were laid scattered on the substrate. There was no parental care. The diameter of newly laid eggs varied from 1.0 to 1.1 mm. Based on spawning in a clean aquarium, plus the number of eggs and larvae in the parental home-aquarium, the minimum clutch-size was calculated at ca. 120. It was impossible to establish whether all the eggs had been fertilized or not, nor whether both or only one of the males participated.

On comparing reproductive traits (number and sizes of eggs, and sizes of reproducing females) in epigean trichomycterids and the troglobitic *Trichomycterus chaberti* and *I. passensis* (Table 1), in the case of the latter, these traits fall well within the range already observed for epigean species as a whole. The troglobitic *T. chaberti* showed a
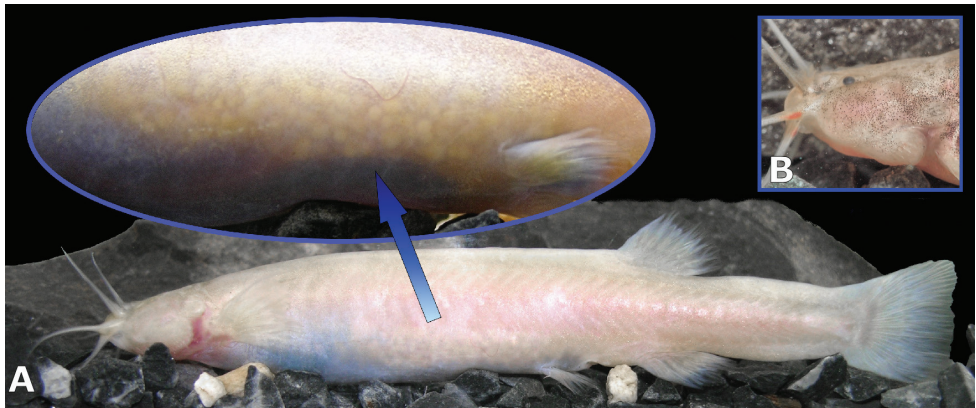
**Figure 1. A** *Ituglanis passens* mature female (73.1 mm SL), eggs seen by transparency **B** detail of male head. Photos author: Alexandre Lopes Camargo.
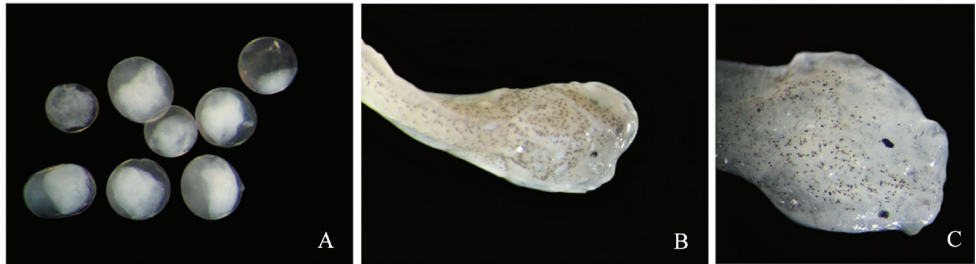


**Figure 2. A** *Ituglanis passens*, newly laid eggs **B** newly hatched free-swimming larva, with visible eyes and melanophores **C** same, in zoom. Photos author: Sandro Secutti.

**Table 1.** Data on numbers of eggs per female, egg diameters (in mm) and respective sizes of female trichomycterids (• standard length, •• total length, in mm) from literature and the present study for *Ituglanis passens* from Passa Três Cave; N – sample sizes; * troglobitic species.

| Species | N° eggs | Egg sizes | Female sizes | N | References |
|---|---|---|---|---|---|
| *Eremophilus mutisii* | 1820–27950 | 4.0–5.0 | 210–300•• | 35 | González and Rosado 2005 |
| *Trichomycterus corduvense* | 2947 | 0.45–0.49 | – | – | Marraro et al. 2005 |
| *Trichomycterus* sp. | ~73 | 1.83 | +42• | 17 | Rondineli et al. 2009 |
| *T. areolatus* | 110–1460 | 1.51–2.1 | 55–98.5•• | 43 | Manriquez et al. 1988 |
| *T. caliense* | 170–223 | 1.24–1.74 | 83• | 37 | Roman-Valencia 2001 |
| *T. cf. barbouri* | 79–231 | 0.80–1.49 | 37.3–61.0• | 34 | Pouilly and Miranda 2003 |
| *T. chaberti* * | 42 | 1.52 | 44–68.8• | 4 | Pouilly and Miranda 2003 |
| *Ituglanis passens* * | ~120 | 1.0–1.1 | 73.1• | 1 | Present data |

tendency for larger and less numerous eggs. On the other hand, compared with epigean and other troglobitic trichomycterids, *I. passens* lays less eggs, but of the same size as other epigean relatives, whereas the epigean *Trichomycterus* sp. from the Passa Cinco stream, Corumbataí basin (SE Brazil), lays even less eggs than *I. passens*, albeit larger than those of *T. chaberti*.

Newly hatched larvae in the parental aquarium were observed to swim actively in midwater and near the surface. There was no avoidance of illumination. When disturbed in any way, they quickly sank to the bottom, to hide under rocks or among pebbles. Larvae and juveniles kept in isolation were less active, mostly remaining stationary on the bottom, under rocks or in corners, except when feeding. When offered live food, juveniles began to forage, actively exploring the substrate and walls, and swimming up to the surface in a vertical position to forage among mob-wool yarns and floating pieces.

## Development and eye regression

Measurements of standard length (SL), head length (HL) and the diameters of both eyes throughout the study are shown in Table 2. The exception is individual 12, which survived for 724 days and reached 29.9 mm SL. Newly hatched larvae (Fig. 2B, C) consistently showed 7.6 mm SL and 1.5 mm HL (Day 1 in Table 2). Nevertheless, heterogeneous growth among larvae was observed from Day 6 on. Just five days after the first measurements, SL varied from 7.65 to 8.30 mm, and HL from 1.7 to 2.0 mm. Growth rates were not consistent, varying throughout the study.

Larval eyes, black spots without distinguishable pupils and measuring ca. 0.1 mm in diameter (Fig. 3), remained visible throughout the study. Contrary to what occurred with the parental female, no visible sign of regression was noted in the longest-living fish (individual 12). Its daily growth (SL) is shown in Figure 4. The average rate was 1.24 mm month $^{-1}$. Nonetheless, the pace varied. From the 40th day on, and until the 370th there was a considerable slowdown. This was followed by an acceleration until day 600, and finally decceleration. This configures an S-shaped-growth-curve in the first 2 years, instead of a C-shaped one.

Curiously, with individual 12 and until the 370th day, no perceptible regression in eye diameter (decrease in size in relation to SL) occurred. In the second year, the increase in eye-size in relation to SL growth was slower. Furthermore, this became asymmetrical, with the right eye beginning to grow faster. However, this tendency reverted around the 550th day (Figs 4, 5). When the surviving juvenile reached ca. 15 mm SL (day 180), both eyes started presenting downward movements, as observed among fishes in general. Less than one month later, the left eye presented a small change in structure, losing the typical round-shape (Fig. 3). This would be the result of fluctuating asymmetry in eye-size, observed from the 350th on (Fig. 5), without dominance in size in either the left or the right. At 370 days, diameters were around 4.5% of SL.

Individual variability in growth rates of standard length and the left eye diameter, observed in the three longest-living catfishes, is illustrated in Figures 6, 7, respectively. A further comparison of differences as to standard lengths and diameters of not only of the left eye (Fig. 8a), but also of the right (Fig. 8b), showed variable asymmetry, but no dominance on either side (Figure 8).

The larvae skin is whitish with black melanophores all over the dorsum (Figs 2B, C, 3). The number increases with growth, with the skin gradually acquiring a yellowish coloration, as in the adults.

**Table 2.** Standard lengths, head sizes, diameter of left and right eyes, respectively (in mm), of 11 larvae of *Ituglanis passensis* raised in laboratory until death (data on larva 12, that survived for 724 days, are not shown).

| Larvae / Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7.60/1.5/0.45/0.45 | 7.60/1.5/0.45/0.45 | 7.60/1.5/0.40/0.25 | 7.60/1.5/0.40/0.30 | 7.60/1.5/0.4/0.40 | 7.60/1.5/0.45/0.45 | 7.60/1.5/0.35/0.40 | 7.60/1.4/0.40/0.40 | 7.60/1.65/0.45/0.45 | 7.60/1.5/0.45/0.35 | 7.60/1.5/0.45/0.45 |
| 6 | 7.65/2.0/0.45/0.45 | 7.90/2.0/0.45/0.45 | 8.15/1.9/0.40/0.25 | 8.15/2.0/0.40/0.30 | 7.65/1.7/0.40/0.40 | 7.65/1.7/0.45/0.45 | 7.70/1.8/0.40/0.40 | 7.70/1.8/0.40/0.40 | 7.90/1.7/0.40/0.40 | 8.30/2.0/0.45/0.35 | 8.15/1.7/0.45/0.45 |
| 7 | 7.65/2.0/0.45/0.45 | 7.90/2.0/0.45/0.45 | 8.17/1.9/0.40/0.25 | 8.16/2.0/0.40/0.30 | 7.80/2.0/0.40/0.40 | 7.80/1.7/0.45/0.45 | 8.31/2.0/0.35/0.40 | 7.75/1.8/0.40/0.40 | 8.00/1.8/0.40/0.40 | 8.30/2.0/0.45/0.35 | 8.15/1.8/0.45/0.45 |
| 9 | 7.70/2.0/0.45/0.45 | 7.90/2.0/0.45/0.45 | 8.18/1.9/0.40/0.25 | 8.17/2.0/0.40/0.30 | 7.90/2.0/0.40/0.40 | 7.90/1.85/0.45/0.45 | 7.90/1.85/0.45/0.45 | 7.80/1.8/0.40/0.40 | 8.00/1.8/0.40/0.40 | 8.35/2.0/0.45/0.35 | 8.20/1.8/0.45/0.45 |
| 12 | | 7.95/2.0/0.45/0.45 | 8.18/1.9/0.40/0.25 | 8.17/2.0/0.40/0.30 | 8.20/2.0/0.40/0.40 | 8.20/1.85/0.45/0.45 | 8.40/2.0/0.35/0.40 | 8.12/1.8/0.40/0.40 | 8.00/1.8/0.40/0.40 | 8.40/2.0/0.45/0.35 | 8.20/2.0/0.45/0.45 |
| 13 | | | 8.19/2.0/0.40/0.25 | 8.18/2.0/0.40/0.30 | 8.30/2.0/0.40/0.40 | 8.30/1.85/0.45/0.45 | 8.50/2.0/0.35/0.40 | 8.00/1.8/0.40/0.40 | 8.14/1.8/0.40/0.40 | 8.50/2.0/0.45/0.35 | 8.25/2.0/0.45/0.45 |
| 14 | | | 8.20/2.0/0.40/0.25 | 8.20/2.0/0.40/0.30 | 8.40/2.0/0.40/0.40 | 8.40/1.85/0.45/0.45 | 8.50/2.0/0.35/0.40 | 8.00/1.8/0.40/0.40 | 8.17/1.8/0.40/0.40 | 8.50/2.0/0.45/0.35 | 8.25/2.0/0.45/0.45 |
| 15 | | | | | 8.45/2.0/0.40/0.40 | 8.45/1.85/0.45/0.45 | 8.55/2.0/0.35/0.40 | 8.00/1.8/0.40/0.40 | 8.20/1.8/0.40/0.40 | 8.55/2.0/0.45/0.35 | 8.3/2.0/0.50/0.50 |
| 16 | | | | | 8.50/2.1/0.35/0.40 | 8.50/1.85/0.45/0.45 | 8.60/2.0/0.35/0.40 | 8.00/1.8/0.40/0.45 | 8.23/1.8/0.40/0.40 | 8.60/2.1/0.45/0.40 | 8.3/2.0/0.50/0.50 |
| 21 | | | | | | | 8.80/2.0/0.35/0.40 | 8.20/1.8/0.40/0.45 | 8.24/1.8/0.40/0.40 | 8.90/2.1/0.45/0.40 | 8.35/2.5/0.55/0.55 |
| 22 | | | | | | | | 8.30/1.8/0.40/0.40 | 8.25/1.8/0.40/0.40 | 8.90/2.1/0.45/0.40 | 8.4/2.5/0.55/0.55 |
| 23 | | | | | | | | 8.40/2.0/0.40/0.35 | 8.30/1.8/0.40/0.40 | 9.00/2.2/0.45/0.45 | 8.5/2.5/0.55/0.55 |
| 27 | | | | | | | | | 8.80/1.8/0.45/0.40 | 9.73/2.2/0.45/0.45 | 8.8/2.5/0.60/0.60 |
| 29 | | | | | | | | | 9.00/2.0/0.40/0.35 | 9.75/2.2/0.45/0.45 | 9.00/3.0/0.60/0.60 |
| 35 | | | | | | | | | | 9.90/2.2/0.45/0.45 | 11.00/3.0/0.65/0.65 |
| 43 | | | | | | | | | | 10.23/2.2/0.45/0.45 | 15.20/3.0/0.65/0.65 |
| 46 | | | | | | | | | | 10.24/2.2/0.45/0.45 | 15.23/3.0/0.70/0.70 |
| 52 | | | | | | | | | | 10.40/2.2/0.45/0.45 | 15.21/3.5/0.70/0.70 |
| 57 | | | | | | | | | | 10.72/2.2/0.45/0.45 | 15.25/3.5/0.75/0.75 |
| 59 | | | | | | | | | | | 15.32/3.5/0.70/0.65 |
| 61 | | | | | | | | | | | 15.32/3.5/0.70/0.65 |
| 65 | | | | | | | | | | | 15.32/3.5/0.70/0.65 |
| 68 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 73 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 74 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 80 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 82 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 87 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 89 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
| 94 | | | | | | | | | | | 15.32/3.5/0.65/0.65 |
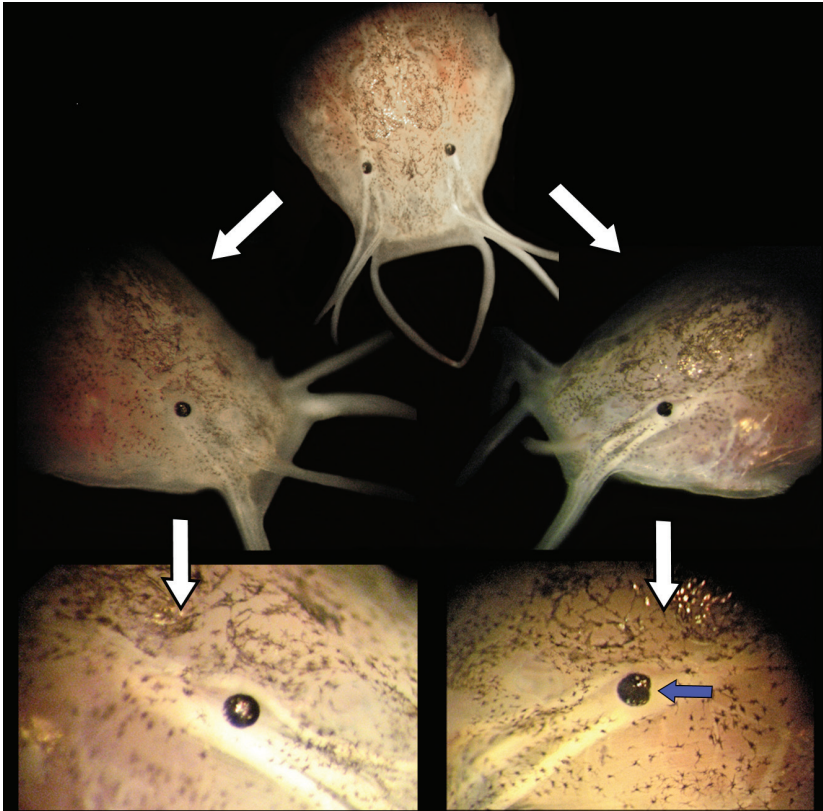
**Figure 3.** Juvenile *Ituglanis passensis* (200 days, 15.4 mm SL), detail of head and eyes showing melanophores and asymmetry in eyes shape. Arrow…. degeneration of round shape in left eye. Photo author: Sandro Secutti.
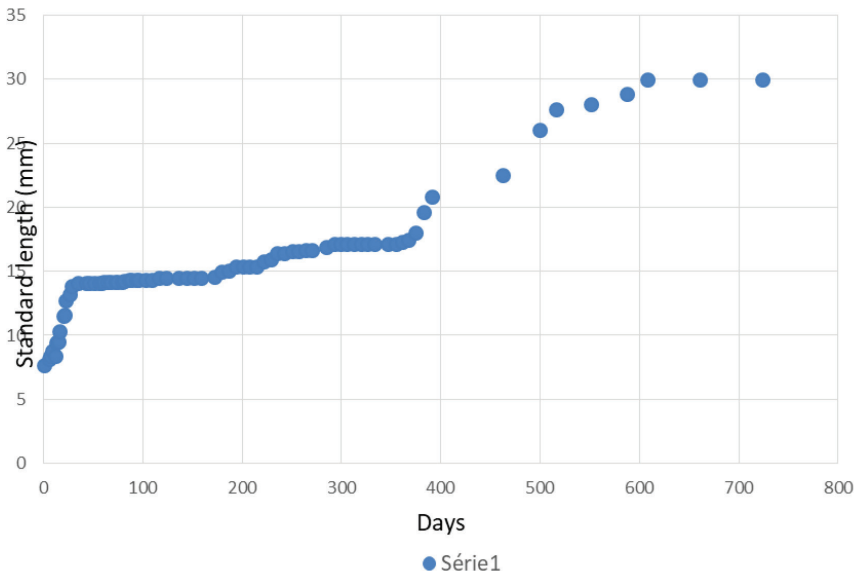


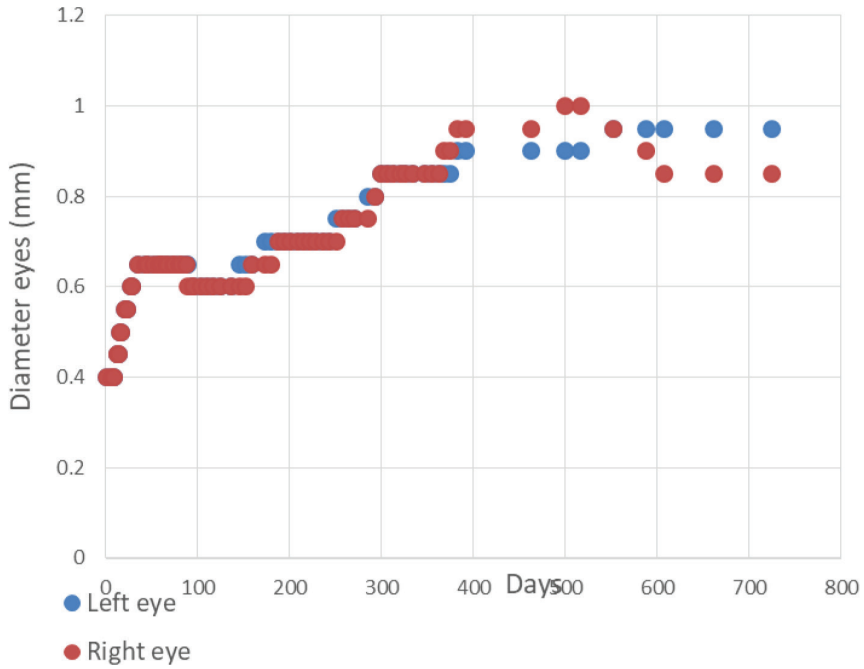**Figure 4.** Size (standard length, in mm) *versus* age (in days) in specimen 12.

**Figure 5.** Eyes size (diameter, in mm) *versus* age (in days) in specimen 12.
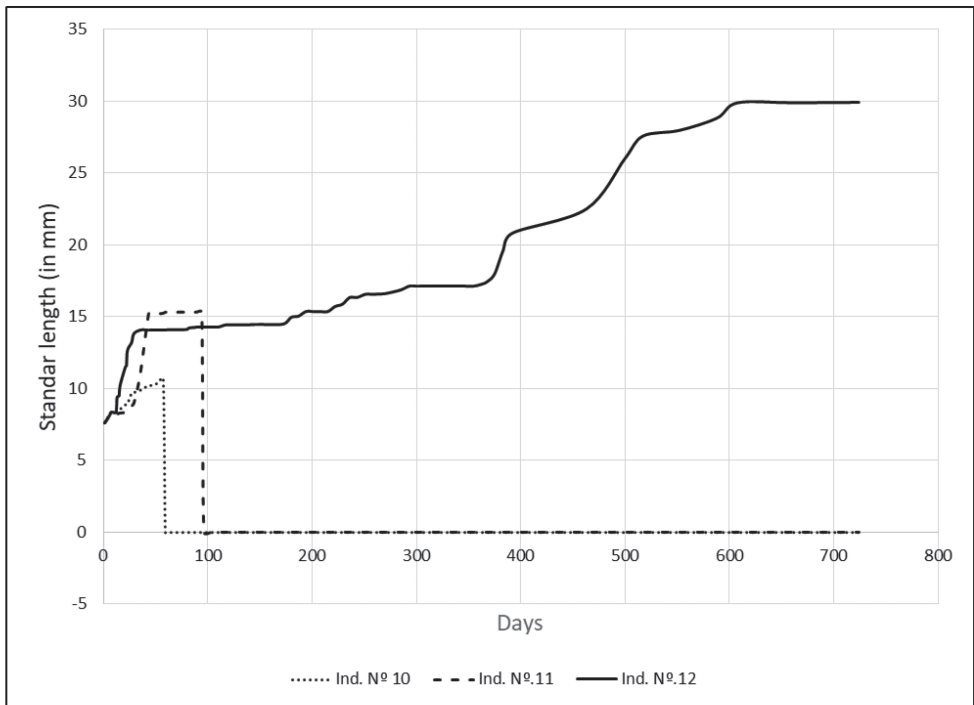


**Figure 6.** Standard length growth along the study period in individuals 10, 11 and 12.
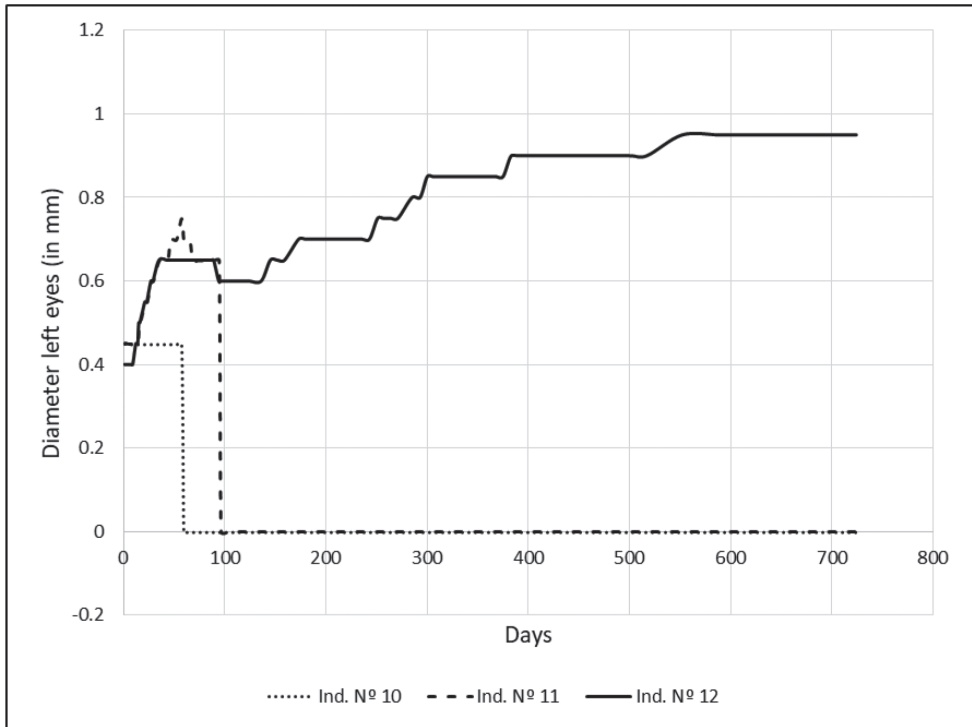
**Figure 7.** Diameter of left eye *versus* time in individuals 10, 11 and 12.

## Discussion

### Reproduction and life span

Seasonal reproduction has been recorded in several troglobitic fishes, usually during the high wet season, as is the case of the Brazilian catfishes, *Pimelodella spelaea* and *Trichomycterus itacarambiensis*, the USAn amblyopsids *Amblyopsis spelaea* and *A. rosae*, and Mexican and Cuban bythitids (*Ogilbia pearse*, *Lucifuga dentatus*, *L. subterraneus*). Counterwise, the Somalian phreatobic *Uegitglanis zammaranoi* reproduces during the warm dry season (Trajano 2001a).

Seasonal reproduction in troglobitic trichomycterids is based on differences in the proportion of mature females, which is higher in the dry season than in the beginning of the rainy, the case of *T. chaberti* (Pouilly and Miranda 2003). In *T. itacarambiensis*, the higher proportion in the beginning than at the end of the dry season, is accompanied by a parallel decrease in the mean size of oocytes, probably due to a progressive depletion in food resources (Trajano 1997b). A reproductive peak at the end of the rainy season was reported for *Rhamdiopsis krugi*, from Chapada Diamantina (BA), characterized by well-defined rain cycles (Mendes 1995; cited as *Imparfinis* sp.). Data on *I. passensis* show reproductive peaks during the rainy season, indicated in the laboratory by slight increases in water temperature (up to 2 °C).
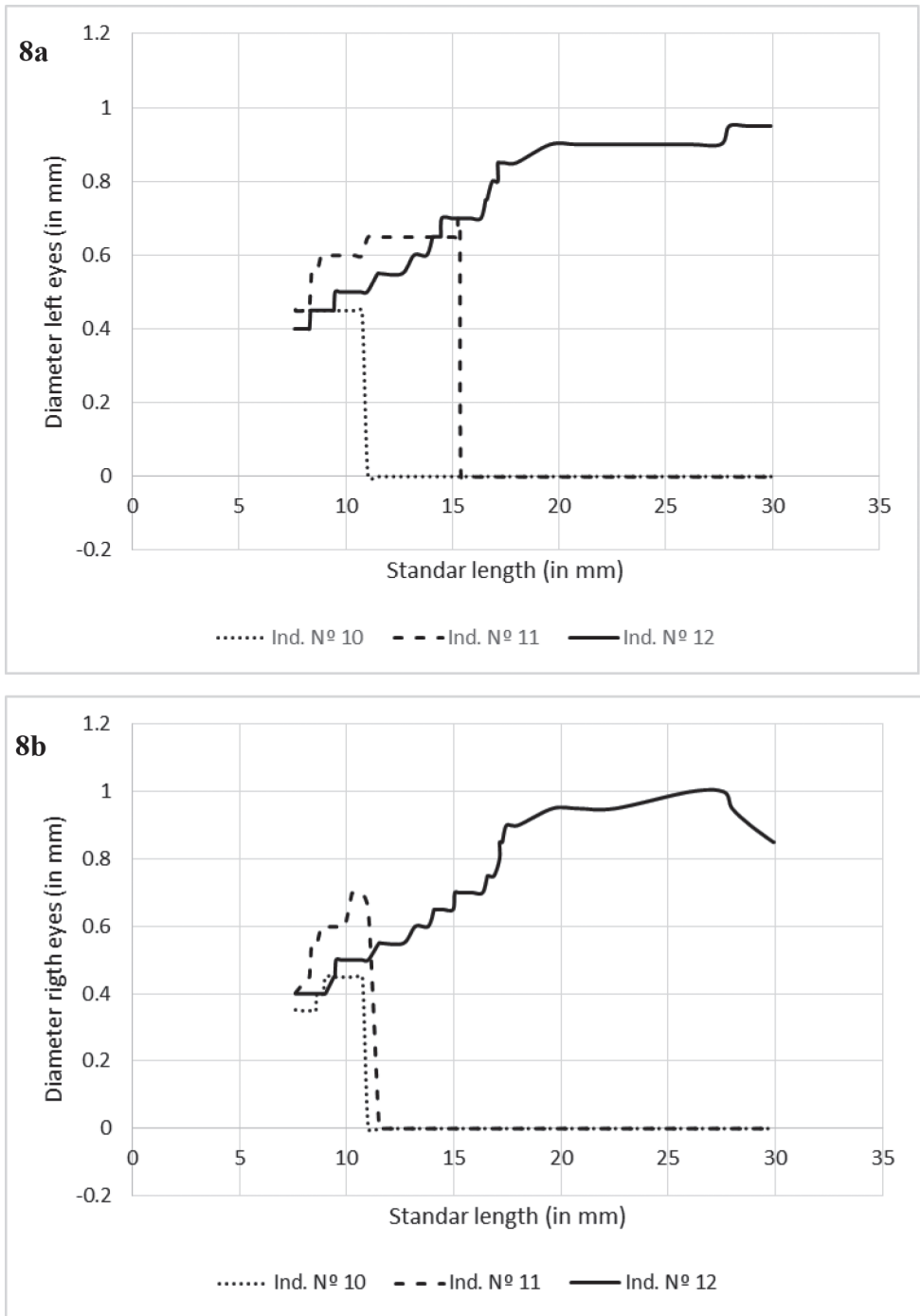
**Figure 8.** Diameter of left (**8a**) and right (**8b**) eyes *versus* standard length in individuals 10, 11 and 12.

The *I. passensis* female under study presented intercalated spawning, like the epigean *Trichomycteus corduvense* (Marraro 2005) and *Trichomycterus* sp. from the Passa Cinco stream (Rondileni et al. 2009), but not *T. areolatus* (Manriquez et al. 1988). Parceled spawning and a prolonged reproductive period are frequent in fishes from tropical and subtropical zones, where seasonality is not so accentuated as in the temperate (Nikolsky 1963). Hence, it is probably a plesiomorphic trait of *I. passensis*, not clearly correlated with a subterranean life.

Studies based on large samples (N > 30) reveal profuse individual variability in reproductive characters, the case of *T. areolatus*, *T. caliense* and *T. cf. barbouri* (Table 1). Further studies are required to clarify life-cycle patterns in trichomycterids in general, and troglobitic species in particular.

When caught in May, 2000, the studied individuals were at least 40 mm long. Based on growth-rates observed in the laboratory, at that time they were probably at least 3–4 years old. Subsequently, they survived for almost another 10 years in aquaria, thus indicating a longevity of more than 10 years, a high life-span for such a relatively small fish. Based on mark-recapture techniques, the estimated average longevity of the same sized troglobitic *T. itacarambiensis* was seven years (Trajano 1997a), and of the larger troglobitic heptapterid *Pimelodella kronei* (mean maximum size = 154 mm) 10–15 (Trajano 1991). When compared to epigean relatives, these life-spans, as part of K-selected life cycles, are higher (Trajano 2001a). Even considering that life expectancy may be higher under protected laboratory conditions (absence of predators, plus regular and predictable feeding), longevity of at least seven years in the natural habitat, as in the case of *T. itacarambiensis*, is a reasonable hypothesis.

Our results are consistent with previous observations based on specimens collected in 1988, and kept under conditions similar to those of the present study. Adult *I. passensis* catfish are shy and cryptobiotic, hiding most of the time under rocks or buried in the substrate, whether in the cave habitat or in aquaria. Agonistic interactions were rare, consisting of rapid pursuing and twisting movements. In the summer of 1989/1990, a couple kept in a 40-l aquarium with external filter and two rocky shelters, initiated reproductive behavior. Over several weeks, the male (83 mm SL) already in the same den as the female, nervously pursued her away from the shelter, sometimes touching her anal region with its snout. This behavior continued even after the first juveniles appeared (February, 1990), whereupon both adults were transferred to another aquarium. The eight surviving juveniles (5 to 12 mm TL) had well-developed and apparently normally structured eyes (Trajano and Souza 1994: fig. 1).

## Growth and eye regression

The presence of eyes in newly hatched larvae, as reported here for *I. passensis* and apparently a general trait in troglomorphic vertebrates, has been observed in organisms as diverse as the European salamander *Proteus anguinus* (*e.g.*, Durand 1973), the USAn

amblyopsids (Niemiller and Poulson 2010), the African cyprinid *Phreatichthys andruzzii* (Aden 2008; Soares and Niemiller 2020), the characid *Astyanax* (Wilkens and Strecker 2017) and the heptapterid catfish *Rhamdia zongolicensis* (Wilkens 2001), the latter two from Mexico. Among Brazilian troglobitic fishes, eyed alevins of *Glaphyropoma spinosum* (Trichomycteridae) were collected in the cave habitat (Rizzato and Bichuette 2013: fig. 6). Incidently, ontogenetic regression in the laboratory was described for *Ancistrus cryptophthalmus* (Secutti and Trajano 2009),

Two non-mutually exclusive explanations have been proposed for the precocious presence of eyes in the larvae of troglomorphic vertebrates. 1) Since the eye anlage plays an essential role in inducing craniofacial formation in fishes, eyes undergo accelerated development during the early stages (Langecker 2000). 2) Cells contributing to the retina, as well as the rest of the secondary prosencephalon (hypothalamus plus telencephalon), are intermingled in the early developmental stages. At one point there is a strong and overall constraint in the process, which antecedes eye-development alone, to so facilitate reaching the ideal point for playing their essential role in the shaping of a correct forebrain (Pottin et al. 2011).

Heterogeneous growth, i.e., the significant variation in growth rates among individuals of the same age and living in the same habitat, seems to be a common phenomenon among fishes. It was first noticed in fish farming, and recorded for adults in mark-recapture studies of Brazilian subterranean fishes, including occasions of negative growth (*Pimelodella kronei*, Trajano 1991; *Trichomycteus itacarambiensis*, Trajano 1997a; *A.cryptophthalmus*, Trajano and Bichuette 2007; *Rhamdia enfurnada* and *P. spelaea*, Trajano unpubl. data; and the amblyopsid *Amblyopsis rosae*, Brown and Johnson 2001). Heterogeneous growth may also take place among larvae and juveniles, as herein reported for *I. passensis*. This was also observed in the laboratory in the trichomycterid *Trichomycterus dali* from the Serra da Bodoquena karst area. However, the absence of individual variations in growth rates between isolated juveniles and those kept collectively indicates that neither genetics nor social interactions can unambiguously account for this (Cordeiro and Trajano, unpubl data).

Fluctuating asymmetry in eye-size, as herein reported for *I. passensis* from the 350th day on, was also observed for *A. cryptophthalmus* from the 180th (Secutti and Trajano 2009). No left-right asymmetry was noted in other Brazilian subterranean fishes characterized by intrapopulation variability, viz., the heptapterids *Pimelodella kronei*, *Rhamdia infurnada* and *Rhamdia* undescribed sp. from the Serra da Bodoquena karst area (Trajano and Bichuette 2010). On the contrary, troglobitic Amblyopsids present this asymmetry (Niemiller and Poulson 2010), and in the Mexican catfish, *R. zongolicensis*, it is extensively common among adults (Wilkens and Strecker 2017).

Although the degeneration process had started by the end of the first year (beginning of asymmetry, change of shape in the left eye), the last surviving *I. passensis* juvenile still had external eyes, visible as black spots, after around 720 days (ca. 30 mm SL). The eyes (diameters around 3% of standard length) presented fluctuating asymmetry. In adults, externally visible eyes are, at the maximum, 1% of SL (Bichuette 2003). Thus, apparently the process of eye regression was still proceeding after two years (for

an estimated lifespan of 7+ years in the laboratory), with body-sizes already having reached 40–50% of the maximum recorded for the species (60–70 mm). Thus, in comparison with other studied troglobitic fishes, this may be considered as late regression.

Timing of eye-regression among species is extremely variable, and apparently taxonomically uncorrelated. Definitive eye regression in the Mexican troglobitic *Astyanax* from the Sabinos Cave starts when fishes are ca. 15 mm long (less than one third of the average maximum size in this population). They present temporary stops, or even a decline, in eye-growth during the development phase (Wilkens and Strecker 2017), as described here for *I. passensis*.

Late ontogenetic regression, as observed for *I. passensis*, has also been recorded for *Ancistrus cryptophthalmus.* In this case, regression started in 480/500-day-old juveniles, 40–45 mm SL (Secutti and Trajano 2009). On the other hand, early eye-regression was described for the highly troglomorphic fishes *Phreatichthys andruzzi*, from Somalia (maximum eye development was reached in 36 hours, followed by very fast regression – Berti et al. 2001) and the Brazilian *Trichomycterus dali* (Cordeiro and Trajano pers. obs.). Further studies involving a larger number of species belonging to various taxonomic groups are required, to test possible correlations between the duration of eye-regression and the degree of troglomorphism, as a means of measuring time of isolation in subterranean habitats (Wilkens 1982, but also see Trajano 2007).

## Evidence for the Neutral Mutation Theory: data on Brazilian subterranean fishes

The main mechanisms supposedly involved in eye-regression are natural selection (energy economy, pleiotropy) and the accumulation of neutral mutations (for review see Wilkens and Strecker 2017).

Generality, as applied to the energy-economy-model, has been refuted, when considering troglomorphic populations dwelling in habitats where food is plentiful, viz.,troglobitic plant-hoppers and internal parasites. The presence of troglomorphic species in tropical caves, generally assumed to be food-rich, is frequently cited as an argument against this model. However, our experience in Brazil shows that the food-rich-tropical-caves paradigm is a myth. Bat caves are uncommon and mostly restricted to areas with scarce rocky shelters, such as sandstone and small karst areas (Trajano 1985). Moreover, eutrophy is a present-day ecological condition, whereas troglomorphy is the result of past historical processes (Trajano 2001b), which may have occurred under very distinct ecological conditions. The opening of large entrances favoring the input of great amounts of nutrients is a late speleogenetic event in karst areas (Ford and Williams 2007). This would be the case of the Passa Três Cave, which opens to the surface through an upper sinkhole. The large amount of organic matter, mostly vegetal debris, carried into the cave during flash floods would explain the high population densities of *I. passensis* and the syntopic *Ancistrus cryptophthalmus* (Trajano and Bichuette 2007), although this factor is probably unrelated to the higher degree of eye-regression in the later, when compared to other high density cave populations found in base-level streams.

Pleiotropic hypotheses on eye development, based on a selection of unrelated traits, are mostly the outcome of molecular studies of Mexican tetra characids, *Astyanax* spp. By cross analysis and quantitative trait loci mapping, Wilkens and Strecker (2017) presented compelling evidence against these hypotheses. Moreover, the mosaic distribution of molecular and behavioral character states in these fishes is inconsistent with this model.

Molecular studies of the cyprinids *Sinocyclocheilus anophthalmus* from China, and of *Phreatichthys andruzzii* from Somalia, point to eye-regression mechanisms different from those affecting the Mexican tetras, thereby indicating that independent evolution of eye degeneration in cave fishes could occur through other genetic mechanisms (Meng et al. 2013; Soares and Niemiller 2020).

The Neutral Mutation Theory on regression in structures, functions and behaviors that are no longer subject to selective pressures (i.e., that cease being adaptive), was proposed for troglomorphic fishes 85 years ago by the German geneticist C. Kosswig (Wilkens and Strecker 2017). A growing body of evidence has been accumulated ever since. Contrary to pleiotropic hypotheses, mostly *ad hoc*, this is a general theory, as foreseen by Darwin to explain the regression of characters that lose their functions in various and unrelated taxa.

A generally observable feature of functionless regressive traits in troglobites is their long-lasting genetic and phenotypic variability, higher than in epigean relatives and not expected for those under selection. This was reported for many troglobitic species, including the Mexican tetras, and involved molecular, morphological and behavioral aspects (Wilkens and Strecker 2017). The mosaic distribution of troglomorphic character states, including morphological such as eyes and melanic pigmentation, and behavioral traits, intra- e inter-populations, is also a common phenomenon among Brazilian subterranean fishes, thereby providing evidence of independent evolution (Trajano and Bichuette 2010).

The late eye regression, the fluctuating asymmetry and the mosaic intrapopulation variability in adult *I. passensis* catfish provide further evidence for the neutral mutation theory as a general model to explain character regression in troglobites.

## Fishes from the São Domingos karst area – a comparison

The São Domingos karst area is distinguished worldwide by its rich subterranean ichthyofauna, comprising troglophilic populations and several troglobitic lineages in seven described species (Trajano 2021). However, compared to fishes from other Brazilian karst areas, troglomorphism varies from low (slightly, yet significantly reduced eyes and little depigmentation – *Pimelodella spelaea* and *Eigenmannia vicentespelaea*) to medium (*Ancistrus cryptophthalmus* and four *Ituglanis* spp.). This is in contrast to karst areas in the semiarid Bahia State, Serra da Bodoquena (MS) and Jaiba (MG), where the most troglomorphic, homogeneously depigmented and anophthalmic populations occur.

On the other hand, the relative paucity of troglobitic invertebrates, plus location in the Cerrado core area, subject to less accentuated paleoclimatic fluctuations than in sev-

eral other Brazilian karst areas, indicate that the paleoclimatic model alone would not account for the troglobitic-fish taxonomic diversity found in São Domingos. Presumedly, topographic isolation due to alluvial downcutting by fast-flowing rivers resulted in a regional lowering of the water table and the presence of waterfalls, dolines and karst canyons (Bichuette and Trajano 2004). Hence, it is not surprising that lineages from higher, older habitats, such as the epikarst and vadose water courses, e.g. *A. cryptophthalmus* from the Passa três Cave and *Ituglanis* spp., including *I. passensis*, are more troglomorphic than those living in base-level streams (*E. vicentespelaea* and *A. cryptophthalmus* from the Angélica, Bezerra and São Vicente caves), due to a longer time in isolation.

Highly troglomorphic subterranean fishes tend to become arrhythmic, loosing photophobic reactions and cryptobiotic habits (Parzefall and Trajano 2010), besides laying fewer and larger eggs than close epigean relatives (Culver 1982; Trajano 2001a). None of this was noted in *I. passensis*.

## Acknowledgements

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