# Reproductive Strategy of the Annual Fish *Leptopanchax Opalescens* (Rivulidae) and Trade-Off Between Egg Size and Maximum Body Length in Temporary Wetlands

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## Abstract

*Leptopanchax opalescens* is a critically endangered small annual fish. Reproductive traits of this species were studied to improve our understanding of the strategies that facilitate the occupation of temporary wetlands. We compiled egg diameter and maximum total length data from 136 neotropical killifishes (Rivulidae) to establish comparisons between species with different life histories. We tested the hypothesis that annual killifishes have smaller body sizes and eggs than non-annual killifishes, which may be associated with different life spans and embryonic diapause. Fish were collected from the Guandu River Hydrographic Region (southeastern Brazil). DNA barcoding was employed to confirm the species' identity. The phases of gonadal development and spawn type were described using histological techniques. Egg size and fecundity were determined. Females with batch spawning and males with continuous spawning were detected. The batch fecundity ranged from 22 to 32 vitellogenic oocytes (mean $27 \pm 7$ SD). Maximum body size was similar between the two life cycles ($p = 0.24$), but egg size was smaller for annual killifishes ($p < 0.001$). Spawning in batches, synchronous modal development of oocytes, continued production of sperm in males, and a complex process of embryonic diapause are reproductive traits that favor the resilience of *L. opalescens* and other annual fish in temporary wetlands. We conclude that body size is not related to lifespan and that factors underlying the selection of different egg sizes between annual and non-annual killifish species may be associated with different life history strategies to deal with stressful habitats.

**Keywords** Seasonal fish · Freshwater ecosystems · Killifish · Offspring size · Cyprinodontiformes

## Resumo

*Leptopanchax opalescens* é um pequeno peixe anual criticamente ameaçado. Características reprodutivas desta espécie foram estudadas para compreender as estratégias que facilitam a ocupação de áreas úmidas temporárias. Compilamos dados do diâmetro de ovo e comprimento total máximo de 136 rivulídeos neotropicais para estabelecer comparações entre espécies com diferentes histórias de vida. Testamos a hipótese de que rivulídeos anuais apresentam tamanho corporal e ovos menores do que rivulídeos não anuais, o que pode estar associado a diferentes tempos de vida e à diapausa embrionária. Peixes foram coletados na Região Hidrográfica do Rio Guandu (sudeste do Brasil). DNA foi empregado para confirmar a identidade da

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espécie. Fases do desenvolvimento gonadal e tipo de desova foram descritos com técnicas histológicas. O tamanho do ovo e fecundidade foram determinados. Foram detectadas fêmeas com desova em lotes e machos com produção contínua de esperma. A fecundidade do lote variou entre 22–32 ($27 \pm 7$) ovócitos vitelogênicos. O tamanho corporal máximo foi semelhante entre os rivulídeos ($p = 0.24$), mas o tamanho dos ovos foi menor para espécies anuais ($p < 0.001$). Desova em lotes, desenvolvimento modal síncrono dos ovócitos, produção contínua de esperma em machos, e um complexo processo de diapausa embrionária são características reprodutivas que favorecem a resiliência de *L. opalescens* e outros peixes anuais em áreas úmidas temporárias. Concluímos que o tamanho do corpo não está relacionado ao tempo de vida, e que fatores subjacentes a seleção de diferentes tamanhos de ovos entre espécies anuais e não anuais podem estar associados a diferentes estratégias de história de vida para lidar com habitats estressantes.

## Introduction

Rivulidae (Cyprinodontiformes) is the eighth most speciose fish family in the World, with 471 valid species (Fricke et al. 2022), and ranks first in the number of threatened species in the Neotropical Region, where they occur. The threat is such that in a megadiverse country like Brazil, a single family of freshwater fish (i.e., Rivulidae) has a higher number of threatened species ($\approx 130$ spp.) than all classes of marine fish combined (Actinopterygii + Chondrichthyes + Myxini = 97 spp.) (ICMBio 2018). Habitat loss is the main threat to rivulids. The swamps and temporary wetlands where many of these animals inhabit have been drastically destroyed, due to deforestation, drainage, and landfilling resulting from agricultural and urban expansion (Loureiro et al. 2018; Castro and Polaz 2020). Loss of temporary wetlands from land alterations has significant ecological and social consequences (Calhoun et al. 2017), but freshwater research, policy, and conservation have historically neglected these habitats, focusing instead on larger water bodies and flagship species (Junk et al. 2014; Grasel et al. 2018; Hill et al. 2021).

Rivulids exhibit a variety of reproductive strategies, some of them unique among vertebrates. The genus *Kryptolebias* Costa 2004 for example, includes the only two self-fertilizing hermaphroditic vertebrates capable of generating homozygous clones (Earley et al. 2012). About 55% (259 spp.) of rivulid species are annual (or seasonal) fishes, with their life history linked to the hydrological cycle. In the rainy season, these fish copulate or fertilize the eggs directly in the substrate (Berois et al. 2016). In the dry season, their habitats dry up, leading to the death of all fish. Cyclic desiccation of habitats selected short life cycles (Žák et al. 2021) and complex embryonic diapause processes (Furness 2016),

in which eggs await specific environmental cues to hatch in the next rainy season (Polačik et al. 2021). Some eggs are resistant enough to continue their development even after spending more than 30 h inside a bird's digestive system (Silva et al. 2019). These peculiarities in the life cycle can lead to the absence of overlapping generations, i.e., the progenitors and offspring do may not coexist.

In recent decades, studies on the reproductive biology of annual rivulids addressed aspects such as reproductive behavior (e.g., Berois et al. 2016; Evangelista et al. 2021), period and size at the onset of maturity (Arenzon et al. 1999), batch fecundity (Gonçalves et al. 2011; Volcan et al. 2011), oogenesis and spermatogenesis (Arezo et al. 2007), eggs size and surface morphology (Thompson et al. 2017). Recently, Domínguez-Castanedo et al. (2022) first observed functional hermaphroditism in an annual killifish. Although the aforementioned studies have made important contributions to the field, basic reproductive aspects are still unknown for most annual fish species. The genus *Leptopanchax* Costa (2016), for example, comprises six valid species endemic to the Atlantic Forest biome (Costa 2016). Four out of the six species are listed as Critically Endangered (CR) in the Red Book of Brazil's Endangered Fauna (ICMBio 2018), including one possibly extinct species (Costa 2019). Information on the reproductive traits of *Leptopanchax* species is minimal and diffuse (e.g., Costa and Leal 2009; Guedes et al. 2020). The scarcity of reproductive information on *Leptopanchax* species and other annual rivulids is a bottleneck that makes it difficult for conservation strategies of species in imminent threat of extinction.

Offspring size has been used as a predictor of fitness and parental energy investment in species with different life histories (e.g., Winemiller and Rose 1992; Marshall et al. 2018; Zandona et al. 2021; Iglesias-Rios et al. 2022). Optimal egg size theory suggests that populations evolve egg size balancing the trade-off between egg size and fecundity in order to maximize reproductive yield (Smith and Fretwell 1974). A relevant and little-discussed issue is that different species may have similar egg diameters and batch fecundity (Vazzoler 1996), but differ drastically in other biological traits, including maximum body weight and length. The body size is a predictor of the availability of energy to invest in reproduction (Barneche et al. 2018a). Therefore, the biological significance of egg size and fecundity for one species may not necessarily be the same for another (Bonisławska et al. 2001), especially with regard to the trade-off between the allocation of reproductive energy vs. somatic growth. One approach to this question is to balance the absolute fecundity by the length or weight of the individual (Wotton and Smith 2015; Rizzo and Bazzoli 2020). However, in the case of eggs size, the approach with absolute diameters remains dominant (e.g., Duarte and Alcaraz 1989; Feiner et al. 2016; Barneche et al. 2018b; Marshall et al. 2018; Iglesias-Rios et al. 2022), which is a useful strategy to differentiate reproductive

traits between species, but which can mask the reproductive energy investment in eggs in species that show different patterns of somatic growth investment.

We studied the reproductive traits of *Leptopanchax opalescens* (Myers, 1942) to determine the strategies that facilitate the occupation of habitats with extreme hydrological variations, such as temporary wetlands. Spawn type, phases of gonadal development, fecundity, egg size, microanatomy of the zona pellucida, and molecular information (DNA barcoding *sensu* Hebert et al. 2003a) are provided for the first time for this species. Additionally, we compiled mean oocyte diameter (egg size) and maximum total length (TLmax) data from 136 species of Neotropical killifishes (Rivulidae) as a proxy to compare the trade-off between the allocation of reproductive energy vs. growth between species with distinct life histories. We tested the hypothesis that killifishes (Rivulidae) with distinct life histories (annual vs. non-annual) have different egg sizes and maximum body length. We expect that: (1) annual fish will have smaller body sizes due to a short lifespan and limited by the hydrological cycle (Žák et al. 2021); (2) Non-annual fish have larger egg sizes due to the absence of embryonic diapause and the consequent lower tolerance to hypoxia (Podrabsky et al. 2007), which could be offset by the increase in egg size (Einum et al. 2002).

## Methods

### Fish Sampling

The study area comprises a temporary pool located in the Guandu River Hydrographic Region (22°42.35'S, 43°41.59'W), a coastal drainage of the Sepetiba Bay (State of Rio de Janeiro, Brazil). Fish samplings were carried out between November 2019 and June 2021. Fish were collected with a hand net with an oval shape (50 × 40 cm, 1 mm mesh panel). Specimens were anesthetized with hydrochloride benzocaine (50 mg/l), euthanized in situ, and fixed in 10% formalin (for morphology) or anhydrous ethanol (for DNA sequencing). *Leptopanchax opalescens* (Fig. 1) is rare, threatened with extinction, and protected by national laws, so we chose to use a maximum of 20 specimens for reproductive and DNA analysis. The specimens were collected under license of IBAMA/ICMBio (authorization #10,707) and vouchers were deposited in the Ichthyological Collection of the Fish Ecology Laboratory of the Universidade Federal Rural do Rio de Janeiro (LEP-UFRRJ 2576–2578; gbif.org/occurrence/2,628,729,394) and in the Ichthyological Collection of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ 51,967; 52,701).



**Fig. 1** Female and male of *Leptopanchax opalescens* from Guandu River Hydrographic Region (coastal drainages of the Sepetiba Bay, State of Rio de Janeiro, Brazil). Scale bar: 5 mm

## DNA Barcoding

Muscular tissue from specimens ($N = 4$) preserved in anhydrous ethanol was processed for DNA extraction following the salting out method (Miller et al. 1988). DNA quality was verified with standard agarose gel electrophoresis, and DNA concentration was measured with a NanoDrop ND-2000 spectrophotometer. Partial sequences of the Cytochrome Oxidase Subunit I (COI) gene were amplified with Polymerase Chain Reaction (PCR) using primers FishF6notail: 5'-ACYAAY CACAAAGAYATTGGCA-3', and FishR7notail: 5'-TAR ACTTCTGGRTGDCCRAAGAAYCA-3' (Jennings et al. 2019). The PCR protocol used the following thermal cycle: denaturation at 94 °C/30 s, primer hybridization at 49 °C/45 s, and nucleotide extension at 72 °C/45 s; this sequence was repeated 35 times in a loop, and the reaction was terminated with 72 °C/10 min and 4 °C/5 min. Quality of amplified products was verified with 2% agarose-gel electrophoresis. PCR products were purified using Exo-SAP (Handy et al. 2011) or PEG (Lis 1980; Jennings 2017), and bidirectionally sequenced with the Sanger method, where a BigDyeTM Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems) was used for labeling, purified again with ethanol precipitation, and loaded on an automatic sequencer ABI3730xl (Applied Biosystems) at Fundação Oswaldo Cruz. The resulting chromatograms were aligned to a reference sequence using the GENEIOUS v.6 software (geneious.com) and manually edited to ensure codon alignment and to verify base calls. Sample details are listed in Online Resource 1, along with GenBank Accession codes. Sequences were aligned with Muscle (Edgar 2004) under default parameters in Mega X (Kumar et al. 2018). Calculation of genetic distances were performed with Mega X under Kimura 2-parameter (K2P) model (Kimura 1980)

following Pereira et al. (2013). Variance estimations were obtained by the analytical method available in Mega X.

## Reproduction Traits

Specimens ($N = 14$) were decalcified whole in a 7% EDTA solution (ethylenediamine tetraacetic acid) for 7 days. Subsequently, they were submitted to the remaining steps (1 h each) of routine histological processing that included: dehydration (series of ethanol in increasing concentrations − 70° GL to 100° GL), diaphanization in xylene, impregnation, and inclusion in paraffin-Histosec (Merck, Germany) at 60ºC to obtain serial histological sections of 5 µm thickness in an automatic microtome (RMC Boeckeler - MR3). Histological sections were stained with Harris Hematoxylin and Eosin (HE) and then dehydrated in an increasing alcohol series and cleared in xylene. Histological slides were mounted with Entellan® (Merck). The maturation phases, spawn type, cells of oogenesis and spermatogenesis lineage were identified and described following the terminologies proposed by Brown-Peterson et al. (2011).

To assess fecundity, ovaries from spawning capable females ($N = 2$) were removed from the visceral cavity, weighted, and kept in Gilson's solution until complete detachment of oocytes from epithelial and ovarian follicles. All eggs were counted and measured (diameter, in µm) in a Sedgewick-Rafter counting camera. Microanatomy of the zona pellucida was examined under scanning electron microscopy (Hitachi TM3000). The batch fecundity (BF), i.e., the number of eggs produced in a single spawning batch, was established from the counting of vitellogenic oocytes (Rizzo and Bazzoli 2020). The spawning type was inferred based on the distribution of the modes of oocyte diameters, and on the oocyte developmental stages and the presence of post-ovulatory follicles and atretic oocytes (Brown-Peterson et al. 2011; McBride et al. 2015).

## Trade-off Between Egg Size and Maximum Body Length

We compiled a dataset of mean egg diameter and maximum total length (TLmax) based on the literature of 136 killifishes (90 annual; 46 non-annual) of the family Rivulidae (e.g., Thompson et al. 2017; Eckerström-Liedholm et al. 2017; Online Resource 2). Species were categorized into different life history strategies (annual and non-annual), according to their ability to perform long-term embryonic diapause (Loureiro et al. 2018). For species in which egg diameter was given as a range, the midpoint was used. Some references had the maximum standard length (SLmax) instead of the maximum total length (TLmax). In these cases, and for standardization, we inserted lateral photographs of the species acquired in Fishbase (Froese and Pauly 2022) in

the ImageJ (v. 1.8) software to determine the ratio between SL and TL. From this ratio, we corrected the SLmax to an estimated TLmax. To avoid the occurrence of synonym in our database, we check the species names following the Eschmeyer Fish Catalog (Fricke et al. 2022).

## Statistical Analysis

Comparisons of maximum body size (TLmax) between annual and non-annual fish were performed with a Wilcoxon-Mann-Whitney test ($p < 0.05$) using the package 'ggstatsplot' (Patil 2021). To determine whether egg size (dependent variable) varies between the life cycle (independent variable), a generalized linear mixed model (GLMM) assuming a gaussian error distribution (*logit* function) was used. The model's fixed factor was the life cycle (2 levels: annual; non-annual), while the TLmax ($\log_{10}$) nested in the fish genus was used as a random factor to account for possible allometric and phylogenetic effects. An ANOVA (Type II Wald chi-square test) was used to test differences between the categories of the independent variable at the 0.05% level. TLmax was lagoratimized to meet the model's parametric assumptions. Normality ($W = 0.98$, $p = 0.058$) and homogeneity of variance ($F = 0.19$; $p = 0.668$) of the model were checked using Shapiro-Wilk and Levene's tests, respectively. Residual diagnoses were checked using the *simulateResiduals* function in the *DHARMa* package (Hartig 2022). All analyses were conducted in an R environment (R Core Team, 2022).

## Results

### DNA Barcoding and Species Confirmation

We generated four sequences of 656 base pairs (bp) of the barcode region of COI, all identical. This unique haplotype of *Leptopanchax opalescens* was aligned to homologous haplotypes of *L. aureoguttatus* (Cruz, 1974), and *L. citrinipinnis* (Costa, Lacerda and Tanizaki, 1988), the only species of *Leptopanchax* with sequences made available in GenBank to date (Table 1 and Online Resource 1). The sequences in the alignment were reduced to 536 bp to avoid missing data in the genetic distance analysis. The pairwise K2P distances among these species varied from 11.0 to 21.8% and showed that *L. opalescens* is more related to *L. citrinipinnis* than *L. aureoguttatus* (Table 1).

### Histology of Ovaries

The ovaries are of a cavity cystovarian type, i.e., with the ovigerous lamellae projecting inwards from the ovarian wall into the lumen (Fig. 2). Different cell stages of the oogenic

**Table 1** Pairwise estimates of evolutionary sequence divergence (K2P genetic distance) among the three (of six) species of *Leptopanchax* sequenced to date. COI haplotypes correspond to those listed in Online Resource 1. Distances are given as percent (%) below the diagonal. Variance estimation is shown above the diagonal

|  | *L. aureoguttatus* | *L. citrinipinnis* | *L. opalescens* |
|---|---|---|---|
| *L. aureoguttatus* |  | 0.023 | 0.022 |
| *L. citrinipinnis* | 21.8% |  | 0.015 |
| *L. opalescens* | 20.3% | 11.0% |  |

lineage were identified, and characterized the presence of four phases (immature, developing, spawning capable and regressing) of ovarian development. The immature phase was characterized by the exclusive presence of oogonia, primary growth, and cortical alveolar oocytes (Fig. 2a). The developing phase presented oocytes in different phases of development as cortical alveolar, and early vitellogenic oocytes (primary - Vtg1) and in secondary growth (Vtg2; Fig. 2b, c). The spawning capable phase predominantly had vitellogenic oocytes with a nucleus evident and layers of the follicular envelope as the basal membrane (BM) and small microvilli (MV) of zona radiata (Fig. 2d). Oocytes in advanced stages of development have mushroom-like projections and polygonal grooves in the zona pellucida, with a single projection at the center of each polygon (Fig. 3). The regressing phase showed disorganized structure with the presence of postovulatory follicles and atresia, characterized by the disappearance of the zona radiata in degenerating and resorbing oocytes (Fig. 2h, i). The presence of different cellular stages of the oogenic lineage between the different stages of maturation reveals that the reproductive cycle of females is discontinuous and spawning in batches.

## Histology of Testes

Spermiogenesis follows a cystic model in the seminiferous tubules, with the germinal epithelium is continuous in peripheral cysts and discontinuous in the opening to the lumen (Fig. 4). Different cell stages of the spermatogenic lineage were identified, and characterized the presence of four phases (immature, developing, spawning capable, regressing) of testes development. The immature phase was characterized by prevalence of primary (Sg1) and secondary spermatogonia (Sg2) with early formation of seminiferous tubules (Fig. 4a). The developing phase was characterized by presence of all cells of the spermatogenic lineage (Sg1, Sg2, Sc1, Sc2, St and Sz), besides the formation of lumen and cysts undergoing active spermatogenesis (Fig. 4b, c). The spawning-capable phase consisted of spermatozoa (Sz) in the lumen of the lobules of the seminiferous tubules and spermatids cysts in which the wall is thicker; spermatogonias (Sg1, Sg2), spermatocytes (Sc1, Sc2) may be present

(Fig. 4d). The regressing phase was identified by reduced stores of Sz in the lumen of disorganized lobules (Fig. 4e, f). This leads us to characterize the cycle as continuous with splitted spermiation.

## Fecundity

The total number of oocytes present in the gonads (regardless of the stage of development) ranged from 91 to 99 (mean $95 \pm 5.65$ SD) in females of 23.0 and 24.5 mm TL (Table 2). The diameter of oocytes ranged from 75 to 1,050 mm ($402.7 \pm 224$ mm). The batch fecundity (only vitellogenic oocytes) ranged from 22 to 32 ($27 \pm 7$), corresponding to oocytes with diameter > 500 mm (Table 2). Table 2 summarizes reproductive traits of *L. opalescens* resulting from this and other studies. *Leptopanchax opalescens* exhibited a high sex ratio for females (2.8:1; Guedes et al. 2020), and males reach higher mean total length ($20.4 \pm 4.1$ mm TL).

## Trade-Off Between Egg Size - Maximum Body Length

The egg size and maximum total length (TLmax) of the 90 annual killifishes varied from 0.52 to 2.2 ($1.26 \pm 0.47$ mm) and from 20 to 220 mm ($62.2 \pm 30.3$ mm TL), respectively. The 46 non-annual killifishes ranged from 0.91 to 2.2 mm ($1.67 \pm 0.26$ mm) and from 34 to 100 mm ($61.6 \pm 15.3$ mm TL). The maximum body size of killifish did not show significant differences (Wilcoxon-Mann-Whitney; Fig. 5a; $p = 0.24$) between life cycle, while egg size was smaller in annual killifish (GLMM, $\chi 2 = 26.4$; $p < 0.001$; Fig. 5b).

## Discussion

This study compiles evidence that reproductive traits favor the resilience of the annual fish *Leptopanchax opalescens* in habitats with extreme cyclical hydrological variations, such as temporary wetlands. Spawn type, stages of gonadal development, and reproductive characteristics (fecundity) have not been previously reported for species of the genus *Leptopanchax*. In addition, we found that the maximum body size did not differ between the type of life cycle (annual vs. non-annual) and that these different strategies to deal with the stressful environment may affect the egg size.

### DNA Barcoding

The genus *Leptopanchax* currently comprise six valid species (Costa 2019), but only a few genes of *L. aureoguttatus*, and *L. citrinipinnis* have been sequenced to date for evolutionary studies of Rivulidae (Murphy and Collier
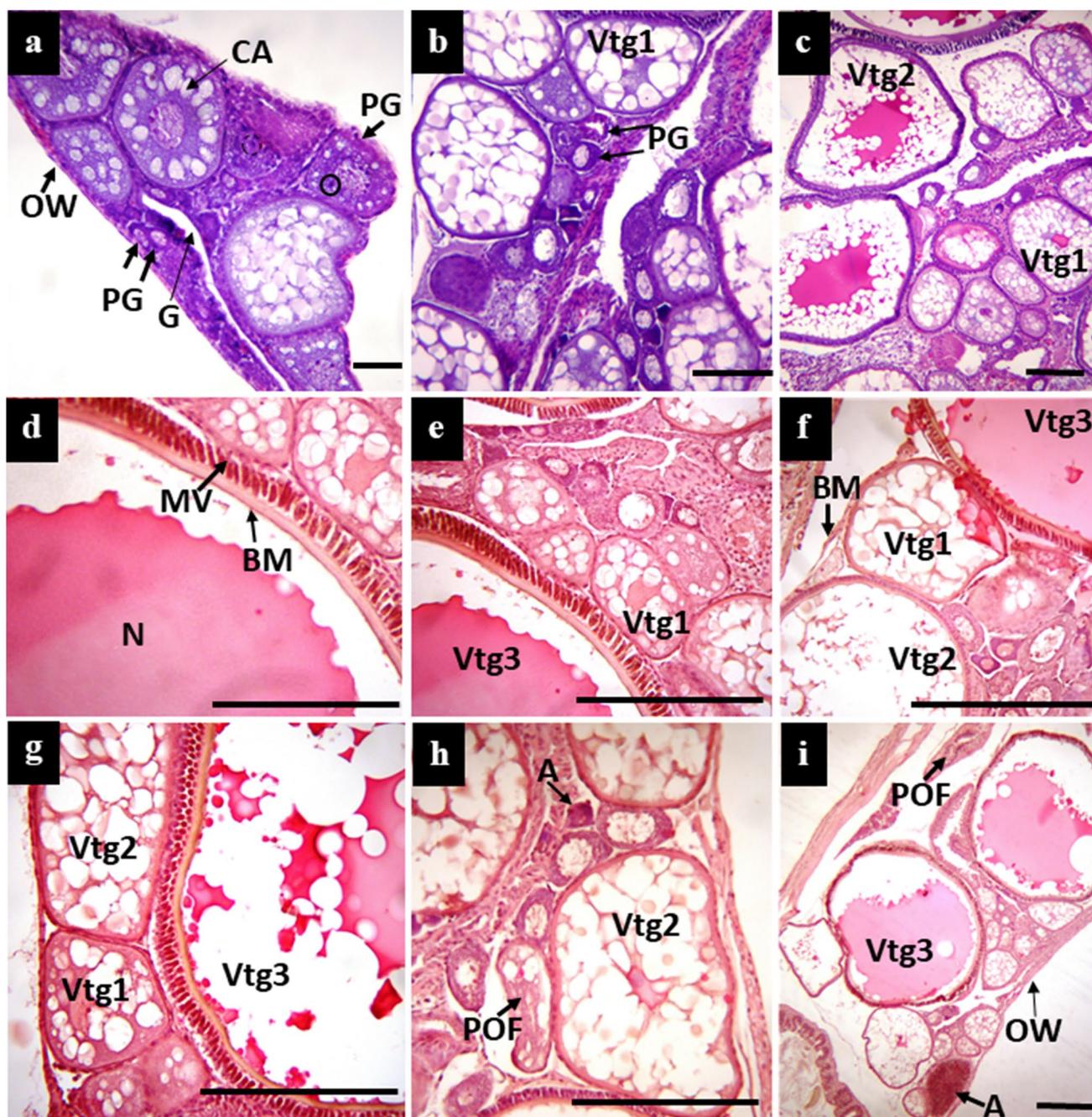
**Fig. 2** Images of ovaries of *Leptopanchax opalescens* in different phases of gonadal maturation: Immature (**a**), Developing (**b**, **c**), Spawning capable (**d**, **e**, **f**, **g**), Regressing (**h**, **i**). Codes: Atresia (A), primary growth (PG), ovarian wall (OW), gonoduct (G), cortical alveolar (CA), primary vitellogenic (Vtg1), secondary vitellogenic (Vtg2), tertiary vitellogenic (Vtg3); nucleus (N), basal membrane (BM), small microvilli (MV), Postovulatory follicle (POF). Staining: haematoxylin and eosin (HE). Scale bar: 100 μm

1997; Murphy et al. 1999; Costa et al. 2016). In addition to their use in phylogenetic studies, these genes may be useful to delimit *Leptopanchax* species through DNA barcoding methods (Hebert et al. 2003a), as an alternative to the use of coloration of living males as the main diagnostic feature (Costa 2019). To reduce the molecular information

gap in *Leptopanchax*, here we sequenced samples of *L. opalescens* from Guandu River Hydrographic Region.

Specimens with COI genetic distances less than 2% are considered conspecific following DNA barcoding standards for fish (Hebert et al. 2003a, b; Pereira et al. 2013). Following this criterion, the comparison of sequences available so

**Fig. 3** Unfertilized eggs of *Leptopanchax opalescens*. Note (i) modal development of oocytes; (ii) formation of mushroom-like projections and polygonal grooves in the zona pellucida. Scale bar: 100 mm
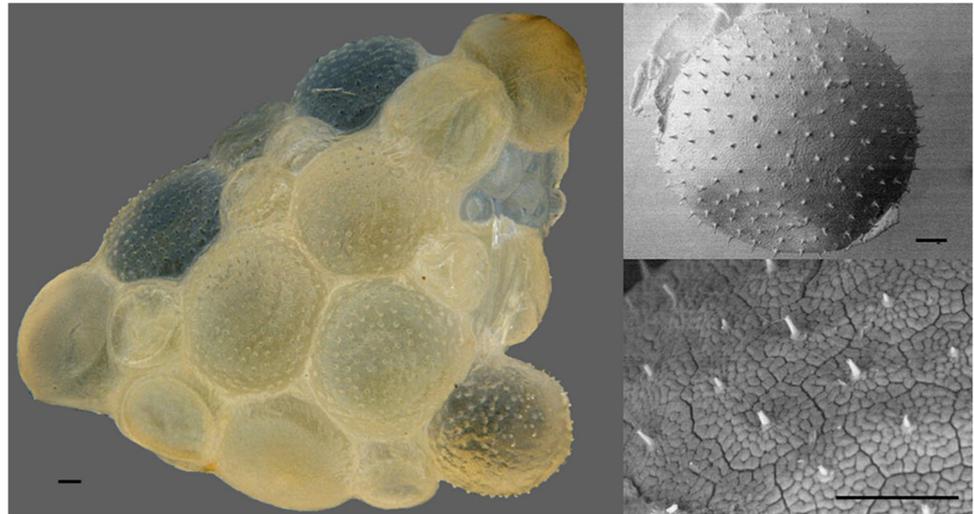
far supports the morphological hypothesis that *L. aureoguttatus*, *L. citrinipinnis*, and *L. opalescens* are distinct species, despite the coloration similarity and geographic proximity of *L. opalescens* and *L. citrinippinis* (Costa 2019). The specimens of *L. opalescens* used in the present study are from the Sepetiba Bay drainage, while previous samples associated to this taxon are from the Guanabara Bay drainage. Morphometric and meristic data from the *L. opalescens* populations are overlapped between these different basins (Guedes et al. 2020), but the populations show small color variations difficult to analyze by photographs taken with different parameters (Costa 2019; Souto-Santos et al. 2021). Therefore, it is desirable that specimens from the type locality of *L. opalescens* (or at least from some locality associated with Guanabara Bay) be sequenced and compared with the Sepetíba sequences made available here.
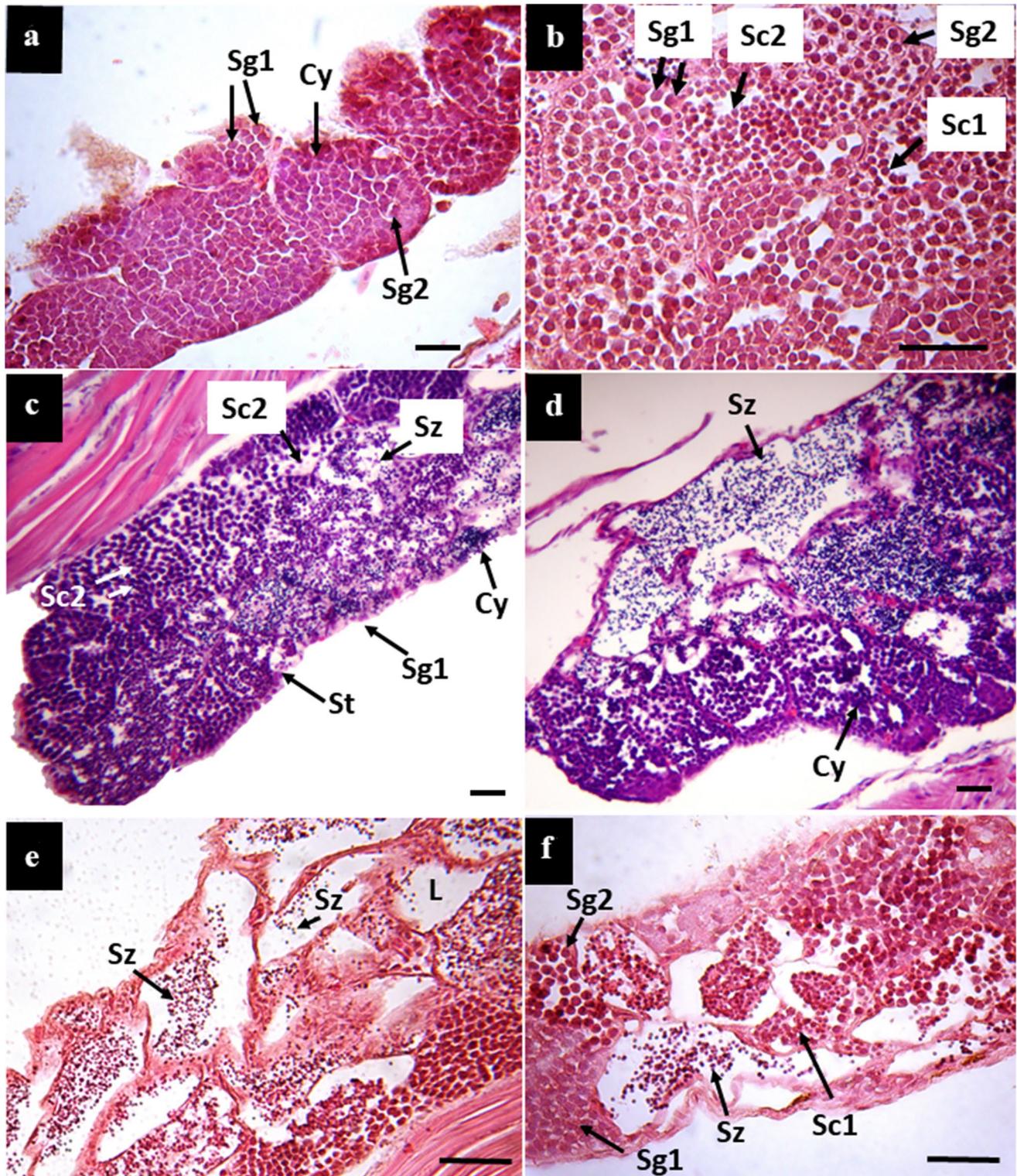
## Spawn Type and Phases of Maturation

The reproductive cycle in females of *L. opalescens* is discontinuous and spawning in batches, indicated by the presence of oocytes at different stages of development and post-ovulatory follicles in specimens capable of spawning. Similar histological results were observed for other rivulids, such as the annuals *Austrolebias charrua* (Arezo et al. 2007), *Millerichthys robustus* (Domínguez-Castanedo et al. 2017), and non-annuals *Melanorivulus aff. punctatus* (Cassel et al. 2013) and *Atlantirivulus riograndensis* (Cavalheiro and Fialho 2015). According to the scale of gonad development proposed by Brown-Peterson et al. (2011), L. *opalescens* has four stages of the reproductive cycle: immature, developing, spawning capable, and regressing. The regressing phase showed disorganized structure with presence of postovulatory follicles and atresia, characterized by the disappearance of the zona radiata in degenerating and resorbing oocytes.

However, the regressing phase did not represent the interruption of the reproductive cycle due to the simultaneous presence of oocytes capable of spawning.

In males, the histological organization of the testes corresponds to the restricted lobular pattern because spermatogonia are confined to the distal end of the lobules (Uribe et al. 2015). Restricted spermatogonial testis type is characteristic of all Atherinomorpha (which includes Cyprinodontiformes) and is considered a feature of more derived taxa (Parenti and Grier 2004). Reproduction of *L. opalescences* was characteristic of species with a continuous cycle and splitted spermiation, due to the consistent presence of sperm capable of being ejaculated in the mature phases. A continued production of sperm has also been reported for other rivulids, such as *Millerichthys robustus* (Domínguez-Castanedo and Uribe 2019), *Melanorivulus aff. punctatus* (Cassel et al. 2013) e *Austrolebias charrua* (Arezo et al. 2007). These characteristics are important in the ecological context, as males will be able to readily release sperm for fertilization whenever females produce new batches of eggs.
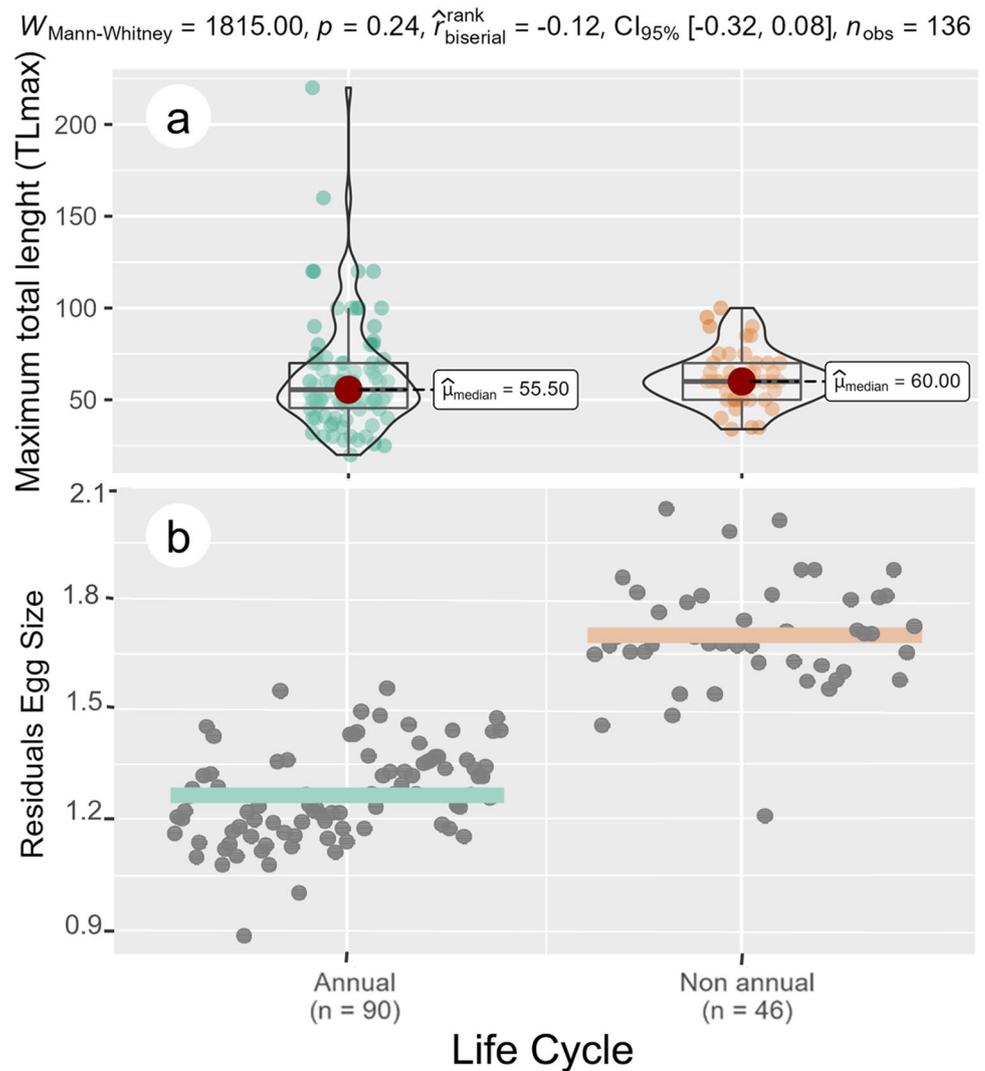
*Leptopanchax opalescens* inhabit temporary wetlands that experience abrupt variations in their areas due to fluctuations in water level (Guedes et al. 2020; Souto-Santos et al. 2021). In these variable and unpredictable habitats, spawning in batches means that during the beginning of the flooding period and at the end of the wet season, egg deposition must occur closer to the center of the swamps, while in the period of maximum flooding, egg deposition may occur in areas more away from the center, i.e., in the water-fluctuating ecotones. Therefore, spawning in batches in habitats in continuous expansion and retraction may determine an asynchrony in time under embryonic diapause within the population, consequently causing an asynchrony in the hatching of eggs of the next generation of annual fish. According to Lowe-McConnell (1987), asynchrony in offspring development

**Fig. 4** Images of testes of *Leptopanchax opalescens* in different phases of maturation. Immature (**a**), Developing (**b**, **c**), Spawning capable (**d**), Regressing (**e**, **f**). Code: primary spermatogonia (Sg1), secondary spermatogonia (Sg2), cysts (Cy), primary spermatocyte (Sc1), secondary spermatocyte (Sc2), spermatid (St), spermatozoa (Sz), lumen (L). Staining: haematoxylin and eosin (HE). Scale bar: 50 μm

**Fig. 5** Comparisons of the maximum body length (**a** Wilcoxon-Mann-Whitney); egg size partial residuals (**b** GLMM-generalized linear mixed models) of killifishes between different life cycles (annual vs. non-annual). Boxplots represent the interquartile range of the distribution, with the red circle representing the mean. The overlay fiddle plot represents the density of data at each value, with wider sections indicating a greater density of observations

$W_{\text{Mann-Whitney}} = 1815.00$, $p = 0.24$, $\hat{r}_{\text{biserial}}^{\text{rank}} = -0.12$, $\text{CI}_{95\%}$ [-0.32, 0.08], $n_{\text{obs}} = 136$



## Egg Size and Zona Pellucida Morphology

*Leptopanchax opalescens* eggs reached a maximum diameter of 1050 µm, similar to that observed for other *Leptopanchax* species (maximum 1005 µm; Costa and Leal 2009), and within the diameter range (688–2104 µm) observed for another 60 annual or non-annual killifishes (Thompson et al. 2017). Determining why annual species have eggs with different sizes is not a trivial task. Congeneric annual species of the family Nothobranchiidae can coexist in the same pools but may present eggs with significantly different sizes (Reichard 2016). Fish can exhibit great variability in egg size among species, and between and within populations. Parental care, environmental quality, fecundity, temperature,

increases the chance of survival by reducing potential predation on offspring and intraspecific competition for food and shelter.

latitude, predation, resource availability, size, and age of females are some factors that, isolated or in interaction, have been identified as influencing egg size in fish (e.g., Duarte and Alcaraz 1989; Einum et al. 2002; Vrtílek and Reichard 2015; Feiner et al. 2016; Barneche et al. 2018b; Iglesias-Rios et al. 2022).

The zona pellucida of mature eggs of *L. opalescens* featured mushroom-like projections similar to other species in the genera *Leptopanchax* and *Notholebias* (Costa and Leal 2009; Thompson et al. 2017). *Leptopanchax opalescens* presented a single mushroom-like projection in the centre of each polygonal groove, identical to *L. citrinipinnis* (Costa and Leal 2009). This corroborates the genetic similarity (DNA barcoding) between these two species in this study. Wourms and Sheldon (1976) hypothesized that these mushroom-like projections on the zona pellucida of *Notholebias* constitutes a chorionic respiratory system since there is a network of channels leading to hollow spikes that may

**Table 2** Summary of reproductive traits of *Leptopanchax opalescens*, Minimum–Maximum (Mean ± standard deviation)

| Reproductive traits | Sex | Description | Source |
|---|---|---|---|
| 1. Sexual dimorphism | | | |
| 1.1 Chromatic | | | Guedes et al. 2020 |
| | Males | Very striking color, well-delimited dark-red stripe on distal margins of dorsal and anal fins | |
| | Females | Light brown body and hyaline fins | |
| 1.2 Size | | Mean size of males was significantly larger than females | Guedes et al. 2020 |
| | Males | 13.0–29.5 (20.4 ± 4.1) mm TL | present study |
| | Females | 11.0–32.0 (18.8 ± 3.8) mm TL | present study |
| 2. Sex ratio | | 2.8 female: 1 male | Guedes et al. 2020 |
| 3. Fertilization | | External | Costa (2008) |
| 4. Development | | External | Costa (2008) |
| 5. Type sparning | | | |
| | Female | Batch spawners | present study |
| | Male | Continuous | present study |
| 6. Parental care | | No care | present study |
| 7. Eggs | | | |
| 7.1 Total number | | 91–99 (95 ± 5.65) | present study |
| 7.2 Total diameter | | 75–1050 (402 ± 224) μm | present study |
| 7.3 Vitelogenic diameter | | 500–1050 (700 ± 225) μm | present study |
| 7.4 Oocyte Development | | Synchronous modal | present study |
| 7.5 Zona Pellucida | | Mushroom-like projections and polygonal grooves | present study |
| 8. Fecundity | | | |
| 8.1 Batch (BF) | | 22–32 (27 ± 7.0) | present study |
| 9. Strategy | | Iteroparous | present study |
| | | Income breeding (*sensu* McBride et al. 2015) | present study |

function as egg-like aeropiles, similar to insect eggs. This may be an adaptation for annual fishes since a thick, hard, and consequently poorly oxygen-permeable zona pellucida may be necessary to prevent desiccation (Thompson et al. 2017).

## Fecundity

*Leptopanchax opalescens* showed reduced batch fecundity (27 ± 7.0 eggs), like other annual species, such as *Cynopoecilus melanotaenia* (19 ± 26 eggs; Gonçalves et al. 2011) and *Austrolebias nigrofasciatus* (21.5 ± 12 eggs; Volcan et al. 2011). Although our fecundity results are similar to other annual fish species mentioned above, our sample number is small, and may not express the range of variability within and between populations of *L. opalescens*. Fecundity is a currently known reproductive parameter for less than 10 species (e.g., Volcan et al. 2011; Gonçalves et al. 2011; Schalk et al. 2014; Cavalheiro and Fialho 2015) among the 471 species of Rivulidae that occur in the Neotropical Region. Optimal egg size theory suggests that populations evolve a particular egg size that balances the trade-off between egg size and fecundity to maximize reproductive

yield (Smith and Fretwell 1974). In other words, larger eggs come at a cost of reducing the number of eggs, which is supported by the findings of this study. In addition to batch fecundity, it is necessary to consider the number of spawning events throughout the breeding season (Wotton and Smith 2015). We do not have information on the number of reproductive events of *L. opalescens*, but the African Nothobranchiidae and Neotropical Rivulidae annual fish may have daily or weekly spawning events, lasting until senescence (e.g., Polačik et al. 2016; Volcan et al. 2011).

## Trade-Off Between Egg Size and Maximum Body Length

Maximum body size did not differ significantly among Neotropical killifishes (family Rivulidae) with annual and non-annual life cycles, a pattern that does not corroborate our initial expectation. Annual species have a shorter life cycle (often < 1 year) and are intrinsically related to the hydrological cycle (Žák et al. 2021), so it would be reasonable to imagine that the lower longevity could limit the maximum size that the species reach. Maximum body sizes similar to those in the present study were also reported by

Eckerström-Liedholm et al. ([2017](#)), who include African killifishes (family Nothobranchidae) in their analyses. In general, both annual and non-annual species of Rivulidae have a small body size (62 ± 32 mm in our database), a characteristic shared with many taxa of the Order Cyprinodontiformes (Malabarba and Malabarba [2020](#)). However, there are cases of annual species with exceptionally large size, such as *Austrolebias elongatus* (Steindachner 1881), which is the largest species of Cyprinodontiformes, reaching up to 22 cm in total length (Froese and Pauly [2022](#)).

Our results indicate that non-annual killifish have a larger egg size than annual species. This means that non-annual fish have a greater reproductive investment in offspring fitness that is disproportionate to the allocation capacity of the maternal reserve. Energy is a finite resource. In iteroparous species, there is a trade-off in the allocation of resources to two primary biological functions: somatic growth and reproduction (Hirshfield and Tinkle [1975](#)). Offspring size is just one axis on which parental investment can vary, but it has great potential to capture maternal energy investment in offspring (Pettersen et al. [2018](#); Marshall et al. [2018](#)). In this sense, the production of larger eggs has a cost of limiting investment in somatic growth of non-annual killifishes. Therefore, the higher reproductive energy expenditure in non-annual species, counterbalanced by the lower longevity of annual species, may have contributed to species with different life histories having similar maximum body sizes.

Annual killifish occur in temporary environments, while non-annual species inhabit more perennial environments, however both environments are commonly shallow, and experience wide ranges of temperature variation, and acidic and hypoxic waters (Berois et al. [2016](#); Earley et al. [2012](#); Furness [2016](#); Podrabsky et al. [2016](#)). The co-occurrence between annual and non-annual species is common (ICMBio [2018](#); Volcan and Guadagnin [2020](#)). In environments with low oxygen content, selection occurs against small eggs, caused by the reduced capacity of these eggs to deal with hypoxia possibly related to lower energy reserves (Einum et al. [2002](#)). A fundamental difference among killifishes is that annual species have embryos with extreme tolerance to hypoxia or anoxia (Podrabsky et al. [2007](#)). Survival in hypoxia/anoxia is possible due to the embryonic diapause process, which culminates in developmental arrest, metabolic depression, and a G1 cell cycle arrest (Podrabsky et al. [2016](#)). In the absence of diapause and underlying mechanisms to cope with hypoxia, embryo survival of non-annual killifishes could be increased with increasing offspring size. Indeed, evidence suggests that for species without embryonic diapause, the optimal investment in offspring size increases as environmental quality decreases (Rollinson and Hutchings [2013](#); Riesch et al. [2014](#); Santi et al. [2021](#)). Therefore, a factor underlying the selection of different egg sizes between annual and non-annual killifish species detected in the present study may be associated with different life history strategies to deal with stressful habitats.

## Conclusion

In conclusion, spawning in batches, synchronous modal development of oocytes, continued production of sperm in males, and a complex process of embryonic diapause are reproductive traits that favor the resilience of *L. opalescens* and other annual fish in temporary wetlands. The presence of embryonic diapause in the life cycle of killifish can affect egg size. Embryonic diapause can result in a reduction in egg size due to unique morpho-physiological adaptations to cope with the cyclic desiccation of habitats. Our data matrix (Online Resource 2) includes annual killifish species of at least two independent lineages (Murphy et al. [1999](#)). The non-monophyly of the annualism highlights the environmental pressure for the convergence of the patterns found. The increase in egg size in killifish without diapause (non-annuals) has the potential to increase offspring fitness, but at a higher energy cost that can affect species' somatic growth. Therefore, the higher reproductive energy expenditure in non-annual species, counterbalanced by the lower longevity of annual species, may have contributed to species with different life histories having similar maximum body sizes.

**Author Contributions** Gustavo Henrique Soares Guedes: Conceptualization, investigation, methodology, formal analysis, writing – original draft. Iracema David Gomes: Methodology, formal analysis, review. Aparecida Alves do Nascimento: Methodology, formal analysis, review. Marcia Cristina Costa de Azevedo: Formal analysis, review. Igor Cavalcanti de Araújo Souto-Santos: Methodology, formal analysis, writing – original draft. Paulo Andreas Buckup: Writing – review, supervision. Francisco Gerson Araújo: Conceptualization, writing – review, supervision, funding acquisition.

**Data Availability** The data is available at Supplementary information.

## Declarations

**Ethics Approvals** The fish collection was authorized by the Brazilian Environment Agency (SISBIO/ IBAMA/ICMBio, Permission 560 #10707). The authors state that the research was conducted according to ethical standards.

**Competing Interests** The authors have no relevant financial or non-financial interests to disclose.

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