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First reproductive data from the F1 generation of the Amazonian killifish *Anablepsoides ornatus*

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ABSTRACT

We provide initial findings on reproductive performance of the poorly known killifish, *Anablepsoides ornatus*, from the Rio Negro basin. We recorded fecundity, spawning frequency, egg diameter, fertilization rate, duration of embryonic development, and hatching success from the F1 generation for 92 days. Additionally, we evaluated the fish preferences for either green or black spawning substrates. Four spawning pairs produced a total of 53 eggs (diameter = 1,428.0±98.5 µm). The average fertilization and hatching rate ranged from 54.7 to 87.8% among pairs. The preference for the black substrate was significantly higher. The duration of embryonic development ranged from 16 to 72 days, and the interval between spawnings ranged from 6.5 to 14.5 days among pairs. Our findings provide a baseline for future studies on the ecology of the species, thereby enhancing our understanding of its reproductive biology, informing conservation practices and the potential for culturing in the ornamental fish trade.

KEYWORDS: non-annual rivulid, reproductive performance, spawning substrate

Primeiros dados reprodutivos da geração F1 do killifish amazônico *Anablepsoides ornatus*

RESUMO

Apresentamos os primeiros dados sobre o desempenho reprodutivo de um killifish pouco estudado da bacia do rio Negro, *Anablepsoides ornatus*. Registramos a fecundidade, frequência de desova, diâmetro dos ovos, taxa de fertilização, duração do desenvolvimento embrionário e sucesso de eclosão da geração F1, durante 92 dias. Além disso, avaliamos a preferência dos peixes por substratos de desova verdes ou pretos. Quatro casais produziram 53 ovos (diâmetro= 1428,0±98,5 µm). A taxa média de fertilização e eclosão variou de 54,7 a 87,8% entre os casais. A preferência pelo substrato preto foi significativamente maior. A duração do desenvolvimento embrionário variou de 16 a 72 dias, e o intervalo entre desovas de 6,5 a 14,5 dias entre os casais. Nossos achados fornecem uma base para futuros estudos sobre a ecologia da espécie, compreensão de sua biologia reprodutiva, e informam práticas de conservação bem como o potencial de criação para o comércio de peixes ornamentais.

PALAVRAS-CHAVE: rivulídeo não anual, desempenho reprodutivo, substrato de desova

Anablepsoides ornatus (Garman 1895) is a non-annual Amazonian killifish that can reach a total length of between 30 and 40 mm and exhibits sexual dimorphism. This species inhabits small streams within the forest, temporary ponds that form along the sides of streams, and sunlit, open areas near larger riverbanks (Zuanon 2015). Since killifish typically inhabit small water bodies, the main threats often include anthropogenic activities related to deforestation, as well as crop and cattle farming (Godoy *et al.* 2025). Still, due to the considerable diversity that is increasingly being recognized, this neglected group of fish is critical from the perspective of biological conservation.

Conservation efforts in many species are often based on a solid knowledge of their reproduction. So far, Amazonian killifish have received especially little attention in this regard. Moreover, similarly to many other killifish species, their embryonic development is expected to be relatively long and complex. In brief, killifish embryos may develop directly without halting or enter up to three stages of developmental dormancy, such as embryonic diapause (e.g. Wourms 1972).

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This capability may vary within a single genus or even among populations of a single species (e.g. Borisov *et al*. 2023).

Despite the large number of species described in the genus *Anablepsoides*, to date, there is no consistent information on their reproductive biology. Thus, for the first time, we investigated the reproductive characteristics of *A. ornatus* to obtain fundamental data for its future conservation and potential utilization in the ornamental fish trade. We conducted laboratory experiments that provided data on their reproductive performance in captivity over a 92-day period.

Collection of parental fish for the laboratory strain and authorization for scientific activities (SISBio - No. 81950-1) was obtained from the Brazilian government *Ministerio do Meio Ambiente*. The study was approved by the Ethics Committee on the Use of Animals (No. 008/2022) of Nilton Lins University (UNL), Manaus, Brazil.

Parental stocks of *A. ornatus* were captured in *Área de proteção Ambiental da Margem Esquerda do Rio Negro, setor Tarumã-Açu/Tarumã-Mirim, Manaus, Brazil*. The sampling site at -2°57'58.31" S, -60°11'50.46" W corresponded to an area with first- and second-order streams, characterised by an average depth of 23.1±6.2 cm, with a muddy substrate in open areas and accumulation of dead leaves in the white-sand forest understory. The stream water exhibited an acidic character, with a pH value of 5.3 ± 0.6, average temperature of 27.1 ± 6.2 °C, total dissolved solids of 34.9 ± 23.9 ppm and electrical conductivity of 60.2 ± 43.3 µS cm^{-1}. The concentrations of ammonia and nitrite were both found to be below 0.25 ppm and 0.03 ppm, respectively. At the collection points, the water quality parameters were measured as baseline data for adjusting laboratory conditions. The captured fish (n= 60) were transported to the Laboratory of Reproduction and Molecular Biology of Aquatic Organisms (LRBMOA/UNL). They were quarantined for four weeks. Based on the description of sexual dimorphism, nine pairs were chosen (Figure 1a). Each parental pair was housed in a 3 L tank, fitted with an internal air-powered filter and a spawning mop (see below) as a substrate for spawning. The fish were fed three times a day with a commercial ornamental fish feed containing 47.5% crude protein (amount equivalent to 3% of the animals' weight). Grindal worms (*Enchytraeus albidus*) and newly hatched *Artemia* nauplii were offered once a day. The nine parental pairs produced 19 viable larvae (F1 generation). The larvae were raised in 7 L tanks, fed with hatched *Artemia* nauplii three times a day for 30 days. Subsequently, the juveniles were fed in the same manner as the broodstock and reached maturity after 5 months, evidenced by clear sexual dimorphism.

Four F1 *A. ornatus* pairs were selected for the evaluation of their reproductive performance. The males (total length = 2.7 ± 0.1 cm, and weight = 194.5 ± 33.4 mg) and females (total length = 2.5 ± 0.1 cm, and weight =144.1 ± 12.6 mg) were housed in four tanks (12 L), filled with 5 L of water, equipped with an air-driven filter for mechanical and biological filtration and an airstone. The water supplied to the tanks originated from an artesian well and was further supplemented with distilled water to maintain the pH and electrical conductivity of the water in the fish's natural habitat (see above). The photoperiod was set to 12L: 12D (12 hours of light and 12 hours of dark).

To maintain water quality, organic debris were removed by siphoning them out each day. Every week, water parameters including temperature (27.9±0.9°C), pH (5.4±0.7), electrical conductivity (84.2±25.1 µS cm^{-1}), and total dissolved solids (44.3±14.4 ppm) were measured using a multiprobe (Combo 5 model, ASKO, Brazil). Total ammonia (0.001±0.0006 mg L^{-1}) and nitrite (0.049±0.109 mg L^{-1}) levels were monitored using a photocolorimeter (ACQUA, Alfakit, Brazil). Partial water changes (30%) were performed once a week in each tank.

The fish were fed according to the feeding protocol previously defined for the nine broodstock pairs mentioned above. To evaluate whether *A. ornatus* shows a color preference for a spawning substrate, a single green and black "killifish spawning mop" was placed in each experimental tank. Both the green and black mops consisted of 20 acrylic wool threads, approximately 2 mm in diameter and 20 cm long, tied together at one end and kept afloat by a piece of Styrofoam® (Figure 1b). The color choice was based on previous studies showing color selectivity in killifish oviposition (e.g. Okyere *et al*. 2021; Thoré & Merckx 2023) and its ecological relevance: green represents vegetation and black represents leaf litter or shaded substrate, typical of the species' natural environment.

Each morning, all tanks were thoroughly checked for the presence of eggs in the spawning mops (Figure 1c). The eggs found were hand-picked, counted, and photographed (N=12) under a binocular microscope (BEL Photonics®, Italy) equipped with a camera (MicronScientific, Brazil) to record

Figure 1. (A) female (♀) and male (♂) of *Anablepsoides ornatus* specimens born in the laboratory; **(B)** green and black spawning mops; **(C)** eggs (white arrows) adhered to the black substrate; **(D)** fertilized egg at an early stage of embryonic development.

the diameter of the eggs (Figure 1d). To evaluate reproductive performance, the interval between spawnings, the number of eggs per female, and the fertilization rate were determined. The eggs with the perivitelline space present were scored as fertilized (e.g. Wourms 1972). All fertilized eggs were placed on a transparent 96-well ELISA plate, one egg per well. 300 µL of Yamamoto's solution was added to each well, and the eggs were incubated at 26°C. Each week, 2/3 of the volume of Yamamoto's solution was changed in each well. Throughout the incubation, we recorded the incubation time, which is the duration of embryonic development until hatching. Additionally, we also recorded the hatching rate (%) based on the presence of a hatched fry in the well. The eggs were checked twice daily.

The reproductive performance datasets did not meet the assumptions of normality (Shapiro-Wilk) and homoscedasticity (Levene). The Kruskal-Wallis nonparametric test was performed, followed by Dunn's test to compare the means. The Mann-Whitney test was used for the spawning substrate color preference. All tests had a significance level of 5% and were performed using R software version 4.4.1 (R Core Team, 2024).

The four F1 *A. ornatus* pairs produced a total of 35 clutches, containing 53 eggs (Table 1), over approximately 92 days, resulting in an average of 0.1 eggs per pair per day. The mean egg diameter in the analyzed sample (n=12) was 1,428.0 ± 98.5 µm. Overall, egg production was low compared to other killifish species, whether annual or non-annual. Killifish typically spawn daily, with a single non-annual female laying at least a couple of eggs per day (e.g. Shibatta 2005). In contrast, annuals commonly produce 20-60 eggs per day (Vrtílek *et al*. 2017), reaching more than 100 eggs per clutch under optimal conditions (Blažek *et al*. 2013).

Table 1. Reproductive performance average values (and minimum and maximum ranges) of four pairs of *Anablepsoides ornatus* evaluated during 92 days under laboratory conditions.

Reproductive performance indices	Breeding Pairs				
	1	2	3	4	*P*-value*
Number of spawns	11	12	5	7	-
Total eggs	15	24	5	9	-
Fertilization rate (%)	87.8 (50-100)	74.0 (0-100)	60.0 (0-100)	54.7 (0-100)	0.606
Hatching rate (%)	87.8 (50-100)	65.7 (0-100)	60.0 (0-100)	54.7 (0-100)	0.479
Number of eggs/spawning	1.4 (1-3)	2.0 (1-7)	1.0 (1-1)	1.3 (1-2)	0.223
Interval between spawning (days)	9.8 (1-38)	6.5 (1-41)	14.5 (2-33)	12.6 (2-33)	0.324
Incubation time of eggs (days)	28.3 (16-75)	38.7 (18-75)	39.0 (26-45)	42.4 (29-61)	0.05

*The P-values refer to the comparison between the four breeding pairs for each reproductive performance index, performed using the nonparametric Kruskal-Wallis test.

A. ornatus might be an "outlier" killifish species with a naturally low fecundity. There is virtually no information available on this species, except for its description, and its taxonomic status remains unresolved. Alternatively, the low egg yield in our experiment could have been caused by suboptimal conditions in captivity, which were unsupportive of the fish's overall reproductive performance. For example, egg production in laboratory killifish cultures is closely related to the quantity and quality of food (e.g., Blažek *et al*. 2013; Papa *et al*. 2015). Although our experimental *A. ornatus* was fed at a rate (three times a day) which was higher than what is usually recommended for laboratory killifishes (twice a day or less, e.g. Podrabsky 1999; Žák *et al*. 2020), it is still possible that the fish were underfed. Many killifish species require large doses of natural, live food to reach maximum output in terms of growth and reproduction and do not perform optimally when forced to accept artificial fish food (e.g. Papa *et al*. 2015; Žák *et al*. 2020). Apart from nutrition, which is the most obvious factor influencing reproductive performance, other factors, such as water quality and temperature, may also have played a role, given that there was considerable variation in water parameters in the natural environment, as shown in the data presented above. Further follow-up field and laboratory studies are needed to determine the optimal ecological niche of *A. ornatus*.

The incubation period of *A. ornatus* eggs, from spawning to hatching, ranged from 16 to 75 days (Table 1), indicating a more than four-fold difference between the shortest and longest durations of development in individual embryos. Significant variation in the length of embryonic development under constant environmental conditions (here 26°C) is characteristic, especially for the semi-annual and annual killifish species. Any diapause entry presents potential for profound variability in the time ultimately taken for the completion of embryonic development (e.g., Wourms 1972; Borisov *et al*. 2023). The diapause in killifish is a physiological adaptation to marginal water habitats (pools) that undergo periodic desiccations, a phenomenon also observed in our population of *A. ornatus* from the Tarumã-Açu/Tarumã-Mirim sector near Manaus. The marginal pools used by this species are formed in the vicinity of the streams in the rainy season, when the stream margins experience a period of inundation that extends for approximately six months, followed by a period of desiccation in the absence of precipitation. Consequently, the embryonic development of *A. ornatus* is likely to involve a developmental halt, either in the form of a diapause II type typical for annual killifish (e.g. Wourms 1972) or the species may be showing the "delayed hatching" dormancy type, characteristic for semi-annuals (e.g. Wourms 1972; Borisov et al. 2023). However, our experimental approach did not involve detailed recordings of the developmental pace. A specialized, dedicated study is needed to characterize the precise mode of embryonic development in *A. ornatus*.

Fertilization rate and hatching success varied to some extent among the four parental pairs (Table 1), but the recorded values were generally comparable to literature data obtained on other killifish species in captivity (e.g. Podrabsky 1999; Shibatta 2005; Vrtílek *et al*. 2017).

Experimental fish strongly preferred (P = 0.041) black material mops (44 clutches) over the green mops (9 clutches), with nearly five times more eggs deposited in the former. A similar behavior was also observed in the African species *Aplocheilichthys spilauchen*, which laid about twice as many eggs into a black mop compared to green, blue, and white mops (Okyere *et al*. 2021). Annually, bottom-spawning killifish also prefer a black spawning substrate over paler options (Thoré and Merckx 2023), suggesting that the tendency to oviposit in the darkest substrate available is a general trait in killifish.

For the first time, we surveyed basic reproductive traits of *A. ornatus*. The results of this study reveal previously unknown aspects of the reproductive biology of *A. ornatus*, suggesting that its low fertility may be related both to the natural characteristics of the species and to the limitations of captivity, such as inadequate nutrition or stress. The substantial variation in incubation time suggests the potential for the implementation of strategies such as diapause or delayed hatching, which have been observed in species adapted to temporary habitats.

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