Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

A preliminary approach to embryonic development of *Austrolebias wolterstorffi*, an endangered neotropical annual fish species

Desenvolvimento embrionário de *Austrolebias wolterstorffi*, uma espécie de peixe anual neotropical criticamente ameaçada de extinção

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Abstract

Annual fish live exclusively in temporary wetlands and are among the shortest-lived vertebrates in the world. These fish persist in these habitats due to drought-resistant eggs, that which, through diapauses are able to detect stimuli from the environment to start the development processes. They are also able to direct their embryonic development in different trajectories with different development times. Our objective in this paper was to describe the different stages of embryonic development of *Austrolebias wolterstorffi*, a critically endangered annual fish. A total of 27 stages of embryonic development were identified for the two observed developmental pathways (direct and diapause 2). Of these 27 developmental stages observed, 24 were identical between the two types of trajectories and three different. A total of 90% of the embryos that completed their development proceeded normally, without pauses. One embryo had a different development from the others, entering diapause 2, with a pause in development for 48 h. Although the embryonic development of *A. wolterstorffi* is similar to that of other Neotropical annual fish species, the diapause 2 occurs when the embryo has a large body size and a well-developed brain, indicating that the lack of embryonic information of the genus *Austrolebias* may hide characteristics still undescribed or even different survival strategies than what has been observed for other annual fish.

Keywords: aging, accelerated growth, diapause, drought resistance, temporary ponds.

Resumo

Os peixes anuais vivem exclusivamente em áreas úmidas temporárias e estão entre os vertebrados de vida mais curta do mundo. Esses peixes persistem nesses habitats devido a ovos resistentes à seca, que, por meio de diapausas, são capazes de detectar estímulos do ambiente para iniciar os processos de desenvolvimento embrionário. Eles também são capazes de direcionar seu desenvolvimento embrionário em diferentes trajetórias com diferentes tempos de desenvolvimento. O objetivo deste trabalho foi descrever os diferentes estágios do desenvolvimento embrionário de *Austrolebias wolterstorffi*, um peixe anual criticamente ameaçado. Um total de 27 estágios de desenvolvimento embrionário foram identificados para as duas trajetórias de desenvolvimento observadas (direto e diapausa 2). Desses 27 estágios de desenvolvimento observados, 24 eram idênticos entre os dois tipos de trajetórias e três diferentes. Um total de 90% dos embriões que completaram seu desenvolvimento seguiu normalmente, sem pausas. Um embrião teve um desenvolvimento diferente dos demais, entrando na diapausa 2, com pausa no desenvolvimento de 48h. Embora o desenvolvimento embrionário de *A. wolterstorffi* seja semelhante ao de outras espécies de peixes anuais neotropicais, a diapausa 2 ocorre quando o embrião tem um corpo grande e um cérebro bem desenvolvido, indicando que a falta de informação embrionária do gênero *Austrolebias* pode escondem características ainda não descritas ou mesmo estratégias de sobrevivência diferentes das observadas para outros peixes anuais.

Palavras-chave: envelhecimento, crescimento acelerado, diapausa, resistência à seca, lagoas temporárias.

1. Introduction

Annual fish are a complex fish group that live exclusively in temporary wetlands and are among the shortest-lived vertebrates (Cellerino et al., 2016). Annual fish can survive in extreme hydrological habitats (such as long dry periods) due to unique characteristics, which include accelerated growth to sexual maturity (Genade et al., 2005), high

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Received: January 17, 2023 – Accepted: May 4, 2023

breeding investment and diapausing eggs (Berois et al., 2014). Annual fish eggs are deposited in sediment and remain buried during the dry season (Vaz-Ferreira et al., 1966). With the pond re-flooding, eggs hatch and juveniles become sexually mature in a few weeks, repopulating the substrate with new viable eggs (Volcan et al., 2012; Fonseca et al., 2013).

Egg resistance to drought is mainly due to diapause mechanisms (Berois et al., 2014), with three facultative pauses in embryonic development to face extreme and challenging environmental conditions (Wourms, 1972a, b, c; Furness et al., 2015). The occurrence of three embryonic phases (D1, D2 and D3) in a single taxon is unique to annual fish (Podrabsky and Hand, 1999), with different levels of resistance to environmental challenges (Wourms, 1972a, b, c). Podrabsky et al. (2010) states that there are at least two distinct trajectories in the embryonic development of annual fish. The direct developmental trajectory and the diapause 2 trajectory allow modifying the development timing of annual fish embryo structures according to maternal and environmental cues (Podrabsky et al., 2010; Furness et al., 2015).

Diapause mechanisms and life history traits of annual fish evolved independently in the Neotropics (family Rivulidae) and Africa (family Nothobranchiidae), and probably several times in South America (Furness et al., 2015; Furness, 2016; Podrabsky et al., 2017). In the Neotropics, complete post-diapause 1 embryonic development has been documented for species of the genus *Austrofundulus* - *Austrofundulus myersi* (Wourms, 1972a, b, c) and *Austrofundulus limnaeus* (Podrabsky et al., 1997; Podrabsky and Hand, 1999; Podrabsky et al., 2017), *Cynopoecilus* (Arenzon et al., 2002) but never for the genus *Austrolebias*. The genus *Austrofundulus* is found in tropical regions unlike the genus *Austrolebias*, which occurs mostly in the subtropical climate (Loureiro et al., 2018). This geographical difference can lead to differences in the embryonic development of these species, which highlights the need for comparative studies.

Studies describing the embryonic development of annual fish species are extremely important as these fish are being consolidated as reference organisms for research on senescence, aging, embryonic development and pharmacology (Di Cicco et al., 2011; Berois et al., 2014; Lucas-Sánchez et al., 2014; Harel et al., 2015; Podrabsky et al., 2017; Godoy et al., 2019, 2020, 2021). These fish are distinguished from other model organisms due to their unique life characteristics, many of which are influenced during the embryonic period (Berois et al., 2014). For instance, in *Nothobranchius furzeri*, an African annual fish, the embryonic developmental trajectory exerts a strong influence on post-hatch life characteristics (Polačik et al., 2014).

Understanding the embryonic aspects of these species is extremely important for their ecology and conservation, since most species of Rivulidae are extremely rare and endangered in Brazil (ICMBIO, 2018; Volcan and Lanés, 2018). Among Neotropical annual killifishes, the genus Austrolebias is the third most representative for the Rivulidae family (Costa, 2006), with species distributed in southern Bolivia, southern and Midwestern Brazil, Paraguay, Uruguay and northern and northeastern Argentina (Costa, 2010; Loureiro et al., 2018). Nonetheless, there are no studies describing the embryonic development of genus Austrolebias. In order to contribute to advance on Neotropical killifish biology, our objective was to describes part of the different stages of embryonic development of *Austrolebias wolterstorffi*.

2. Material and Methods

2.1. Study species

Austrolebias wolterstorffi inhabits shallow and vegetated temporary wetlands (depths lower than 60 cm) in areas where dry periods vary from 4 to 8 months (Lanés et al., 2016; Volcan et al., 2019). These species may co-occur with other annual fish species, such as *Cynopoecilus* spp. and the *Austrolebias adloffi* species group. In these assemblages, *A. woltersforffi* reaches the largest sizes and lowest abundances (Volcan et al., 2019) probably due to higher energy demand (Laufer et al., 2009; Arim et al., 2010). The State Decree of Rio Grande do Sul (51,797 - 2014) determines the species *Austrolebias wolterstorffi* in the category of Critically Endangered.

2.2. Sampling and breeding

Two individuals of *Austrolebias wolterstorffi* (female 6.2 cm CT; male 8.4 cm CT) were collected with an aquatic net (D-shaped hand net 60 x 30 x30 cm, 2 mm between nodes) in June 2019 in a temporary wetland of Camaquã River floodplain, in the municipality of Cristal, Rio Grande do Sul (RS), southern Brazil.

The sampled fish were placed in a bucket with water from the habitat and transferred to the zoological park of the Fundação Zoobotânica do Rio Grande do Sul in Sapucaia do Sul city (RS). There, they were kept in a 54-liter aquarium (60 x 30 x 30 cm), under standardized breeding conditions (20°C, 12:12 hour light/dark cycle). The aquarium had constant aeration, spawning substrate composed of coconut fiber and aquatic vegetation, simulating the natural environment. Partial water changes were performed weekly to maintain water quality. Breeder fish were fed zooplankton (microcrustaceans: Cladocera) and annelids twice a day. The methodologies used for the *A. wolterstorffi* maintenance and breeding were adapted from Berois et al. (2012, 2014) and Fonseca et al. (2018).

2.3. Experiment design

After 10 days in the aquarium, the substrate was removed, and sixteen fertilized eggs were selected for the experiment. Fertilization of the eggs was verified by the presence of perivitelline space. All eggs obtained in the substrate were in diapause 1. Diapause 1 is the stage where the blastomeres are still large and randomly distributed within the egg. During this period, the lipid droplets coalesce into a single large drop (Podrabsky et al., 2017). Diapause 2 was characterized by 38-42 somite pairs and reduced heart rate. Diapause 3 was characterized by the complete formation of the embryo and ready for hatching.

The eggs were kept individually in 10 ml plastic containers, in Yamamoto's solution [NaCl, 0.75%, KCl, 0.02%, CaCl2.02%], in the dark and at temperature of 18ºC. All eggs were daily analyzed under an optical microscope, cleaned with tissues used for contact lenses, and then photographed with a Xiaomi Note 8 smartphone 64 mega pixels camera with microscope adapter for evaluating the egg developmental stage. The classification of each stage of embryonic development followed Wourms (1972a, b, c) and Podrabsky et al. (2017).

3. Results

Austrolebias wolterstorffi eggs had an average size of 2.07 mm (± 0.03), spherical shape and color varying from translucent to dark amber. The mean time for complete development was 63.7 days, ranging from 53 to 78 days. At the beginning of the development, the yolk is mobile and each time the egg was turned, the lipid droplet projected upwards, moving the entire internal content of the egg.

At the end of the experiment, 62.5% (n=10) of the eggs were completely developed. The other 6 eggs (37.5%) were not viable, being recognized by the development of fungus in its envelope or chorion. Only one embryo did not use the escape path (individuals that escape diapause 2), showing a pause in development as well as characteristics clearly attributed to diapause 2. A total of 27 stages of embryonic development were identified (Figures 1, 2 and 3) for the two observed developmental pathways (escape and diapause 2). Of these 27 developmental stages observed, 24 were identical between the two types of trajectories and three different (Figure 2).

Figure 1 shows the 10 early stages of *Austrolebias wolterstorffi* embryonic development post diapause 1 and development time after the incubation period starts (in days).

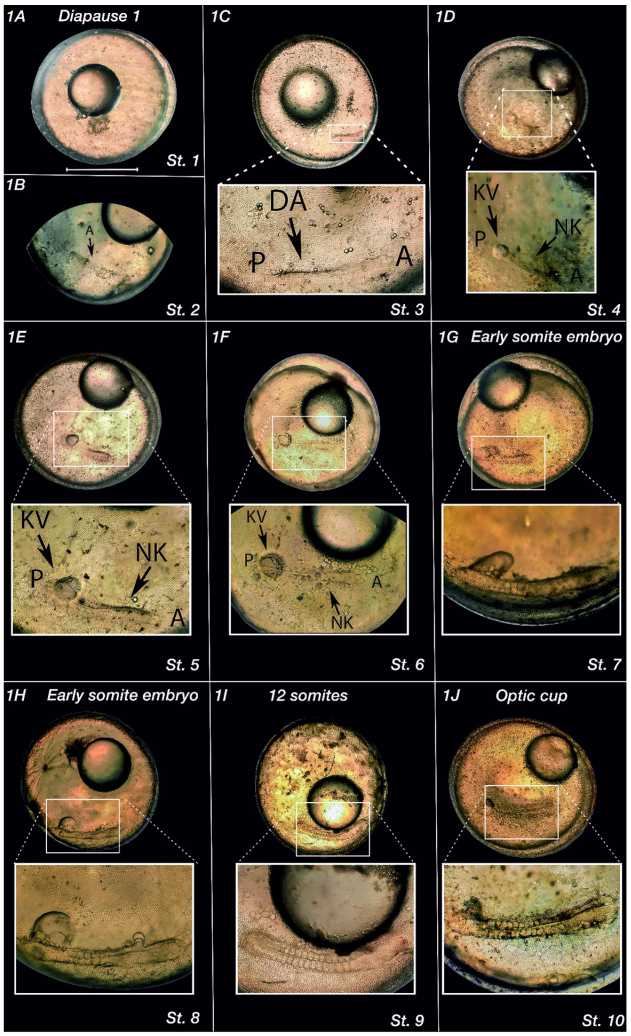

Figure 1. Early stages (St 1 to St10) of embryonic development of *Austrolebias wolterstorffi* post diapause 1. **A**- Future anterior region of the embryo; **P**- Future posterior region of the embryo; **DA**- Definitive embryonic axis; **NK**- Neural Keel; **KV** - Kupffer's vesicle. Scale: 1mm.

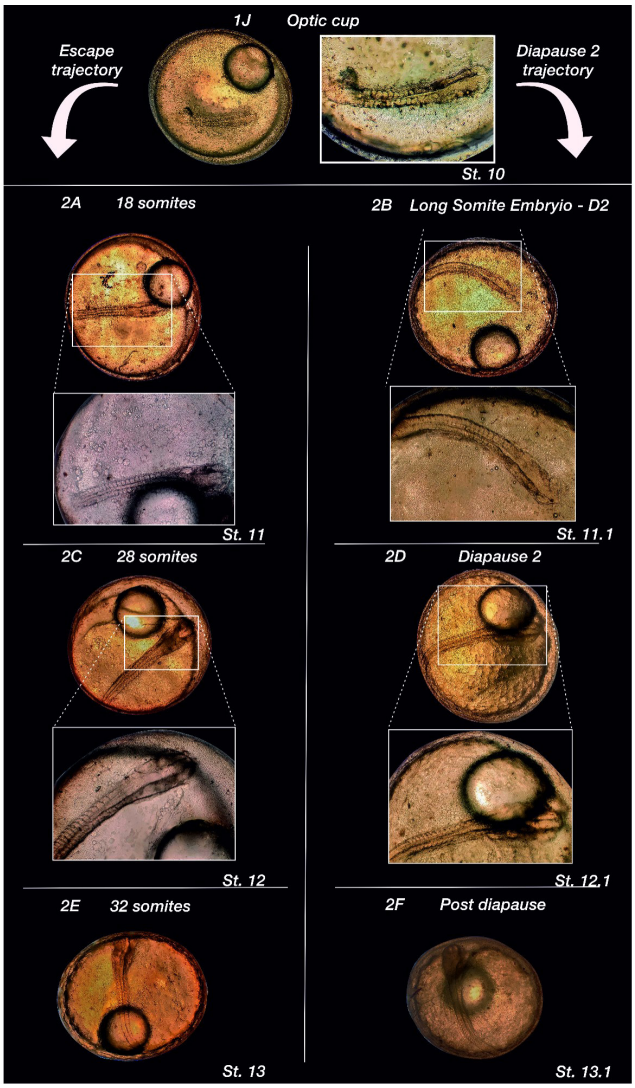

Figure 2. *Austrolebias wolterstorffi* escape and diapause 2 trajectories.

Below we describe the initial development stages and time (in days) shown in Figure 1.

Stage 1 (Figure 1A): (0 days) *Diapause 1*. The egg has a single large lipid droplet that is mobile and moves along with the egg and large rounded blastomeres randomly distributed throughout the yolk.

Stage 2 (Figure 1B): (1 day) At this stage it is possible to visualize the beginning of axis formation after reaggregation. Most of the cells are accumulating and starting to form a reaggregate, and droplets accumulating under or near the aggregate are visible. The lipid droplets remain mobile.

Stage 3 (Figure 1C): (1 day) Cells accumulate forming an axis in the middle of the elongated reaggregate. The anterior and posterior regions remain undifferentiated, with irregular margins.

Stage 4 (Figure 1D): (2 days) Neural keel in formation and beginning of Kupffer's vesicle formation, located in the posterior region, just below the embryo.

Stage 5 (Figure 1E): (2 days) The embryo has a larger size and width than the previous stage (D). Kupffer's vesicle increased in size. The anterior and posterior regions are not still defined.

Stage 6 (Figure 1F): (3 days) The anterior region of the neural keel is wider and has a blade shape. The posterior region remains thinner. Kupffer's vesicle has its largest size so far.

Stage 7 (Figure 1G): (3 days) *Early Somite Embryo.* The first pairs of somites appear, just after the Kupffer's vesicle, lateral to the neural keel. At the anterior end, a head fold begins to form. Kupffer's vesicle is oval in shape. At this stage, the lipid droplet and yolk are fixed inside the

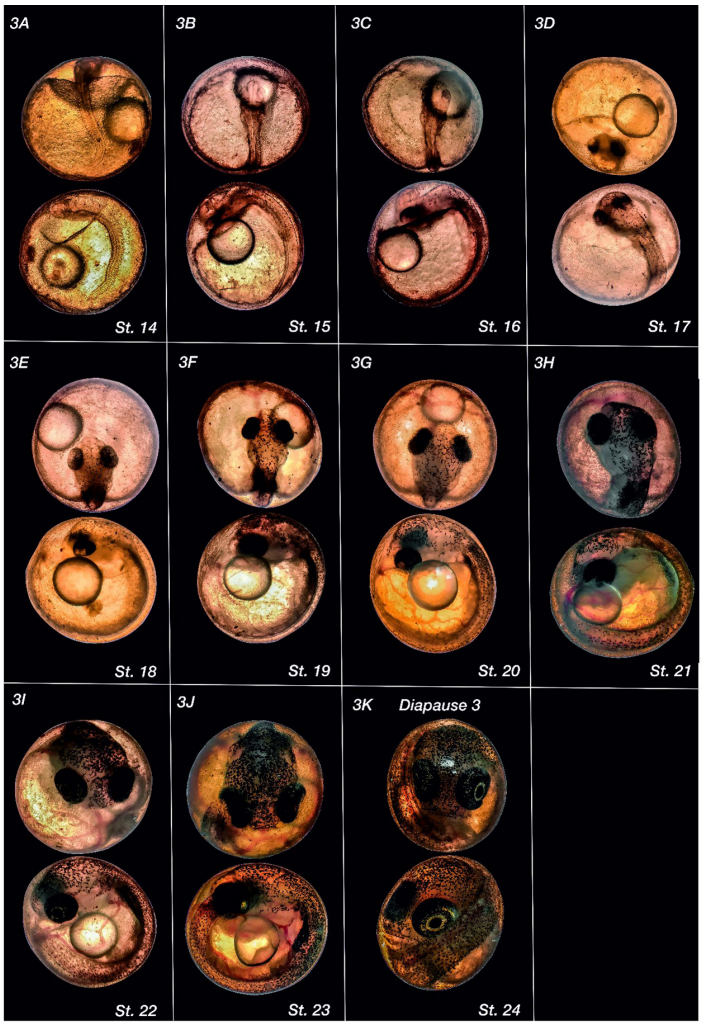

Figure 3. *Austrolebias wolterstorffi* final stages of embryonic development.

egg, and the embryo is well visualized. A lateral mesoderm plate is apparent on both sides of the embryo as a thin band.

Stage 8 (Figure 1H): (4 days) *Early Somite Embryo.* The embryo now has 4 to 6 pairs of somites and the anterior region, previously rounded, begins to show a sharp tip. Kupffer's vesicle is round in shape. The lateral mesoderm plate is thicker.

Stage 9 (Figure 1I): (7 days) *10 to 12 Somites.* The number of somite pairs has increased to 10 or 12. The head region becomes triangular in shape. Two horns begin to appear on either side of the neural keel.

Stage 10 (Figure 1J): (8 days) *Optic Cup.* The optic vesicles are formed and the embryo has between 12 and 15 pairs of somites. Small lateral folds of the neural tube begin to form. It is possible to differentiate the subregions of the future brain, and the groove between the midbrain and hindbrain is formed. The Kupffer's vesicle is still visible at the posterior end of the embryo, but is reduced in size.

After the formation of the optic vesicle, the embryo can follow its development through two trajectories: (1) embryos that completed their development without pauses and (2) embryos entering diapause 2. A total of 90% of the embryos that completed their development proceeded normally, without pauses. One embryo had a different development from the others, that enter to diapause 2, with a pause in development for 48 h.

Figure 2 shows the difference of the three stages found in the direct developmental trajectory and diapause 2 in *Austrolebias wolterstorffi* and development time after the incubation period starts (in days).

Below we describe the three stages shown in Figure 2.

Stage 11 (Figure 2A): (12 days) *18 Somites.* The embryo has 18 to 20 pairs of somites. The head is wider and the otic vesicles are forming.

Stage 11.1 (Figure 2B): (11 days) *Pre-Diapause* 2. The embryo had a fast initial development. The

head width did not change after the formation of otic vesicles. The only visible change was the increase in the number of somites, from 15 to 28-32 pairs. Heartbeat starts (8 to 12 contractions per minute) and some immature blood cells are seen circulating.

Stage 12 (Figure 2C): (20 days) *28 Somites.* The embryo has 28 pairs of somites. Start of heartbeat (8 to 12 contractions per minute). The heart is tubular and is located above the head. Immature (light colored) blood cells can be seen circulating. Otic vesicle is clearly visible and optic lobes are located to the side of the body.

Stage 12.1 (Figure 2D): (15 days) *Diapause 2.* The embryo now has 40 pairs of somites. The head is wider than the previous stage and the optic lobe is turned to the side of the body. The heartbeat stopped for 48 hours. Blood cells no longer circulate, making it possible to see them inside the vessels.

Stage 13 (Figure 2E): (26 days) *32 Somites.* The embryo increased in size, with 32 pairs of somites. Heart rate increased to 45 beats per minute.

Stage 13.2 (Figure 2E): (17 days) *Post-Diapause 2.* The diapause is interrupted and the embryo resumes its development. The number of somites remains the same (36 to 40 pairs), and the heart starts beating slowly again (8-12 beats/min). Blood cells return to circulation.

Figure 3 shows the 11 final stages (stage 14 to stage 24) of *Austrolebias wolterstorffi* embryonic development and developmental time after the incubation period started (in days).

Below we describe the 11 final stages (stage 14 to stage 24) shown in Figure 3.

Stage 14 (Figure 3A): (30 days) Anterior somites take on a chevron shape. From this stage, no differences were observed between diapause 2 and escape embryos.

Stage 15 (Figure 3B): (33 days) The embryo covers a little less than half the egg's circumference. The first melanocytes appear in the form of spots on the back and begin to extend to the tail and head.

Stage 16 (Figure 3C): (37 days) The embryo has increased in size and width, but remains less than half the circumference of the egg. Eye's pigmentation begins, from the upper outer part to the center. Body melanocytes have enlarged and extended to the head and tail, but maintain the form of spots.

Stage 17 (Figure 3D): (39 days) The embryo's head shows a round shape. Melanocytes enlarge and begin to bind together and form a net-like structure. Heart has 41 beats/minute.

Stage 18 (Figure 3E): (41 days) The embryo now covers half the egg circumference. The formation of the intestinal tube begins.

Stage 19 (Figure 3F): (44 days) The eyes had a large increase when compared to the previous stage. The head shows a rounded shape. Blood cells are red. Heart rate increased to 55 beats/min.

Stage 20 (Figure 3G): (45 days) The embryo covers three quarters of the egg circumference. The eye begins to show a reflective coloring.

Stage 21 (Figure 3H): (50 days) The embryo's eyes become golden. Jaws formation begins.

Stage 22 (Figure 3I): (55 days) The embryo covers almost the entire circumference of the egg. The jaw is fully formed and mobile. The eyes have a well-formed golden ring. Embryos appear to be more sensitive to light under the microscope at this stage, reacting when exposed.

Stage 23 (Figure 3J): (58 days) The tip of the tail extends over the head until it reaches the eye. The melanocytes have increased and the embryo has most of its black color.

Stage 24 (Figure 3K): (63 days) The embryo is ready to hatch. The tail now surpasses the eyes and is tucked over the head. The lipid droplet remains visible in the embryo's abdomen. Heart rate is at 82 beats/min, but speeds up when handled.

The embryo that followed the trajectory of diapause 2 showed fastest embryonic development at all stages when compared with the other embryos, until diapause 2 (Stage 12.1). This embryo differed from the others due to an increase in the number of somites (from 15 pairs to 32 pairs) and the beginning of the heartbeat. The escape embryos showed cephalic enlargement and formation of auditory vesicles before the increase in somite number. Although the pause was only 48 h, it was the last to show body and eye pigmentation, reflective eyes, and diapause 3 characteristics. The development time of the embryo that entered diapause 2 was 78 days, 25.58% longer than the other embryos. The Figure 4 that show the timeline in days and the number of stages of both trajectories (diapause 2 and escape).

4. Discussion

This study provides data on the post-diapause 1 embryonic development of *Austrolebias wolterstorffi*, a Neotropical annual fish of high conservation concern. Most of the data on embryonic development for annual fish from the neotropics is available for the genus *Austrolebias* (Berois et al., 2014). Also, the information on developmental stages focused only on the early development and diapause 1 (Arezo et al., 2005; Arezo et al., 2017). Information available on diapause 2 and other developmental stages of Neotropical annual fish are rare (Berois et al., 2014).

The average size of *Austrolebias wolterstorffi* eggs was 2.07 mm (± 0.03). Reports for other *Austrolebias* species show smaller eggs, such as those for *A. nigrofasciatus*, 1.51 mm (±0.12) (Volcan et al., 2011) and *A. viarius*, 1.7 mm (Arezo et al., 2005) (see Table 1). The larger size of *A. wolterstorffi* eggs are likely the result of the, also larger, body size of this species. Larger annual fish species produce disproportionately larger eggs (Eckerström-Liedholm et al., 2017). Visible internal mobile content in the early stages has also been observed for *Austrofundulus limnaeus* (Podrabsky et al., 2017).

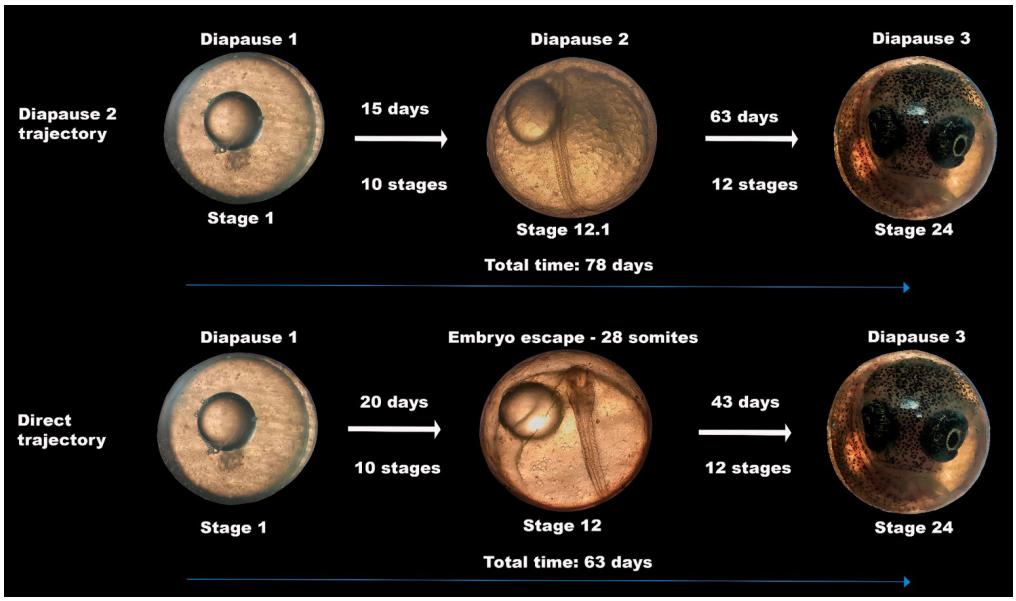

Figure 4. The timeline in days and the number of stages of two trajectories (diapause 2 and escape) of embryonic development of *Austrolebias wolterstorffi*.

Table 1. Embryonic development of three annual fish species.

Annual fish species	Optic cup	Otic vesicle	Heartbeat (escape embryo)	Blood cells	Reflexive eyes
Austrolebias wolterstorffi	12-15 pairs of somites	18 pairs of somites	28 pairs of somites	28 pairs of somites	Embryo covers almost the entire circumference of the egg
Austrolebias viarius (Arezo et al., 2005)	3-15 pairs of somites	3-15 pairs de somites	-	-	-
Austroundulus limnaeus (Podrabsky et al., 2017)	10 pairs of somites	25-28 pairs of somites	12-15 pairs of somites	38 pairs of somites	Embryo covers three quarters of the egg's circumference

In this study, we characterize 24 embryonic stages post diapause 1. The embryonic development and number of developmental stages of *A. wolterstorffi* is similar to those of other species, such as *A. limnaeus* (Podrabsky et al., 2017), although there was variation in the formation of some structures at different developmental stages (see Table 1). There is some degree of similarity in the appearance of melanocytes and the golden colour of the eyes in both *A. wolterstorffi* and *A. limnaeus*, the embryos of the former being more pigmented than the ones from the latter towards the end of the development.

The differentiation in embryonic development that occurred for the embryo that entered diapause 2 in the present study (an "elongated" embryo with a greater number of somites and imperceptible changes in the cephalic region) was also observed in *Austrofundulus limnaeus* previous diapause 2 (Podrabsky et al., 2017). This could be a strategy for embryos going into diapause 2 to save resources before entering diapause (Furness et al., 2015).

The embryo of *Austrolebias wolterstorffi* that entered diapause 2 reached head and body size larger than *Austrofundulus limnaeus*, pausing its development for 48 h at this advanced stage. *Austrofundulus limnaeus* embryos in diapause 2 continued with reduced cephalic size, showing evolution of these structures only after leaving diapause 2. It has been argued that the heart and the sensory organs associated with the head are energetically demanding structures to maintain for long periods (Elia, 1992).

The embryos of annual fish are able to follow the escape trajectory or diapause 2. This choice strategy favors the survival of the fish egg bank in intermittent ponds (Furness et al., 2015). Our results show the importance of a diapause 2 to enable embryos to have different development times within the same egg bank. The development time of the embryo that entered in diapause 2 was 25.58% longer than the other embryos. This led to a delay of 20 days for the completion of the embryonic development. Such delayed can be advantageous in dealing with unpredictable habitats,

such as temporary wetlands, where flash floods can trigger a hatching response from fish but unable to maintain surface water for long enough to keep the population viable throughout the cycle, On the other hand, under favorable conditions, direct development anticipates the hatching of embryos, allowing the establishment of two generations over a single station of surface water from a temporary pond (Furness et al., 2015).

The high number of embryos that passed through the escape trajectory (90%) may be related to the fact that the eggs were kept in controlled laboratory conditions, without the unpredictable and harsh conditions of the natural habitat that would stimulate the entry into diapause 2 (Furness et al., 2015). In fact, *Austrolebias nigrofasciatus* embryos, when kept in different culture media, show a highly variable rate of individuals that escape diapause 2 (Fonseca et al., 2018). Keeping the eggs in Yamamoto's solution was chosen as it prevents fungal infections in the eggs. Fonseca et al. (2018) observed that this culture medium had the highest number of embryos completing their development (93% survival). Our study had 65% of the embryos completing their development and we attribute this relatively lower number to the high exposure of eggs to the microscope (daily).

Austrolebias wolterstorffi is a rare and critically endangered species (Lanés et al., 2016), and there are no studies that characterize the entire trajectory of the embryonic development of an *Austrolebias* species after diapause 1 with information on the steps that precede diapause 2, start of heartbeat and formation of the main structures. Further studies focusing on the embryonic development of *Austrolebias* in the laboratory and in the field are necessary to further advance the knowledge on this genus embriology. The embryonic development of annual fish has a strong influence on life characteristics of the adult fish (Polačik et al., 2014) and this knowledge is essential for population conservation and management. The time it takes to form structures and complete the embryonic development of *A. wolterstorffi* was this far unknown, and the information gathered in this study allows the estimation of the stage at which eggs are found in nature, improving the efficiency of methodologies for egg sampling and observation in the field.

Information on the biology and conservation status of annual fish is still limited (Nascimento et al., 2015). From a conservation standpoint, knowledge of the embryonic development of annual fish allows decision makers to have a starting point to begin us to understand how a range of threats, from climate change to use of pesticides, will influence the survival of these species. In addition to that, such information will help the management of populations kept in captivity with the intention of reintroduction in the wild or the establishment of gene banks.

Acknowledgements

The authors are grateful to private landowners for granting access to their properties, Zoobotany Foundation of Rio Grande do Sul for providing room to carry out the experiments and ICMBio for obtaining the license. This research was authorized by IBAMA/ICMBio (SISBIO process number 68290-3) and UNISINOS Ethic Committee (PPECEUA 12.2019). This research was supported by CNPq - Conselho Nacional de Desenvolvimento Científico (grant number 474892/2013-1). CNPq granted Master and PhD scholarships to PHOH, VW and RG. CAPES supported LEKL with a doctoral fellowship. LM hold Research Productivity Grants from CNPq.

Ethics approval statement

This research was authorized by IBAMA/ICMBio (SISBIO process number 68290-3) and UNISINOS Ethic Committee (PPECEUA 12.2019).

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