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Embryonic Development of Natural Annual Killifish populations of the genus Austrolebias: Evolutionary parallelism and the role of environment

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ORIGINAL ARTICLE

Embryonic development of natural annual killifish populations of the genus *Austrolebias*: Evolutionary parallelism and the role of environment

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Abstract

1. Repeated, independent emergence of the same trait within different phylogenetic lineages is termed parallel evolution. It typically occurs as a result of similar selective pressures. Annual killifish have adapted to survive in the extreme habitat of temporary pools on three continents and present an especially amenable system for studying fundamental principles of evolutionary parallelism. When the pools dry, annual killifish embryos survive through the dry phase in the bottom substrate in a stage of dormancy—a diapause. The diapause is a complex set of three different developmental stages, none of which is obligate, thus leading to a multitude of potential developmental trajectories. While the intricacy of the killifishes' embryonic development has been thoroughly studied in the laboratory, information on their natural development is virtually absent. We hypothesised that the natural development of annual killifishes is largely synchronised and governed by ambient conditions as shown in the lineage of the African genus *Nothobranchius*.
2. We sampled wild embryo banks of the South American genus *Austrolebias*, which evolved its diapause independently of the African lineage. We sampled during two consecutive dry seasons, using both longitudinal and snapshot monitoring, and conducted transplant experiments to determine the extent of the evolutionary parallelism and role of the environment in *Austrolebias* spp. embryo development.
3. Main habitat phases were characterised by largely synchronised embryo banks. Different inter-seasonal or local environmental conditions were reflected in a different developmental profile of the embryo banks, suggesting a high degree of environmental control.
4. We found striking similarity in the habitat phase–embryo stage associations between the two lineages. The diapause in the two annual killifish lineages represents a unique example of evolutionary parallelism, with the analogy manifested in very close detail. We highlight the similarity of the selective forces in the two

genera despite the different geographic origins, climate zones and reversed seasonality. The repeatedly occurring strict association of the same developmental stages with the same habitat conditions suggests a limited array of developmental settings that can be applied to cope with the given environmental challenges.

KEYWORDS
bet-hedging, convergence, embryo ecology, environmental cue, unpredictable conditions

1 | INTRODUCTION

Evolutionary parallelism refers to the evolution of similar traits or structures in evolutionary independent organismal lineages. The similarities shared between the lineages are analogous—they evolved without existence of a common ancestor possessing such a trait. Parallel evolution arises as a consequence of similar selective pressures (environmental or ecological) encountered by organisms across space and time (e.g. Losos, 2011; Rundle et al., 2000; Scheffer & Van Nes, 2006).

Environmental signalling is a one-way information pathway through which environmental variables influence cellular and organismal function. Further, environmental signals are often transient and occur with a high level of variation and a certain amount of unpredictability. The signals' impacts and subsequent organismal responses are typically the most profound in embryos, which may respond to environmental changes through altered development of plastic traits that result in life-long effects (e.g. McLachlan, 2001). However, the developmental environment may not always provide signals that induce adaptive responses, and thus selection pressure is probably very high during early life exposures to environmental challenges.

Freshwater temporary pools occurring in subtropical and temperate zones across the continents represent replicated, seasonal ecosystems (Williams, 2006), well suited for the study of evolutionary parallelisms and environmental signalling (Furness et al., 2015, 2018; Hrbek & Larson, 1999; Winemiller & Adite, 1997). They are spatially small and thus abundant, while the seasonality—loss of water during the dry season—translates as a very substantial environmental change. The seasonal shift is especially challenging to aquatic organisms and they are under strong selection for strategies to cope with habitat desiccation. Aquatic animals that exploit temporary pools must either migrate when the ponds dry, or evolve adaptations which allow them stay in situ, that is drought-resistant stages. Most commonly, the drought-resistant stage is an embryo (Brendonck & De Meester, 2003; Drummond et al., 2015; Wourms, 1972a, 1972b), facing seasonal shifts in a suite of environmental factors, some of which may act as signals or cues.

Annual killifish is a rare group of fishes that has evolved adaptations for long-term persistence in temporary pools. They are small, extremely fast growing, and adults deposit drought-resistant eggs in the bottom substrate before the pool goes dry. The embryos hatch when the pool is flooded again at the onset of the next rainy season (Berois et al., 2015; Cellerino et al., 2016; Wildekamp, 2004; Wourms, 1972a, 1972b). Because the dry period normally lasts for several months (often longer), annual killifish have evolved embryonic dormancy—a system of three facultative diapauses termed diapause I (DI), diapause II (DII), and diapause III (DIII) (Wourms, 1972a), although recent work indicates that the system may be more even more complex (Polačik & Vrtílek, 2023). At the level of the individual, each of the diapauses can be skipped, where entry into and exit from any of the diapauses are independent. The developmental stages when the embryo enters a diapause are firmly defined. DI may be entered early in the development, before any embryonic axis is formed. DII occurs in the middle of the development, when approximately 38 pairs of somites and foundations for several organ systems are established. DIII takes place in a fully developed embryo. All three stages of diapause are characterised by both a metabolic and developmental arrest (Podrabsky et al., 2017; Podrabsky & Hand, 1999; Wourms, 1972a, 1972b).

Interestingly, ample evidence suggests that this life history pattern in annual killifishes has multiple, independent evolutionary origins (Furness, 2016; Furness et al., 2015, 2018; Hrbek & Larson, 1999). Even in lineages inhabiting different continents, the physiological underpinnings of diapause and the developmental timing are conspicuously similar, with the entire diapause system replicated in detail. The three diapauses are even entered at the same embryonic stages (Furness, 2016; Furness et al., 2015, 2018; Wourms, 1972a). This unusual evolutionary parallelism is hypothesised to be due to the intrinsic properties of embryos at these specific stages in development that maximises resilience and minimises the chances of abnormal developmental outcomes (Furness et al., 2015, 2018; Wourms, 1972a).

Development in annual killifishes has been traditionally perceived as a process where an interplay of phenotypic plasticity and intrinsic bet-hedging determine the actual developmental trajectory for each individual embryo (Furness et al., 2015). A high degree of developmental asynchrony in laboratory-reared embryos suggests a leading role for bet-hedging (e.g. Pinceel et al., 2015; Polačik et al., 2017; Vrtílek et al., 2020). Developmental asynchrony ensures that at least part of the progeny matches actual conditions for survival and reproduction in a habitat with environmental signals that may be unreliable. For example, a partial flooding of the pool could provide a *false* hatching cue and result in fish hatching into conditions that are not consistent with survival and reproduction. The expectation for a strong role of bet-hedging in natural development of annual killifishes therefore seems supported, but data on wild populations were unavailable until recently.

We conducted a follow up study on our previous survey of the natural development of African annual killifishes of the genus *Nothobranchius* (Polačik et al., 2021), which have evolved diapause independently of the American lineage (Hrbek & Larson, 1999). In *Nothobranchius* spp., we revealed that embryonic development in the wild is largely synchronised with a clear association to the wet or dry conditions in the habitats, a result that is in stark contrast to results from laboratory observations and experiments (e.g. Furness et al., 2015; Pinceel et al., 2015; Polačik et al., 2017, 2018). Thus, we concluded that ecological factors in the wild provide reliable environmental signals that facilitate developmental synchrony in the population. These signals must either be absent or overridden under the artificial conditions imposed in laboratory environments.

Here, we provide the first evaluation on the natural development of South American annual killifishes of the genus *Austrolebias*. We sampled several species in two distant regions of Uruguay using longitudinal and snapshot sampling over two seasons with contrasting climatic conditions. Consequently, we were able to compare characteristic embryo developmental profiles across the same sites under different ambient conditions. We further performed two field experiments to better understand factors that may affect the development. The data collected in South America were then compared with our previous data on the independently evolved *Nothobranchius* lineage from East Africa (Polačik et al., 2021). We tested whether: (1) the course of embryonic development in *Austrolebias* is determined by actual environmental conditions as in *Nothobranchius*; and (2) the embryos of *Austrolebias* reside in DI, DII, and DIII under similar seasonal conditions as observed in other killifish lineages.

2 | METHODS

2.1 | Study fish and their habitats

Our study period covered the entire dry season of 2018/2019 and the end of the dry season 2019/2020 (Table 1). In the temperate climatic zone of South America, the dry season is represented by the summer period (November–March) when water evaporation from the pools normally exceeds rainfall water deposition. However, the dry season of 2018–2019 was extremely rich in rainfall due to an El Niño climatic cycle and the study pools remained flooded (total amount of rainfall more than four times higher than usual). This atypical dry season prompted an additional sampling campaign in the following dry season of 2019/2020 to collect data on developmental profiles in dry substrate.

A total of seven *Austrolebias* spp. sites were sampled, located near the town Villa Soriano (−33.3966°S, −58.3200°W) in the inland Province of Soriano, southwest Uruguay (five pools) and near the town La Coronilla (−33.8973S, −53.5169W) in the coastal Province of Rocha, northeast Uruguay (two pools; Figure 1). All the inland pools were inhabited by *Austrolebias bellottii* (Steindachner, 1881), sometimes accompanied by *Austrolebias elongatus* (Steindachner, 1881) and *Austrolebias nigripinnis* (Regan, 1912) (García, Loureiro, et al., 2019, García, Smith, et al., 2019). The two coastal pools were inhabited by *Austrolebias charrua* Costa & Cheffe, 2001 and *Austrolebias luteoflammulatus* Vaz-Ferreira, Sierra de Soriano & Scaglia de Paulete, 1965 (Table 1). All the study pools were shallow (maximum depth 50cm), located in open grassland, ranging in surface area from 50 to 900m^2. The vegetation was represented by littoral and aquatic vegetation during the wet phase and terrestrial grasses during the dry phase. The bottom substrate consisted of dark, clay-rich soil with a high organic matter content (*c.* 10%–50%) such as plant roots, various organic debris and negligible amount of sand (estimated as <1%; Figure 2).

Ambient temperature is recognised as an important environmental factor influencing development and survival of annual killifish embryos (e.g. Markofsky & Matias, 1977; Romney et al., 2018). We used temperature data loggers (HOBO Pendant® Temperature Data Logger UA-002-08, Onset, Bourne, MA, U.S.A.) to continually monitor substrate temperature in three study pools throughout the entire period of embryonic development. A set of three data loggers per site was exposed at the depths of 5, 10, and 20cm in mid-November 2018 and recovered in late April 2019.

TABLE 1 Details on the sites sampled for *Austrolebias* spp. embryos in the dry season of 2018/2019 (white background) and in February 2020 (grey shaded column).

Site	Region	Latitude	Longitude	Species	Nov 1	Nov 2	Dec 1	Dec 2	Jan	Feb	Mar 1	Mar 2	Apr 1	Apr 2	Feb 20
1	Inland	33.3975°	58.3387°	Ab, An	fl	fl	fl	fl	fl(f)	fl	fl	fl	fl	fl	d
2	Inland	33.4164°	58.3385°	Ab, An, Ae	fl	fl	fl	fl	fl	fl	fl	fl	fl	fl	d
3	Inland	33.4427°	58.3104°	Ab, An	fl	fl	fl	fl	fl	fl	fl	fl	fl(f)	fl	d
4	Inland	33.4811°	58.3349°	Ab, An, Ae	fl	fl	fl	fl	fl	d	d	fl(f)	fl(f)	d	d
5	Inland	33.4820°	58.3316°	Ab, Ae	fl	fl	fl	fl	fl(f)	fl	fl	fl	fl	fl	d
6	Coast	33.8963°	53.5154°	Ac, Al	–	–	–	–	–	fl	–	–	–	–	fl(f)
7	Coast	33.9199°	53.5431°	Ac, Al	–	–	–	–	–	–	–	–	–	–	d

Note: Two columns for the same month indicate two sampling trips, early and late in the month.

Abbreviations: Ab, *Austrolebias bellottii*; Ac, *Austrolebias charrua*; Ae, *Austrolebias elongatus*; Al, *Austrolebias luteoflammulatus*; An, *Austrolebias nigripinnis*; d, dry site; f, presence of juvenile fish; fl, flooded site.

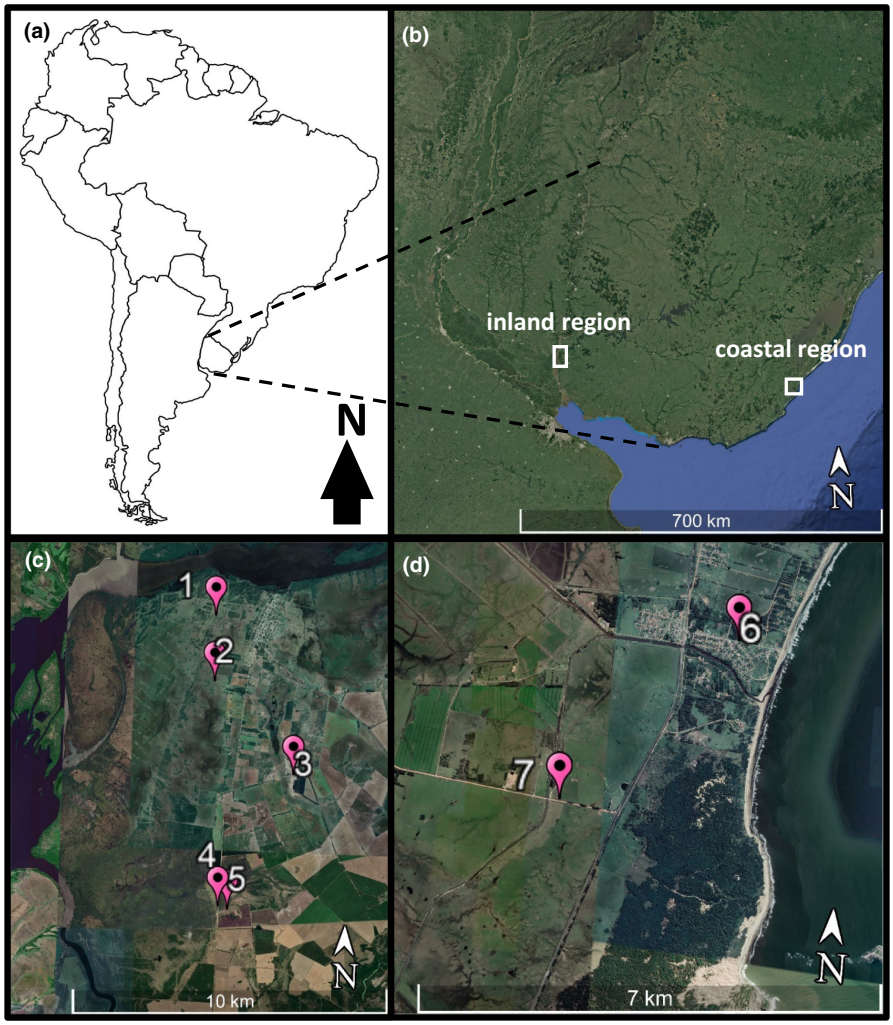

FIGURE 1 Schematic map of South America with the indicated location of the state Uruguay (a). Location of the sampled inland region and coastal region (b). Location of five pools sampled within the inland region (c). Location of two pools sampled within the coastal region (d). See Table 1 for the GPS coordinates of individual pools.

Ambient humidity influences killifish embryo survival (Podrabsky et al., 2001) and development (Van Dooren & Varela-Lasheras, 2018). To monitor substrate moisture in a pool without water, three data loggers (Hobo H21-USB Micro Station equipped with 10HS soil moisture sensor) were deployed in expectation of impending pool desiccation at sites 3 and 4 (15 February 2019) and at site 2 (3 March 2019). Local conditions allowed positioning of datalogger probes directly into embryo sampling spots for sites 2 and 4 (private property, suitable terrain, see Embryo sampling below). However, to avoid cow trampling and decrease its conspicuousness on public property, the probe for site 3 had to be installed nearby under a fence in a bump of soil about 10 cm higher than the bottom of the pool. The data loggers were recovered on 2 April 2019.

2.2 | Embryo sampling method and data collection

Briefly, *Austrolebias* spp. embryo banks were sampled by liquifying the mud/bottom substrate and passing it through a fine mesh stainless steel sieve. Embryos were retained in the sieve and collected by eye in the field.

For the bottom substrate collection, we targeted the central, deepest parts of each pool to minimise the risk of searching an area previously not flooded (i.e. without eggs). We sampled roughly the same parts of each pool to be able to track any potential developmental shift over time. Bottom substrate to the maximum depth 15 cm was collected using a shovel (inundated pool) or a pickaxe (dry pool). The amount of the substrate processed per a sampling event and pool varied and depended on local embryo density. We primarily aimed to maintain sample continuity across successive sampling events.

The method of embryo extraction from the substrate through the fine mesh sieve was the same as used by our team previously (see Polačik et al., 2021). The collected embryos were cleaned of debris to improve visibility of embryonic structures. Developmental stage was determined by examination using Specwell M0616-E 6×16 (LS & S) and BoliOptics 40-400× (Cucamonga, CA, U.S.A.) portable light microscopes. The embryos were sorted into one of five categories Podrabsky et al. (2017): (1) DI embryo (embryo lacking any visible organs, potentially including pre-DI embryos); (2) post-DI—pre-DII embryo (segmented embryonic axis formed, DII stage not yet reached); (3) DII embryo (middle of the embryonic development, between 30 and 40 somite pairs formed); (4)

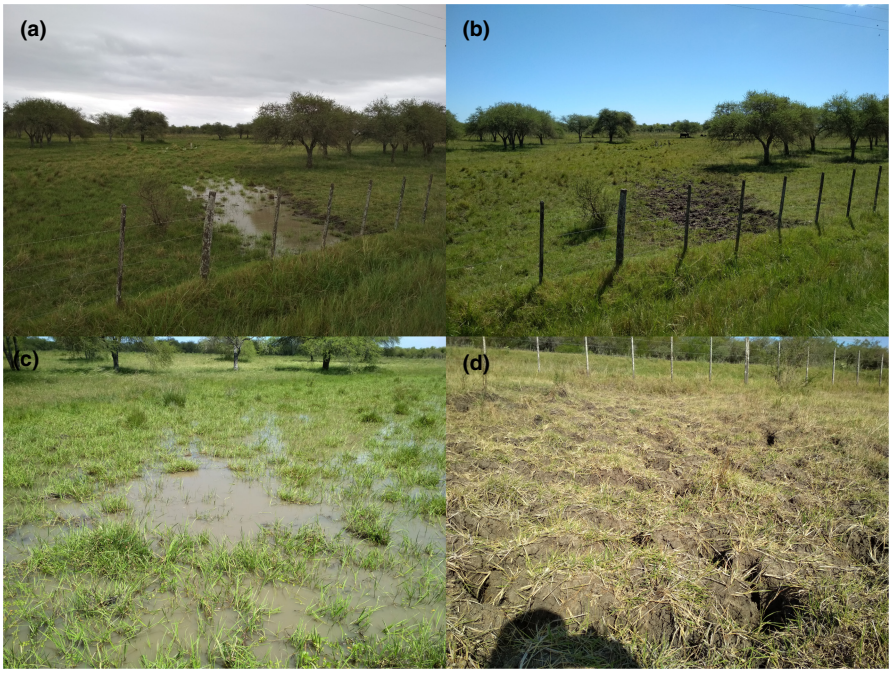

FIGURE 2 Example of an *Austrolebias* spp. habitat. General appearance (a) and a close up (c) of a flooded pool. General appearance (b) and a close up (d) of a dry pool.

post-DII–pre-DIII embryo (embryo progressed past DII stage but not yet fully developed, potentially including the developmental stasis sensu Polačik & Vrtílek, 2023); and (5) DIII embryo (development completed, pre-hatching stage).

Pilot sampling revealed that embryos were distributed in the substrate across a range of depths. We evaluated vertical embryo distribution in all sampling sites in the inland region in two successive seasons—February 2019 (site 2, 3 and 4) and February 2020 (sites 1 and 5). We counted the number of embryos in 5-cm substrate layers (0–5 cm, 5–10 cm, 10–15 cm) within squares covering an area of 30 × 30 cm. Three replicated samples (three squares) were obtained and separately processed for each site sampled in 2019. A single vertical sample was collected from the sites sampled in 2020.

We attempted to assign the collected embryos to a respective killifish species based on the long-term adult fish species occurrence during the past flood periods (García, Loureiro, et al., 2019, García, Smith, et al., 2019) combined with the differences in egg size. For the inland region, we preserved a subsample of embryos pooled from all five sampled pools in 4% formaldehyde, measured their size (Polačik et al., 2021; Vrtílek & Reichard, 2015) and performed a confirmatory analysis, based on size frequency distribution of each species. The analysis showed that only the large eggs of *A. elongatus* could be unambiguously distinguished because of size overlap in the rest of the embryos (*A. bellottii* and *A. nigripinnis*). No subsample was preserved and measured for the coastal region due to the much lower number of collected embryos and expected strong dominance of *A. charrua* in the local killifish community. Nevertheless, we are confident that all five *Austrolebias* species expected to occur in the sampled sites are included in our embryo bank samples as they all were present when a sample of the live collected wild embryos ($N = c.$ 450 individuals) were hatched and grown in the laboratory (inland species: *A. bellottii c.* 90%; *A. nigripinnis* 10% [*A. elongatus* embryos were clearly distinguishable and thus excluded from the hatched sample]; coastal species: *c.* 95% *A. charrua*, 5% *A. luteoflammulatus*).

2.3 | Fish sampling

Continual flooding during the dry season of 2018/2019 prompted attempts to obtain data on the potential presence of juvenile or adult *Austrolebias* spp. in the flooded habitats. We sampled the pools in January, late March and early April 2019 using dip netting, a sampling technique well proven for the use in annual killifish communities (for details on the method see e.g. García, Loureiro, et al., 2019; Reichard et al., 2009).

2.4 | Field incubation experiment

We experimentally tested the role of environmental on embryonic development through long-term incubation of embryos collected in captivity and transplanted into natural killifish habitats. Experimental embryos originated from 20 pairs of parental *A. bellottii* collected at site 4. In the laboratory, fish were housed at even densities in seven aerated tanks (volume 15–25 L) and maintained on an ad libitum diet of live *Tubifex* sp. Annual killifishes spawn each day and their oocyte turnover is very fast (e.g. Blažek et al., 2013). To reduce the effects of previous environmental exposures on egg production and quality, embryos produced during the first 21 days of captivity were not used in this study.

For collection of embryos, we placed all fish into a single, heavily aerated 40-L aquarium with the entire bottom covered with a 1.5 cm thick layer of coconut fibres as the spawning substrate. After 4 days of spawning the substrate was removed and left to dry for 24 h. Subsequently, 180 embryos were hand-picked from the substrate

and split into six experimental batches of 30 embryos each. All collected embryos were in pre-DI or DI stages of development. Embryo batches were spread into a ball of water-soaked natural bottom substrate taken from the same site as the parental fish. The *mud ball* was additionally wrapped into a fine mesh fabric to allow for full moisture permeability.

The six *mud balls* were buried in three different study pools at different depths (site 2–5 cm; site 3–10 cm; site 4–15 cm) on 22 December 2018. Two batches were buried at each site to allow for two sampling time points without the need for replacing the embryos after sampling. This avoided the potential for confounding effects of substrate disturbance on development. The first batch from each of the three sites was recovered after 55 days (February 2019). The second batch was recovered after 88 days from site 4 (March 2019) and after 102 days (April 2019) from sites 2 and 3. The embryos were washed out of the substrate and staged as when processing the natural samples.

2.5 | Laboratory incubation experiment

We tested the effect of a change in incubation conditions on field-obtained embryos transferred to the laboratory. We collected wild embryos residing in DI and incubated them in an aquatic medium in the laboratory.

Wild embryos were collected as a part of our regular sampling campaigns in site 3 (Batch I; 16 January 2019) and site 2 (Batch II; 14 February 2019). Each batch consisted of 24 embryos staged as DI immediately after their collection and subsequently stored in water at naturally fluctuating temperature (*c*. 18–30°C) during transport to the lab. Batch I was transferred to the laboratory 48 h after collection and incubated in the dark at room temperature (*c*. 22–27°C). Batch II was transferred to the laboratory 24 h after collection and incubated at 25°C in an incubator in the dark. Developmental stage was evaluated 10 and 17 days post-collection for Batch I, and 9, 14, and 22 days post-collection for Batch II.

2.6 | Data analysis

We statistically tested the effect of depth on the number and developmental profile of embryos in R environment v 4.0.0. (R Core Team, 2020). To analyse relationship between egg number and substrate depth, we used generalised mixed effect model (function *glmer* from package *lme4* v 1.1.21.; Bates et al., 2015) with Poisson distribution and random intercept of site. The developmental profile association with substrate depth was tested with cumulative link mixed effects model (function *clmm* from package *ordinal* v 2019.12-10; Christensen, 2019) using logit link and site as random intercept.

3 | RESULTS

3.1 | Environmental conditions in embryo banks

The *Austrolebias* spp. Embryo banks in both study regions experienced contrasting conditions in the dry season of 2018/2019 compared to the dry season of 2019/2020. In the 2018/2019 dry season, the rainfall was exceptionally high (Figure 3). As a consequence, all but one study pool contained at least a shallow water column throughout the entire 6-month survey (the inland region). Site 4 dried out for a brief period in late February and early March 2019 (Figure 4). The situation was probably similar in the coastal region (Figure 3).

In contrast, the dry season of 2019/2020 was more typical with only 27.1% of total seasonal rainfall (for the period November 2019–February 2020) of the previous season in the inland region and 43.3% in the coastal region (Figure 3). In the inland region, all the study sites were dry during our sampling in February 2020. In the coastal region, study site 6 was flooded but site 7 was without water, with a high level of soil moisture.

Temperature in the pool substrate followed seasonal air temperature changes, ranging from approximately 16 to 30°C. The

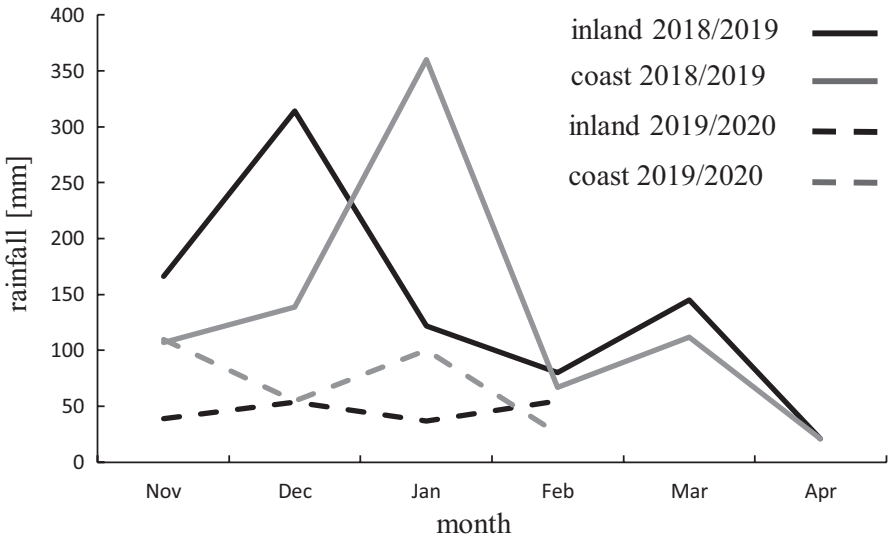

FIGURE 3 Monthly rainfall totals in the sampled regions in the dry seasons of 2018/2019 and 2019/2020. Data from the inland region courtesy of El Curupí cattle farm in Villa Soriano, Uruguay. Data for the coastal region are public data of the Uruguayan Meteorological Institute for the regional centre, city of Rocha.

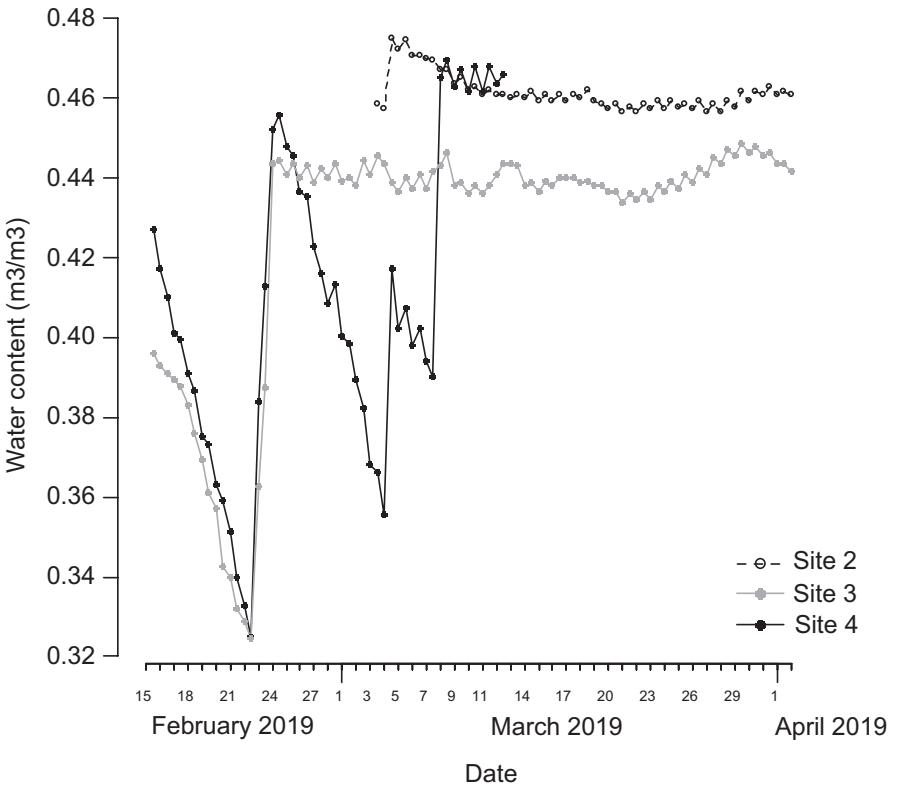

FIGURE 4 Bottom substrate moisture measured by continuous datalogging in three sites of the inland region in February–March 2019. The datalogger in site 4 malfunctioned on 13 March 2019 due to a flood.

course of the changes was similar across the monitored sites. A slight difference occurred in temperature extremes and the extent of daily amplitudes experienced across the sites, but temperature stability in the pool substrate increased with depth (Figure 5).

3.2 | Natural embryo development

A total of 2,212 *Austrolebias* spp. embryos were collected during our survey. Only 1% of all embryos could be unambiguously assigned to a species (*A. elongatus*) based on their contrasting egg size and the region of occurrence. Species determination of the smaller embryos was impossible due to the egg size overlap amongst co-occurring species. Nevertheless, the results presented for the inland region are largely applicable to the dominant *A. bellottii* and for the coastal region to the dominant *A. charrua* (see Section 2).

No significant shift in the developmental status of the sampled embryo banks was observed in the inland region over the entire season of 2018/2019 when a water column was continuously present. The embryo banks maintained a stable developmental profile dominated by DI embryos (76.7%–95.8%) followed by DII embryos (4.2%–13.7%). Overall, the proportion of transitional or post-DII stages was negligible. The only exception was site 4, which briefly desiccated at the end of the dry season and this drying was followed by an increase in the proportion of post-DI stages (Figure 6). The data collected from the flooded site 6 in the coastal region in February 2019 revealed the same developmental pattern as in the inland region (94.4% DI; 5.6% DII embryos; Figure 6).

A completely reversed developmental pattern was found at the end of the following dry season (February 2020) when the sampled sites were mostly dry. In the inland region, the embryo banks progressed much further in their development compared to the previous year, with the embryo banks now dominated by DII embryos (74.3%) while the proportion of DI embryos was very low (2.7%). The coastal region showed a pattern identical to the inland region with 73.9% of embryos in DII and only 2.9% embryos in DI. The proportion of post-DII stages also increased in both regions in a very similar manner (inland region: 18.1%; coastal region: 20.3%; Figure 6).

3.3 | Vertical distribution of embryos

Embryos were consistently found throughout all sampled depths of the bottom substrate column (15 cm) in all five sites for which vertical distribution of embryos was examined. No clear difference in embryo density was found between the examined substrate layers across all sites (log-Likelihood ratio test, interaction depth × year: $\chi^2 = 4.97$, $p = 0.083$, log-Likelihood ratio test, depth: $\chi^2 = 5.64$, $p = 0.060$; Figure 7).

Embryo developmental profiles did not differ across the sampled layers within a site (analysis of deviance, depth: $\chi^2 = 3.98$, $p = 0.137$), suggesting a lack of influence in substrate depth on developmental progression. Consistent with the general inter-annual differences, the developmental stage profiles were similar within a season but contrasted between the two seasons (Figure 7).

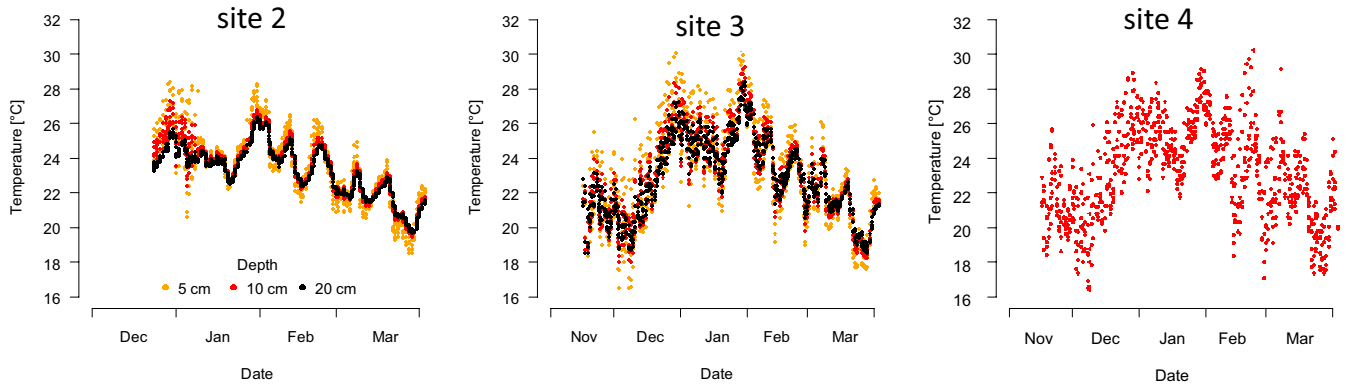

FIGURE 5 Bottom substrate temperature measured by continuous data logging at three different depths in three sites of the inland region between December 2018 and March 2019. In site 4, only the datalogger exposed at 10 cm was recovered.

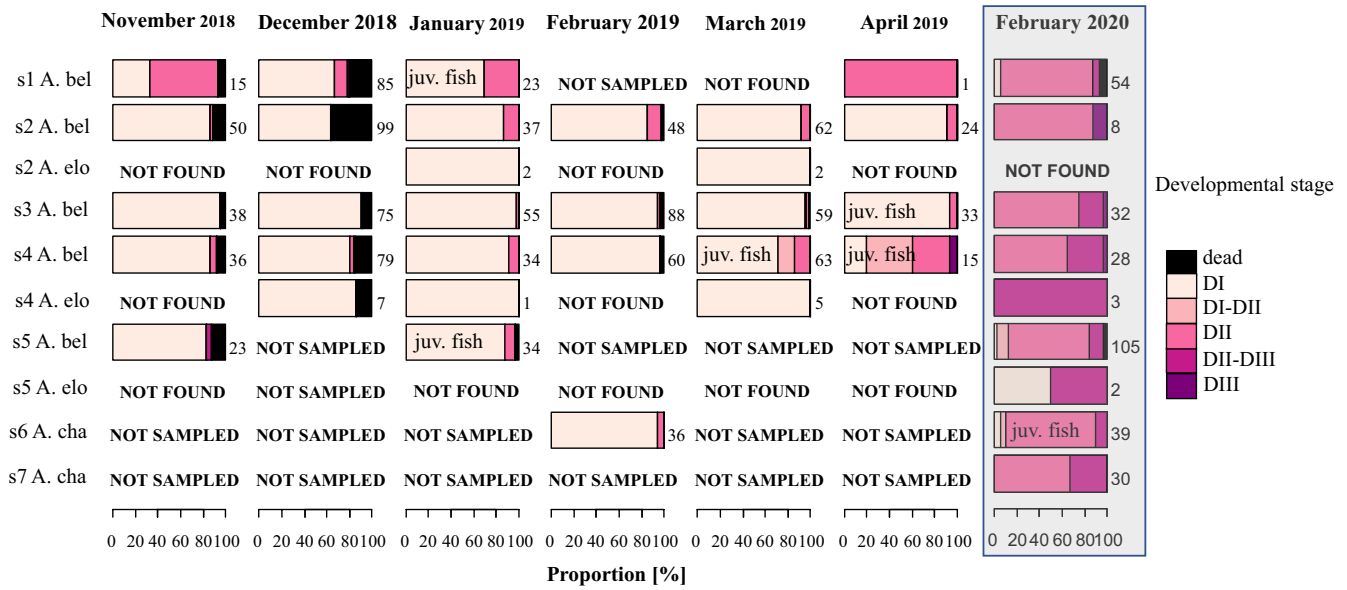

FIGURE 6 Longitudinal and snapshot data on developmental profiles of *Austrolebias* spp. embryo banks sampled during the dry season of 2018/2019 and 2019/2020. A. bel = *Austrolebias bellottii*, A. elo = *Austrolebias elongatus*, A. cha = *Austrolebias charrua*. Occurrence of juvenile fish is highlighted in respective columns.

3.4 | Transfer experiments

All live DI embryos collected in the wild exited DI after their removal from the natural substrate. In Batch I, 100% of embryos survived until the first check (day 9) when all of them were already in a post-DI stage. In Batch II, the total survival was lower (79.2%). Most embryos exited DI soon after the transfer but a minor proportion took more time to resume development. Nevertheless, all surviving embryos finally exited DI by the third check (day 22). Notably, all embryos that exited DI in Batch II bypassed DII and developed directly towards DIII (Figure 8a).

Captive-spawned embryos that were transferred to the wild and incubated under natural conditions showed the same developmental pattern as the respective, fully wild embryo banks. The vast majority of these embryos resided in DI, and a small proportion in DII when they were recovered from the natural substrate. Overall, 83.3% of the exposed embryos were successfully recovered (survival range per batch of 30 embryos: 76.7%–86.7%). Exposure site and incubation depth did not influence the developmental pattern. Developmental progress of embryos recovered earlier in the season did not appear to differ from those recovered approximately a month later (Figure 8b).

3.5 | Occurrence of juvenile fish

Longitudinally monitored pools were regularly sampled for the presence of fish because they remained flooded throughout the whole 2018/2019 dry season. Juvenile fish appeared in four of the total five inland pools between January and April 2019 (Table 1). They were mostly juveniles of *A. bellottii* (four pools), but juvenile *A. elongatus* (two pools) and *A. nigripinnis* (single pool) were also recorded.

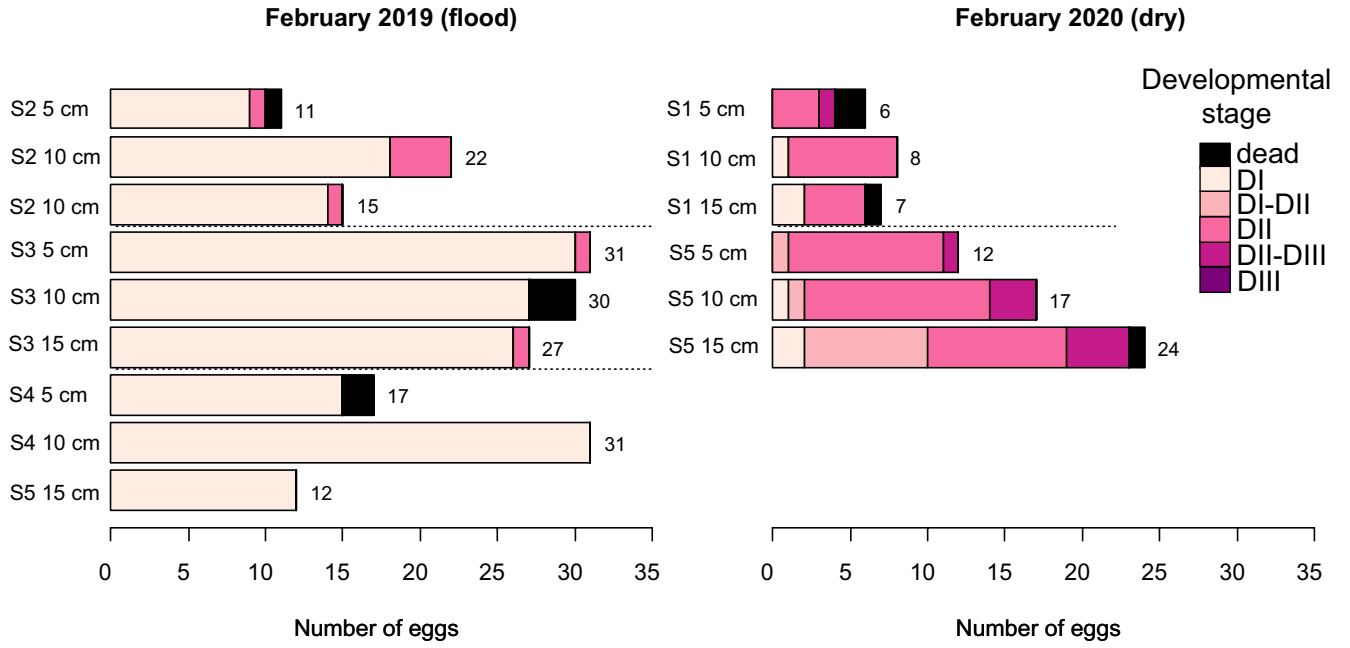

FIGURE 7 Vertical embryo distribution and developmental profiles in five embryo banks of *Austrolebias* spp. across two successive dry seasons with contrasting rainfall.

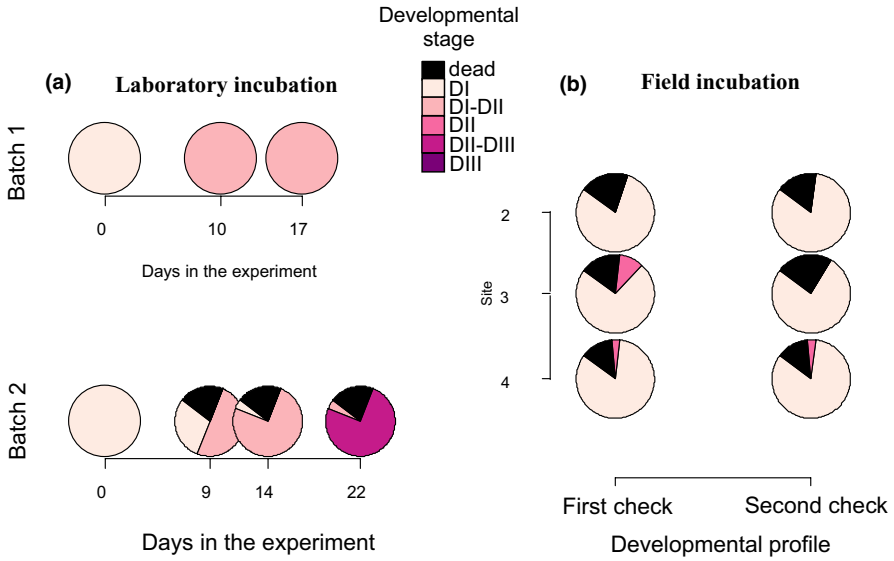

FIGURE 8 Transfer experiments of embryos from natural and laboratory environments. (a) Results of laboratory incubation of DI *Austrolebias bellottii* embryos collected in the wild. (b) Results of incubation of captive-spawned *A. bellottii* embryos transferred to natural habitats.

The size of all juveniles ranged from 1.5 to 2.5 cm total size. Snapshot sampling in February 2020 revealed 1.0–1.5 cm juveniles of the dominant *A. charrua* and a small proportion of *A. luteoflammulatus* in the single flooded site (Table 1).

4 | DISCUSSION

Developmental profiles of annual killifish embryos were closely associated with environmental conditions. Inundated pools supported embryo banks largely in DI, while stages of DII and beyond were characteristic for the same pools following desiccation. Drying of the pool prompted an advance in the developmental profile of the embryo bank. Captivity-spawned embryos transferred to natural habitats followed developmental trajectories of embryos naturally deposited in those habitats. Wild DI embryos removed from their natural setting and incubated in the laboratory exited diapause and resumed development.

Multiple lines of evidence suggest substantial environmental control over embryonic development in the examined *Austrolebias* spp. Our sampling of the natural embryo banks revealed a number of interesting patterns. First, general developmental patterns in the same habitats differed substantially between two successive dry seasons with contrasting rainfall patterns. In February 2019 during the continually flooded El Niño dry season of 2018/2019, the vast majority of embryos resided in DI while embryo banks in the identical sites composed of DII or more advanced stages in February 2020 when the ponds dried (Figures 3 and 6). Loss

of water in site 4 in March–February 2019 (Figure 4; Table 1) and probably also in site 6 in 2020 was followed by clear developmental progress in the embryo bank. Despite the flooding of site 6 at the time of our sampling in February 2020, site 6 had been recently dry (information from local people). This recent dry period probably facilitated the mass occurrence of a single cohort of juvenile annual killifish with the estimated age of about 10 days (Table 1). Second, the observation that developmental advance occurs only after a desiccation event is consistent with our data on the development of *Nothobranchius* fishes from Africa in 2018, where moist conditions in a single pool maintained a completely different developmental profile compared to all other dry sites (Polačik et al., 2021). Finally, juvenile *Austrolebias* spp. occurred in some pools (e.g. site 1, site 5 in January 2019, Figure 6) despite no observed progress in development of the monitored embryo banks. Sampling in the middle of the pool (see Section 2) provided a very stable profile of early stages in the parts of the pools used for the substrate collection (Figure 6). Thus, the unexplained appearance of juvenile fish (see Section 3) must have come from embryos that completed their development elsewhere within the same pool, probably in marginal areas with intermittently dry microconditions (Watters, 2009). The fully developed embryos then probably hatched during one of the water level fluctuation events caused by ever-recurring rains.

Additional evidence corroborating the crucial role of the environment came from the transfer experiments. Wild-collected embryos exited DI when removed from the wet mud and transferred to the laboratory. Exit from DI was likely to have been triggered by presence of oxygen in the laboratory incubation medium, or perhaps the absence of adult fish in the water (Inglima et al., 1981; Levels et al., 1986; Peters, 1963). Conversely, *A. bellottii* embryos spawned in captivity and transferred to natural habitats mirrored the developmental trajectory of naturally deposited embryos and remained largely in DI. In theory, any functional environmental control over developmental trajectory or progression should be favoured in evolution as an adaptive response to a changing environment over the alternative model of reproductive bet-hedging (see Section 1). Development of progeny that follow reliable environmental cues and maintain the most suitable stage for a given phase of the habitat (DI—Furness et al., 2015; DII—Podrabsky et al., 2001) should increase individual fitness by preventing the wasting of energy and fecundity on bet-hedged offspring that are mismatched with actual conditions and unlikely to survive and reproduce.

Embryonic development under natural conditions shares strikingly similar patterns across large geographic and phylogenetic distances. In this study, we recorded parallel development across embryo banks of several *Austrolebias* species in distant regions during similar seasonal habitat phases (Figure 6). Very similar, phase-specific diapause stages were also revealed by our extensive research in East Africa in *Nothobranchius* spp. (Polačik et al., 2021) and in the North American annual killifish *Millerichthys robustus* (Domínguez-Castanedo et al., 2017). Notably, ample evidence suggests that diapause has evolved independently in the South American (*Austrolebias*) and African (*Nothobranchius*) evolutionary lineages (Furness et al., 2015; Hrbek & Larson, 1999). The diapause has also evolved multiple times within the Family Rivulidae (Furness et al., 2018) where the genera *Austrolebias* and *Millerichthys* might share a common ancestor. However, the relationships are not yet fully resolved (e.g. Loureiro et al., 2018). The most striking characteristics shared across the lineages and geography is the linkage between DI and the inundated phase of the habitat, and between D2 and/or post-D2 stages and the dry habitat phase (this study; Domínguez-Castanedo et al., 2017; Polačik et al., 2021). The marked similarity across distant geographies, different taxa and even independent evolutionary lineages can be interpreted as an ecological parallelism in selective pressures and adaptive responses.

Selective pressure on the killifish embryos in temporary pools in general is probably more globally similar than so far acknowledged. Temporary pools are characterised by extensive seasonal fluctuation, but their fundamental characteristics, such as the shifting wet and dry phases, gradual filling and desiccation, and hypoxic substrate remain very similar worldwide (Williams, 2006). For instance, similar selective forces repeatedly resulted in characteristic body size structuring in adult killifish communities (Canavero et al., 2014; Helmstetter et al., 2020). Consequently, we hypothesise that the selective pressures imposed by temporary pool seasonality acting on embryos of annual killifish are very similar regardless of geography.

Similar adaptive responses to this intense selection pressure are supported by the characteristic developmental stages observed for a given seasonal phase of a temporary pool (roughly, flood = DI, dry = DII and beyond). Phase-specific developmental stage repeatability is probably a result of specific developmental stages being best suited to cope with ambient conditions during those seasonal phases (Furness, 2016; Wourms, 1972a). Laboratory experiments support this hypothesis, with DI embryos exhibiting tolerance of anoxia and the ability to buffer cell damage and loss without compromising developmental outcomes (e.g. Wagner & Podrabsky, 2015a, 2015b) and D2 embryos surviving for almost 2 years in highly desiccating conditions (Zajic et al., 2020). Alternatively, the similarity is so striking that it might put into question the independent origin of the diapause in annual killifishes (Murphy & Collier, 1997). Mammalian diapause, as an analogical example, was also long believed to have evolved multiple times, but relatively recent evidence points to a basal origin (Fenelon & Renfree, 2018; Lindenfors et al., 2003; Ptak et al., 2012).

Apart from the general similarity in the main developmental patterns, minor alterations did occur between the African and North American versus the South American modes of natural annual killifish development. A small but relatively stable percentage of DII embryos was found in most *Austrolebias* pools during the persisting flood (Figure 6). In contrast, flooded *Nothobranchius* spp. (Polačik et al., 2021) and *M. robustus* (Domínguez-Castanedo et al., 2017) embryo banks were uniform, consisting exclusively of DI embryos. This difference may stem from biological differences in the examined genus, different nature and duration of local environmental conditions, or a combination of both factors. On one hand, the occurrence

of DII embryos during inundation may be a form of a bet-hedging strategy, where some of the *Austrolebias* spp. embryos are intrinsically programmed to skip DI. This hypothesis is supported by the results of our transfer experiment, where some captive-spawned embryos soon proceeded to DII despite sharing the same conditions with the embryos stopping in DI (Figure 8b). On the other hand, the removal of the naturally spawned embryos from the bottom substrate was followed by exit from DI (Figure 8a). This lends support to the role of the environment as an alternative to bet-hedging. Embryo position in the bottom substrate is not stable but dynamic due to cattle herds entering and mixing the substrate with their hoofed legs (Polačik et al., 2021). The extent of the mixing is especially substantial in the examined *Austrolebias* spp. habitats as demonstrated by vertical embryo distribution where the embryos were distributed evenly at least to a depth of 15 cm (Figure 7a,b). Thus, the DI–DII developmental dichotomy might have originated through some DI embryos (temporary) exposed to conditions outside the substrate (oxygen available, Polačik et al., 2021). The embryos that reached DII, however, did not proceed further while the flood was persisting (Figure 6). A combination of laboratory and field experiments is needed to disentangle the specific role of potential ecological triggers for maintenance or exit from the respective diapauses.

5 | CONCLUSIONS

Our study on the natural embryonic development of South American annual killifish include five *Austrolebias* species, different geographic regions, two successive dry seasons, longitudinal and snapshot samplings of natural habitats, and field experiments. The extremity of the very wet El Niño dry season 2018/2019, combined with the normal character of the following dry season of 2019/2020, revealed environmental conditions as a principal driver of natural developmental progression. Additional evidence from the experimental work and particular coincidental contrasts observed in the wild (e.g. the developmental progress in site 4 after its brief desiccation) corroborates the overall picture of the critical role of the environment in controlling annual killifish development. Specifically, a conclusive body of evidence has accumulated to support the strong relationship between wet conditions and DI and, conversely, between substrate desiccation and DII (this study, Domínguez-Castanedo et al., 2017; Polačik et al., 2021). Certain seasonal phases and stages of natural embryonic development still leave space for intrinsic programming to influence developmental outcomes, but even in those cases an alternative environmental explanation exists. Although the ecosystem of temporary pools has been traditionally regarded as too unpredictable to offer reliable environmental cues (Cellerino et al., 2016; Furness et al., 2015; Moshgani & Van Dooren, 2011; Polačik et al., 2017, 2018; Wourms, 1972a, 1972b), our current data indicate that at least the main seasonal phases present anchoring signal elements for the embryos to follow.

Repeated, independent evolution of the killifish diapause is a widely accepted view (Costa, 1990; Furness et al., 2015, 2018; Hrbek & Larson, 1999). Virtually identical diapause systems with the embryos halting at the very same developmental points are attributed to the strong selection towards the most stable and resistant embryonic stages that occur at *natural windows* for developmental arrest and resilience (Furness, 2016; Wourms, 1972a). Notably, the only other example of embryonic diapause in fish, the embryos of the Asian autumn-spawning bitterling species (Cyprinidae), also halt their development throughout the winter in a stage that closely resembles the DII in annual killifishes (Kawamura & Uehara, 2005; Kim et al., 2018). Our data on the natural course of development in *Austrolebias* spp. provide additional details and show that the diapause stages are applied in the same context across geographic regions, species and independent evolutionary lineages (this study, Polačik et al., 2021). We are unaware of any other example of an evolutionary parallelism with such complex, multi-level similarities.

AUTHOR CONTRIBUTIONS

Conceptualisation: Matej Polačik, Milan Vrtílek, Jason E. Podrabsky. *Developing methods*: Matej Polačik, Jason E. Podrabsky. *Conducting the research*: Matej Polačik, Daniel García, Maria J. Arezo, Jason E. Podrabsky, Nicolas Papa, Hellen Schlueb, Daniel Blanco. *Data analysis*: Milan Vrtílek, Matej Polačik. *Preparation of figures and tables*: Milan Vrtílek, Matej Polačik. *Data interpretation*: Matej Polačik, Milan Vrtílek, Jason E. Podrabsky. *Writing*: Matej Polačik, Milan Vrtílek, Jason E. Podrabsky, Daniel García, Maria J. Arezo, Nicolas Papa, Hellen Schlueb, Daniel Blanco.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in FigShare at https://figshare.com/s/3fd9f635644ac533ad49, reference number DOI: 10.6084/m9.figshare.23508408.

ETHICS STATEMENT

The study was conducted in accordance with the research and ethical approval No. 18502769 issued by the Ministry of Housing, Territorial Planning and Environment of Uruguay.

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REFERENCES

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Berois, N., Garcia, G., & De Sá, R. O. (2015). *Annual fishes: Life history strategy, diversity, and evolution*. CRC Press.

Blažek, R., Polačik, M., & Reichard, M. (2013). Rapid growth, early maturation and short generation time in African annual fishes. *EvoDevo*, *4*(1), 1–7.

Brendonck, L., & De Meester, L. (2003). Egg banks in freshwater zooplankton: Evolutionary and ecological archives in the sediment. *Hydrobiologia*, *491*(1), 65–84.

Canavero, A., Hernández, D., Zarucki, M., & Arim, M. (2014). Patterns of co-occurrences in a killifish metacommunity are more related with body size than with species identity. *Austral Ecology*, *39*(4), 455–461.

Cellerino, A., Valenzano, D. R., & Reichard, M. (2016). From the bush to the bench: The annual *Nothobranchius* fishes as a new model system in biology. *Biological Reviews*, *91*(2), 511–533.

Christensen, R. H. B. (2019). *Ordinal—regression models for ordinal data. R Package Version 2019.12-10*.

Costa, W. J. E. M. (1990). Análise filogenética da família Rivulidae (Cyprinodontiformes, Aplocheiloidei). *Revista Brasileira de Biologia*, *50*, 65–82.

Domínguez-Castanedo, O., Uribe, M. C., & Rosales-Torres, A. M. (2017). Life history strategies of annual killifish *Millerichthys robustus* (Cyprinodontiformes: Cynolebiidae) in a seasonally ephemeral water body in Veracruz, México. *Environmental Biology of Fishes*, *100*(8), 995–1006.

Drummond, L. R., McIntosh, A. R., & Larned, S. T. (2015). Invertebrate community dynamics and insect emergence in response to pool drying in a temporary river. *Freshwater Biology*, *60*(8), 1596–1612.

Fenelon, J. C., & Renfree, M. B. (2018). The history of the discovery of embryonic diapause in mammals. *Biology of Reproduction*, *99*(1), 242–251.

Furness, A. I. (2016). The evolution of an annual life cycle in killifish: Adaptation to ephemeral aquatic environments through embryonic diapause. *Biological Reviews*, *91*(3), 796–812.

Furness, A. I., Lee, K., & Reznick, D. N. (2015). Adaptation in a variable environment: Phenotypic plasticity and bet-hedging during egg diapause and hatching in an annual killifish. *Evolution*, *69*(6), 1461–1475.

Furness, A. I., Reznick, D. N., Tatarenkov, A., & Avise, J. C. (2018). The evolution of diapause in *Rivulus* (Laimosemion). *Zoological Journal of the Linnean Society*, *184*(3), 773–790.

García, D., Loureiro, M., Machín, E., & Reichard, M. (2019). Species co-occurrence and population dynamics in annual fish assemblages in the lower Río Uruguay basin. *Environmental Biology of Fishes*, *102*(4), 569–580.

García, D., Smith, C., Machín, E., Loureiro, M., & Reichard, M. (2019). Changing patterns of growth in a changing planet: How a shift in phenology affects critical life-history traits in annual fishes. *Freshwater Biology*, *64*(10), 1848–1858.

Helmstetter, A. J., Papadopulos, A. S., Igea, J., & Van Dooren, T. J. (2020). Trait evolution and historical biogeography shape assemblages of annual killifish. *Journal of Biogeography*, *47*(9), 1955–1965.

Hrbek, T., & Larson, A. (1999). The evolution of diapause in the killifish family Rivulidae (Atherinomorpha, Cyprinodontiformes): A molecular phylogenetic and biogeographic perspective. *Evolution*, *53*(4), 1200–1216.

Inglima, K., Perlmutter, A., & Markofsky, J. (1981). Reversible stage-specific embryonic inhibition mediated by the presence of adults in the annual fish *Nothobranchius guentheri*. *Journal of Experimental Zoology*, *215*(1), 23–33.

Kawamura, K., & Uehara, K. (2005). Effects of temperature on free-embryonic diapause in the autumn-spawning bitterling *Acheilognathus rhombeus* (Teleostei: Cyprinidae). *Journal of Fish Biology*, *67*(3), 684–695.

Kim, H. S., Choi, H. S., & Park, J. Y. (2018). Embryonic development characteristics and host mussel utilization of flat bitterling *Acheilognathus rhombeus* (Cyprinidae) during winter in Korea. *Environmental Biology of Fishes*, *101*(1), 55–66.

Levels, P. J., Gubbels, R. E., & Denucé, J. M. (1986). Oxygen consumption during embryonic development of the annual fish *Nothobranchius korthausae* with special reference to diapause. *Comparative Biochemistry and Physiology. A, Comparative Physiology*, *84*(4), 767–770.

Lindenfors, P., Dalèn, L., & Angerbjörn, A. (2003). The monophyletic origin of delayed implantation in carnivores and its implications. *Evolution*, *57*(8), 1952–1956.

Losos, J. B. (2011). Convergence, adaptation, and constraint. *Evolution: International Journal of Organic Evolution*, *65*(7), 1827–1840.

Loureiro, M., Sá, R. D., Serra, S. W., Alonso, F., Lanés, L. E. K., Volcan, M. V., Calviño, P., Nielsen, D., Duarte, A., & Garcia, G. (2018). Review of the family Rivulidae (Cyprinodontiformes, Aplocheiloidei) and a molecular and morphological phylogeny of the annual fish genus *Austrolebias* Costa 1998. *Neotropical Ichthyology*, *16*, 1–20.

Markofsky, J., & Matias, J. R. (1977). The effects of temperature and season of collection on the onset and duration of diapause in embryos of the annual fish *Nothobranchius guentheri*. *Journal of Experimental Zoology*, *202*(1), 49–56.

McLachlan, J. A. (2001). Environmental signaling: What embryos and evolution teach us about endocrine disrupting chemicals. *Endocrine Reviews*, *22*(3), 319–341.

Moshgani, M., & Van Dooren, T. J. (2011). Maternal and paternal contributions to egg size and egg number variation in the blackfin pearl killifish *Austrolebias nigripinnis*. *Evolutionary Ecology*, *25*(5), 1179–1195.

Murphy, W. J., & Collier, G. E. (1997). A molecular phylogeny for aplocheiloid fishes (Atherinomorpha, Cyprinodontiformes): The role of vicariance and the origins of annualism. *Molecular Biology and Evolution*, *14*(8), 790–799.

Peters, N. (1963). Embryonale Anpassungen oviparer Zahnkarpfen aus periodisch austrocknenden Gewässern. *Internationale Revue der Gesamten Hydrobiologie Und Hydrographie*, *48*(2), 257–313.

Pinceel, T., Vanschoenwinkel, B., Deckers, P., Grégoir, A., Ver Eecke, T., & Brendonck, L. (2015). Early and late developmental arrest as complementary embryonic bet-hedging strategies in African killifish. *Biological Journal of the Linnean Society*, *114*(4), 941–948.

Podrabsky, J. E., Carpenter, J. F., & Hand, S. C. (2001). Survival of water stress in annual fish embryos: Dehydration avoidance and egg envelope amyloid fibers. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *280*(1), R123–R131.

Podrabsky, J. E., & Hand, S. C. (1999). The bioenergetics of embryonic diapause in an annual killifish, *Austrofundulus limnaeus*. *Journal of Experimental Biology*, *202*(19), 2567–2580.

Podrabsky, J. E., Riggs, C. L., Romney, A. L., Woll, S. C., Wagner, J. T., Culpepper, K. M., & Cleaver, T. G. (2017). Embryonic development of the annual killifish *Austrofundulus limnaeus*: An emerging model for ecological and evolutionary developmental biology research and instruction. *Developmental Dynamics*, *246*(11), 779–801.

Polačik, M., Reichard, M., & Vrtílek, M. (2018). Local variation in embryo development rate in annual fish. *Journal of Fish Biology*, *92*(5), 1359–1370.

Polačik, M., Smith, C., & Reichard, M. (2017). Maternal source of variability in the embryo development of an annual killifish. *Journal of Evolutionary Biology*, *30*(4), 738–749.

Polačik, M., & Vrtílek, M. (2023). Cryptic stasis during the development of Nothobranchius furzeri suggests new stages of dormancy outside of the typical three diapauses of annual killifishes. *Environmental Biology of Fishes*, *106*(3), 575–583.

Polačik, M., Vrtílek, M., Reichard, M., Žák, J., Blažek, R., & Podrabsky, J. (2021). Embryo ecology: Developmental synchrony and asynchrony in the embryonic development of wild annual fish populations. *Ecology and Evolution*, *11*(9), 4945–4956.

Ptak, G. E., Tacconi, E., Czernik, M., Toschi, P., Modlinski, J. A., & Loi, P. (2012). Embryonic diapause is conserved across mammals. *PLoS One*, *7*(3), e33027.

R Core Team. (2020). *R: A language and environment for statistical computing. v. 4.0.0*. R Foundation for Statistical Computing.

Reichard, M., Polačik, M., & Sedláček, O. (2009). Distribution, colour polymorphism and habitat use of the African killifish *Nothobranchius furzeri*, the vertebrate with the shortest life span. *Journal of Fish Biology*, *74*(1), 198–212.

Romney, A. L., Davis, E. M., Corona, M. M., Wagner, J. T., & Podrabsky, J. E. (2018). Temperature-dependent vitamin D signaling regulates developmental trajectory associated with diapause in an annual killifish. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(50), 12763–12768.

Rundle, H. D., Nagel, L., Boughman, J. W., & Schluter, D. (2000). Natural selection and parallel speciation in sympatric sticklebacks. *Science*, *287*(5451), 306–308.

Scheffer, M., & Van Nes, E. H. (2006). Self-organized similarity, the evolutionary emergence of groups of similar species. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(16), 6230–6235.

Van Dooren, T. J., & Varela-Lasheras, I. (2018). Embryonal life histories: Desiccation plasticity and diapause in the Argentinean pearlfish *Austrolebias bellottii*. *Ecology and Evolution*, *8*(22), 11246–11260.

Vrtílek, M., & Reichard, M. (2015). Highly plastic resource allocation to growth and reproduction in females of an African annual fish. *Ecology of Freshwater Fish*, *24*(4), 616–628.

Vrtílek, M., Van Dooren, T. J., & Beaudard, M. (2020). Egg size does not universally predict embryonic resources and hatchling size across annual killifish species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *249*, 110769.

Wagner, J. T., & Podrabsky, J. E. (2015a). Gene expression patterns that support novel developmental stress buffering in embryos of the annual killifish *Austrofundulus limnaeus*. *EvoDevo*, *6*(1), 1–13.

Wagner, J. T., & Podrabsky, J. E. (2015b). Extreme tolerance and developmental buffering of UV-C induced DNA damage in embryos of the annual killifish *Austrofundulus limnaeus*. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *323*(1), 10–30.

Watters, B. R. (2009). The ecology and distribution of *Nothobranchius* fishes. *Journal of American Killifish Association*, *42*(2), 37–76.

Wildekamp, R. H. (2004). *A world of killies: Atlas of the oviparous Cyprinodontiform fishes of the world* (Vol. *4*). American Killifish Association.

Williams, D. D. (2006). *The biology of temporary waters*. Oxford University Press.

Winemiller, K. O., & Adite, A. (1997). Convergent evolution of weakly electric fishes from floodplain habitats in Africa and South America. *Environmental Biology of Fishes*, *49*(2), 175–186.

Wourms, J. P. (1972a). The developmental biology of annual fishes. III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. *Journal of Experimental Zoology*, *182*(3), 389–414.

Wourms, J. P. (1972b). Developmental biology of annual fishes. I. Stages in the normal development of *Austrofundulus myersi* dahl. *Journal of Experimental Zoology*, *182*(2), 143–167.

Zajic, D. E., Nicholson, J. P., & Podrabsky, J. E. (2020). No water, no problem: Stage-specific metabolic responses to dehydration stress in annual killifish embryos. *Journal of Experimental Biology*, *223*(18), jeb231985.

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