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Annual Killifish Adaptations to Ephemeral Environments: Diapause I in Two *Austrolebias* Species

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Background: Many organisms are able to survive in extreme environments by entering a state of dormancy. In dormancy, vital activities are reduced until environmental conditions are compatible with active life. Annual killifishes show a special developmental pattern characterized by a phase of dispersion-reaggregation of the blastomeres that separates epiboly from organogenesis, and the capability to enter dormancy in diapause. High tolerance to environmental stress confers annual killifish embryos the condition of extremophiles. At present, the questions of our research group are focused on the understanding of the mechanisms involved in diapause regulation through an interdisciplinary approach. As a first step, it is necessary to characterize diapauses at morphological and physiological levels and to evaluate induction cues under laboratory conditions. In this context, we characterized diapause I in two *Austrolebias* species. **Results:** Our experimental approach to induce diapause I was successful and revealed the co-existence of two diapause I phenotypes named A and B instead of one. These phenotypes showed a tendency for lower total extractable RNA content compared with active developmental stages (80–100% epiboly and early reaggregate). **Conclusions:** These phenotypes are alternative diapause I stages and may have ecological relevance because both were found in embryos in natural ponds. *Developmental Dynamics 246:848–857, 2017*. © 2017 Wiley Periodicals, Inc.

Key words: annual killifish; diapause I; development

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Introduction

The conquest of almost all Earth's regions has been achieved because living organisms are able to invade and persist in extreme environments: e.g., deserts, salt marshes, heated hydrothermal regions, and polar ice. Tolerance to a variety of stresses is often supported by entrance into a state of dormancy. In dormancy, vital activities or development are reduced until environmental conditions are compatible with the active life of organisms. A diversity of organisms of different evolutionary trajectories use special states of dormancy, such as: programmed arrest during development in yeast spores, cysts of *Artemia*, plant seeds, diapauses described in annual killifish embryos, cryptobiosis in tardigrades and rotifers, and hibernation in mammals (Lubzens et al., 2010). Progress in clarifying the mechanisms involved in metabolic arrest, cell cycle control, and signaling pathways controlling gene expression patterns, as well as evidence of epigenetic contributions to diapause regulation in nematodes, crustaceans, insects, and fish were recently reviewed. These comparative data illustrate that diapause across all species and developmental stages is not regulated by a single suite of mechanisms, although some pathways appear to be shared by many lineages. Thus, it is essential to generate more information about how environmental cues are coupled to signal transduction cascades that initiate and generate diapause phenotypes (Hand et al., 2016).

Annual killifish (Cyprinodontiformes, Aplocheloidei) are a group of freshwater teleost found in Africa and America. They show a fascinating survival strategy experiencing the harshest conditions a fish can deal with; every dry season the temporary ponds they inhabit dry up. Therefore, the entire juvenile and adult population dies while the embryos, both dormant and actively developing, remain buried in the bottom mud until the next rainy season. Once the dry ponds are again flooded, embryos hatch and juveniles reach sexual maturity within a few weeks. Therefore, species survival in a given location is entirely embryo dependent (Wourms, 1972a–c; Errea and Danulat, 2001; Arezo et al., 2007; Berois et al., 2012; Blažek et al., 2013). Embryo survival during the rainy season is likely reliant on tolerance of long periods of hypoxia or anoxia (Podrabsky et al., 2011; Anderson and Podrabsky, 2014), whereas survival through the dry season is possibly supported by both tolerance of hypoxia/anoxia and the ability to resist environmental water loss (Podrabsky et al., 2001, 2010a).

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Noteworthy, embryos are able to tolerate rather harsh environments while retaining the ability to sense and respond to critical environmental cues such as temperature and hypoxia (Podrabsky et al., 2016a)

Persistence in this sort of environment requires evolution of reproductive and developmental strategies that are tightly coupled to their annual life cycle. These strategies are "shaped" to exploit the production of viable embryos in the face of environmental extremes, and have resulted in annual killifishes being perhaps the most remarkable extremophile organisms among vertebrates. They display high diversity, and their speciation patterns probably are the result of evolutionary forces acting at individual, population, and ecological levels (reviewed by Berois et al., 2012). This kind of life cycle, condensed in a few months, evolved in a variety of invertebrates but is uncommon in vertebrates (Wourms, 1972c; Furness, 2016).

Annual killifish share several characteristics with other widely used model fish species such as: oviparity, transparency of eggs and embryos, and easy maintenance in the laboratory (Berois et al., 2012). Nevertheless, they display two unique developmental features. First, a phase of blastomere dispersion over the yolk surface and subsequent reaggregation of these cells is interposed between cleavage and internalization of endomesoderm. Primary body axis formation takes place in this reaggregate. Thus, epiboly is temporally and spatially uncoupled from axis formation unlike nonannual teleosts. Second, annual killifish embryos can undergo reversible arrests during the dispersed phase (diapause I), during somitogenesis (diapause II), and at the prehatching stage (diapause III) (Peters, 1963; Wourms, 1972a,c). All diapauses are physiologically different (Wourms, 1972c) and react differentially to the same environmental signal (e.g., hypoxia induces and prolongs diapause I and II but terminates diapause III: Peters, 1963).

Plasticity for developmental delay in nonannual rivulids (Cyprinodontiformes) suggests that this feature might have been present in the shared ancestor of annual and nonannual South American killifish and that the evolution of plasticity could have played a role in the emergence of the diapauses (Varela-Lasheras and Van Dooren, 2014). The existence of diapause has been considered an ancestral feature, lost several times during evolution of nonannual killifishes (Murphy and Collier, 1997). However, recently Furness et al. (2015a) suggested that diapause evolved by convergent evolution.

Diapause is defined as a predetermined arrest of development regulated by endogenous factors and environmental signals that predict future conditions, such as temperature and photoperiod. It is a specific adaptation occurring in a particular stage in the life cycle of the species, and is crucial to enduring extreme environments (Roberts, 1978; Podrabsky and Hand, 2015). A reversible arrest involves a suspension or a major delay of morphological growth and development that differs from quiescence because in the latter development can be temporally inhibited by an unfavorable environment at any stage. Only extrinsic factors are responsible for induction and termination of quiescence (Andrewartha, 1952).

Diapause was traditionally defined as obligate (induced independently of environmental cues) or facultative (induced by external cues) (Andrewartha, 1952; Wourms, 1972c; Roberts, 1978). However, Podrabsky et al. (2016b) suggested that diapause in annual killifishes may always be facultative. Diapauses in annual killifish embryos are characterized by a profound metabolic depression (Levels et al., 1986a; Podrabsky and Hand, 1999). Growing evidence suggests that insulin-like signaling pathways, involved in cell-cycle arrest at the G_1 phase, play a central role in diapause regulation in a variety of lineages representing a universal manner to control cellular physiology of metabolic depression (Podrabsky and Hand, 2015). Dolfi et al. (2015) found microRNA expression involved in diapause regulation in annual killifish and comparative transcriptomics between diapause III embryos and free-living larvae of the South American *Nematolebias whitei* identified 945 differentially expressed genes (Thompson and Ortí, 2016). They found similar transcriptional patterns among *N. whitei* and other diapausing animals in a small set of genes associated with stress resistance, circadian rhythm, and metabolism (Thompson and Ortí, 2016).

Annual killifish embryos either develop continuously, "escape eggs," or pass through variable periods of arrest. This physiological adaptation generates alternative developmental trajectories defined as the "multiplier effect" (Wourms, 1972c). Moreover, spontaneous or induced developmental arrests can be experienced even in embryos of the same clutch cultured under constant standardized laboratory conditions. Peters (1963) and Wourms (1972c) suggested that this feature enables annual killifishes to maintain permanent populations in temporary ponds because development is greatly extended for variable lengths of time through the dry season. Entry into diapause I and II or following the direct trajectory to diapause III or hatching is dependent on a variety of factors including maternal effects such as female age (demonstrated for entrance into diapause II; Podrabsky et al., 2010b), photoperiod (Markofsky and Matias, 1977; Markofsky et al., 1979; Levels and Denucé, 1988; Podrabsky and Hand, 1999), temperature (Wourms, 1972c; Markofsky and Matias, 1977; Levels and Denucé, 1988; Podrabsky et al., 2010b), hypoxia (Peters, 1963), presence of adults (Inglima et al., 1981), and intrinsic genetic variability (Wourms, 1972c).

The mechanisms, probably epigenetic, involved in the integration and transfer of information from adults to embryos are unknown. The production of diapausing embryos may depend on maternal packaging into the embryo of an RNA, protein, or small molecules, while the absence of this signal could lead to escape embryo production (Podrabsky et al., 2016b). Recently, transcriptomic analyses of the South American *Austrofundulus limnaeus* one- to two-cell stage embryos suggest maternal programming of developmental trajectory through alternatively spliced, trajectory-specific, mRNAs of genes expressed in both phenotypes and antisense sncRNAs (Romney and Podrabsky, 2017). This hypothesis is supported by the conclusion of Furness et al. (2015a) that the direct developing pathway is typical of nonannual killifish (and teleosts in general) and is possibly the ancestral state, suggesting an exciting area for future studies. However, it is important to note that, although maternal influences may program the embryo for one trajectory, environmental cues experienced by the embryo can override those maternal influences (Levels and Denucé 1988; Podrabsky et al., 2016b).

The embryo's response to environmental factors (phenotypic plasticity) allows for suitable reactions to prevailing conditions, in a probabilistic sense. Moreover, embryos from the same clutch cultured in conditions similar to natural ponds, but without appropriate seasonal cues show variability in diapause entrance, duration, and hatching. This is consistent with diversified bet-hedging, a risk-spreading strategy that maximizes fitness and produces an extensive array of phenotypes. Thus, the annual killifish life history strategy appears to be a combination of adaptive phenotypic plasticity and bet-hedging (Furness et al., 2015b).

Diapause I, as previously mentioned, may occur during the dispersed cell phase in embryos either spontaneously or be induced by exposure to low temperatures, hypoxia / anoxia, habitat desiccation or diffusible factors secreted by adult fish (Peters, 1963; Wourms, 1972a–c; Markofsky and Matias, 1977; Inglima et al., 1981 Genade et al., 2005). Embryos left in the bottom of tanks covered by peat moss enter diapause I (Inglima et al., 1981; Arezo et al., 2005). It has been suggested that the tank bottom is hypoxic, similar to natural substrate conditions in which *Nothobranchius* embryos are placed. These environmental conditions induce diapause I, that if induced by hypoxia, could last until the total drying of the pond when shrinkage cracks the vertisol soil and creates aerobic conditions allowing development to proceed and reach diapause II (Peters, 1963; Watters, 2009).

These observations are in agreement with findings of Dominguez-Castanedo et al. (2013) in the basal annual rivulid *Millerichthys robustus* from Mexico where the entire population of embryos extracted from the completely dried soil were in diapause I. In this species, it appears that most of embryonic life is spent in this developmental arrest (Dominguez-Castanedo et al., 2013). In contrast, there is extensive evidence that embryos in diapause II of *A. limnaeus* are extremely tolerant of environmental stresses compared with diapause I or III, and that arrest in diapause II contributes most to the duration of developmental arrest under laboratory conditions (Podrabsky et al., 2010a*)*. Consequently, embryos in diapause I and II can endure dry seasons lasting at least 6 months in *Nothobranchius furzeri* (Furness et al., 2015b) and 2 months in *Austrolebias charrua* (Arezo et al., 2007) a South American annual killifish. In diapause III, the fully developed embryo may remain arrested for shorter periods until the establishment of necessary conditions to hatch (Furness et al., 2015b).

Wourms (1972b) suggested that embryos of annual killifishes avoid exposure to harsh environmental conditions by escaping in time. Diapause I may act as a protective mechanism arresting development at an insensitive stage (dispersed phase) immediately preceding a period of developmental sensitivity (gastrulation and axis formation). Dispersed cells could be undifferentiated developmentally equivalent blastomeres. This hypothesis is consistent with what was assumed about some teleosts: blastomeres are totipotent (Wourms, 1972b). During environmental stress, injured cells could be replaced by mitotic proliferation of surrounding cells without interfering with the formation of a normal embryo (Wourms, 1972b). Conversely, in *Austrolebias charrua* the expression of Vasa protein, the most documented molecular marker of teleosts germ cell population (Lin et al., 2012), is first differentially expressed during epiboly (Arezo et al., 2016).

These results indicate that blastomeres at the dispersed phase are not totipotent, as previously claimed (Wourms, 1972b) and have undergone at least the primary determination process that separates the germ cell population from future somatic cells in this annual killifish species. Instead, dispersed blastomeres appear to be pluripotent. The first experimental support for blastomere pluripotency came from a recent study. Dynamic spatiotemporal gene expression patterns of the conserved pluripotency cell markers *oct4, sox2, sox3, chordin, noggin*, and *follistatin* support a role for an extended period of pluripotency during the dispersion–reaggregation phases of development in the annual killifish *A. limnaeus*. These results suggest that *A. limnaeus* pluripotent embryonic cells begin to acquire specific identities after the dispersion–reaggregation is completed and gastrulation begins (Wagner and Podrabsky, 2015a).

Furthermore, embryos of *A. limnaeus* in the dispersed phase exhibit exceptional tolerance to DNA damage induced by ultraviolet radiation compared with embryos irradiated during early somitogenesis. Dispersed phase embryos are more frequently able to develop normally after the same doses of UV radiation. These findings are one of the most interesting conclusions of the studies on radiation exposure in *A. limnaeus* embryos (Wagner and Podrabsky, 2015). Taken together these results are the first partial experimental support for Wourms' (1972b) hypothesis (Wagner and Podrabsky, 2015a,b) but it is important to note that blastomeres are pluripotent, not totipotent cells as Wourms (1972b) originally assumed.

The dispersion–reaggregation phases of development and the ability to enter diapause always co-occur in annual killifishes leading to the hypothesis that the molecular mechanisms involved in both processes could be in some way linked (Wagner and Podrabsky, 2015a). There is scarce information to support this hypothesis. Particularly, in the South American genus *Austrolebias*, there is very limited published information. What little is known comes from early studies from many decades ago. Peters (1963) induced entrance in diapause I in *A. nigripinnis* and Wourms (1972c) briefly reported the same in *A. bellottii* as a consequence of partial desiccation under simulated natural conditions.

The questions of our research group are focused on using an interdisciplinary approach to understand the genetic and environmental mechanisms involved in the regulation of diapauses as special adaptations for the exploitation of ephemeral environments. We are analyzing the conditions needed for diapause induction and possible differential expression patterns of proteins in prediapause and diapausing embryos in laboratory and natural conditions. In particular, we are focused on diapause I because this arrest occurs at the dispersed cell phase, the unique and remarkable derived feature specific to annual killifishes (Wourms, 1972c; Dolfi et al., 2014).

Austrolebias (Cyprinodontiformes: Rivulidae) is distributed in the La Plata-Paraná and Patos-Merín basins. The genus comprises 42 species (reviewed by Loureiro et al., 2016) divided in five groups (García et al., 2002; Costa, 2006). Most species are endemic to very restricted areas. Their location and limited distribution, two parameters used by IUCN in the Red List Assessments, together with the severe land-use transformation that is taking place in the area (deforestation, rice and soy crops, open-sky mining, and tourism) place them in the Endangered category (Loureiro et al., 2016). *Austrolebias viarius* (Vaz-Ferreira et al., 1964), an endemic Uruguayan species, and *Austrolebias charrua* (Costa y Cheffe, 2001) shared with Brazil belong to the *Austrolebias adloffi* group, a well-supported clade (García, 2006). Their distribution in lowlands includes "Bañados del Este," an eastern Uruguayan Biosphere Reserve and Ramsar Site (Probides, 1999).

Characterization of diapause I at morphological and molecular levels is essential to provide insights into this fascinating evolutionary adaptation. Thus, as a first comprehensive approach, it is necessary to evaluate induction cues for diapause I in laboratory and natural conditions. In this context, the aim of the present work is to establish morphological features of diapause I and establish standardized laboratory conditions to induce this arrest in two annual killifish species: *Austrolebias viarius* and *A. charrua*.

TABLE 1. Summary of the Experimental Series of *Austrolebias* Embryos Cultured at 19 °C[a]. L: laboratory hatched adults, F: field collected adults used to obtain embryos cultured in Yamamoto solution or in adult presence, DIA: diapause I phenotype A and DIB: diapause I phenotype B.

Experimental series	Experimental condition 19 °C	Initial embryo number	Final embryo number	Viability %	Induction %	DIA %	DIB %
A. viarius 1 (L)	Yamamoto solution	223	118	59,2	21,2	57,7	42,3
	Induced by adults	294	121	41,1	100	79,3	20,7
A. viarius 2 (F)	Yamamoto solution	71	38	53,5	21	87,5	12,5
	Induced by adults	185	100	54	100	51	49
A. charrua 1 (L)	Yamamoto solution	392	193	49,2	2,1	50	50
	Induced by adults	427	227	53,6	100	47,6	52,4
A. charrua 2 (L)	Yamamoto solution	209	173	82,8	3,5	33,3	66,7
	Induced by adults	164	38	23,2	100	42,1	57,9
A. charrua 3 (F)	Yamamoto solution	40	20	50	15	33,3	66.7
	Induced by adults	217	112	51,6	100	25	75

Results and Discussion

Embryos from *Austrolebias viarius* and *A. charrua* between 1 to 16 cells were divided in two groups in all experimental series. Control groups were maintained in Yamamoto's solution and the induced experimental groups were kept in peat moss located in tanks with adults present. Developmental rates and embryo survival are equivalent in Yamamoto's solution or reverse osmosis filtered water reconstituted with marine salt in tanks at 19 °C. After 30 days in culture, a final total number of 1.140 embryos (377 *A. viarius* and 763 *A. charrua*) were observed as viable and classified in this study. The duration of 30 days of induction to confirm diapause I entrance was based on a previous report from preliminary observations of three annual killifish species (Wourms 1972c). Viability ranged from 23.2% to 82.8% (Table 1). Viability percentages reported here were within the range previously described in teleosts (33 to 93%, Fossum, 1988) except for *A. charrua* embryos in experimental series 2 induced by adults (23.2%, see Table 1). The highest vulnerability to mortality occurs during the blastula and gastrula stages in all species studied (Bunn and Webb, 2000).

In control conditions, viability exhibited a mean value of 58.8% while embryos left in the presence of adults had a mean viability of 45.2%. Most of the control viable embryos were at different stages of advanced reaggregation and somitogenesis. A subpopulation of this group (2.1% to 21.2%) were classified as diapause I phenotypes A and B (Table 1) described below. To assess if this subpopulation corresponded to spontaneous diapause I, they were transferred at 25 °C in Yamamoto's solution. At 42 days, all viable embryos resumed the development and were at different stages of somitogenesis and organogenesis. Embryos of *Austrolebias* species cultured in relatively stable control conditions (19 °C, Yamamoto's solution) were able to follow heterogeneous development rates. In the analyzed population, some were found in diapause I phenotypes A and B and others followed direct-developing trajectory to advanced reaggregation and somitic stages at the end of the 30-day experiments.

We conclude that control embryos spontaneously entered diapause I (both phenotype A and B) because all viable embryos transferred at 25 °C resumed development and were found at different stages of somitogenesis and organogenesis after 42 days. This information suggests that exit from diapause I would not be expected to be a synchronous event across the population of embryos. Spontaneous diapause I is extremely rare or absent in *Austrolebias nigripinnis* (Peters, 1963), *Austrofundulus myersi, Nothobranchius* (Wourms, 1972a), *Aphyosemion, Cynopoecilus melanotaenia* (Wourms, 1972c), *A. viarius* (Arezo et al., 2005), and *A. limnaeus* (Wagner and Podrabsky, 2015a) embryos cultured at 25 °C. In contrast, spontaneous arrest at diapause I occurs in *Rachovia brevis* cultured at 25 °C (Wourms, 1972c). At 19 °C, we observed 21% spontaneous diapause entrance in *A. viarius* in both experimental series (Table 1).

Conversely, in all experimental series, diapause I was successfully induced in 100% of viable embryos by the presence of adults in both species. The obtained results demonstrated that the implemented protocol was effective in these annual killifish species. Embryos cultured in the presence of adults displayed one of two alternative diapause I phenotypes (Table 1). Phenotype A embryos exhibited cells in a dispersed configuration but rounded in shape (Fig. 1A) compared with elongated actively migrating cells typically observed during the dispersed cell phase (80–100% epiboly; Fig. 1C). Phenotype B embryos showed loosely arrayed rounded cells in close proximity or in contact (Fig. 1B). This phenotype can be distinguished from the early reaggregation phase because the latter consists of cells that display an elongated and actively migrating morphology (Fig. 1D). A common feature observed in both phenotypes *in vivo* is that the cells are more difficult to distinguish than cells actively migrating in the dispersed and early reaggregation phases using brightfield microscopy (Fig. 1).

To compare amounts of total RNA in actively developing embryos (80–100% epiboly and early reaggregate) and arrested embryos in diapause I (phenotypes A and B), average concentration of total RNA is reported for each developmental stage. A total of 15 samples showed satisfactory integrity and concentration (Bioanalyzer RIN 7,2–9; 13–35 ng/µl). Four and three independent biological replicates *per* stage were done in *A. viarius* and *A. charrua* embryos, respectively. Total amounts of extractable RNA in each analyzed developmental stage revealed a tendency for both arrested diapause I phenotypes to exhibit lower total RNA concentrations compared with active developmental stages (80–100% epiboly and early reaggregate). Moreover, diapause I phenotype A and B embryos exhibited similar amounts of total RNA. To illustrate these results, Figure 2 shows *A. viarius* total RNA amounts in each developmental stage.

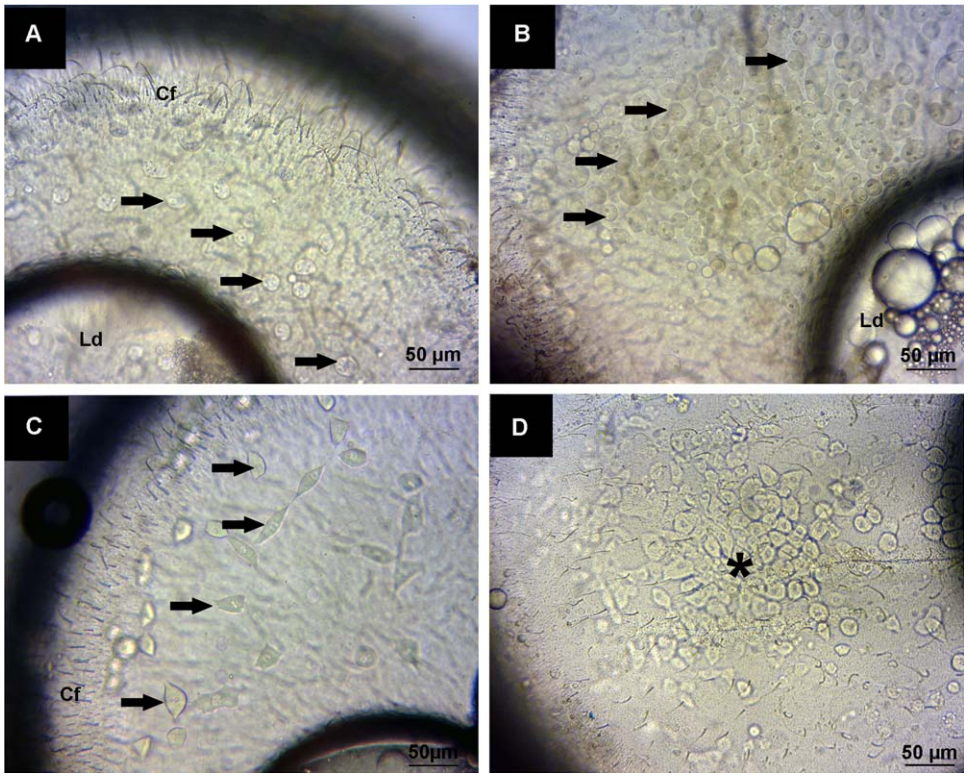
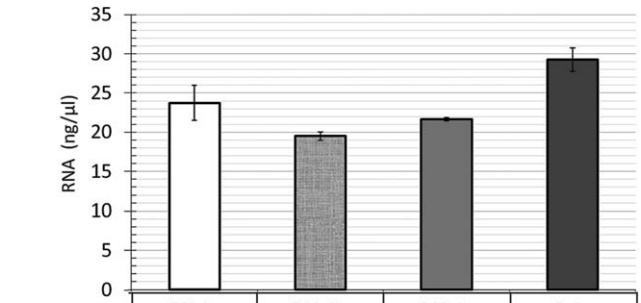


Fig. 1. Developmental stages found in both *Austrolebias* species analyzed in the experimental series. **A**: *A. viarius* diapause I phenotype A. **(B)** *A. viarius* diapause I phenotype B. **(C)** Dispersed cell phase (80–100% epiboly). **(D)** Early reaggregate; asterisk: reaggregation zone. Arrows, blastomeres; Cf: chorion filaments; Ld: lipid droplet



	EPI Av	DIA Av	DIB Av	R Av
RNA (ng/µl)	23,8	19,5	21,7	29,3
SEM	2,2	0,5	0,2	1,5

Fig. 2. Average concentration of *Austrolebias viarius* total RNA from 80 to 100% epiboly (EPI Av), diapause I phenotype A (DIA Av), diapause I phenotype B (DIB Av) and early reaggregates (R Av). SEM, standard error mean value.

In this study, we found and describe, for the first time, an alternative diapause I phenotype (diapause I B) induced by the applied experimental conditions. Phenotype A in *A. charrua* and *A. viarius* was similar to the dispersed phase I (stage 20) while phenotype B appeared to be analogous to dispersed phase II (stage 21) typified by a region of increased cell density loosely arrayed in close proximity or in contact as observed in *Austrofundulus myersi* by Wourms (1972a). In this work, Wourms (1972a) stated that diapause I can occur only in dispersed phase I (stage 20), but we found that embryos at stage 21 (Wourms, 1972a) also enter into diapause I (phenotype B). We suggest that diapause I displays two alternative phenotypes A and B that would respond differentially to environmental cues. The existence of two diapause I phenotypes could result in an increased diversity of responses to environmental cues providing additional diversity among the population of embryos and enriching the multiplier effect proposed by Wourms (1972c).

Previous work in the African annual killifish *Nothobranchius korthausae* suggested that homogenates from ovaries of the same species contain a pheromone-like agent capable of inducing a prolongation of reaggregation as well as diapause I of short duration (Levels et al., 1986b). We propose that embryos observed by Levels et al. (1986b) in delayed reaggregation phase were embryos undergoing phenotype B diapause I.

Considering the percentages of embryos observed in both phenotypes, *A. viarius* showed mean values of 68,9% of embryos expressing phenotype A and 31.1% phenotype B, whereas *A. charrua* exhibited mean values of 38.5% expressing phenotype A and 61.5% phenotype B. Regarding this difference in percentage among phenotypes, *A. viarius* in both experimental series and *A. charrua* in experimental series 2 and 3 display an opposite distribution favoring phenotype A in *A. viarius* and phenotype B in *A. charrua* (Table 1). In accordance with these findings, previous work established that *A. charrua* populations exhibit high genetic variation (García, 2006) at several levels compared with *A. viarius* (García et al., 2009). Thus, additional experiments must be replicated using individuals from the entire distribution of *A. charrua.*

Remarkably, preliminary field data of *A. charrua* embryos, during 78 days in the natural environment showed the presence of both phenotypes of diapause I ($n = 15$). The embryos were maintained in the pond in the same containers designed for

diapause I induction laboratory experimental series used in this study. Phenotype A embryos represented 80% (n = 12) and phenotype B 20% (n = 3) of the population (Arezo et al., unpublished). Although the number of embryos is low, it seems to reflect the same tendency observed in *A. viarius*. It is important to note that *A. charrua* adults used in the experimental series 2 and 3 of this work were collected in the same pond (the same genetic pool) where the experimental field assay of diapause I induction was implemented.

Nevertheless, in the African *Nothobranchius korthausae*, Levels and Denucé (1988) documented a first developmental arrest during the dispersed phase but it was most commonly observed during the reaggregation phase in embryos cultured at temperatures below 16 °C. These findings show the same pattern reported here in *A. charrua* experimental series 2 and 3. It is important to note that these results were obtained under laboratory conditions. When working with organisms in captivity, caution should be taken when extrapolating experimental results to natural populations because of variations in stress level, diet composition, predator abundance, and others factors that could affect experimental results (Cellerino et al., 2015).

Incubation temperatures below 20 °C induce entrance into diapause I in several annual killifish species (Wourms, 1972a; Levels and Denucé, 1988). However, the environmental relevance of this temperature effect must be interpreted with detailed information about the thermal environment experienced by embryos under natural conditions (Podrabsky et al., 2016a). It is very important to note that, although maternal influences may program the embryo for one trajectory, environmental cues experienced by the embryo can override those maternal influences (Levels and Denucé, 1988; Podrabsky et al., 2016b). This fact is clearly observed in our results. For both species and in all experimental series, a striking difference was seen in the percentage of embryos entering diapause I when cultured in Yamamoto's solution compared with those cultured in the presence of adults (see Table 1).

In control conditions, the unique environmental cue is temperature: 19 °C. This temperature is not necessarily informative with respect to the timing of the seasons because it was registered in two instances in the year, either in Autumn (at the beginning of rainy season) or Spring (at the end of the rainy season) in eastern ponds in Uruguay (Van Dooren, personal communication 2009). At this temperature, only 2 to 21% of embryos entered diapause I. On the other hand, embryos cultured in laboratory in the presence of adults were potentially exposed to at least three environmental cues: a chemical factor secreted by adults (Inglima et al., 1981), hypoxia generated by peat moss (Peters, 1963; this study), and temperature below 20 °C (19 °C). In this situation, our results showed that 100% of the viable embryo population entered diapause I.

Temperature is the most variable and critical environmental variable that adult annual killifishes must manage. It influences other environmental factors (i.e., oxygen availability and pH) and has a significant effect on the rate of biological processes such as cellular and organismal metabolism. Temperatures in temporary pond environments exhibits a high degree of variation, therefore, annual killifishes are tolerant to a wide range of temperatures (eurythermal). Daily temperatures for annual killifishes from northern South America and Africa, ranges from 20 to 40 °C (Podrabsky et al., 1998; Podrabsky and Somero, 2003; Reichard, 2010). In southern South America, annual killifish face even larger temperatures fluctuations and must tolerate temperatures below 10 °C.

For example, *Austrolebias nigrofasciatus* from southern Brazil is exposed to temperature ranges of 9 to 42 °C (Volcan et al., 2011). For *Austrolebias viarius*, habitat temperatures range from 6.0 to 28.8 °C (Errea and Danulat, 2001) and for *Austrolebias charrua* from 7.0 to 28.8 °C (Van Dooren, personal communication, 2009) However, the thermal environment experienced by embryos is unknown. It is highly probable that temperature fluctuations are attenuated in the pond sediments. Detailed field experiments and sampling are needed to understand the thermal environment experienced by embryos during their life cycle (Podrabsky et al., 2016a).

Results presented in this work and preliminary field data (Arezo et al., unpublished) suggest that diapause I in both *Austrolebias* species is obligate in natural conditions. This assumption is based on the known natural occurrence of at least three of the environmental cues considered in our experimental series that induced 100% entrance into diapause I: presence of adults, hypoxia in the soil, and reduced temperatures. These observations are in agreement with Podrabsky et al. (2016b) who suggested that diapause is likely always facultative in annual killifishes, with environmental inductors being unique to each species. Our results are also in agreement with the observations of Peters (1963) and Watters (2009) concerning the natural substrate conditions found in the African *Nothobranchius* environment that induces and maintains diapause I until the total drying of the pond, and with the report from Dominguez-Castanedo et al. (2013) that for *Millerichthys robustus* all embryos extracted from the completely dried soil were in diapause I.

Our results indicate that *A. viarius* and *A. charrua*, as reported for *Nothobranchius furzeri* (Furness et al., 2015b), appear to exhibit a combination of phenotypic plasticity to environmental signals (presence of adults) and an intrinsic variability in response to temperature (bet-hedging) as adaptations to environmental unpredictability. There are no generalizations that characterize the habitat of all species of annual killifish, but the most common element is one wet and one dry season each year with no generation superposition (reviewed by Furness, 2016). This is the most common situation documented for *Austrolebias*. Nevertheless, *Austrolebias* face environmental variability at two levels: within and between seasons as recently described in *N. furzeri* (Furness et al., 2015b). Polačik et al. (2014) stated that African ponds inhabited by *N. furzeri* may fill, dry, and refill during a single wet season. This situation was also the case for *Austrolebias* species inhabiting ponds in western (Loureiro, personal communication, 2015) and eastern ponds (Passos, personal communication, 2015) of Uruguay during 2015 rainy season.

Annual killifishes exhibit a wide range of environmental stress tolerances to temperature, dehydration, anoxia, oxidative stress, hypoxia, ultraviolet radiation. Despite the extraordinary tolerance seen in diapause stages and even in developing embryos, these fishes are threatened with extinction due to human disturbance and global climate change. Thus, it is essential to develop field studies to characterize their environment and better understand how these embryos survive in such conditions and how close they are to the limits of their environmental tolerances (Podrabsky et al., 2016a; Volcan et al., 2016).

Detailed examination of the molecular mechanisms that control entrance into and exit from diapause is interesting for two reasons. First, in terms of basic knowledge it contributes to the understanding of mechanisms for controlling cell proliferation and metabolism in fish species that exploit extreme conditions,

and that have an as of yet unresolved evolutionary history with respect to the origins of the annual life history and their unique abilities to exploit marginal and terrestrial habitats. Second, in terms of applied knowledge, their high tolerance to environmental stress factors make them an example of extremophile vertebrates. The identification of the main molecular cues underlying diapause that increases cellular tolerance to stress could bring new insights to explore similar situations that cause tissue damage in other vertebrates. The understanding of long-term embryo tolerance to anoxia could be a good model to search for therapies to human pathologies generated by anoxia/hypoxia and other cellular situations.

Conclusions

Embryos were successfully induced to enter diapause I under the selected experimental conditions in two species of *Austrolebias*. Two alternative phenotypes of diapause I, A and B, were identified and morphologically described in *A. viarius* and *A. charrua*. These results have ecological relevance because both diapause I phenotypes were also found in *A. charrua* embryos maintained in natural ponds with a similar distribution to the *A. viarius* experimental series. We consider that the existence of these two alternative diapause I phenotypes contributes additional diversity in developmental rates improving the control of embryonic responses to highly variable environmental conditions. Diapause I occurs during the unique dispersion-reaggregation phase of development in annual killifishes, and its ecological significance has been demonstrated for *Austrolebias, Millerichthys robustus*, and *Nothobranchius*. However, data on diapause I are scarce in the literature, and the work presented here contributes new basic knowledge to continue exploring this key developmental arrest in annual killifishes.

Experimental Procedures

Diapause I Induction

To evaluate optimal conditions needed to induce diapause I in *Austrolebias* and to further characterize this developmental arrest, we designed experimental series using embryos obtained from two *Austrolebias* species. The protocols used in this work were approved by the Animal Experimentation Committee from the Universidad de la República, CHEA (Comisión Honoraria de Experimentación Animal; protocol code 240011-002308-14).

Austrolebias viarius experimental series 1

Adult females and males of *Austrolebias viarius* were obtained from hatched larvae in laboratory conditions during 2015. The parents were collected in 2014 (Barra de Valizas, Departamento de Rocha, Uruguay: latitude −34.35, longitude −53.78). Adults were kept in 20-L tanks, filled with reverse osmosis filtered water reconstituted with marine salt (Tetra Marine Salt Pro, 300 μS, pH 7–7.5) with continuous air bubbling. Fish were exposed to natural light, and water temperature was maintained at 19 °C. Water was partially changed every 5 days. Specimens were fed once a day with live bloodworms. Spawning was obtained naturally from fish groups consisting of one male/two females isolated in tanks using a container filled with glass spheres (500 μm diameter Thomas Scientific, Swedesboro, NJ) on the bottom.

A total of 517 fertilized eggs between 1- and 16-cell stage were collected. Eggs were divided in two groups and were incubated at 19 °C under a natural photoperiod. Group 1: control, were kept in Yamamoto's solution (Yamamoto, 1967) and group 2 were kept in glass bottles with peat moss at the bottom sealed by a porous mesh. Inside these containers dissolved oxygen was 0.02 g/L (Horiba pH/DO meter D-25).These embryos were placed in a tank containing seven males and three females of *A. viarius*. The experimental induction of diapause I was performed for 30 days since Wourms (1972c) stated that duration of obligate diapause I is 30 days. Embryos were checked individually by placing each one, in a drop of Yamamoto's solution, in a 1.7-mm deep-chamber created by layering electrical tape on a microscopic slide. A coverglass was gently placed over the chamber that touched the top of the egg allowing rotation and observation under a light microscope. Live embryos were examined, classified according to Arezo et al. (2005) and photographed using an Olympus Vanox microscope.

Austrolebias viarius experimental series 2

Adult females and males of *Austrolebias viarius* were collected in 2016 from Barra de Valizas (Departamento de Rocha, Uruguay: latitude −34.35, longitude −53.78) and were maintained under the same conditions described in Experimental series 1. A total of 256 fertilized eggs between 1- and 16-cell stage were collected, divided in two groups and treated as described in experimental series 1.

Austrolebias charrua experimental series 1

Adult females and males of *Austrolebias charrua* were obtained from hatched larvae in laboratory conditions during 2015. The parents were collected in 2014 (La Coronilla, Departamento de Rocha, Uruguay: latitude −33.92, longitude −53.52) and were maintained under the same conditions described in Experimental series 1. A total of 819 fertilized eggs between 1- and 16-cell stage were collected, divided in two groups and treated as described in experimental series 1.

Austrolebias charrua experimental series 2

Adult females and males of *Austrolebias charrua* were obtained from hatched larvae in laboratory conditions in 2016. The parents were collected in 2015 (La Charqueada Departamento de Treinta y Tres, Uruguay: latitude −33.15, longitude −53.53). Adults were maintained under the same conditions described in Experimental series 1. A total of 373 fertilized eggs between 1- and 16-cell stage were collected, divided in two groups and treated as described in experimental series 1.

Austrolebias charrua experimental series 3

Adult females and males of *Austrolebias charrua* collected in 2016 from La Charqueada (Departamento de Treinta y Tres, Uruguay: latitude −33.15, longitude −53.53) and were maintained under the same conditions described in Experimental series 1. A total of 257 fertilized eggs between 1- and 16-cell stage were collected, divided in two groups and treated as described in experimental series 1.

Total RNA Quantification

Stages selected and analyzed in quadruplicate in *A. viarius* were epiboly (n = 11), diapause IA (n = 11), diapause IB (n = 11), reaggregate (annual killifish gastrula) (n = 11). To confirm the observations made in *A. viarius*, the same developmental stages were examined in triplicate in *A. charrua*. Total RNA was extracted with Trizol Reagent (Invitrogen) using the manufacturer's instructions. To normalize the amount of RNA retrieved, 340 µl aqueous phase was obtained from each sample, the RNA was precipitated with 350 µl dextran blue/isopropanol 0,02 µg/ml for optimal co-precipitation of RNA (Papa et al., in preparation), washed in 1 ml 70% ethanol, and resuspended in 30 µl AMRESCO water. RNA quality was initially inspected by electrophoresis in a 0.5% GelRed (BIOTIUM) stained 1% agarose gel using 1XTAE (Tris-Acetate-EDTA). Later, RNA concentration, quality, and integrity were analyzed using a Bioanalyzer 2100 (Agilent, supplementary material).

Statistical Analyses

To compare quantification results, samples were grouped by stage (Epiboly, Diapause IA, Diapause IB and Reaggregated) and parametric and nonparametric tests were performed with GraphPad Prism® 6.0 (www.graphpad.com) to determine if there were significant differences between the different groups.

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Ethics Approval and Consent to Participate

Not applicable.

Availability of Data and Material

All datasets on which the conclusions of the manuscript rely on are presented in the main paper.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

María José Arezo was involved in the conception of the project, the design, acquisition, analysis and interpretation of the data, as well as the writing of the manuscript. Nicolás Papa participated in the design, acquisition, analysis, and interpretation of data and the writing of the manuscript. Nibia Berois participated in the conception of the project, acquisition of data, and the writing and revisions of the manuscript. Graciela Clivio and Jimena Montagne were mainly involved in acquisition and interpretation of data, and Soledad De la Piedra, helped in the preliminary experimental set up for diapause I induction and troubleshooting of experimental procedures.

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