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Instituto de Ciências Biológicas
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AMADURECIMENTO PRECOCE E VIDA CURTA: COMO O ENVELHECIMENTO RÁPIDO AFETA A REPRODUÇÃO DOS PEIXES ANUAIS?

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RESUMO

Os peixes anuais habitam pequenas áreas úmidas temporárias que secam sazonalmente. A sobrevivência em áreas úmidas temporárias requer adaptações fisiológicas, morfológicas e/ou do ciclo de vida. Para sobreviver nesses ambientes, os peixes anuais apresentam crescimento acelerado, maturidade sexual precoce e alto investimento em reprodução, com ovos resistentes a seca capazes de entrar em diapausa conforme as condições ambientais. Além disso, apresentam cobertura de apostas em suas estratégias de desenvolvimento e eclosão, permitindo-lhes lidar com a imprevisibilidade do hidroperíodo das áreas úmidas temporárias. Nesse sentido, investigamos no primeiro capítulo a fecundidade dos peixes anuais *Matylebias cyaneus* e *Cynopoecilus nigrovittatus* individualmente e em interação in situ durante todo o hidroperíodo (inundação precoce, secagem e inundação tardia) de uma lagoa temporária. Nossos resultados mostram que as espécies apresentam reprodução parcelada ao longo de todo ciclo de vida. O tratamento monoespecífico de *Matylebias cyaneus* não apresentou efeito sobre a fecundidade em relação ao tamanho corporal, peso e fase de inundação. No tratamento monoespecífico de *Cynopoecilus nigrovittatus*, houve efeito positivo do tamanho corporal das fêmeas e do período de amostragem sobre o número de ovos, que foi maior na fase tardia da cheia. No tratamento interespecífico, *M. cyaneus* depositou menos ovos na fase inicial de cheia quando comparado ao tratamento monoespecífico, e *C. nigrovittatus* apresentou redução no número de ovos, considerando todo o ciclo hidrológico. No segundo capítulo testamos se *Matilebias cyaneus* poderia "mudar suas apostas" dinamicamente em resposta a ciclos hidrológicos variados, combinando procedimentos de campo e de laboratório para avaliar ajustes nos estágios de desenvolvimento embrionário e eclosão. Quando comparados, os embriões de um cenário de secagem no inverno (espera-se uma reinundação em breve) versus um cenário de secagem na primavera (espera-se uma reinundação posterior) exibiram uma proporção maior de escape da diapausa I, tempos de desenvolvimento mais curtos e taxas de eclosão mais altas.

Palavras-chave: Peixes anuais; reprodução; áreas úmidas temporárias; ciclo de vida;

ABSTRACT

Annual fish inhabit small temporary wet areas that dry up seasonally. Survival in temporary wet areas requires physiological, morphological, and/or life cycle adaptations. To survive in these environments, annual fish exhibit accelerated growth, early sexual maturity, and a high investment in reproduction, with drought-resistant eggs capable of entering diapause according to environmental conditions. Additionally, they employ a hedging strategy in their development and hatching, allowing them to cope with the unpredictability of the hydroperiod in temporary wet areas. In this regard, in the first chapter, we investigated the fecundity of the annual fish *Matylebias cyaneus* and *Cynopoecilus nigrovittatus* individually and in interaction in situ throughout the hydroperiod (early flooding, drying, and late flooding) of a temporary pond. Our results show that the species exhibit staggered reproduction throughout their life cycle. The monospecific treatment of *Matylebias cyaneus* had no effect on fecundity in relation to body size, weight, and flooding phase. In the monospecific treatment of *Cynopoecilus nigrovittatus*, there was a positive effect of female body size and sampling period on the number of eggs, which was higher in the late flooding phase. In the interspecific treatment, *M. cyaneus* deposited fewer eggs in the early flooding phase compared to the monospecific treatment, and *C. nigrovittatus* showed a reduction in the number of eggs throughout the hydrological cycle. In the second chapter, we tested whether *Matylebias cyaneus* could dynamically "change its bets" in response to varied hydrological cycles, combining field and laboratory procedures to assess adjustments in embryonic development stages and hatching. When compared, embryos from a winter drying scenario (expecting imminent re-flooding) versus a spring drying scenario (expecting later re-flooding) exhibited a higher proportion of diapause I escape, shorter development times, and higher hatching rates.

Key-words: Annual fish; reproduction; temporary wetlands; life cycle;

APRESENTAÇÃO

Esta dissertação intitulada “Amadurecimento precoce e vida curta: como o envelhecimento rápido afeta a reprodução de peixes anuais” foi elaborada como parte dos requisitos para a obtenção do título de Mestre em Biologia de Ambientes Aquáticos Continentais da Universidade Federal de Rio Grande – FURG.

Apesar de um recente apelo para compreender o envelhecimento dos peixes anuais e sua utilização como modelo biológico de envelhecimento, ainda são escassas informações sobre sua ecologia, reprodução e ciclo de vida. Dessa forma, este estudo teve como objetivo avaliar como o envelhecimento rápido dos peixes anuais afeta sua reprodução, investigando se o número de ovos depositados por fêmeas das espécies *Cynopoecilus nigrovitattus* e *Matilebias cyaneus* muda ao longo do ciclo de vida e se os embriões de *Matilebias cyaneus* mudam suas estratégias de desenvolvimento conforme o momento em que os ovos foram depositados.

A dissertação está estruturada em dois capítulos em formato de manuscrito científico. No primeiro manuscrito, nós apresentamos os resultados obtidos através de experimentos realizados em campo, submetido e publicado em 2023 no periódico científico Wetlands (A2). O segundo manuscrito apresenta resultados obtidos através de experimentos realizados em laboratório, e submetido para o periódico científico The American Naturalist (A1).

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INTRODUÇÃO GERAL

Os peixes anuais vivem exclusivamente em áreas úmidas temporárias (COSTA, 2002). Esses habitats são caracterizados por possuírem uma fase seca e outra úmida (WILLIANS, 2003), tornando-os ambientes imprevisíveis (WILLIANS, 2006). Por apresentar tamanho reduzido, pouca profundidade (MALTCHIK, 2003; VOLCAN; GONÇALVES; GUADAGNIN, 2013) e variações físicas e químicas significativas (FONSECA et al., 2013), a sobrevivência dos peixes anuais nesses ambientes depende de adaptações fisiológicas, morfológicas e/ou de ciclo de vida (WIGGINS; MACKAY; SMITH, 1980). Muitos táxons aquáticos, como rotíferos e microcrustáceos, produzem estruturas resistentes à seca para garantir sua sobrevivência, como bancos de ovos em dormência que permanecem no substrato e eclodem em condições favoráveis (VENDRAMIN et al. 2021). Por sua vez, os peixes anuais possuem diferentes estratégias de sobrevivência que estão relacionadas com características únicas dentre os vertebrados (WALFORD; LIU, 1965; LIU; WALFORD, 1969; COSTA, 2006, PODRABSKY; HAND, 1999), que podem ser observadas em diferentes momentos do seu ciclo de vida.

O extremo sul do Brasil (Rio Grande do Sul) possui uma alta representatividade de espécies de peixes anuais, sendo considerado um centro de endemismo (LANÉS, 2011). Nessa região, as áreas úmidas temporárias são abundantes (MALTCHIK et al., 2003) e costumam alternar períodos de inundação (outono a primavera) e seca (verão) (MALTCHIK et al., 2024). Com o início das chuvas no outono e inundação das áreas úmidas temporárias, os peixes anuais eclodem (março – maio) (LANÉS, 2011). Após a eclosão, os alevinos apresentam crescimento acelerado e atingem a maturidade sexual em 6-8 semanas (WALFORD; LIU, 1965; LIU; WALFORD, 1969, COSTA, 2006). Os peixes anuais apresentam dimorfismo sexual, com os machos apresentando tamanho maior e padrões de coloração mais brilhantes quando comparados à coloração acastanhada das fêmeas (GARCIA et al., 2004; LAUFER et al., 2009). Apresentam complexo comportamento reprodutivo (GARCIA et al., 2008) e os seus ovos são depositados no substrato diariamente até a sua morte (VAZ-FERREIRA et al., 1966; ERREA & DANULAT, 2001; GONÇALVES; SOUZA; VOLCAN, 2011).

Diante das condições variáveis das áreas úmidas temporárias, reproduzir continuamente após a maturidade sexual até a senilidade ou morte pode ser uma importante estratégia para manutenção das espécies de peixes anuais, pois resulta em uma grande disponibilidade de ovos no substrato (GONÇALVES; SOUZA; VOLCAN, 2011). No entanto, pouco se sabe sobre sua reprodução em ambiente natural (ARENZON; PERET; BOHRER,1999; SHIBATTA, 2005), ou quantos ovos uma fêmea é capaz de produzir.

Com a morte dos peixes adultos, a população de peixes anuais está restrita ao banco de ovos presente no sedimento. Os ovos de peixes anuais são resistentes a seca e possuem diapausas fortemente influenciadas pelas condições ambientais (PODRABSKY; GARRETT; KOHL, 2010; FURNESS, 2016). A diapausa é caracterizada por uma interrupção no desenvolvimento associada com a redução acentuada na taxa metabólica e, nos peixes anuais, ela é dividida em três fases (diapausa I, II e III) (WOURMS, 1972; PODRABSKY; HAND, 1999), sendo que cada uma apresenta diferentes graus de resistência e funções (WOURMS, 1972; PODRABSKY; HAND, 1999; AREZO; PEREIRO; BEROIS, 2005; GENADE et al., 2005; PODRABSKY; GARRETT; KOHL, 2010; PRI-TAL et al., 2011).

A diapausa I ocorre no estágio de dispersão dos blastômeros, antes da formação do eixo embrionário. Nesta fase, os blastômeros dispersam completamente em torno do vitelo, podendo permanecer desta forma até que um sinal ambiental seja percebido, fazendo com que voltem a se agregar (WOURMS, 1972). Estudos identificaram que a presença de peixes adultos na água leva à entrada de diapausa I (AREZO; PEREIRO; BEROIS, 2005; FONSECA et al., 2018). Essa característica pode estar relacionada com a sincronia do banco de ovos, como já observado para peixes anuais africanos (POLACIK et al., 2021).

A diapausa II ocorre quando os embriões já apresentam desenvolvimento, com batimentos cardíacos e elementos do sistema nervoso em formação (WOURMS, 1972). Alguns embriões podem apresentar uma trajetória alternativa à entrada em diapausa II, chamada estratégia de escape, em que eles se desenvolvem de forma direta sem passar pela diapausa II (PODRABSKY et al., 2017). A entrada, saída e/ou estratégia alternativa podem ser controladas por fatores bióticos e abióticos. Fatores bióticos como caracteres genéticos (PODRABSKY & HAND, 1999) e pistas maternas são exemplos (PRI-TAL et al.,

2011). Podrabsky et al., (2010) observaram que a idade das fêmeas pode alterar as proporções de embriões que entram em escape em um peixe anual Neotropical (*Austrofundulus limaneus*) - fêmeas mais jovens geram mais embriões que evitam a entrada em diapausa II. Para insetos, os hormônios possuem um papel importante na regulação da entrada em diapausa (MOUSSEAU e DINGLE, 1991). As alterações hormonais que ocorrem com o envelhecimento podem ser um fator que regula a trajetória de desenvolvimento embrionário em peixes anuais (PRI-TAL et al., 2011).

Porém, a entrada e saída da diapausa II pode ser moldada também por fatores abióticos como fotoperíodo, temperatura e disponibilidade de oxigênio (PODRABSKY; HAND, 1999; ARENZON; PERET; BOHRER, 2001; PODRABSKY; CULPEPPER, 2012). A diapausa III ocorre quando os embriões estão com seu desenvolvimento completo (WOURMS, 1972), esperando os estímulos da chuva para iniciar o processo de eclosão. Estudos recentes demonstraram que os ovos nesse estágio de desenvolvimento são capazes de identificar a presença de hormônios de predadores na água e atrasar sua eclosão (GODOY et al., 2021).

A ocorrência e duração de cada fase de diapausa é regulada por fatores bióticos e abióticos, variando individualmente e entre espécies (WOURMS, 1972; PODRABSKY; HAND, 1999; AREZZO; PEREIRO; BEROIS, 2005; PRI-TAL et al., 2011). Ovos colocados ao mesmo tempo e mantidos nas mesmas condições podem apresentar embriões com tempos distintos de desenvolvimento (PODRABSKY et al., 2017), sugerindo que outros fatores podem influenciar as diferentes trajetórias de desenvolvimento embrionário em peixes anuais. As infinitas possibilidades de desenvolvimento embrionário foram definidas como “mecanismo multiplicador” (WOURMS, 1972). Este mecanismo possui extrema importância na manutenção da população, pois se todo banco de embriões apresentasse a mesma trajetória de desenvolvimento, uma chuva passageira poderia gerar um processo de eclosão síncrono e a dessecação prematura do habitat levaria à morte de toda a população (PRI-TAL et al., 2011).

Uma maneira pela qual as espécies podem lidar com a estocasticidade das áreas úmidas temporárias é através de estratégias de distribuição de risco (através de bet-hedging), quando existe a produção de uma ampla gama de fenótipos ou uma distribuição de características “seguras” (SIMONS e JOHNSTON, 1997). Essa estratégia pode levar a uma redução da aptidão dentro de uma temporada, quando

somente parte dos indivíduos apresentam características fenotípicas para sobreviver as imprevisibilidades, mas permitir a aptidão à longo prazo (FURNEES et al., 2015). A variação no desenvolvimento do embrião de peixe anual e atraso na eclosão são estratégias reconhecidas de proteção para maximizar a sobrevivência em peixes anuais africanos (FURNESS et al., 2015; PINCEEL et al., 2015; POLAČIK et al., 2018). A variação no desenvolvimento do embrião como uma estratégia de cobertura de apostas garante que uma proporção do pool de ovos esteja sempre pronta para eclodir e corresponda às condições contemporâneas (SIMONS, 2011). Segundo Furness et al., (2015), mesmo embriões de peixes anuais em DIII (aguardando o estímulo da chuva para eclodir), são capazes de eclodir de forma parcelada, resultando em um conjunto diversificado de eclosões em diferentes eventos de inundação.

Dessa forma, todas as características citadas anteriormente são importantes para que os peixes anuais obtenham sucesso nas áreas úmidas temporárias, repetindo seu ciclo anualmente. As mesmas características os tornam excelentes modelos biológicos em estudos de laboratório (POLAČIK & REICHARD, 2010), sendo utilizados em estudos de envelhecimento (GODOY et al., 2019; 2020; CASTRO et al., 2021). Este trabalho visa preencher lacunas referente a reprodução dos peixes anuais na natureza e investigar suas estratégias embrionárias. No primeiro capítulo, nós investigamos a fecundidade dos peixes anuais *Matilebias cyaneus* e *Cynopoecilus nigrovittatus* individualmente e em interação *in situ* durante todo o hidroperíodo (inundação precoce, secagem e inundação tardia) de uma área úmida temporária. No segundo capítulo, nós testamos se *Matilebias cyaneus* poderia "mudar suas apostas" dinamicamente em resposta a ciclos hidrológicos variados, combinando procedimentos de campo e de laboratório para avaliar ajustes nos seus estágios de desenvolvimento embrionário e eclosão.

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CAPÍTULO 1 - Egg production of annual fish *Austrolebias cyaneus* and *Cynopoecilus nigrovittatus* occurs throughout their entire life cycle to survive in a temporary wetland

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Abstract

Annual fishes inhabit small temporary ponds that dry up seasonally. Survival in temporary ponds requires specific physiological, morphological, and/or life cycle adaptations. Annual fishes exhibit a complex reproductive strategy with resistant eggs to survive droughts. Here, we investigate the fecundity of the annual fishes *Austrolebias cyaneus* and *Cynopoecilus nigrovittatus* individually and in interaction in situ throughout the hydroperiod (early inundation, drying, and late inundation) of a temporary pond. The monospecific treatment of *Austrolebias cyaneus* showed no effect on fecundity regarding body size, weight, and flooding phase. In the monospecific treatment of *Cynopoecilus nigrovittatus*, there was a positive effect of female body size and sampling period on the number of eggs, which was higher in the late flooding phase. In the interspecific treatment, *Austrolebias* laid fewer eggs in the early flooding phase when compared to the monospecific treatment, and *Cynopoecilus nigrovittatus* showed a reduction in the number of eggs, considering the entire hydrological cycle. These results obtained from wild populations should help to fill the knowledge gap on biological traits, which impairs the understanding of the ecology of annual killifish in small temporary wetlands.

Keywords: *Austrolebias*, *Cynopoecilus*, diapause, ecological interactions, fish eggs, Neotropical region.

Introduction

Annual fishes of the Rivulidae family are among the most diverse clades of the Neotropical ichthyofauna, with distinct evolutionary, biological, and ecological characteristics (Loureiro et al. 2018). Species of annual fishes in this family inhabit small temporary ponds that dry up seasonally, resulting in the death of the entire adult population (Lanés et al. 2014). Survival in such a peculiar and challenging habitat requires specific physiological, morphological, and/or life cycle adaptations (Wiggins et al. 1980).

Annual fishes are fast life-cycle organisms (Blazek et al. 2013; Berois et al. 2014; Vrtílek et al. 2018; Godoy et al. 2019) that exhibit high environmental plasticity, but are sensitive to anthropogenic alterations of their habitats (Reis et al. 2003; Rosa and Lima 2008). These characteristics determine the degree of vulnerability and threat to most of their species (Volcan and Lanés 2018).

Annual fishes exhibit a complex reproductive strategy with resistant eggs that go through different stages of embryonic development to survive droughts (Wourms 1972). Additionally, they have the ability to change their developmental trajectory according to environmental conditions (Podrabsky et al. 2016; Godoy et al. 2021). In addition to drought, temporary aquatic ecosystems pose other challenges, such as limited space and time for growth, sexual maturation, and reproduction (Loureiro et al. 2016). In this sense, annual fishes invest heavily in growth to reach sexual maturity before the environment dries up (Vrtílek et al. 2018), and reproduction, with staggered spawning from early sexual maturity to death (Vaz-Ferreira et al. 1964; Gonçalves et al. 2011).

The Rivulidae exhibit unique and varied reproductive characteristics (Loureiro et al. 2018). Although most species have external fertilization, there are uncommon reproductive modes among vertebrates. For example, two species of *Kryptolebias* Costa are the only known vertebrates capable of self-fertilization (Avise and Tatarenkov 2015; Costa et al. 2017). The genus *Austrolebias* Costa has complex reproductive behavior, involving courtship, external fertilization, and egg deposition on the substrate (Garcia et al. 2008), and the genus *Cynopoecilus* Regan has morphological adaptations for internal insemination (Costa et al. 2017).

The *Austrolebias* genus is represented by approximately 50 species (Volcan and Severo-Neto 2019), distributed from Bolivia, Brazil, Paraguay, Uruguay, and Argentina (Costa 2010; Volcan and Severo-Neto 2019), while the genus *Cynopoecilus* has 7

described species, all restricted to Southern Brazil and Uruguay (Ferrer et al. 2014; Costa et al. 2017). *Austrolebias* and *Cynopoecilus* are the annual fish genera found in southern Brazil (Lanés et al. 2014), often sharing the environment in a syntopic and sympatric manner (Volcan et al. 2011; Lanés et al. 2018). The coexistence of annual fish in very restricted environments may be due to differences in embryonic development and hatching time of coexisting species, as well as the difference in sizes throughout the temporary wetland cycle (Volcan and Guadagnin 2020). This ecological/evolutionary adjustment in embryonic development and growth may be an ecological-evolutionary mechanism to avoid competitive exclusion, allowing for the occurrence of species with similar bio-ecological characteristics in wetlands.

Studies on these genera have included studies to their reproductive behavior (Belote and Costa 2004; García et al. 2008), aging characteristics (Godoy et al. 2019, 2020; Castro et al. 2021), population dynamics (Lanés et al. 2014; Lanés et al. 2016; Volcan et al. 2018), ex-situ reproduction (Volcan et al. 2013), and gonadal analyses (Arenzon et al. 1999; Schalk et al. 2014). However, data on *in situ* fecundity and studies on the influence of environmental conditions that annual fish face in the natural environment are still scarce (Volcan et al. 2011; Lanés et al. 2018; Volcan and Guadagnin 2020).

Our main goal was to evaluate the fecundity of *Austrolebias cyaneus* and *Cynopoecilus nigrovittatus* throughout the hydroperiod (early inundation, drying, and late inundation) of a temporary pond. Fecundity was studied by examining the total number of eggs produced. We also correlated egg production with the size and weight of fish from both species. Furthermore, we analyzed the fecundity of *Austrolebias cyaneus* in the presence of *Cynopoecilus nigrovittatus* and vice versa. Our approach involves exploratory analysis to infer the underlying factors affecting egg production in fish of the Rivulidae family.

Material and methods

Study area

The study area is located in the municipality of General Câmara, in the geomorphological province of the Central Depression in the state of Rio Grande do Sul, southern Brazil. The site is in floodplain of the Jacuí River, within the Guaíba River hydrographic region, Laguna dos Patos hydrographic ecoregion. The region's average annual temperature varies between 16 and 18°C, with higher values in the summer and lower in the winter, and temperatures below zero are very rare. The average annual precipitation is 1600 mm (Maluf 2000; Alvarez et al. 2013).

The studied pond is inserted in a matrix of altered natural fields subject to cattle grazing, with a predominance of various grasses and shrubby vegetation (*Mimosa bimucronata*). The pond had crystal clear/tea-colored water, muddy substrate, and dense aquatic vegetation of floating, submerged, and emergent macrophytes (*Echinodorus grandiflorus, Eleocharis minima, Nymphoides indica, Sagittaria* sp*., Hydrocotyle* sp.). In autumn, at the end of April, the temporary pond filled with surface water with the onset of precipitation. Annual fish reach sexual maturity between 6-8 weeks (Costa 2006) and they die before the pool dries up, mainly males (Lanés et al. 2016). In this sense, the experimental period was designed to cover the sexual maturation of populations and allow the capture of the minimum necessary number of individuals during periods when annual fish populations are usually more abundant (Lanés et al. 2016). In June 2022, the pond had surface water, reducing its wet area in July-August, and re-flooding with new precipitation in September. In the following months, with the reduction of the number of individuals, it was not possible to capture a minimum number of individuals, mainly males. The temporary ponds of the region, habitats of

466 annual fish populations, remain flooded usually between 6-8 months of the year, from
467 late autumn to late spring (Lanés et al. 2016, 2018).
468
469 **Sampling design**
470
471 A total of three samplings were performed throughout three hydrological phases of
472 2022: June (early inundation phase), August (drying phase), and September (late
473 inundation phase). In each sampling period, a total of 80 individuals were collected,
474 consisting of 40 individuals (20 males and 20 females) of *Austrolebias cyaneus* and 40
475 individuals (20 males and 20 females) of *Cynopoecilus nigrovittatus*, were collected
476 using a D-shaped hand net (60 x 30 x30 cm, 2 mm mesh). In order to evaluate the
477 fecundity of the two annual fish species, an *in-situ* experiment was conducted with three
478 treatments: (i) ten buckets (3.6L) with only pairs of *A. cyaneus*, (ii) ten buckets (3.6L)
479 with only pairs of *C. nigrovittatus*, and (iii) ten buckets (3.6L) containing pairs of both
480 species. One pair was separated and maintained per bucket for egg deposition,
481 facilitating their collection and storage.
482 Each bucket contained sediment from the pond and was perforated to allow for
483 water exchange with the habitat and food intake. In the field, the eggs were sieved
484 (1mm) and stored in Falcon plastic tubes (50ml) at for 24 hours (day 1), 48 hours (day
485 2), and 72 hours (day 3) in each sampling period. In the laboratory, the eggs of
486 *Austrolebias cyaneus* and *Cynopoecilus nigrovittatus* were counted using a
487 stereomicroscope. For *Austrolebias cyaneus*, fertilization was verified through the
488 perivitelline space, as described by Fonseca et al. (2018). For *Cynopoecilus*
489 *nigrovittatus*, which has internal fertilization, the eggs were considered inviable when if
490 presenting fungal contamination within the first 24 hours.

The physical-chemical parameters of the water (pH, electrical conductivity, dissolved oxygen (OD), temperature, total dissolved solids (TDS), and oxidation-reduction potential (ORP) were measured using a Horiba Multiparameter U-10 probe, and the depth was measured using a millimeter ruler. The measurement of environmental variables was performed three times in different areas of the temporary pond during each sampling period. The mean values obtained in each seasonal sampling campaign were used in the data analysis.

All individuals were measured using a millimeter ruler (mm) to obtain total length (TL) and standard length (SL) and had their mass measured using a precision balance (0.1g). The individuals were sexed based on morphology and coloration according to Costa (2002, 2006). To analyze egg predation in the interspecific treatment, all individuals of *Austrolebias cyaneus* and *Cynopoecilus nigrovittatus* were preserved using 70% ethanol to assess the presence of chorion in their stomachs and intestines. All procedures employed were ethically reviewed and approved by the Ethics Committee (PPECEUA 12.2019) of UNISINOS.

Data analysis

We used the total number of eggs laid over a 72-hour period. We used generalized linear models (GLM) to evaluate the effect of sampling on the total number of eggs produced by both species separately (monospecific treatment) and in interaction (interspecific treatment). In the monospecific and interspecific treatments, weight and standard length of females and males of each species were included in GLMs as covariates. We used variance inflation factors (VIF; Legendre and Legendre 2012) to detect collinearity between variables. We established a threshold value of 5 and retained

516 variables with VIF below this value (Zuur et al. 2010). This procedure resulted in the
517 retaining of weight and standard length of females and males in GLMs. In these models,
518 we used negative binomial distribution and log-link function after diagnostic tests
519 showed no overdispersion for this distribution. In addition, generalized linear models
520 (GLM) with binomial error distribution and 'logit' link function were used to evaluate
521 the effect of sampling on spawning occurrence (0 = without spawning, 1 = with
522 spawning) in monospecific (only for *C. nigrovittatus*) and interspecific treatments. The
523 influence of weight and standard length of females and males on spawning occurrence
524 was tested only for *C. nigrovittatus*. Statistical significances of the GLMs were assessed
525 using Wald Chi-Square test statistic. Significant interactions were investigated with a
526 Tukey's post hoc test. All analyses were performed using the functions *glm.nb* of the
527 package lme4 (Bates et al. 2015) and *vif* of the package car (Fox and Weisberg 2019)
528 for R (R Development Core Team 2020).
529

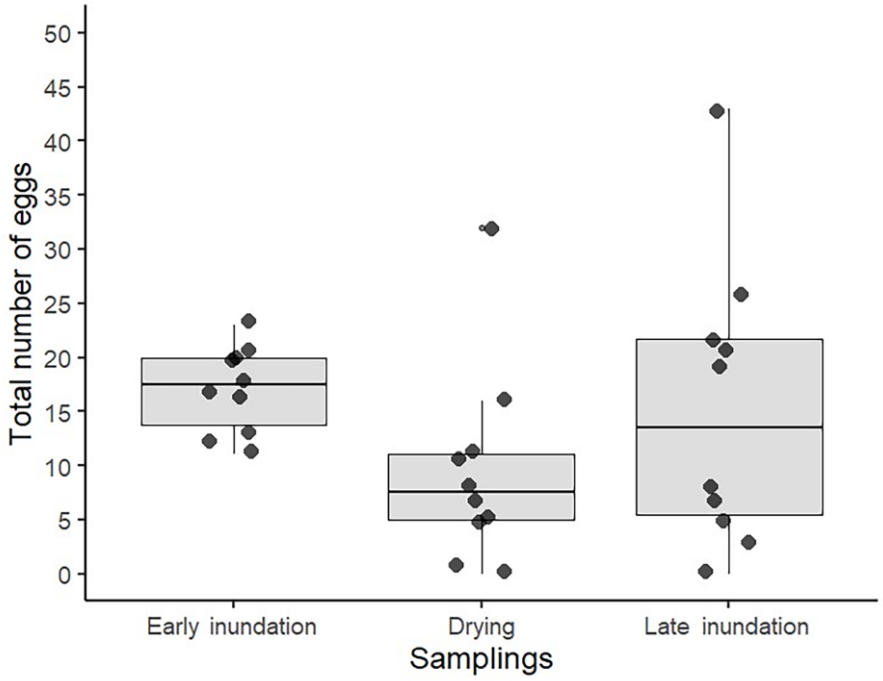
530 **Results**

531
532 The values of the means and standard deviation of the water physicochemical variables
533 over the study (early inundation, drying, late inundation) are listed in Table 1.

534
535 ***Monospecific treatment: Austrolebias cyaneus***
536

537 A total of 421 eggs of *Austrolebias cyaneus* were collected during the study period, with
538 355 fertilized and 66 unfertilized eggs. The total number of eggs produced in the early
539 inundation phase was 171 distributed by all females (40.62%; mean ± standard
540 deviation = 17.1 ± 4.1), followed by 96 eggs produced by nine females in the drying
541 phase (22.80%; mean ± standard deviation = 10.7 ± 9.1) and 154 eggs produced by nine
542 females in the late inundation phase (36.58%; mean ± standard deviation = 17.1 ± 12.9).

543 The total number of eggs of *A. cyaneus* did not vary among the studied samplings
544 (GLM, $P = 0.183$) (see Supporting Information Online Resource 1; Fig. 1). The weight
545 and standard length of females and males did not influence the total number of eggs
546 produced by the species (GLM, $P > 0.05$) (Online Resources 1 and 2).



547

548 Figure 1. Total number of eggs laid by *Austrolebias cyaneus* in each sampling of the hydrological cycle.
549

550

551 ***Monospecific treatment: Cynopoecilus nigrovittatus***

552

553 A total of 195 eggs of *Cynopoecilus nigrovittatus* were collected during the study
554 period, with 176 fertilized and 19 unfertilized eggs. The total number of eggs produced
555 during the early inundation phase was 16 distributed by four females (8.20%; mean ±
556 standard deviation = 4.0 ± 3.8), followed by 59 eggs produced by eight females in the
557 drying phase (30.26%; mean ± standard deviation = 7.4 ± 6.3) and 120 eggs produced
558 by eight females in the late inundation phase (61.54%; mean ± standard deviation = 15
559 ± 15.8). The total number of eggs of *C. nigrovittatus* varied throughout the study period,

being higher in the late inundation phase than in the early inundation phase (GLM, P = 0.008) (Online Resource 1; Fig. 2).

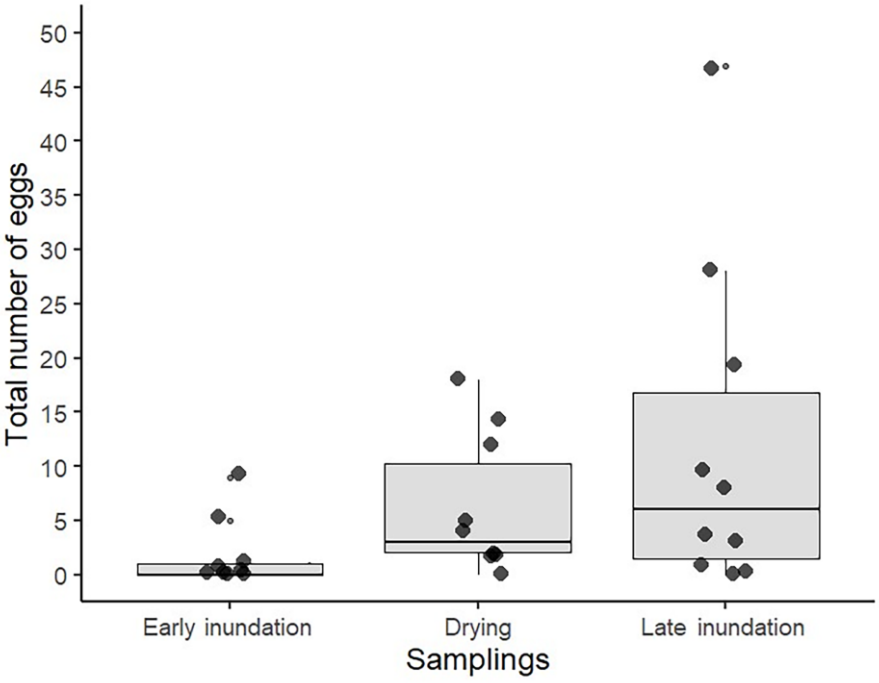


Figure 2. Total number of eggs laid by *Cynopoecilus nigrovittatus* in each sampling of the hydrological cycle.

However, the occurrence of spawning (presence/absence of the eggs) did not vary among the studied samplings (GLM, P = 0.094; Online Resource 3). The standard length of females positively influenced the total number of eggs and the spawning occurrence, with larger females spawned more frequently and laid more eggs than smaller ones (GLM, P = 0.010; Online Resources 1, 2 and 3; Fig. 3; Fig. 4).

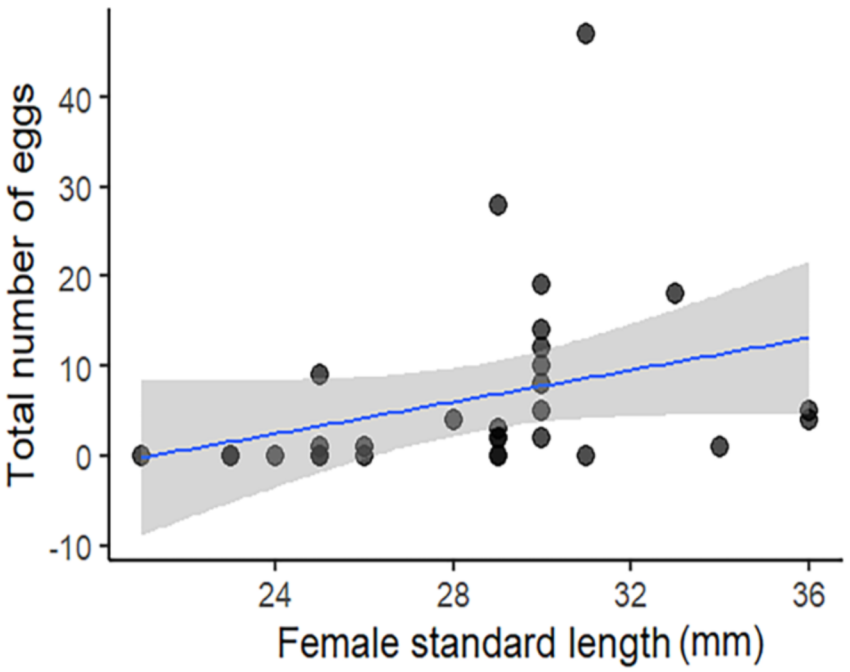


Figure 3. Total number of eggs laid by *Cynopoecilus nigrovittatus* in relation to female standard length.

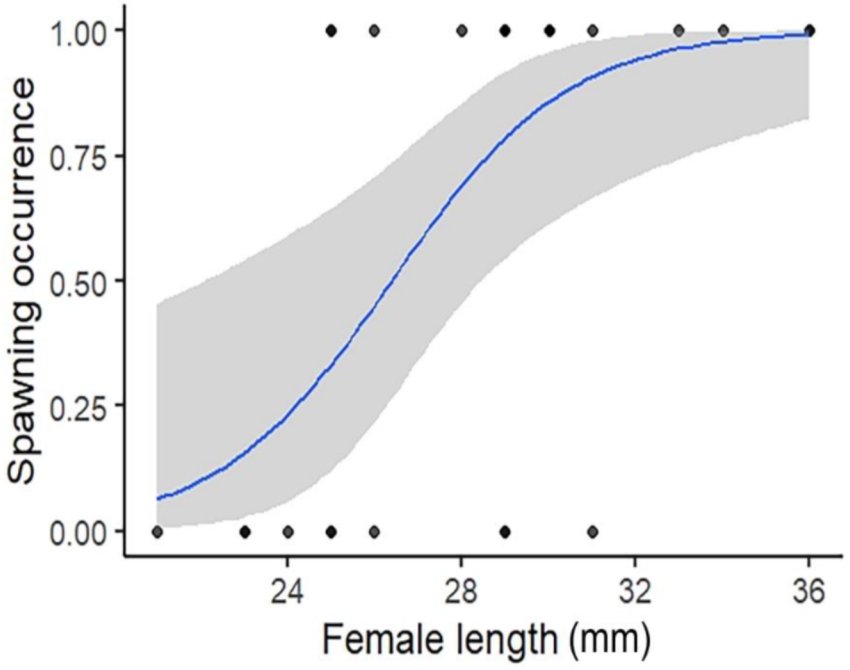


Figure 4. Spawning occurrence (presence/absence of the eggs) of *Cynopoecilus nigrovitattus* in relation to female standard length.

Interspecific treatment: Austrolebias cyaneus and Cynopoecilus nigrovittatus

A total of 213 eggs of *A. cyaneus* (165 fertilized and 48 unfertilized) and 173 eggs of *C. nigrovittatus* (159 viable and 14 nonviable) were collected during the study period. The total number of *A. cyaneus* eggs with the presence of *C. nigrovittatus* was significantly lower only in the early inundation phase when compared to the treatment where *A. cyaneus* was alone (GLM, P <0.001; Online Resource 4, Fig. 5a). In the other samplings, the interaction between the species did not influence the number of eggs of *A. cyaneus* (Fig. 5a).

5a. **5b.**

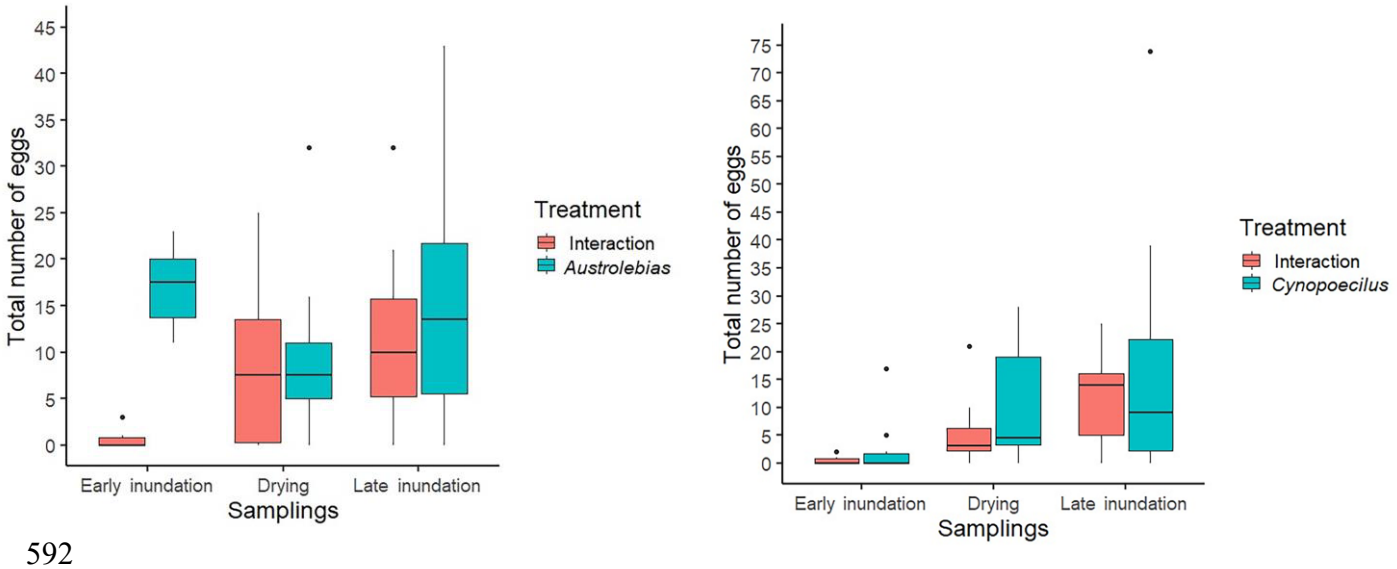


Figure 5a. Total number of eggs laid by *Austrolebias cyaneus* in interaction with *Cynopoecilus nigrovittatus* (Interaction), and in the monospecific treatment (*Austrolebias*). **Figure 5b**. Total number of eggs laid by *Cynopoecilus nigrovittatus* in interaction with *Austrolebias cyaneus* (Interaction), and in the monospecific treatment (*Cynopoecilus*).

The interaction between both species influenced the spawning occurrence of *A. cyaneus*, since no spawnings were more frequent with the presence of *C. nigrovittatus* when

compared to the monospecific treatment, mainly in the early inundation phase (GLM, $P = 0.032$; Online Resource 4, Fig. 6a).

Figure 6a.

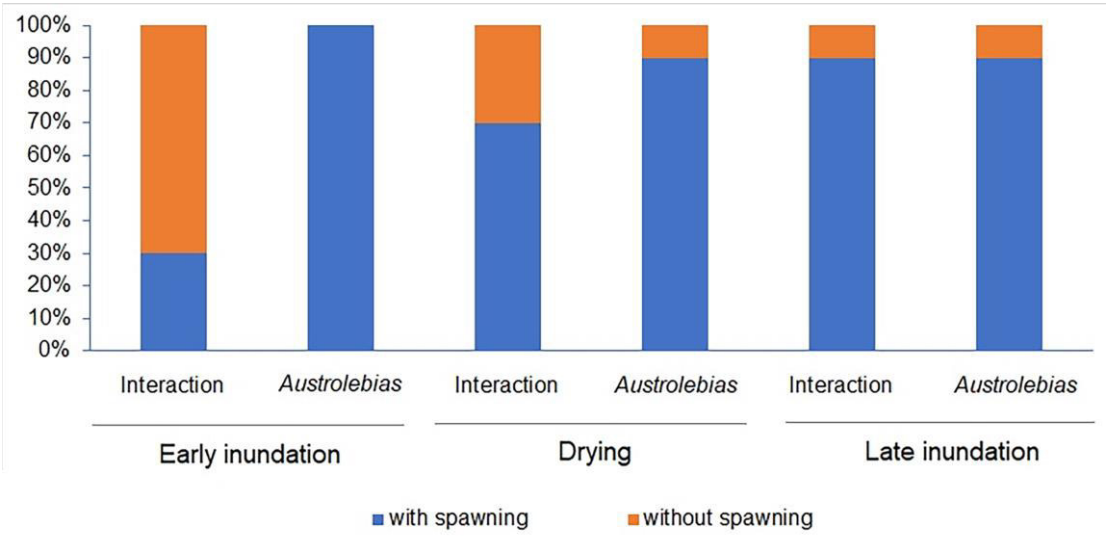


Figure 6b.

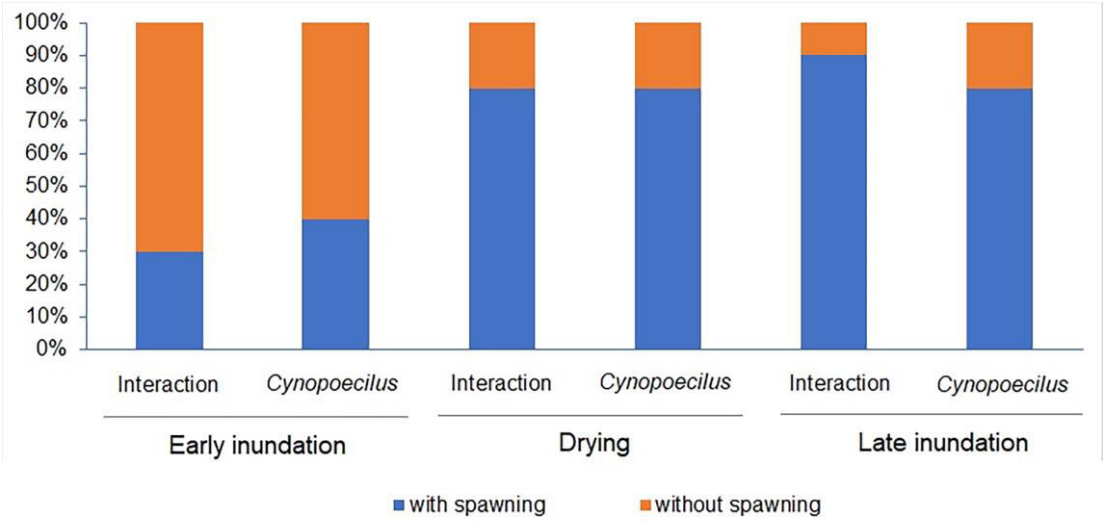


Figure 6a. Spawning occurrence (with and without spawning proportion) of *Austrolebias cyaneus* in interaction with *Cynopoecilus nigrovitattus* (Interaction), and in the monospecific treatment (*Austrolebias*). **Figure 6b.** Spawning occurrence (with and without spawning proportion) of *Cynopoecilus nigrovitattus* in interaction with *Austrolebias cyaneus* (Interaction), and in the monospecific treatment (*Cynopoecilus*).

The number of *C. nigrovittatus* eggs with the presence of *A. cyaneus* was lower when compared to the treatment where *C. nigrovittatus* was alone, considering the

616 entire hydrological cycle (GLM, $P = 0.027$; Online Resource 4, Fig. 5b). The number of
617 eggs was higher in the drying and late inundation phases than in the early inundation
618 phase (*C. nigrovittatus* with the presence of *A. cyaneus*), and in the late inundation
619 phase than in the early inundation phase (*C. nigrovittatus* alone) (GLM, $P = 0.030$;
620 Online Resource 4, Fig. 5b). However, the presence of *A. cyaneus* did not affect the
621 number of eggs of *C. nigrovittatus* in each sampling (Fig. 5b). In a similar way, the
622 interaction between both species did not influence the spawning occurrence of *C.*
623 *nigrovittatus* over the samplings (GLM, $P = 0.580$; Online Resource 4, Fig. 6b). The
624 lack of chorions in the stomachs and intestines of all *Austrolebias cyaneus* and
625 *Cynopoecilus nigrovittatus* individuals indicated the absence of egg predation by both
626 species during the studied period.
627
628 **Discussion**
629
630 Here, we provide the first information on in situ fecundity *for Austrolebias*
631 *cyaneus* and *Cynopoecilus nigrovittatus*, annual fish species that co-occur in a
632 temporary pond in southern Brazil. Our results indicate that there are reproductive
633 differences between *A. cyaneus* and *C. nigrovittatus*. Both species exhibit continuous
634 reproduction, as previously observed for other annual fish (Shibatta 2005; Arezo et al.
635 2007; Gonçalves et al. 2011), and it may be a strategy to increase the chance of survival
636 in the unpredictability of temporary wetlands (Shibatta 2005). However, *A. cyaneus*
637 exhibited constant egg deposition throughout the study period, and the body size of *C.*
638 *nigrovittatus* positively influenced the number of eggs, with greater deposition at the
639 end of the cycle. Furthermore, in the first collection (flood-beginning), most females of

640 *C. nigrovittatus* (60%) did not lay eggs, suggesting the sexual maturity of *C.*
641 *nigrovittatus* may be more later than *A. cyaneus*.

642 Although the studied genera have similar natural history traits, such as annual
643 life cycle, drought-resistant eggs, and accelerated growth (Lanés et al. 2016; Volcan et
644 al. 2018), they exhibit distinct reproductive characteristics. *Austrolebias* has external
645 reproduction, with complex reproductive behavior and egg deposition in the sediment
646 immediately after mating (Garcia et al. 2008), while *Cynopoecilus* has internal
647 fertilization with egg deposits in the water column without the obligatory presence of a
648 male (Costa 1995; Costa et al. 2017). The occupation of different reproductive niches
649 within the temporary wetland may favor the coexistence of *Austrolebias* and
650 *Cynopoecilus*. Studies suggest that the coexistence of annual fish is possible through
651 spatial segregation within wetlands (Nico and Thomerson 1989; Costa 2002). In
652 addition, other non-annual fish species also show an increase in fecundity with
653 increasing female body mass (Barneche et al. 2018). Thibault and Schultz (1978) argued
654 that internal fertilization evolved through modifications in the reproductive system
655 related to the number and size of oocytes. For example, large but few oocytes were
656 observed for *C. fulgens* (Arenzon et al. 1999).

657 The weekly approximate average of eggs laid by *Austrolebias cyaneus* was 35
658 eggs, similar to what has been found for other Rivulidae species. Shibata (2005) found a
659 daily average of about three eggs/day for the species *Simpsonichthys boitonei* in
660 captivity. For other *Austrolebias* species, Calviño (2005) observed a weekly average
661 fecundity of 57 eggs/female for *A. toba*, while Volcan et al. (2012) found a weekly
662 average of 30 eggs per female for *A. nigrofasciatus*, both studies conducted in captivity.
663 The fecundity of wild and *in situ* individuals was tested by Volcan et al. (2011) for *A.*
664 *nigrofasciatus*, where females had an oviposition of 21.5 eggs per week, with lower

665 fecundity than in captivity (Volcan et al. 2012), and a large variation in weekly
666 fecundity ranging from 3 to 39 eggs per female/week. The total number of eggs laid by
667 *Austrolebias cyaneus* in the monospecific treatment of our study did not vary
668 throughout the phases. Every campaign, our broodstock were collected in their natural
669 environment, which may have led to the homogeneity of egg-laying at different phases
670 of the cycle. For *A. nigrofasciatus*, the authors kept the fish confined for four
671 consecutive weeks and reported that prolonged confinement can harm the reproduction
672 of the fish, such as decreasing the size of the eggs (Volcan et al. 2011). The exchange of
673 broodstock throughout the cycle may provide a more precise assessment of the
674 fecundity of females at the time of collection.

675 For *C. nigrovittatus*, our results showed egg laying in all stages of the study,
676 with an increase in fecundity at the end of the cycle. Generally, the reproductive aspects
677 of fish are positively related to body size (Wootton and Smith 2015), including in
678 annual fish (Schalk et al. 2014). Arenzon et al. (1999) observed that females of *C.*
679 *fulgens* have more mature oocytes as they grow. Reproductive studies on the
680 *Cynopoecilus* genus have been carried out through gonadal analyses. Gonçalves et al.
681 (2011) found mature ovaries ranging from 2-157 oocytes for *Cynopoecilus*
682 *melanotaenia*. Arenzon et al. (1999) found 49-219 mature oocytes for *C. fulgens*.
683 Reproductive studies with fecundity data of *Cynopoecilus*, or closely related species, for
684 comparative purposes are non-existent (Gonçalves et al. 2011). Our work elucidates the
685 first results of oviposition for the *Cynopoecilus* genus.

686 When the species *Austrolebias cyaneus* and *Cynopoecilus nigrovittatus* were in
687 interaction, there was a reduction in the fecundity of *Austrolebias* only at the beginning
688 of the cycle compared to the monospecific treatment. For *Cynopoecilus nigrovittatus*,
689 there was a reduction in fecundity when in co-occurrence considering the entire

690 hydrological cycle, but the interaction between species did not affect the number of eggs
691 of *C. nigrovittatus* in each sampling. The co-occurrence of annual fish in a single
692 wetland area suggests the existence of ecological relationships between the species
693 (Costa 2009; Canavero et al. 2014) and may be possible mechanisms responsible for
694 creating variability and diversity within the pools (D'Anatro and Loureiro 2005).
695 However, understanding how species coexist is still a fundamental challenge (Volcan et
696 al. 2018).

697 Our results show that the species *Austrolebias cyaneus* and *Cynopoecilus*
698 *nigrovittatus* exhibit egg laying throughout their entire life cycle. Annual fish usually
699 reproduce until their senility or death (Arenzon et al. 1999; Gonçalves et al. 2011),
700 forming a stock of eggs in the substrate that can, along with different stages of
701 embryonic development, protect the species from a single massive hatching during a
702 very short rainy period (Arenzon et al. 1999). This continuous effort in egg production
703 is evolutionarily considered a way to ensure the persistence of these species in
704 ecosystems with high environmental stochasticity that are so extreme and unpredictable,
705 such as temporary wetlands (Vrtílek and Reichard 2015).

706 Annual killifish exhibit a unique life history among vertebrates (Berois et al.
707 2012; Blažek et al. 2013; Cellerino et al. 2015). There are several ongoing studies on
708 basic aspects of the reproductive biology of these species. Even simple information such
709 as fecundity is lacking for most species of annual killifish in the neotropical region. In
710 this sense, we present the first data on reproductive aspects of *A. cyaneus* and *C.*
711 *nigrovittatus*. These results obtained from wild populations should help to fill the
712 knowledge gap on biological traits, which impairs the understanding of the ecology of
713 annual killifish in small temporary wetlands.

714 Finally, our study was carried out in just one temporary pond. The lack of
715 studies in other habitats was due to the difficulty in finding other temporary ponds with
716 the presence of the two species studied. *A. cyaneus* has been observed so far in only 6
717 other ponds in the world and in none of them co-occurs with *C. nigrovittatus*. These
718 data are unique for science, and it is the first study to survey in situ fertility for the
719 genera studied.

720

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Variables/Samplings	Early inundation	Drying	Late inundation
Area (m^2)	6,709	5,459	7,373
Water temperature (°C)	17.6±0.9	19.5±2.2	21.6±3.7
pH	5.4±0.4	5.4±0.8	5.8±0.4
Oxidation-reduction potential	223.7±42.1	147.0±95.3	132.3±33.5
Electrical conductivity (Ms/cm)	0.3±0.4	0.04±0.01	0.1±0.1
Dissolved oxygen (mg/L O_2)	6.9±2.1	6.3±2.5	9.7±2.2
Total dissolved solids	0.02±0.01	0.02±0.01	0.05±0.03
Water depth (cm)	48.0±8.7	40.0±4.1	57.0±13.4

Tabela 1. Mean ± standard deviation of the water physical and chemical variables of the study pond throughout the hydrological phases.

CAPÍTULO 2

CAPÍTULO 2 - Changing your bets when the game changes: adjustments in the egg development of a Neotropical killifish from temporary wetlands under different environmental flooding scenarios

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Abstract

Organisms facing variable and unpredictable environmental conditions often employ bet-hedging strategies, a risk-spreading approach that involves diversifying "bets" across different possible scenarios. While bet hedging may temporarily reduce fitness, it minimizes long-term temporal variance and enhances overall fitness. We aimed to investigate a refined form of bet hedging. In this sense, are organisms capable of adjust their initial bets in response to changing probabilities of future outcomes? Our research focused on seasonal killifish, known for its adaptations to temporary wetlands including desiccation-resistant eggs capable of undergoing up to three developmental arrests known as diapauses. Killifish exhibit bet hedging in their development and hatching strategies, enabling them to cope with stochastic filling and drying of their habitats. We tested whether *Matilebias cyaneus* from the Pampasic region could dynamically "change their bets" in response to varying hydrological cycles by combining field and laboratory procedures to assess adjustments in embryo development stages. When compared, embryos from a winter drying scenario (soon reflooding expected) versus a spring drying scenario (later reflooding expected) exhibited a greater proportion escaping diapause I, shorter developmental times, and higher hatching rates. This confirmed our hypothesis, suggesting physiological or environmental cues play a role in these adaptive responses.

Keywords: annual fish, bet hedging, diapause, temporary ponds, drought, survival strategy

Introduction

In a casino roulette wheel, a prudent gambler minimizes losses by spreading bets across multiple options rather than concentrating all chips on a single number. This strategy, known as bet hedging, reduces the risk of total loss on a single spin and increases the likelihood of smaller, albeit more frequent, payouts. Similarly, organisms habitats may present variable unpredictable environmental conditions over a fixed range, (i.e. wet years, and dry years), this may be a challenge for a fixed phenotypic set of characters optimal for only one of those scenarios so an evolutionary strategy analogue to the casino roulette example would be to have offspring with different sets of characters best fitted for the different possible conditions (Oloffson et al., 2009; Gianella et al., 2021). Although this strategy may reduce the fitness at the short term (i.e., lowered arithmetic mean), it reduces the temporal variance in the long-term and enhances fitness over longer timescales (Oloffson et al., 2009; Furness et al., 2015).

A refinement of this strategy would involve the casino player having access to additional information during the game, enabling them to adjust their bets. For instance, if the player could modify their bets mid-game upon discovering that the roulette wheel was rigged and that a particular number was more likely to land, they could increase their chances of success. In the presence of new information altering the probabilities of potential future outcomes, being able to adapt their bets accordingly would enhance their likelihood of success compared to being constrained to their initial bets. We aim to investigate this type of refinement of bet-hedging strategies in our research, to see if species can use environmental signals and fine-tuning their “bets” at specific point of their life cycle in response to changing conditions to different possible scenarios.

In this study, we explore this intriguing hypothesis by focusing on seasonal killifishes (Cyprinodontiformes; Aplocheiloidei). These fish inhabit temporary wetlands that

1007 expose them to peculiar and uncommon environmental conditions, such as extended periods
1008 of drought (Costa, 2002). African and South American seasonal killifish species,
1009 respectively represented by the families Nothobranchiidae and Rivulidae, present
1010 remarkable adaptations to such ephemeral aquatic environment such as accelerated growth,
1011 early sexual maturity (Pinceel et al., 2015; Polačik et al., 2018; Godoy et al., 2023), and
1012 desiccation-resistant eggs capable of undergoing up to three stages of development arrest
1013 known as diapauses (Wourms, 1972a, 1972b, 1972c). Diapause consists of a period of
1014 extremely low metabolic, cellular, and developmental activity, which enables embryos to
1015 endure adverse conditions (Podrabsky et al., 2017).

1016 Furness *et al*. (2015) demonstrated that seasonal killifish eggs vary at multiple levels
1017 of their development (e.g., trajectory, development time, hatching timing) consistent with
1018 bet-hedging. For instance, not all diapausing eggs in a single wetland are in the same
1019 developmental stage or hatch after individual flood events (Domínguez-Castanedo et al.,
1020 2022, Polačik et al., 2021; 2023), resulting in a diverse set of hatching times (Pinceel et al.,
1021 2015; Polačik et al., 2017; 2018). This strategy for example copes with short aquatic phases
1022 (filling and drying in a very short time) when not all eggs will hatch and therefore,
1023 increasing overall descendants survival probability, as a subset of eggs remains viable for
1024 hatching in the next flooding event (Wourms, 1972a, 1972b, 1972c). Generally killifish
1025 habitat have a more or less regular period of rains, for example in the Pampasic region it is
1026 generally in autumn and spring while presenting a long dry summer and a short medium dry
1027 winter (i.e.: Alonso et al. 2016) which may vary between dry and wet years in their extents,
1028 therefore presenting one or two cycles per year, depending if winter drying is complete or
1029 only partial. Despite the knowledge that flood events are key hatching cues for killifish eggs
1030 (Garcia et al., 2019), whether this fish can adjust their development for hatching after a short
1031 (winter) or long (summer) dry cycle according to the point of the year when the drying of

the pond occurs is enigmatic. Individual flood events in these wetlands were related to the population structure of killifishes of the region, suggesting that flood dynamics affect the development of the egg bank of this fauna (Lanés et al. 2016; Garcia et al., 2019). Recent studies also showed that eggs from Neotropical killifishes adjust several aspects of their development under variable conditions (Godoy et al. 2023). Therefore, here we want to test this hypothesis to see if *Matilebias cyaneus* a killifish endemic to temporary wetlands of the Pampasic region (Volcan et al., 2011) "changes its bets" in a changing scenario. For this we combined field and laboratory procedures to test whether embryos adjust their development stage (trajectory, development time and hatching patterns) accordingly over the hydrological cycle.

In a winter drying scenario, characterized by a short aquatic phase with filling in autumn, we anticipate a refilling of the wetland in the short term during the spring rainy period. In contrast, in a later drying scenario occurring in late spring, marked by a long aquatic phase with only partial drying in winter, a prolonged dry period in summer is expected, with the next flooding likely to occur in the following year's autumn. Therefore, it is expected that embryos from the winter drying scenario, when compared to those from the late drying scenario, will exhibit the following differences: 1) a greater proportion of embryos escaping diapause I, 2) shorter developmental times, and 3) a higher hatching proportion across a series of sequential simulated floodings. This expectation is supported by the reasoning that embryos from the late drying scenario should be more likely to delay hatching and avoid responding to short-term rains during the hot summer that are likely to result in unsuccessful recruitments.

By exploring the reproductive adaptations of killifish in response to the highly variable conditions of their temporary wetland habitats, our research aims to shed light on the fascinating interplay between bet hedging and environmental fluctuations, offering

valuable insights into the survival strategies of organisms in unpredictable environments. Furthermore, this study may uncover a refined "bet hedging 2.0" strategy, where organisms demonstrate the ability to adjust their reproductive decisions dynamically, optimizing their fitness in ever-changing surroundings.

Material and methods

Study species and ecosystems

Matilebias cyaneus (Amato 1987) is a Neotropical killifish species native to the Pampas region. It resides in temporary wetlands within the Jacuí River basin, part of the Laguna dos Patos system, situated in Río Grande do Sul, Brazil. The region experiences a subtropical climate with an annual precipitation of approximately 1600 mm, with a higher concentration during the cooler months (July to November) (Volcan et al., 2011).

Sampling procedures

To examine the hatching rates and development trajectories of *M. cyaneus* fluctuating environmental conditions, we conducted three sampling events in 2022. The first sampling event occurred in June, during the initial superficial water phase, marking the beginning of inundation. The second sampling took place in August, when the water surface was diminishing, representing the drying phase (But still with water and sexually active animals). Lastly, the third sampling was carried out in September, following a reflooding event triggered by new precipitation. The first collection campaign was conducted in June as it was the time when the annual fish reached sexual maturity, and in October, it was not possible to collect males. Typically, annual fish populations experience a decline in males towards the end of their cycle (Lanés et al., 2016).

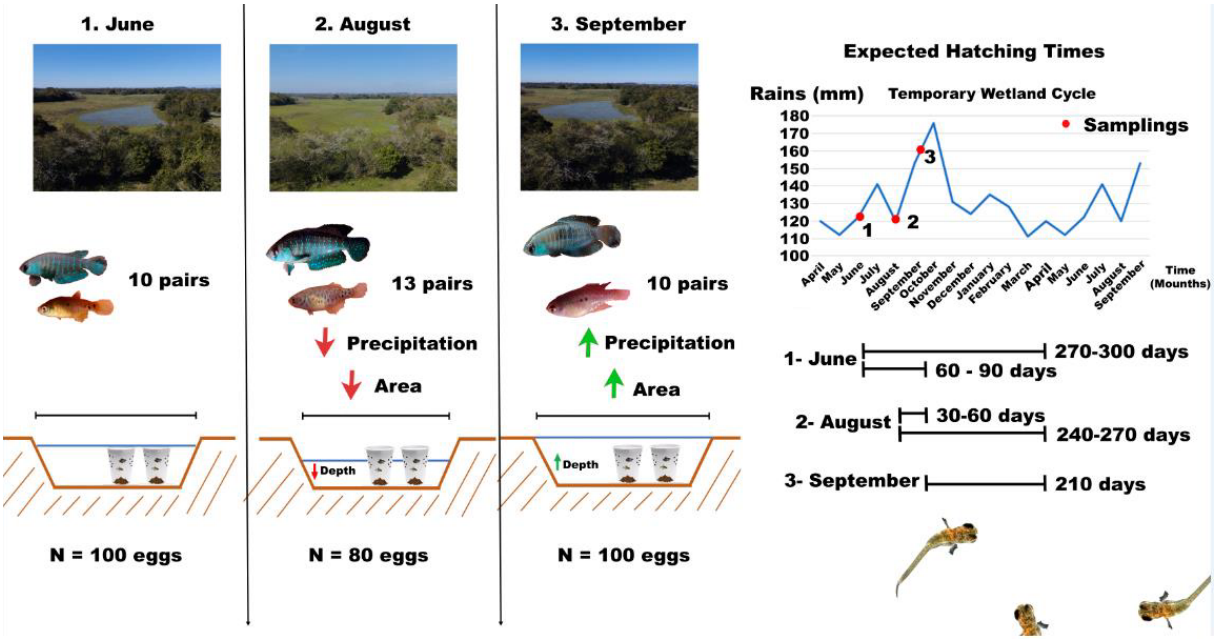


Figura 1.Schematic representation of the temporary wetland filling drying cycles and sampling methodology, evidencing the logical basis of the hypothesis development and the expected results.

In each sampling event, fishes were placed in pairs, consisting of a male and a female, within a box to facilitate egg collection. In total, we collected 66 fish individuals: 20 individuals (10 pairs) in June, 26 individuals (13 pairs) in August, and 20 individuals (10 pairs) in September. For each sampling period, we collected an equal number of males and females. *M. cyaneus* shows strong sexual dimorphism, with males being larger and more brightly colored than females and possessing the base of the dorsal and anal fins longer than females. These sexually dimorphic characteristics were used to differentiate and sex the specimens in the field (Costa, 2006) (see Fig 1).

Egg collection and incubation

In each sampling event, eggs were collected over three consecutive days (24, 48 and 72 h) from each box. The eggs were obtained from the plastic containers (3.6 L) in which the paired fish were kept. The containers were designed to simulate natural egg-laying

conditions and contained sediment from the fish's native environment. To allow water exchange and food supply, we opened holes in each container. The collected eggs were stored in Falcon-type plastic tubes (50 ml) containing water from the natural environment and transported to the laboratory. In the laboratory, each egg was examined under stereomicroscope to verify fertilization by the presence of the perivitelline space, following Fonseca et al., (2018).

Experiment (1): Assessment of development trajectory and time

To test the prediction as to whether killifish eggs laid at different periods of a temporary wetland's flooding-drying cycle vary in their development trajectories and total development time, we carried out a two-step assessment:

In the assessment of development trajectories, 120 eggs were used to investigate diapause I. These eggs were individually placed in Falcon tubes (15 ml) with water from the natural environment and kept in the dark at 24 degrees Celsius for 30 days. After 30 days, there were 100 eggs from June, 80 eggs from August, and 100 eggs from September remaining for the analyses. In the second stage, the eggs used in the diapause I experiment were transferred to bacterial culture microplates (48-well plate) with Yamamoto solution. The eggs were maintained at 24°C in the dark, and their developmental stage was assessed weekly under a stereomicroscope.

Experiment (2): Assessment of hatching rates

To test our hypothesis regarding whether killifish eggs laid at different times during a temporary wetland's flooding-drying cycle vary in their hatching strategies in relation to their hatching fractions, we utilized a subset of 135 eggs that had reached diapause III (45 eggs from each sampling campaign).

All the eggs were individually maintained in bacterial culture microplates (16-well plate) within a climate-controlled room at a temperature of 24 degrees Celsius and kept in darkness, on a substrate from the natural environment, which remained dry for 30 days to simulate the dry season. We subjected the eggs to five sequential hydration events, aiming to simulate floods in the natural environment (1st hydration: 30 dry days; 2nd hydration: 51 dry days; 3rd hydration: 81 dry days; 4th hydration: 131 dry days; 5th hydration: 175 dry days). The inundations were conducted using deionized water (2 cm above the substrate) for a duration of 24 hours. Hatched fries were counted under a stereomicroscope, and unhatched eggs were returned to the pre-established dry periods, followed by rehydration.

Data analysis

Experiment (1): Assessment of development trajectory and time

We first annotated the development trajectory employed by each egg, which encompassed one of four possibilities:

(iv)skipping diapause I/skipping diapause II;

(ii) skipping diapause I/entering diapause II;

(iii) entering diapause I/skipping diapause II, and;

(iv) entering diapause I/entering diapause II

To test eggs laid at different periods of a temporary wetland’s flooding-drying cycle vary, we conducted a multinomial logistic regression with a logit link function. The significance

1156 of the model was assessed using the likelihood ratio test. Additionally, we performed three
1157 post-hoc multinomial logistic regressions (one for each pair of temporary cycle phase
1158 levels), and these pairwise differences were corrected using Holm's sequential Bonferroni
1159 procedure.

1160 To test whether the total development time of killifish eggs vary in relation to the
1161 period of a temporary wetland's flooding-drying cycle and development trajectory, we used
1162 generalized linear mixed models (GLMM) with a negative binomial error distribution. The
1163 response variable in this analysis was the number of days from fertilization to diapause III.
1164 Female identity was incorporated as a random-effect component in the model to account for
1165 individual differences. For this analysis, we excluded embryos that did not complete their
1166 developmental trajectory and, therefore, the dataset included N = 225 embryos.

1167

1168 **Experiment (2): Assessment of hatching rates**

1169

1170 To assess potential differences in hatching rates among eggs laid at different periods
1171 of the temporary wetland's flooding-drying cycle showed variable hatching fractions across
1172 the sequence of five inundation events, we applied loglinear models for tests of association.
1173 To examine the temporal variation in embryonic development strategies, we analyzed the
1174 developmental trajectories of embryos deposited at different time points during the flooded
1175 phase. We calculated proportions of embryos exhibiting different developmental outcomes,
1176 such as escape from diapause I and II, delayed development, or mortality. To explore
1177 potential maternal effects, we correlated the observed developmental patterns with the
1178 recorded environmental variables, including temperature and humidity. Statistical analyses,
1179 such as correlation tests or regression models, were performed to determine the relationships
1180 between these variables. Furthermore, to assess the general trends and patterns in embryonic
1181 development, we conducted comparative analyses among different temporal intervals within

1182 the flooding phase. This allowed us to identify any significant differences in developmental
1183 trajectories and survival rates.
1184

1185 ## Results

1186 ### Development trajectories

1187
1188 Eggs laid at different periods of the temporary wetland's flooding-drying cycle
1189 showed variable trajectories, as revealed by an analysis of variance based on multinomial
1190 logistic regression (χ^2 = 36.64, $p < 0.001$). Post-hoc multinomial logistic regressions
1191 indicated significant differences in the proportion of eggs using each development trajectory
1192 among early- and mid-laid eggs (χ^2 = 41.52, $p < 0.001$), early- and late-laid eggs (χ^2 = 77.51,
1193 $p < 0.001$) and mid- and late-laid eggs ($\chi 2$ = 12.36, $p < 0.006$). A higher number of early-
1194 laid eggs skipped both diapause I and II, while a higher number of mid- and late-laid eggs
1195 entered diapause I and skipped diapause II (Fig. 2).
1196

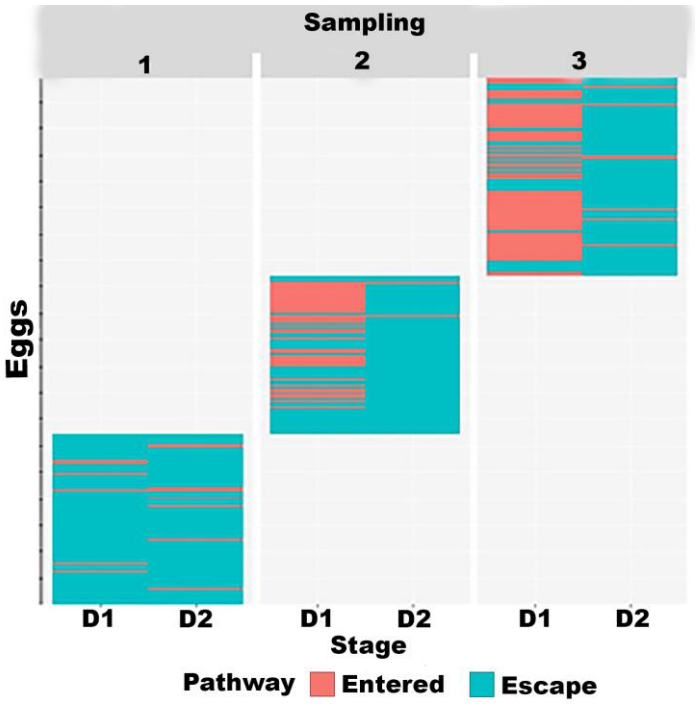


Figura 2. A higher number of early-laid eggs skipped both diapause I and II, while a higher number of mid- and late-laid eggs entered diapause I and skipped diapause II.

Development time

The interaction between periods of the temporary wetland's flooding-drying cycle and trajectory significantly influenced total development time (Table 1). Early-laid eggs that skipped both diapause I and II exhibited shorter development time compared to mid- and late-laid eggs that entered diapause I (Fig. 3).

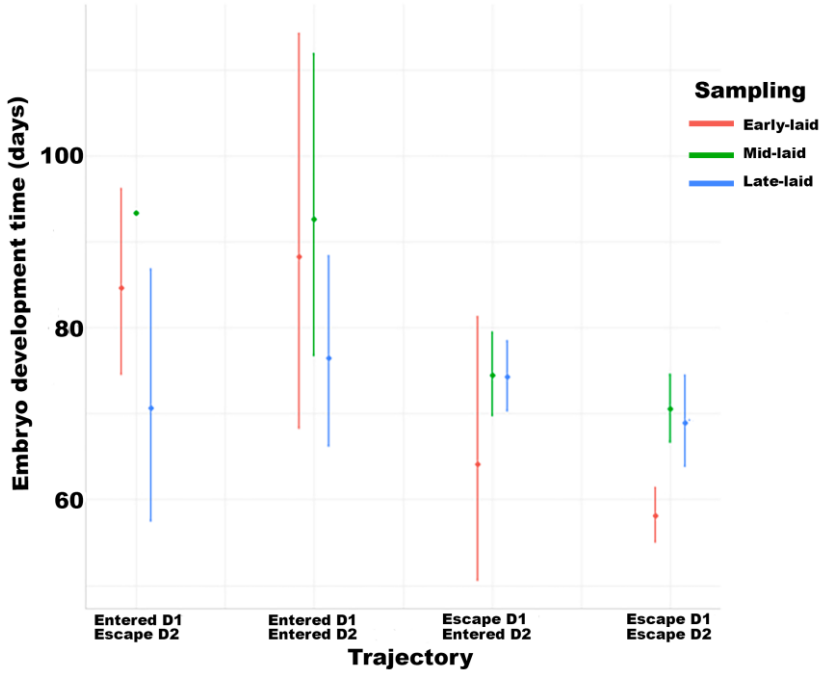
Table fixed effects

Chisq	Chisq	Df	*P*
Trajectory	37.642	3	<0.001
Temporary cycle phase	25.246	2	<0.001
Temporary cycle phase*Trajectory	12.262	5	0.031

Table 1. The interaction between periods of the temporary wetland's flooding-drying cycle and trajectory significantly influenced total development time

1212 Table fixed effects

1213



1214

1215 **Figura 3. Early-laid eggs that skipped both diapause I and II exhibited shorter development time**
1216 **compared to mid- and late-laid eggs that entered diapause I.**
1217

Hatching rates

Eggs laid at different periods of the temporary wetland's flooding-drying cycle showed variable hatching rates across inundation events ($\chi 2 = 151.11$, $p < 0.001$). Pearson residuals indicated that early- and mid-laid eggs exhibited higher hatching rates in the first inundation events, while embryos from mid- and late-laid eggs avoided hatching in the third inundation event (Fig. 4).

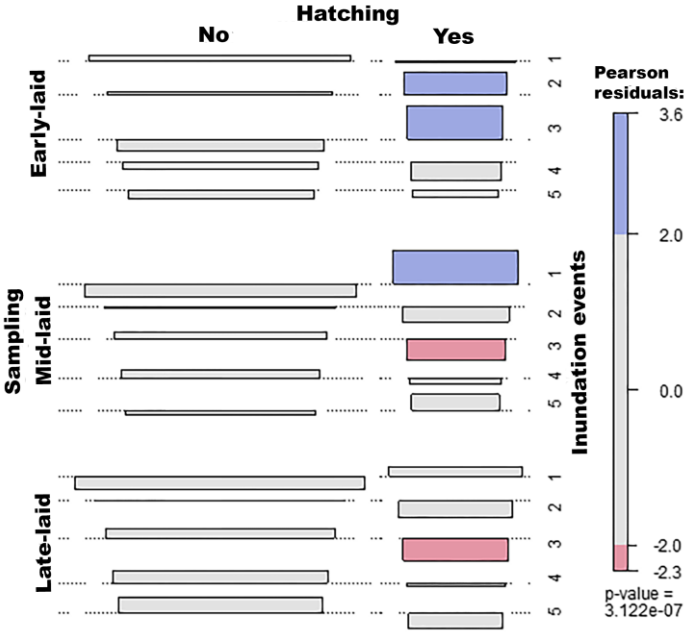


Figura 4. Pearson residuals indicated that early- and mid-laid eggs exhibited higher hatching rates in the first inundation events, while embryos from mid- and late-laid eggs avoided hatching in the third inundation event.

Discussion

In this study, we combined field-based and laboratory procedures to test whether the eggs of a Neotropical killifish (*Matilebias cyaneus*) laid at different periods of a temporary wetland's flooding-drying cycle adjust specific aspects of their development (trajectory, development time and hatching) to different expected flooding scenarios.

Given the interannual variability in the duration of the flooding season in the temporary wetlands of the South American Pampa (as discussed by Alonso et al., 2016; Lanés et al., 2016; Volcan et al., 2020), the emergence of 'bet-hedging' strategies can be anticipated in response to these unpredictable conditions, aiming to mitigate the depletion of offspring due to the risk of premature inundation events, as highlighted by Simons (2011). In this context, eggs laid and dried early in the flooding season, typically occurring in June and prior to partial desiccation during the winter months (July-August), are expected to hatch promptly with the onset of the following spring flood. Consequently, these early-laid eggs exhibit distinct developmental trajectories compared to eggs deposited later in the flooding season, typically around September. The latter eggs are expected to endure an extended dry period during the summer months and hatch in the subsequent autumn. Conversely, as the flooding season draws to a close, it is anticipated that all embryos will enter diapause (as observed by Furness et al., 2015), primarily due to the expectation of an extended dry season during the summer, with precipitation projected for the following year's autumn, and consequently, individuals hatching in response to eventual summer rains are at risk of perishing before reaching sexual maturity.

Here, early-laid eggs skipped diapause I and had shorter development times compared to mid- and late-laid eggs. Those findings are coarsely consistent with the predictions by Podrabsky *et al*., (2010) and Furness *et al*., (2015) on the adjustment of development trajectory in seasonal killifishes in relation to time of the year, as the autumn-

1257 laid eggs could take advantage of likely additional flood events until to spring, while the
1258 late-laid eggs (September, toward end of the flooding season) would be expected to enter
1259 diapause, as the wetland would only refill in April. Our results are consistent to field-based
1260 reports of additional cohorts in seasonal killifish populations developing after flash floods in
1261 mid-winter (Lanés et al., 2016; Garcia et al., 2019).

1262 Some insects in temperate regions show the ability to switch from direct
1263 development eggs (capable of completing a second generation) to producing diapause eggs
1264 that survive the cold winter (Cohen, 1970; Taylor, 1980; Bradford and Roff, 1993).
1265 Similarly, embryos of seasonal fish that develop more rapidly could take advantage of
1266 additional floods and reproduce before the puddles dry up. We found that early-laid eggs
1267 exhibited shorter development times compared to late-laid eggs that used different
1268 development trajectories. In this context, the production of offspring with variable
1269 development length is thus consistent with bet-hedging (Stearns, 1976). This could be
1270 beneficial because acceleration ensures successful hatching within the narrower window of
1271 suitable conditions, potentially enhancing their survival chances. Furthermore, consistently
1272 with our hypothesis, for the African killifish species *Nothobranchius furzeri*, it has been
1273 demonstrated that faster-developing embryos exhibit faster post-hatching growth, reaching
1274 sexual maturity early, and aging more rapidly compared to slower-developing embryos
1275 (Polačik et al., 2014). This suggests that each flooding event may provide seasonal killifish
1276 with a new opportunity to replenish the egg bank in the sediment.

1277 Moreover, our study shed light on the timing and nature of the transition from direct
1278 development to diapause in egg clutches of *M. cyaneus*. This is because the heterogeneity in
1279 season length raises the question about how eggs switch from direct development to
1280 diapause (Furness et al., 2015). Our results showed that the transition in the development
1281 pathways in the egg clutches of *M. cyaneus* was gradual (not bang-bang). Such gradual

1282 transition is line with previous field-monitoring studies showing that killifish eggs (same
1283 'age') tend to develop synchronously over a considerable part of the flooding season
1284 (Polačik et al., 2021).
1285

1286 **Hatching rates**

1287

1288 Under a bet-hedging perspective, one could expect that partial hatching (i.e., the
1289 hatching of only a subset of the egg bank after each hydration event) evolved as a risk-
1290 spreading strategy that avoids depletion of the entire egg bank after "false start" (Furness et
1291 al., 2015). Given the chance of false start of flooding seasons in ephemeral wetlands, one
1292 could thus expect variable hatching fractions after individual floods (despite complete
1293 embryo development) (Furness et al., 2015). Authors evidenced that not all killifish
1294 diapausing eggs hatch after individual floods (Domínguez-Castanedo et al., 2022; Polačik et
1295 al., 2021; 2023). Consequently, if bet-hedging regarding the relationship between eggs laid
1296 at different periods of a temporary wetland's hydrological cycle and sequential hydration
1297 cues hold true, most part of the eggs laid early in the flooding season would require fewer
1298 hydration cues (and thus hatch in the first floods), and our results mostly corroborated this
1299 prediction as hatching strategy is variable among eggs with different ages across a series of
1300 inundation events.

1301 Hatching fractions of mid- and early-laid eggs were positively associated with the
1302 first inundation events, while hatching fractions of mid- and late-laid eggs were negatively
1303 associated with the third inundation event. This strongly suggests that killifish eggs vary in
1304 their hatching strategies over the flooding-drying cycle of a wetland's hydrological cycle
1305 and point out for an increase in the 'hedge' towards the late-flooding season, even under
1306 controlled-environment settings. Maternal age seems like a probable explanation to

influence hatching rates, possibly indicating a strategy to avoid unfavorable conditions. Such variable hatching strategies detected in controlled-environment setting agree with the notion that laboratory conditions are typically insufficient to overcome the genetic and epigenetic underpinning and the intrinsic developmental code prevails (Polačik et al., 2021).

In conclusion, the availability of good quality pool duration data presents an opportunity to predict the percentage of embryos expected to hatch at each flooding event within the population. This opens avenues for further research to investigate the interplay between environmental cues, maternal effects, and within-season hatching strategies. Understanding the underlying mechanisms and triggers that lead to the adjustment of development pathways and hatching rates in response to environmental fluctuations can provide valuable insights into the adaptive responses of organisms to variable habitats.

Our field-based study on seasonal killifish, *Matilebias cyaneus*, makes significant contributions to the understanding of bet-hedging strategies in response to unpredictable environmental conditions. The observed adjustment of development pathways, embryo development time, and hatching rates align with previous hypotheses and call for further ecological investigations to comprehensively understand the adaptive strategies employed by these fish in their variable habitats. Our research advances the understanding of environmental signaling in killifish, highlighting remarkable adaptations that enable them to thrive in fluctuating and challenging environments of temporary wetlands.

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ANEXOS

SERVIÇO PÚBLICO FEDERAL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG
PROPESP - COMISSÃO DE ÉTICA EM USO ANIMAL

PARECER N° 48, DE 30 DE MARÇO DE 2023

Certificamos que o projeto intitulado "Amadurecimento precoce e vida curta: como o envelhecimento rápido afeta a reprodução dos peixes anuais?", protocolo n^0 23116.003720/2023-20, sob a responsabilidade de Leonardo Maltchik Garcia
- que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 29 de março de 2023 (Ata 004/2023).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq022/2022
COLABORADORES AUTORIZADOS A MANIPULAR OS ANIMAIS	Vinicius Weber, Pedro Henrique de Oliveira Hoffmann, Giliandro Gonçalves Silva, Robson Souza Godoy
VIGÊNCIA DO PROJETO	01/01/2024
ESPÉCIE / GRUPOS TAXONÔMICOS	*Austrolebias* sp., *Austrolebias cyaneus*, *Cynopoecilus* sp., *Cynopoecilus nigrovittatus*

NÚMERO DE ANIMAIS	300 ovos de *Austrolebias* sp. e *Cynopoecilus* sp. 30 machos e 30 fêmeas de *Austrolebias cyaneus* e *Cynopoecilus nigrovittatus*
N SOLICITAÇÃO / AUTORIZAÇÃO SISBIO	83141-1 e 68290-8

ATIVIDADE(S)	(X) CAPTURA (X) COLETA DE ESPÉCIMES ( ) MARCAÇÃO ( ) OUTRAS:
LOCAL(is) REALIZAÇÃO ATIVIDADES	Município de General Câmara, RS
ENVIO DE RELATÓRIO PARCIAL	-
ENVIO DO RELATÓRIO FINAL	Janeiro de 2024

Documento assinado eletronicamente por Marcio de Azevedo Figueiredo , Servidor, em 30/03/2023, às 11:59, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Referência: Caso responda este documento Parecer, indicar o Processo nº 23116.003720/2023-20 SEI nº 003685

PARECER 02.2022

A Comissão de Ética no Uso de Animais – CEUA da Universidade do Vale do Rio dos Sinos - UNISINOS analisou o adendo ao projeto abaixo descrito:

Código de protocolo: PPECEUA 12.2019 (adendo)

Versão: 05.2022

Título: "ECOLOGIA DE OVOS DE PEIXES ANUAIS ENDÊMICOS DO EXTREMO SUL DO BRASIL: IMPLICAÇÕES PARA SUA CONSERVAÇÃO, DISTRIBUIÇÃO E DIS- PERSÃO DE ESPÉCIES".

Coordenador: Profa. Cristina Stenert Maltchik Garcia

Departamento: PPG em Biologia – Laboratório de Ecologia e Conservação de EcossistemasAquáticos - UNISINOS

DECISÃO da CEUA: as modificações/novas informações no projeto, foram APROVADAS, por estarem adequadas ética e metodologicamente e de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com a Diretriz Brasileira para o Cuidado e a Utilizaçãode Animais para Fins Científicos e Didáticos – DBCA e com a Resolução UNISINOS 04/2013.

O proponente deverá encaminhar relatório final sobre o andamento do projeto à CEUA – UNISINOS, comunicar à mesma qualquer alteração na equipe ou na metodologia prevista, com vistas ao preenchimento do relatório anual da CEUA junto ao CONCEA.

São Leopoldo, 06 de maio de 2022.

Tanise Gemelli
Coordenadora CEUA - UNISINOS

ATA DE DEFESA DE DISSERTAÇÃO DE MESTRADO EM BIOLOGIA DE AMBIENTES AQUÁTICOS CONTINENTAIS – Nº 002/2024

Às 14:00h (quatorze horas) do dia 04 (quatro) do mês de abril de 2024 (dois mil e vinte e quatro), via Webconferência no endereço eletrônico: (https://conferenciaweb.rnp.br/sala/glauco-cesar-dias-delevedove) reuniram-se docentes, discentes e comunidade em geral, para a Defesa Pública da Dissertação de Mestrado do acadêmico **Vinicius Weber**. A Dissertação intitulada **"AMADURECIMENTO PRECOCE E VIDA CURTA: COMO O ENVELHECIMENTO RÁPIDO AFETA A REPRODUÇÃO DOS PEIXES ANUAIS?"** foi avaliada pela Banca Examinadora composta pelo Prof. Leonardo Maltchik Garcia (Orientador); Profª. Dra. Edélti Faria Albertoni (FURG) e Prof. Dr. Vitor Hugo Valiati (UNISINOS). Após a defesa e arguição pública, a Banca Examinadora reuniu-se, para deliberação final, e considerou o acadêmico **APROVADO**. Desta forma, o acadêmico concluiu mais uma das etapas necessárias para a obtenção do grau de **MESTRE EM BIOLOGIA DE AMBIENTES AQUÁTICOS CONTINENTAIS**. Nada mais havendo a tratar, às 17:00h (dezessete horas) foi lavrada a presente ata, que lida e aprovada, foi assinada pelos membros da Banca Examinadora, pela Acadêmica e pelo Coordenador adjunto do Curso.

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Prof. Leonardo Maltchik Garcia

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Profª. Dra. Edélti Faria Albertoni

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Prof. Dr. Vitor Hugo Valiati

Prof. Dr. Junior Borella
Coordenador adjunto do Curso