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Convergent evolution of alternative developmental trajectories associated with diapause in African and South American killifish

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Annual killifish adapted to life in seasonally ephemeral water-bodies exhibit desiccation resistant eggs that can undergo diapause, a period of developmental arrest, enabling them to traverse the otherwise inhospitable dry season. Environmental cues that potentially indicate the season can govern whether eggs enter a stage of diapause mid-way through development or skip this diapause and instead undergo direct development. We report, based on construction of a supermatrix phylogenetic tree of the order Cyprinodontiformes and a battery of comparative analyses, that the ability to produce diapause eggs evolved independently at least six times within African and South American killifish. We then show in species representative of these lineages that embryos entering diapause display significant reduction in development of the cranial region and circulatory system relative to direct-developing embryos. This divergence along alternative developmental pathways begins mid-way through development, well before diapause is entered, during a period of purported maximum developmental constraint (the phylotypic period). Finally, we show that entering diapause is accompanied by a dramatic reduction in metabolic rate and concomitant increase in long-term embryo survival. Morphological divergence during the phylotypic period thus allows embryos undergoing diapause to conserve energy by shunting resources away from energetically costly organs thereby increasing survival chances in an environment that necessitates remaining dormant, buried in the soil and surrounded by an eggshell for much of the year. Our results indicate that adaptation to seasonal aquatic environments in annual killifish imposes strong selection during the embryo stage leading to marked diversification during this otherwise conserved period of vertebrate development.

1. Introduction

There are several competing models purporting to explain patterns of embryonic conservation, or lack thereof [1–6]. The most influential is the hourglass model of development, which posits a phylotypic period (a bottleneck of reduced phenotypic divergence that occurs mid-embryogenesis) bracketed by periods of increased divergence earlier and later in development [1,4]. According to Raff [4], early development is characterized by significant flexibility as initial axial patterning is established via localized processes. Likewise, late development is readily modified as the body is already divided into separate modules or organ primordia, which although complex within themselves, can operate relatively independently of each other. During mid-development, when organogenesis occurs, there is an increase in genetic and developmental interactions between developing modules, which constrains evolutionary change and leads to a conserved phylotypic period [4]. In the *Origin of Species* [7], Darwin emphasized that development, particularly early on, tends to be conserved because embryos reside in an egg or their mother's womb and hence are not typically exposed to strong selection; however, he went

on to note that any period can be modified and exhibit adaptation if there is strong selection (see also [3,5]). This view of embryology focuses on the role of selection rather than constraint; developmental conservatism reflects a dearth of selection rather than constraint *per se*.

The production of discrete alternative phenotypes from the same genotype (polyphenism) provides an under-used opportunity to contrast these alternative views of vertebrate embryology and examine whether the phylotypic stage is constrained or can instead be subject to phenotypic divergence along alternative pathways if there is selection for such divergence. We show that, under the right circumstances, even the narrow bottleneck of the hour glass (the phylotypic period) can be subject to selection and, as a consequence, will evolve to exhibit intraspecific divergence.

Aquatic organisms living in habitats that regularly or periodically dry have a limited number of life-history strategies [8,9]. Mobile organisms can leave a deteriorating environment in search of better quality habitat patches, while those that do not have this option must survive either as dormant adults or by producing resilient embryos capable of withstanding prolonged desiccation [8,9]. The production of embryos (seeds, cysts and eggs) capable of traversing unfavourable conditions uninhabitable by the adult stage is common in plants and invertebrates but has only rarely evolved in vertebrates [10–13]. Perhaps the most prominent adaptation of such embryos is diapause—developmental arrest accompanied by a reduction in metabolic rate [14]. Diapausing embryos face a number of adaptive challenges including arresting cellular, developmental and metabolic processes, conserving energy for long-term survival on a limited pre-packaged nutrient supply, maintaining homoeostasis in the face of diverse environmental challenges (e.g. lack of water or oxygen, temperature fluctuations), then resuming development and hatching at the appropriate time and in the appropriate conditions [13–16]. These challenges are predicted to impart strong selective pressures on the embryo stage whenever diapause has evolved.

Some killifish are able to persist in ephemeral aquatic environments by producing diapausing eggs that remain viable in the soil long after the ponds have dried and the adult fish have perished. In habitats that have distinct wet and dry seasons, where pools regularly dry and refill each year, this has been referred to as an annual life cycle [13]. In annual killifish, eggs are capable of entering diapause at specific stages during embryology—termed I, II and III [17]. Diapause I occurs early in development before the somite-embryo has formed during a dispersed cell phase, which is unique to annual killifish [17]. Although embryos have been induced to enter this state through low temperatures or hypoxia [17,18], embryos reared under our standard laboratory conditions did not undergo diapause I. Diapause II occurs after the formation of the embryonic axis, in embryos possessing approximately 38 pairs of somites and the beginnings of several organ systems [13,17]. It is during this diapause that embryos are most resistant to temperature extremes, desiccation and oxygen deprivation [14]. Lastly, diapause III occurs when the embryo is fully developed and capable of hatching. The end result of arrest at one or more of these three stages is that eggs can have a greatly extended development that allows them to traverse the dry season, when adult fish have perished. Embryos, even of the same clutch, routinely follow different developmental

trajectories [13,18] which we term the diapause and direct-developing pathways. Direct-developing eggs skip diapause II and instead undergo continuous development until diapause III is reached, whereas those that enter diapause II exhibit developmental arrest and may not resume development and reach diapause III for a variable length of time, in some cases well over a year. Whether embryos follow the diapause or direct-developing pathway is sensitive to a variety of factors including maternal effects, temperature, light levels and presence of adult fish, but also exhibits a measure of intrinsic variability [14,18]. This strategy probably represents a combination of adaptive phenotypic plasticity to prevailing environmental conditions that may indicate relative timing in the year and a risk-spreading strategy known as bet-hedging [19–21].

Here, we address several inter-related questions on the evolution of diapause and its developmental ramifications. Annual killifish, found across large swaths of Africa and South America, fall into several distinct clades [10,12,22]. It has yet to be conclusively determined whether this pattern is best explained by multiple independent origins of diapause, one ancestral origin with multiple subsequent losses, or a more complicated pattern involving both losses and gains [10,12]. We address these alternative hypotheses through construction of a supermatrix phylogeny of order Cyprinodontiformes, character mapping of diapause II (the most prominent stage of developmental arrest, and largely synonymous with an annual life history), ancestral state reconstructions and comparative analyses. Next, we formally test the hypothesis that diapause evolves in response to the colonization of seasonally ephemeral aquatic environments by examining the correlation between diapause and ecological habitat across the killifish phylogeny. We then ask whether diapause is associated with the evolution of alternative developmental pathways [18] by characterizing the embryology of representative annual and non-annual killifish species. Lastly, we probe the adaptive significance of these alternative developmental pathways in the annual species *Nothobranchius furzeri*. Specifically, we test and find that phenotypic divergence associated with entrance into diapause II reduces embryonic metabolic rate and increases long-term survival prospects relative to embryos that follow the direct-developing pathway.

2. Material and methods

(a) Tree construction

Killifish capable of producing diapause eggs (i.e. an annual life cycle) are found within the order Cyprinodontiformes, suborder Aplocheiloidei (637 currently recognized species). We generated a molecular phylogeny of the group using supermatrix tree construction methods [23]. First, we identified molecular sequence data from 269 species of Apocheiloidei killifish and 42 outgroup taxa from NCBI. We used GENEIOUS v. 5.4.6 [24] to download seven mitochondrial and two nuclear genes from GenBank, and constructed a 10 960 base pair supermatrix for these 311 taxa. Sequences were aligned using MUSCLE [25] in GENEIOUS v. 5.4.6 [24] followed by manual adjustment in SE-AL [26]. The concatenated dataset was analysed using nine partitions (12S + 16S, D loop, Cox1, cytochrome b, ND1, ND2, tRNAs Val-Ile-Gln-Met-Trp-Ala-Asn-Cys-Tyr, Rag1, 28S.). The maximum-likelihood (ML) tree was estimated using RAxML7.2.7 (CIPRES platform; [27]), with a GTRCAT + Γ model of molecular

evolution for each of the nine partitions, 500 bootstrap replicates, randomized maximum parsimony starting trees and the fast hill-climbing algorithm with all free parameters estimated. Methodological details regarding molecular dating (timetree) analyses can be found in the electronic supplementary material.

(b) Ancestral state reconstructions

Each species in the phylogeny was scored for presence or absence of diapause II based upon direct observation (A.F.) or reporting in the literature. We performed parsimony and ML ancestral state reconstructions in MESQUITE v. 2.75 [28] using default settings. Likelihood reconstructions were based upon categorical presence or absence of diapause II with marginal probabilities estimated with model Mk1. This model is a k-state generalization of the Jukes–Cantor model in which all state changes are equally probable [29]. Ancestral state reconstructions can positively mislead if extinction or speciation rates are significantly correlated with the evolution of the trait being reconstructed [30]. Diversification rates were analysed using binary trait speciation and extinction [31] in the R package DIVERSITREE [32] to discount this possibility as a source of bias (electronic supplementary material).

(c) Correlation between diapause II and habitat

Each species in the phylogeny was scored for type of aquatic habitat in which it typically resides (permanent, seasonal or marginal). SIMMAP v. 1.5 [33] was used to determine whether diapause II and habitat covary with each other across the phylogeny. This programme implements Bayesian mutational mapping [34,35] and estimates two statistics, d_{ij} and m_{ij}, each a measure of covariation between character states i and j.

(d) Divergence associated with developmental trajectory

Representative species were selected from several clades that according to our ancestral state reconstructions independently evolved diapause. These include species from the South American Rivulidae (*Austrofundulus leoghnei*, *Austrolebias nigripinnis* and *Rivulus* (*Laimosemion*) *tecminae*) and the African Nothobranchiidae (*Callopanchax occidentalis*, *Fundulopanchax deltaensis*, *N. furzeri* and *Nothobranchius korthausae*). In addition, we examined embryos from five non-annual species that are distributed throughout the tree (*Fundulopanchax gardneri*, *Fundulopanchax scheeli*, *Rivulus* (*Anablepsoides*) *hartii*, *Pachypanchax playfarii* and *Oryzias latipes*). Breeding adults of these 12 species were maintained in stock aquaria and provided with spawning substrate. Embryos were collected daily at regular intervals and incubated individually in 24 or 48 well tissue culture plates containing Yamomoto's solution [36] or compacted peat moss. Eggs were incubated at light and temperature regimes such that (for annual species) some eggs entered diapause and others followed the direct-developing pathway, generally 25°C and 12 L : 12 D cycle. Each day embryos were transferred to a depression slide using a plastic pipette and viewed under an Olympus BH-2 compound microscope. The number of somite pairs were counted as a way of staging embryos [17]. Embryo heart rate was measured (beats per minute), and with the aid of a coverslip the embryo rotated such that a clear flat image of the head region could be photographed with a Nikon D3100 camera attached to the microscope. Measurements of embryo head morphology were made with IMAGEJ software [37]. The variables measured were head width at optic cups, head width at otic vesicles and head length (electronic supplementary material, figure S5). Embryos were tracked throughout development so that developmental trajectory could be determined (direct-developing or diapause).

(e) Statistical analyses

We used linear mixed models implemented in the R [38] package *nlme* [39] to examine the effect of developmental trajectory on morphological and physiological divergence during embryology for each of the studied species. Developmental stage (number of somite pairs), developmental trajectory (diapause versus direct-developing) and their interaction were entered as fixed effects. Repeated measurements were taken on eggs as they progressed through development (i.e. longitudinal dataset). To account for this non-independence [40], random effects for egg identity (individual intercept and slope allowed to vary) were included. In electronic supplementary material, table S7, we report the fixed effect parameter estimates produced by restricted ML estimation. In cases where the full model failed to converge due to overparametrization, the random slope effect was excluded and the model rerun with only random intercepts.

(f) Measurement of egg metabolic rate (oxygen consumption) in *Nothobranchius furzeri*

Eggs were obtained through natural spawning activity of males and females held in stock tanks. Beginning 14 days post-fertilization (approx. 20 somite stage) each batch of eggs was subdivided into separate groups on the basis of developmental trajectory (diapause versus direct-developing). Oxygen consumption was measured on batches of 8–96 embryos (37.0 ± 2.0 s.e.) of known age, developmental stage and trajectory using a polarographic oxygen microelectrode (Clark style, Instech Laboratories) connected to a YSI Model 5300 Biological Oxygen Monitor (YSI Incorporated, Yellow Springs Instrument Co., Inc.). The tip of the micro electrode was secured inside a water-jacketed 600 µl closed-system respirometry chamber (Batch Cell Chamber, Instech Laboratories) filled with Yamamoto's solution held to a constant temperature of 20°C. Oxygen readings were hand recorded in 1 min intervals. For each trial, per cent O_2 values were plotted as a function of time. The slope (% O_2 consumed min^{-1}) of the best-fit linear regression line was converted to an absolute measure of O_2 consumption (pmol s^{-1} embryo^{-1}) accounting for the volume of the respirometry chamber, the solubility of dissolved oxygen in Yamamoto's solution and the number of eggs in the trial.

(g) Long-term embryo survival

To determine whether developmental pathway influenced long-term embryo survival, we performed a longitudinal laboratory study. *Nothobranchius furzeri* embryos were collected daily and distributed individually into the wells of 48 well tissue culture plates ($n = 24$) containing Yamamoto's solution. These plates were incubated under different combinations of light and temperature as part of a study on phenotypic plasticity. Embryos were observed once or twice weekly under a dissecting microscope and developmental trajectory scored. Monitoring of developmental progress was continued until all embryos either hatched or perished. For the survival analyses, we excluded embryos that perished before developmental trajectory could be determined, and embryos that hatched during the course of incubation. Results are qualitatively the same regardless of whether such groups of embryos were included. We used the Kaplan–Meier model for survival analysis and a Log Rank (Mantel–Cox) test to determine whether survival distributions differed as a function of developmental trajectory (diapause versus direct-developing).

3. Results

Our molecular phylogeny of killifish (figure 1) reveals that taxa with diapause fall into at least six distinct clades in the African family Nothobrachiidae and the South American
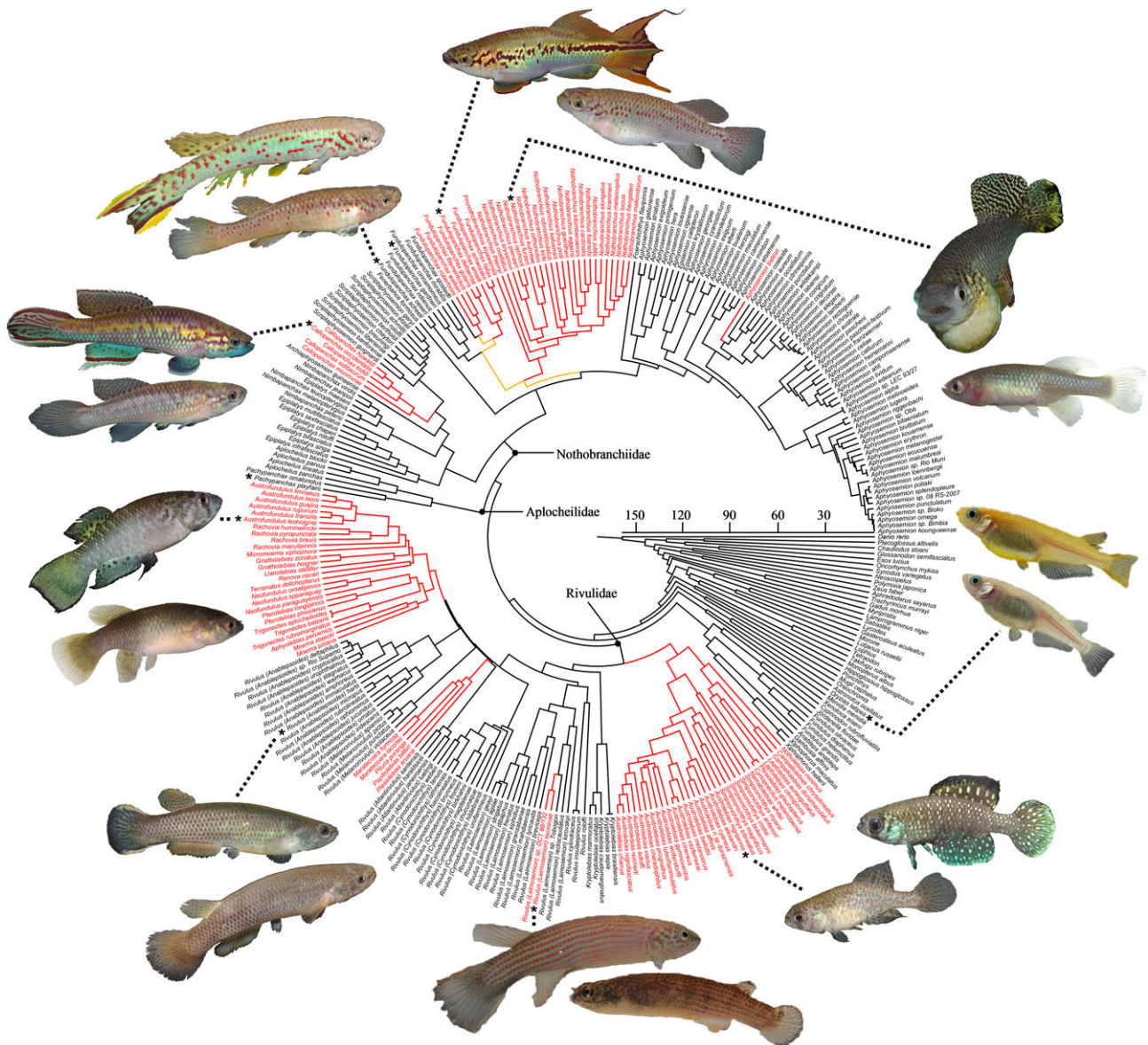
Figure 1. Timetree (independent rates, hard-bounded constraints) of Aplocheiloidei killifish with 42 teleost outgroup taxa. Species highlighted in red have embryos capable of undergoing diapause II, while those in black do not. Parsimony and ML ancestral state reconstructions indicate multiple independent origins of diapause. Branch colours correspond to likelihood ancestral state reconstructions, with red indicating diapause II (proportional likelihood greater than 20 : 1, $p < 0.05$), black absence of diapause II (proportional likelihood greater than 20 : 1, $p < 0.05$), and orange equivocal reconstruction (proportional likelihood less than 20 : 1 for either state). The starred taxa are species (males and females pictured) in which embryo development was studied. Scale bar = millions of years before present.

Rivulidae. Both ML and parsimony ancestral state reconstructions support multiple independent origins of diapause within killifish (figure 1 and electronic supplementary material, figure S1). Furthermore, diapause in killifish is strongly tied to a certain selective environment—seasonally ephemeral water bodies (SɪᴍMᴀᴘ 1.5, correlation analysis $p <$ 0.0000001; electronic supplementary material, table S6). We measured embryo head size and heart rate as a function of developmental stage (pairs of somites) and developmental trajectory (diapause or direct-developing) in seven species that represent at least five independent origins of diapause II, plus five species of killifish that lack diapause II (figure 1 and table 1). In annual species, embryos that entered diapause II became conspicuously different in appearance from direct-developing embryos. This divergence began during mid-embryogenesis, well before diapause was entered, as originally reported for *Austrofundulus limnaeus* [18]. For example, in *N. furzeri* direct-developing embryos exhibit a significantly faster rate of head growth and higher heart rate relative to embryos that become

committed to entering diapause (trajectory × somite interaction, all $p < 0.0001$). A nearly identical pattern of divergence was observed in *Austrofundulus leohgnei* embryos (figures 2 and 3), *N. korthause*, *Austrolebias nigripinnis* and *R. (Laimosemion) tecminae* (electronic supplementary material, figure S4). In the annuals *C. occidentalis* and *F. deltaensis*, all embryos followed the diapause trajectory (electronic supplementary material, figures S4 and S6). These diapause embryos revealed head dimension and heart rate trajectories similar to diapause embryos from the other annual species; specifically, the relationship between somite number and the measured variables was relatively flat preceding diapause. Embryos of the five representative non-annual species (*F. gardneri*, *F. scheeli*, *O. latipes*, *P. playfairii* and *R. (Anablepsoides) hartii*) exhibited a single pathway characterized by continuous development that appeared equivalent to the direct-developing pathway found in annual killifish (electronic supplementary material, figure S5). Specifically, embryo heart rate, head width and head length increased linearly as a function of stage of development (somite pairs).

Table 1. Summary of evidence regarding the presence of diapause II and existence of alternative developmental pathways characterized by phenotypic divergence in 12 representative killifish species (phylogenetic placement indicated in figure 1). (Further details are provided in the electronic supplementary material.)

dspecies	diapause II	alternative developmental pathways observed	significant divergence in head width	significant divergence in head length	significant divergence in heart rate	electronic supplementary material
Austrofundulus leohoignei	yes	yes	yes	yes	yes	figures S3, S4 and table S7
Austrolebias nigripinnis	yes	yes	yes	yes	yes	figures S3, S4 and table S7
Callopanchax occidentalis	yes	no[a]	—	—	—	figures S3, S4 and table S7
Fundulopanchax deltaensis	yes	no[a]	—	—	—	figures S3, S6 and table S7
Fundulopanchax gardneri	no	no	—	—	—	figures S3, S5 and table S7
Fundulopanchax scheeli	no	no	—	—	—	figures S3, S5 and table S7
Nothobranchius furzeri	yes	yes	yes	yes	yes	figures S3, S4 and table S7
Nothobranchius korthausae	yes	yes	yes	yes	yes	figures S3, S4 and table S7
Oryzias latipes	no	no	—	—	—	figures S3, S5 and table S7
Pachypanchax playfairii	no	no	—	—	—	figures S3, S5 and table S7
Rivulus (Anablepsoides) hartii	no	no	—	—	—	figures S3, S5 and table S7
Rivulus (Laimosemion) tecminae	yes	yes	yes	yes	yes	figures S3, S4 and table S7

[a]All embryos entered diapause II. See the electronic supplementary material for additional details.

We tested the adaptive hypothesis that morphological divergence preceding entry into diapause reduces investment in structures that are energetically costly by measuring metabolic rate (oxygen consumption) in groups of *N. furzeri* embryos of known stage and developmental trajectory over the time-course of development (figure 4). We found a pattern of divergence in metabolic rate which mirrors that observed in head dimensions and heart rate. The metabolic rate of embryos entering diapause II remained low while that of direct-developing embryos increased dramatically (figure 4). Lastly, we demonstrate that under laboratory rearing conditions in Yamamoto's embryo incubation medium *N. furzeri* embryos following the diapause II trajectory are able to survive for significantly longer periods than those that follow the direct-developing pathway (figure 5).

4. Discussion

Convergent evolution, when different lineages independently evolve similar phenotypic characteristics, is indicative of adaptation by natural selection [41]. Is the evolution of diapause in killifish due to convergence? Our ancestral state reconstructions strongly indicate multiple independent origins of diapause within killifish and there are several reasons that suggest the feasibility of this evolutionary scenario. There are limited life-history solutions for fishes to survive in ephemeral water bodies. The African lung fish (*Protopterus*), which across much of their range coexist with annual killifish in the same ephemeral pools, exhibits one such strategy—long-lived adults bury into the soil, secrete a slime coat that hardens into a cocoon and aestivate until the rainy season returns [42]. Annual killifish exhibit the opposite life cycle, that of short-lived adults with embryos that undergo diapause and survive the dry season. Killifish are found in seasonally ephemeral aquatic habitats scattered across large portions of Africa and South America that have very different geologic histories [10,12]. Furthermore, there are several instances of a single species or several species with diapause nested within an otherwise non-annual clade (figure 1). Taken together, this evidence suggests that the

evolution of diapause and transition to an annual life history evolved repeatedly as killifish invaded waters that periodically desiccate [10,22]. The developmental stages where diapause occurs may represent the most stable or insensitive points in the developmental process so that Aplocheiloidei killifish are pre-adapted towards evolving diapause at these stages [13].

The direct-developing pathway in annual killifish is typical of non-annual killifish and teleosts in general, and thus probably represents the ancestral condition. The morphological and physiological divergence preceding diapause II is novel, and in need of adaptive explanation. One prominent challenge associated with remaining dormant for many months is energy conservation, particularly when a finite and limited nutrient supply (in the form of yolk) is available [15]. The heart and sensory organs associated with the head (brain, eyes, etc.) are anatomical structures that may be energetically costly to maintain for long periods [43], especially if development is arrested at a stage where these structures are already partially developed, as is the case with arrest in diapause II. That embryos following the diapause trajectory show a heart rate that is undetectable, sporadic or significantly reduced and an under-developed cranial region (relative to direct-developing embryos) suggests that divergence may function to reduce early investment in energetically costly structures in preparation for a long period of developmental arrest (i.e. a reduction in maintenance costs). The pattern of extremely reduced metabolic rate preceding and following entrance into diapause II is consistent with this hypothesis (figure 4). The final piece of evidence in general congruence with this interpretation is that embryos which enter diapause II are able to survive for longer periods than embryos which are direct-developing and proceed directly to diapause III (figure 5).

Given a particular set of circumstances, including temporal or spatial variation, an extreme case of adaptive developmental plasticity—the evolution of discrete alternative phenotypes or polyphenisms—can evolve [20]. In vertebrates, discrete alternative phenotypes are often brought about by environmentally induced triggers that cause changes in the timing of developmental events in the larval, juvenile or adult phase. Examples include the cannibalistic and omnivorous trophic
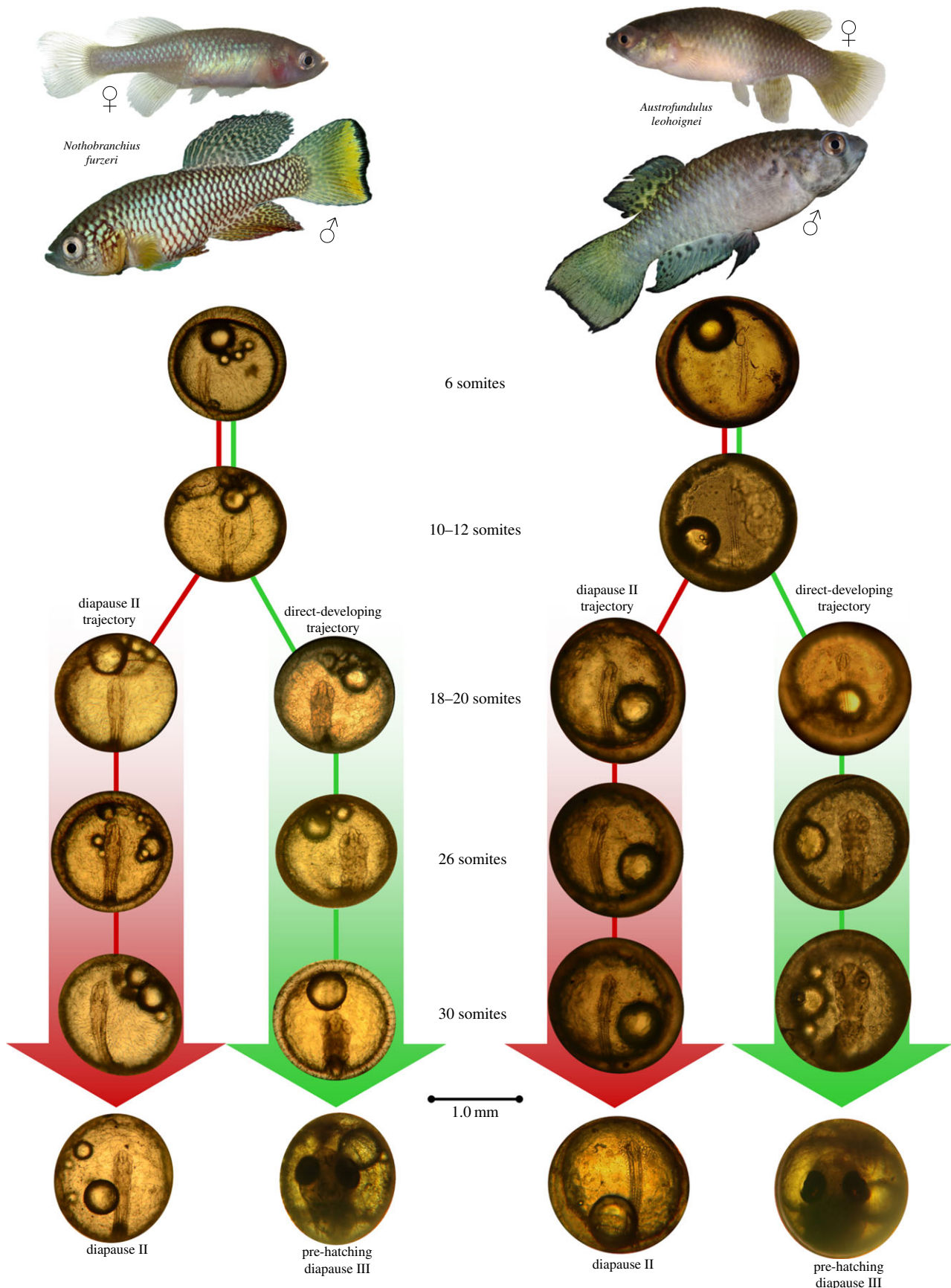
Figure 2. The African species *Nothobranchius furzeri* (left column) and the South American species *Austrofundulus leohoignei* (right column) exhibit similar patterns of embryological divergence depending upon developmental trajectory. In each species, diapause II is entered around the 38-somite stage, yet morphological divergence in the head region is readily apparent well before this stage is reached. Figure design modified after [18].

morphs in spadefoot toad tadpoles, paedomorphosis in tiger salamanders and adult sex change in bluehead wrasse [44]. In annual killifish, the transitory existence of ephemeral pools and inherent uncertainty associated with pool duration and beginning of the rainy season have selected for a developmental system poised to generate significant variation in the time-course of development and hatching [13]. Our results emphasize the importance of natural selection in generating
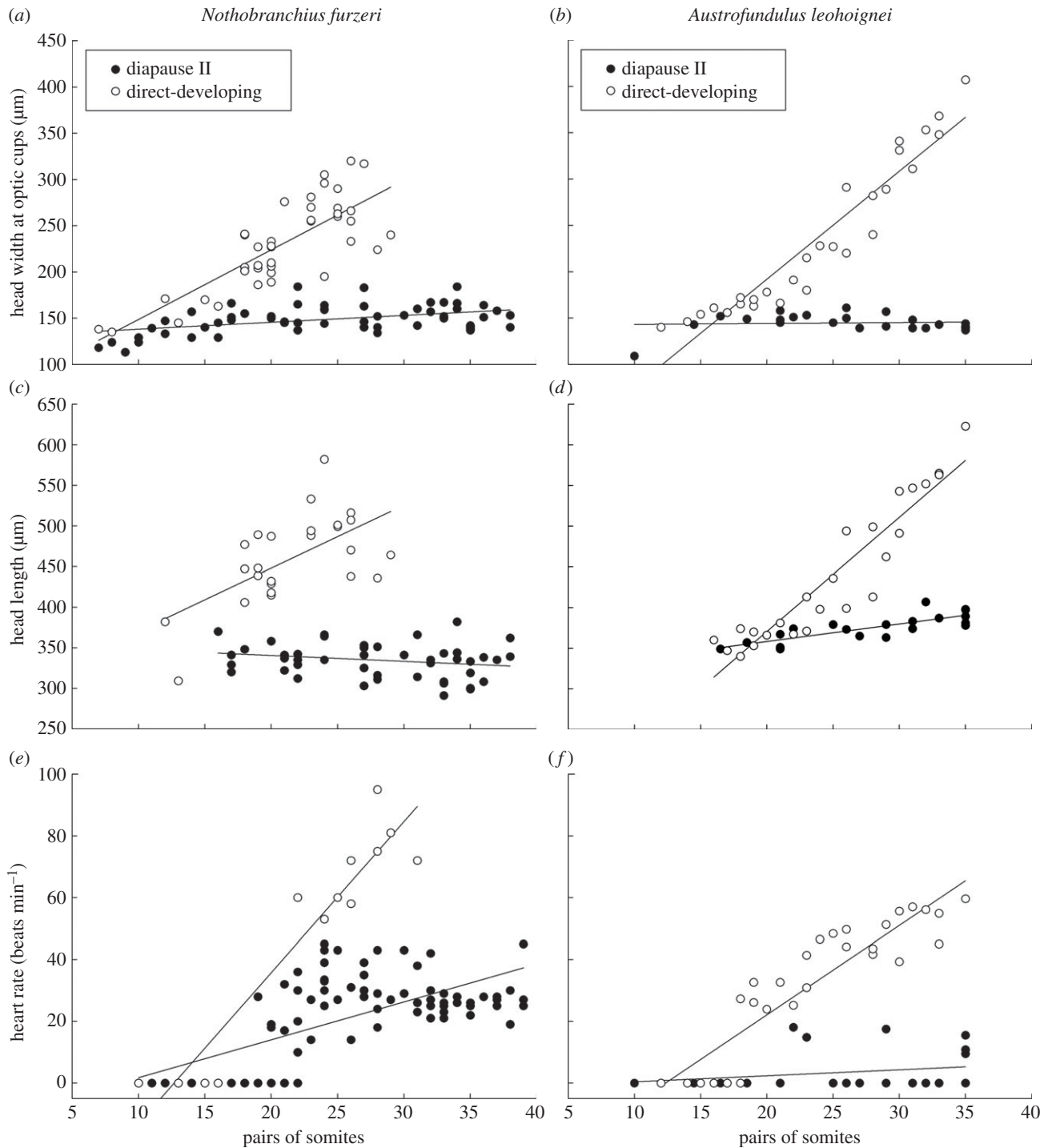
Figure 3. *Nothobranchius furzeri* and *Austrofundulus leohoignei* exhibit similar patterns of morphological and physiological divergence along alternative developmental trajectories. For each of the dependent variables including head width at optic cups (*a,b*), head length (*c,d*) and heart rate (*e,f*), linear mixed models indicate significant divergence (trajectory × somite interaction, all *p* < 0.0001).

marked intraspecific diversification during embryology. The alternative developmental pathways in killifish are remarkable in that phenotypic divergence begins mid-embryogenesis, during a period of development that is supposedly highly conserved among vertebrates [1,4], but see [6], and is nearly identical in phenotypic pattern among species that evolved diapause independently in response to similar selective environments (seasonal aquatic habitat) across two continents.

Divergence early in development in association with alternative developmental pathways may be widespread, particularly in invertebrates. Species that have diapause, exhibit different morphs, display strong sexual dimorphism or have alternative mating strategies may follow different

developmental trajectories that extend prior to birth or hatching. For example, in many taxa, chemical alarm cues can elicit anti-predator behaviours [45] or inducible defences [46]. Most studies have focused on the effect of exposure during the juvenile or adult stage [45]. Yet, several recent studies indicate that embryos may be just as responsive to environmental conditions or cues, and this can lead to an adaptive matching between phenotype and the (expected) future environment via effects on post-embryonic behaviour [47] or morphology [48]. Alternative developmental trajectories, or developmental plasticity in general, can induce post-hatching phenotypic differences that have large effects on fitness and are adaptive given predicted future environments [49], but
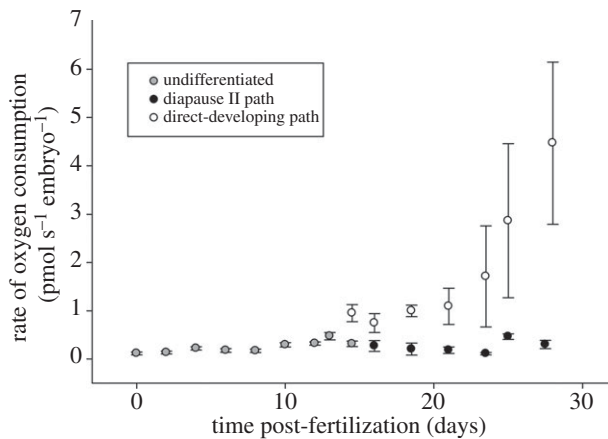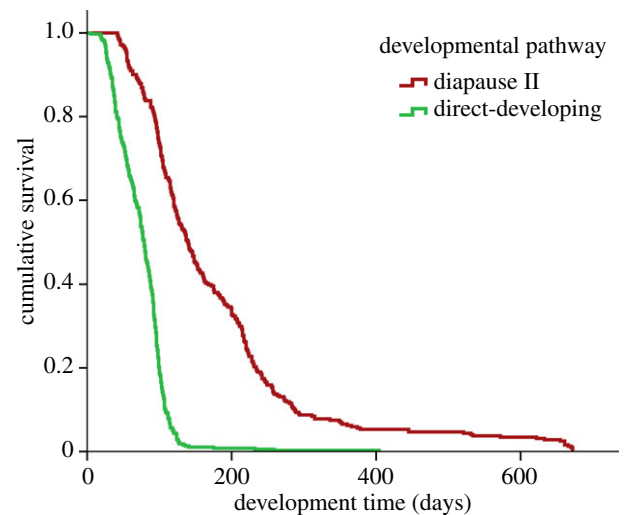
Figure 4. The rate of oxygen consumption in *Nothobranchius furzeri* eggs over the course of development. Beginning 14 days post-fertilization, batches of embryos were subdivided according to developmental trajectory prior to measurement. Embryos following the direct-developing trajectory reach a stage of development where they are capable of hatching (DIII) 24–26 days post-fertilization. Symbols represent the mean ± s.e. for three to seven batches of eggs.

Figure 5. Plot of cumulative survival as a function of development time in *Nothobranchius furzeri* embryos. Embryos following the diapause II developmental pathway ($n = 321$) exhibit longer survival time than embryos following the direct-developing pathway ($n = 387$) when incubated in Yamamoto's solution in a laboratory environment (Kaplan–Meier method, Mantel–Cox test, $\chi^2 = 331.3$, $p < 0.00001$).

may prove maladaptive if mismatched with environment [50]. In annual killifish, divergence prior to diapause apparently facilitates long-term egg survival, but it may be beneficial to minimize the effect of developmental pathway on post-hatching phenotype [51]. Embryos that enter diapause eventually resume development, ultimately coming full circle and reaching the same pre-hatching stage as direct-developing embryos, albeit via an extended stop-over in diapause that temporally separates the two groups of embryos. Whether following different developmental pathways has lasting effects on post-hatching phenotype, or if the developmental system has effectively buffered embryos from potential negative consequences of remaining dormant for long periods has recently been addressed. Polacik *et al.* [52] have shown that the developmental pathway followed has significant effects on fishes' post-hatching life histories.

Darwin [7] proposed that adaptive diversification is the product of selection and the conservation of some life stages relative to others may be a by-product of the reduced role that natural selection has played in shaping some phases of development. The hourglass model [4] posits constraint is the cause of a mid-embryonic phylotypic period characterized by reduced phenotypic divergence. Here, we have shown that even if such constraints exist, they can be

repeatedly overcome given strong selection on the embryo stage in harsh environments. More generally, our results invite further consideration of the role of selection in shaping different periods of embryology [3,5,6], particularly in species that exhibit alternative phenotypes.

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