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The evolution of diapause in *Rivulus* (*Laimosemion*)

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Annual killifish that are adapted to life in aquatic habitat that dries seasonally have evolved desiccation-resistant eggs capable of undergoing diapause, i.e. developmental arrest, at specific stages during embryology. Although noted for their remarkable ability to live at the land–water interface, species in the genus *Rivulus* are considered to be non-annual killifish that exhibit typical teleost developmental patterns and no embryonic diapause. Here, we combine a molecular phylogeny with an embryological study in order to demonstrate an independent origin of mid-embryonic diapause within a clade of *Rivulus* (subgenus *Laimosemion*) that inhabits small streams or savannah pools. We also observed that some of these species exhibit a short dispersed cell phase separating epiboly and the formation of the embryo axis, which is a feature of development observed only in annual killifish. Lastly, we incubated embryos of *Laimosemion* species and outgroup taxa in both water and peat moss and observed that on peat moss the embryos of all species are capable of delaying hatching for > 10 days, but when water incubated there are significant differences among species in the duration of this delay before hatching. We hypothesize that the preferred microhabitat of this clade of killifish exposes their embryos to periodic desiccation, creating selection in favour of embryonic diapause.

ADDITIONAL KEYWORDS: annual life cycle – Cyprinodontiformes – delayed hatching – embryology – killifish – *Laimosemion* – *Owiyeye* – phylogeny.

INTRODUCTION

A successful comparative approach to the study of biological complexity, such as complex organs or reproductive modes, has been first to find taxonomic groups that exhibit variation in the degree of complexity, meaning those that exhibit plausible evolutionary intermediates along the trajectory from trait absence to full trait expression. For many complex traits, such as flight and morphological adaptations to flight, intermediate forms have gone extinct, and evolutionary scenarios are often developed with paleontological data (Xu *et al.*, 2011; Lee & Worthy, 2012). However, our ability to understand how and why complex traits evolve is magnified if intermediate steps exist among closely related extant species. This is because we can gain insights into the type of selection that caused the evolution of these traits and the incremental development of complexity. First, time-calibrated molecular phylogenies can be generated to estimate the time frame over which the complexity has evolved (Kumar & Hedges, 1998; Filipski *et al.*, 2014). Second, comparative methods can be used to infer the sequence of events that led to the evolution of the full trait. Furthermore, hypotheses regarding correlated character evolution can be tested and ancestral character states reconstructed to infer how many times the trait arose (Felsenstein, 1985; Harvey & Pagel, 1991; Schluter *et al.*, 1997; Pagel, 1999; Reznick, Mateos & Springer, 2002; Avise, 2006). Once this foundation has been laid, follow-up experiments can be used to test hypotheses regarding conditions that might have favoured evolution of the complex feature (Pollux *et al.*, 2009; Weber & Agrawal, 2012). This is the general approach we are pursuing with regard to the evolution of diapause (developmental arrest during embryology) in killifishes.

The evolution of embryonic diapause permits population persistence in harsh environments that are habitable by the adult phase for only a portion of the year (Wiggins, Mackay & Smith, 1980; Williams, 1985).

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Much is known about the ecological importance, geographical variation and hormonal and physiological control of diapause, but there has been little work on the evolution of diapause. Two reasons for this paucity are: (1) for most species in which diapause occurs, the egg shell is opaque such that embryos are not readily observable; and (2) sometimes all species in a relevant taxon display diapause as an obligate stage in the life cycle, hence the group lacks the variation that could yield clues to how the phenomenon evolved. Killifish that are adapted to ephemeral aquatic environments circumvent both of these difficulties. Available evidence suggests multiple independent origins of diapause, thus providing the raw material for comparative studies (Furness *et al.*, 2015b); and the embryos are abundant, easily obtained and translucent, thus facilitating observational studies of development.

Background on diapause in killifishes

Killifish that are adapted to life in temporary bodies of water are found across large portions of Africa, South America (Murphy & Collier, 1997; Cellerino, Valenzano & Reichard, 2016; Furness, 2016) and Mexico (Dominguez-Castanedo, Mosqueda-Cabrera & Valdesalici, 2013). Owing to the regular or periodic drying of their aquatic habitat, certain species have evolved desiccation-resistant eggs capable of undergoing diapause at specific embryological stages (Wourms, 1972a, b, c; Podrabsky *et al.*, 2017). As their aquatic environments dry out and the adults die, the developing eggs remain buried in the soil in diapause until the next rainy season. In habitats that have distinct wet and dry seasons, where pools regularly dry each year, this suite of traits has been referred to as an annual life cycle. One of the defining features of annual killifish is their unique embryology characterized by discrete and conserved stages at which development can be halted, termed diapause I, II and III by Wourms (1972a).

Diapause I occurs during a feature of development that has been described as unique to annual killifish, a dispersed cell phase, which occurs after epiboly but before the formation of the somite embryo (Wourms, 1972b). During this period, flattened and aggregated blastoderm cells disperse such that the embryo appears completely empty. Depending upon environmental conditions, this dispersed cell phase can last from several days to many months before cells re-aggregate and eventually form the embryonic axis. Harsh environmental conditions, such as cold temperatures and/or lack of oxygen, greatly increase the prominence and length of diapause I during the dispersed cell phase (Levels & Denuce, 1988; Podrabsky, Tingaud-Sequeira & Cerda, 2010b).

Diapause II occurs in embryos possessing ~38 pairs of somites and the beginnings of several organ systems. It is during this diapause that embryos are most resistant to temperature extremes, desiccation and oxygen deprivation (Matias & Markofsky, 1978; Matias, 1982; Podrabsky & Hand, 1999; Podrabsky *et al.*, 2010b). Environmental conditions during embryo incubation influence whether annual killifish embryos enter into diapause II or instead follow the direct-developing pathway and proceed directly to the prehatching stage (Podrabsky, Garrett & Kohl, 2010a; Furness, Lee & Reznick, 2015a).

Diapause III occurs at late prehatching when the embryo is fully developed and is accompanied by a slowing of heart rate and metabolic rate (Levels, Gubbels & Denucé, 1986; Podrabsky & Hand, 1999). Annual and non-annual killifish alike can exhibit something superficially similar to diapause III. However, in non-annual killifish it is generally referred to as delayed hatching rather than diapause because embryos usually remain in this state for a shorter period, remain metabolically active, and it may not be expressed by all embryos (Wourms, 1972c; Verla-Lasheras & Van Dooren, 2014; Furness, 2016; Martin & Podrabsky, 2017; Thompson *et al.*, 2017). Although all species of annual killifish apparently have embryos capable of arresting development in one or more of the three diapause stages (I, II and III), different species exhibit variation in the propensity and regularity with which their embryos enter into each diapause (Wourms, 1972c; Simpson, 1979).

In killifish there has been limited work on the sequence of evolutionary events leading to these diapause stages, whether species with intermediate stages exist, or if there are precursors in outgroup taxa. A comparative examination of development in a group that contains the full range of variation is needed. Intermediates on the path to an annual life cycle (characterized by diapause I, II and III) would come in the form of species that exhibit only a subset of diapause or proto-diapause stages. Given the existence of such intermediates and a phylogeny of the group, it should be possible to construct an evolutionary scenario to get from typical teleost development characterized by no diapause to the acquisition of an annual life cycle with all three diapause stages.

Furness *et al.* (2015b) created a molecular phylogeny of Aplocheiloidei killifish using supermatrix tree construction methods (de Queiroz & Gatesy, 2007), mapped the presence or absence of diapause II (the most prominent stage of developmental arrest in annual killifish) onto this tree and performed parsimony and maximum likelihood ancestral state reconstructions. Their results suggested multiple independent origins within clades of killifish found in both Africa and South America, including some at relatively low taxonomic levels (i.e.

a single species or several species with diapause nested within otherwise non-annual genera). These results support the argument that the evolution of diapause and transition to an annual life cycle can occur readily and rapidly given appropriate ecological selection pressures, such as the colonization of ephemeral aquatic environments or the origin of such environments as a consequence of environmental change. Wourms (1972c) hypothesized and provided some descriptive and comparative evidence that the specific periods of development where diapause I, II and III occur might represent periods of discontinuity and natural windows in which to halt development. This could be construed as one possible explanation for the repeated appearance (i.e. convergent evolution) of the same diapause stages in multiple lineages of annual killifish that may have last shared a common ancestor > 100 Mya, before the break-up of Africa and South America (Murphy & Collier, 1997; Costa, 2013). A related hypothesis is that the ancestor of all extant Aplocheiloidei killifish residing on the Pangaea supercontinent exhibited diapause I, II and III. Diapause was subsequently lost, and upon re-colonization of ephemeral waters this ancient diapause pathway was reactivated in multiple descendant clades spread across the now separated African and South American continents. The repeated evolution of a 'supersoldier' subcaste in ants by means of an ancestral developmental pathway provides a fascinating example of this phenomenon (Rajakumar *et al.*, 2012).

Here we focus on the evolution of diapause within the killifish genus *Rivulus* (subgenus *Laimosemion*). The genus *Rivulus* represents a large (> 150 species), widely distributed (most of Central and South America and the Caribbean) and morphologically conservative group of killifish. Species in this genus exhibit remarkable abilities to live at the land–water interface. For example, *Rivulus* species are noted for their ability to survive out of water for long periods, travel through damp leaf litter to colonize new aquatic habitat and even forage terrestrially (Seghers, 1978; Sayer & Davenport, 1991; Brosset, 2003; Turko & Wright, 2015; Furness, 2016). With few exceptions, *Rivulus* are considered 'non-annual' killifish exhibiting typical teleost development patterns (i.e. no diapause). However, beginning with the suggestion by Thomerson & Taphorn (1992) of an annual life cycle for *Rivulus nicoi*, there have been several credible reports of species collected in ephemeral pools, producing diapause eggs or having eggs with a long incubation period: *Rivulus* (*Laimosemion*) *tecminae*, *Rivulus* (*Laimosemion*) *nicoi*, *Rivulus* (*Laimosemion*) sp. Tobogán (now *Rivulus* (*Laimosemion*) *tomasi*) and *Rivulus* (*Laimosemion*) sp. Maroa (Thomerson, Nico & Taphorn, 1992; Thomerson & Taphorn, 1992; Hrbek & Taphorn, 2010; Vermeulen, 2011; Vermeulen, Valdesalici & Garcia-Gil, 2013). The nature of these apparent instances of diapause have not been the subject of detailed study, and it is not known whether they occur, in all cases, at the same stages of development as has been reported in other annual South American killifish. These reports are particularly important because, if confirmed, they would almost certainly represent recent and independent origins of diapause (I, II and III) in this otherwise non-annual group that inhabits marginal aquatic habitat.

A collection expedition to the Orinoco drainage basin, Amazonas territory, Venezuela targeted these potentially annual *Rivulus* species. Here we report on the developmental biology of these species, gathered through laboratory study. Specifically, we test whether any *Rivulus* (*Laimosemion*) species exhibit embryonic diapause and, if so, whether it occurs at the same stage(s) of development as in other annual killifish. We combine these data with a molecular phylogeny of the group and perform ancestral state reconstructions in order to make inferences regarding the evolution of diapause I, II and III. We provide a descriptive narrative of how diapause might evolve by documenting the intermediate steps on the path to complexity.

MATERIAL AND METHODS

Taxonomic notes

The taxonomy of the genus *Rivulus* is in a state of flux, with a recent call for well-supported subgenera (*Anablepsoides*, *Atlantirivulus*, *Cynodonichthys*, *Laimosemion* and *Melanorivulus*) to be elevated to genera (Costa, 2011) and with the generic name *Rivulus* being retained by a handful of species found on the Caribbean islands of Cuba and Hispaniola. This decision has proved controversial (Huber, 2012), in large measure because the higher-level relationships among these subgenera remain unresolved (Hrbek & Larson, 1999; Murphy, Thomerson & Collier, 1999; Costa, 2011; Huber, 2012; Furness *et al.*, 2015b) and the iconic name *Rivulus*, which formerly applied to > 150 species, is relegated to only three species: *cylindraceus/insulaepinorum*, *berovidesi* and *roloffi*. The crucial issue in determining whether the traditional taxonomic genus known as *Rivulus* can remain intact is whether it is monophyletic; that is, whether each of the well-supported subgenera, which Costa (2011) elevated to generic level status, form a monophyletic group. Although several studies have suggested that *Rivulus* is paraphyletic or polyphyletic, such relationships are taxonomically unstable and characterized by weak bootstrap support (Hrbek & Larson, 1999; Murphy *et al.*, 1999; Hrbek, Pereira de Deus & Pires Farias, 2004; Costa,

2011; Huber, 2012; Furness *et al.*, 2015b). Until the higher-level taxonomy of *Rivulus* is put on more solid footing, we continue to refer to *Rivulus* as the traditional taxonomic grouping that includes all subgenera (*Anablepsoides*, *Atlantirivulus*, *Cynodonichthys*, *Laimosemion*, *Melanorivulus* and *Rivulus*), and we explicitly denote when we are referring to a given subgenus.

Field collection trip

In August 2012, the first author and five others travelled to the Orinoco river drainage, Amazonas territory, Venezuela with the goal of collecting the reportedly annual *R.* (*L.*) *tecminae*, *R.* (*L.*) *nicoi* and several congeners (*R.* (*L.*) *tomasi*, *R.* (*L.*) sp. Maroa). Seventeen sites were visited and *Rivulus* collected from 15. Sampling included floodplains and tributaries to the Orinoco, Ventuari, Atabapo and Temi rivers between the cities of Puerto Ayacucho in the north and Maroa in the south. Collection locality information can be found in Table 1 and Figure 1. At each site, *Rivulus* were collected from small creeks or isolated pools using hand nets. Live stock from seven sites was transported to the Universty of California, Riverside for study of embryological development; specimens from additional populations were preserved in 95% ethanol or formalin, with lots being deposited at the UNELLEZ Museum of Natural History in Guanare, Venezuela.

Laboratory study of *Rivulus* embryonic development

Twelve populations of *Rivulus* (each consisting of between three and ten individuals) were maintained in 38-litre aquaria in the vivarium facilities at the University of California, Riverside. The fish room was kept on a 12 h light–12 h dark cycle, and air temperature was maintained at 25 °C. Feeding was *ad libitum*. Filtration was provided by air-driven sponge filters, and tanks were given 50% partial water changes approximately monthly. During mating, annual killifish deposit embryos within the bottom substrate (Wourms, 1972c; Simpson, 1979). In contrast, non-annual killifish have been reported to deposit embryos amongst aquatic vegetation, leaf litter, organic debris and plant root masses (Breder & Rosen, 1966; Simpson, 1979; Fraser *et al.*, 1999). Initially, we did not know what the optimal spawning substrate was for each population. In each tank, we thus provided three types of spawning substrates that encompassed a range of natural variation: (1) clear plastic Tupperware container with ~2 cm of very fine-grained brown river sand; (2) dark-brown yarn mop; and (3) green floating plastic plants. These three spawning substrates were located in the bottom (sand), mid-level/bottom (mop) and surface (plant) layers of the water column. Embryos were collected every 1–7 days, examined under a dissecting microscope to ensure viability, and placed into plastic Petri dishes or the wells of 24-well tissue culture plates containing either moist peat

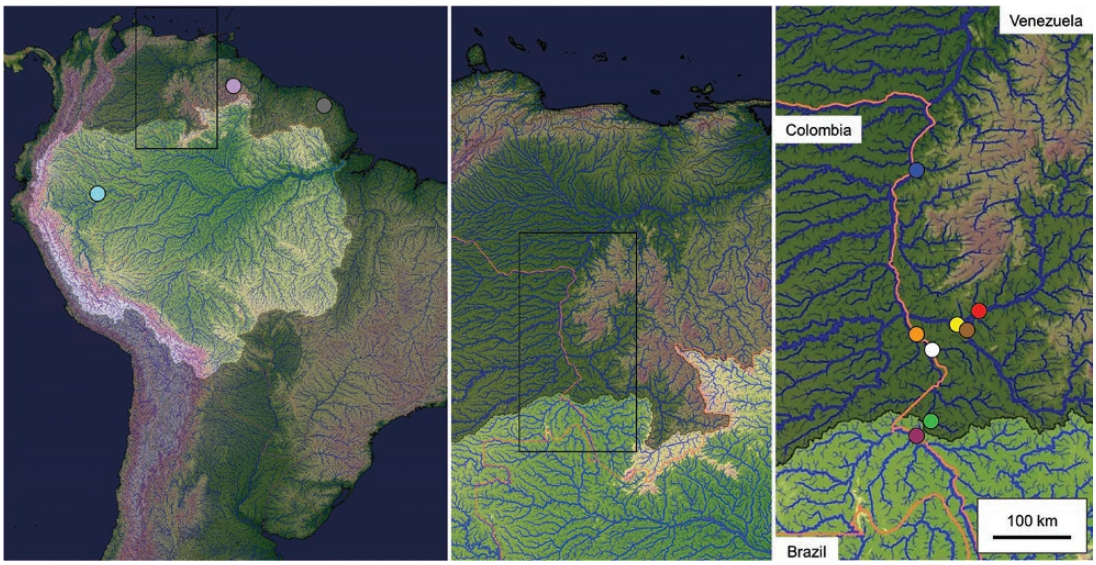

Figure 1. Map of South America overlaid with major river networks (NASA image created by Jesse Allen using data provided by University of Maryland and HydroSHEDS). In all panels, the light region is the drainage basin of the Amazon river. The drainage basin of the Orinoco river is directly north of the Amazon basin and encompasses the majority of our Venezuelan collection localities. Pink lines denote country boundaries. Coloured circles correspond to collection localities detailed in Table 1.

Table 1. Collection locality and identity of *Rivulus* (*Laimosemion*) and outgroup taxa that were studied herein

Locality	Site code	Killifish species
Sabanita, Venezuela	IVE 12-01	*Rivulus* (*Laimosemion*) *tecminae*
Maroa, Venezuela	IVE 12-02	*Rivulus* (*Laimosemion*) sp. Atabapo and sp. Maroa
Yavita, Venezuela	IVE 12-06	*Rivulus* (*Laimosemion*) sp. Maroa and sp. Yavita
Guarinuma, Venezuela	IVE 12-07	*Rivulus* (*Laimosemion*) sp. Atabapo
Macuruco, Venezuela	IVE 12-09	*Rivulus* (*Laimosemion*) sp. Atabapo
Chipiro, Venezuela	IVE 12-13	*Rivulus* (*Laimosemion*) sp. Atabapo
Ventuari, Venezuela	IVE 12-14	*Rivulus* (*Laimosemion*) sp. Ventuari
Tobogan, Venezuela	IVE 12-16	*Rivulus* (*Laimosemion*) *tomasi*
Iquitos, Peru	–	*Rivulus* (*Laimosemion*) *rectocaudatus*
French Guiana	RN2	*Rivulus* (*Laimosemion*) *xiphidius*
San Rafael de Kamoiran	THV 2010-10	*Rivulus* (*Laimosemion*) *gransabanae*
Naranjo river, Trinidad	–	*Rivulus* (*Anablepsoides*) *hartii*
Sugarloaf Key, USA	–	*Kryptolebias marmoratus*

Colours correspond to localities indicated on the map in Figure 1 and the phylogeny in Figure 6.

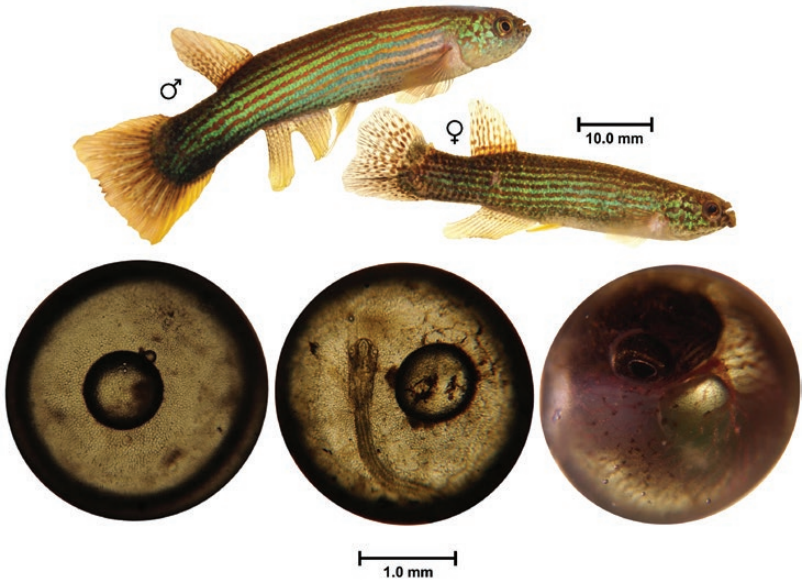

Figure 2. Male and female *Rivulus* (*Laimosemion*) sp. Atabapo from the Maroa locality. Embryos that are in the dispersed cell stage, diapause II and the prehatching stage (i.e. delayed hatching or diapause III).

moss or water. These dishes or trays were incubated at one of two temperatures, 20 or 25 °C. The goal of our study was to test whether *Rivulus* (*Laimosemion*) species exhibit embryonic diapause. Different annual killifish species exhibit variation in how readily each diapause is entered (i.e. whether each stage is a facultative or obligate feature of development) and their length, both of which can be influenced by incubation conditions (Podrabsky *et al.*, 2010b; Furness *et al.*, 2015a; Pinceel *et al.*, 2015). For example, in the South American annual killifish *Austrofundulus limnaeus*, all embryos entered mid-embryonic diapause at the cooler temperature of 20 °C, ~80% entered mid-embryonic diapause at the intermediate temperature of 25 °C, and all embryos skipped mid-embryonic diapause at the warmer temperature of 30 °C (Podrabsky *et al.*, 2010a). In the present study, we chose to incubate embryos at two temperatures and on two incubation media in case diapause was expressed in only a subset of these conditions. Specifically, we reared *Rivulus* embryos at a cooler (20 °C) and moderate (25 °C) temperature in case diapause expression was temperature dependent. Embryos were incubated on damp peat moss or in water because these are common

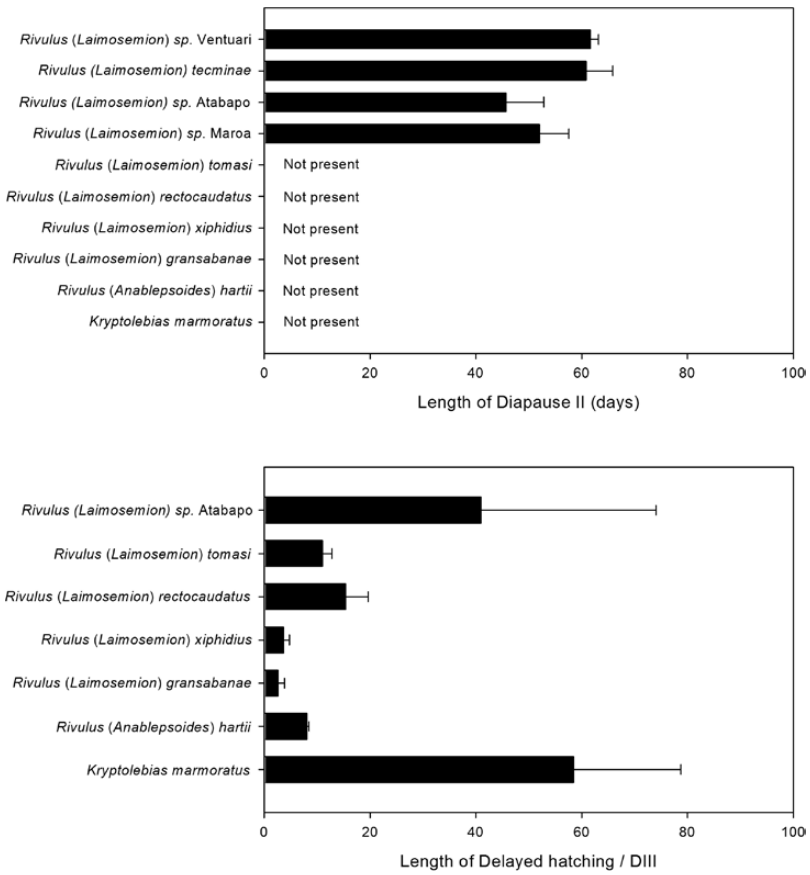

Figure 3. Top panel, duration of diapause II (mean + SEM) for embryos incubated in water and on peat moss. This is the number of days for which embryos arrested development in the late somite stage. The duration of diapause II did not differ among the four species that exhibited diapause II (generalized least-squares model that accounts for unequal variance among species: $F = 2.418$ $P = 0.083$). Bottom panel, duration of delayed hatching/diapause III (DIII; mean + SEM) for embryos incubated in water. This is the number of days from first reaching the prehatching stage (i.e. when development was complete) until embryo hatch. The duration of delayed hatching/diapause III differed significantly among species ($F = 7.3$, $P < 0.0001$). *Rivulus* (*Laimosemion*) *gransabanae* showed the shortest period of delayed hatching after development was complete (2.57 ± 1.27 days); therefore, this species is used as a baseline for comparison with the other species (i.e. the intercept in the generalized least-squares model). *Kryptolebias marmoratus*, *Rivulus* (*Anablepsoides*) *hartii*, *Rivulus* (*Laimosemion*) *rectocaudatus* and *Rivulus* (*Laimosemion*) *tomasi* had significantly longer periods of delayed hatching/diapause III than *R.* (*L.*) *gransabanae* ($P < 0.05$). *Rivulus* (*Laimosemion*) *xiphidius* and *Rivulus* (*Laimosemion*) sp. Atabapo did not differ significantly from *R.* (*L.*) *gransabanae*. In the case of *R.* (*L.*) sp. Atabapo, this is attributable to the extremely high variance and small sample size.

incubation methods for annual and non-annual killifish embryos, respectively. However, testing how incubation conditions affect diapause length was not the primary focus of our study. Thus, we primarily limit ourselves to conclusions regarding the presence or absence of diapause.

After collection, individual embryos were tracked through time, and the stage of development was scored and recorded periodically (on average every 5.3 days) according to the development guide for the annual killifish *Austrofundulus myersi* (Wourms, 1972a). Embryo developmental stage was determined by means of examination under a dissecting or compound microscope. Embryos were periodically transferred to a depression slide and, with the aid of a coverslip, rotated such that a clear flat image of the head region could be photographed with a Nikon D3100 camera attached to an Olympus BH-2 compound microscope. The dispersed cell phase was evident as a short period (< 10 days) between epiboly and the formation of the embryonic axis during which the embryo appeared clear because cells had dispersed and formed no visible aggregation or a loose aggregation (Supporting Information, Figs S1–S7). Given the lengthy arrest during the dispersed cell

Figure 4. Representative embryos that have arrested development in diapause II (38–42 somites).

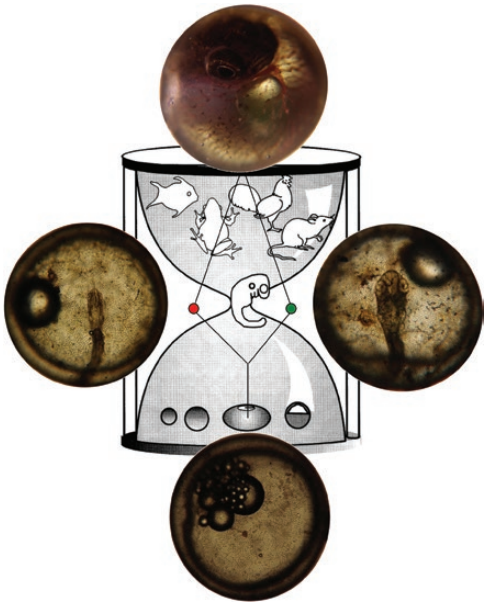

Figure 5. Embryological divergence along alternative developmental pathways in *Rivulus* (*Laimosemion*) sp. Atabapo. The embryo on the left has entered diapause II at the 38-somite stage, and the embryo on the right is at the same stage of development but has skipped diapause II and remained direct developing. Morphological and physiological divergence begins before the stage at which diapause II is entered, during a period of supposed developmental constraint when the organ systems are being developed, represented as the narrow bottleneck of the hourglass model (see also Furness *et al.*, 2015b). This 'phylotypic period' is reportedly conserved among vertebrates, yet within single species of killifish prominent developmental divergence occurs. Hourglass drawing taken from Richardson *et al.* (1997).

phase (i.e. diapause I) reported in the annual killifish *Terranatos dolichopterus* (20–30 days) and *Rachovia brevis* (mean 83 days) (Wourms, 1972c), the relatively short dispersed cell phase observed in some *Rivulus* taxa seems consistent with a failure to enter diapause I. A threshold of 10 days was therefore chosen as a criterion with which to score whether diapause I was entered. If the dispersed cell phase lasted > 10 days, it would have been considered diapause I; given our incubation conditions, this was not observed in any of the studied species. Diapause II was evident as a long-term (weeks to several months) arrest of development at the 38–42 somite stage. Lastly, delayed hatching/diapause III was evident as water-incubated embryos that had completed development (exhibited a golden ring around the iris of the eye; see Fig. 2) yet remained viable and unhatched for ≥ 10 days.

In addition to scoring the presence or absence of each diapause stage, the duration of the dispersed cell phase, diapause II and diapause III was calculated for all embryos in which the entrance and exit dates could be determined accurately. In general, observation windows were too wide relative to the short length of the dispersed cell phase (when present) to obtain an accurate estimate of duration, which precluded formal statistical analysis. The duration of diapause II and III was analysed using generalized least-squares models while accounting for unequal variance among species. We analysed the proportion of embryos that exhibited each diapause stage as a function of species, temperature and incubation medium using generalized linear models with a binomial distribution and pairwise proportion tests. Further details regarding diapause scoring guidelines and statistical analyses can be found in the Supporting Information.

PHYLOGENETIC PROCEDURES

Taxon sampling

We included all species within the *Owiyeye* clade of *Laimosemion* (Costa, 2006) with which we were able to obtain specimens for sequencing, relying mainly on those obtained from our field collection trip. We also included all other species in *Laimosemion* (Costa, 2006, 2011) for which genetic data were available in

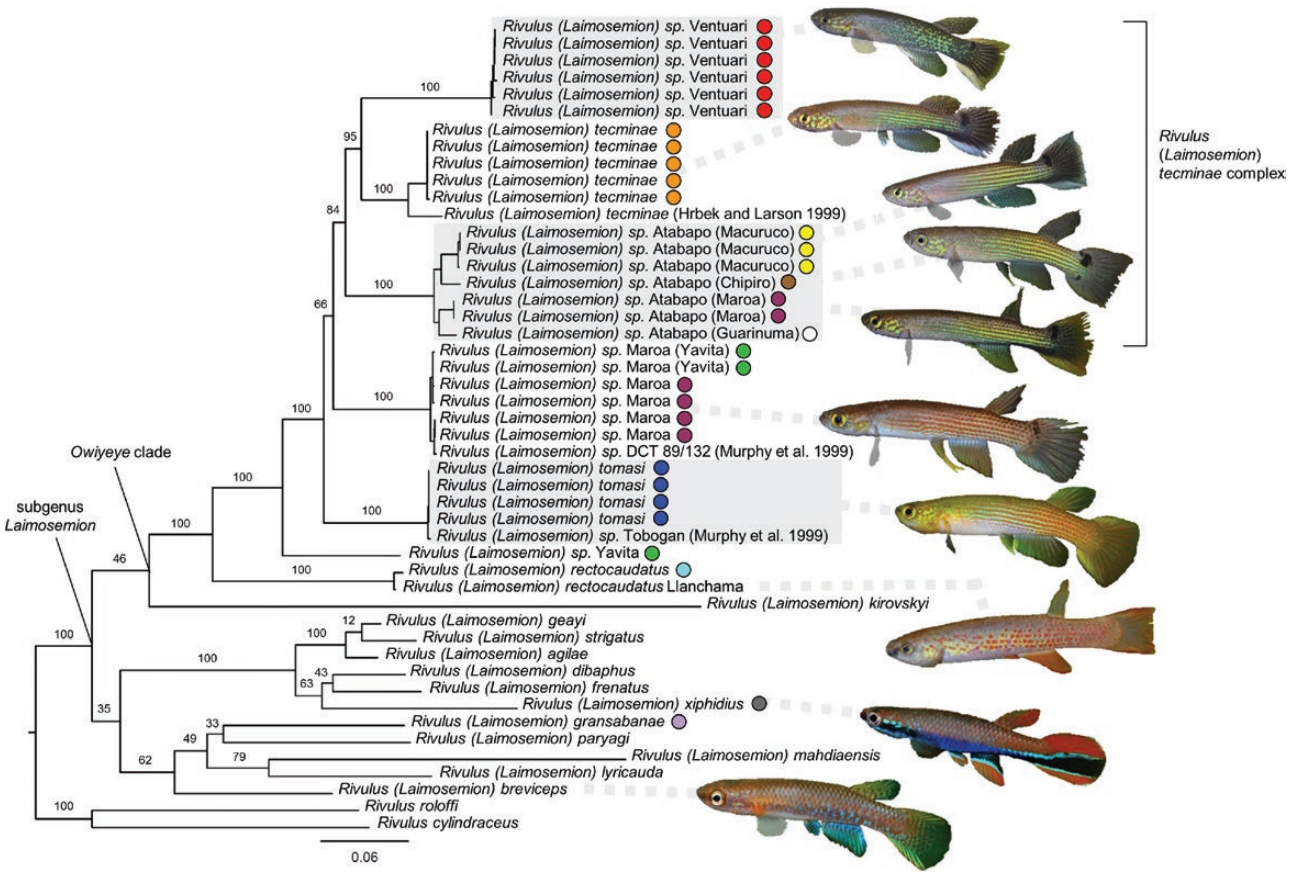

Figure 6. Maximum likelihood phylogeny (ln likelihood = –25470.81745) obtained with RAxML for the combined data set with six partitions. Maximum likelihood bootstrap support percentages are shown above branches. Coloured circles on branch tips denote collection localities detailed in Table 1 and depicted in Figure 1. Clade shading highlights well-supported clusters that might correspond to species. Fish pictures are representative males of the denoted population/species and were kindly provided by Frans Vermeulen.

GenBank (see Supporting Information, Tables S1, S2). Two species of *Rivulus* (*cylindraceus* and *roloffi*) were included as outgroups.

Gene sampling

The *AccuPrep* genomic DNA extraction kit from Bioneer or the proteinase K isolation of total DNA procedure (Milligan, 1998) was used to extract genomic DNA from muscle tissue of 95% ethanol-preserved specimens. Five mitochondrial gene regions were amplified. These included fragments of 12S, 16S, cytochrome oxidase 1 (*co1*), and cytochrome *b* (*cytb*). The final region included the 3′ end of $tRNA^{Ile}$, $tRNA^{Gln}$ (full), $tRNA^{Met}$ (full), the complete NADH dehydrogenase subunit 2 gene (*nd2*), $tRNA^{Trp}$ (full), $tRNA^{Ala}$ (full), $tRNA^{Asn}$ (full), $tRNA^{Cys}$ (full) and $tRNA^{Tyr}$ (full).

Sequences for polymerase chain reaction (PCR) primers used in this study have been published previously (summarized in Supporting Information, Table S3) and include those for 12S (Kocher *et al.*, 1989), 16S (Palumbi *et al.*, 2002), *co1* (Murphy *et al.*, 1999), *cytb* (Kocher *et al.*, 1989; Meyer *et al.*, 1990) and *nd2* (Hrbek & Larson, 1999). The PCRs were carried out using the following protocol: initial denaturation at 95 °C for 5 min; 32–36 cycles of 40 s at 95 °C (denaturation), 40 s at 45–60 °C (annealing) and 40 s to 1 min 50 s (depending upon amplicon length) at 72 °C (extension); with a final extension time of 7 min at 72 °C. Five microlitres of PCR products were run on 1% agarose gels to confirm a DNA band of appropriate size, after which the remainder of the PCR product (20 μL) was sent for purification and sequencing in both directions at Beckman Coulter Genomics. GenBank accession numbers for new and previously published sequences are given in the Supporting Information (Table S1).

DNA alignments and data compatibility

DNA alignments were performed using the Clustal algorithm implemented in Mega 6.0 (Tamura *et al.*, 2013), with gap opening and extension penalties of 15 and six, respectively. Slight alignment modifications were made by eye, and 18 bp of alignment ambiguous

Table 2. Data on presence or absence of diapause I, dispersed cell phase, diapause II and delayed hatching/diapause III

Species	Diapause I	Proportion of dispersed cell phase	Diapause II	Proportion of diapause II	Duration of diapause II [days; mean (range)]	Proportion of delayed hatching/ diapause III	Duration of delayed hatching/ diapause III [days; mean (range)]
Rivulus (*Laimosemion*) sp. Ventuari	No	6/6	Yes	9/9	61.7 (57–64)		
Rivulus (*Laimosemion*) *tecminae*	No	6/6	Yes	14/14	60.9 (43–81)		
Rivulus (*Laimosemion*) sp. Atabapo	No	37/37	Yes	30/37	45.7 (14–142)	1/2	41.0 (8–74)
Rivulus (*Laimosemion*) sp. Maroa	No	5/5	Yes	8/8	52.0 (35–64)		
Rivulus (*Laimosemion*) *tomasi*	No	39/47	No	0/62		5/10	11.0 (5–18)
Rivulus (*Laimosemion*) *rectocaudatus*	No	43/51	No	0/44		9/19	15.4 (2–66)
Rivulus (*Laimosemion*) *gransabanae*	No	0/15	No	0/13		0/7	2.6 (0–7)
Rivulus (*Laimosemion*) *xiphidius*	No	0/36	No	0/31		2/16	3.6 (0–14)
Rivulus (*Anablepsoides*) *hartii*	No	0/21	No	0/29		6/27	8.0 (3–14)
Kryptolebias marmoratus	No	0/16	No	0/56		28/29	58.5 (23–108)

Delayed hatching/diapause III refers to water-incubated embryos only, whereas for other diapause stages water- and peat-incubated embryos are pooled. In the second to last column, delayed hatching/diapause III was scored as present if an embryo survived for ≥ 10 days (after development was complete) before hatching or going bad and as absent if the embryo hatched within 10 days after the completion of development. In the last column, the length of delayed hatching/diapause III is defined as the number of days from first reaching prehatching until embryo hatch. Only embryos in which a given diapause stage (or the critical developmental window) was observed directly and could be scored unambiguously were included. This quality control, in part, explains unequal sample sizes across rows. A more detailed summary table and details regarding diapause scoring guidelines and statistical analyses can be found in the Supporting Information, Table S7.

region was removed from the 16S gene fragment and thus excluded from phylogenetic analysis. Protein-coding genes (*co1*, *cytb* and *nd2*) were translated to amino acids to ensure an intact reading frame. SequenceMatrix (Vaidya, Lohman & Meier, 2011) was used to concatenate individual final alignments. The concatenated data set (3462 bp) was analysed using six partitions (12S, 16S, *co1*, *cytb*, *nd2* and tRNAs). jModelTest (Posada, 2008) was used to determine whether a gamma distribution should be included for each of the partitions based on Bayesian information criterion (Supporting Information, Table S4). The partition-homogeneity test (Swofford, 2002) with 100 replicate heuristic searches (P = 0.760) suggested that it was appropriate to combine individual gene regions into a single multigene data set.

Phylogenetic analyses

PAUP 4.0b10 (Swofford, 2002) and RAxML 8.0.24 (Stamatakis, 2014) were used to perform maximum parsimony and maximum likelihood analyses. All gaps were treated as missing data. Maximum parsimony analysis in PAUP used heuristic searches to find the shortest tree(s). Bootstrap analyses (500 replications) used the tree bisection–reconnection (TBR) branch-swapping algorithm, with starting trees obtained via stepwise addition. Maximum likelihood analyses with RAxML, carried out on the CIPRES platform (Miller, Pfeiffer & Schwartz, 2010), used the GTRCAT model of molecular evolution for each of the six partitions, 500 bootstrap replicates, randomized maximum parsimony starting trees, and the fast hill-climbing algorithm with all free parameters estimated.

Ancestral state reconstructions

Parsimony (unordered) and maximum likelihood ancestral state reconstructions, implemented in Mesquite version 2.75 (Maddison & Maddison, 2011), were used to estimate ancestral states for the presence or absence of diapause II. Likelihood reconstructions were based upon categorical presence or absence, with marginal probabilities estimated with an asymmetrical

two-parameter Markov *k*-state model, allowing two different rates of change between states. Terminal taxa included all species in the *Laimosemion* clade for which we gathered data on presence or absence of diapause plus two outgroup taxa, *Rivulus* (*Anablepsoides*) *hartii* and *Kryptolebias marmoratus*.

RESULTS

RIVULUS EMBRYONIC DEVELOPMENT

Through observation of embryos over the time course of development, we were able to confirm that several named and currently unnamed *Rivulus* species exhibit the dispersed cell phase, diapause II and delayed hatching/diapause III. These results are presented in Table 2 and described briefly below.

No embryos from any species exhibited diapause I. However, a brief dispersed cell phase separating epiboly from the formation of the embryonic axis (Supporting Information, Figs S4–S6) was observed in all species of the *Owiyeye* clade of *Laimosemion* that were examined, but not the outgroup taxa, *Rivulus* (*Laimosemion*) *xiphidius* (Supporting Information, Fig. S3), *Rivulus* (*Laimosemion*) *gransabanae*, *Rivulus* (*Anablepsoides*) *hartii* (Supporting Information, Figs S1, S2) and *K. marmoratus*. The dispersed cell period was short, lasting between 1 and 7 days. In other annual species, harsh environmental conditions, such as lack of oxygen, chemical factors produced by adult fish and cold temperatures (either alone or in some combination), tend to induce diapause I during the dispersed cell phase, whereas in benign conditions (such as those of a laboratory with regular embryo collection and incubation in fresh medium) it is a less frequent and lengthy occurrence (Levels & Denuce, 1988; Podrabsky *et al.*, 2010b; Furness *et al.*, 2015a). Although diapause I was not observed during the dispersed cell phase in any of the *Rivulus* species studied here, it is possible that diapause I might occur given different harsher incubation conditions.

Diapause II was clearly observed in the *R.* (*L.*) *tecminae* complex (sp. Ventuari, *tecminae* and sp. Atabapo) and *Rivulus* (*L.*) sp. Maroa (Figs 3, 4), but not in *R.* (*L.*) *tomasi*, *R.* (*L.*) *rectocaudatus*, *R.* (*L.*) *xiphidius*, *R.* (*L.*) *gransabanae*, *R.* (*A.*) *hartii* and *K. marmoratus*. Pairwise tests of the proportion of embryos that exhibited diapause II were performed; all pairwise comparisons among species with and without diapause II were highly significant ($P < 0.0001$). Embryos that halted development at the diapause II stage did so when they reached 38–42 somite pairs, the same stage at which other annual killifish from South America and Africa halt development (Wourms, 1972c). Of the species that exhibited diapause II, only *R.* sp. Atabapo exhibited variation among embryos in whether diapause II was entered (30 of 37 embryos exhibited diapause II and seven of 37 skipped diapause II). These alternative developmental trajectories have been termed the 'diapause' and 'direct-developing' or

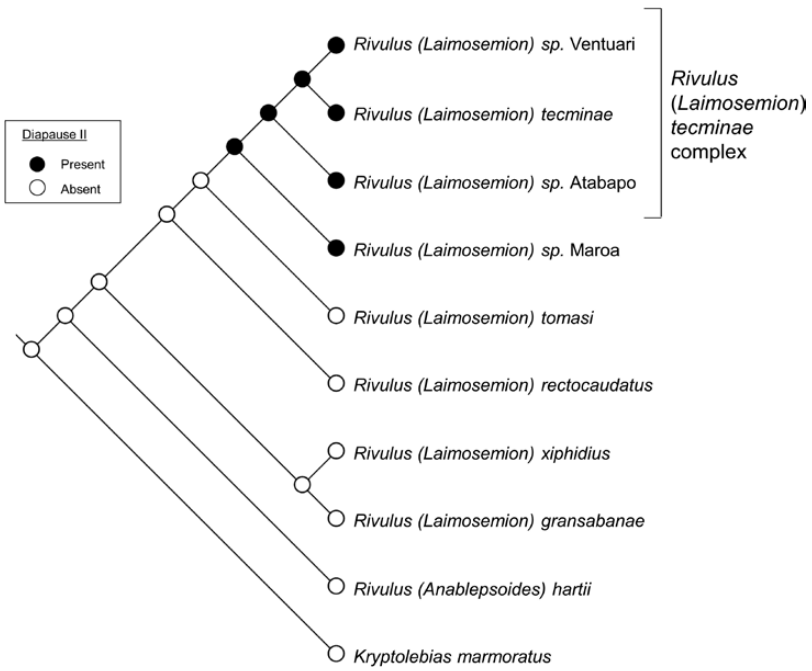

Figure 7. Parsimony reconstruction of mid-embryonic diapause (i.e. diapause II) in *Rivulus* (*Laimosemion*).

'escape' pathways (Podrabsky *et al.*, 2010a; Furness *et al.*, 2015b). Furness *et al.* (2015b) demonstrated that *R.* (*L.*) sp. Atabapo embryos following the direct-developing pathway exhibit significantly more robust head morphologies relative to embryos at an identical stage of development that entered diapause II (Fig. 5; supplementary material of Furness *et al.*, 2015b). This divergence in morphology began at ~20 somites, well before the 38–42 somite stage at which development was arrested in diapause II embryos.

All species examined had embryos capable of surviving on peat moss without hatching for > 10 days after development was complete. This is probably the case because such conditions are generally unsuitable for hatching and therefore required for survival. This phenomenon could therefore realistically be called delayed hatching. The degree to which hatching is postponed when it could presumably occur at any time (i.e. water incubation) might therefore be a more realistic measure of the potential for diapause III. Among species, there was significant variation in the proportion of water-incubated embryos that delayed hatching for > 10 days (general linear model analysis with binomial distribution: $\chi^2 = 59.6$, $P < 0.0001$) and in the duration of this delay (generalized least-squares analysis: $F = 7.3$, $P < 0.0001$; Fig. 3). However, there was no apparent phylogenetic trend to this variation given that the longest delays were observed in *R.* (*L.*) sp. Atabapo and the outgroup taxa *K. marmoratus*.

Phylogenetic analyses

The combined alignment included a total of 3462 bp, of which 1923 bp were constant, 293 bp were variable but parsimony uninformative, and 1246 bp were parsimony informative. Figure 6 shows the maximum likelihood phylogeny and Supporting Information, Figure S8 the maximum parsimony phylogeny (5048 steps). The maximum likelihood and maximum parsimony trees were largely congruent (identical topologies with regard to the ingroup taxa sequenced in the present study) but with differences in the placement of *Rivulus* (*Laimosemion*) *kirovskyi* and some outgroup *Laimosemion* taxa derived from GenBank. The sub-genus *Laimosemion* was recovered as strongly monophyletic. Within *Laimosemion*, there was a basal split between a clade that has been named *Owiyeye* (Costa, 2006) that includes the species of interest with regard to the evolution of diapause, and the rest of *Laimosemion*.

Ancestral state reconstructions

Figure 7 shows parsimony ancestral state reconstructions for diapause II. The parsimony reconstruction suggests that diapause II evolved in the common ancestor of *R.* (*L.*) sp. Ventuari, *R.* (*L.*) *tecminae*, *R.* (*L.*) sp. Atabapo and *R.* (*L.*) sp. Maroa. Maximum likelihood reconstructions also support this general evolutionary scenario, but with considerably less certainty (Supporting Information, Table S5).

DISCUSSION

Evolution of diapause

All *Laimosemion* taxa in the *Owiyeye* clade that were studied exhibited a dispersed cell phase separating epiboly and the formation of the embryo axis that lasted between ~1 day and 1 week. Owing to the transient nature of this phase, it is clear that embryos did not halt development during this short period (i.e. they did not enter diapause I). However, having a dispersed cell phase seems to be a requirement for halting development in diapause I (Wourms, 1972c) and, based upon the response in other annual killifish, exposing embryos to harsh environmental conditions from an early stage greatly increases the likelihood of diapause I (reviewed by Podrabsky *et al.*, 2010b). The dispersed cell phase has not, to our knowledge, been reported in other *Rivulus* species and was not apparent in *R.* (*L.*) *xiphidius* and *R.* (*L.*) *gransabanae* (present study), *R.* (*A.*) *hartii* (present study; Wourms, 1972b), *Rivulus cylindraceus* (Wourms, 1972b) and *K. marmoratus* (Mourabit *et al.*, 2011). This phase proved challenging to score accurately, particularly for the species *R.* (*L.*) *tomasi* and *R.* (*L.*) *rectocaudatus*. These two species appear to exhibit a very short dispersed cell stage (often < 24 h), and its expression may be affected by incubation conditions, which may explain its variable appearance (sometimes scored as present and other times absent). However, when observed frequently enough during the relevant window of development, at least some embryos of these species appeared to exhibit a short period between epiboly and the formation of the embryo axis during which the blastomere cells were scattered across the embryo surface in various degrees of aggregation.

The expression of diapause II in the *R.* (*L.*) *tecminae* complex and *R.* (*L.*) sp. Maroa is remarkably similar to that observed in other South American and African annual killifish (Furness *et al.*, 2015b). Embryos entered this state at the same 38–42 somite stage of development (Fig. 4). Furthermore, *R.* (*L.*) sp. Atabapo embryos exhibited alternative developmental pathways associated with either entering or directly bypassing diapause II. Embryos that become committed to entering diapause II begin to diverge morphologically and physiologically (around the 20 somite stage) from direct-developing embryos such

that by the time diapause II is entered these embryos are strikingly different in appearance (Furness *et al.*, 2015b). Specifically, embryos that entered diapause II exhibited reduced head sizes and rudimentary optic cups relative to embryos at an identical stage of development that skipped diapause II (Fig. 5). Direct-developing embryos proceed until development is complete and then either hatch or enter diapause III, whereas diapause II embryos halt development at the late somite stage for weeks to months, then eventually resume development and reach the diapause III stage. It is remarkable that this prominent morphological and physiological divergence occurs within a single species during organogenesis, which is a period of development supposedly highly conserved among all vertebrates. The phenotypes associated with the diapause II pathway (small head, rudimentary heart) reduce energetic costs (Furness *et al.*, 2015b). If an embryo must arrest development for many months and survive on a limited yolk supply at a stage of development in which several prominent organ systems already exist, then reducing investment in such organ systems will reduce long-term maintenance costs. It seems that these savings are achieved by following an alternative developmental pathway.

Several authors have drawn a distinction between delayed hatching and diapause (Martin, 1999; Podrabsky *et al.*, 2010b; Martin & Podrabsky, 2017). Delayed hatching is considered a short-term phenomenon, often induced by unfavourable conditions, characterized by an embryonic metabolic rate that remains high, and with embryos hatching immediately after conditions become favourable (i.e. after inundation by water). In contrast, diapause is considered a longer-term phenomenon, which can occur spontaneously (even in the absence of inducing conditions), is characterized by a reduction in metabolic rate and is endogenously maintained. As applied to killifish (particularly those in the sub-order Aplocheiloidei), the implication is that non-annual species (including *Rivulus*) exhibit delayed hatching, whereas annual species exhibit something that warrants a separate name, i.e. diapause III. Elsewhere it has been argued that these phenomena may represent ends of a continuum rather than dichotomous categories, with what is termed diapause III being a more intense, longer-duration and more frequent version of delayed hatching (Wourms, 1972c; Furness, 2016). In *R.* (*A.*) *hartii*, a small subset of embryos that were incubated in water were found to exhibit a spontaneous delayed hatching response lasting up to several weeks (Furness, 2016). However, when embryos were reared on a drier substrate (moist peat moss), all embryos exhibited this delay. Thus, delayed hatching appears spontaneously at a relatively low level when conditions are ideal for hatching, but given unsuitable hatching conditions (in which it is required for survival), it is virtually obligate. In the present study, we incubated the embryos of several *Laimosemion* species and outgroup taxa in both water and peat moss and observed that on peat moss the embryos of all species are capable of delaying hatching, but when water incubated there are highly significant differences among species in the duration of this delay before hatching. In species such as *R.* (*L.*) *xiphidius* and *R.* (*L.*) *gransabane*, this delay exhibited a mean value of < 5 days, whereas in *K. marmoratus* it was close to 60 days, with other species falling between these values. It is unclear to us whether a threshold can be applied that clearly delineates delayed hatching from diapause III based upon duration alone. But other criteria, such as metabolic rate, rate of yolk usage, heart rate or even gene expression patterns, can parse differences (Thompson *et al.*, 2017). The phenomenon of delayed hatching is apparently fairly widespread within the genus *Rivulus* and is expressed more prominently in certain taxa than others (probably dependent on ecology). Furthermore, *K. marmoratus* is capable of exhibiting prominent delays before hatching, so the phenomenon may extend throughout the New World Rivulidae and perhaps to the base of all Aplocheiloidei killifish (see also Furness, 2016).

Annual life cycle vs. diapause, selection pressures and habitat use in *Rivulus*

An annual life cycle is characterized by growth, maturity, reproduction and complete population turnover within a single calendar year. Usually, a seasonal environmental factor, such as harsh winter or lack of water, truncates the adult life-history stage on a regular basis. The species examined here inhabit a region characterized by high rainfall and relatively mild seasonality (Pulwarty, Barry & Riehl, 1992). We suggest that the *Rivulus* (*Laimosemion*) species that exhibit diapause II may not exhibit a true annual life cycle comparable to killifish from parts of South America and Africa with strong seasonal precipitation. Why has diapause evolved in these fish?

We hypothesize that the preferred microhabitat of this clade of killifish exposes their embryos to periodic pool desiccation, creating selection in favour of extended diapause. *Rivulus* collection localities were small pools or puddles in either open savannah patches or closed-canopy forest. The common habitat characteristics of all sites where *Rivulus* were collected included the following: (1) small, shallow, stagnant pools or puddles; (2) large quantities of leaf litter and other debris to provide cover; (3) few, if any, other fish species present and no large-bodied species; and (4) other nearby pools or flowing water (up to several hundred metres away). It is clear from behavioural observations of *Rivulus* in the field and laboratory and from numerous anecdotal

reports that *Rivulus* are adept at moving over land to colonize isolated pools or puddles that are proximate to more permanent source aquatic habitat (Seghers, 1978; Sayer & Davenport, 1991; Brosset, 2003; Turko & Wright, 2015; Furness, 2016). By flipping or crawling over damp leaf litter, *Rivulus* species are evidently capable of travelling fairly long distances for fish of such small size (typically < 100 mm). We envision this clade of *Rivulus* as using a habitat composed of dynamically reshuffling forest and savannah pools where juveniles and adults are constantly moving about this network as some pools dry out and others are created, perhaps occasionally (during dry spells) seeking refuge in flowing water or getting caught in too isolated a pool and perishing. Embryos spawned in these shallow pools and puddles, even in a region that has high rainfall, may be required to withstand periodic desiccation while buried amongst the bottom substrate and damp leaf litter. In this manner, there may be the necessary selection pressures in place for delaying development by means of embryonic diapause. Interestingly, Costa & Bragança (2013) report that: 'Whereas Amazon killifishes of the genus [*Rivulus*] *Anablepsoides* are frequent in ichthyological collections because they occur in streams and lakes at the same place as other freshwater teleosts, species of *Owiyeye*, even when sympatric to species of *Anablepsoides*, are rarely found because they inhabit isolated shallow pools not sampled by most fish collectors'. A more detailed comparative examination of ecology and habitat use in this group is warranted.

Examination of spawning substrate preference in relationship to diapause shows that egg-laying behaviour is part of adaptation to ephemeral environments (Simpson, 1979). Annual killifish that exhibit diapause I, II and III and reside in seasonally ephemeral savannah pools deposit embryos within the bottom substrate during spawning (Wourms, 1972c; Simpson, 1979). This is essential because embryos must be encased within the soil substrate that forms the dry bed of the pool bottom to be able to survive the dry season. In contrast, non-annual killifish have been reported to deposit embryos among leaf litter, aquatic vegetation and root masses that dangle into the water. The *R.* (*L.*) *tecminae* complex, which exhibit the dispersed cell phase, diapause II and (maybe) diapause III, spawn entirely in the mid- to bottom layers of the water column (sand or mop) (Supporting Information, Table S6). In contrast, *R.* (*L.*) sp. Maroa, *R.* (*L.*) *tomasi*, *R.* (*L.*) *rectocaudatus*, *R.* (*L.*) *xiphidius* and *R.* (*L.*) *gransabanae* regularly spawn in the surface layer (plants) in addition to the mid-layer (mop) (Supporting Information, Table S6). The correlation between diapause and spawning substrate suggests that the emergence of the dispersed cell stage and mid-embryonic diapause (II) are associated with a progression towards more bottom-substrate spawning.

Limitations of the present study

Our goal was to document the presence or absence of different diapause stages in each population or species, such that inferences could be made regarding their evolution. Given that embryo incubation conditions affect diapause expression, we chose a range of incubation conditions that included two temperatures and two incubation media. When present, the length of diapause II was substantial, lasting for a mean of 45.7–61.7 days and would be near impossible to miss given our observation windows of a mean of 5.3 days. It remains possible that diapause might be revealed in species for which we did not see diapause given different incubation conditions or larger embryo sample sizes. Furthermore, our study was not ideally suited to testing how incubation conditions affect diapause length because embryo sample sizes were not uniformly large for each species and among the different incubation treatments (Supporting Information, Table S7). Therefore, we refrain from drawing conclusions regarding the duration of diapause II as a function of incubation conditions or trying to distinguish between delayed hatching and diapause III for those species with a long prehatching delay.

How does incomplete taxon sampling affect conclusions regarding the evolution of diapause? Our phylogenetic tree included 15 of 29 species in subgenus *Laimosemion* and several putatively undescribed species. Taxon sampling in the *Owiyeye* subclade of *Laimosemion* was limited by the fact that many of these species are known only from the species description; no tissues or genetic data are available, and certainly no live fish for spawning and studies of developmental biology. However, the primary conclusion we derive (i.e. an independent origin of diapause II in *Owiyeye*) is robust even with limited taxon sampling. This is because other *Rivulus* species do not exhibit diapause II, or at least are not reported to do so. Thus the (derived) species in *Owiyeye* that have diapause II are well nested within a clade (subgenus *Laimosemion*) containing dozens of outgroup species that do not have diapause, and *Laimosemion* is nested within *Rivulus*, which contains hundreds of species not reported to exhibit diapause. This strongly suggests that there was an independent origin of this trait (diapause II) within *Owiyeye*. No developmental data are available for the additional species of *Owiyeye* not included here. If some of these species are eventually found to have diapause II, then conclusions may have to be modified to reflect a more complicated pattern of diapause evolution. However, the fact that there has been an independent origin of diapause II within the clade would remain unchanged.

We sequenced five mitochondrial gene segments and used these as the basis for reconstructing the phylogeny of this group. Mitochondrial genes were used because the focus of our study was at a low taxonomic level, and mitochondrial DNA generally exhibits greater variation than nuclear DNA. Furthermore, these same genes have been sequenced in other *Laimosemion* species and made available in GenBank, allowing us to include these species as outgroup taxa. However, we recognize the potential drawbacks of inferring a phylogeny on the basis of only mitochondrial genes. Specifically, the five mitochondrial gene segments sequenced herein are tightly linked and could therefore be thought of as a single locus, with the recovered phylogeny potentially differing from the true species-level phylogeny.

Conclusions and future work

Thirteen of 29 species (45%) within the subgenus *Laimosemion* have been described since the year 2000 (Supporting Information, Table S2). This trend shows no sign of slowing and highlights the considerable diversity that remains to be discovered and described. From a natural history perspective, *Rivulus* are a fascinating group of fishes that live at the terrestrial–aquatic interface. Here we have described an independent origin of the dispersed cell and mid-embryonic diapause stages within this otherwise 'non-annual' killifish clade. The nature of the developmental diversity in this genus presents the raw material for developing an evolutionary scenario for the acquisition of an annual life cycle, through intermediate stages. With regard to patterns of diapause observed herein, we can divide killifish into four broad categories: (1) species with typical teleost development exhibiting no diapause and limited capacity for delayed hatching; (2) species that exhibit more prominent delayed hatching and/or diapause III; (3) species that exhibit the dispersed cell phase (and possibly the potential for diapause I) and delayed hatching/diapause III; and (4) species that exhibit the dispersed cell phase (and possibly the potential for diapause I), diapause II and delayed hatching/diapause III. In this evolutionary progression, species in categories 2 and 3 might represent intermediates in the evolution of the full diapause sequence (i.e. annual life cycle). However, simply scoring the presence or absence of diapause I, II and III necessarily underestimates the range of variation present within each type of diapause. Specifically, there is variation in the propensity and regularity with which the embryos of different species enter into each diapause and the length of time spent in diapause before resuming development. The evolution of plasticity might play a significant role in determining whether diapause entry is facultative or an obligate feature of development. For example, a facultative delay in hatching brought about by infrequent but harsh environmental conditions could, over the course of many generations of exposure to such conditions, become a regular developmental feature (Verla-Lasheras & Van Dooren, 2014). When this within-diapause variation is considered, there becomes an even greater degree of gradation between the above categories. This evolutionary scenario suggests a number of future research targets. Is delayed hatching ancestral to all killifish, and is there a significant gulf between what has been termed delayed hatching and diapause III? How widespread is the dispersed cell phase, and is this feature always synonymous with diapause I? Is developmental diversity analogous to that described here found in other clades of killifish that inhabit similar habitats? Furthermore, the elucidation of sister taxa that differ in the presence or absence of different stages of embryonic diapause (and can be bred and maintained in the laboratory) offers promise for a genomic examination of embryonic diapause in a comparative evolutionary framework (Thompson & Ortí, 2016; Thompson *et al.*, 2017). Lastly, within killifish there is ample opportunity for studies of natural history relating habitat usage, behaviour and development.

DATA ACCESSIBILITY

Phylogenetic data, including taxon sampling and GenBank accession numbers, are available in Supporting Information. Data used to create all figures and tables (aligned DNA sequence file and diapause developmental data) are archived in Dryad (https://doi.org/10.5061/dryad.8p8t27d).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Early developmental sequence of a *Rivulus* (*Anablepsoides*) *hartii* embryo (20 °C, water incubation), photographed at ×40 magnification. A, when the embryo was first collected and photographed it was undergoing epiboly. B, epiboly; 2.5 h post-collection. C, epiboly; 5 h post-collection. D, dense cellular aggregate; 10 h post-collection. E, embryo axis; 21 h post-collection. F, embryo axis; 24 h post-collection. G, first somites forming; 29 h post-collection. H, six somites; 34 h post-collection. I, ten somites; 45 h post-collection. J, 13 somites; 49 h post-collection. K, 14 somites; 54 h post-collection. L, mid- to late-somite embryo; 3 days post-collection. Note the absence of a dispersed cell phase, which would have manifested between epiboly and the formation of the embryonic axis (i.e. between C and E).

Figure S2. Early developmental sequence of a *Rivulus* (*Anablepsoides*) *hartii* embryo (25 °C, peat incubation), photographed at ×40 magnification. A, when the embryo was first collected and photographed it was at the blastula stage. B, epiboly; 17 h post-collection. C, epiboly; 24 h post-collection. D, embryo axis; 32 h post-collection. E, six somites; 45 h post-collection. F, ten somites; 52 h post-collection. G, 18 somites; 68 h post-collection. H, mid- to late-somite embryo; 89.5 h post-collection.

Figure S3. Early developmental sequence of a *Rivulus* (*Laimosemion*) *xiphidius* embryo (25 °C, peat incubation), photographed at ×40 and ×100 magnification. A1, A2, when the embryo was first collected and photographed it was at the flattened blastula stage. B1, B2, epiboly; 17 h post-collection. C1, C2, aggregate; 23 h post-collection. D1, D2, embryo axis; 32 h post-collection. E, four somites; 45 h post-collection. F, eight somites; 52 h post-collection. G, 16 somites; 68 h post-collection. H, 24 somites; 89.5 h post-collection. I, mid- to late-somite embryo; 98 h post-collection. J, late-somite embryo; 114 h post-collection. K, fin buds; 141 h post-collection. L, fin buds; 163 h post-collection.

Figure S4. Early developmental sequence of a *Rivulus* (*Laimosemion*) *tomasi* embryo (25 °C, peat incubation), photographed at ×40 magnification with ×100 insets. A, when the embryo was first collected and photographed it was at the six-cell stage. B, blastula; 17 h post-collection. C, flat blastula; 23 h post-collection. D, epiboly; 32 h post-collection. E, dispersed/slight aggregate; 45 h post-collection. F, dispersed/slight aggregate; 52 h post-collection. G, extremely faint embryo axis; 68 h post-collection. H, embryo axis; 89.5 h post-collection. I, five somites; 98 h post-collection. J, 11 somites; 114 h post-collection. K, 19 somites; 141 h post-collection. L, 25 somites; 163 h post-collection.

Figure S5. Early developmental sequence of a *Rivulus* (*Laimosemion*) *rectocaudatus* embryo (20 °C, water incubation). A1–A3, when embryo was first collected and photographed it was in the dispersed cell stage; ×40, ×100 and ×400 magnification. B1–B3, dispersed cell stage; ×40, ×100 and ×400 magnification; 2 h post-collection. C1–C3, dispersed cell stage; ×40, ×100 and ×400 magnification; 4.5 h post-collection. D, dispersed cell stage, ×40 magnification; 10 h post-collection. E, dispersed cell stage, ×40 magnification; 21 h post-collection. F, embryo axis, ×40 magnification; 24 h post-collection. G, embryo axis, ×40 magnification; 29 h post-collection. H, embryo axis, ×40 magnification; 34 h post-collection. I, seven somites, ×40 magnification; 49 h post-collection.

Figure S6. Early developmental sequence of a *Rivulus* (*Laimosemion*) *rectocaudatus* embryo (20 °C, water incubation), photographed at ×40 magnification (insets are ×100 magnification). A, when the embryo was first collected and photographed it was at the blastula stage. B, blastula; 2 h post-collection. C, blastula; 4.5 h post-collection. D, blastula; 10 h post-collection. E, epiboly; 20 h post-collection. F, epiboly; 24 h post-collection. G, epiboly; 29 h post-collection. H, dispersed; 34 h post-collection. I, dispersed; 45 h post-collection. J, dispersed; 49 h post-collection. K, dispersed; 54 h post-collection. L, two somites; 68 h post-collection. M, seven somites; 82.5 h post-collection. N, 11 somites; 95 h post-collection.

Figure S7. Early developmental sequence of a *Rivulus* (*Laimosemion*) *rectocaudatus* embryo (25 °C, peat incubation), photographed at ×40 and ×100. A, when the embryo was first collected and photographed it was at the flat blastula stage. B1, B2, epiboly; 17 h post-collection. C1, C2, epiboly; 23 h post-collection. D1, D2, aggregate; 32 h post-collection. E1, E2, aggregate; 45 h post-collection. F1, F2, embryo axis; 52 h post-collection. G, three somites; 68 h post-collection. H, 11 somites; 89.5 h post-collection. I, 14 somites; 98 h post-collection. J, 19 somites; 114 h post-collection. K, 27 somites; 141 h post-collection.

Figure S8. Maximum parsimony tree (5048 steps) obtained with PAUP 4.0b10 (Swofford, 2002). Bootstrap values > 50 are shown.

Table S1. Gene accession numbers for sequences used in this study.

Table S2. Currently recognized species in *Rivulus* (*Laimosemion*).

Table S3. Sequences for primers used in polymerase chain reaction amplifications.

Table S4. Models of molecular evolution chosen by jModeltest (Posada, 2008) on the basis of Bayesian information criterion.

Table S5. Proportional likelihood values of reconstructed ancestral states for diapause II (Asymmetric 2 parameter model).

Table S6. Preferred spawning substrate for *Rivulus* (*Laimosemion*) species/populations reared in the laboratory. The number of embryos deposited in sand (bottom), spawning mop (bottom to mid-level) and floating plant (surface) are indicated.

Table S7. Data on presence or absence of diapause I, the dispersed cell phase, diapause II and delayed hatching/diapause III as a function of species, incubation medium and incubation temperature (see below, 'Diapause scoring guidelines, analyses, and results' for definitions). For embryos incubated on peat moss, the duration of delayed hatching is the number of days from the completion of development until embryos perished or were hatched by being placed into water. For water-incubated embryos, the duration of delayed hatching/diapause III is the number of days from the completion of development until hatching occurred.