

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 (TLmax) data from 132 neotropical freshwater fish (83 genera, 43 families) to establish comparisons with this species. We used the egg diameter / TLmax ratio to test the hypothesis that annual fish in temporary wetlands have relatively larger eggs than non-annual species from perennial habitats (lakes, rivers). Fish were collected from Guandu River drainages (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 with microscopic analysis. Females with batch spawning and males with continuous spawning were detected. The batch fecundity ranged from 22–32 vitellogenic oocytes (mean 27 ± 7 SD). Annual species presented greater relative egg size than perennial species ($p < 0.001$), which is an indication of greater reproductive investment at the expense of somatic growth in temporary wetlands. Larger eggs are advantageous for annual fish as their lifespan is limited, and they can allocate a greater amount of yolk reserves for long periods buried in the substrate under embryonic diapause. Large relative oocytes, 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 resilience of *L. opalescens* and other annual fish in temporary wetlands.

1 Introduction

Rivulidae (Cyprinodontiformes) is the eighth most speciose fish family in the World, with 471 valid species (Fricke et al. 2022), and the first in 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 (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 the 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 hours 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 not coexist.

In recent decades, studies on 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 reproductive traits of *Leptopanchax* species is minimal and diffuse (e.g., Costa and Leal 2009; Guedes et al. 2020). The scarcity of reproductive information of *Leptopanchax* species and other annual rivulids is a bottleneck that makes it difficult 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; 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 such as the catfish *Hypophthalmus edentatus* Spix & Agassiz, 1829 and the characid *Psalidodon fasciatus* (Cuvier, 1819) 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 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.

In this study, the reproductive traits of *Leptopanchax opalescens* (Myers, 1942) were studied to improve our understanding of 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 (TL_{max}) data from 132 species (83 genera, 43 families) of Neotropical freshwater fish. We used the egg size / TL_{max} ratio as proxy to compare the trade-off between the allocation of reproductive energy vs. growth between species with distinct life histories (annual vs. perennial). Fish with higher egg size / TL_{max} ratios present (1) relatively large eggs in relation to the body, and (2) post-hatch offspring larger and closer to the beginning of the reproductive cycle, i.e., the size at first maturation. These characteristics may be a proxy for greater maternal investment in offspring fitness (Pettersen et al. 2017; Marshall et al. 2018) at the expense of maternal somatic growth. The tested hypothesis was that annual fish in temporary wetlands have relatively larger eggs than non-annual species from perennial habitats (e.g., lakes, rivers). Relatively larger eggs in annual fish may be associated with: (1) short lifespan and limited by the hydrological cycle (Žák et al. 2021); (2) greater ability to obtain oxygen to develop embryos in hypoxic habitats (Einum et al. 2002) typical of temporary ponds; (3) embryonic diapause may require greater storage of nutrients, lipids and water to ensure metabolism (Riddle and Hu 2021), and consequent embryo survival, which can last for months and sometimes even years in dry habitats.

2 Methods

2.1 Fish sampling

The study area comprises a temporary pool located in the Guandu River basin (22°42.35'S, 43°41.59'W), a costal 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 x 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 #10707) and vouchers were deposited at the Ichthyological Collection of the Fish Ecology Laboratory of the Universidade Federal Rural do Rio de Janeiro (LEP-UFRRJ 2576–2578; gbif.org/occurrence/2628729394) and in at Ichthyological Collection of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ 51967; 52701).

3.2 DNA barcoding

Muscular tissue from specimens (N = 4) preserved in anhydrous ethanol were 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'-ACYAAYCACAAAGAYATTGGCA-3', and FishR7notail: 5'-TARACTTCTGGRTGDC CRAAGAAYCA-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 BigDye™ 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.

3.3 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 hour 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 60°C 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 (Online Resource 2). Microanatomy of the zona pellucida was examined under scanning electron microscopy (Hitachi TM3000). The bath 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. 2013).

3.4 Trade-off between egg size and maximum body length

We compiled a dataset of mean egg diameter and maximum total length based on the literature (TL_{max}) of 48 annual/seasonal species of the Family Rivulidae (13 genera) that occur in temporary wetlands (Thompson et al. 2017). This information was compared with that of another 84 perennial species (68

genera, 39 families) that occur in Neotropical rivers, streams and reservoirs (e.g., Vazzoler 1996, Orsi 2010, Rizzo and Bazzoli 2020; Online Resource 3). 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 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. A Wilcoxon-Mann-Whitney test ($p < 0.01$) was used to test the hypothesis that annual fish in temporary wetlands have relatively larger eggs (egg size / TLmax ratio) than non-annual species from perennial habitats. Analyzes were conducted in R environment (R Core Team, 2022) using the package 'ggstatsplot' (Patil 2021). Data are available at Online Resource 3.

3 Results

3.1 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).

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.

| | <i>L. aureoguttatus</i> | <i>L. citrinipinnis</i> | <i>L. opalescens</i> |
|-------------------------|-------------------------|-------------------------|----------------------|
| <i>L. aureoguttatus</i> | | 0.023 | 0.022 |
| <i>L. citrinipinnis</i> | 21.8% | | 0.015 |
| <i>L. opalescens</i> | 20.3% | 11.0% | |

3.2 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 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 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 nucleus evident and layers of the follicular envelope as 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 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.

3.3 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. X). Different cell stages of the spermatogenic lineage were identified, and characterized the presence of five phases (immature, developing, spawning capable, regressing, regenerating) 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.

3.4 Fecundity, and trade-off between egg size - maximum body

The total number of oocytes present in the gonads (regardless of the stage of development) ranged from 91 to 99 (mean 95 ± 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 μm (402.7 ± 224 μm). The bath fecundity (only vitellogenic oocytes) ranged from 22 to 32 (27 ± 7), corresponding to oocytes with diameter > 500 μm (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 ± 4.1 mm TL). The absolute egg size and LTmax of the 48 annual species varied from 0.52 to 2.1 (0.98 ± 0.3 mm) and from 20 to 220 mm (56.1 ± 30.8 mm), respectively. The 84 non-annual species in perennial habitats range from 0.32 to 4.36 mm (1.4 ± 0.9 mm) and from 49 to 1660 mm (381 ± 294 mm TL). Annual fishes inhabiting temporary wetlands have an egg size / TLmax ratio (mean 1.9×10^{-2}) significantly higher ($p < 0.0001$; Fig. 5) than other non-annual species in perennial habitats (5.6×10^{-3}).

Table 2

Summary of reproductive traits of *Leptopanchax opalescens*, Minimum–Maximum (Mean \pm 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 \pm 4.1) mm TL | present study |
| | Females | 11.0–32.0 (18.8 \pm 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 spawning | | | |
| | 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 \pm 5.65) | present study |
| 7.2 Total diameter | | 75–1050 (402 \pm 224) μ m | present study |
| 7.3 Vitelogenic diameter | | 500–1050 (700 \pm 225) μ m | |

| Reproductive traits | Sex | Description | Source |
|------------------------|-----|---|---------------|
| 7.4 Oocyte Development | | Synchronous modal | present study |
| 7.5 Zona Pellucida | | Mushroom-like projections and polygonal grooves | present study |
| 8. Fecundity | | | |
| 8.1 Bath (BF) | | 22–32 (27 ± 7.0) | present study |
| 9. Strategy | | Iteroparous | present study |
| | | Income breeding (<i>sensu</i> McBride et al. 2013) | present study |

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 previously been reported for species of the genus *Leptopanchax*. In addition, we compile evidence that the eggs of annual fishes inhabiting temporary wetlands are relatively larger than those of perennial freshwater species.

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 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 drainage.

Specimens with COI genetic distances less than 2% are considered conspecific following DNA barcoding standards for fish (Hebert et al. 2003a, 2003b, Pereira et al. 2013). Following this criterion, the comparison of sequences available so 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 Sepetiba 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 dry 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 increases the chance of survival by reducing potential predation on offspring and intraspecific competition for food and shelter.

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, 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; Vrtilek 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. 2015). *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 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. 2015).

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). 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

Analyzes based on absolute diameter of fish eggs demonstrated the occurrence of smaller sizes in more seasonal and less predictable environments (Winemiller and Rose 1992; Morrongiello et al. 2012). However, when the diameter of the eggs is weighted by the species' maximum body size (egg size/TLmax ratio) different perspectives arise. We found that fish with an annual life cycle inhabiting unpredictable habitats (i.e, temporary wetlands) have relatively larger eggs ($p < 0.001$) than other freshwater perennial species that occur in more hydrologically stable environments (i.e., rivers, lakes and reservoirs). This means that annual fish have a greater reproductive investment in offspring fitness disproportionate to the maternal reserve allocation capacity. Females with smaller reserves draw more heavily on exogenous resources, closer to the income breeding strategy (McBride et al. 2013). This implies in a rapid conversion in the acquired energy from feeding to use to spawn (Aristizabal 2007).

Eggs of annual fish undergo embryonic diapause, a process that requires a larger size to storage of nutrients, lipids and water for long periods buried in the substrate (Riddle and Hu 2021). Large eggs increase survival during the pre- and post-hatch stages especially in environments with low dissolved oxygen (Einum et al. 2002) typical of temporary wetlands. Larger eggs contain more yolk, produce larger larvae with reduced susceptibility to starvation, lower risk of predation, and greater food capture abilities (Wotton and Smith 2015). The energy cost of bath spawning of larger eggs is disproportionately expensive for annual fishes due to their reduced body size (56 mm on average). Preparation for reproduction can cause oxidative stress and affect maternal self-maintenance (Godoy et al. 2020) and consequently somatic growth. However, this seems to be an effective strategy of annual fishes to (1) increase embryo survival in unpredictable habitats that can remain without water for months, and (2) increase offspring fitness in larval stages, as free-living time is limited by the time constraints imposed by the hydrological cycle.

Conclusions

In summary, *L. opalescens* and other annual fish have large relative (TL weighted) oocytes, spawning in batches, synchronous modal development of oocytes, continued production of sperm in males, and a complex process of embryonic diapause. The egg size / TLmax ratio was a useful proxy to detect greater maternal investment in offspring fitness at the expense of somatic growth among species with different life histories. We found that fish with annual or seasonal life cycle have relatively larger eggs than other perennial Neotropical freshwater species. Relatively larger eggs in annual fish may be associated with (1) limited lifespan and the consequent need for a rapid post-hatch reproductive start; (2) hypoxic habitats may select for larger eggs; (3) the embryonic diapause process may require greater stock reserves to keep the embryo viable during months without water in temporary wetlands. These reproductive characteristics require high maternal energy investment, but increase the survival of the offspring and ensure the resilience of populations of annual fishes in habitats with extreme cyclical hydrological variations.

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Data Availability: The data is available at Supplementary information.

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.

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Figures



Figure 1

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

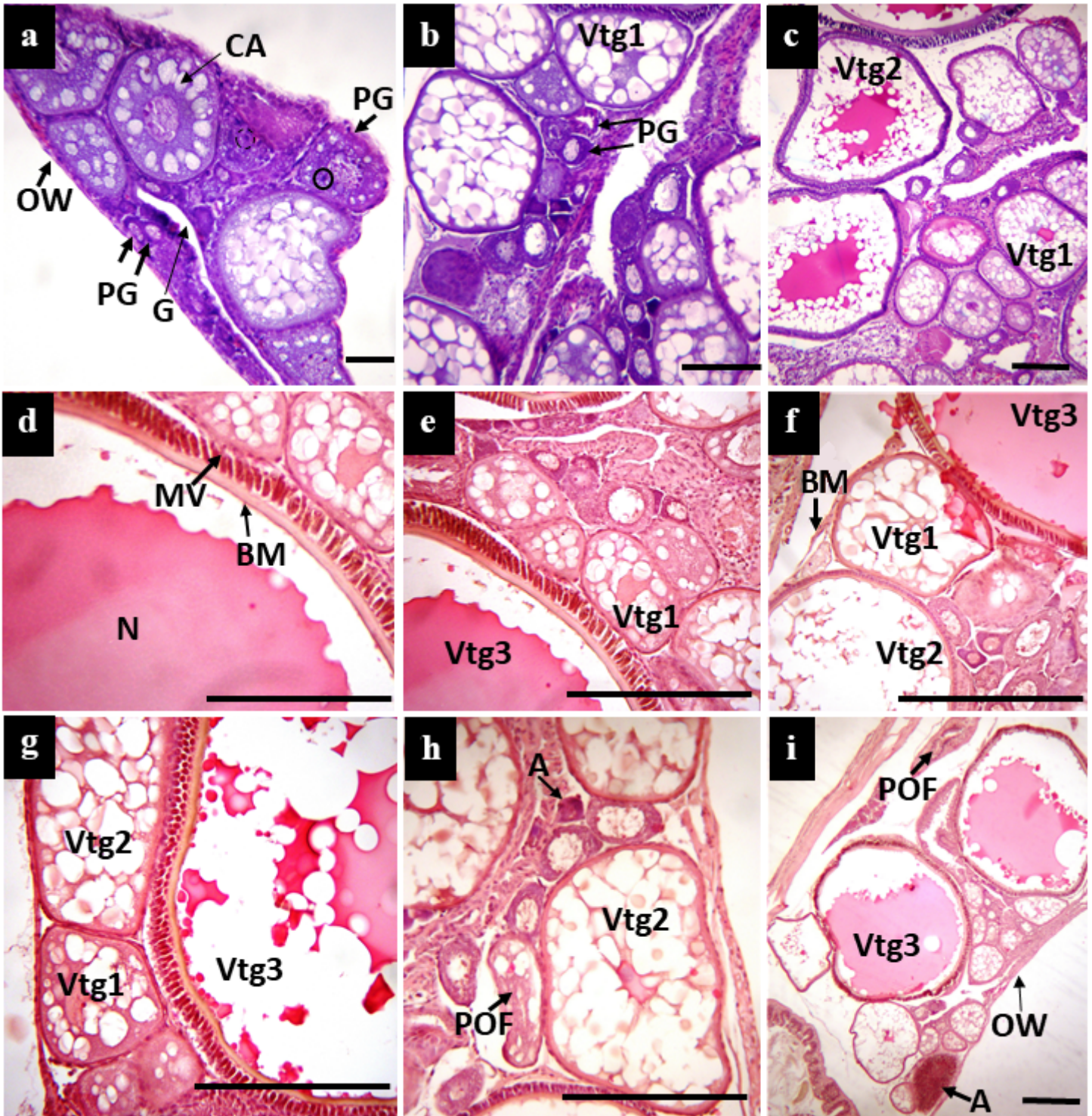


Figure 2

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 μ m

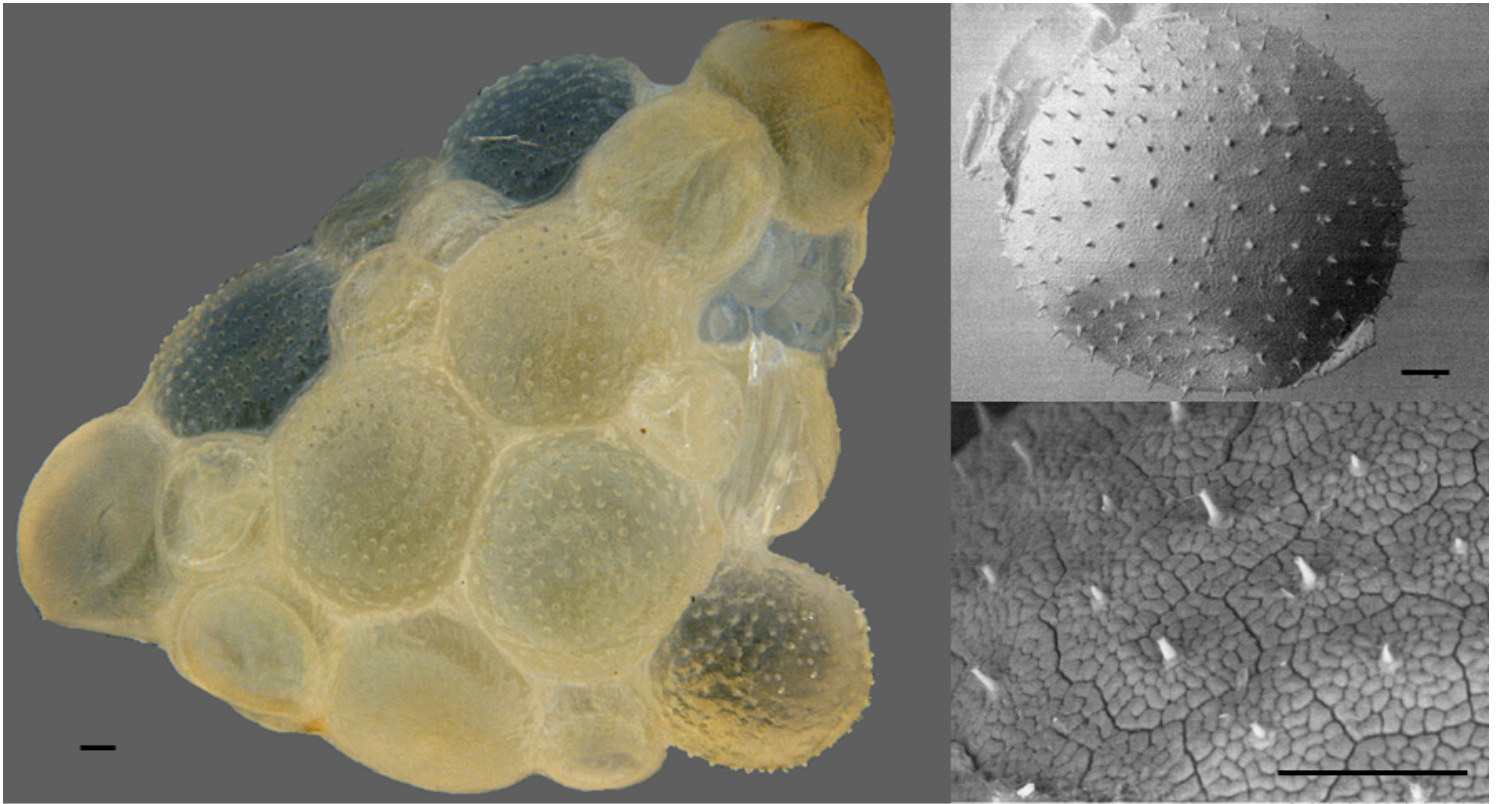


Figure 3

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 μm

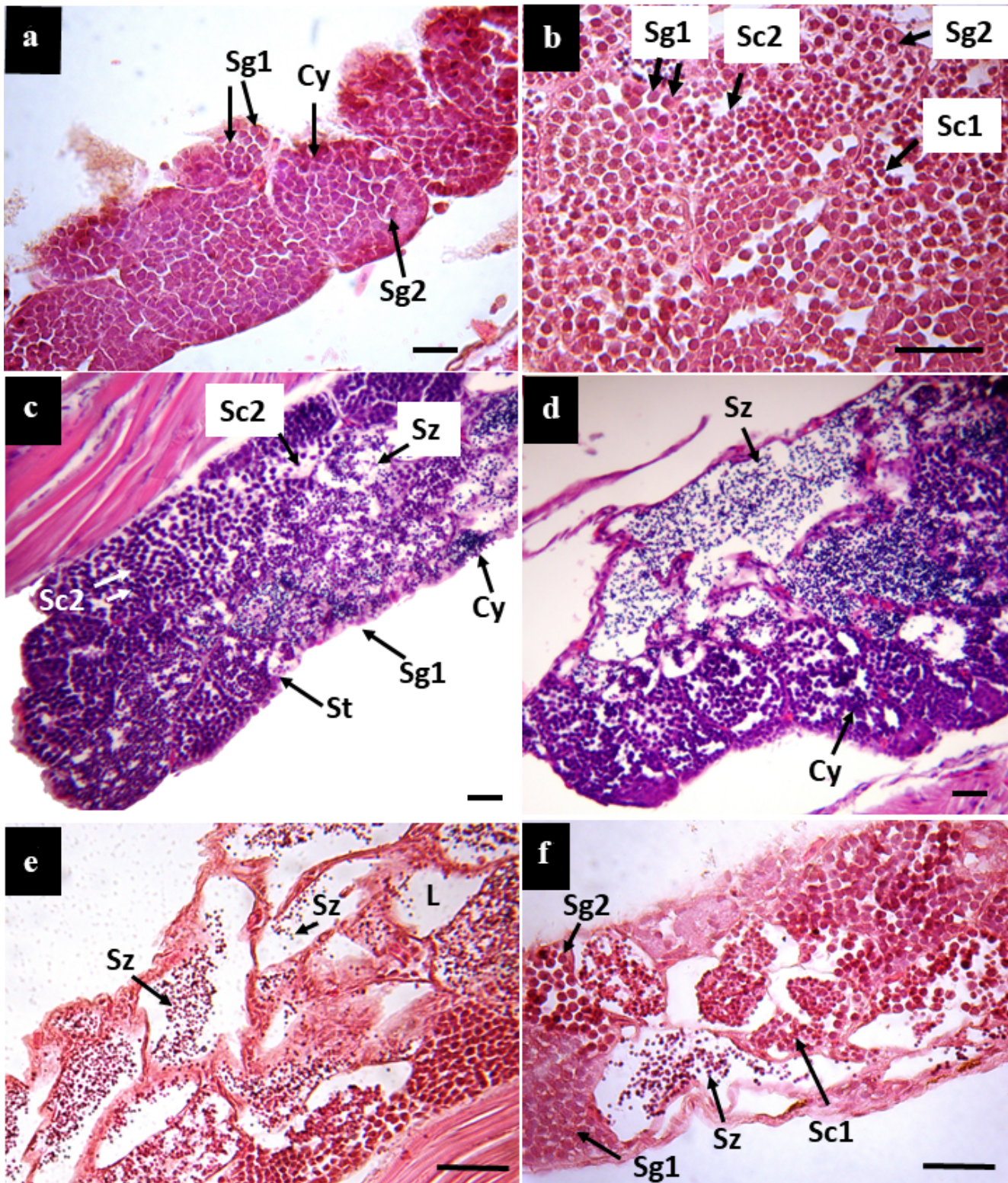


Figure 4

Images of testes of *Leptopanchax opalescens* in different phases of gonadal 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 μ m

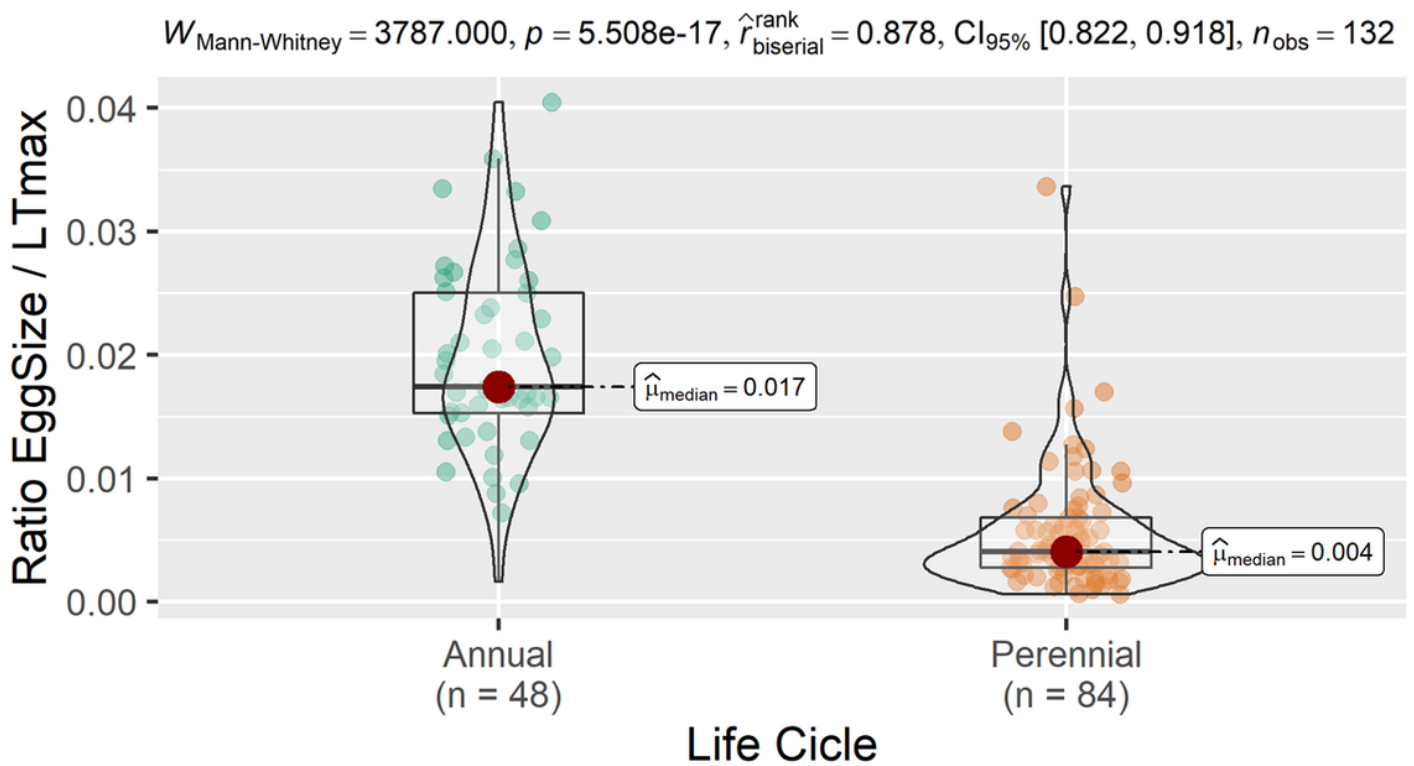


Figure 5

Egg size / TLmax ratio of annual fish in temporary wetlands and non-annual fish in perennial habitats. 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 greater density of observations

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