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The Evolution of an Annual Life Cycle in Killifish: Adaptation to Seasonally Ephemeral Aquatic Habitat Across Two Continents

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by

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ABSTRACT OF THE DISSERTATION

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by

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Across the tree of life there is a great deal of variation in life cycles, life histories, and reproductive strategies. Explaining this diversity in terms of selective pressures and ecological conditions is one of the grand challenges within the field of evolutionary biology. Studying the extremes, or the ends on this spectrum of life cycle variation, can provide a fuller understanding of how these different strategies evolve and allow for population persistence. My dissertation research is united by two themes – the evolution of reproductive mode, and adaptation to ephemeral and variable environments. Killifish, small oviparous fishes within the Order Cyprinodontiformes, have evolved an annual life cycle and are adapted to life in seasonally ephemeral aquatic habitats. The most prominent adaptation of these short-lived killifish are embryos capable of undergoing diapause (halting development) at one or more of three different stages during embryology and remaining buried in the soil for much of the year. In this dissertation I combine a phylogenetic, comparative, and experimental approach to study the evolution of this life cycle and how through embryonic diapause these fish have adapted to ephemeral and variable aquatic habitat.

In chapter one I demonstrate convergent evolution of alternative developmental trajectories associated with diapause in African and South American killifish species. Adaptation to seasonal aquatic environments in annual killifish imposes strong selection during the embryo stage leading to marked diversification during a mid-embryogenesis period that is otherwise highly conserved during vertebrate development. In chapter two, I demonstrate that the embryos of an annual killifish *Nothboranchius furzeri* exhibit a combination of phenotypic plasticity and bet-hedging (a risk spreading strategy). Specifically, whether embryos enter diapause is influenced by environmental factors (temperature and light level) that vary seasonally but also exhibits a measure of intrinsic variability, even after controlling genetics and environment. In chapter three, I compile available evidence from the literature and my own comparative experiments and provide a plausible scenario for how an annual life cycle evolved through intermediate steps. Killifish are found in aquatic habitats that span a continuum from permanent and stable to seasonal and variable, thus providing a useful system in which to piece together the evolutionary history of this life cycle using natural comparative variation embedded in a phylogenetic context.

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Introduction

Organisms adapted to life in ephemeral environments face formidable survival challenges and special reproductive modes or strategies are required if such niche space is to be utilized (Wiggins et al. 1980; Williams 1985). Mobile organisms can simply leave a deteriorating environment and search for better quality habitat patches. Organisms that are unable to travel such distances can sometimes survive during the adult phase by burying into the soil and remaining dormant until conditions improve. This is the strategy used by spadefoot toads in the American Southwest (McClanahan Jr 1967) or African lungfish (Fishman et al. 1986). The sort of opposite life history is referred to as an annual life cycle. Here growth, maturity, and reproduction take place within a single calendar year, with the production of propagules such as seeds or eggs that are capable of surviving harsh seasonal conditions that are uninhabitable by juveniles or adults (Evans and Dennehy 2005). An annual life cycle is probably best associated with desert annual plants in which seeds remain dormant for most of the year in an underground seed bank (Venable 2007). However, an analogous life cycle has evolved independently many times in very different groups of organisms including small crustaceans that live in ephemeral pools in arid regions and produce hardy cysts (Simovich and Hathaway 1997), Daphnia or water fleas that live in either temporary or permanent aquatic habitat and produce dormant embryos as a way of surviving harsh environmental conditions such as desiccation of their environment or intense predation by fish (Dudycha and Tessier 1999; Ślusarczyk 1995), and many insects in temperate zones that produce dormant eggs in response to cues indicating the onset of winter and freezing temperatures which kill off the adult phase (Bradford and Roff 1997; Fielding 2006; Hopper 1999). Furthermore, an annual life cycle has evolved in vertebrates (once in a chameleon on the island of Madagascar - Karsten et al. 2008) and in killifish adapted to life in seasonally ephemeral aquatic environments through the production of embryos capable of undergoing diapause or

developmental arrest (Wourms 1972). For my dissertation I use killifish as a vertebrate system to address hypotheses regarding the evolution of this reproductive mode, the role of selection and constraint during embryology, and how diapause allows for adaptation to variable environments.

Study system

Killifish, small oviparous fishes in the Order Cyprinodontiformes, have adapted to life in ephemeral pools and streams and are found across large portions of Africa and South America in habitats ranging from open savannah to forest (Hrbek and Larson 1999; Murphy and Collier 1997; Murphy and Collier 1999). Due to the regular or periodic drying of their aquatic habitat, these fish have evolved eggs capable of undergoing prolonged diapause or developmental arrest at specific stages (Wourms 1972). As their aquatic environment dries out and the fish die, the developing eggs can remain buried in the soil, in diapause, until the following rainy season (Podrabsky et al. 2010b; Watters 2009; Wourms 1972). Another characteristic feature of such killifish is an abbreviated life cycle characterized by fast growth, early maturity, and rapid senescence (Genade et al. 2005). In habitats that have distinct wet and dry seasons, where such pools regularly dry each year this suite of traits has been referred to as an annual life cycle. Annual killifish represent one extreme on the spectrum of life history variation in vertebrates and offer advantages for studying ecological and evolutionary questions pertaining to developmental biology, phenotypic plasticity, physiological tolerance, and adaptation to extreme environments.

Dissertation chapters

The purpose of this dissertation is to investigate the evolution of an annual life cycle with embryonic diapause and adaptations to living in seasonal aquatic environments in killifish. Specifically, I address the following questions: 1.) Has diapause evolved independently in multiple clades, and is there convergent evolution of underlying developmental pathways associated with entering or skipping diapause? 2.) Does entrance into diapause and hatching time

exhibit plasticity to environmental cues that may indicate timing in the season, and / or do embryos exhibit variability in these traits, consistent with a risk spreading strategy? And 3.) How did this annual life cycle evolve through intermediate stages? Are there traits or precursors among non-annual killifish that facilitated this transition?

In a multi-faceted first chapter I demonstrate convergent evolution of alternative developmental pathways associated with diapause in African and South American annual killifish. I first create a molecular supermatrix phylogeny of killifish, then perform detailed comparative studies of development in twelve species (7 annual, 5 non-annual), and lastly, test the adaptive significance of developmental divergence preceding diapause in one representative species of annual killifish, *Nothobranchius furzeri*. There have been two prominent molecular phylogenies of killifish (Hrbek and Larson 1999; Murphy and Collier 1997). Murphy and Collier (1997) found support for a single early origin of the annual life history prior to the break-up of Africa and South America, followed by an ancestral loss, with several reversions. While the analyses of Hrbek and Larson (1999) supported several gains of diapause in the predominantly South American Rivulidae family. Building upon this prior work I incorporate all available molecular data available on Genbank and build a maximum likelihood supermatrix phylogeny of Aplocheiloidei killifish. Each species in the phylogeny was scored for presence or absence of diapause II, the most prominent stage of developmental arrest in annual killifish, based upon reporting in the literature or examination of living embryos. Ancestral state reconstructions performed on the combined phylogeny and diapause data set strongly suggest multiple independent origins of diapause in both African and South American species.

Annual killifish have embryos capable of entering into or skipping diapause. Working with the South American annual killifish *Austrofundulus limnaeus* (Podrabsky et al. 2010a) described how embryos destined to enter into diapause II mid way through development exhibit

morphological and physiological divergence prior to this arrest, compared to embryos destined to skip this stage of diapause and remain 'direct-developing.' I tested whether this phenomenon was found in representative species from clades that the ancestral state reconstructions indicate evolved diapause II independently. In seven different species of annual killifish representing at least five clades that independently evolved diapause a pattern of divergence during embryology was clearly found, and such patterns were absent in five non-annual killifish tested. Specifically, embryos destined to enter diapause show a significant reduction in development of the head region and slowed heart rate relative to embryos that are destined to skip diapause. In other words, embryos that will eventually enter diapause do not simply halt development at the proper stage, rather they diverge morphologically and physiologically prior to the cessation of development, as if in preparation for what is coming. Typically development within a species is highly conserved and canalized, especially during mid-embryogenesis, when a prominent developmental theory - the hourglass model (Raff 1996) posits the existence of a 'phylotypic stage' characterized by reduced phenotypic divergence. The convergent pattern of intraspecific divergence in annual killifish embryos following alternative developmental pathways argues for the primacy of natural selection in overcoming constraint. Adaptation to seasonal aquatic environments in annual killifish across two continents has led to marked diversification during this otherwise conserved period of vertebrate development. Lastly, I tested the adaptive significance of such divergence by measuring metabolic rate and long-term embryo survival in *Nothobranchius furzeri*. Results are consistent with the interpretation that morphological divergence during the phylotypic period allows embryos undergoing diapause II 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 egg shell for much of the year.

In chapter two, I develop and test contrasting predictions regarding two different modes of adaptation to environmental variability using annual killifish embryos. Annual fish live in an environment where the rainy season can be highly unpredictable both within and across seasons (Watters 2009). Within a season, rain may come in pulses such that pools which support fish have multiple inundations and dryings (Polacik et al. 2011; Watters 2009). Across seasons a given year may have such low or sporadic rainfall that no fish are able to successfully reproduce (Podrabsky et al. 1998; Wourms 1972). If all eggs of an annual fish were ready to hatch at approximately the same time, and the year was atypical in its rain patterns, then the entire batch of eggs could be destroyed. Specifically, I examine whether embryos of the annual killifish *Nothobranchius furzeri* exhibit phenotypic plasticity to environmental cues in 'deciding' whether to enter diapause, and / or whether embryos exhibit diversification bet-hedging and exhibit variability in whether diapause is entered and hatching is initiated (analogous to a seed bank in annual plants). Theory predicts that reliable cues indicative of future environmental conditions can favor plasticity, while unreliable cues tend to favor the risk spreading strategy of diversification bet-hedging (Wong and Ackerly 2005). To test for phenotypic plasticity, I exposed embryos to combinations of light and temperature that mimic different times during the season and scored embryos for whether they entered into or skipped diapause. To make a case for bet-hedging the following predictions were tested 1) eggs from the same female and clutch, treated in the same manner, are capable of undergoing heterogonous development rates (i.e. some enter and some skip diapause) 2) Eggs that are not ready to hatch remain viable for long periods 3) Not all eggs hatch during the first rainfall event (i.e. when wetted the first time).

Both sets of predictions found support. Given laboratory incubation conditions, cool temperatures, which are associated with the dry season, induced nearly all embryos to enter into long phases of arrest at the diapause II stage, while warm temperatures induced the direct-

developing pathway. Light level only had an affect at intermediate temperatures, suggesting that it is perhaps a less reliable cue than temperature. Consistent with bet-hedging, embryos of known parentage, incubated under moderate condition were capable of either entering diapause II or following the direct-developing pathway. Furthermore, fully developed embryos exhibited a great deal of variation in hatching time. These results suggest that given intermediate levels of environmental predictability a combination of phenotypic plasticity and bet-hedging can be optimal (Kaplan and Cooper 1984; Moran 1992; Wong and Ackerly 2005).

In chapter three, I review and synthesize habitat, behavioral, developmental, and life-historical variation in killifish in order to present a plausible scenario for how an annual life cycle likely evolved through a series of intermediate stages. A successful approach to studying the evolution of complex traits or life cycles has been to harness comparative variation among living species and use this variation in a phylogenetic context to make inferences, perform experiments, or test hypotheses for the evolution of the full trait (Pollux et al. 2009; Weber and Agrawal 2012). Different species of killifish are found across a gradient of habitats ranging from permanent and stable to seasonal and variable. Non-annual killifish that inhabit marginal habitats that are subject to periodic short-term desiccation risk are adapted to life at the aquatic-terrestrial interface. I demonstrate that the fully-developed embryos of non-annual killifish are capable of exhibiting a delayed hatching response, that appears functionally equivalent to the last diapause stage of annual species. This delayed hatching and desiccation resistance allow embryos to survive short periods of dry-down and may represent precursors or pre-adaptations that allowed non-annual killifish to exploit marginal aquatic habitats subject to periodic desiccation thus setting the stage for the colonization of seasonally ephemeral waters and the evolution of diapause II and a true annual life cycle.

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

Abstract

There are contrasting views of the role of selection versus constraint during vertebrate embryology. The influential hourglass model posits that reduced phenotypic divergence during a mid-embryonic phylotypic period is the product of constraint imposed by organogenesis and the patterning of the adult body plan. An alterative view is that all periods of development are potentially subject to adaptation and diversification; conservatism reflects a dearth of selection. Annual killifish adapted to life in seasonally ephemeral water-bodies exhibit desiccation resistant eggs that can undergo diapause, developmental arrest, enabling them to traverse the otherwise inhospitable dry season. I characterize the convergent evolution of alternative developmental trajectories associated with diapause in lineages of killifish that independently adapted to seasonally ephemeral aquatic habitats across two continents. Embryos destined to enter diapause mid-way through development diverge morphologically and physiologically compared to direct-developing embryos that skip diapause. This divergence begins prior to and during the phylotypic period. My results thus emphasize that even this most conserved period of development can be modified by natural selection, resulting in prominent intraspecific diversification during vertebrate embryology.

Introduction

There are several competing models purporting to explain patterns of embryonic conservation, or lack thereof (Duboule 1994; Kalinka and Tomancak 2012; Poe and Wake 2004; Raff 1996; Richardson 1999; Richardson et al. 1997). The most influential is the hourglass model of development, which posits a phylotypic period (reduced phenotypic divergence that occurs mid-embryogenesis) bracketed by periods of increased divergence earlier and later in development (Duboule 1994; Raff 1996). According to Raff (1996) 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, that 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 leading to a conserved phylotypic period. A contrasting view of embryology, foreshadowed by Darwin (1859), focuses on the role of selection and posits that all stages of development are potentially subject to adaptive diversification (Poe and Wake 2004; Richardson et al. 1997).

The production of discrete alternative phenotypes from the same genotype (polyphenism) provides an under-utilized 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. Through construction of a supermatrix phylogenetic tree of the order Cyprinodontiformes and a battery of comparative analyses I first demonstrate and then characterize the convergent evolution of alternative developmental trajectories associated with embryonic diapause/direct-development in lineages of killifish that have independently adapted to seasonally ephemeral aquatic habitats

across two continents. I 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. Lastly, I demonstrate that the morphological and physiological divergence along alternative developmental pathways functions to reduce investment in energetically costly organs thereby increasing survival chances in an environment that necessitates remaining dormant, buried in the soil, and surrounded by an egg shell for much of the year.

Aquatic organisms living in habitats that regularly or periodically dry have a limited number of life history strategies (Williams 1985). 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 (Williams 1985). The production of embryos (seeds, cysts, eggs) capable of traversing unfavorable conditions uninhabitable by the adult stage, is common in plants and invertebrates but has only rarely evolved in vertebrates (Hrbek and Larson 1999; Karsten et al. 2008; Murphy and Collier 1997; Wourms 1972b). Perhaps the most prominent adaptation of such embryos is diapause - developmental arrest accompanied by a reduction in metabolic rate (Podrabsky et al. 2010b). 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 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 (Hahn and Denlinger 2011; Podrabsky et al. 2010b; Tauber et al. 1986; Wourms 1972b). 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 (Wourms 1972b). In annual killifish, eggs are capable of entering diapause at specific stages during embryology - termed I, II, and III (Wourms 1972a). Diapause I occurs early in development before the somite-embryo has formed during a dispersed cell phase, which is unique to annual killifish (Wourms 1972a). Although embryos have been induced to enter this state through low temperatures or hypoxia (Podrabsky et al. 2010a; Wourms 1972a) 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 (Wourms 1972a; Wourms 1972b). It is during this diapause that embryos are most resistant to temperature extremes, desiccation, and oxygen deprivation (Podrabsky et al. 2010b). 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 (Podrabsky et al. 2010a; Wourms 1972b) termed the diapause and direct-developing pathways. Direct developing eggs skip diapause II and instead undergo continuous development until (DIII) is reached, while those that enter diapause II exhibit developmental arrest and may not resume development and reach the pre-hatching stage (DIII) 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 (Podrabsky et al. 2010a; Podrabsky et al. 2010b). This

strategy likely represents a combination of adaptive phenotypic plasticity and a risk-spreading strategy known as bet-hedging (Kaplan and Cooper 1984; Moran 1992; Wong and Ackerly 2005).

Materials and Methods (Abbreviated)

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). I generated a molecular phylogeny of the group using supermatrix tree construction methods (de Queiroz and Gatesy 2007). First, I identified molecular sequence data from 269 species of Apocheiloidei killifish and 42 outgroup taxa from NCBI. I used Geneious version 5.4.6 to download seven mitochondrial and two nuclear genes from Genbank, and constructed a 10960 base pair supermatrix for these 311 taxa. Sequences were aligned using MUSCLE (Edgar 2004) in Geneious version 5.4.6 followed by manual adjustment in Se-Al (Rambaut 1996). The concatenated data set was analyzed 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; Miller et al. 2010), GTRCAT + G model of molecular evolution for each of the nine partitions, with 500 bootstrap replicates, randomized MP starting trees, and the fast hill-climbing algorithm with all free parameters estimated.

Ancestral state reconstructions

Each species in the phylogeny was scored for presence or absence of diapause II. I performed parsimony and maximum likelihood ancestral state reconstructions in Mesquite version 2.75 (Maddison and Maddison 2011) 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 (Lewis 2001). Ancestral state reconstructions can positively mislead if extinction or speciation rates are significantly correlated with the evolution of the trait being reconstructed (Gamble et al. 2012). Diversification rates were analyzed using BiSSE: Binary trait speciation and extinction (Maddison et al. 2007) in diversitree (FitzJohn 2012) to discount this possibility as a source of bias.

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 version 1.5 (Bollback 2006) was used to determine if diapause II and habitat covary with each other across the phylogeny. This program implements Bayesian mutational mapping (Huelsenbeck et al. 2003; Nielsen 2002) and estimates two statistics, d_{ij} and m_{ij}, each a measure of covariation between character states *i* and *j*.

Divergence associated with developmental trajectory

Representative species were selected from several clades that according to my ancestral state reconstructions independently evolved diapause. These include species from the South American Rivulidae (*Austrofundulus leoghnei*, *Austrolebias nigripinnis,* and *Rivulus tecminae*) and the African Nothobranchiidae (*Callopanchax occidentalis*, *Fundulopanchax deltaensis*, *Nothobranchius furzeri* and *Nothobranchius korthausae*). In addition I examined embryos from five nonannual species that are distributed throughout the tree (*Fundulopanchax gardneri*, *Fundulopanchax scheeli*, *Rivulus hartii*, *Pachypanchax playfarii*, and *Oryzias latipes*). Breeding adults of these twelve 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 (Rembold et al. 2006) 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 12:12 (light: dark). 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 (Wourms 1972a). 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 (Schneider et al. 2012). The variables measured were: head width at optic cups, head width at otic vesicles, and head length - tip of snout to base of otic vesicles (Figure S1.7). Embryos were tracked throughout development so that developmental trajectory could be determined (direct-developing or diapause).

Statistical analyses

I used linear mixed models implemented in the R (R Core Team 2013) package *nlme* (Pinheiro et al. 2010) 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 data set). To account for this non-independence (Bolker et al. 2009) random effects for egg identity (individual intercept and slope allowed to vary) were included. In Table S1.7 I report the fixed effect parameter estimates produced by restricted maximum likelihood estimation. In cases where the full-model failed to converge due to overparameterization, the random slope effect was excluded, and the model rerun with only random intercepts.

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 (~20 somite stage) each batch of eggs was sub-divided into separate groupings on the basis of developmental trajectory (diapause vs. direct-developing). Oxygen consumption was measured on batches of 8 - 96 embryos (37.0 +/- 2.0 SE) 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 ul 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 one minute intervals. For each trial, percent 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 sec^{-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.

Long-term embryo survival

In order to determine whether developmental pathway influenced long-term embryo survival I 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 I 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. I 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).

Results

My molecular phylogeny of killifish (Figure 1.1) reveals that taxa with diapause fall into at least six distinct clades in the African family Nothobrachiidae and the South American Rivulidae. Both maximum likelihood and parsimony ancestral state reconstructions support multiple independent origins of diapause within killifish (Figure 1.1, S1.6). Furthermore diapause in killifish is strongly tied to a certain selective environment - seasonally ephemeral water bodies (SIMMAP 1.5, correlation analysis $P<0.0000001$, Table S1.6). I 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 origns of diapause II, plus five species of killifish that lack diapause II (Figure 1.1). In annual species, embryos destined to enter 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* (Podrabsky et al. 2010a). For example, in *Nothobranchius furzeri* direct-developing embryos exhibit a significantly faster rate of head growth and higher heart rate relative to embryos destined to enter diapause (trajectory x somite interaction, all $P<0.0001$). A nearly identical pattern of divergence was observed in *Austrofundulus leohgnei* embryos (Figure 1.2 and 1.3), *Nothobranchius korthause*, *Austrolebias nigripinnis*, and *Rivulus* (*Laimosemion*) *tecminae* (Figure S1.9). In the annuals *Callopanchax occidentalis* and *Fundulopanchax deltaensis*, all embryos followed the diapause trajectory

(Figure S1.9, S1.11). 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 (*Fundulopanchax gardneri, Fundulopanchax scheeli, Oryzias latipes, Pachypanchax playfairii*, and *Rivulus (Anablepsoides) hartii*) exhibited a single pathway characterized by continuous development that appeared equivalent to the direct-developing pathway found in annual killifish (Figure S1.10). Specifically, embryo heart rate, head width, and head length increased linearly as a function of stage of development (somite pairs).

I 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 *Nothobranchius furzeri* embryos of known stage and developmental trajectory over the time-course of development (Figure 1.4). I found a pattern of divergence in metabolic rate that 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 1.4). Lastly, I demonstrate that under laboratory rearing conditions in Yamamoto's embryo incubation medium *Nothobranchius furzeri* embryos following the diapause II trajectory are able to survive for significantly longer periods than those that follow the direct-developing pathway (Figure 1.5).

Discussion

Convergent evolution, when different lineages independently evolve similar phenotypic characteristics, is indicative of adaptation by natural selection (Schluter and Nagel 1995). Is the evolution of diapause in killifish due to convergence? My 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 fish 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, secret a slime coat that hardens into a cocoon, and aestivate until the rainy season returns (Fishman et al. 1986). 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 (Hrbek and Larson 1999; Murphy and Collier 1997). Furthermore there are several instances of a single species or several species with diapause nested within an otherwise non-annual clade (Figure 1.1). Taken together this evidence suggests that the evolution of diapause and transition to an annual life history evolved repeatedly as killifish invade waters which periodically desiccate (Hrbek and Larson 1999; Murphy et al. 1999a). 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 toward evolving diapause at these stages (Wourms 1972b).

The direct-developing pathway in annual killifish is typical of non-annual killifish and teleosts in general, and thus likely 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 (Hahn and Denlinger 2011). 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 (Elia 1992), 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 1.4). The final piece of evidence in general congruence with this interpretation is that embryos that enter diapause II are able to survive for longer periods than embryos that are direct-developing and proceed directly to diapause III (Figure 1.5).

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 (Moran 1992). 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 morphs in spadefoot toad tadpoles, paedomorphosis in tiger salamanders, and adult sex change in bluehead wrasse (Moczek et al. 2011). 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 (Wourms 1972b). These results emphasize the importance of natural selection in generating marked intraspecific diversification during embryology; the alternative developmental pathways in killifish are remarkable in that phenotypic divergence begins mid-embryogenesis, during a development period that is supposedly highly conserved among vertebrates (Duboule 1994; Raff 1996), but see Richardson (1997), 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 behaviors (Ferrari et al. 2010) or inducible defenses (Tollrian and Harvell 1999). Most studies have focused on the effect of exposure during the juvenile or adult stage (Ferrari et al. 2010). 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 behavior (Mathis et al. 2008) or morphology (Laforsch and Tollrian 2004). 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 (Rabus and Laforsch 2011), but may prove maladaptive if mismatched with environment (Bateson et al. 2004). 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 (Pechenik 2006). 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. (2014) have shown that the developmental pathway followed has significant effects on fishes post-hatching life histories.

Conclusion

Darwin (1859) 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 (Raff 1996) posits constraint is the cause of a mid-embryonic phylotypic period characterized by reduced phenotypic divergence. Here, I 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, these results invite further consideration of the role of selection in shaping different periods of embryology (Poe and Wake 2004; Richardson 1999; Richardson et al. 1997), particularly in species that exhibt alternative phenotypes.

Figure 1.1. Timetree (independent rates, hard-bounded constraints) of the 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 maximum likelihood ancestral state reconstructions indicate multiple independent origins of diapause. Branch colors 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.

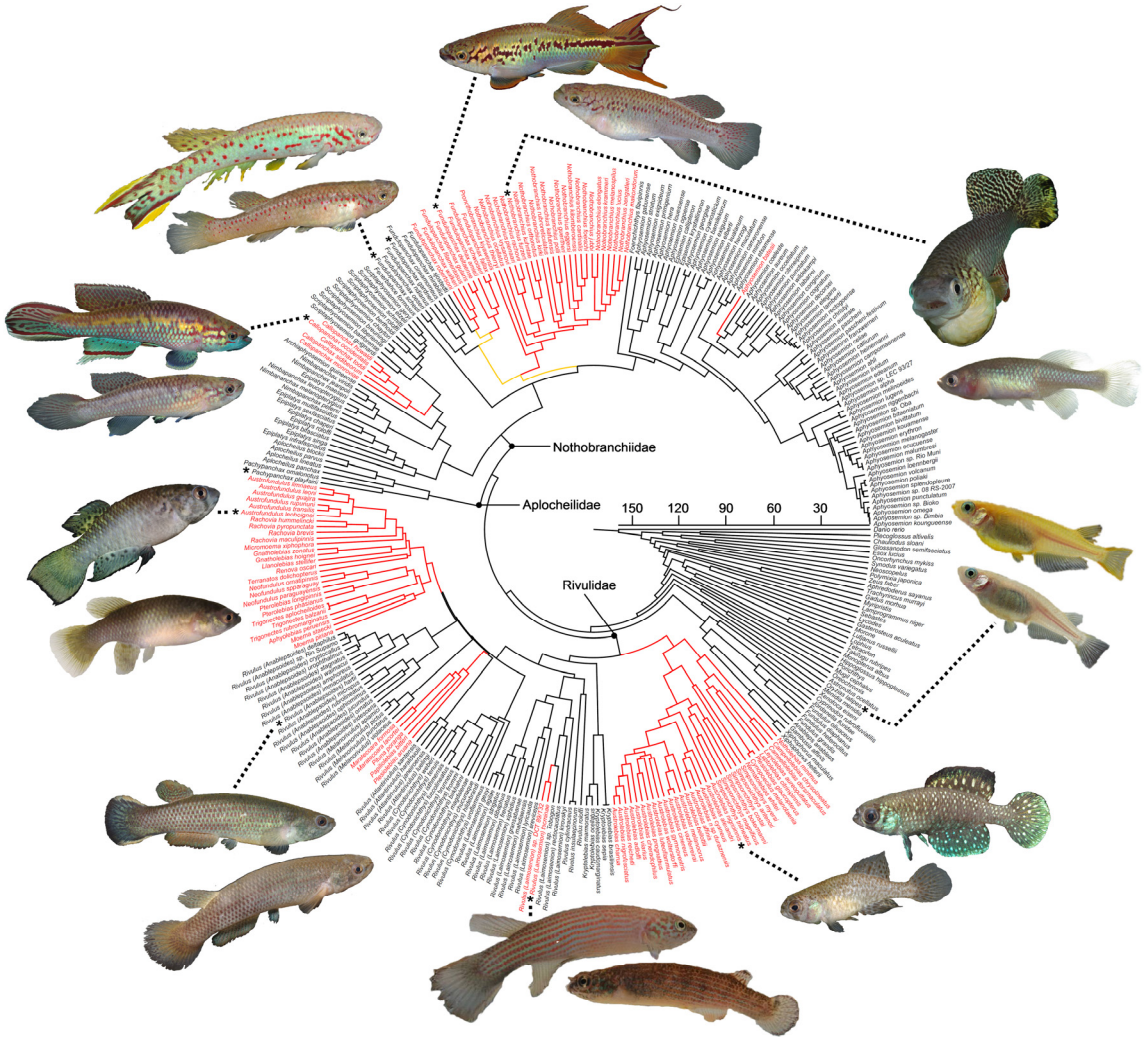


Figure 1.2. The African species *Nothobranchius furzeri* (left column) and the South American species *Austrofundulus leohoignei* (right column) exhibit similar patterns of embryological divergence depending upon developmental trajectory. In each species, diapause II is entered around the 38 somite stage, yet morphological divergence in the head region is readily apparent well before this stage is reached.

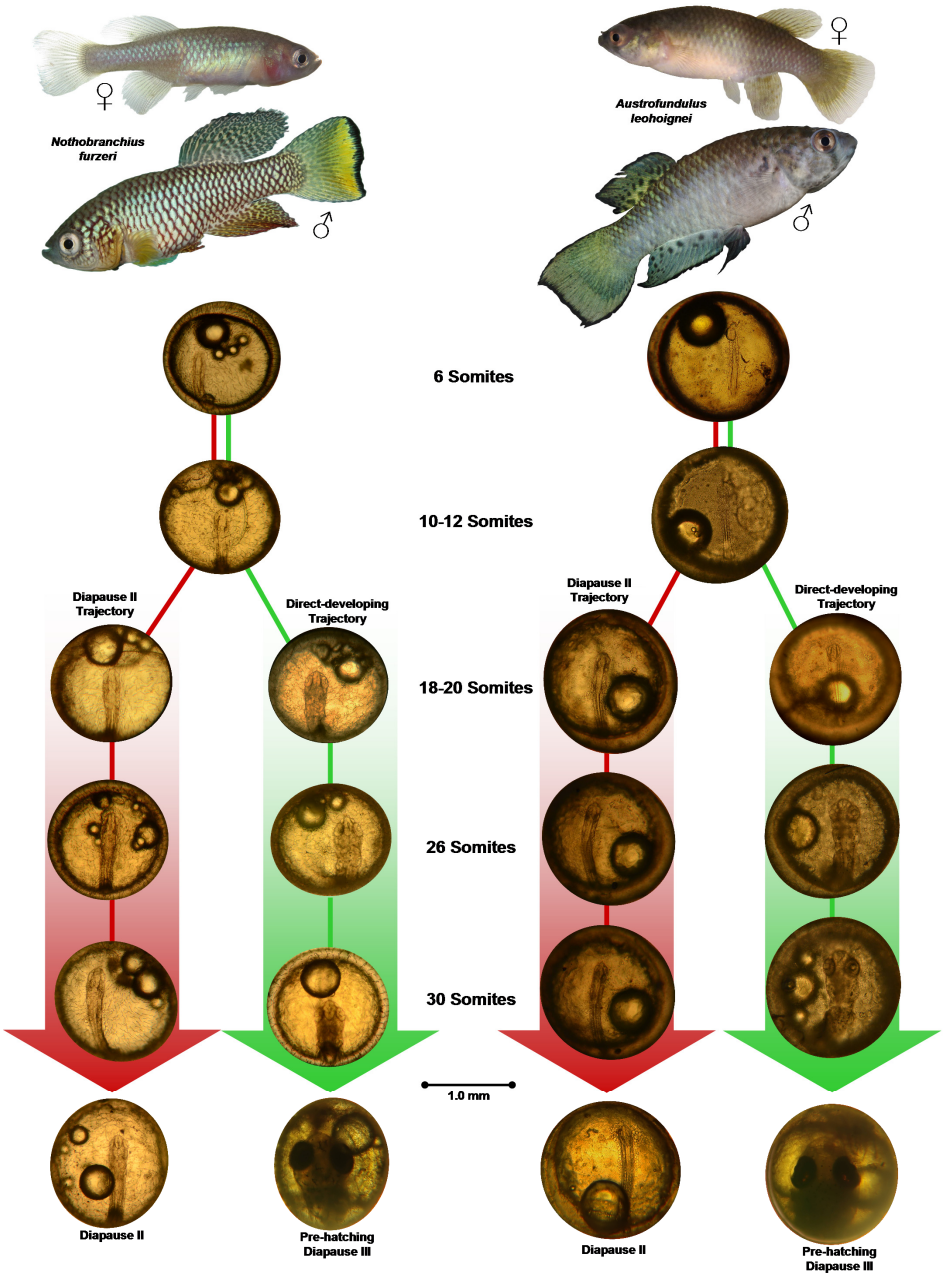


Figure 1.3. *Nothobranchius furzeri* and *Austrofundulus leohoignei* exhibit similar patterns of embryological divergence depending upon developmental trajectory. For each of the dependent variables (head width at optic cups, head length, and heart rate) linear mixed models indicate significant divergence along alternative developmental trajectories (trajectory x somite interaction, all $P<0.0001$).

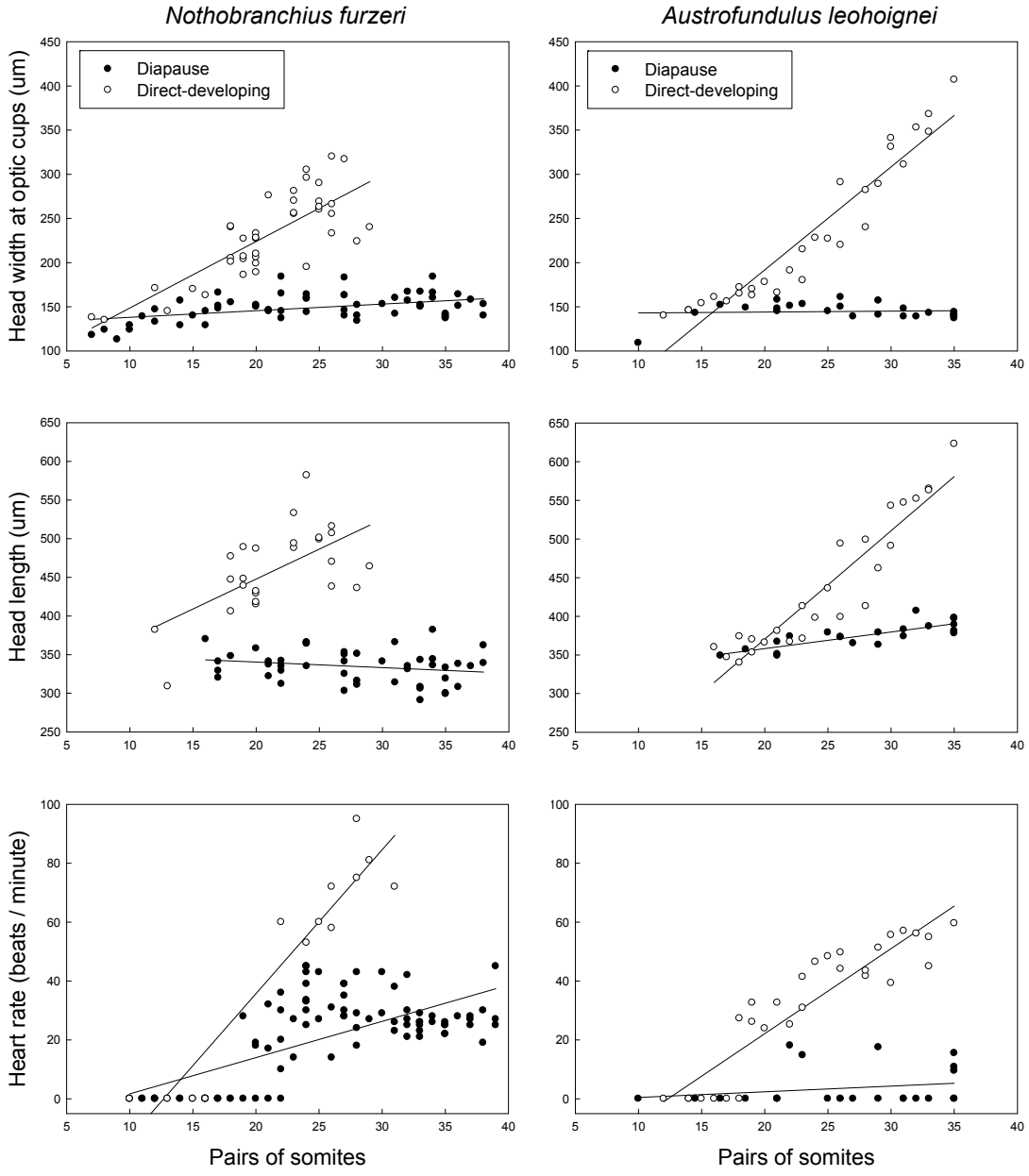


Figure 1.4. Rate of oxygen consumption over the time-course of development in the eggs of *Nothobranchius furzeri*. Beginning 14 days post-fertilization batches of embryos were subdivided according to developmental trajectory prior to measurement. Embryos following the direct-developing trajectory reach a stage of development where they are capable of hatching (DIII) 24-26 days post-fertilization. Symbols represent the mean +/- SE for 3-7 batches of eggs (independent spawning events).

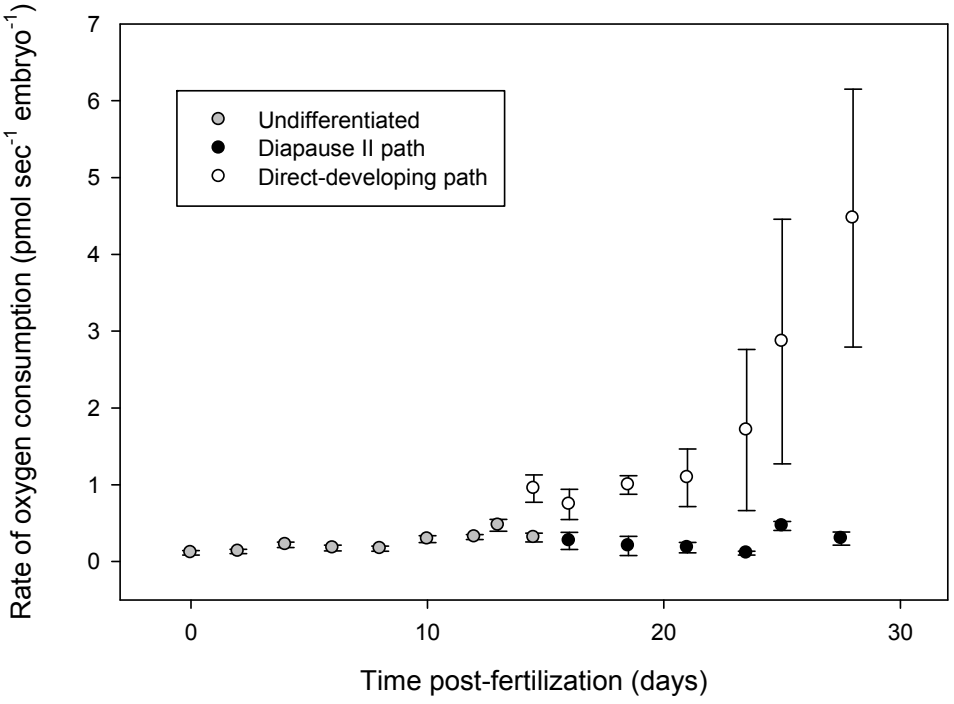
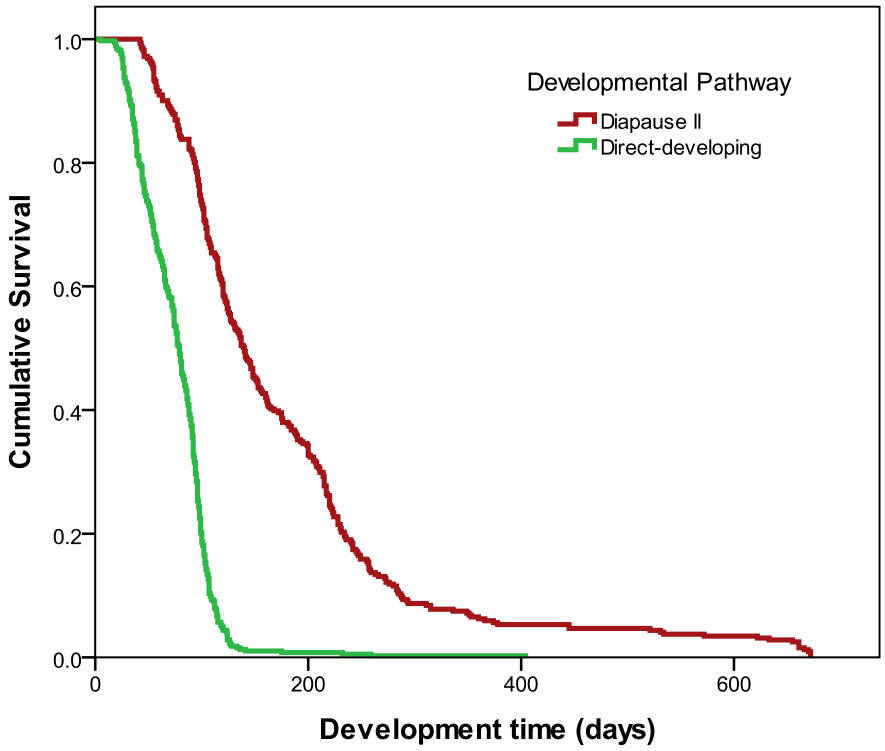


Figure 1.5. Plot of cumulative survival as a function of development time in *Nothobranchius furzeri* embryos. Embryos following the diapause II developmental pathway (n=321) exhibit longer survival time than embryos following the direct-developing pathway (n=387) when incubated in Yamamoto's solution in a laboratory environment (Kaplan-Meier method, Mantel-Cox Test, $\chi^2 = 331.3$, $P<0.00001$).



Supplementary Information

Materials and Methods

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). I generated a molecular phylogeny of the group using supermatrix tree construction methods (de Queiroz and Gatesy 2007). First, I identified molecular sequence data from 269 species of Apocheiloidei killifish and 42 outgroup taxa from NCBI. I used Geneious version 5.4.6 to download seven mitochondrial (12S, 16S, Cox1, Cyt b, D loop, ND1, ND2, as well as tRNAs Val-Ile-Gln-Met-Trp-Ala-Asn-Cys-Tyr) and two nuclear genes (28S, Rag1) from Genbank, and constructed a 10960 base pair supermatrix for these 311 taxa. Sequences were aligned using MUSCLE (Edgar 2004) in Geneious version 5.4.6 (Biomatters) followed by manual adjustment in Se-Al (Rambaut 1996). Taxonomic ambiguity was resolved in reference to original literature, Fishbase (Froese and Pauly), and the Catalog of Fishes (Eschmeyer and Fricke 2012). Individual gene trees were created using maximum likelihood (RAxML 7.2.7; GTRCAT + G model of molecular evolution; Stamatakis 2006) on the CIPRES platform (Miller et al. 2010) and examined to ensure species with multiple sequences came out monophyletic. For the species-level analysis I generally retained the longest exemplar sequence. When multiple different genes were available for a given species an effort was made to keep together those sequences generated from the same specimen. NUMTs (mitochondrial sequences incorporated into the nuclear genome) were deleted, as were certain sequences with multiple ambiguous regions (many Ns). A few species were eliminated from the analysis due to lack of gene overlap with other species in the matrix. Protein coding gene segments were translated into amino acids in Se-Al (Rambaut 1996)

to verify placement of gaps to avoid breaking up translated proteins. Visually identified alignment ambiguous regions were excluded from final phylogenetic analyses (1365 bp). SequenceMatrix (Vaidya et al. 2011) was used to concatenate the individual final alignments (ambiguous regions removed). The concatenated data set (10960 bp) was analyzed using nine partitions (12S + 16S, D loop, Cox1, Cytochrome b, ND1, ND2, tRNAs Val-Ile-Gln-Met-Trp-Ala-Asn-Cys-Tyr, Rag1, 28S.). jModelTest (Guindon and Gascuel 2003; Posada 2008) was used to determine if a gamma distribution should be included for each of the partitions based on the Akaike Information Criterion (Table S1.1). The maximum likelihood (ML) tree was estimated using RAxML7.2.7 (CIPRES platform; Miller et al. 2010) using the GTRCAT + G model of molecular evolution for each of the nine partitions, with 500 bootstrap replicates, randomized MP starting trees, and the fast hill-climbing algorithm with all free parameters estimated.

Molecular dating (timetree) analyses were performed with the MCMCTree program in PAML 4.5 (Yang 2007). MCMCTree implements the MCMC algorithms of Rannala and Yang (2007). Analyses were performed with both autocorrelated and independent rates models. Each of the nine data partitions was allowed to have its own GTR + Γ model of sequence evolution. One time unit was set to equal 100 million years. Analyses were performed with cleandata = 0. Shape (α) and scale (β) parameters for the gamma prior of the overall rate parameter μ (i.e., rgene_gamma in mcmctree) were 1 and 3.37, respectively. Calculations for the shape and scale parameters of the gamma prior for the rate-drift parameter assumed an age of 157.5 million years for the most recent common ancestor of Otocephala. Chains were run for 100,000 generations after a burn-in of 10,000 generations, and were sampled every 20 generations. Analyses were performed with both hard-bounded and soft-bounded constraints. Soft-bounded analyses allocated 2.5% of the prior distribution to each tail. Minimum ages for ten different nodes, including the root of the tree, were based on the oldest crown fossils that are assignable to each clade.

Maximum ages were based on stratigraphic bounding, phylogenetic bracketing, and phylogenetic uncertainty as in Meredith et al. (2011). Minimum and maximum constraints are summarized in Table S1.2.

Diapause character scoring

I scored killifish species for presence or absence of diapause II rather than an annual life cycle. Embryos of a given species either are or are not capable of arresting development at the diapause II stage - making it a discrete trait. I agree with Simpson (1979) "Originally, annual killifishes were defined by their ecology and the nature of their overt life history, but knowledge of their specialized development now permits more precise segregation from other teleosts."

Diapause is traditionally defined as developmental arrest with an accompanying slowing of metabolic rate (Podrabsky and Hand 1999). Killifish that reside in temporary bodies of water are able to persist through the production of eggs that are capable of undergoing diapause at specific stages during embryology - these stages have been termed diapause I, II, and III (Wourms 1972a). Diapause II occurs after the formation of the embryonic axis, in embryos possessing 38 pairs of somites and is the most prominent stage of developmental arrest in annual killifish. Embryos of annual species spontaneously enter this state under a wide range of environmental conditions, embryos are capable of arresting at this stage for many months, and it is at this stage that they are most impervious to environmental insult (Matias 1982; Matias and Markofsky 1978; Podrabsky and Hand 1999; Podrabsky et al. 2010b).

The mechanistic details of diapause have been observed and described in a limited number of African and South American killifish species, and it has been found that the embryological time points (stages where diapause can occur) are remarkably similar. However, there are many more killifish species than have undergone detailed developmental studies of diapause. Whether a species has an annual life cycle (and by logical inference is capable of

producing diapause eggs) has been inferred from a variety of indirect evidence including: 1) occurrence in aquatic habitats that regularly dry, 2) association with known annual species, 3) general morphology and behavior, and 4) an extended egg incubation period as judged by aquarium observation. In regards to this last point, a large hobbyist literature reports average egg incubation times for captive reared killifish species. Aquarists typically store killifish eggs in bags of damp peat moss for a number of months before water is added. When this occurs, eggs that have reached the pre-hatching stage (DIII) are capable of hatching. The peat moss is then re-dried for storage and successive re-wettings occur until most eggs have hatched. Although this method of incubation allows one to determine whether a given species has an extended egg incubation period, the eggs are not directly observed during development so it is not possible to ascertain which if any diapause stages were entered. In regards to character scoring I used a total evidence approach in which each species in the phylogeny was scored for presence or absence of diapause II based upon direct observation (AF) or the general consensus based upon reporting in the literature (Costa 1998; Hrbek and Larson 1999; Murphy and Collier 1997; Murphy and Collier 1999; Murphy et al. 1999a; Murphy et al. 1999b; Simpson 1979; Thomerson and Taphorn 1992; Wourms 1972b).

Diversitree analyses

Ancestral state reconstructions can positively mislead if extinction or speciation rates are significantly correlated with the evolution of the trait being reconstructed (Gamble et al. 2012). Diversification rates were analyzed using BiSSE: Binary trait speciation and extinction (Maddison et al. 2007) in diversitree (FitzJohn 2012) implemented in R (R Core Team 2013) to discount this possibility as a source of bias. In diversitree eight BiSSE analyses were performed using the four full-taxa time-calibrated phylogenies (autocorrelated and independent rates with both hard and soft bounded constraints). BiSSE calculations assume phylogenies are complete.

To account and correct for incomplete taxon sampling two alternative procedures were used. In both methods the fraction of extant species in the phylogeny (out of the total known from the group) is specified. In the 'sampling fraction' procedure it is assumed that the given tree contains a random sample of extant species with respect to character state (diapause II presence or absence) and this fraction is specified. The 'incomplete sampling' procedure assumes that the species included in the phylogeny are not random but instead vary with character state. This is specified such that the BiSSE calculations account for this uneven sampling. To test the hypothesis of single versus multiple origins I compared the fit of a model constrained such that there is one single origin of diapause (while allowing other parameters such as trait specific speciation and extinction rates to freely vary) to the model whose only difference is that this constraint is removed (Table S1.3, S1.4).

Habitat scoring

Each species in the phylogeny was scored for type of aquatic habitat in which it typically resides, with three possible character states: permanent, seasonal, and marginal. Permanent water bodies exist year round and do not dry out (i.e. are not seasonal). Seasonal water bodies regularly dry out on an annual basis. Marginal is a catch-all term for aquatic habitats that fall between the extremes of permanent and seasonal. This latter category includes small rivulets, forest pools, swamps, and floodplains (often adjacent to permanent aquatic habitat) that may experience temporal variation in drying. Data were derived from genera-level habitat descriptions from Huber (2000), species level from Wildekamp (2004), supplemented with data available from the Killifish of West Africa (http://www.aka.org/wak/) and It Rains Fishes (http://www.itrainsfishes.net/content/) websites.

Correlation analysis

SIMMAP version 1.5 (Bollback 2006) was used to determine if diapause and habitat covary with each other across the phylogeny. This program implements Bayesian mutational mapping (Huelsenbeck et al. 2003; Nielsen 2002) and estimates two statistics, d_{ij} and m_{ij}, each a measure of covariation between character states *i* and *j*. d_{ij} is a measure of the association (frequency of occurrence on the phylogeny) between the individual character state i and j. m_{ij} is a statistic similar to the mutual information content statistic and evaluates the correlation between character states i and j as the fraction of time one state is associated with another.

As input for the correlation analysis I used the full taxa RAxML tree and my character matrix (Table S1.9) in which each species was scored for presence or absence of diapause II, and habitat (permanent, marginal, seasonal). The substitution rate for each of the two standard characters is modeled with a gamma distribution governed by parameters α and β. For two-state characters, a bias parameter, described by the single α parameter is also required. Priors on these parameter estimates were estimated using the two-step procedure recommended by Bollback (2006). Briefly, MCMC analyses with default settings (100,000 cycles, sampling frequency=200, 10% burnin, rate upper bound = 1000) were used to sample parameter space after which this output file was analyzed in R using the sumprmcmc.r script provided with SIMMAP. These analyses resulted in best-fit gamma and beta estimates, which were used as priors for the subsequent correlation analysis (Table S1.5).

The correlation analysis was set up to have an Observed and Predictive sample size of 2000 with prior draw n=1. The predictive samples are used to statistically test the null hypothesis that the characters evolve independently and association between character states is due to chance rather than correlated evolution. The settings and priors used in the correlation analysis are given in Table S1.5.

Measurement of egg metabolic rate (oxygen consumption)

Eggs were obtained through natural spawning activity of *Nothobranchius furzeri* males and females held in stock tanks. The spawning substrate consisted of a plastic container (spawning tray) filled with approximately one cm of fine-grained river sand. When eggs were to be collected, this container was removed and the sand sifted through a metal screen. Eggs, which remained on the screen, were collected with a plastic pipette, rinsed in distilled water, and stored in Yamamoto's embryo medium.

Over the course of several months, *Nothobranchius furzeri* eggs were collected daily ensuring that, because fertilization occurred within a 24-hour window, eggs were of known age (days post-fertilization) and a relatively tight range of developmental stages. Batches of eggs were incubated at 20°C in dishes containing Yamamoto's solution. At least twice per week, unviable embryos were removed and incubation medium was changed. Prior to use in oxygen consumption trials, embryos in a given batch were examined under a microscope so that developmental stage could be assayed (Wourms 1972a). Given incubation conditions some embryos followed the diapause pathway while others were direct-developing. Beginning 14 days post-fertilization (~20 somite stage) each batch of eggs was sub-divided into separate groupings on the basis of developmental trajectory (diapause vs. direct-developing). Trajectory was determined through microscopic examination of each egg; an open circulatory system and relatively large head is characteristic of direct-developing embryos, and an under-developed circulatory system and reduced head size characteristic of embryos on the diapause pathway (Figure 1.2). Prior to day 14, developmental pathway could not be determined, and all eggs were categorized as undifferentiated. Oxygen consumption was measured on batches of 8 - 96 embryos (37.0 +/- 2.0 SE) 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 ul 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 one minute intervals. Trials lasted 40 minutes or until the percentage of oxygen in the chamber reached 50%. After trial completion, each batch of eggs was pipetted onto a Kimwipe, gently blotted, and weighed to the nearest 0.1 mg. New air-saturated incubation medium was used for each trial. Electrode maintenance, including replacing the membrane and rechloriding the surface layer were performed as necessary according to manufacturer's instruction.

For each trial, percent 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 sec^{-1} $embryo^{-1}$) accounting for the volume of the respirometry chamber, the solubility of dissolved oxygen in Yamamoto's solution, and the number of embryos in the trial.

Supplementary information on results and interpretation

Phylogeny and ancestral state reconstructions

The three families that make up the Aplocheiloidei killifish - the South American Rivuluidae, African Nothobranchidae, and Aplocheilidae of Madagascar, Seychelles, and South Asia - each came out as well supported monophyletic groups (Figure S1.6). Furthermore, the sub-Order Aplocheiloidei came out as a well supported monophyletic clade within Cyprinodontiformes. Both maximum likelihood and parsimony ancestral state reconstructions support multiple independent origins of diapause within Aplocheiloidei killifish (Figure S1.6). The two equally parsimonious reconstructions, assuming equal weighting toward gains and losses of the trait, are either nine independent origins or seven origins and two losses, respectively. Maximum likelihood reconstructions indicate six statistically significant independent origins of

diapause. In both likelihood and parsimony reconstructions uncertainty was involved in ancestral states of the genus *Fundulopanchax* and the clade composed of *Fundulopanchax* and *Nothobranchius*. This is due to the fact that the genus *Fundulopanchax* contains species with and without diapause and has a more complicated pattern of diapause evolution than is apparent in other groups. Furthermore, *Fundulopanchax* species, native to Central and Western Africa, live in forest pools and habitats that may periodically dry, and several of these species appear intermediate between true annual species, with diapause, and those that exhibit typical teleost development patterns. There are even species in this genus, for example *Fundulopachax gardneri*, that exhibit intraspecific variation in the ability to express diapause II (Dobroruka 1990; Hrbek and Larson 1999; Kroll 1984; Peters 1963; Wourms 1972b). *Fundulopanchax gardneri* did not express diapause II under our, Kroll's (1984), or Wourms' (1972b) laboratory conditions but did in Dobroruka's (1990) and Peters' (1963) study. Different populations of *Fundulopanchax gardneri* are likely polymorphic for presence or absence of diapause II - perhaps exhibiting fine scale adaptation to environmental conditions (Hrbek and Larson 1999), or alternatively diapause II might be expressed under a narrow range of environmental conditions only present in some studies. Despite the challenges of diapause character scoring in this transitional development group the general conclusions regarding at least six independent origins of diapause across Aplocheiloidei killifish are robust to alternative forms of character scoring in the genus *Fundulopanchax*.

Within the Aplocheiloidei killifish there are currently 637 valid species, of which 159, or 25.0% have been described within the past 10 years (Eschmeyer and Fricke 2012). As the development characteristics and habitat of these species are described more instances of diapause II within otherwise non-annual clades may well be found; this would strengthen the argument for

multiple independent origins of diapause and further solidify the contention that diapause and an annual life history "is evolutionarily more plastic than once thought" (Murphy et al. 1999a).

Diversitree

AIC and lnLik (by means of Chi-square test) were compared between the full model which allowed for multiple transitions between character states versus a model constrained such that diapause II evolved once at the base of the tree. This later scenario requires numerous losses of diapause II. In all eight analyses the full model performed significantly better (lnLik $P<0.000000000000001$), providing support for multiple independent origins (Table S1.4).

Correlation analysis

Table S1.6 shows the results of the correlation analysis between presence or absence of diapause II and habitat. The presence of diapause was positively associated with inhabitation of seasonal environments ($P<0.000001$).

Taxonomic notes

Nothobranchiidae.

The genus *Episemion* came out nested within *Aphyosemion*, although the nodes placing it in this position have weak bootstrap support. *Epiplatys maeseni* came out strongly placed within the newly erected genus *Nimbapanchax* (Sonnenberg and Busch 2009).

Rivulidae.

The genus *Rachovia* and *Moema* each came out paraphyletic. The species *Plesiolebias aruana* and *Plesiolebias glaucopterus* came out in very disparate areas of the tree, but each well-supported (raising the possibility that one of these species was misidentified or the genus is in need of taxomonic revision). *Simpsonichthys myersi* and *Nematolebias whitei* came out as well-supported sister taxa distinct from the otherwise monophyletic genus *Simpsonichthys*. Based upon the larger molecular data set analyzed here, Costa's (2011) revision of *Rivulus* and elevation of

subgenera to generic level status appears to be justified. *Laimosemion*, *Cynodonichthys*, *Atlantirivulus*, *Melanorivulus*, and *Anablepsoides* represent well-supported monophyletic clades. However, the relationship between these clades and the placement of the Plesiolebiatini clade of annual killifish (represented in the tree by the species *Maratecoara formosa, Maratecoara lacortei, Pituna poranga, Papiliolebias bitteri,* and *Plesiolebias aruana*) remains largely unknown. Here, the Plesiolebiatini clade is sister to *Atlantirivulus* but this relationship has very weak bootstrap support and in general this clade is unstable with respect to position, as in the original phylogenetic analyses including these species (Hrbek and Larson 1999). Given additional molecular data it is possible the Plesiolebiatini clade could shift position and become sister to the well-supported annual clade composed of the genera (*Austrofundulus, Rachovia, Micromoema, Gnatholebias, Llanolebias, Renova, Terranatos, Neofundulus, Pterolebias, Trigonectes, Aphyolebias,* and *Moema*), thus reducing the number of independent origins of diapuase by one.

Divergence along alternative developmental pathways

I quantified embryo head morphology and heart rate as a function of developmental trajectory in representative species from several clades that the ancestral state reconstructions indicate evolved diapause independently (Figure S1.9). In addition, I measured the same set of traits on closely related species that lack diapause (Figure S1.10). In the annual species *Nothobranchius furzeri*, *Nothobranchius korthausae*, *Austrofundulus leohgnei*, *Austrolebias nigripinnis*, and *Rivulus (Laimosemion) tecminae* embryos destined to enter diapause II became conspicuously different in appearance from direct-developing embryos - and this divergence began very early in development, during the formation of the primary embryonic axis, well before diapause was entered. Direct-developing embryos exhibited a significantly faster rate of head growth and higher heart rate relative to embryos destined to enter diapause (trajectory x somite interaction, all $P<0.0001$; Table S1.7).

I was only able to obtain diapause embryos from *Callopanchax occidentalis* despite attempting to induce direct-development by varying incubation conditions. It has been indicated that diapause II is an obligate feature of development for this genus (Murphy and Collier 1997; Simpson 1979). However, a myriad of factors are known to influence developmental trajectory in the embryos of annual killifish (Podrabsky et al. 2010b) and it is possible that embryos of this species just were not particularly sensitive to the range of cues manipulated (temperature and light level) but perhaps are to other cues. Measurements were made on diapause embryos of *Callopanchax occidentalis* and they exhibited a head size and heart rate trajectory similar to that of diapause embryos from other species. That is, the relationship between pairs of somites and the measured variables was flat, indicating that head size remained relatively constant leading up to diapause entry (Figure S1.9).

Embryos of the five representative non-annual species that were examined did not have diapause II. Rather such embryos exhibited a single pathway characterized by continuous development that appeared equivalent to the direct-developing pathway found in annual killfish. Embryo heart rate, head width, and head length increased linearly as a function of stage of development, in contrast to the flat-line pattern seen preceding diapause in embryos of annual species (Figure S1.10).

Determining developmental pathway was complicated in *Fundulopanchax deltaensis*. Given incubation conditions nearly all *Fundulopanchax deltaensis* embryos exhibited an arrest of development around the 38 somite stage. This arrest, after which development was resumed, was often very brief, making it difficult to say with certainty whether diapause II was entered, or if such embryos were actually direct-developing. In an attempt to account for this uncertainty I analyzed a full and reduced data set. In the full data set embryos were scored as 'diapause trajectory' if they exhibited an arrest in development (regardless of length), and in the reduced

data set embryos with ambiguous trajectories (those that apparently entered diapause for only a few days) were excluded. Nonetheless, *Fundulopanchax deltaensis* embryos following the alternative developmental trajectories did not exhibit strong morphological divergence. Apparent direct-developing embryos had slightly larger head size than those following the diapause trajectory - an effect that was more pronounced in the reduced data set (Figure S1.11, Table S1.7).

Fundulopanchax deltaensis is native to a region that has a relatively long wet season and mild seasonality (Table S1.8) which was initially taken to suggest that divergence along alternative developmental pathways may evolve after the evolution of diapause and is tied to the ecology of the species, with less prominent divergence being associated with short periods of diapause II. However, further consideration of the data in a comparative context allows for an alternative explanation. All *Fundulopanchax deltaensis* embryos likely entered diapause II around the 38 somite stage, albeit in some cases for a very short period of time. By this interpretation, embryos that immediately escaped from a very brief hiatus in diapause II, and continued development, were scored as direct-developing (even in the reduced data set) whereas those that remained in diapause II for a longer period were scored as following the diapause trajectory. Consistent with this interpretation, even the supposed direct-developing embryos of *Fundulopanchax deltaensis* exhibit a relatively flat relationship between somite number and head size (Figure S1.11), unlike the strong positive relationship exhibited by direct-developing embryos of every other species (Figure S1.9, S1.10). Furthermore, strong embryonic divergence in head size (driven by supposed direct-developing embryos showing a dramatic increase) began to occur around the 38 somite stage (when counting somites could no longer be used as a way of accurately scoring development). This is consistent with such embryos having exited diapause II and thus beginning to strongly diverge in head size relative to embryos remaining in this state of

arrest. If the above interpretation is correct, which I now favor, then it appears embryos that spent a brief period in diapause II exhibit slightly more robust head morphologies early in development relative to embryos that remain in diapause for a longer period (Figure S1.11).

There were subtle differences among species in the stage at which development was arrested. In *Rivulus tecminae*, the diapause II arrest consistently occurred around the 42 somite stage. In *Austrolebias nigripinnis* arrest occurred at the 32-34 somite stage. In the other species with diapause, arrest occurred when the embryo reached mid to upper 30s somite pair number. The evolutionary significance of this variation is unknown.

Metabolic rate

Direct-developing and diapause embryos of *Nothboranchius furzeri* exhibited divergence in rate of oxygen consumption that largely mirrored the divergence seen in head size and heart rate (Figure 1.4).

Long-term embryo survival

The survival distributions for direct-developing and diapause embryos were significantly different (Kaplan-Meier method, Mantel-Cox Test, $\chi^2 = 331.3$, $P<0.00001$). Specifically, *Nothobranchius furzeri* embryos following the diapause II developmental pathway (n=321) exhibit longer survival time than embryos following the direct-developing pathway (n=387).

Supplementary Figures

Figure S1.6. Maximum likelihood supermatrix phylogeny with 311 terminal taxa. Numbers on branches are bootstrap support values. Color coding of taxa names on branch tips indicate character states with red being presence of diapause II, and black being absence. Branch colors 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). Scale bar = substitutions per site.


Danio rerio
Plecoglossus altivelis
Chaulíodus sloani
Glossanodon semifasciatus
Esox lucius
Oncorhynchus mykiss
Synodus variegatus
Neoscopelus
Polymixia japonica
Zeus faber
Aphredoderus sayanus
Trachyrincus murrayi
Gadus morhua
Myripristis
Lamprogrammus niger
Sebastes
Lycodes
Gasterosteus aculeatus
Morone
Lutjanus russellii
Lophius
Tetragodon
Takifugu rubripes
Monopterus albus
Hippoglossus hippoglossus
Porichthys
Mugil cephalus
Oreochromis
Astronotus ocellatus
Oryzias latipes
Menidia menidia
Xenotoca eiseni
Cyprinodon rubrofluviatilis
Jordanella floridae
Fundulus olivaceus
Fundulus diaphanus
Fundulus heteroclitus
Fundulus grandis
Anableps anableps
Gambusia affinis
Xiphophorus maculatus
Xiphophorus helleri
Notholebias minimus
Campellolebias chrysolineatus
Campellolebias dorsimaculatus
Leptolebias aureoguttatus
Leptolebias citrinipinnis
Plesiolebias glaucopterus
Cynopoecilus melanotaenia
Nematolebias whitei
Simpsonichthys myersi
Simpsonichthys antenori
Simpsonichthys costai
Simpsonichthys bokermanni
Simpsonichthys magnificus
Simpsonichthys trilineatus
Austrolebias nigripinnis
Austrolebias alexandri
Austrolebias cyaneus
Austrolebias sp. duraznensis
Austrolebias affinis
Austrolebias bellottii
Austrolebias melanoorus
Austrolebias vazferreirai
Austrolebias cinereus
Austrolebias gymnoventris
Austrolebias wolterstorffi
Austrolebias luteoflammulatus
Austrolebias prognathus
Austrolebias cheradophilus
Austrolebias viarius
Austrolebias adloffi
Austrolebias reicherti
Austrolebias nigrofasciatus
Austrolebias charrua
Kryptolebias brasiliensis
Kryptolebias sepia
Kryptolebias caudomarginatus
Kryptolebias ocellatus
Kryptolebias marmoratus
Rivulus roloffi
Rivulus insulaepinorum
Rivulus cylindraceus
Rivulus (Laimosemion) kirovskyi
Rivulus (Laimosemion) rectocaudatus
Rivulus (Laimosemion) sp. Tobogon
Rivulus (Laimosemion) tecminae
Rivulus (Laimosemion) sp. DCT 89-132
Rivulus (Laimosemion) breviceps
Rivulus (Laimosemion) lyricauda
Rivulus (Laimosemion) mahdiaensis
Rivulus (Laimosemion) gransabanae
Rivulus (Laimosemion) xiphidius
Rivulus (Laimosemion) frenatus
Rivulus (Laimosemion) dibaphus
Rivulus (Laimosemion) strigatus
Rivulus (Laimosemion) agilae
Rivulus (Laimosemion) geayi
Rivulus (Cynodonichthys) uroflammeus
Rivulus (Cynodonichthys) hildebrandi
Rivulus (Cynodonichthys) chucunaque
Rivulus (Cynodonichthys) magdalenae
Rivulus (Cynodonichthys) birkhahni
Rivulus (Cynodonichthys) frommi
Rivulus (Cynodonichthys) brunneus
Rivulus (Cynodonichthys) fuscolineatus
Rivulus (Cynodonichthys) isthmensis
Rivulus (Cynodonichthys) tenuis
Rivulus (Cynodonichthys) weberi
Rivulus (Atlantirivulus) luelingi
Rivulus (Atlantirivulus) janeiroensis
Rivulus (Atlantirivulus) haraldsiolii
Rivulus (Atlantirivulus) santensis
Plesiolebias aruana
Papiliolebias bitteri
Pituna poranga
Maratecoara lacortei
Maratecoara formosa
Rivulus (Melanorivulus) violaceus
Rivulus (Melanorivulus) punctatus
Rivulus (Melanorivulus) pictus
Rivulus (Melanorivulus) apiamici
Rivulus (Anablepsoides) iridescens
Rivulus (Anablepsoides) ornatus
Rivulus (Anablepsoides) jucundus
Rivulus (Anablepsoides) ophiomimus
Rivulus (Anablepsoides) rubrolineatus
Rivulus (Anablepsoides) micropus
Rivulus (Anablepsoides) hartii
Rivulus (Anablepsoides) immaculatus
Rivulus (Anablepsoides) amphoreus
Rivulus (Anablepsoides) waimacui
Rivulus (Anablepsoides) stagnatus
Rivulus (Anablepsoides) urophthalmus
Rivulus (Anablepsoides) cryptocallus
Rivulus (Anablepsoides) sp. Rio Supamo
Rivulus (Anablepsoides) deltaphilus
Moema piriana
Moema staecki
Aphyolebias peruensis
Trigonectes rubromarginatus
Trigonectes balzanii
Trigonectes aplocheiloides
Pterolebias phasianus
Pterolebias longipinnis
Neofundulus paraguayensis
Neofundulus sp. paraguay
Neofundulus ornatipinnis
Terranatos dolichopterus
Renova oscari
Llanolebias stellifer
Gnatholebias hoignei
Gnatholebias zonatus
Micromoema xiphophora
Rachovia maculipinnis
Rachovia brevis
Rachovia pyropunctata
Rachovia hummelincki
Austrofundulus leohoignei
Austrofundulus transilis
Austrofundulus rupununi
Austrofundulus guajira
Austrofundulus leoni
Austrofundulus limnaeus
Pachypanchax playfairii
Pachypanchax omalonotus
Aplocheilus panchax
Aplocheilus lineatus
Aplocheilus parvus
Aplocheilus blockii
Epiplatys infrafasciatus
Epiplatys singa
Epiplatys bifasciatus
Epiplatys roloffi
Epiplatys chaperi
Epiplatys sexfasciatus
Epiplatys multifasciatus
Nimbapanchax petersi
Nimbapanchax melanopterygius
Nimbapanchax leucopterygius
Epiplatys maeseni
Nimbapanchax jeanpoli
Nimbapanchax viridis
Archiaphyosemion guineense
Callopanchax monroviae
Callopanchax sidibeorum
Callopanchax toddi
Callopanchax occidentalis
Callopanchax huwaldi
Scriptaphyosemion guignardi
Scriptaphyosemion banforense
Scriptaphyosemion liberiense
Scriptaphyosemion breuningi
Scriptaphyosemion chaytori
Scriptaphyosemion bertholdi
Scriptaphyosemion roloffi
Scriptaphyosemion schmitti
Scriptaphyosemion etzeli
Scriptaphyosemion geryi
Fenerbahce formosus
Fundulopanchax scheeli
Fundulopanchax oeseri
Fundulopanchax ndianus
Fundulopanchax gardneri
Fundulopanchax cinnamomeus
Fundulopanchax mirabilis
Fundulopanchax sjostedti
Fundulopanchax robertsoni
Fundulopanchax walkeri
Fundulopanchax filamentosus
Fundulopanchax gularis
Fundulopanchax deltaense
Fundulopanchax amieti
Fundulopanchax schwoiseri
Fundulopanchax fallax
Pronothobranchius kiyawensis
Nothobranchius thierryi
Nothobranchius ocellatus
Nothobranchius krysanovi
Nothobranchius pienaari
Nothobranchius rachovii
Nothobranchius furzeri
Nothobranchius kuhntae
Nothobranchius orthonotus
Nothobranchius kirki
Nothobranchius rubroreticulatus
Nothobranchius kafuensis
Nothobranchius polli
Nothobranchius guentheri
Nothobranchius eggersi
Nothobranchius kilomberoensis
Nothobranchius cardinalis
Nothobranchius foerschi
Nothobranchius jubbi
Nothobranchius ugandensis
Nothobranchius krammeri
Nothobranchius melanospilus
Nothobranchius lucius
Nothobranchius hengstleri
Nothobranchius makondorum
Foerschichthys flavipinnis
Aphyosemion gabunense
Aphyosemion striatum
Aphyosemion exigoideum
Aphyosemion primigenium
Aphyosemion hera
Aphyosemion louessense
Aphyosemion ogoense
Episemion callipteron
Episemion krystallinoron
Aphyosemion georgiae
Aphyosemion cyanostictum
Aphyosemion bamilekorum
Aphyosemion exiguum
Aphyosemion elberti
Aphyosemion bualanum
Aphyosemion herzogi
Aphyosemion maculatum
Aphyosemion cameronense
Aphyosemion mimbon
Aphyosemion etsamense
Aphyosemion batesii
Aphyosemion coeleste
Aphyosemion aureum
Aphyosemion occellatum
Aphyosemion citrineipinnis
Aphyosemion punctatum
Aphyosemion wildekampi
Aphyosemion labarrei
Aphyosemion congicum
Aphyosemion cognatum
Aphyosemion decorsei
Aphyosemion elegans
Aphyosemion lamberti
Aphyosemion rectogoense
Aphyosemion christyi
Aphyosemion australe
Aphyosemion pascheni
Aphyosemion paschenifestivum
Aphyosemion franzwerneri
Aphyosemion celiae
Aphyosemion calliurum
Aphyosemion heinemanni
Aphyosemion campomaanense
Aphyosemion ahli
Aphyosemion lividum
Aphyosemion edeanum
Aphyosemion sp. LEC 93/27
Aphyosemion alpha
Aphyosemion melinoides
Aphyosemion lugens
Aphyosemion riggenbachi
Aphyosemion sp. Oba
Aphyosemion bitaeniatum
Aphyosemion bivittatum
Aphyosemion kouamense
Aphyosemion erythron
Aphyosemion melanogaster
Aphyosemion ecucuense
Aphyosemion malumbresi
Aphyosemion sp. Rio Muni
Aphyosemion loennbergii
Aphyosemion volcanum
Aphyosemion poliaki
Aphyosemion splendopleure
Aphyosemion sp. 08 RS-2007
Aphyosemion punctulatum
Aphyosemion sp. Bioko
Aphyosemion omega
Aphyosemion sp. Bimbia
Aphyosemion koungueense
0.09

Figure S1.7. Embryo morphological measurements (*Nothobranchius furzeri*). 1 - head width at optic cups. 2 - head width at otic vesicles. 3- head length.

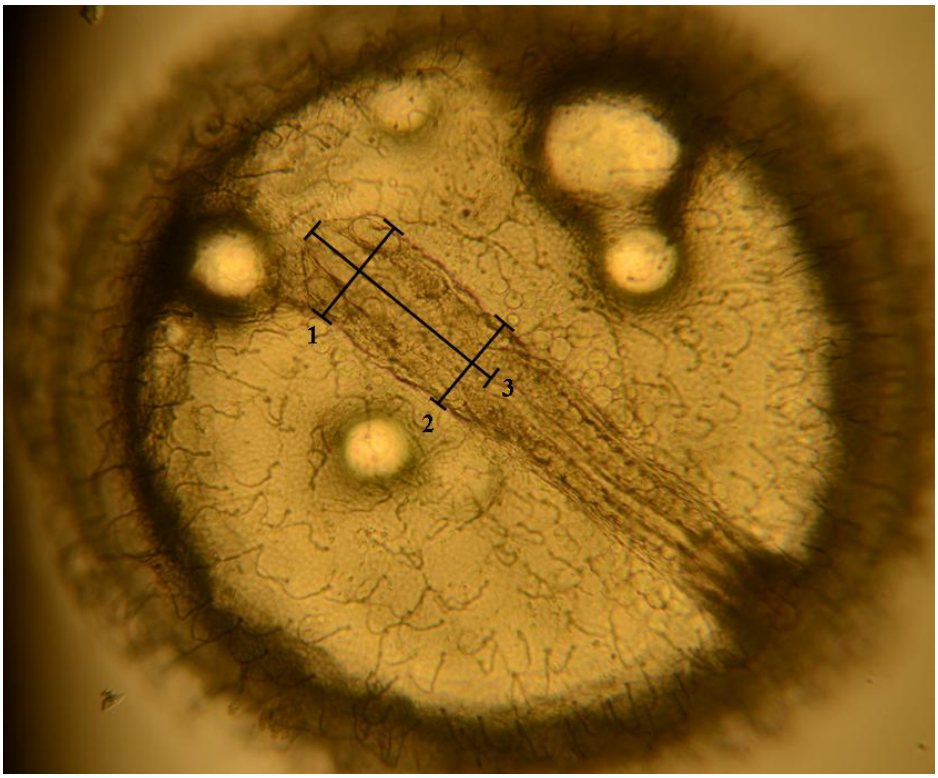


Figure S1.8. Graphs depicting the relationship between somite number and head width at optic cups (first row), head width at otic vesicles (second row), head length (third row), and heart rate (fourth row). The first column depicts direct-developing embryos from all species. The second column depicts direct-developing embryos from non-annual species. The third column depicts direct-developing embryos from annual species. The fourth column depicts diapause embryos from annual species.

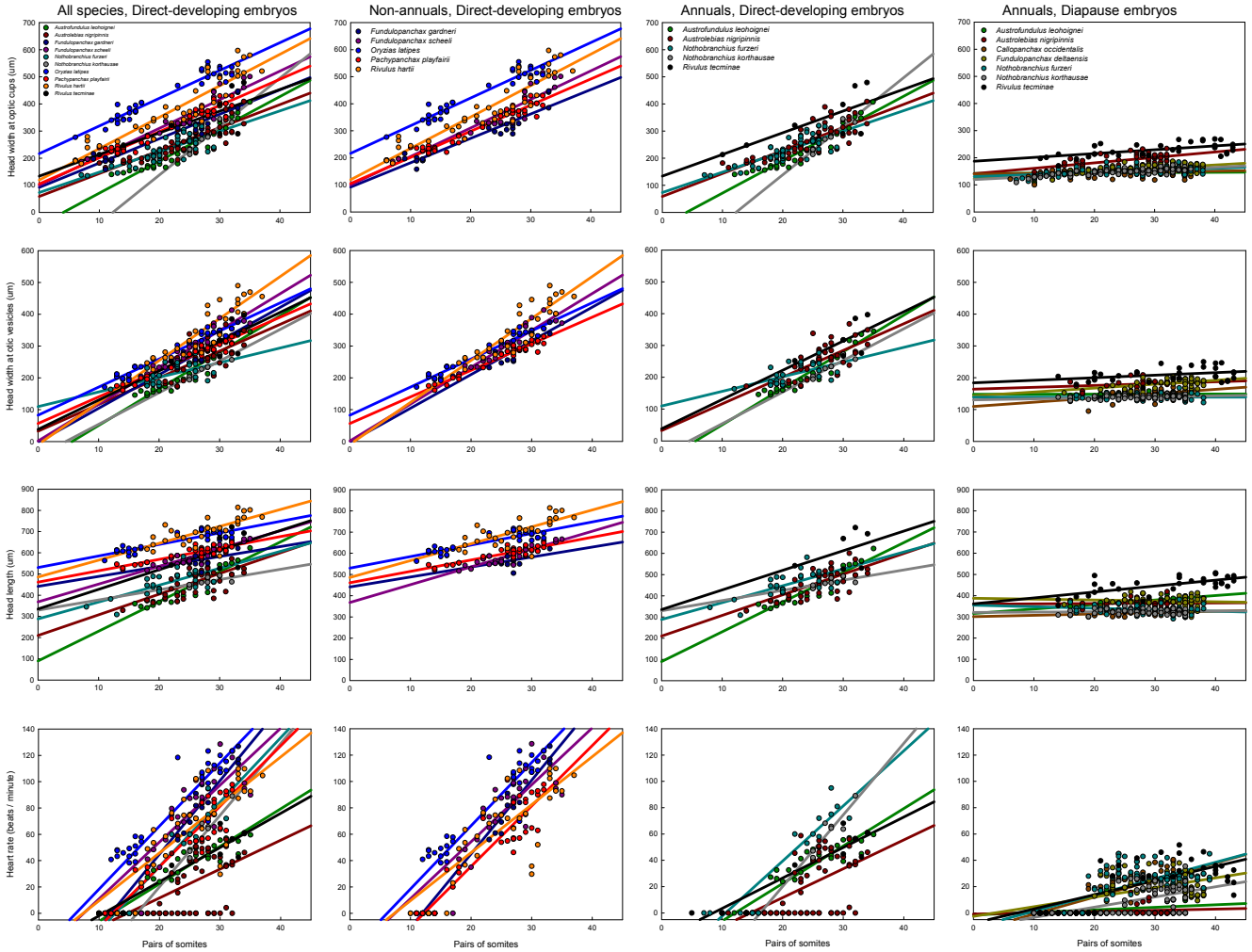


Figure S1.9. Graphs depicting the relationship between somite number and head width at optic cups (upper left panel), head width at otic vesicles (upper right panel), head length (lower left panel), and heart rate (lower right panel) for each annual killifish species.

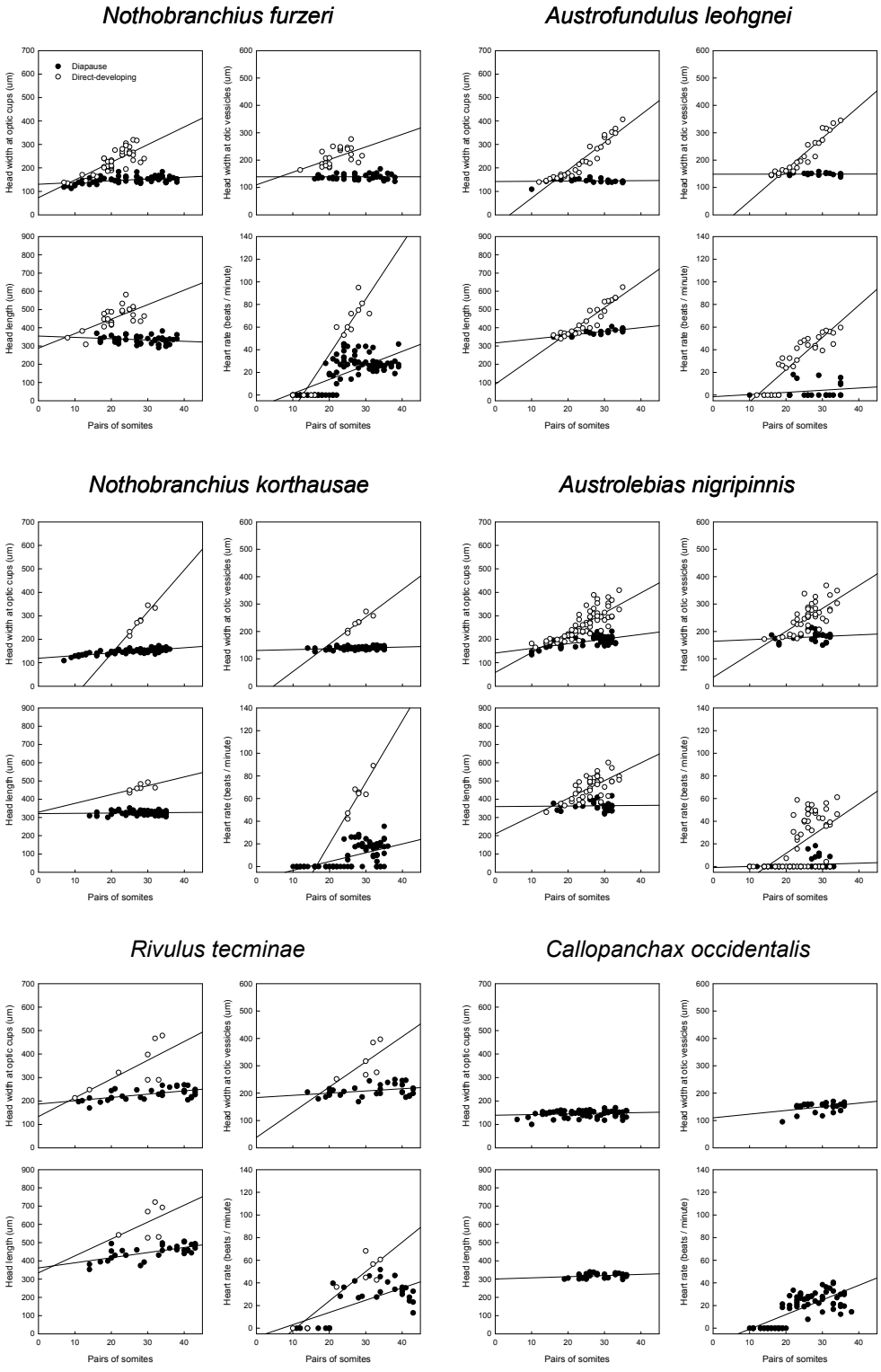


Figure S1.10. Graphs depicting the relationship between somite number and head width at optic cups (upper left panel), head width at otic vesicles (upper right panel), head length (lower left panel), and heart rate (lower right panel) for each non-annual killifish species.

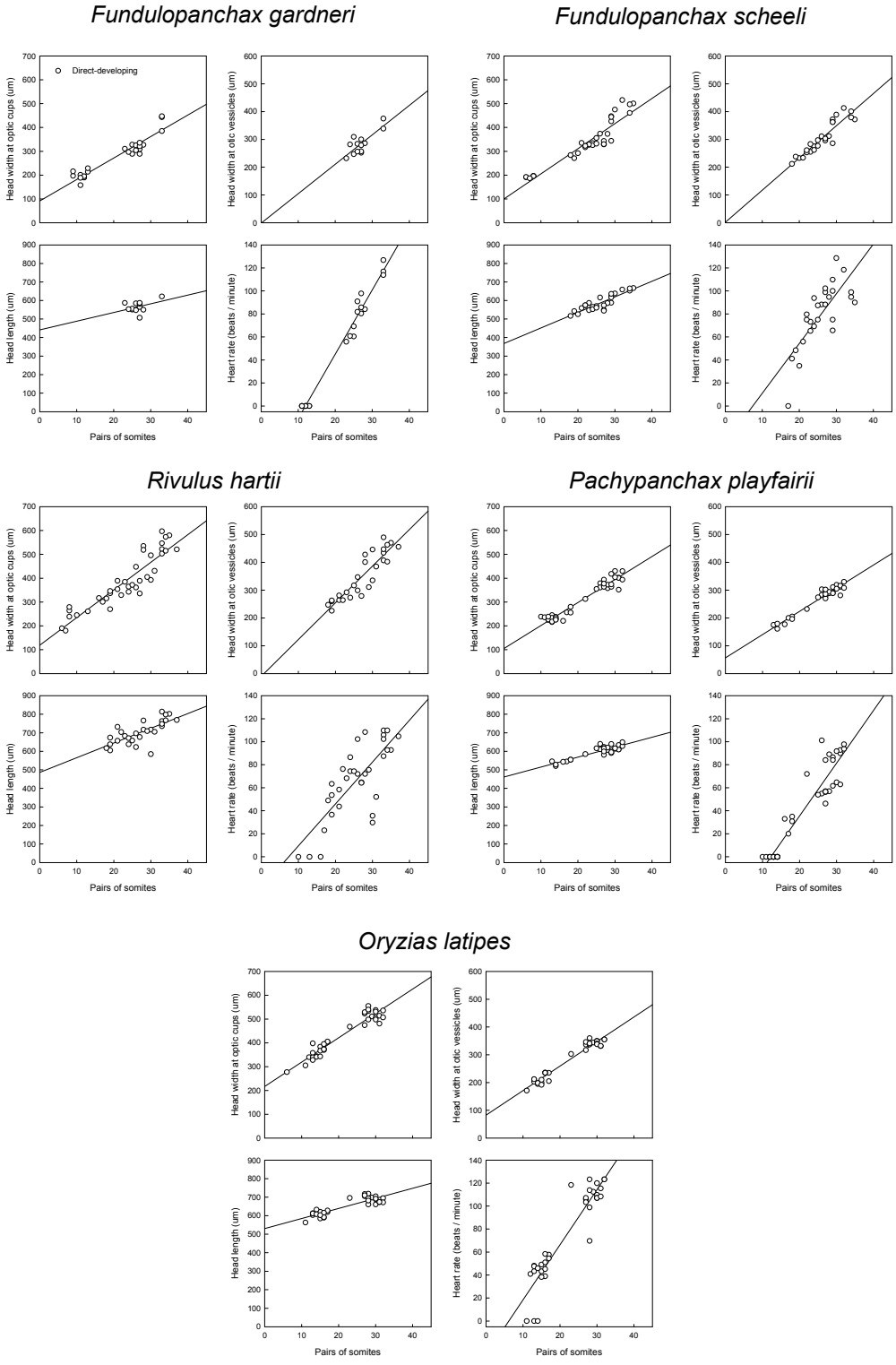
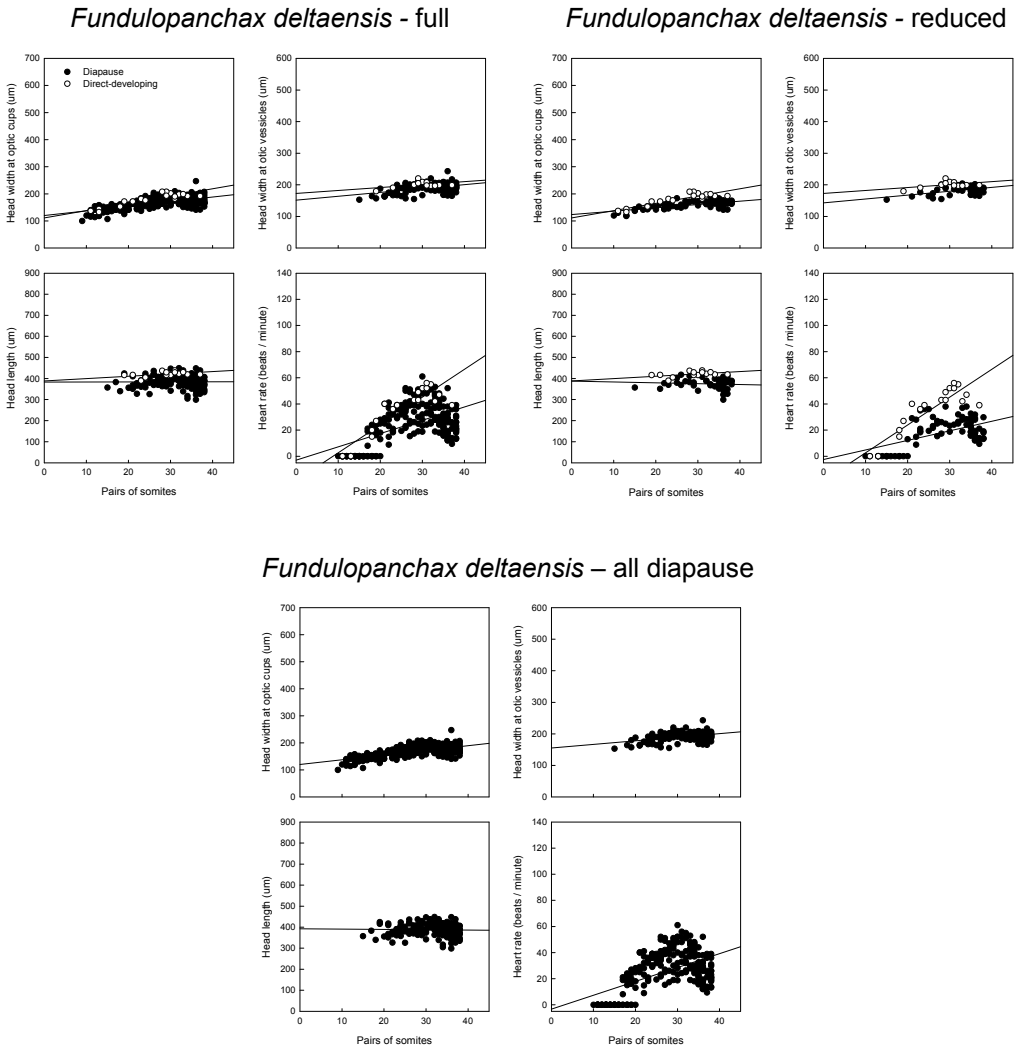


Figure S1.11. Graphs depicting the relationship between somite number and head width at optic cups (upper left panel), head width at otic vesicles (upper right panel), head length (lower left panel), and heart rate (lower right panel) for *Fundulopanchax deltaensis*. Definitively determining whether embryos entered into diapause II or were direct-developing was challenging for this species (see text). The interpretation I favor (lower panel) is that all embryos entered diapause II, albeit in some cases for a very brief period of time before resuming development.



Supplementary Tables

Table S1.1. Models of molecular evolution chosen by jModeltest (Posada 2008) on the basis of Akaike Information Criterion.

Partition	Model
28S	GTR+G
Cox1	GTR+G
Cyt b	GTR+G
ND1	GTR+G
ND2	GTR+G
Rag1	TIM2+G
12S+16S	TIM2+G
D loop	TPM3uf+G
tRNAs (Val-Ile-Gln-Met-Trp-Ala-Asn-Cys-Tyr)	TPM2uf+G

Table S1.2. Minimum and maximum constraints used in molecular dating (timetree) analyses.

Fossil taxa	Node in tree	Minimum age (Ma)	Maximum age (Ma)
Esociformes + Salmoniformes	(Esox lucius + Oncorhynchus mykiss)	70.0	126.0
Neoteleostei	(Polymixia japonica + (Zeus faber + Aphredoderus sayanus))	124.0	154.8
Macrouridae to Gadidae (in Gadiformes)	(Trachyrincus murrayi + Gadus morhua)	58.5	131.5
Zoarcoidea + Gasterosteiformes	(Lycodes + Gasterosteus aculeatus)	13.0	84.2
Moronidae + Lutjanidae	(Morone + Lutjanus russellii)	47.0	84.2
Tetraodontiformes + Lophiiformes	(Lophius + (Tetraodon + Takifugu rubripes))	96.9	126.0
Tetraodontidae	(Tetraodon + Takifugu rubripes)	32.3	56.0
Cichlidae	(Oreochromis + Astronotus ocellatus)	40.2	100.5
Poeciliidae + Anablepidae	(Anableps anableps + (Gambusia affinis (Xiphophorus maculatus + Xiphophorus hellerii)))	39.9	71.2
Otocephala = Ostarioclupeomorpha (Ostariophysi + Clupeomorpha)	Root of tree	149.9	165.2

Table S1.3. Summary of BiSSE models in diversitree that are relevant to testing the hypothesis of multiple (full model) versus single (trait of interest evolved once) origins of diapause. λ = speciation rate, μ = extinction rate, q = transition between states; state 0 = no diapause II, state 1 = diapause II present.

Model	Df	Estimated Parameters	Constraints
Full	6	λ1, λ0, μ1, μ0, q01, q10	none
Trait of interest evolved once	5	λ1, λ0, μ1, μ0, q10	q01 = 0

Table S1.4. AIC and lnLik comparison of full model and model where diapause was constrained to have evolved once (single origin) for each of the four trees. Analyses were performed in diversitree. Auto / IR and Hard / Soft refers to autocorrelated and independent rates with either hard or soft bounded constraints.

	Full model		Trait of interest evolved once			
Data set / Tree	**lnLik**	**AIC**	**lnLik**	**AIC**	**ChiSq**	**Pr(>|Chi|)**
Incomplete Sampling Set Hard Auto	64.733	-117.466	34.011	-58.022	61.444	<0.0001
Incomplete Sampling Set Hard IR	21.361	-30.723	-13.566	37.131	69.854	<0.0001
Incomplete Sampling Set Soft Auto	64.979	-117.958	33.827	-57.653	62.305	<0.0001
Incomplete Sampling Set Soft IR	24.995	-37.991	-10.027	30.055	70.046	<0.0001
Sampling Fraction Hard Auto	77.366	-142.731	45.856	-81.711	63.02	<0.0001
Sampling Fraction Hard IR	33.337	-54.675	-1.921	13.843	70.518	<0.0001
Sampling Fraction Soft IR	37.212	-62.424	2.005	5.991	70.415	<0.0001
Sampling Fraction Soft Auto	77.222	-142.444	45.227	-80.454	63.99	<0.0001

Table S1.5. Settings and priors used in the correlation analysis performed in SIMMAP 1.5 (Bollback 2006).

Character 1: presence / absence of Diapause II (2 states)
Bias parameter: $\alpha = 4.3890$, $k = 31$ Rate parameter: Gamma distribution prior, $\alpha = 4.0070$, $\beta = 0.5080$, $k = 100$ Rescale tree length State ordering: unordered
Character 2: habitat (3 states)
Bias parameter: Empirical prior Rate parameter: Gamma distribution prior, $\alpha = 7.9670$, $\beta = 0.9870$, $k = 100$ Rescale tree length State ordering: linear

Table S1.6. Correlation results for diapause and habitat.

Statistic	Description	Observed Value	*P*-Value	Observed sample size	Predictive sample size
M		0.53694	0.000000	2000	2000
m(0,0)	m(no diapause, permanent)	0.139743	0.004000	2000	2000
m(0,1)	m(no diapause, marginal)	0.12533	0.001000	2000	2000
m(0,2)	m(no diapause, seasonal)	-0.017191	0.001000	2000	2000
m(1,0)	m(diapause, permanent)	-0.011012	0.088000	2000	2000
m(1,1)	m(diapause, marginal)	-0.037711	0.003500	2000	2000
m(1,2)	m(diapause, seasonal)	0.33778	0.000000	2000	2000
D		0.862942	0.000000	2000	2000
d(0,0)	d(no diapause, permanent)	0.112387	0.006500	2000	2000
d(0,1)	d(no diapause, marginal)	0.103348	0.000500	2000	2000
d(0,2)	d(no diapause, seasonal)	-0.215736	0.000000	2000	2000
d(1,0)	d(diapause, permanent)	-0.112388	0.006500	2000	2000
d(1,1)	d(diapause, marginal)	-0.103348	0.000500	2000	2000
d(1,2)	d(diapause, seasonal)	0.215735	0.000000	2000	2000

Values of m_{ij} and d_{ij} are positive when given character states occur together more frequently than expected by chance (i.e. the null).

Table S1.7. Linear mixed models implemented in the R (R Core Team 2013) package *nlme* (Pinheiro et al. 2010) were used 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 data set). To account for this non-independence (Bolker et al. 2009) random effects for egg identity (individual intercept and slope allowed to vary) were included. Here I report the fixed effect parameter estimates produced by restricted maximum likelihood estimation. In cases where the full-model failed to converge due to overparameterization, the random slope effect (~Somites|Subject) was excluded, and the model rerun with only random intercepts (~1|Subject).

	Head width at optic cups			Head width at otic vessicles			Head length			Heart rate		
	Estimate	SE	*t*	Estimate	SE	*t*	Estimate	SE	*t*	Estimate	SE	*t*
Austrofundulus leohgnei												
Fixed effects												
Intercept	142.13	15.82	8.98***	148.17	16.94	8.75***	316.16	24.81	12.74***	-1.48	6.16	-0.24
Trajectory	-189.22	21.77	-8.69**	-212.88	22.99	-9.259**	-226.02	33.21	-6.81**	-32.98	8.47	-3.89*
Somites	0.097	0.58	0.17	0.014	0.60	0.024	2.1159	0.8738	2.421*	0.19	0.23	0.85
Trajectory x Somites	11.76	0.84	14.00***	11.49	0.86	13.42***	11.90	1.23	9.70***	2.65	0.37	8.11***
Random effects	~1|Subject			~Somites|Subject			~1|Subject			~Somites|Subject		
Nothobranchius furzeri												
Fixed effects												
Intercept	128.94	7.80	16.53***	138.58	9.77	14.18***	366.63	16.13	22.72***	-10.99	3.64	-3.02**
Trajectory	-42.95	14.27	-3.01**	-65.82	19.26	-3.42**	-74.63	25.3	-2.95**	-51.01	11.48	-4.44***
Somites	0.81	0.24	3.42**	0.014	0.29	0.046	-1.02	0.50	-2.05*	1.24	0.14	8.95***
Trajectory x Somites	6.21	0.58	10.74***	6.14	0.78	7.89***	8.98	0.97	9.24***	3.65	0.48	7.54***
Random effects	~1|Subject			~Somites|Subject			~Somites|Subject			~Somites|Subject		
Nothobranchius korthausae												
Fixed effects												
Intercept	120.38	3.15	38.23***	132.77	4.13	32.15***	321.91	9.10	35.36***	-11.05	3.61	-3.06**
Trajectory	-377.89	33.35	-11.33***	-204.83	24.38	-8.40***	17.79	48.06	0.37	-76.79	37.71	-2.036+
Somites	1.069	0.14	7.89***	0.23	0.13	1.81+	0.11	0.26	0.43	0.78	0.13	5.91***
Trajectory x Somites	18.17	1.20	15.11***	10.66	0.86	12.40***	4.31	1.67	2.58*	4.64	1.35	3.44***
Random effects	~Somites|Subject			~Somites|Subject			~Somites|Subject			~1|Subject		
Fundulopanchax deltaensis (full)												
Fixed effects												
Intercept	115.84	2.88	40.22***	140.68	6.73	20.91***	351.27	9.72	36.14***	-6.42	2.83	-2.27*
Trajectory	-7.83	9.60	-0.81	22.63	23.39	0.97	40.14	28.99	1.38	-12.91	9.49	-1.36
Somites	1.85	0.12	15.25***	1.55	0.22	7.22***	0.91	0.26	3.51***	1.14	0.088	12.92***
Trajectory x Somites	1.00	0.42	2.37*	-0.26	0.79	-0.33	0.050	0.83	0.060	1.02	0.32	3.19**
Random effects	~Somites|Subject			~Somites|Subject			~1|Subject			~1|Subject		
Fundulopanchax deltaensis (reduced)												
Fixed effects												
Intercept	121.19	4.30	28.17***	115.40	9.34	12.36***	387.84	18.39	21.09***	-4.63	3.58	-1.29
Trajectory	-11.43	8.89	-1.29	50.84	17.95	2.83*	-1.82	32.74	-0.055	-14.84	7.57	-1.96+
Somites	1.34	0.12	10.78***	2.07	0.26	7.83***	-0.31	0.54	-0.58	0.81	0.13	6.42***
Trajectory x Somites	1.42	0.28	5.05***	-0.89	0.55	-1.63	1.47	1.02	1.43	1.36	0.296	4.58***
Random effects	~1|Subject			~1|Subject			~Somites|Subject			~Somites|Subject		
Austrolebias nigripinnis												
Fixed effects												
Intercept	143.74	14.36	10.01***	155.15	24.36	6.37***	327.78	32.86	9.97***	-1.01	7.97	-0.13
Trajectory	-112.44	20.13	-5.585***	-188.35	35.11	-5.36***	-205.84	46.89	-4.39***	-32.84	11.34	-2.90**
Somites	1.85	0.72	2.56*	0.81	0.93	0.87	1.18	1.093	1.080	0.10	0.30	0.33
Trajectory x Somites	7.76	0.98	7.91***	10.11	1.35	7.49***	11.89	1.63	7.32***	2.19	0.44	5.00***
Random effects	~Somites|Subject			~Somites|Subject			~1|Subject			~1|Subject		
Rivulus tecminae												
Fixed effects												
Intercept	192.81	14.58	13.22***	188.06	10.65	17.66***	392.27	21.07	18.62***	-5.86	7.00	-0.84
Trajectory	-82.65	35.90	-2.30*	-178.42	31.32	-5.70***	-118.47	68.31	-1.73	-22.67	15.95	-1.42
Somites	1.066	0.62	1.73+	0.41	0.49	0.83	1.86	0.55	3.38**	0.94	0.22	4.26***
Trajectory x Somites	8.16	1.41	5.80***	9.98	1.23	8.12***	9.96	2.06	4.83***	1.71	0.54	3.13**
Random effects	~Somites|Subject			~Somites|Subject			~1|Subject			~1|Subject		
Callopanchax occidentalis												
Fixed effects												
Intercept	136.34	5.41	25.22***	119.30	10.47	11.40***	301.86	13.46	22.43***	-14.15	3.50	-4.04***
Somites	0.37	0.22	1.66	0.98	0.31	3.20**	0.61	0.47	1.30	1.29	0.13	10.00***
Random effects	~Somites|Subject			~1|Subject			~Somites|Subject			~1|Subject		
Fundulopanchax gardneri												
Fixed effects												
Intercept	102.29	10.27	9.96***	17.62	29.30	0.60	441.46	88.68	4.98***	-67.68	3.69	-18.36***
Somites	8.35	0.54	15.51***	9.80	1.064	9.21+	4.70	3.33	1.41	5.63	0.16	35.68***
Random effects	~Somites|Subject			~1|Subject			n/s			~1|Subject		
Fundulopanchax scheeli												
Fixed effects												
Intercept	99.69	18.39	5.42***	-6.95	22.68	-0.31	363.29	19.67	18.47***	-32.21	18.78	-1.71
Somites	10.54	0.75	14.066***	11.87	0.85	14.02***	8.49	0.73	11.67***	4.32	0.71	6.06***
Random effects	~1|Subject			~1|Subject			~Somites|Subject			~1|Subject		
Oryzias latipes												
Fixed effects												
Intercept	211.98	7.39	28.70***	82.63	8.32	9.93***	534.01	10.78	49.52***	-29.64	8.13	-3.64**
Somites	10.48	0.39	26.86***	8.85	0.36	24.83***	5.36	0.43	12.56***	4.80	0.35	13.54***
Random effects	~Somites|Subject			~Somites|Subject			~1|Subject			~1|Subject		
Pachypanchax playfairii												
Fixed effects												
Intercept	103.84	8.48	12.24***	56.42	10.84	5.20***	456.11	11.44	39.86***	-56.17	5.82	-9.65***
Somites	9.70	0.37	26.58***	8.36	0.42	19.91***	5.57	0.43	13.01***	4.59	0.25	18.06***
Random effects	~1|Subject			~1|Subject			~1|Subject			~1|Subject		
Rivulus hartii												
Fixed effects												
Intercept	113.08	21.07	5.37***	-9.49	35.09	-0.27	475.18	32.59	14.58***	-25.34	12.59	-2.01+
Somites	11.82	0.84	14.073***	13.20	1.26	10.50***	8.30	1.15	7.23***	3.62	0.47	7.71***
Random effects	~1|Subject			~Somites|Subject			~Somites|Subject			~1|Subject		

***P <0.001; **P <0.01; *P <0.05; +P <0.1.

Table S1.8. Summary of killifish species habitat data. All environmental variables derived from New LocClim 1.10, Local Climate Estimator, Food and Agriculture Organization of the United Nations. Length of growing season = Days per year when Precipitation / Potential Evapotranspiration (PET) > 0.5. Aridity Index = Ratio of annual precipitation to annual PET. Precipitation deficit = Amount of annual rain missing to fully compensate PET, where positive values mean water deficit and negative values water surplus.

Species	Locality	Collection code	Collection year	Latitude	Longitude	Length of growing season (days)	Growing season	Aridity index	Precipitation Deficit (mm/year)	Mean annual precipitation (mm/year)	Budyko Climate Class
Nothobranchius furzeri	Near Chefu river on Mozambique / Zimbabwe border	ZMZ 10-01	2010	-21.816	31.931	107	23 Nov - 9 Mar	0.33	1007	493	Semiarid
Austrfundulus leohoignei	3.2 km from town of Sanare, Venezuela	VEN 09/2	2009	9.740	-69.650	127, 81	13 Apr - 17 Aug, 14 Sep - 3 Dec	0.49	822	791	Steppe
Austrolebias nigripinnis	Near town of Villa Soriano, Uruguay	Villa Soriano	~2010	-33.401	-58.320	291	13 Feb - 30 Nov	0.75	299	914	Steppe
Nothobranchius korthausae	Mafia Island, Tanzania	Kiziko TAN 05-47	2005	-7.859	39.797	245	26 Oct - 27 Jun	1.18	-281	1877	Forest
Fundulopanchax deltaensis	Niger Delta, Nigeria	-	-	5.110	6.168	298	9 Feb - 3 Dec	2.04	-1309	2567	Forest
Callopanchax occidentalis (cf. *toddi*)	Kabak, Guinea	GM 08/13	2008	9.333	-13.400	203	4 May - 22 Nov	2.75	-2406	3777	Forest
Fundulopanchax gardneri gardneri	Telemu, Nigeria	NTC 07-3	2007	9.356	4.093	217	30 March - 1 Nov	0.76	366	1184	Steppe
Fundulopanchax scheeli	-	-	-	7.658	7.602	231	25 March - 10 Nov	0.95	65	1385	Steppe
Rivulus hartii	Naranjo River, Trinidad	-	2009	10.610	-61.235	254	2 May - 10 Jan	1.4	-533	1876	Forest
Pachypanchax playfairii	-	-	-	-4.762	55.387	365	-	1.38	-594	2172	Forest
Rivulus tecminae	Macuruco, Venezuela	IVE 12-09	2012	3.929	-67.042	274	17 March - 15 December	1.77	-988	2269	Forest
Oryzias latipes	-	-	-	34.390	136.532	365	-	2.4	-1366	2340	Forest

Table S1.9. Taxon sampling, character file, and accession numbers.

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
1	*Anableps anableps*	0	Permanent	EF017456	EF017456			AF449341	AF449341	AF449341
2	*Aphredoderus sayanus*	0	Permanent	NC_004372	NC_004372	NC_004372	NC_004372	NC_004372	NC_004372	NC_004372
3	*Aphyolebias peruensis*	1	Seasonal	AF092340	AF092340		AF002569		AF092407	AF092407
4	*Aphyosemion ahli*	0	Marginal	DQ278267						EF417000
5	*Aphyosemion alpha*	0	Marginal	DQ278418						
6	*Aphyosemion aureum*	0	Marginal	AF002385						
7	*Aphyosemion australe*	0	Marginal	AF002367			AAU73245			EF417018
8	*Aphyosemion bamilekorum*	0	Marginal							
9	*Aphyosemion batesii*	1	Marginal	AF002350						
10	*Aphyosemion bitaeniatum*	0	Marginal	DQ278264						
11	*Aphyosemion bivittatum*	0	Marginal	DQ278348			ABU73246			
12	*Aphyosemion bualanum*	0	Marginal	AF002370						
13	*Aphyosemion calliurum*	0	Marginal	EU282844						EF417020
14	*Aphyosemion cameronense*	0	Marginal	AF002382						
15	*Aphyosemion campomaanense*	0	Marginal							
16	*Aphyosemion celiae*	0	Marginal	AF002369						EF417036
17	*Aphyosemion christyi*	0	Marginal	AF002390						
18	*Aphyosemion citrineipinnis*	0	Marginal	AF002386						
19	*Aphyosemion coeleste*	0	Marginal	AF002387						
20	*Aphyosemion cognatum*	0	Marginal	AF002391						
21	*Aphyosemion congicum*	0	Marginal	AF002398						
22	*Aphyosemion cyanostictum*	0	Marginal	AF002372						
23	*Aphyosemion decorsei*	0	Marginal	AF002393						
24	*Aphyosemion ecucuense*	0	Marginal							
25	*Aphyosemion edeanum*	0	Marginal	EU282850						EF417039
26	*Aphyosemion elberti*	0	Marginal	AF092288	AF092288				AF092355	AF092355
27	*Aphyosemion elegans*	0	Marginal	AF002396						
28	*Aphyosemion erythron*	0	Marginal							
29	*Aphyosemion etsamense*	0	Marginal							

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
30	*Aphyosemion exigoideum*	0	Marginal	AF002376						
31	*Aphyosemion exiguum*	0	Marginal	AF002371						
32	*Aphyosemion franzwerneri*	0	Marginal							EF417043
33	*Aphyosemion gabunense*	0	Marginal	AF002377						
34	*Aphyosemion georgiae*	0	Marginal							
35	*Aphyosemion heinemanni*	0	Marginal							EF417045
36	*Aphyosemion hera*	0	Marginal	DQ286834						
37	*Aphyosemion herzogi*	0	Marginal							
38	*Aphyosemion kouamense*	0	Marginal	EF063374						
39	*Aphyosemion koungueense*	0	Marginal							
40	*Aphyosemion labarrei*	0	Marginal	AF002389						
41	*Aphyosemion lamberti*	0	Marginal	AF002397						
42	*Aphyosemion lividum*	0	Marginal	EU282847						
43	*Aphyosemion loennbergii*	0	Marginal	DQ278337						
44	*Aphyosemion louessense*	0	Marginal	AF002378						
45	*Aphyosemion lugens*	0	Marginal	DQ278406						
46	*Aphyosemion maculatum*	0	Marginal	AF002383						
47	*Aphyosemion malumbresi*	0	Marginal	EF063378						
48	*Aphyosemion melanogaster*	0	Marginal	DQ278365						
49	*Aphyosemion melinoeides*	0	Marginal							
50	*Aphyosemion mimbon*	0	Marginal	AF002384						
51	*Aphyosemion occellatum*	0	Marginal	AF002388						
52	*Aphyosemion ogoense*	0	Marginal	AF002379						
53	*Aphyosemion omega*	0	Marginal							
54	*Aphyosemion pascheni*	0	Marginal							EF417046
55	*Aphyosemion pascheni-festivum*	0	Marginal	EU282841						EF417040
56	*Aphyosemion poliaki*	0	Marginal	DQ278273						
57	*Aphyosemion primigenium*	0	Marginal	AF002380						
58	*Aphyosemion punctatum*	0	Marginal	AF002400						
59	*Aphyosemion punctulatum*	0	Marginal	DQ278399						

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
60	*Aphyosemion rectogoense*	0	Marginal	AF002399						
61	*Aphyosemion riggenbachi*	0	Marginal	DQ278289						
62	*Aphyosemion sp. 08 RS-2007*	0	Marginal							
63	*Aphyosemion sp. Bimbia*	0	Marginal							
64	*Aphyosemion sp. Bioko*	0	Marginal							
65	*Aphyosemion sp. LEC 93/27*	0	Marginal	AF002375						
66	*Aphyosemion sp. Oba*	0	Marginal	EF185307						
67	*Aphyosemion sp. Rio Muni*	0	Marginal	DQ278414						
68	*Aphyosemion splendopleure*	0	Marginal	DQ278311						
69	*Aphyosemion striatum*	0	Marginal	AF002381						
70	*Aphyosemion volcanum*	0	Marginal	DQ278395						
71	*Aphyosemion wildekampi*	0	Marginal	AF002401						
72	*Aplocheilus blockii*	0	Permanent				EF591764			
73	*Aplocheilus lineatus*	0	Permanent	ALU73260			ALU73235			
74	*Aplocheilus panchax*	0	Permanent	NC_011176	NC_011176	NC_011176	NC_011176	NC_011176	NC_011176	NC_011176
75	*Aplocheilus parvus*	0	Permanent				EF591765			
76	*Archiaphyosemion guineense*	0	Marginal	AF000688			FJ872031		FJ872058	
77	*Astronotus ocellatus*	0	Permanent	NC_009058	NC_009058	NC_009058	NC_009058	NC_009058	NC_009058	NC_009058
78	*Austrofundulus guajira*	1	Seasonal	AY850673	AY850673			AY850650	AY850650	
79	*Austrofundulus leohoignei*	1	Seasonal	AY850679	AY850679			AY850656	AY850656	
80	*Austrofundulus leoni*	1	Seasonal	AY850677	AY850677			AY850654	AY850654	
81	*Austrofundulus limnaeus*	1	Seasonal	AY850670	AY850670		ALU73254	AY850647	AY850647	
82	*Austrofundulus rupununi*	1	Seasonal	AY850681	AY850681			AY850658	AY850658	
83	*Austrofundulus transilis*	1	Seasonal	AY850684	AY850684		AF002521	AY850661	AY850661	
84	*Austrolebias adloffi*	1	Seasonal	AF244412			AF244443			
85	*Austrolebias affinis*	1	Seasonal	CAU41801			AF244437			
86	*Austrolebias alexandri*	1	Seasonal	AF092302	AF092302				AF092369	AF092369
87	*Austrolebias bellottii*	1	Seasonal	AF092303	AF092303		AF244442		AF092370	AF092370
88	*Austrolebias charrua*	1	Seasonal							
89	*Austrolebias cheradophilus*	1	Seasonal	AF244424			AF244431			

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
90	*Austrolebias cinereus*	1	Seasonal				AF244439			
91	*Austrolebias cyaneus*	1	Seasonal	AF244426			AF244445			
92	*Austrolebias gymnoventris*	1	Seasonal	AF244418			AF244438			
93	*Austrolebias luteoflammulatus*	1	Seasonal				AF244444			
94	*Austrolebias melanoorus*	1	Seasonal							
95	*Austrolebias nigripinnis*	1	Seasonal	AF244415			AF244432			
96	*Austrolebias nigrofasciatus*	1	Seasonal							
97	*Austrolebias prognathus*	1	Seasonal	AF244425						
98	*Austrolebias reicherti*	1	Seasonal							
99	*Austrolebias sp. duraznensis*	1	Seasonal	AF244414			AF244433			
100	*Austrolebias vazferreirai*	1	Seasonal	AF244421			AF244435			
101	*Austrolebias viarius*	1	Seasonal	AF243423			AF244441			
102	*Austrolebias wolterstorffi*	1	Seasonal				AF244434			
103	*Callopanchax huwaldi*	1	Seasonal	AF000676						
104	*Callopanchax monroviae*	1	Seasonal				GU553013			
105	*Callopanchax occidentalis*	1	Seasonal	AF000674			FJ872034		AF092360	AF092360
106	*Callopanchax sidibeorum*	1	Seasonal	AF000678			GU553012			
107	*Callopanchax toddi*	1	Seasonal	AF000677			GU553010			
108	*Campellolebias chrysolineatus*	1	Seasonal	AF002413			AF002515			
109	*Campellolebias dorsimaculatus*	1	Seasonal	AF092295	AF092295		AF002516		AF092362	AF092362
110	*Chauliodus sloani*	0	Permanent	AP002915	AP002915	AP002915	AP002915	AP002915	AP002915	AP002915
111	*Cynopoecilus melanotaenia*	1	Seasonal	AF092296	AF092296				AF092363	AF092363
112	*Cyprinodon rubrofluviatilis*	0	Permanent	NC_009125	NC_009125	NC_009125	NC_009125	NC_009125	NC_009125	NC_009125
113	*Danio rerio*	0	Permanent	NC_002333	NC_002333	NC_002333	NC_002333	NC_002333	NC_002333	NC_002333
114	*Epiplatys bifasciatus*	0	Marginal							
115	*Epiplatys chaperi*	0	Marginal	ECU73265			ECU73240			
116	*Epiplatys infrafasciatus*	0	Marginal							
117	*Epiplatys maeseni*	0	Marginal	RMU73268						
118	*Epiplatys multifasciatus*	0	Marginal	EBU73264			EBU73239			
119	*Epiplatys roloffi*	0	Marginal	ERU73266			ERU73241			

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
120	*Epiplatys sexfasciatus*	0	Marginal				FJ872035		FJ872061	
121	*Epiplatys singa*	0	Marginal	AF092291	AF092291				AF092358	AF092358
122	*Episemion callipteron*	0	Marginal							
123	*Episemion krystallinoron*	0	Marginal							
124	*Esox lucius*	0	Permanent	NC_004593	NC_004593	NC_004593	NC_004593	NC_004593	NC_004593	NC_004593
125	*Fenerbahce formosus*	0	Marginal	AF002402			AF002404			
126	*Foerschichthys flavipinnis*	0	Marginal	AF002403			AF002407			
127	*Fundulopanchax amieti*	1	Marginal	AF002359			AF002341			
128	*Fundulopanchax cinnamomeus*	0	Marginal	AF002361			AF002343			
129	*Fundulopanchax deltaense*	1	Marginal	AF002354			AF002337			
130	*Fundulopanchax fallax*	1	Marginal	AF002355			AF002338			
131	*Fundulopanchax filamentosus*	1	Marginal	AF002351			AF002334			
132	*Fundulopanchax gardneri*	0	Marginal	AF092289	AF092289		AF002344		AF092356	AF092356
133	*Fundulopanchax gularis*	1	Marginal	AF002356			AF002339			
134	*Fundulopanchax mirabilis*	0	Marginal	FMU73272			FMU73247			
135	*Fundulopanchax ndianus*	0	Marginal	AF002360			AF002342			
136	*Fundulopanchax oeseri*	0	Marginal	AF002364			AF002345			
137	*Fundulopanchax robertsoni*	1	Marginal	AF002352			AF002335			
138	*Fundulopanchax scheeli*	0	Marginal	AF002365			AF002346			
139	*Fundulopanchax schwoiseri*	1	Marginal	AF002357			AF002340			
140	*Fundulopanchax sjostedti*	0	Marginal	FSU73273			FSU73248			
141	*Fundulopanchax walkeri*	1	Marginal	AF002353			AF002336			
142	*Fundulus diaphanus*	0	Permanent	NC_012361	NC_012361	NC_012361	NC_012361	NC_012361	NC_012361	NC_012361
143	*Fundulus grandis*	0	Permanent	NC_012377	NC_012377	NC_012377	NC_012377	NC_012377	NC_012377	NC_012377
144	*Fundulus heteroclitus*	0	Permanent	NC_012312	NC_012312	NC_012312	NC_012312	NC_012312	NC_012312	NC_012312
145	*Fundulus olivaceus*	0	Permanent	NC_011380	NC_011380	NC_011380	NC_011380	NC_011380	NC_011380	NC_011380
146	*Gadus morhua*	0	Permanent	NC_002081	NC_002081	NC_002081	NC_002081	NC_002081	NC_002081	NC_002081
147	*Gambusia affinis*	0	Permanent	NC_004388	NC_004388	NC_004388	NC_004388	NC_004388	NC_004388	NC_004388
148	*Gasterosteus aculeatus*	0	Permanent	NC_003174	NC_003174	NC_003174	NC_003174	NC_003174	NC_003174	NC_003174
149	*Glossanodon semifasciatus*	0	Permanent	AP004105	AP004105	AP004105	AP004105	AP004105	AP004105	AP004105

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
150	*Gnatholebias hoignei*	1	Seasonal	EF455701	EF455701		AF002523			
151	*Gnatholebias zonatus*	1	Seasonal	AF092352	AF092352		AF002524		AF092419	AF092419
152	*Hippoglossus hippoglossus*	0	Permanent	NC_009709	NC_009709	NC_009709	NC_009709	NC_009709	NC_009709	NC_009709
153	*Jordanella floridae*	0	Permanent	NC_011387	NC_011387	NC_011387	NC_011387	NC_011387	NC_011387	NC_011387
154	*Kryptolebias brasiliensis*	0	Marginal	AY946281	AY946281				AY946276	AY946276
155	*Kryptolebias caudomarginatus*	0	Marginal	AF092294	AF092294		AF002530		AF092361	AF092361
156	*Kryptolebias marmoratus*	0	Marginal	NC_003290	NC_003290	NC_003290	NC_003290	NC_003290	NC_003290	NC_003290
157	*Kryptolebias ocellatus*	0	Marginal	AF002430			AF002532			
158	*Kryptolebias sepia*	0	Marginal	AY946277	AY946277				AY946272	AY946272
159	*Lamprogrammus niger*	0	Permanent	NC_004378	NC_004378	NC_004378	NC_004378	NC_004378	NC_004378	NC_004378
160	*Leptolebias aureoguttatus*	1	Seasonal	AF002411			AF002513			
161	*Leptolebias citrinipinnis*	1	Seasonal	LCU73277			LCU73253			
162	*Llanolebias stellifer*	1	Seasonal	AF092353	AF092353				AF092420	AF092420
163	*Lophius (americanus & piscatorius)*	0	Permanent	NC_004380	NC_004380	NC_004380	NC_004380	NC_004380	NC_004380	NC_004380
164	*Lutjanus russellii*	0	Permanent	NC_010963	NC_010963	NC_010963	NC_010963	NC_010963	NC_010963	NC_010963
165	*Lycodes (toyamensis & diapterus & brevipes)*	0	Permanent	NC_004409	NC_004409	NC_004409	NC_004409	NC_004409	NC_004409	NC_004409
166	*Maratecoara formosa*	1	Seasonal	AF092344	AF092344				AF092411	AF092411
167	*Maratecoara lacortei*	1	Seasonal	AF092343	AF092343		AF002517		AF092410	AF092410
168	*Menidia menidia*	0	Permanent	NC_011174	NC_011174	NC_011174	NC_011174	NC_011174	NC_011174	NC_011174
169	*Micromoema xiphophora*	1	Seasonal	AF092351	AF092351		AF002525		AF092418	AF092418
170	*Moema piriana*	1	Seasonal	AF002457			AF002570			
171	*Moema staecki*	1	Seasonal	AF092339	AF092339		AF002571		AF092406	AF092406
172	*Monopterus albus*	0	Permanent	NC_003192	NC_003192	NC_003192	NC_003192	NC_003192	NC_003192	NC_003192
173	*Morone (saxatilis & chrysops)*	0	Permanent	NC_014353	NC_014353	NC_014353	NC_014353	NC_014353	NC_014353	NC_014353
174	*Mugil cephalus*	0	Permanent	NC_003182	NC_003182	NC_003182	NC_003182	NC_003182	NC_003182	NC_003182
175	*Myripristis (berndti & botche)*	0	Permanent	NC_003189	NC_003189	NC_003189	NC_003189	NC_003189	NC_003189	NC_003189
176	*Nematolebias whitei*	1	Seasonal	AF092298	AF092298		AF002511		AF092365	AF092365
177	*Neofundulus ornatipinnis*	1	Seasonal	AF092336	AF092336				AF092403	AF092403
178	*Neofundulus paraguayensis*	1	Seasonal	AF092338	AF092338		AF002573		AF092405	AF092405
179	*Neofundulus sp. Paraguay*	1	Seasonal	AF092337	AF092337				AF092404	AF092404

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
180	*Neoscopelus (microchir & macrolepidotus)*	0	Permanent	NC_003180	NC_003180	NC_003180	NC_003180	NC_003180	NC_003180	NC_003180
181	*Nimbapanchax jeanpoli*	0	Marginal				FJ872029		FJ872057	
182	*Nimbapanchax leucopterygius*	0	Marginal				FJ872026		FJ872054	
183	*Nimbapanchax melanopterygius*	0	Marginal				FJ872024		FJ872052	
184	*Nimbapanchax petersi*	0	Marginal	AF000690			FJ872030			
185	*Nimbapanchax viridis*	0	Marginal	AF000691			FJ872027		FJ872055	
186	*Nothobranchius cardinalis*	1	Seasonal							
187	*Nothobranchius eggersi*	1	Seasonal		GU138042	GU138042			GU138045	
188	*Nothobranchius elongatus*	1	Seasonal		EU401647	EU401647			EU182591	
189	*Nothobranchius foerschi*	1	Seasonal							
190	*Nothobranchius furzeri*	1	Seasonal	NC_011814	NC_011814	NC_011814	NC_011814	NC_011814	NC_011814	NC_011814
191	*Nothobranchius guentheri*	1	Seasonal							
192	*Nothobranchius hengstleri*	1	Seasonal		EU401666	EU401666			EU401646	
193	*Nothobranchius jubbi*	1	Seasonal		EU401651	EU401651			EU401628	
194	*Nothobranchius kafuensis*	1	Seasonal	NKU73274 & GU138041	NKU73274 & GU138041	GU138041	NKU73249		GU138059	
195	*Nothobranchius kilomberoensis*	1	Seasonal							
196	*Nothobranchius kirki*	1	Seasonal	NKU73275			NKU73250			
197	*Nothobranchius krammeri*	1	Seasonal							
198	*Nothobranchius krysanovi*	1	Seasonal		GU138040	GU138040			GU138058	
199	*Nothobranchius kuhntae*	1	Seasonal							
200	*Nothobranchius lucius*	1	Seasonal		EU401650	EU401650			EU401631	
201	*Nothobranchius makondorum*	1	Seasonal		EU401654	EU401654			EU401633	
202	*Nothobranchius melanospilus*	1	Seasonal		EU401659	EU401659			EU401639	
203	*Nothobranchius ocellatus*	1	Seasonal		EU401663	EU401663			EU401643	
204	*Nothobranchius orthonotus*	1	Seasonal		EU401664	EU401664			EU401644	
205	*Nothobranchius pienaari*	1	Seasonal		GU138038	GU138038			GU138056	
206	*Nothobranchius polli*	1	Seasonal		GU138033	GU138033			GU138051	
207	*Nothobranchius rachovii*	1	Seasonal		GU138044	GU138044			GU138050	
208	*Nothobranchius rubroreticulatus*	1	Seasonal		GU138032	GU138032			GU138047	

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
209	*Nothobranchius thierryi*	1	Seasonal	AF002347			AF002405			
210	*Notholebias minimus*	1	Seasonal	AF092297	AF092297		AF002514		AF092364	AF092364
211	*Oncorhynchus mykiss*	0	Permanent	NC_001717	NC_001717	NC_001717	NC_001717	NC_001717	NC_001717	NC_001717
212	*Oreochromis (niloticus & tanganicae & aureus)*	0	Permanent	NC_013663	NC_013663	NC_013663	NC_013663	NC_013663	NC_013663	NC_013663
213	*Oryzias latipes*	0	Permanent	NC_004387	NC_004387	NC_004387	NC_004387	NC_004387	NC_004387	NC_004387
214	*Pachypanchax omalonotus*	0	Permanent	PHU73262			PHU73237			
215	*Pachypanchax playfairii*	0	Permanent	PPU73263			DQ532927			
216	*Papiliolebias bitteri*	1	Seasonal	AF092341	AF092341		AF002520		AF092408	AF092408
217	*Pituna poranga*	1	Seasonal	AF092345	AF092345		AF002518		AF092412	AF092412
218	*Plecoglossus altivelis*	0	Permanent	NC_002734	NC_002734	NC_002734	NC_002734	NC_002734	NC_002734	NC_002734
219	*Plesiolebias aruana*	1	Seasonal	AF092342	AF092342		AF002519		AF092409	AF092409
220	*Plesiolebias glaucopterus*	1	Seasonal	AF244429						
221	*Polymixia japonica*	0	Permanent	NC_002648	NC_002648	NC_002648	NC_002648	NC_002648	NC_002648	NC_002648
222	*Porichthys (myriaster & notatus)*	0	Permanent	NC_006920	NC_006920	NC_006920	NC_006920	NC_006920	NC_006920	NC_006920
223	*Pronothobranchius kiyawensis*	1	Seasonal	AF002348 & EU401665	AF002348 & EU401665	EU401665	AF002406		EU401645	
224	*Pterolebias longipinnis*	1	Seasonal	AF002426			AF002528		AF092415	AF092415
225	*Pterolebias phasianus*	1	Seasonal	AF092347	AF092347		AF002529		AF092414	AF092414
226	*Rachovia brevis*	1	Seasonal	AY850665	AY850665			AY850640	AY850640	AY850640
227	*Rachovia hummelincki*	1	Seasonal					AY850642	AY850642	AY850642
228	*Rachovia maculipinnis*	1	Seasonal	AY850664	AY850664		AF002522	AY850639	AY850639	AY850639
229	*Rachovia pyropunctata*	1	Seasonal	AY850666	AY850666			AY850641	AY850641	AY850641
230	*Renova oscari*	1	Seasonal	AF092346	AF092346		AF002527		AF092413	AF092413
231	*Rivulus (Anablepsoides) amphoreus*	0	Marginal	RAU41795			AF002550			
232	*Rivulus (Anablepsoides) cryptocallus*	0	Marginal	AF092327	AF092327				AF092394	AF092394
233	*Rivulus (Anablepsoides) deltaphilus*	0	Marginal	AF092328	AF092328		AF002548		AF092395	AF092395
234	*Rivulus (Anablepsoides) hartii*	0	Marginal	AF092326	AF092326		AF002551		AF092393	HQ405400 & AF092393
235	*Rivulus (Anablepsoides) immaculatus*	0	Marginal	RIU41797			AF002552			
236	*Rivulus (Anablepsoides) iridescens*	0	Marginal	AF092324	AF092324				AF092391	AF092391
237	*Rivulus (Anablepsoides) jucundus*	0	Marginal	AF092325	AF092325		AF002545		AF092392	AF092392

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
238	*Rivulus (Anablepsoides) micropus*	0	Marginal	AY578722	AY578722				AY578714	AY578714
239	*Rivulus (Anablepsoides) ophiomimus*	0	Marginal	AF092332	AF092332		AF002546		AF092399	AF092399
240	*Rivulus (Anablepsoides) ornatus*	0	Marginal	AF092323	AF092323				AF092390	AF092390
241	*Rivulus (Anablepsoides) rubrolineatus*	0	Marginal	AF002443			AF002547			
242	*Rivulus (Anablepsoides) sp. Rio Supamo*	0	Marginal	AF092329	AF092329		AF002549		AF092396	AF092396
243	*Rivulus (Anablepsoides) stagnatus*	0	Marginal	AF092331	AF092331		RSU73255		AF092398	AF092398
244	*Rivulus (Anablepsoides) urophthalmus*	0	Marginal	AY946278	AY946278				AY946273	AY946273
245	*Rivulus (Anablepsoides) waimacui*	0	Marginal	AF092330	AF092330				AF092397	AF092397
246	*Rivulus (Atlantirivulus) haraldsiolii*	0	Marginal	AF092312	AF092312				AF092379	AF092379
247	*Rivulus (Atlantirivulus) janeiroensis*	0	Marginal	AF002450			AF002563			
248	*Rivulus (Atlantirivulus) luelingi*	0	Marginal	AF092314	AF092314		AF002564		AF092381	AF092381
249	*Rivulus (Atlantirivulus) santensis*	0	Marginal	AF092313	AF092313		AF002565		AF092380	AF092380
250	*Rivulus (Cynodonichthys) birkhahni*	0	Marginal	AF002448			AF002557			
251	*Rivulus (Cynodonichthys) brunneus*	0	Marginal	AF092316	AF092316				AF092383	AF092383
252	*Rivulus (Cynodonichthys) chucunaque*	0	Marginal	AF002447			AF002556			
253	*Rivulus (Cynodonichthys) frommi*	0	Marginal	AF092317	AF092317				AF092384	AF092384
254	*Rivulus (Cynodonichthys) fuscolineatus*	0	Marginal	RFU41786			AF002562			
255	*Rivulus (Cynodonichthys) hildebrandi*	0	Marginal	RHU41791			AF002553			
256	*Rivulus (Cynodonichthys) isthmensis*	0	Marginal	RIU41785			AF002561			
257	*Rivulus (Cynodonichthys) magdalenae*	0	Marginal	AF092315	AF092315		AF002555		AF092382	AF092382
258	*Rivulus (Cynodonichthys) tenuis*	0	Marginal	RTU41788			AF002558		AF092386	EU751964 & AF092386
259	*Rivulus (Cynodonichthys) uroflammeus*	0	Marginal	RUU41792			AF002554			
260	*Rivulus (Cynodonichthys) weberi*	0	Marginal	AF092318	AF092318		AF002560		AF092385	AF092385
261	*Rivulus (Laimosemion) agilae*	0	Marginal	AF092310	AF092310		AF002536		AF092377	AF092377
262	*Rivulus (Laimosemion) breviceps*	0	Marginal	AF092309	AF092309				AF092376	AF092376
263	*Rivulus (Laimosemion) dibaphus*	0	Marginal	AY578726	AY578726				AY578718	AY578718
264	*Rivulus (Laimosemion) frenatus*	0	Marginal	AF002435			AF002539		AF092378	AF092378
265	*Rivulus (Laimosemion) geayi*	0	Marginal	AF002433			AF002537		AY946274	AY946274
266	*Rivulus (Laimosemion) gransabanae*	0	Marginal	AF092308	AF092308				AF092375	AF092375

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
267	*Rivulus (Laimosemion) kirovskyi*	0	Marginal	AY578719	AY578719				AY578711	AY578711
268	*Rivulus (Laimosemion) lyricauda*	0	Marginal	AY578725	AY578725		AF002543		AY578717	AY578717
269	*Rivulus (Laimosemion) mahdiaensis*	0	Marginal	DQ501248			DQ501249			
270	*Rivulus (Laimosemion) rectocaudatus*	0	Marginal	AY578724	AY578724		AF002544		AY578716	AY578716
271	*Rivulus (Laimosemion) sp. DCT 89-132*	1	Marginal	AF002438			AF002542			
272	*Rivulus (Laimosemion) sp. Tobogon*	0	Marginal	AF092306	AF092306		AF002541		AF092373	AF092373
273	*Rivulus (Laimosemion) strigatus*	0	Marginal	AY578721	AY578721		AF002538		AY578713	AY578713
274	*Rivulus (Laimosemion) tecminae*	1	Marginal	AF092307	AF092307				AF092374	AF092374
275	*Rivulus (Laimosemion) xiphidius*	0	Marginal	AF002436			AF002540			
276	*Rivulus (Melanorivulus) apiamici*	0	Marginal	AF002453			AF002566			
277	*Rivulus (Melanorivulus) pictus*	0	Marginal	AF092321	AF092321				AF092388	AF092388
278	*Rivulus (Melanorivulus) punctatus*	0	Marginal	AF092322	AF092322		AF002567		AF092389	AF092389
279	*Rivulus (Melanorivulus) violaceus*	0	Marginal	AF092320	AF092320		AF002568		AF092387	AF092387
280	*Rivulus cylindraceus*	0	Marginal	AF092304	AF092304		AF002533		AF092371	FN544251 & AF092371
281	*Rivulus insulaepinorum*	0	Marginal							FN545681
282	*Rivulus roloffi*	0	Marginal	AF092305	AF092305		AF002534		AF092372	AF092372
283	*Scriptaphyosemion banforense*	0	Marginal	AF000679						
284	*Scriptaphyosemion bertholdi*	0	Marginal	AF000680						
285	*Scriptaphyosemion breuningi*	0	Marginal	AF000681						
286	*Scriptaphyosemion chaytori*	0	Marginal	AF000682						
287	*Scriptaphyosemion etzeli*	0	Marginal	AF000685						
288	*Scriptaphyosemion geryi*	0	Marginal	AF092292	AF092292		FJ872033		AF092359	AF092359
289	*Scriptaphyosemion guignardi*	0	Marginal	EF455700	EF455700			EF455703	EF455703	EF455703
290	*Scriptaphyosemion liberiense*	0	Marginal	AF000684						
291	*Scriptaphyosemion roloffi*	0	Marginal	AF000686						
292	*Scriptaphyosemion schmitti*	0	Marginal	AF000683						
293	*Sebastes (ruberrimus & elongatus)*	0	Permanent	EU008930	EU008930	EU008930	EU008930		EU008930	EU008930
294	*Simpsonichthys antenori*	1	Seasonal	CAU73276			CAU73252			
295	*Simpsonichthys bokermanni*	1	Seasonal	AF092300	AF092300				AF092367	AF092367

	Species	Diapause II	Aquatic habitat	12S	16S 5' end	16S middle	16S 3' end	ND1	ND2	Cox1 5' end
296	*Simpsonichthys costai*	1	Seasonal	AF002410			AF002512			
297	*Simpsonichthys magnificus*	1	Seasonal	AF092301	AF092301				AF092368	AF092368
298	*Simpsonichthys myersi*	1	Seasonal	AF092299	AF092299				AF092366	AF092366
299	*Simpsonichthys trilineatus*	1	Seasonal	AF244428						
300	*Synodus variegatus*	0	Permanent	NC_007228	NC_007228	NC_007228	NC_007228	NC_007228	NC_007228	NC_007228
301	*Takifugu rubripes*	0	Permanent	NC_004299	NC_004299	NC_004299	NC_004299	NC_004299	NC_004299	NC_004299
302	*Terranatos dolichopterus*	1	Seasonal	AF092354	AF092354		AF002526		AF092421	AF092421
303	*Tetraodon (nigroviridis & fluviatilis)*	0	Permanent	NC_007176	NC_007176	NC_007176	NC_007176	NC_007176	NC_007176	NC_007176
304	*Trachyrincus murrayi*	0	Permanent	NC_008224	NC_008224	NC_008224	NC_008224	NC_008224	NC_008224	NC_008224
305	*Trigonectes aplocheiloides*	1	Seasonal	AF092333	AF092333				AF092400	AF092400
306	*Trigonectes balzanii*	1	Seasonal	AF092334	AF092334		AF002572		AF092401	AF092401
307	*Trigonectes rubromarginatus*	1	Seasonal	AF092335	AF092335		TRU73257		AF092402	AF092402
308	*Xenotoca eiseni*	0	Permanent	NC_011381	NC_011381	NC_011381	NC_011381	NC_011381	NC_011381	NC_011381
309	*Xiphophorus hellerii*	0	Permanent	NC_013089	NC_013089	NC_013089	NC_013089	NC_013089	NC_013089	NC_013089
310	*Xiphophorus maculatus*	0	Permanent	NC_011379	NC_011379	NC_011379	NC_011379	NC_011379	NC_011379	NC_011379
311	*Zeus faber*	0	Permanent	NC_003190	NC_003190	NC_003190	NC_003190	NC_003190	NC_003190	NC_003190

Table S1.9 (continued). Taxon sampling, character file, and accession numbers.

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
1	*Anableps anableps*		EF017508	AF449341	AF449341	EF017456		EF017405	
2	*Aphredoderus sayanus*	NC_004372	NC_004372	NC_004372	NC_004372	NC_004372		FJ215201	DQ028169
3	*Aphyolebias peruensis*	AF002638	AF002506	AF092407	AF092407	AF092340		EF455718	
4	*Aphyosemion ahli*	DQ267386 & EF417000	EU272807		EF417000		DQ284716		EF417231
5	*Aphyosemion alpha*	DQ267387	EU056925				DQ284717		
6	*Aphyosemion aureum*		AF002317						
7	*Aphyosemion australe*	EF417018	EU272816		EF417018				EF417249
8	*Aphyosemion bamilekorum*		DQ981778						
9	*Aphyosemion batesii*		AF002286						

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
10	*Aphyosemion bitaeniatum*	DQ267390	DQ522281				DQ284720		
11	*Aphyosemion bivittatum*	DQ267392	DQ522261				DQ284722		
12	*Aphyosemion bualanum*		AF002303						
13	*Aphyosemion calliurum*	EU282842 & EF417020	DQ981781		EF417020		EU282843		EF417257
14	*Aphyosemion cameronense*		AY748280						
15	*Aphyosemion campomaanense*		EU272811						
16	*Aphyosemion celiae*	EF417036	EU885234		EF417036				EF417271
17	*Aphyosemion christyi*		AF002322						
18	*Aphyosemion citrineipinnis*		AF002318						
19	*Aphyosemion coeleste*		AF002319						
20	*Aphyosemion cognatum*		AF002323						
21	*Aphyosemion congicum*		AF002330						
22	*Aphyosemion cyanostictum*		AF002305						
23	*Aphyosemion decorsei*		AF002325						
24	*Aphyosemion ecucuense*		EU249508						
25	*Aphyosemion edeanum*	EU282848 & EF417039	EU272815		EF417039		EU282849		EF417247
26	*Aphyosemion elberti*		DQ981776	AF092355	AF092355	AF092288			
27	*Aphyosemion elegans*		AF002328						
28	*Aphyosemion erythron*		EU249499						
29	*Aphyosemion etsamense*		AY748294						
30	*Aphyosemion exigoideum*		AF002308						
31	*Aphyosemion exiguum*		DQ981777						
32	*Aphyosemion franzwerneri*	EF417043	EU885237		EF417043				EF417275
33	*Aphyosemion gabunense*		AF002309						
34	*Aphyosemion georgiae*		DQ981775						
35	*Aphyosemion heinemanni*	EF417045	EU885236		EF417045				EF417248
36	*Aphyosemion hera*								
37	*Aphyosemion herzogi*		EU885235						

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
38	*Aphyosemion kouamense*	EF063365	EU056928				EF143412		
39	*Aphyosemion koungueense*		EU056927						
40	*Aphyosemion labarrei*		AF002321						
41	*Aphyosemion lamberti*		AF002329						
42	*Aphyosemion lividum*	EU282845					EU282846		
43	*Aphyosemion loennbergii*	DQ267320	DQ342225				DQ284650		
44	*Aphyosemion louessense*		AF002310						
45	*Aphyosemion lugens*	DQ267354	EU885232				DQ284684		
46	*Aphyosemion maculatum*		AF002315						
47	*Aphyosemion malumbresi*	EF063369	EU249501				EF143415		
48	*Aphyosemion melanogaster*	DQ267300	EU249513				DQ284630		
49	*Aphyosemion melinoeides*		EU056933						
50	*Aphyosemion mimbon*		AY748287						
51	*Aphyosemion occellatum*		AF002320						
52	*Aphyosemion ogoense*		AF002311						
53	*Aphyosemion omega*		EU056934						
54	*Aphyosemion pascheni*	EF417046			EF417046				EF417277
55	*Aphyosemion pascheni-festivum*	EU282839 & EF417040			EF417040		EU282840		EF417272
56	*Aphyosemion poliaki*	DQ267275	EU056935				DQ284605		
57	*Aphyosemion primigenium*		AF002312						
58	*Aphyosemion punctatum*		AF002332						
59	*Aphyosemion punctulatum*	DQ267362	EU056936				DQ284692		
60	*Aphyosemion rectogoense*		AF002331						
61	*Aphyosemion riggenbachi*	DQ267409	DQ342220				DQ284738		
62	*Aphyosemion sp. 08 RS-2007*		EU056938						
63	*Aphyosemion sp. Bimbia*		EU056939						
64	*Aphyosemion sp. Bioko*		EU056940						
65	*Aphyosemion sp. LEC 93/27*		AF002307						
66	*Aphyosemion sp. Oba*	EF185309							

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
67	*Aphyosemion sp. Rio Muni*	DQ267397					DQ284727		
68	*Aphyosemion splendopleure*	DQ267268	EU056942				DQ284598		
69	*Aphyosemion striatum*		AF002313						
70	*Aphyosemion volcanum*	DQ267284	AF002306				DQ284614		
71	*Aphyosemion wildekampi*		AF002333						
72	*Aplocheilus blockii*								
73	*Aplocheilus lineatus*		ALU73282						
74	*Aplocheilus panchax*	NC_011176	NC_011176	NC_011176	NC_011176	NC_011176		EF455705	EF417190
75	*Aplocheilus parvus*								
76	*Archiaphyosemion guineense*		AF000711	FJ872058					FJ872045
77	*Astronotus ocellatus*	NC_009058	NC_009058	NC_009058	NC_009058	NC_009058		EF095671	
78	*Austrofundulus guajira*			AY850650	AY850650	AY850673			
79	*Austrofundulus leohoignei*			AY850656	AY850656	AY850679			
80	*Austrofundulus leoni*			AY850654	AY850654	AY850677			
81	*Austrofundulus limnaeus*	AF002589	ALU73300	AY850647	AY850647	AY850670			
82	*Austrofundulus rupununi*			AY850658	AY850658	AY850681			
83	*Austrofundulus transilis*		AF002469	AY850661	AY850661	AY850684		EF455715	
84	*Austrolebias adloffi*		AF245009						
85	*Austrolebias affinis*	AF002579	AF245464						
86	*Austrolebias alexandri*		AF245011	AF092369	AF092369	AF092302			
87	*Austrolebias bellottii*		AF245007	AF092370	AF092370	AF092303			
88	*Austrolebias charrua*		AY724390						
89	*Austrolebias cheradophilus*		AF245467						
90	*Austrolebias cinereus*		AF245005						
91	*Austrolebias cyaneus*		AF245461						
92	*Austrolebias gymnoventris*		AF245463						
93	*Austrolebias luteoflammulatus*		AF245459						
94	*Austrolebias melanoorus*		AY724373						
95	*Austrolebias nigripinnis*		AF245013						
96	*Austrolebias nigrofasciatus*		AY724407						

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
97	*Austrolebias prognathus*		AF245458						
98	*Austrolebias reicherti*		AY724392						
99	*Austrolebias sp. duraznensis*		AF245012						
100	*Austrolebias vazferreirai*		AF245457						
101	*Austrolebias viarius*		AF245456						
102	*Austrolebias wolterstorffi*		AF245014						
103	*Callopanchax huwaldi*		AF000698						
104	*Callopanchax monroviae*								
105	*Callopanchax occidentalis*		AF000696	AF092360	AF092360				FJ872048
106	*Callopanchax sidibeorum*		AF000700						
107	*Callopanchax toddi*		AF000699						
108	*Campellolebias chrysolineatus*		AF002464						
109	*Campellolebias dorsimaculatus*	AF002584	AF002465	AF092362	AF092362	AF092295			
110	*Chauliodus sloani*	AP002915	AP002915	AP002915	AP002915	AP002915		GQ860327	
111	*Cynopoecilus melanotaenia*		AF245465	AF092363	AF092363	AF092296			
112	*Cyprinodon rubrofluviatilis*	NC_009125	NC_009125	NC_009125	NC_009125	NC_009125			
113	*Danio rerio*	NC_002333	NC_002333	NC_002333	NC_002333	NC_002333		NM131389	AF398343
114	*Epiplatys bifasciatus*								EF417193
115	*Epiplatys chaperi*		AF000693						
116	*Epiplatys infrafasciatus*		DQ981783						
117	*Epiplatys maeseni*		AF000712						
118	*Epiplatys multifasciatus*		AF000692						
119	*Epiplatys roloffi*		AF000694						
120	*Epiplatys sexfasciatus*			FJ872061					FJ872049
121	*Epiplatys singa*			AF092358	AF092358	AF092291			
122	*Episemion callipteron*		DQ981774						
123	*Episemion krystallinoron*		DQ981769						
124	*Esox lucius*	NC_004593	NC_004593	NC_004593	NC_004593	NC_004593		AY380542	AY158056
125	*Fenerbahce formosus*		AF002408						
126	*Foerschichthys flavipinnis*		AF002409						

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
127	*Fundulopanchax amieti*		AF002294						
128	*Fundulopanchax cinnamomeus*		AF002296						
129	*Fundulopanchax deltaense*		AF002290						
130	*Fundulopanchax fallax*		AF002291						
131	*Fundulopanchax filamentosus*		AF002287						DQ533033
132	*Fundulopanchax gardneri*		AF002297	AF092356	AF092356	AF092289			
133	*Fundulopanchax gularis*		AF002292						
134	*Fundulopanchax mirabilis*		FMU73294						
135	*Fundulopanchax ndianus*		AF002295						
136	*Fundulopanchax oeseri*		AF002298						
137	*Fundulopanchax robertsoni*		AF002288						
138	*Fundulopanchax scheeli*		AF002299						
139	*Fundulopanchax schwoiseri*		AF002293						
140	*Fundulopanchax sjostedti*		DQ981782						
141	*Fundulopanchax walkeri*		AF002289						
142	*Fundulus diaphanus*	NC_012361	NC_012361	NC_012361	NC_012361	NC_012361		GQ119877	
143	*Fundulus grandis*	NC_012377	NC_012377	NC_012377	NC_012377	NC_012377		GQ119886	
144	*Fundulus heteroclitus*	NC_012312	NC_012312	NC_012312	NC_012312	NC_012312		GQ119890	AY655692
145	*Fundulus olivaceus*	NC_011380	NC_011380	NC_011380	NC_011380	NC_011380		GQ119912	
146	*Gadus morhua*	NC_002081	NC_002081	NC_002081	NC_002081	NC_002081		AF369064	
147	*Gambusia affinis*	NC_004388	NC_004388	NC_004388	NC_004388	NC_004388		EF017411	AF152163
148	*Gasterosteus aculeatus*	NC_003174	NC_003174	NC_003174	NC_003174	NC_003174		EF033039	DQ028178
149	*Glossanodon semifasciatus*	AP004105	AP004105	AP004105	AP004105	AP004105			
150	*Gnatholebias hoignei*		AF002471			EF455701		EF455712	
151	*Gnatholebias zonatus*			AF092419	AF092419	AF092352		EF455711	
152	*Hippoglossus hippoglossus*	NC_009709	NC_009709	NC_009709	NC_009709	NC_009709		FJ769824	EU057700
153	*Jordanella floridae*	NC_011387	NC_011387	NC_011387	NC_011387	NC_011387			DQ533046
154	*Kryptolebias brasiliensis*			AY946276	AY946276	AY946281		EF455707	
155	*Kryptolebias caudomarginatus*		AF002478	AF092361	AF092361	AF092294			
156	*Kryptolebias marmoratus*	NC_003290	NC_003290	NC_003290	NC_003290	NC_003290			

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
157	*Kryptolebias ocellatus*	AF002599	AF002480						
158	*Kryptolebias sepia*			AY946272	AY946272	AY946277			
159	*Lamprogrammus niger*	NC_004378	NC_004378	NC_004378	NC_004378	NC_004378			
160	*Leptolebias aureoguttatus*	AF002581	AF002462						
161	*Leptolebias citrinipinnis*	AF002582	LCU73299						
162	*Llanolebias stellifer*			AF092420	AF092420	AF092353		EF455713	
163	*Lophius (americanus & piscatorius)*	NC_004380	NC_004380	NC_004380	NC_004380	NC_004380		AY308786	AY372751
164	*Lutjanus russellii*	NC_010963	NC_010963	NC_010963	NC_010963	NC_010963		EU627667	
165	*Lycodes (toyamensis & diapterus & brevipes)*	NC_004409	NC_004409	NC_004409	NC_004409	NC_004409		EU167890	AY539166
166	*Maratecoara formosa*			AF092411	AF092411	AF092344			
167	*Maratecoara lacortei*	AF002585	AF002466	AF092410	AF092410	AF092343			
168	*Menidia menidia*	NC_011174	NC_011174	NC_011174	NC_011174	NC_011174		AY430225	AY539074
169	*Micromoema xiphophora*			AF092418	AF092418	AF092351		EF455720	
170	*Moema piriana*	AF002639	AF002507						
171	*Moema staecki*	AF002640		AF092406	AF092406	AF092339			
172	*Monopterus albus*	NC_003192	NC_003192	NC_003192	NC_003192	NC_003192			DQ533072
173	*Morone (saxatilis & chrysops)*	NC_014353	NC_014353	NC_014353	NC_014353	NC_014353		AY308767	AY539150
174	*Mugil cephalus*	NC_003182	NC_003182	NC_003182	NC_003182	NC_003182		EF095639	AY655693
175	*Myripristis (berndti & botche)*	NC_003189	NC_003189	NC_003189	NC_003189	NC_003189			AY141517S
176	*Nematolebias whitei*	AF002577	CWU09130	AF092365	AF092365	AF092298			
177	*Neofundulus ornatipinnis*			AF092403	AF092403	AF092336			
178	*Neofundulus paraguayensis*	AF002643	AF002510	AF092405	AF092405	AF092338		EF455722	
179	*Neofundulus sp. Paraguay*			AF092404	AF092404	AF092337			
180	*Neoscopelus (microchir & macrolepidotus)*	NC_003180	NC_003180	NC_003180	NC_003180	NC_003180		EU366727	DQ533075
181	*Nimbapanchax jeanpoli*			FJ872057					FJ872043
182	*Nimbapanchax leucopterygius*			FJ872054					FJ872040
183	*Nimbapanchax melanopterygius*			FJ872052					FJ872038
184	*Nimbapanchax petersi*		AF000714						FJ872044
185	*Nimbapanchax viridis*		AF000715	FJ872055					FJ872041
186	*Nothobranchius cardinalis*	EF464711							

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
187	*Nothobranchius eggersi*	EF464686				GU138042			
188	*Nothobranchius elongatus*					EU401647			
189	*Nothobranchius foerschi*	EF464687							
190	*Nothobranchius furzeri*	NC_011814	NC_011814	NC_011814	NC_011814	NC_011814			EU780557
191	*Nothobranchius guentheri*	EF464692							
192	*Nothobranchius hengstleri*	EF464709				EU401666			
193	*Nothobranchius jubbi*					EU401651			
194	*Nothobranchius kafuensis*					NKU73274 & GU138041			
195	*Nothobranchius kilomberoensis*	EF464693							
196	*Nothobranchius kirki*	AF002575	NKU73297						
197	*Nothobranchius krammeri*	EF464707							
198	*Nothobranchius krysanovi*	EF464700				GU138040			
199	*Nothobranchius kuhntae*	EF464695							
200	*Nothobranchius lucius*					EU401650			
201	*Nothobranchius makondorum*	EF464713				EU401654			
202	*Nothobranchius melanospilus*	EF464712				EU401659			
203	*Nothobranchius ocellatus*					EU401663			
204	*Nothobranchius orthonotus*	EF464696				EU401664			
205	*Nothobranchius pienaari*	EF464702				GU138038			
206	*Nothobranchius polli*					GU138033			
207	*Nothobranchius rachovii*	EF464703				GU138044			
208	*Nothobranchius rubroreticulatus*					GU138032			
209	*Nothobranchius thierryi*		AF002284						
210	*Notholebias minimus*	AF002583	AF002463	AF092364	AF092364	AF092297			
211	*Oncorhynchus mykiss*	NC_001717	NC_001717	NC_001717	NC_001717	NC_001717		NM001124737	OMU34341
212	*Oreochromis (niloticus & tanganicae & aureus)*	NC_013663	NC_013663	NC_013663	NC_013663	NC_013663		DQ012223	GU289229
213	*Oryzias latipes*	NC_004387	NC_004387	NC_004387	NC_004387	NC_004387		EF095641	AF398344
214	*Pachypanchax omalonotus*		PHU73284						
215	*Pachypanchax playfairii*		PPU73285						DQ533086

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
216	*Papiliolebias bitteri*	AF002588		AF092408	AF092408	AF092341			
217	*Pituna poranga*	AF002586	AF002467	AF092412	AF092412	AF092345			
218	*Plecoglossus altivelis*	NC_002734	NC_002734	NC_002734	NC_002734	NC_002734		AY380536	
219	*Plesiolebias aruana*	AF002587	AF002468	AF092409	AF092409	AF092342			
220	*Plesiolebias glaucopterus*		AF245468						
221	*Polymixia japonica*	NC_002648	NC_002648	NC_002648	NC_002648	NC_002648		AY308765	DQ533098
222	*Porichthys (myriaster & notatus)*	NC_006920	NC_006920			NC_006920			DQ533101
223	*Pronothobranchius kiyawensis*	EF464705	AF002285			AF002348 & EU401665			
224	*Pterolebias longipinnis*	AF002595	AF002476	AF092415	AF092415			EF455709	EF417191
225	*Pterolebias phasianus*	AF002596	AF002477	AF092414	AF092414	AF092347		EF455710	
226	*Rachovia brevis*			AY850640	AY850640	AY850665			
227	*Rachovia hummelincki*			AY850642	AY850642				
228	*Rachovia maculipinnis*	AF002590	AF002470	AY850639	AY850639	AY850664		EF455714	
229	*Rachovia pyropunctata*			AY850641	AY850641	AY850666			
230	*Renova oscari*	AF002594	AF002475	AF092413	AF092413	AF092346		EF455721	
231	*Rivulus (Anablepsoides) amphoreus*		RAU41777						
232	*Rivulus (Anablepsoides) cryptocallus*		RCU41776	AF092394	AF092394	AF092327			
233	*Rivulus (Anablepsoides) deltaphilus*	AF002616	AF002494	AF092395	AF092395	AF092328			
234	*Rivulus (Anablepsoides) hartii*	AF002619	HQ612222	AF092393	AF092393	AF092326			
235	*Rivulus (Anablepsoides) immaculatus*	AF002620	RIU41779						
236	*Rivulus (Anablepsoides) iridescens*			AF092391	AF092391	AF092324			
237	*Rivulus (Anablepsoides) jucundus*	AF002612	AF002491	AF092392	AF092392	AF092325			
238	*Rivulus (Anablepsoides) micropus*			AY578714	AY578714	AY578722			
239	*Rivulus (Anablepsoides) ophiomimus*	AF002613	AF002492	AF092399	AF092399	AF092332			
240	*Rivulus (Anablepsoides) ornatus*			AF092390	AF092390	AF092323			
241	*Rivulus (Anablepsoides) rubrolineatus*	AF002614	AF002493						
242	*Rivulus (Anablepsoides) sp. Rio Supamo*		AF002495	AF092396	AF092396	AF092329			
243	*Rivulus (Anablepsoides) stagnatus*	AF002615	RSU41774	AF092398	AF092398	AF092331			
244	*Rivulus (Anablepsoides) urophthalmus*			AY946273	AY946273	AY946278			

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
245	*Rivulus (Anablepsoides) waimacui*			AF092397	AF092397	AF092330			
246	*Rivulus (Atlantirivulus) haraldsiolii*			AF092379	AF092379	AF092312			
247	*Rivulus (Atlantirivulus) janeiroensis*	AF002632	AF002500						
248	*Rivulus (Atlantirivulus) luelingi*	AF002633	AF002501	AF092381	AF092381	AF092314			
249	*Rivulus (Atlantirivulus) santensis*	AF002634	AF002502	AF092380	AF092380	AF092313		EF455708	
250	*Rivulus (Cynodonichthys) birkhahni*	AF002625	AF002498						
251	*Rivulus (Cynodonichthys) brunneus*			AF092383	AF092383	AF092316			
252	*Rivulus (Cynodonichthys) chucunaque*	AF002624	AF002497						
253	*Rivulus (Cynodonichthys) frommi*			AF092384	AF092384	AF092317			
254	*Rivulus (Cynodonichthys) fuscolineatus*	AF002631	RFU41770						
255	*Rivulus (Cynodonichthys) hildebrandi*	AF002621	RHU44746						
256	*Rivulus (Cynodonichthys) isthmensis*	AF002630	RIU41769						
257	*Rivulus (Cynodonichthys) magdalenae*	AF002623	RMU41773	AF092382	AF092382	AF092315			
258	*Rivulus (Cynodonichthys) tenuis*	AF002626	RTU41771	AF092386	AF092386				
259	*Rivulus (Cynodonichthys) uroflammeus*	AF002622	RUU41775						
260	*Rivulus (Cynodonichthys) weberi*	AF002629	RWU41768	AF092385	AF092385	AF092318			
261	*Rivulus (Laimosemion) agilae*	AF002603	AF002482	AF092377	AF092377	AF092310			
262	*Rivulus (Laimosemion) breviceps*			AF092376	AF092376	AF092309			
263	*Rivulus (Laimosemion) dibaphus*			AY578718	AY578718	AY578726			
264	*Rivulus (Laimosemion) frenatus*	AF002606	AF002485	AF092378	AF092378				
265	*Rivulus (Laimosemion) geayi*	AF002604	AF002483	AY946274	AY946274				
266	*Rivulus (Laimosemion) gransabanae*			AF092375	AF092375	AF092308			
267	*Rivulus (Laimosemion) kirovskyi*			AY578711	AY578711	AY578719			
268	*Rivulus (Laimosemion) lyricauda*	AF002610	AF002489	AY578717	AY578717	AY578725			
269	*Rivulus (Laimosemion) mahdiaensis*		DQ501250						
270	*Rivulus (Laimosemion) rectocaudatus*	AF002611	AF002490	AY578716	AY578716	AY578724			
271	*Rivulus (Laimosemion) sp. DCT 89-132*	AF002609	AF002488						
272	*Rivulus (Laimosemion) sp. Tobogon*	AF002608	AF002487	AF092373	AF092373	AF092306			
273	*Rivulus (Laimosemion) strigatus*	AF002605	AF002484	AY578713	AY578713	AY578721			
274	*Rivulus (Laimosemion) tecminae*			AF092374	AF092374	AF092307			

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
275	*Rivulus (Laimosemion) xiphidius*	AF002607	AF002486						EF417192
276	*Rivulus (Melanorivulus) apiamici*	AF002635	AF002503						
277	*Rivulus (Melanorivulus) pictus*			AF092388	AF092388	AF092321			
278	*Rivulus (Melanorivulus) punctatus*	AF002636	AF002504	AF092389	AF092389	AF092322			
279	*Rivulus (Melanorivulus) violaceus*	AF002637	AF002505	AF092387	AF092387	AF092320			
280	*Rivulus cylindraceus*	AF002601	RCU41781	AF092371	AF092371	AF092304			
281	*Rivulus insulaepinorum*	FN545681							
282	*Rivulus roloffi*	AF002602	RRU41780	AF092372	AF092372	AF092305			
283	*Scriptaphyosemion banforense*		AF000701						
284	*Scriptaphyosemion bertholdi*		AF000702						
285	*Scriptaphyosemion breuningi*		AF000703						
286	*Scriptaphyosemion chaytori*		AF000704						
287	*Scriptaphyosemion etzeli*		AF000707						
288	*Scriptaphyosemion geryi*	AF002574	AF000708	AF092359	AF092359	AF092292			FJ872047
289	*Scriptaphyosemion guignardi*		AF000710	EF455703	EF455703	EF455700		EF455706	
290	*Scriptaphyosemion liberiense*		AF000706						
291	*Scriptaphyosemion roloffi*		AF000709						
292	*Scriptaphyosemion schmitti*		AF000705						
293	*Sebastes (ruberrimus & elongatus)*	EU008930	EU008930		EU008930	EU008930			AY539081
294	*Simpsonichthys antenori*	AF002580	CAU73298						
295	*Simpsonichthys bokermanni*			AF092367	AF092367	AF092300			
296	*Simpsonichthys costai*	AF002578	AF002461						
297	*Simpsonichthys magnificus*			AF092368	AF092368	AF092301			
298	*Simpsonichthys myersi*			AF092366	AF092366	AF092299			
299	*Simpsonichthys trilineatus*								
300	*Synodus variegatus*	NC_007228	NC_007228	NC_007228	NC_007228	NC_007228			DQ533129
301	*Takifugu rubripes*	NC_004299	NC_004299	NC_004299	NC_004299	NC_004299		AY700363	
302	*Terranatos dolichopterus*	AF002593	AF002474	AF092421	AF092421	AF092354		EF455716	
303	*Tetraodon (nigroviridis & fluviatilis)*	NC_007176	NC_007176	NC_007176	NC_007176	NC_007176		AY700355	AJ270041
304	*Trachyrincus murrayi*	NC_008224	NC_008224	NC_008224	NC_008224	NC_008224		FJ215297	AY372709

	Species	Cox1 3' end	cytb	tRNA-Ile-Gln-Met	tRNA-Trp-Asn-Cys-Tyr	tRNA-Val	d-loop	Rag1	28S
305	*Trigonectes aplocheiloides*			AF092400	AF092400	AF092333			
306	*Trigonectes balzanii*		AF002509	AF092401	AF092401	AF092334			
307	*Trigonectes rubromarginatus*	AF002642	TRU73301	AF092402	AF092402	AF092335		EF455723	
308	*Xenotoca eiseni*	NC_011381	NC_011381	NC_011381	NC_011381	NC_011381			
309	*Xiphophorus hellerii*	NC_013089	NC_013089	NC_013089	NC_013089	NC_013089		EF017445	
310	*Xiphophorus maculatus*	NC_011379	NC_011379	NC_011379	NC_011379	NC_011379		EF017448	
311	*Zeus faber*	NC_003190	NC_003190	NC_003190	NC_003190	NC_003190		FJ215202	DQ028175

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Adaptation in a variable environment: phenotypic plasticity and bet-hedging during egg diapause and hatching in an annual killifish

Abstract

Two ways in which organisms adapt to changing environments are phenotypic plasticity and bet-hedging. Here I experimentally test which of these modes of adaptation are at play in killifish that have evolved an annual life cycle. These fish are able to persist in ephemeral pools, that completely dry each season, through the production of eggs that can remain in developmental arrest or diapause, buried in the soil, until the following rainy season. Consistent with bet-hedging (a risk spreading strategy), the eggs of the annual killifish *Nothobranchius furzeri* exhibit variation at multiple levels - whether or not different stages of diapause are entered, for how long diapause is entered, and the timing of hatching - and this variation persists after controlling for both genetic and environmental sources of variation. However, phenotypic plasticity is also present in that the proportion of eggs that enter diapause is influenced by environmental factors (temperature and light level) that vary seasonally. In nature there is typically a large parameter zone where environmental cues are somewhat correlated with seasonality, but not perfectly so, such that it may be advantageous to have a combination of both bet-hedging and plasticity.

Introduction

Different environmental conditions are likely to favor different modes of evolutionary response (Salinas and Munch 2012; Simons 2011; Wong and Ackerly 2005). Two modes of adaptation to variable environments are phenotypic plasticity and bet-hedging. A bet hedging strategy maximizes (geometric) fitness multiplicatively across generations either through the production of a broad array of phenotypes (diversification bet-hedging) or a narrowly unimodal distribution of 'safe' trait values (conservative bet-hedging) (Simons and Johnston 1997). Phenotypic plasticity is the phenomenon whereby a genotype produces different phenotypes depending upon environment (Ghalambor et al. 2007) thus allowing an organism to have higher fitness than if the trait value was fixed. Whether phenotypic plasticity or bet-hedging is expected to evolve hinges critically upon the reliability of cues indicating future conditions (Wong and Ackerly 2005) and costs (Relyea 2002). Bet-hedging is expected to evolve in unpredictable environments for which reliable cues indicative of future conditions are lacking (Simons 2011). In other words, in an unpredictable world organisms are selected to 'assume' the worst case scenario and evolve a 'risk-spreading' or 'safe' reaction norm or strategy, which can lead to reduced within season fitness (relative to a non-bet-hedging strategy), but increased long term geometric mean fitness. Alternatively, if perfectly reliable cue(s) exist indicating future conditions, organisms will be under selection to produce the most appropriate phenotype so as to achieve higher fitness - that is, phenotypic plasticity (Cohen 1967; Wong and Ackerly 2005). However, natural environments may vary in their predictability (Colwell 1974) in a way that may select for a combination of bet-hedging and plasticity (Cohen 1967; Kaplan and Cooper 1984; Moran 1992; Wong and Ackerly 2005). This combination of strategies has been explained in several equivalent ways, including 'adaptive coin flipping' (Kaplan and Cooper 1984) and diversification bet-hedging around the norm of reaction (Wong and Ackerly 2005).

Many temperate zone insects produce diapause eggs in response to changing environmental cues indicating the onset of winter. Because the date of first frost will vary among years the insect must optimize the switch from producing direct developing eggs potentially capable of completing a second generation, to producing diapause eggs capable of surviving the winter (Cohen 1970; Taylor 1980). As the probability of winter's onset increases (decreasing daylight hours) so does the proportion of diapause eggs that are produced (Bradford and Roff 1993; Bradford and Roff 1997). When environmental cues are imperfect predictors of future environment a graded shift from the production of direct developing to diapause eggs is expected. In other words a measure of bet-hedging is expected in addition to phenotypic plasticity - ensuring that at least some offspring will survive (Cohen 1966; Kaplan and Cooper 1984; Moran 1992).

In addition to variability in whether diapause is entered, a staggered emergence from diapause (i.e. hatching or germination) could represent a bet-hedging strategy against unpredictability in season beginning date (Leon 1985; Philippi et al. 2001; Simons and Johnston 1997; Venable 1989). Simons and Johnston (1997) present the results of a simulation model set in the context of an annual plant found in a temperate region. The date of final spring frost varies among years. If a seed germinates early, and a frost occurs after it has germinated it will die. If a seed germinates very late, then it will avoid frost, but will have lower fecundity at the end of the season since it started growing late. When season beginning date was made more predictable the optimal germination strategy is to produce seeds that all germinate around the same date, right after the final frost. In contrast, when date of final frost is highly unpredictable between years a bet hedging strategy is favored where seeds germinate over a broad distribution of time. This way no matter what time the season starts there are some seeds able to capitalize.

There are other seasonal patterns that organisms have adapted to besides winter, especially in the large portion of the world where winter freezing does not occur. Killifish adapted to life in ephemeral aquatic habitats are found across large portions of Africa and South America in habitats ranging from open savannah to forest (Hrbek and Larson 1999; Murphy and Collier 1997). Due to the regular or periodic drying of their aquatic habitat, these fish have evolved eggs capable of undergoing prolonged diapause or reversible developmental arrest at specific stages during development (Peters 1963; Wourms 1972a; Wourms 1972b; Wourms 1972c). As their aquatic environment dries out and the fish die, the developing eggs remain viable, buried in the soil, until the following rainy season (Genade et al. 2005; Watters 2009). In habitats that have distinct wet and dry seasons, where aquatic pools regularly dry each year and hatching, maturation, and reproduction all occur within a single season, this is an annual life cycle. Annual killifish face analogous selection pressures as the annual plants and insects described above. Specifically, the start time and total length of the rainy season shows variation both within and across seasons (Watters 2009). Within a season, rain may come in pulses such that pools which support fish have multiple inundations and dryings (Polacik et al. 2011; Watters 2009). Killifish inhabiting such environments face the risk of false-starts in regards to season beginning - an early rain followed by a period of dryness could cause eggs to hatch only to have the fish perish quickly thereafter. Between seasons the duration of the rainy season and total annual rainfall can also vary (Podrabsky et al. 1998; Polacik et al. 2011; Watters 2009). Given favorable conditions it may be possible for direct-developing embryos to complete a second generation during a single season. At the other end of the spectrum, it is possible that a given year may occasionally have such low or sporadic rainfall that aquatic pools occur too briefly for fish to successfully reproduce (Podrabsky et al. 1998).

The annual killifish studied here, *Nothobranchius furzeri*, is native to savannah habitat of southern Mozambique and Zimbabwe where it inhabits temporary aquatic pools which dry on an annual basis (Figure 2.1). *Nothobranchius furzeri* eggs are capable of undergoing diapause, developmental arrest with an accompanying slowing of metabolic rate (Podrabsky et al. 2010b), at three specific stages during embryology - termed diapause I, II, and III (depicted in Figure 2.2 and 2.3). Diapause I occurs early in development and is brought about by embryos being exposed to harsh environmental conditions, including cold temperatures, hypoxia, and presence of adult fish (Inglima et al. 1981; Levels et al. 1986; Podrabsky et al. 2010b; Wourms 1972c). Diapause II can occur in embryos possessing 38 somite pairs, just after a rudimentary tubular heart and the beginnings of several other organ systems have formed. It is during this diapause that embryos are most resistant to temperature extremes, desiccation, and oxygen deprivation (Matias 1982; Matias and Markofsky 1978; Podrabsky and Hand 1999). Diapause III can occur when the embryo is fully developed and capable of hatching. The embryos of *Nothobranchius furzeri*, and other annual killifish, are capable of either entering into or skipping each phase of developmental arrest giving rise to a diversity of different developmental pathways (Figure 2.3; Wourms 1972c). I refer to embryos that enter into diapause II as following the diapause pathway and those that skip this state and instead proceed directly to diapause III as following the direct-developing pathway (Podrabsky et al. 2010a).

Here I carry out a series of experiments on egg diapause induction and hatching variability designed to test two modes of adaptation to environmental unpredictability, phenotypic plasticity and bet-hedging. I was interested in whether the induction of diapause is sensitive to environmental cues that may indicate timing in the year (phenotypic plasticity), or if it shows intrinsic variability as a strategy to minimize risk of complete reproductive failure in an environment where timing and length of the rainy season can vary by years (bet-hedging). If a

bet-hedging strategy is being employed one would predict variability in developmental pathway and hatching date. The following specific bet-hedging predictions were tested: 1) eggs from the same male- female pair, spawned and fertilized on the same day and treated in an identical manner, are capable of undergoing heterogonous development rates (i.e. some enter diapause and some exhibit direct-development), 2) not all eggs hatch during the first rainfall event (i.e. when wetted the first time) even if development is complete, and 3) eggs that do not hatch remain viable for long periods, and hatch under identical circumstances at a later date.

If phenotypic plasticity were occurring one would expect that whether or not eggs enter diapause would be sensitive to environmental cues that indicate timing in the season. My goal was to select several ecologically relevant environmental cues and expose eggs to combinations of these cues that mimic realistic levels at different times in the season. I obtained long-term monthly precipitation, temperature, and day length data from a weather station 94 kilometers from the *Nothobranchius furzeri* collection locality (Table S2.1). This data (Figure 2.4) allowed me to parameterize cues, or experimental treatments to encompass the range of environmental variability that eggs of *Nothobranchius furzeri* would ordinarily experience in their natural habitat over the course of a year. The dry season is characterized by cool temperatures and reduced day length while the rainy season is associated with warm temperatures and longer days (Figure 2.4). It was therefore anticipated that cooler temperatures and shorter day length would cause embryos to enter diapause and warmer temperatures and longer day length would be associated with direct-development.

Methods

Phenotypic plasticity in response to environmental cues.

The *Nothobranchius furzeri* population used in these laboratory studies were originally collected from an ephemeral pool in the Chefu river drainage in southern Mozambique near the border with Zimbabwe. Mature F2 generation male and female *Nothobranchius furzeri* were maintained in 40 gallon stock tanks in a fish room (25 degrees C, 12 hours light: 12 hours dark daily cycle) and provided with spawning substrate. Eggs were collected daily, over a one month period, and distributed individually into the wells of 48-well tissue culture plates containing Yamamoto's embryo incubation medium (Valenzano et al. 2009). Eggs were incubated at three temperatures (20, 25, and 30° C) crossed with four light treatments (hours light: hours dark - 0:24, 10:14, 12:12, 14:10) for a total of twelve different combinations. The twelve treatment combinations of temperature and light were replicated for a total of 24 environmental chambers that each contained one tray with 48 embryos. Since fish embryos are translucent it is possible to stage them throughout development (Wourms 1972a). Eggs were observed once or twice weekly under a dissecting microscope and scored for if, when, and what stage they entered diapause. Diapause II was apparent as an arrest of development at the 38 somite stage. Direct-developing embryos bypass this arrest and instead continue developing until diapause III is reached. Embryos following the diapause and direct-developing pathway exhibit significant morphological divergence that begins prior to the stage at which embryos enter into diapause II (Podrabsky et al. 2010a; Chapter 1). The date at which diapause II embryos resumed development or 'escaped' from diapause was recorded. Lastly, the date at which eggs either hatched or were no longer viable was recorded. These data provide a longitudinal record of development for embryos incubated at different temperature and light levels.

I analyzed the data in two complementary ways. For each of the dependent variables (percentage of embryos that entered diapause II, days until 'escape' from diapause II, and total development time) I calculated tray means by averaging values for the 48 embryos contained within each tray. I then performed general linear models with temperature, light, and the interaction as fixed effects. This procedure homogenizes variance between groups, but reduces power because each tray becomes the unit of analysis rather than the 48 individual eggs contained within. Alternatively, I used generalized linear models with temperature, light, and their interaction as fixed effects and individual embryos nested within tray as random effects. For the analysis of how temperature and light affect developmental pathway (0=diapause, 1=direct-development) I used a binomial distribution and logit link function, while for the continuous variables (days until 'escape' from Diapause II, and total development time) a gamma distribution and log link function was employed.

Bet-hedging during diapause induction and hatching

Diversification bet-hedging requires variation in phenotype when similar genotypes are exposed to the same environment (Philippi and Seger 1989). Thus, for this experiment I kept track of parentage. I incubated cohorts of eggs of known parentage in a common garden environment and examined phenotypic variance in developmental pathway and timing of hatching.

Five male-female pairings were generated and these fish were reared in separate tanks containing spawning substrate. Eggs were collected daily from each tank and distributed individually into the wells of a 48-well plate containing compacted, moist peat moss. These trays were incubated in a dark cupboard at 25 degrees C. Approximately twice weekly embryos were examined such that developmental pathway - direct developing or diapause - could be ascertained. When eggs first reached Diapause III - indicated by fully developed eyes lined with a

gold iris (Figure 2.2) I attempted to induce hatching by placing them in dishes filled with aquarium water and peat moss extract. Eggs that did not hatch in the 24-hour period were returned to their respective peat-containing well. Identical hatching attempts were repeated weekly thereafter. This iterative pattern was repeated until all eggs had hatched. These hatching trials were designed to simulate what is likely to occur at the beginning of the rainy season - occasional short bursts of rain which briefly inundate embryos followed by a return to drier conditions (incubation on moistened peat moss substrate).

Results

Phenotypic plasticity

Diapause I was transient under the conditions of the experiment. It generally lasted only a few days, sometimes a bit longer in the 20° C treatments. Both temperature and light influenced whether eggs entered diapause II or were direct-developing (GLM analysis, Temperature $F_{2,12}$=399.3, P<0.0001, Light $F_{3,12}$=4.6, P<0.05). At 20 C (a temperature that eggs typically experience during the dry season - see Figure 2.4), virtually all eggs entered into diapause II regardless of light level (Figure 2.5). In contrast, at 30 C (a temperature that would likely only be found in the middle of the wet season) virtually all eggs were direct developing, again regardless of light level. At the temperature of 25 C, a variable proportion of embryos entered diapause while the others were direct-developing (Figure 2.5). It was only at 25 C that light level affected the proportion of embryos that entered diapause - causing the significant Temperature x Light interaction ($F_{6,12}$=3.8, P<0.05). Specifically, in the dark treatment 60.3% of embryos entered diapause II, while at the three light treatments (10, 12, and 14 hours) 26.7, 22.3, and 30.8% of embryos entered diapause, respectively. Thus, temperature was the sole determinant of

developmental pathway at the lowest and highest temperatures. It was only at the intermediate temperature that an effect of light level was observed.

I limited the data set to embryos that entered diapause and asked whether light and temperature had an effect on the number of days until such embryos 'escaped' from diapause II. The mean number of days until 'escape' across all treatments was 126.7 [range: 44 - 639 days]. Temperature, light level, and the temperature x light level interaction were all significant in regards to length of diapause (Generalized LM analysis, all $P<0.01$). Although temperature had a dominant influence on whether diapause was entered, it had a lesser influence on its length (Table S2.2). This result should however be interpreted with caution because few embryos entered diapause at 30 C (Figure 2.5) and because the temperature of the 20 C environmental chambers increased to 25 C (ambient room temperature) for a 72 hour period approximately three months into the experiment which likely caused some embryos to prematurely escape from diapause II. Light level had a significant impact on the length of diapause II with embryos in the dark showing significantly longer periods of arrest. This trend was particularly apparent at the intermediate temperature of 25 C (Table S2.2).

Total development time is the number of days from fertilization until embryos hatch or perish. *Nothobranchius furzeri* embryos that enter diapause II have significantly longer development periods relative to embryos that follow the direct-developing pathway (Figure 2.6) - such a result is found whether the analysis is performed on the subset of embryos that hatched, those that perished, or both categories of embryos. I then asked how temperature, light level, and developmental pathway affected long-term embryo survival. For these survival analyses I 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. The survival distributions for

direct-developing and diapause embryos were significantly different (Kaplan-Meier method, Mantel-Cox Test, $\chi^2 = 331.3$, $P<0.00001$). Specifically, *Nothobranchius furzeri* embryos following the diapause II developmental pathway (n=321) exhibit longer survival time than embryos following the direct-developing pathway (n=387). Embryo survival distributions for the three different temperatures were significantly different (Kaplan-Meier method, Mantel-Cox Test, $\chi^2 = 222.8$, $P<0.00001$). Embryos incubated at 20 degrees (n=218) had a mean survival time of 161.1 (5.6 SE) days, those at 25 degrees (n=230) had mean survival of 137.9 (SE 9.3) days, and those at 30 degrees (n=260) 74.3 (SE 2.6) days. These significant differences are largely due to the effect temperature had on the proportion of embryos that entered diapause II.

Bet-hedging

Nothobranchius furzeri eggs from the same male female pair and clutch, incubated under identical common garden laboratory conditions (25 C, dark, on peat moss) are capable of undergoing heterogonous development rates (i.e. some enter diapause II and some follow direct-developing trajectory) - Figure 2.7. Furthermore, eggs that enter into diapause II remain in this state for variable lengths of time, so the emergence from diapause II is not synchronous. Once embryos reached diapause III not all eggs hatched at the same rate, even though they were at a stage where development was complete and thus presumably physically capable of hatching (Figure 2.7). Lastly, eggs that didn't hatch initially remained viable and hatched under identical conditions experienced at a later date (Figure 2.8). There were significant differences among the five male-female pairs in both the proportion of diapause versus direct-developing embryos (Generalized linear model, binomial distribution and logit link function, Wald $\chi^2 = 23.0$, $P<0.001$) and the number of consecutive once-weekly inundations (hatching attempts) before successful hatching (Generalized linear model, Poisson distribution and log link function, Wald $\chi^2 = 59.3$, $P<0.001$).

Discussion

The life cycle of killifish adapted to life in ephemeral water bodies is more typically associated with annual plants (Venable 2007), invertebrates found in ephemeral aquatic habitats (Hairston and Caceres 1996; Simovich and Hathaway 1997) or insects found in temperate regions with freezing winters (Bradford and Roff 1997; Fielding 2006; Hopper 1999). In annual killifish many of the same questions can be addressed as in these analogous study systems with the added benefit that one can observe development through their translucent eggs. Here, I demonstrate that the eggs of *Nothobranchius furzeri* exhibit a combination of both phenotypic plasticity and bet-hedging strategies.

Phenotypic plasticity

Whether eggs of *Nothobranchius furzeri* enter into diapause or are direct-developing exhibits plasticity to temperature, and at intermediate temperature, day length. There are many environmental cues of varying reliability (experienced by either mother or eggs) that could indicate timing in the year. It is the historical association between environmental cue(s) and timing of year which natural selection would be expected to act upon in determining the optimized reaction norm for entering into or skipping diapause. In temperate zones, the amount of daylight is evidently a quite reliable index of timing in the year and this may preclude the use of other cues. In this system, it stands to reason that temperature might be a more reliable index of timing in the year (seasonality) than day length, and therefore is the cue which eggs respond more strongly too. This offers a potential heuristic explanation for the fact that very warm or very cool incubation temperatures induce nearly all eggs to follow a single developmental trajectory. These strong cues may represent those drawn from either tail of a probability distribution and are thus associated with relative certainty of timing in the year.

Previous work in other annual killifish has demonstrated that a variety of environmental factors influence the probability of entering or skipping diapause (reviewed in Podrabsky et al. 2010b). For example, when the eggs of *Austrofundulus limnaeus*, a South American species of annual killifish, are incubated at 20 C all enter diapause II, at 25 C greater than 90% enter diapause II, and at 30 C nearly all embryos follow the escape or direct-developing trajectory (Podrabsky et al. 2010a). A similar phenomenon has been demonstrated in the African annual *Nothobranchius korthausae* (Levels and Denuce 1988). Furthermore, light level experienced by embryos can have effects on diapause induction and duration; Podrabsky and Hand (1999) and Levels and Denuce (1988) working on *Austrofundulus limnaeus* and *Nothobranchius korthausae* respectively, found that diapause II could be skipped or broken by exposing embryos to longer daylight periods. Inglima et al. (1981) found that the overwhelming majority of *Nothobranchius guentheri* embryos left in unaerated tanks with adult fish entered diapause I or II, whereas eggs collected shortly after spawning didn't. Lastly, maternal age influences the proportion of diapausing vs. direct-developing embryos produced; young females tend to produce a larger fraction of direct-developing embryos than old females (Podrabsky et al. 2010a). The emerging theme from this work is that there is a combination of plasticity to environmental cues and maternal effects that govern whether diapause is entered and for how long. What has been largely lacking is an explicit connection between the environmental cues with which eggs respond and the ecological context with which the fish live and have evolved. One of the challenges is that because so many different potentially interacting cues influence developmental pathway and length of diapause, there is the potential for a disconnect between laboratory and field studies.

Bet-hedging

Simons (2011) created strength of evidence categories for demonstrating bet-hedging. In my study system I identified an environmental factor, duration of aquatic environment, that leads

to a measure of habitat unpredictability. I identified traits (developmental pathway and hatching time) that show a high degree of variance. Lastly, I demonstrated variation in these traits is expressed within the same cohort (while controlling or minimizing genetic and environmental sources of variation). In sum, it has been demonstrated that eggs with the same parentage (genetics), incubated under identical common garden laboratory environment (same environment) are capable of following either the diapause or direct-developing pathways and exhibit variation in hatching time. In the context of the natural history of these fish such variation in development and hatching time is likely an adaptation to environmental unpredictability.

Conclusively demonstrating *adaptive* bet-hedging requires considerable effort, in large part because maximization of geometric mean fitness becomes apparent across generations in response to fluctuating selection (Simons 2009; Simons 2011). Over short time periods, particularly when environmental conditions are average or normal, bet-hedging strategies can appear maladaptive. It is for this reason that traits expressing increased variance are often readily identified - leading one to an examination of diversification bet-hedging. Diversification bet-hedging in its purest sense requires the same genotype to produce different phenotypes under identical environmental conditions (Philippi and Seger 1989), perhaps by means of selection for developmental instability (Simons and Johnston 1997). In sexually reproducing organisms identical genotypes aren't observed thus diversification bet-hedging is usually observed across a cohort of individuals with the same parents. In other words, diversification bet-hedging requires variance in the phenotype or trait to be expressed either within or among cohorts that have the same parentage and not as variation expressed among individuals at the population-level (Simons and Johnston 2003). The later scenario could result from genetic polymorphism - which is not considered bet hedging (Seger and Brockmann 1987).

Environmental uncertainty, the interaction between plasticity and bet-hedging, and natural history

Like other organisms that reside in annual environments, the habitat in which *Nothobranchius furzeri* reside is characterized by both within and between season environmental variability. Consideration of these multiple levels of variability has led to three inter-related questions and modeling efforts: What is optimal annual germination fraction given probability of good versus poor year (Cohen 1966; Venable 1989)? When (at what time of year, or given what cues) should the organism switch from producing direct-developing to diapause eggs given heterogeneity in length of growing season and what is the nature of this transition, graded or bang-bang (Bradford and Roff 1997; Cohen 1970; Halkett et al. 2004; Spencer et al. 2001)? Lastly, what is optimal within year germination strategy given risk of season false-starts, i.e. what percentage of eggs are expected to hatch at each rainfall event (Leon 1985; Philippi et al. 2001; Simons and Johnston 1997; Venable 1989)?

In annual killifish each of these questions appear relevant and because embryos are translucent and development is readily observable the interaction between strategies for coping with between and within season uncertainty comes to the forefront. In addition to generating multiplicative variation through the course of development - termed the multiplier effect by Wourms (1972c), three different diapause stages may allow for the rate of development to be more readily fine-tuned to prevailing environmental conditions than would a single diapause. Entrance into diapause II both greatly lengthens the development period (Figure 2.6), and allows for such embryos to survive for longer periods than embryos following the direct-developing pathway. This may derive from the fact that diapause II embryos (and likely I) have significantly reduced metabolic rate relative to embryos that halt development in diapause III (Chapter 1). Diapause I and II may be specialized in allowing embryos to traverse the rather long gap between rainy seasons. For *Nothobranchius furzeri* such a period is likely to last at least six months (Table S2.1). Diapause III, on the other hand, may represent a means by which a fully developed embryo

can, over a comparatively shorter time-frame, remain 'ready and waiting' for circumstances in which to hatch. In other words Diapause III may function to generate variation in hatching time in response to the within year timing of pool filling, but be relatively ill-suited toward long-term dormancy necessary to survive the comparatively longer dry season.

Given the relatively short-duration environments these fish live, why do embryos follow the direct-developing pathway? Why don't all embryos enter into each diapause as a conservative strategy? There are two possibilities. It isn't known what developmental pathways embryos typically follow in nature. However, it seems likely that embryos are more likely to enter into each successive diapause phase (I, II, and III) when exposed to combinations of cues in nature. For instance, embryos buried in the mud at the bottom of a pool would experience several cues simultaneously including low oxygen levels, temperature, and potentially light and the chemical cues of adult fish (Podrabsky et al. 2010b; Watters 2009). This may cause embryos to more readily enter into diapause, than in a laboratory environment where embryos are collected at regular intervals and incubated under relatively benign conditions.

A second non-mutually exclusive possibility is that it is adaptive for *Nothobranchius furzeri* to have (some) direct-developing eggs early in the season and eggs which enter into diapause late in the season. This would be analogous to temperate zone insects which mid-way through the summer switch from producing direct-developing eggs capable of completing a second generation to producing diapause eggs capable of surviving the freezing winter (Bradford and Roff 1993; Cohen 1970; Taylor 1980). The reason it would be adaptive to have a second generation within a single season is the multiplicative nature of the potential fitness increase. If pool duration is long enough for a second generation to reach maturity then a substantial fitness benefit could accrue to individuals that produced at least some direct-developing embryos early in the season. Or, as is perhaps more likely to be the case given the short and variable aquatic

environments of *Nothboranchius furzeri*, pools may experience several inundations and dryings within a single rain season (Polacik et al. 2014; Polacik et al. 2011). Direct-developing embryos would be in the best position to capitalize on such a scenario.

In the plasticity experiment, both temperature and light influenced whether eggs entered diapause II or were direct-developing, and the direction of the response is consistent with adaptation. That is, the dry season is associated with cool temperatures and short days. At 20 C (a temperature that matches what eggs would experience in the wild during the dry season), virtually all eggs entered into diapause regardless of light level. In a probabilistic sense an egg that finds itself at 20 C is likely in or approaching the dry season and it would be advantageous to enter diapause, because the rainy season isn't expected for many months. Alternatively at 30 C (a temperature likely only to be found during the wet season) virtually all eggs were direct developing, again regardless of light level. An egg that finds itself at this very high temperature is, in a probabilistic sense, almost certainly in the middle of the rainy season, and may benefit from direct-development such that it can quickly attain hatching capability and thus have the potential to reach maturity before the dry season begins and the aquatic environment dries. At the intermediate temperature of 25 C, a variable proportion of embryos entered diapause. Here, light level had an effect; dark treatments induced a higher proportion of embryos to enter into diapause than in light treatments. The likelihood of *Nothobranchius furzeri* being physically capable of completing a second generation is quite high given that this species has an incredibly short generation time and condensed life cycle (Terzibasi et al. 2007). This species can reach sexual maturity in a little as 3 to 4 weeks (Blazek et al. 2013). At 30 C, it took direct-developing embryos approximately 15 days from fertilization until development was complete and hatching capability was reached. Thus a minimum time for two generations to be completed in a single rainy season would be around 60 days (21 days for parental generation to reach maturity

assuming they hatch immediately upon first pool filling + 15 days embryo development time + 21 days for second generation to attain maturity). All 62 species in the genus *Nothobranchius* are considered annual, reside in ephemeral aquatic habitats, and have eggs capable of undergoing diapause (Watters 2009), yet the length of the rain season, and hence duration of aquatic environment exhibits considerable variation (Table S2.1; see also Watters 2009; Polacik et al. 2011). *Nothobranchius furzeri* is found in one of the driest regions over which the genus is distributed (Figure S2.9).

While collecting *Nothobranchius furzeri* over a 1.5 week interval toward the end of the rainy season / beginning of the dry season (April 2010) I was able to make some natural history observations that are relevant to the above discussion. Within a given region, aquatic pools containing adult *Nothobranchius furzeri* exhibited substantial variation in size, likely as a function of drainage catchment size, land topography, frequency of rain in the region, and soil and surrounding vegetation characteristics. Furthermore, given the deliberate timing of our collection trip to coincide with the end of the rainy season, it is probable that I was looking at a selected subset of pools which supported fish, as smaller pools likely dried earlier in the season. Thus although rainfall patterns in a given region can be used as a reasonable proxy of duration of aquatic environments the relevant variable is actually pool duration and this exhibits variation not just temporally but also spatially (Polacik et al. 2011). Seven sites which contained *Nothobranchius* were visited. In six of these sites only adult fishes were found but in one of the pools which contained both *Nothobranchius orthonotus* and *furzeri* I found *Nothobranchius* of very different sizes. This suggests that multiple generations were co-inhabiting the same pool. However, even this is subject to a plausible alternative interpretation, namely that eggs surviving from the previous season hatched over a distribution of times (Figure 2.8) giving rise to fish of very different sizes that all happen to be of the same generation. One mechanism that could

contribute to this would be if pools gradually filled with water over the course of the rainy season, thereby sequentially exposing greater and greater numbers of buried embryos to inundation along with the rising water levels (Polacik et al. 2011; Watters 2009). Age determination based upon otoliths from wild *Nothobranchius furzeri* support the view that hatching date is not completely synchronized within fish from the same pool, and embryos do not hatch immediately after pool filling - as evidenced by the fact that mean hatching date tended to occur during the peak of the rainy season (Polacik et al. 2011).

Embryos that enter into diapause II are capable of surviving for over 600 days (Figure 2.6) or the equivalent of two dry seasons. Furthermore, it seems quite probable that had embryos entered diapause I they would be capable of surviving even longer (Watters 2009). It is unknown whether there are certain years in which pool duration is so short that no fish are able to reach maturity, which would make a multi-year egg bank, and these very long development periods, a necessity. The Pafuri weather station, which is 94 kilometers from the *Nothobranchius furzeri* collection locality, recorded a mean annual precipitation of 423 mm, with a low of 171 mm in 1947 and a high of 865 mm in 1932 (Table S2.1). Without data linking rainfall levels to pool duration over multiple years it is difficult to come to any firm conclusions regarding 1) the probability of years in which pools are of long enough duration that a second generation can be successfully completed prior to dry down, and 2) the probability that pools are of such short duration that no fish are able to reach maturity prior to pool dry down.

Maternal versus egg control of diapause

In several taxonomic groups, such as daphnia, whether eggs are direct-developing or enter diapause is apparently under parental control (i.e. a maternal effect). Here I have shown that when parental conditions were kept constant, conditions experienced by embryos determine whether diapause is entered (phenotypic plasticity). However, other killifish studies demonstrated

that the parental environment, namely photoperiod (Markofsky et al. 1979) and maternal age (Podrabsky et al. 2010a) influenced whether eggs enter diapause (i.e. maternal effects). Currently, we lack information on the interaction between parental effects and offspring (embryo) plasticity. It is possible that environmental conditions experienced by parents modify embryo reaction norms - termed transgenerational plasticity (Salinas and Munch 2012). In the present study, individual male-female pairs 'produced' different proportions of diapause versus direct-developing eggs (Figure 2.7) when embryos were reared in a common garden environment at the intermediate temperature of 25 C. Since environmental effects were standardized (for both parents and embryos) this inter-individual variation indicates that parentage (genetics or parental effects) influences the propensity for eggs to enter diapause. However this effect of parentage on offspring development trajectory was readily overridden by strong environmental cues experienced by the embryo (Figure 2.5; Levels and Denuce 1988). Why is this the case? Are conditions experienced by embryos always able to over-ride parental effects on developmental pathway? The critical issue may be the strength of autocorrelation between environmental cues and seasonality, how easily these cues are perceived by parents and embryos, and the constancy of the relationship between environmental cues and seasonality over evolutionary time (Wong and Ackerly 2005). One point worth mentioning is that environmental information is continually being updated in real time. Thus a mother 'deciding' whether to produce diapause or direct developing eggs will be relying on different (and more outdated) information than an egg buried in the soil. If a mother is capable of predisposing an egg toward one phenotypic pathway or another she must do so at a relatively early period. The egg, while going through development, will be experiencing more up-to-date cues on the nature of the environment and it may be advantageous for individual eggs to use phenotypic plasticity to adjust developmental trajectory (phenotype).

Conclusion

The annual killifish *Nothobranchius furzeri* inhabits ephemeral aquatic pools that dry on an annual basis. This species is capable of persisting in such spatially and temporally heterogeneous environments through a developmental program capable of generating significant variation in the timing over which embryos complete development and ultimately hatch (Wourms 1972a; Wourms 1972b; Wourms 1972c). This is accomplished through three stages of diapause that can vary in length and whether or not a given egg enters into each diapause. This variability is multiplicative through development such that cohorts of embryos regularly reach hatching capability over a distribution of times and do not all hatch at the first suitable opportunity, a phenomena consistent with bet-hedging. Yet, strong environmental cues which may correspond to relative certainty in regards to timing in the season can winnow this variation in a manner that is consistent with adaptive phenotypic plasticity.

Figure 2.1. *Nothobranchius furzeri* habitat in the wet (left panel) and dry (right panel) seasons.

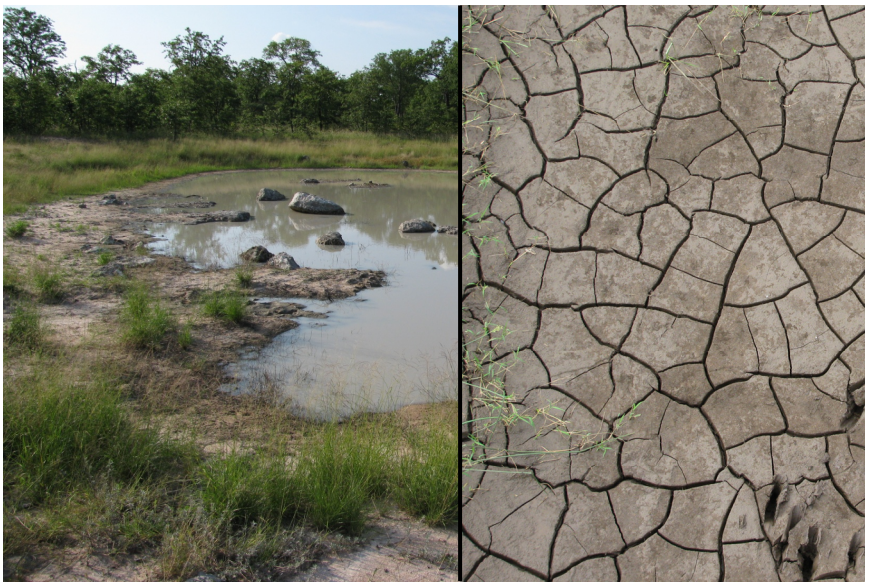


Figure 2.2. *Nothobranchius furzeri* male and female. Embryos that have arrested development in diapause I, II, and III.

Figure 2.3. Diagram depicting alternative developmental pathways for annual killifish embryos. Several days after fertilization all embryos pass through a dispersed cell phase in which development can be arrested (Diapause I). Environmental conditions including hypoxia or cold temperatures have been shown to induce an arrest in or significant lengthening of this phase. However, given incubation conditions in the present study, the dispersed cell phase was rather transient, generally lasting only a few days. After cellular re-aggregation and the formation of the somite-embryo, a point of divergence is reached (yellow circle) where embryos become committed to following either the diapause II pathway (upper trajectory) or direct-developing pathway (lower trajectory). This point is depicted as a bifurcating fork because here embryos following the different pathways begin to diverge morphologically and physiologically. Direct-developing embryos, as the name implies, proceed with development until it is complete and they are capable of hatching given appropriate environmental conditions (lower white circle). Embryos following the diapause II pathway arrest development when they attain approximately 38 somite pairs. After a variable length of time, diapause II embryos 'escape', resume development, and reach the stage where they are capable of hatching (upper white circle). Fully-developed embryos (white circles) are capable of hatching, yet can remain in this 'ready and waiting' state (Diapause III) for variable lengths of time.

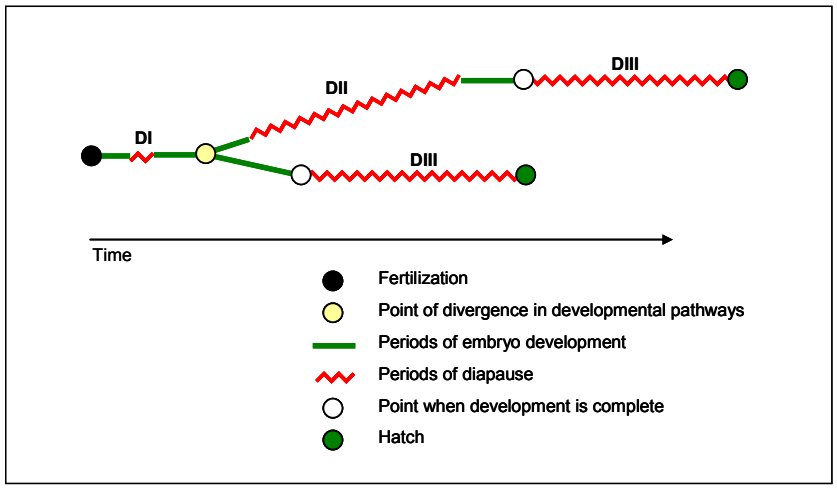


Figure 2.4. Annual variation in precipitation (mm / day), daily mean temperature (°C), and day length (hours / day) estimated for the collection locality (Lat. -21.816, Long. 31.931) of the study species - *Nothobranchius furzeri*. Climate estimations were generated using the Nearest Neighbor interpolation method and long-term data from the ten weather stations nearest to the collection locality, implemented in the New_**Loc**Clim 1.10, Local climate estimator program of the Food and Agriculture Organization of the United Nations. The precipitation and daily mean temperature graphs display 95% confidence levels. At this locality the growing season (Days per year when Precipitation / Potential Evapotranspiration (PET) > 0.5) is from November 23rd to March 9th. Green shading indicates the growing season and red shading the dry season. The growing (rainy) season is characterized by higher temperatures and increased day length.

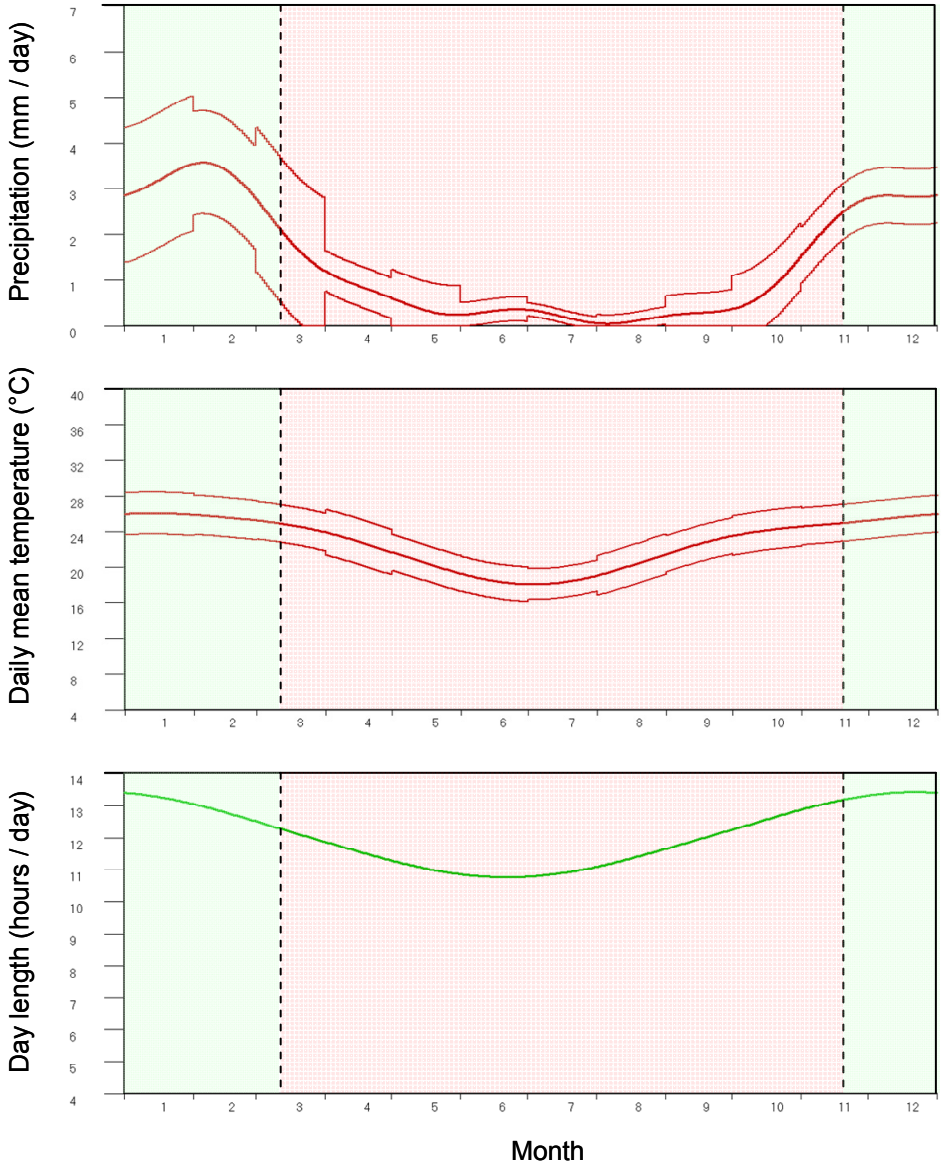


Figure 2.5. Percentage of *Nothobranchius furzeri* embryos that entered diapause II as a function of incubation conditions (Temperature and Day length).

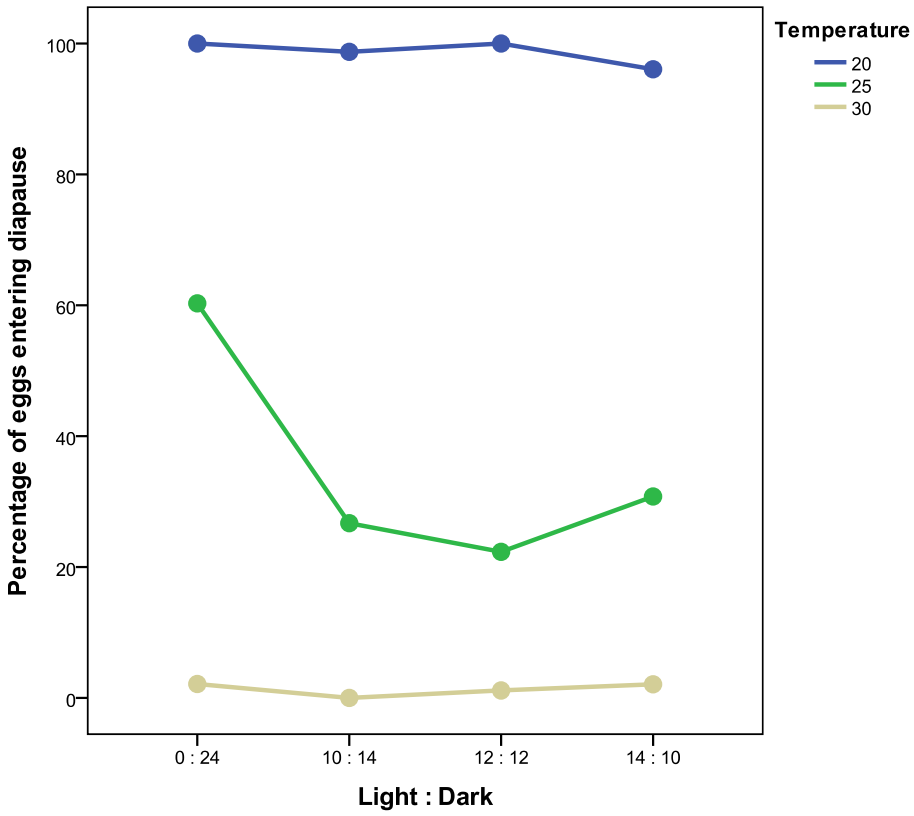


Figure 2.6. Frequency histograms of total development time of *Nothobranchius furzeri* embryos incubated at 20, 25, and 30 degrees Celsius. Total development time is defined as number of days from fertilization until embryo hatched or perished. Embryos that entered diapause II are indicated in red and those that were direct-developing are indicated in green.

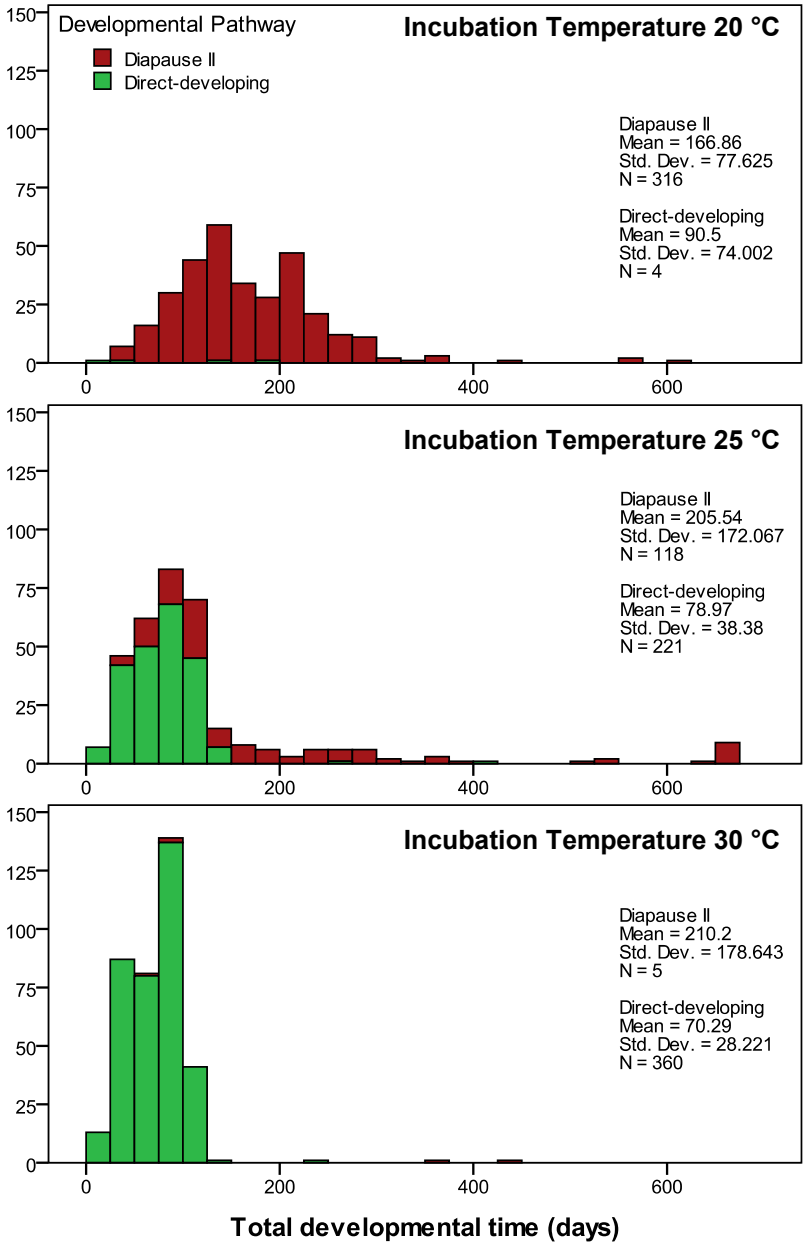


Figure 2.7. (A) *Nothobranchius furzeri* embryos from the same male-female pair (1-5), incubated under identical conditions (25C, dark, peat moss) exhibit diversity in developmental pathway. Dark bars represent diapause II embryos and white bars direct-developing embryos. (B) After embryos reached the pre-hatching stage (DIII) they underwent once-weekly hatching trials in which they were inundated with water for a 24 hour period. Embryos exhibited variation in the number of consecutive trials before hatching.

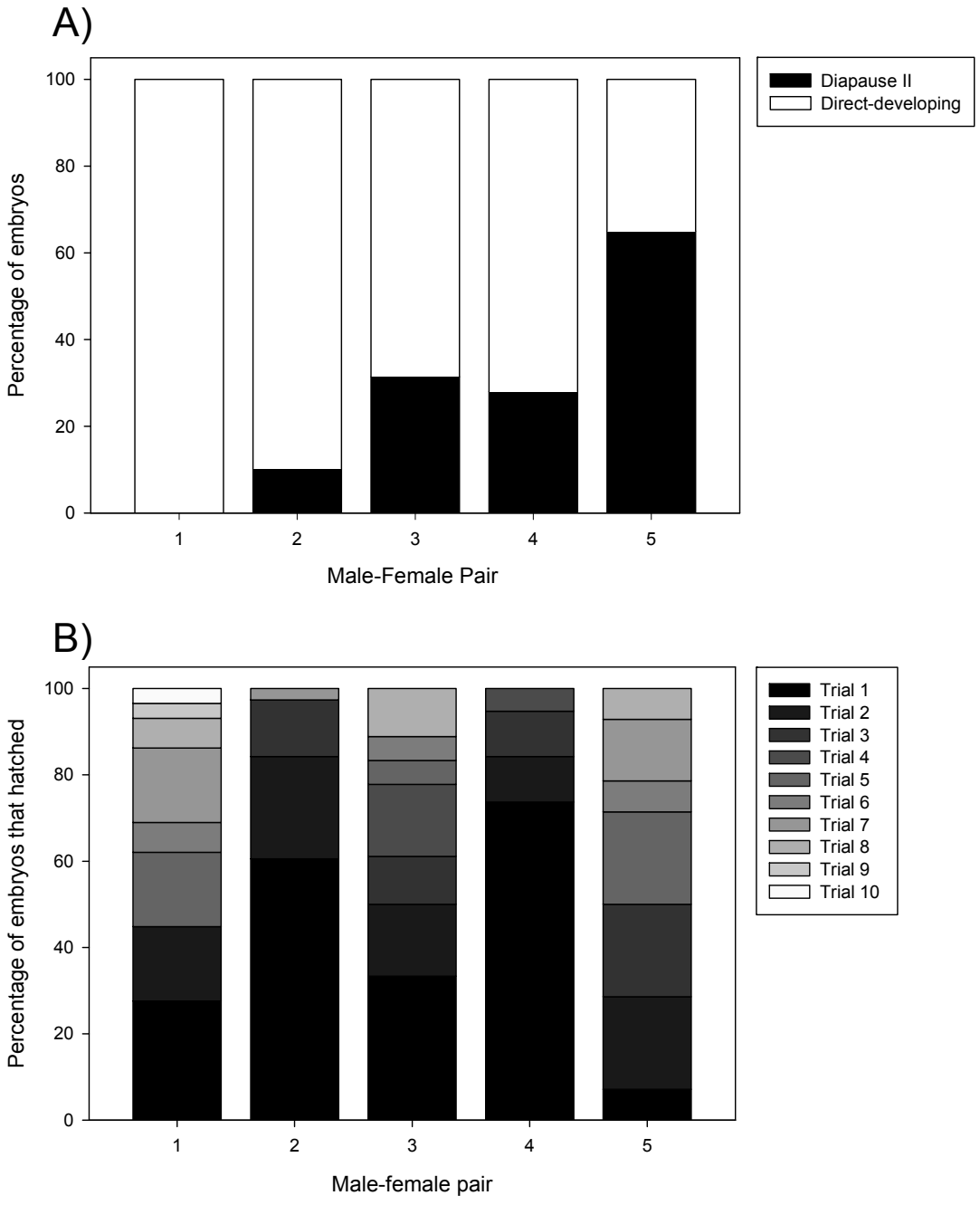
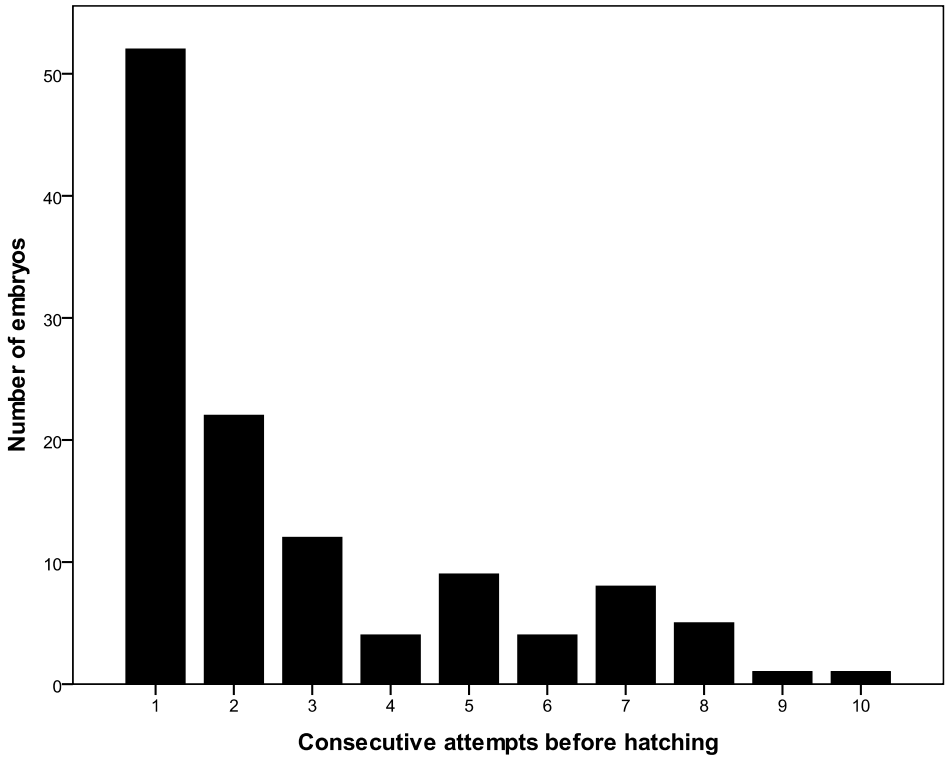


Figure 2.8. Hatching distribution for *Nothobranchius furzeri* embryos that reached the pre-hatching stage (Diapause III). Although fully developed and presumably physically capable of hatching such embryos exhibit variation in the number of consecutive (once weekly) wetting trials or attempts before hatching.



Supplementary Materials

Figure S2.9. Rough approximation of the geographic distribution of the genus *Nothobranchius* (outlined in black lines). The approximate range of *Nothobranchius furzeri* is indicated in red. This species is found in one of the driest regions over which the genus is distributed. *Nothobranchius* distribution data taken from Watters (2009) and map of Africa from the Food and Agriculture Organization of the United Nations (http://www.fao.org/nr/climpag/cropfor/lgp_en.asp).

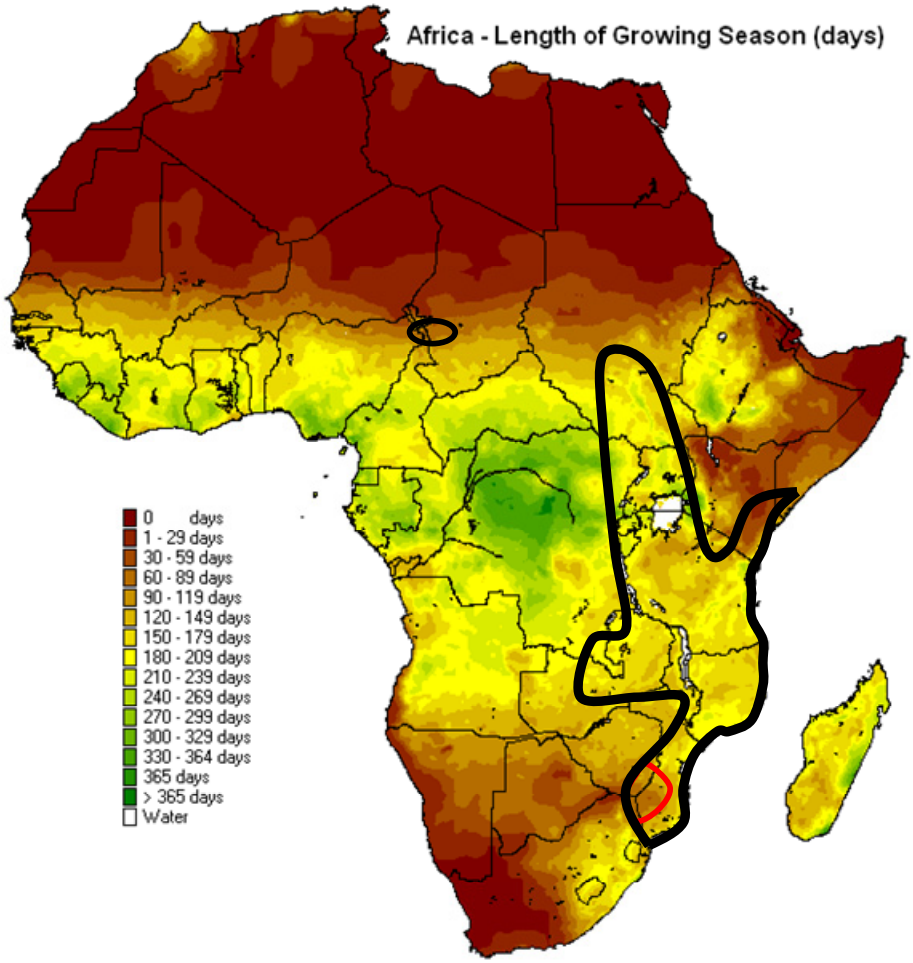


Table S2.1. Annual precipitation data (mm of rainfall per month) for the Pafuri, Mozambique weather station. Station ID: MZ21PFR0. Elevation: 290 meters. Longitude 31.33, Latitude -22.45.

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
1926	100	150	44	3	10	14	1	0	0	0	40	15	377
1927	61	45	32	9	0	0	1	0	0	81	22	81	332
1928	72	0	0	0	0	0	0	12	0	0	79	179	342
1929	69	220	19	0	6	3	0	0	0	17	68	0	402
1930	101	88	251	0	0	0	0	0	0	0	81	325	846
1931	46	38	42	0	0	0	0	30	0	0	84	5	245
1932	86	62	35	114	63	0	0	0	0	25	160	320	865
1933	102	28	15	5	2	0	0	0	0	0	189	38	379
1934	56	30	39	4	0	1	0	0	0	48	NA	28	
1935	149	39	22	0	0	17	0	0	37	40	13	47	364
1936	251	128	65	3	0	0	0	0	0	21	18	15	501
1937	41	154	37	2	0	0	0	0	30	7	12	164	447
1938	107	14	10	142	0	12	0	0	21	0	26	144	476
1939	60	234	45	0	0	0	5	0	28	0	135	29	536
1940	49	18	75	37	0	37	0	0	0	12	69	121	418
1941	13	64	30	0	0	0	0	1	0	16	4	242	370
1942	85	0	30	7	2	24	1	31	16	2	27	46	271
1943	34	88	46	36	0	1	6	20	0	0	31	37	299
1944	152	85	63	15	0	3	0	0	0	25	12	18	373
1945	77	107	97	40	3	0	0	1	0	3	137	29	494
1946	129	20	50	17	6	1	1	0	0	3	24	33	284
1947	9	47	14	3	0	1	4	0	1	16	17	59	171
1948	41	15	180	27	0	0	1	0	0	75	38	39	416
1949	112	87	13	15	35	4	3	0	0	3	53	112	437
1950	9	59	32	93	2	0	0	0	0	3	22	144	364
1951	6	1	26	10	14	0	0	62	4	41	22	53	239
1952	110	1	39	5	8	28	1	0	0	6	40	238	476
1953	252	70	100	57	5	0	1	0	0	4	102	41	632
1954	110	134	17	8	1	2	0	0	0	2	80	55	409
1955	66	121	21	32	4	0	0	0	0	29	80	79	432
1956	4	113	144	4	6	2	0	0	18	1	19	118	429
1957	71	74	21	2	0	0	3	0	5	14	97	90	377
1958	363	35	11	35	29	2	0	0	26	6	23	64	594
1959	142	111	45	4	2	36	7	0	7	12	68	55	489
1960	27	83	27	38	1	28	7	0	0	42	45	53	351
1961	57	100	13	25	5	14	0	6	11	5	20	106	362
1962	54	79	75	23	2	4	0	7	0	20	93	66	423
1963	9	43	8	44	2	1	14	3	0	39	3	66	232
1964	25	69	3	13	0	0	0	1	0	20	43	156	330
1965	46	15	5	0	0	0	0	0	44	4	49	67	230
1966	85	129	19	3	6	1	0	3	14	4	29	60	353
1967	160	89	30	100	0	0	3	0	12	3	20	29	446
1968	22	100	0	61	55	13	0	0	0	2	84	233	570
1969	54	55	81	2	13	1	1	0	39	55	73	107	481
1970	12	52	40	0	0	8	1	0	0	18	103	39	273
1971	99	6	4	33	26	1	0	0	2	38	71	100	380
1972	442	86	34	5	38	0	0	0	0	26	55	9	695
1973	18	1	52	33	6	2	3	0	93	19	13	79	319
1974	20	150	120	7	6	0	4	0	19	0	143	207	676
1975	87	155	15	56	15	1	0	0	0	0	45	75	449
1976	189	135	88	22	76	2	0	2	1	30	31	39	615
1977	37	302	125	6	29	6	0	8	39	14	50	105	721
1994	59.9	23.7	37.7	1.9	0.2	0.3	0.1	0.2	8.6	21.2	16.3	132.1	302.2
1995	35.1	45.9	83.4	37.1	46.3	1.4	0	0.1	0	27.6	9.4	35.2	321.5
1996	286.4	NA	NA	9.6	2.8	0.4	39.5	4.4	1	13.1	8	138.1	
1997	88.5	21.8	96.8	12.5	2.6	0	6.2	0.6	8.1	1.5	36.4	6.3	281.3
1998	114.9	11.1	14.3	1.7	0	0	0	0.8	0	47.6	76.7	105.4	372.5
Average	90.6	74.6	47.9	22.2	9.3	4.8	2.0	3.4	8.5	16.9	53.7	89.1	423.1

Table S2.2. Summary of *Nothobranchius furzeri* embryo response to incubation at different combinations of temperature and light. Percentage column refers to the percent of embryos that entered diapause II or were direct-developing (bypassed diapause II) at the respective combination of temperature and light. Days until escape refers to the number of days until embryos 'escaped' from diapause II (diapause trajectory) or passed the stage at which they would have entered diapause II (direct-developing pathway). Total development time is the number of days until embryos hatched or perished. Estimated marginal means and standard errors were taken from generalized linear models that included temperature, light, and trajectory and their interactions as fixed effects and individual embryos nested within tray as random effects.

				Days until escape		Total development time	
Temperature	**Light**	**Trajectory**	**Percentage**	**Mean**	**Std. Error**	**Mean**	**Std. Error**
20	0 : 24	Diapause	100	125.9	0.1	189.9	2.9
		Direct-developing	0	NA	NA	NA	NA
	10 : 14	Diapause	98.7	106.3	0.9	149.3	19.0
		Direct-developing	1.3	21.0	NA	175.0	NA
	12 :12	Diapause	100	99.9	2.8	148.7	1.2
		Direct-developing	0	NA	NA	NA	NA
	14 : 10	Diapause	96.1	105.1	2.4	177.0	17.1
		Direct-developing	3.9	21.0	NA	62.3	NA
25	0 : 24	Diapause	60.3	237.5	6.4	314.5	32.5
		Direct-developing	39.7	11.7	0.6	89.1	4.8
	10 : 14	Diapause	26.7	164.0	19.2	145.4	17.6
		Direct-developing	73.3	11.6	0.3	65.7	3.4
	12 :12	Diapause	22.3	94.4	16.9	95.7	6.2
		Direct-developing	77.7	11.7	0.1	82.5	7.9
	14 : 10	Diapause	30.8	82.4	11.4	108.6	1.7
		Direct-developing	69.2	11.7	0.3	83.4	5.1
30	0 : 24	Diapause	2.1	273.0	NA	402.5	30.1
		Direct-developing	97.9	8.1	0.2	83.6	1.6
	10 : 14	Diapause	0	NA	NA	NA	NA
		Direct-developing	100	8.0	0.1	69.4	2.2
	12 :12	Diapause	1.1	90.0	NA	98.0	NA
		Direct-developing	98.9	7.8	0.1	63.6	5.0
	14 : 10	Diapause	2.1	53.0	NA	74.0	NA
		Direct-developing	97.9	7.6	0.3	64.1	3.0

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The evolution of an annual life cycle in killifish: adaptation to ephemeral aquatic environments through embryonic diapause

Abstract

An annual life cycle is characterized by growth, maturity, and reproduction condensed into a single, short season favorable to development, with production of embryos (seeds, cysts, or eggs) capable of surviving harsh conditions in which juveniles or adults cannot. More typically associated with plants in desert environments, or temperate-zone insects exposed to freezing winters, the evolution of an annual life cycle in vertebrates is fairly novel. Killifish, small sexually-dimorphic fishes in the Order Cyprinodontiformes, have adapted to seasonally ephemeral water bodies across much of Africa and South America through the independent evolution of an annual life history. These annual killifish produce hardy desiccation resistant eggs that undergo diapause (developmental arrest) and remain buried in the soil for long periods when fish have perished due to the drying of the habitat. Killifish are found in aquatic habitats that span a continuum from permanent and stable to seasonal and variable, thus providing a useful system in which to piece together the evolutionary history of this life cycle using natural comparative variation embedded in a phylogenetic context. In this review I bring together available evidence from a variety of approaches and provide a plausible scenario for how this life cycle evolved. There are a number of features within Aplocheiloidei killifish including their inhabitation of marginal or edge aquatic habitat, their small size and rapid attainment of maturity, and egg properties that make them particularly well suited toward the colonization of ephemeral waters.

Introduction

Aquatic organisms living in habitats that regularly or periodically dry face the same challenges as plants living in arid regions. Special reproductive modes or strategies are required if such niche space is to be utilized (Rzóska 1961; Wiggins et al. 1980; Williams 1985). Unlike plants, some animals are sufficiently mobile to leave a deteriorating environment in search of better quality habitat patches. Those that do not have this option sometimes survive as dormant adults. Aquatic vertebrates such as the African lung fish (*Protopterus*) survive desiccation of their habitat by burying into the soil, secreting a slime coat which hardens into a cocoon, and estivating (Fishman et al. 1986). Similarly, Spadefoot toads of the American southwest survive the dry season by burying into the soil for up to 10 months per year (McClanahan Jr 1967). Other species have an annual life cycle and survive in ephemeral habitats via the production of propagules (cysts, or eggs) capable of undergoing diapause, developmental arrest during embryology. This life cycle in which hatching, growth, and reproduction are condensed into a single short period has evolved in a variety of invertebrates, but rarely in vertebrates.

Killifish, small sexually-dimorphic fish within the Order Cyprinodontiformes, are among the only vertebrates that include species that have evolved annual life cycles. This Order contains 1,264 currently recognized species including many well known live-bearers in the families Poeciliidae, Goodeidae, and Anablepidae as well as killifish, which are oviparous species with external fertilization and are the subject of this review (Figure 3.1). Killifish typically inhabit shallow waters or marginal aquatic habitats (Figure 3.2; Huber 2000; Scheel 1990; Wildekamp 2004). Species found across large portions of Africa (the Nothobranchiidae) and South America (the Rivulidae) have adapted to life in seasonally ephemeral water bodies that dry completely on an annual basis (Hrbek and Larson 1999; Murphy and Collier 1997; Wourms 1972c). The stereotypical case is represented by species that inhabit regions with distinct wet and dry seasons

and live in rather isolated ephemeral pools (Figure 3.3). During the wet season such pools contain fish that quickly reach maturity and spawn continuously thereafter. As the dry season begins the pools dry, all fish perish, and the entire population is represented by eggs that remain in diapause, buried in the soil until the following rainy season. The return of the rains cause eggs to hatch and the cycle begins anew. The most prominent adaptation of these annual killifish species are eggs capable of undergoing prolonged diapause at specific stages during embryology (Peters 1963; Wourms 1972a; Wourms 1972b; Wourms 1972c).

Annual killifish represent one extreme on the spectrum of life history variation in vertebrates and offer advantages for studying ecological and evolutionary questions pertaining to life history evolution and aging, phenotypic plasticity, physiological tolerance, and adaptation to extreme environments. This life cycle is quite remarkable for fish, especially when viewed in isolation from the natural variation that exists within the group. A successful approach to studying the evolution of complex traits or life cycles involves finding groups that exhibit graded variation among extant species, and then performing experiments or comparative studies on transitional forms to make inferences about the evolution of the trait(s) (Weber and Agrawal 2012). Here I take such an approach by documenting and describing the range of habitat, behavioral, developmental, and life-historical variation in killifish adapted to living in dynamically-changing, variable environments at the aquatic-terrestrial interface. I assemble and interpret this variation in a comparative phylogenetic context and present a scenario for how this life cycle has independently evolved several times within African and South American killifish.

Taxonomic distribution of annual life history in killifish

Within the sub-order Aplocheiloidei, there are three families of killifish, the African Nothobranchiidae, the South American Rivulidae and the Aplocheilidae of Madagascar, India, the

Seychelles Islands, and Southeast Asia. The Nothobranchiidae and Rivulidae each contain annual and non-annual clades (Figure 3.1; Hrbek and Larson 1999; Murphy and Collier 1997). Two alternative phylogenetic hypotheses for this pattern of trait distribution are either multiple independent origins of an annual life cycle in African and South American species or, alternatively, a deep ancestral origin in fishes residing on Gondwana prior to the divergence of Africa and the Americas, with one or more subsequent losses of this character state, followed by several reversions to an annual life cycle.

In chapter 1, a supermatrix phylogeny of the Order Cyprinodontiformes based upon seven mitochondrial and two nuclear genes was constructed. Each species in the phylogeny was scored for presence or absence of diapause II - the most prominent stage of developmental arrest and here used synonymously with an annual life cycle. Parsimony and maximum likelihood ancestral state reconstructions were performed. All analyses were congruent in supporting multiple independent origins of diapause II within both the South American Rivulidae and the African Nothobranchiidae, even when taking into account potential differences in rate of speciation or extinction associated with the evolution of diapause. There are a number of patterns that support the feasibility of this evolutionary scenario including: 1) the tight correlation between presence of diapause II and inhabitation of seasonal aquatic environments, 2) the fact that killifish are found in these habitats scattered across regions with very different geologic histories (Costa 2013; Hrbek and Larson 1999; Murphy et al. 1999a; Murphy et al. 1999b), and 3) several instances of a single species or several species with diapause II nested within an otherwise non-annual clade (Brosset 2003; Dominguez-Castanedo et al. 2013; Murphy and Collier 1999; Thomerson and Taphorn 1992). Furthermore, a number of features of non-annual killifish make them well suited toward colonizing and adapting to ephemeral environments.

Adaptations for life in ephemeral aquatic environments in killifish

Some universal themes in the annual plant literature include the evolution of seed dormancy, bet-hedging and plasticity in the timing of germination, and rapid growth and development. I use these adaptations in annual plants adapted to arid or semi-arid regions as a framework to discuss adaptations in killifish that reside in seasonally ephemeral aquatic habitat.

Embryonic diapause

In annual killifish diapause occurs at one or more of three distinct stages during embryology - termed Diapause I, II, and III (Wourms 1972a). These developmental stages correspond to a period early in development (I), mid-way through development (II), and when development is complete (III). The end result of entry into one or more diapause stages is that eggs can have a greatly extended incubation period that allows them to survive the dry season in a dormant state buried in the soil substrate at the bottom of ephemeral pools (Scheel 1990; Simpson 1979). The induction of diapause I and II is sensitive to a variety of environmental factors including oxygen levels, temperature, light levels, and presence of adult fish (reviewed in Podrabsky et al. 2010b). Annual killifish are thus capable of producing eggs that follow different developmental trajectories ranging from direct-developing (proceeding directly to diapause III and hatching) to entering into each successive diapause stage (I, II, and III) in turn (Podrabsky et al. 2010a; Wourms 1972c). Direct-developing embryos of annual killifish behave like those of nonannual killifish. The direct-developing pathway is the ancestral condition in killifish and it is the diapause stages (I and II) that are the derived state found in killifish that inhabit seasonally ephemeral aquatic environments.

Phenotypic plasticity and bet-hedging during diapause induction and hatching

One prominent adaptive challenge for organisms with an annual life cycle is entering and emerging from diapause at the appropriate times such that hatching coincides with a period

favorable for survival and reproduction. Annual killifish often reside in habitats where the timing and duration of the rainy season can vary both within and across years. Yet, environmental cues may exist (such as day length or temperature) that could indicate approximate time in the year (in relation to the rain season). In many annual killifish the probability of entrance into diapause is affected by a variety of environmental factors (such as temperature, light, oxygen level, and even presence of adult fish) preceding each diapause stage (Podrabsky et al. 2010b). This phenotypic plasticity allows embryos to respond appropriately (in a probabilistic sense) to the conditions they find themselves in (Chapter 2). However, embryos of the same clutch, incubated under common garden conditions at intermediate cue levels can exhibit heterogeneity in whether diapause is entered, for how long, and when hatching occurs (Podrabsky et al. 2010a; Chapter 2). This variance is consistent with bet-hedging - a risk spreading strategy that involves not placing all eggs in one basket, and can be viewed as an adaptation to environmental unpredictability itself. This combination of both plasticity to (strong) environmental cues and a measure of intrinsic variability (particularly at 'intermediate' cue levels) is consistent with theory that considers the intermediate reliability of cues indicating future environmental conditions (Cohen 1967; Kaplan and Cooper 1984; Moran 1992; Wong and Ackerly 2005).

Voracious appetite, early maturity, high fecundity, and short lifespan

Adaptation to life in ephemeral pools has put a premium on completing the life cycle during a short interval of time, thereby selecting for an accelerated life history. Annual species have a voracious appetite (Ijumba and Kilama 1991) which translates to a rapid early growth rate, an early maturity, near continuous reproduction once maturity is attained, and high fecundity (Blazek et al. 2013; Haas 1976; Simpson 1979). A byproduct of adapting to short duration environments in which lifespan is truncated each year is a corresponding rapid senescence and short lifespan, even when reared in captivity free of such extrinsic mortality (Genade et al. 2005).

This fact has been exploited, particularly in the genus *Nothobranchius*, where there have been an increasing number of studies documenting rapid aging phenotypes (Hsu et al. 2008; Valdesalici and Cellerino 2003; Valenzano et al. 2006), testing whether a correlation exists between habitat duration and longevity using natural populations (Terzibasi et al. 2008; Tozzini et al. 2013), and seeking to determine the genetic basis of aging (Kirschner et al. 2012; Reichwald et al. 2009). *Nothobranchius* inhabit aquatic environments that typically dry seasonally, and several species have correspondingly short lifespans. Their compressed life cycle increases the number of life history variables available for study within a short interval of time, making them an appropriate vertebrate model organism for aging studies. However, not all annuals are homogenous in exhibiting severely reduced longevity. Some species that live in seasonally ephemeral habitats sometimes can live for several years in a laboratory setting (Liu and Walford 1966; Lucas-Sanchez et al. 2011; Romand and Broche 1983; Simpson 1979).

Embryonic diapause in annual killifish

Here I tackle several questions related to the evolution of embryonic diapause in annual killifish.

Why do embryos enter diapause at the specific developmental stages that they do?

The apparent conservatism of developmental stages at which diapause is entered among African and South American clades (Wourms 1972c) caused some investigators to conclude that the annual life cycle was inherited from a single common ancestor, lost multiple times, and regained upon subsequent exposure to habitats that dry seasonally (Murphy and Collier 1997). Here I present the case for convergent evolution of diapause with diapause I, II and III representing the most stable points in embryology at which to halt development (Wourms 1972c).

Diapause I is brought about by harsh environmental conditions, particularly anoxia, and occurs during a period labeled the dispersed cell phase (Wourms 1972a; Wourms 1972b). This stage of development occurs after the blastula has formed and then dispersed and before cellular reaggregation occurs to form the primary embryonic body axis. According to Wourms (1972b) this stage is unique to annual killifish. This stage typically lasts a few days in a benign laboratory environment. However, incubation at colder temperatures (20 C) can cause this dispersed cell phase to last up to a few weeks in *Nothobranchius furzeri*. Several authors have reported that embryos left at the bottom of the tank buried in a layer of peat moss are more likely to enter diapause I than embryos collected soon after being fertilized and incubated in fresh medium (Arezo et al. 2005; Inglima et al. 1981; Levels et al. 1986b). Conditions at the bottom of the tank are likely to be hypoxic with accumulation of waste. Watters (2009) suggests that the oxygen deficient bottom substrate in which *Nothobranchius* embryos are deposited in nature are likely to induce diapause I. In such conditions, diapause I could last from the time of fertilization until the pool dries and shrinkage cracks in the vertisol soil create aerobic conditions that allow development to proceed to diapause II (Watters 2009). Diapause I may be a stage / means by which the effect of harsh environmental conditions (oxygen deficiency) is minimized if embryos are required to halt development at this early stage (Wourms 1972c). Specifically, the dispersed nature of the cells could act as a buffer such that if some cells are damaged or lost due to stress it would be localized damage that is able to be overcome when cells reaggregate (Wourms 1972c).

Diapause II is the stage that contributes most to the potential duration of developmental arrest (Matias 1982; Matias and Markofsky 1978; Podrabsky et al. 2010b; Scheel 1990). Embryos of most annual species readily enter diapause II under a range of conditions (Podrabsky et al. 2010a; Simpson 1979) and it is also at this stage that embryos are most resistant to extreme environmental conditions. Podrabsky and colleagues present extensive evidence that embryos of

Austrofundulus limnaeus that halt development in Diapause II are extremely robust to environmental stresses including high temperatures (Podrabsky et al. 2010b), lack of oxygen (Podrabsky et al. 2007), salinity (Machado and Podrabsky 2007), and desiccation (Podrabsky et al. 2001) - and furthermore it is at this stage of diapause rather than diapause I or diapause III that embryos are clearly hardiest. From this they conclude "... diapause II embryos are likely the life history stage responsible for long-term survival of environmental dehydration and the persistence of populations of *A. limnaeus* in ephemeral habitats." (Podrabsky et al. 2001).

Diapause II occurs just after the beginnings of many organ systems have been formed, but before they have gotten large and potentially costly to maintain. Embryos that arrest in diapause II possess "38-42 somites, the foundations of the central nervous system, optic cups, olfactory and lens placodes, otic vesicles and a functional tubular heart" (Podrabsky et al. 2010b). The hourglass model of development has been widely influential in developmental biology (Raff 1996) although by no means universally accepted (Richardson 1995). This model posits a phylotypic period mid-way through development that is conserved among all species within a Phylum. This stage corresponds to a period of increased genetic and developmental interactions between developing organ modules, which is thought to constrain evolutionary change. For historical reasons the phylotypic period has been most closely associated with vertebrate embryology (Richardson and Keuck 2002). Character-based descriptions of this period include the pharyngula stage (Ballard 1981), the period between the beginning of neurulation and formation of the last somites (Galis and Metz 2001), and during organogenesis (Irie and Kuratani 2011; Raff 1996). It appears that diapause II occurs just after or near the tail end of the phylotypic period. This may be a sweet spot in which to halt development for long periods. On the one hand the supposedly complex and intricate part of development has just been completed - the body has been separated into separate modules or organ primordia, that although complex within

themselves, can operate relatively independently of each other (Raff 1996). All that is left is the comparatively less complex task of growing and elaborating these organs to full size. On the other hand, the organ systems are still very small and therefore of minimal cost to maintain for long periods. This is evidenced by the fact that *Nothobranchius furzeri* embryos which halt development in diapause II have a 15-fold lower metabolic rate than those that have completed development and arrested at diapause III (Chapter 1). A similar pattern has been found in *Nothobranchius korthauase* (Levels et al. 1986a).

This leads to one of the most interesting insights derived from killifish developmental biology. In species that independently evolved an annual life cycle, embryos following the diapause II developmental pathway display significant reduction in development of the cranial region and circulatory system relative to direct-developing embryos (Podrabsky et al. 2010a; Chapter 1). This divergence along alternative developmental pathways begins early to mid-way through development, well before diapause II is entered, during the phylotypic period of purported maximum developmental constraint (Raff 1996). I suspected that the striking morphological divergence between embryos following the direct-developing versus diapause II pathways allows embryos undergoing diapause II to conserve energy by shunting resources away from energetically costly organs (brain and heart). Such conservation of energy could increase survival chances in an environment that necessitates remaining dormant for much of the year. To test this hypothesis I measured metabolic rate in embryos that either developed directly or entered diapause II. Embryos that followed the diapause II pathway exhibited a pattern of metabolic depression that mirrored the morphological divergence. Furthermore, embryos that entered diapause II were able to survive for significantly longer periods than direct-developing embryos. In a sense, embryos that are going to be entering diapause II may be best suited for long term survival in potentially harsh conditions when they have invested minimally in organ systems that

would be costly to maintain. This requires that diapause II embryos diverge morphologically and physiologically from the standard (ancestral) direct-developing pathway which is characteristic of nonannual killifish and teleosts in general. Taken together these 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.

Why do annual killifish have multiple development stages in which diapause can be entered?

Killifish embryos are translucent such that we can clearly see at what developmental stage and for how long embryos enter diapause. Having multiple points in the developmental progression in which development can be halted gives rise to a range of different developmental trajectories, termed the multiplier effect by Wourms (1972), that can spread risk over a greater period of time. This could prove adaptive in an environment characterized by temporal and spatial uncertainty. This ability to have embryos exhibit such diversity likely represents a combination of adaptive phenotypic plasticity to prevailing environmental conditions that indicate season, and bet-hedging - a risk spreading strategy that involves not placing all eggs in one basket. In a related manner, having three stages of diapause, gives a measure of redundancy to the developmental system. This may allow for finer control in response to environmental conditions. Because environmental conditions preceding each phase of diapause can influence whether it is entered or skipped a higher degree of matching can be attained between appropriate phenotype based on expected future environment (i.e. timing of the rainy season). A third, non-mutually exclusive possibility is that the different diapause stages are specialized for coping with different conditions. Particularly, diapause I and II appear specialized for allowing embryos to hunker down and survive for long periods (between season), whereas diapause III may be ideal for generating short term (within season) variation in hatching time.

Embryos that are fully developed have a limited window of opportunity in which to hatch that lasts at most a few months. Comparing long-term embryo survival in the annual species *Nothobranchius furzeri* as a function of developmental pathway (diapause II versus direct developing) revealed substantial differences; embryos incubated in Yamamoto's solution that entered diapause II survived on average over 100 days longer than embryos treated in an identical manner that followed the direct-developing pathway and halted development at diapause III (Chapter 2). Data on desiccation resistance (Podrabsky et al. 2001), salinity tolerance (Machado and Podrabsky 2007), and anoxia tolerance (Podrabsky et al. 2007) in embryos of *Austrofundulus limnaeus* indicate that diapause II is specialized for long-term embryo survival, the sort required to traverse a rather long dry season that lasts more than a few months (Podrabsky et al. 2010b). In contrast, diapause III may be specialized for traversing relatively short term periods in which conditions are not immediately suitable for hatching, but are expected to become so in the near future.

Why do annual species, which live in ephemeral aquatic habitats that dry each year, retain direct-developing embryos?

If the production of direct-developing embryos was an aberration then natural selection would presumably have canalized development such that diapause I and II would become an obligate feature of development. That direct-development is induced under conditions presumably sometimes found in nature, in most but not all annual killifish (AF personal observation; Simpson 1979; Wourms 1972c) leads me to conclude that this pathway likely has adaptive value at least under certain circumstances. If seasonal aquatic environments are of long enough duration it would be beneficial to have multiple generations within the same season. Substantial fitness benefits would accrue to individuals that produced direct-developing embryos early in the rainy season, and switched to diapause embryos later on, as has been observed in

insects (Bradford and Roff 1993; Bradford and Roff 1997). This advantage would be especially evident in regions where aquatic habitat may only dry completely in particularly poor years. In such cases direct-developing embryos might be the norm, with diapause embryos produced relatively infrequently as a means of bet-hedging. Direct-developing embryos may also be adaptive when pools experience multiple dryings and fillings in the same season, which has been reported to occur (Podrabsky et al. 1998; Podrabsky et al. 2010b; Polacik et al. 2014). In this scenario embryos in diapause III would be able to more quickly capitalize on the refilling of a pool because they could immediately hatch. In contrast, eggs in diapause I or II would have to break diapause and resume development before reaching a stage where hatching capability is reached, hence making it less likely for them to capitalize on such short-duration environments.

Within annual killifish there is a lot of variation in habitat seasonality and duration that could be exploited to test predictions regarding the adaptive significance of entry into diapause in relation to environment. For example environmental cues such as temperature or photoperiod that are seasonal predictors of the rainy and dry season, could serve as cues that influence the frequency with which diapause is entered. Since annual killifish are found across large swaths of Africa and South America the nature of the environmental cues likely vary with geographic region. For example, cool temperatures could signal either an oncoming rainy or dry season, depending on where the species is found. If embryos are responding adaptively to such cues, then one would predict opposite responses - in one species cool temperatures would be expected to induce diapause and in the other direct-development. Another prediction is that populations or species from environments with strong seasonality and shorter rainy seasons have embryos that enter into diapause I and II more readily and under a wider range of environmental conditions than those from environments with less seasonality and longer rainy seasons (Watters 2009).

Habitat, life historical, and embryonic diversity in Cyprinodontifrom killifish that yield clues to how an annual life cycle evolved

One of the grand challenges in the field of evolutionary biology, dating to the time of Darwin, is explaining how complex traits, or life cycles, evolve by incremental steps. A successful approach involves finding groups that exhibit variation among extant species. One can use this variation to make inferences and perform comparative experiments that allow for a better understanding of how the life cycle evolved through intermediate stages.

The hypothesis in brief

The habitats occupied by killifish and their life histories range across a spectrum. One end of the spectrum is defined by the common ancestor of Aplocheiloidei killifish, which likely inhabited permanent water bodies, as do the vast majority of bony fishes and some extant Aplocheiloidei killifish (Figure 3.1). At the other end of the spectrum, annual species live in aquatic environments that dry seasonally, with the species living on as diapausing eggs embedded in the dry sediment that once formed the bottom of the pond. In between these extremes are species that live in habitats that are best described as intermediate. The majority of non-annual killifish in the sub-order Aplocheiloidei live in edge or marginal aquatic habitats such as small brooks, forest pools, floodplains, and swamps (Figure 3.2). Killifish in these intermediate habitats are adept at surviving in poor water quality (Taylor 2012), breathing air (Graham 1997), and even traveling over land to escape deteriorating habitats (Seghers 1978). Eggs deposited in such environments may occasionally be exposed to short-periods of desiccation risk and exhibit properties well suited for survival under such conditions. Many of these species have embryos that are somewhat resistant to desiccation and when fully developed are capable of delaying hatching for days to weeks to months (Scheel 1990; Wildekamp 2004; Wourms 1972c). This phenomena of delayed hatching appears functionally equivalent to Diapause III in annual species.

I propose that having eggs with the ability to survive desiccation and delay hatching represent two adaptations that allowed some species of killifish to colonize marginal or edge aquatic habitats of intermediate unpredictability. These adaptations in turn paved the way for the evolution of more prominent diapause at earlier stages of development, as the ancestors of annual clades pushed the boundaries by colonizing more isolated aquatic habitat and exploiting these new ecological niches.

I (1) assemble and review data regarding habitat use in killifish and show they are found in habitats that grade from permanent to seasonal, (2) present evidence that non-annual killifish embryos can exhibit delayed hatching that is functionally equivalent to diapause III in annuals, (3) demonstrate that delayed hatching is well-suited for relatively short term embryo survival but comparatively ill-suited for long term embryo survival, and (4) present the argument that, once established in marginal aquatic habitats, it is relatively easy to gradually colonize habitats with greater degrees of seasonality, which in turn provides selection for more prominent stages of arrest earlier in development (Diapause I and II), and this process has given rise to convergent evolution of diapause at the most developmentally stable points during embryology.

Habitat use in killifish

Killifish have been described as 'small water' fish that inhabit shallow edge habitats at the periphery of more permanent aquatic water bodies (Scheel 1990). Examples of such habitat include seasonal brooks or creeks that drain to permanent rivers, isolated pools, depressions, and backwaters adjacent to rivers or lakes, and the leading edges of swamps and floodplains that recede considerably on a seasonal basis (Figure 3.2). The initial selective pressure for making the transition to these more temporally unstable habitats could be avoidance of predators (Gilliam and Fraser 2001) or the absence or scarcity of potential competitors (Escalera-Vazquez and Zambrano 2010; Sayer and Davenport 1991). Early in this evolutionary colonization scenario the

close proximity to permanent aquatic water bodies provides a safety net; fish can return to the refuge of permanent water if the marginal habitat deteriorates. Indeed, many extant killifish that inhabit such marginal habitat are adept at surviving at the aquatic-terrestrial interface. Thorough descriptions of such adaptations are available for a few species, mainly in the genera *Kryptolebias* and *Rivulus* (Abel et al. 1987; Gibb et al. 2013; Graham 1997; Lüling 1971; Saul 1975; Sayer and Davenport 1991; Seghers 1978; Seghers and Nielsen 1982). Here I synthesize natural history data on habitat use in killifish. When an annual life cycle is viewed as an endpoint of adaptation to progressively more marginal habitats, a plausible scenario can be constructed as to how an annual life cycle evolved through a series of intermediate steps.

Habitat and life-historical variation within annual killifish

An often implicit assumption, is that the drying of the aquatic habitat causes a complete truncation of lifespan for all killifish in seasonally ephemeral habitats, condensing the life cycle into a single calendar year. This is not always true. Some 'annual' species, such as *Nothobranchius korthausae* on Mafia island off the coast of Tanzania, are found in habitats that may dry in some years but not others (Scheel 1990; Wildekamp 2004) or significantly recede during the dry season but retain low levels of water at the center (Ijumba and Kilama 1991). Ijumba and Kilama (1991) studied a population of *Nothobranchius palmquisti* in a large shallow pond in coastal Tanzania and observed: "During the dry season the pond was observed to dry out except for the central part where a little water, approximately 0.5m in depth, remained." Pools that support several annual killifish in the Orinoco river basin of Venezuela and eastern Colombia are generally shallow, filled with vegetation, and occasionally expansive, but dry on a seasonal basis (Nico and Taphorn 1984). However, these authors sometimes collected fish thought to have been in their second year (apparently because of their exceptionally large size early in the season) implying that the aquatic habitat didn't completely desiccate, enabling some adults to survive the

dry season (Nico and Taphorn 1984 and references therein). Romand and Broche (1983) describe seasonal habitat changes in an ephemeral brook inhabited by *Funulopanchax walkeri* in the Ivory Coast. The authors note a dry period lasting two months (February and March). In late January, when almost no water remained, they found adult fish living in the bottom mud. Some of these fish survived in captivity for more than two years. In February the bottom of the brook was dry but beneath the surface layer was wet mud. After the beginning of the rainy season in April, they found newly hatched fry in the brook but no other size classes suggesting that all adult fish had perished (Romand and Broche 1983).

Some annual killifish that inhabit seasonally ephemeral aquatic habitat may be able to seek refuge in more permanent water bodies during the dry months, depending upon proximity and connectivity. Sonnenberg and Busch (2009) describe the habitat of the type locality for *Callopanchax sidibeorum*, in southwestern Guinea, West Africa. Like other members of the genus, this species is large-bodied with eggs that enter diapause II (Murphy and Collier 1997; Simpson 1979; AF personal observation). The habitat is described as "a depression with small water bodies, ditches and connected pools in a secondary forest adjacent to a small creek." The authors note that there is a seasonal connection between the creek and the stagnant pools and ditches and *Callopanchax* seems to prefer shaded areas with slow flow or standing water. During the rainy season, the pools and ditches are interconnected to the creek but become isolated during the dry season, then eventually completely dry. Since the creek remains year round some adult fish may be able to retreat to this habitat refuge. This latter scenario is supported by several pieces of evidence: 1) observations of the local people (Sonnenberg and Busch 2009), 2) both juvenile and adult fish having been found together, and 3) the interconnectivity and close proximity of annual and permanent aquatic habitat. Furthermore, species in the genus *Callopanchax* are rather large-bodied and can be long-lived in a laboratory environment (AF personal observation)

supporting the notion that they have a different natural history compared to short-lived annuals known to inhabit isolated savannah pools that dry on a seasonal basis (Genade et al. 2005; Podrabsky et al. 1998; Polacik et al. 2011). Even though *C. sidibeorum* may not have a truly annual life cycle characterized by complete population turn-over each year, its embryos likely require entry into diapause as a means of survival because its preferred habitats dry out seasonally. The collection locality of *C. sideibeorum* receives greater than 3000 mm of rainfall per year yet exhibits strong seasonality, with precipitation falling almost entirely between the months of April and November. Furthermore, there were two sympatric non-annual killifish species, *Epiplatys fasciolatus* and *Scriptaphyosemion geryi*, living in this locality (Sonnenberg and Busch 2009). In instances where annual and non-annual killifish are found together one would expect annual species with diapause eggs to utilize peripheral or temporary aquatic habitats subject to seasonal drying whereas non-annual killifish that lack diapause would generally be expected to utilize more stable aquatic habitat. This appears to be the case.

In sum, there are no habitat generalizations that adequately characterize all annual killifish species (Simpson 1979). The simplest and most commonly discussed habitat scenario is that of one wet and dry season annually with complete pool drying and population turn-over each year - as is typically the case with species of the genera *Nothobranchius* (Polacik et al. 2011; Reichard et al. 2009) and *Austrofundulus* (Nico and Taphorn 1984; Podrabsky et al. 1998). However, annual killifish are sometimes found in habitat that doesn't fully dry each year (Ijumba and Kilama 1991; Nico and Taphorn 1984; Scheel 1990), that does dry but is connected to more permanent habitat that allows refuge (Sonnenberg and Busch 2009), that has multiple dryings and fillings within the same season (Podrabsky et al. 1998; Polacik et al. 2014), or that experiences such low or sporadic rainfall that an entire year can pass without there being sufficient water for a complete generation of fish (Pienaar 1968; Simpson 1979; Watters 2009; Wourms 1972c).

Habitat use in non-annual killifish species

Most Aplocheiloidei killifish, including non-annual species, inhabit environments subject to temporal variability and potential short-term desiccation risk. *Rivulus hartii* on the island of Trinidad is representative of this life style, but similar life styles are found in many non-annual killifish (particularly those of the genera *Kryptolebias*, *Rivulus*, *Aphyosemion*, and *Fundulopanchax*). *Rivulus hartii* can be found in streams of various sizes, some with intermittent flow, and forest pools (Figure 3.4; Gilliam and Fraser 2001). The way this species utilizes its habitat is dependent upon the species of fish it co-occurs with (Fraser et al. 1999; Fraser et al. 1995; Gilliam and Fraser 2001; Gilliam et al. 1993). In larger rivers that contain predatory species, *Rivulus* is rarely found in the main river channel and never in open water. Rather, they inhabit edge habitats, often being locally abundant in isolated forest pools and shallow leaf and debris filled backwaters. In smaller tributaries and rivulets that drain into larger rivers, where predator species are usually absent, *Rivulus* are significantly more abundant (Gilliam et al. 1993) and use a much wider range of available habitat, inhabiting both marginal edge habitats as well as the main channel (Fraser et al. 1995). *Rivulus* are thus not adverse to inhabiting open waters, but avoid them when predators are present (Gilliam and Fraser 2001).

Edge habitats, by nature of being on the margins or outskirts of permanent aquatic habitat, are susceptible to periodic desiccation, dependent upon prevailing rainfall patterns (Gilliam and Fraser 2001). *Rivulus hartii*, as is characteristic of the genus, is adept at traversing the complicated patchwork of isolated and semi-isolated shallow pools, depressions, rivulets, and backwaters characteristic of many forest river systems. They do so by flipping or otherwise moving about at night through damp leaf litter (Seghers 1978) earning the common name 'jumping guabine' or 'crazy fish'. It is not uncommon to find several *Rivulus* in each shallow forest depression within a river's floodplain. They are nearly always the only fish species present.

Seghers (1978) found that *Rivulus hartii* placed in an arena of foam rubber saturated in water "explored the arena in a systematic way suggesting the jumps were not random movements." Laboratory and field study confirmed that *Rivulus hartii* readily spawn viable eggs in very shallow water that doesn't fully cover their bodies (Fraser et al. 1999), and eggs are typically deposited in root masses, and leaf litter (Fraser et al. 1999). Shallow forest pools and rivulets, often filled with debris and forest leaf-litter may serve as ideal nurseries for hatchlings and juveniles (Doug Fraser personal communication). If eggs are deposited in pools that experience periodic desiccation, then the fish themselves would leave in search of better quality habitat patches, but the eggs must be able to survive periodic short-term desiccation and delay hatching until conditions are suitable.

Seghers and Nielsen (1982) found that during the dry season *Rivulus hartii* in ephemeral streams congregated at extremely high density in the few remaining pools. In some instances there was complete drying and fish survived buried under moist leaves until the rain season. A similar response has been described for *Rivulus limoncochae* in Ecuador: "Many specimens were obtained when a temporary swamp became dry except for one pool of water in a hole at the base of a fallen tree....Fish were congregated at the perimeter of this pool over a collar of leaf litter." Likewise, (Lüling 1971) described how as the rain season in the Peruvian Amazon draws to an end *Rivulus beniensis* flip about the moist ground and colonize the deepest pools, which are likely to be around the longest. Terrestrial insects make up a significant portion of the diet of *Rivulus hartii* (Fraser et al. 1999; Seghers 1978). They have been reported to take insects from overhanging vegetation up to 14 cm above the water and to forage terrestrially (Seghers 1978). *Rivulus brunneus*, native to Panamanian mangrove forests will leave water to avoid predation by *Hoplias sp.* (Abel et al. 1987), consistent with patterns in *Rivulus hartii*.

Kryptolebias marmoratus is unique among vertebrates as a self-fertilizing hermaphrodite capable of producing genetically identical clonal lineages (Harrington 1961), although some populations retain males (Mackiewicz et al. 2006; Turner et al. 1992). This species is also noted for its amphibious behaviors and inhabitation of tidal mangrove forests subject to periodic and sometimes long term drying. Adults congregate in large numbers in crab holes and hollow mangrove logs that remain spongy and water-saturated for long periods after dry down (Taylor 2012; Taylor et al. 2008). In a laboratory simulation of such conditions adults were able to survive 66 days out of water (Taylor 1990). Furthermore, *Kryptolebias marmoratus* will occasionally forage above the tide line (Abel et al. 1987; Davis et al. 1990; Huehner et al. 1985) and in the laboratory were capable of terrestrially hunting and capturing crickets (Pronko et al. 2013). Captive specimens have been observed to lay eggs on damp mud (Taylor 1990). Investigators have characterized physiological and morphological adaptations that allow this species to survive environmental extremes - dry periods, high temperatures, hydrogen sulfide, and low oxygen (reviewed in Taylor et al. 2012). These adaptations include air-breathing and skin adaptations (Taylor 2012; Wright 2012) that allow for long-term survival out of water (Abel et al. 1987; Taylor 1990; Taylor 2012), insensitivity to high levels of salinity, ammonia, and hydrogen sulfide (Taylor 2012), and a proficient terrestrial locomotion that allows for both feeding and movement between habitats (Gibb et al. 2013; Pronko et al. 2013). Unlike typical fish that flail aimlessly when out of water, *Kryptolebias marmoratus* perches upright on its belly, like a mud skipper, and makes directed and purposeful flipping movements to explore its habitat (Gibb et al. 2013) or switch to a different crab burrow (Taylor 1990).

Although terrestrial or amphibious habits have been documented in relatively few *Rivulus* and *Kryptolebias* species, the general consensus is that all members of these genera are found in marginal habitat that contain few other fish species (Breder and Rosen 1966; Brosset 2003; Costa

2006). This suggests they share the same suite of adaptations - ability to survive drying conditions by taking refuge in available substrate, aptitude at terrestrial locomotion, ability to spawn in shallow water, and eggs that are capable of desiccation resistance and delayed hatching - as the species described above (Abel et al. 1987; Breder and Rosen 1966; Lüling 1971; Scheel 1990; Taylor 2012).

The genera *Rivulus* and *Kryptolebias* are hardly alone among non-annual Aplochieloidei killifish in their penchant for inhabiting forest pools and backwaters, close to and often isolated from small permanently flowing rivers. (Brosset and Lachaise 1995) provide a detailed description of the hierarchical habitat structuring and partitioning characteristics of killifish species, particularly the genus *Aphyosemion*, found in the tropical African rainforest drainages of Gabon. This vivid account of the complex network of structured habitat that includes stagnant pools, permanent rivulets, secondary streamlets, and major streams is remarkably similar to the New World habitat of the genus *Rivulus* (Brosset 2003). Brosset (2003) documented convergence in killifish morphology, behavior, and habitat use in the South American *Rivulus* and African *Aphyosemion* that inhabit similar ecological niches. This is likely due to adaptation to similar environments on two continents with tight correlation between morphology, life-history, and behavior. In particular, species that inhabit intermediate habitats and frequently move about the terrestrial environment appear to have dorsally and ventrally compressed bodies which facilitates maintaining an upright orientation while in shallow water or terrestrial areas (Gibb et al. 2013).

Delayed hatching in non-annual killifish embryos

The embryos of annual killifish can arrest development for long periods at early stages of development or skip such diapause stages and proceed directly to diapause III (Podrabsky et al. 2010a; Wourms 1972c). Furthermore, fully developed (Diapause III) embryos exhibit a great deal of variation in hatching time (Wourms 1972c; Scheel 1990; Chapter 2). This phenomena is

consistent with diversification bet-hedging, a risk spreading strategy that could be adaptive given variation in the timing of pool filling and risk of false-starts in regards to season beginning date (Simons and Johnston 1997). Non-annual killifish that inhabit marginal habitats may experience a lesser degree of unpredictability regarding habitat duration such that eggs may experience short-term periodic desiccation risk. I therefore predicted that non-annual killifish species might have eggs capable of surviving desiccation and the ability to delay hatching for short periods (Wourms 1972c). Further, I predicted that these properties might be ancestral to Aplocheiloidei killifish. I tested these hypotheses using non-annual killifish species representative of some genera that inhabit marginal (*Rivulus hartii*, *Fundulopanchax gardneri*, *Fundulopanchax scheeli*) and more permanent (*Pachypanchax playfairii*) aquatic habitat, as well as a species that likely represents the ancestral condition outside the Order Cyprinodontiformes (*Oryzias latipes*). I tracked cohorts of eggs through development and recorded hatching date such that I could generate and compare the distribution of hatching times among these non-annual killifish species (Figure 3.5, 3.6A; for population locality data see Chapter 1 Supplementary Materials).

Rivulus hartii eggs exhibited a mean hatching time of 23.8 days, but with a highly skewed distribution (Figure 3.6A). Eggs progressed through development in synchrony, with 70% hatching within 25 days. An average of 16 % of eggs delay hatching for more than 40 days, with an extreme value of 69 days. Since *Rivulus* eggs were water-incubated hatching could take place at any time. Eggs that exhibited this 'delayed hatching' were not defective because they produced viable fry that appeared normal. Similar delayed hatching responses were observed in two other non-annual species (*Fundulopanchax gardneri* and *scheeli*) that inhabit small forest creeks and pools in Africa (Figure 3.5). *Pachypanchax playfairii*, a representative non-annual killifish from the family Aplocheilodae that inhabits permanent streams on the Seychelles islands, exhibited an embryo hatching distribution that was less skewed than that of these other three

species (Figure 3.5). *Oryzias latipes* is in the order Beloniformes, which is sister to the order Cyprinodontiformes (Figure 3.1; Setiamarga et al. 2008) and thus represents a reasonable choice of outgroup. Embryos of this species had a hatching distribution that was not significantly different than normal (One-Sample Kolmogorov-Smirnov Test, P=0.096) although there was an apparent delay between when eggs completed development and when they hatched (see Teather et al. 2000). These results accord well with the hypothesis that delayed hatching, which would manifest as a right skewed hatching distribution is a more prevalent phenomenon in species that inhabit waters with greater desiccation risk / unpredictability.

Desiccation resistance, delayed hatching, and survival on peat moss in *Rivulus hartii* embryos

Eggs of *Rivulus hartii* were incubated in wells containing damp peat moss rather than water. This setting mimics what eggs would experience at the bottom of a drying forest pool containing damp soil and leaf litter. Eggs treated in this manner developed continuously until embryos were fully formed, but the vast majority of embryos didn't hatch. They instead remained viable for up to 80 days. The survival distribution of embryos incubated in such conditions is indicated in Figure 3.6C. Just under 80 percent of embryos survived a period of 20 days under these partial desiccation conditions, a number roughly comparable to embryos that were water incubated. Thus, *Rivulus hartii* embryos spawned in shallow water that subsequently dries down, leaving them in moist substrate, are capable of developing normally, delaying hatching once development is complete, and surviving in such a state for several weeks. The embryos of other non-annual killifish are capable of surviving on damp peat moss (Varela-Lasheras and Van Dooren in press), suggesting that the phenomena demonstrated here is more widespread.

Delayed hatching, desiccation resistance, and habitat use as pre-adaptations

"It is suggested that obligate Diapause III may have resulted from the intensification of the “delayed hatching” phenomenon found in some non-annual cyprinodonts and its subsequent

assimilation into the annual fish developmental pathway" (Wourms 1972c). Traditionally diapause has been defined as an endogenously maintained arrest or delay in development accompanied by metabolic depression, as opposed to delayed hatching, a short-term facultative delay in hatching in which metabolic rate remains high and embryos hatch readily as soon as conditions are appropriate (Martin 1999; Podrabsky et al. 2010b; Warkentin 2011). Annual killifish embryos in diapause III decrease metabolic rate over a period of several weeks (Levels et al. 1986a; Podrabsky and Hand 1999; AF unpublished) and can survive in this state for a period of several months (Wourms 1972c; Chapter 2). In non-annual killifish a variable portion of embryos spontaneously delay hatching when development is complete (Figure 3.5, 3.6A) and survive in this state for several weeks (Wourms 1972c). In *Rivulus hartii* embryonic heart rate decreases as time post-fertilization increases (Figure 3.6B), suggesting that there is likely to be a decrease in metabolic rate, since the two are functionally linked (Boyd et al. 1995). A similar heart rate response was observed in *Fundulopanchax gardneri* embryos; heart rate peaked 10-15 days post-fertilization and declined precipitously as the embryos exhibited long periods of delayed hatching (Kroll 1984). Heart rate increased again approximately 50 days post-fertilization in embryos that hadn't yet hatched (Kroll 1984), this uptick likely occurring in preparation for hatching.

The delayed hatching spontaneously exhibited by non-annual killifish eggs represents a functionally equivalent precursor to diapause III in annual species. That is, the 'less intense' and relatively short-term delayed hatching exhibited by non-annual killifish and the prolonged and more frequent diapause III in annual killifish may represent ends of a continuum rather than dichotomous categories. The capacity for short periods of delayed hatching appear to be an intrinsic property of killifish eggs, perhaps initially with no adaptive significance. In species that colonized marginal aquatic habitat this occasional response may have taken on adaptive value and

subsequently became a more regular occurrence. A gradual slowing of heart and metabolic rate presumably came later as a means to conserve yolk resources and remain viable for longer periods when the conditions for hatching were unfavorable.

Several killifish in the family Fundulidae (outside the Aplocheiloidei) spawn eggs in tidal marshes and have eggs that exhibit desiccation resistance and are capable of delayed hatching (Martin 1999; Martin et al. 2004; Podrabsky et al. 2010b; Tingaud-Sequeira et al. 2009). For example, *Fundulus heteroclitus* inhabits saltwater marshes and spawns eggs in protected areas near the high water mark at high tide during the lunar cycle. The eggs then complete development while exposed to the air, and are typically ready to hatch when inundated during the next high tide (Taylor 1999). If these traits (desiccation resistance and delayed hatching) are ancestral in Cyprinodontiform fishes, which they appear to be (Wourms 1972c) then they would represent preadaptations that allowed killifish to exploit periodically desiccating environments thus setting the stage for the colonization of seasonally ephemeral waters and the evolution of diapause I and II and a true annual life cycle.

Conclusion

1. **The evolution of an annual life cycle through intermediate steps.** In presenting a scenario for the evolution of diapause and annual life cycle I have emphasized variation within Aplocheiloidei killifish, the evolutionary precursors in ancestral lineages, and the multiple independent origins of diapause. Far from being isolated evolutionary relics at the end of long lineages, there is much life historical and developmental variation among extant species. This comparative variation, combined with natural replicates, presents opportunities for the comparative study of these traits in a phylogenetic context which could prove valuable both in understanding how this life cycle evolved and also testing hypotheses about function.

2. **Natural history.** Annual killifish provide a unique vertebrate system to study a variety of questions relating to life history evolution, adaptation to environmental unpredictability, and developmental biology. Due to their short lifespan, annual fishes, particularly those of the genus *Nothobranchius*, are rapidly becoming a vertebrate model system for the study of the genetics of aging (Reichwald et al. 2009; Valenzano et al. 2009). Further study of the evolutionary history, ecology, and natural history of annual fish may prove to be of great importance in interpreting patterns of aging and development. It is also likely the case that it will be difficult to make broad generalizations regarding the natural history of all 'annuals' as they inhabit environments that differ in predictability, duration, and permanence. However it is this fact that makes them great subjects for a host of ecological and evolutionary questions that uses this natural variation.

3. **Diversity of reproductive mode within the Order Cyprinodontiformes provides perspective.** Within this order of 1,264 species there is remarkable diversity - internal fertilization, intromittent organs, and live-bearing has evolved a minimum of three times, with the further evolution of placentation (maternal provisioning of offspring during gestation by means of a placenta) and superfetation (gestating multiple broods at different developmental stages) on multiple occasions (Avise 2013; Parenti 2007; Pollux et al. 2009; Reznick et al. 2002). In the Aplocheiloidei killifish, we see desiccation resistant eggs, delayed hatching, and diapause as species adapted to ephemeral aquatic habitat across two continents. There are other North American killifish (family Fundulidae) that spawn in the intertidal zone and have desiccation resistant eggs that complete development aerially (Martin 2014), and yet others (the pupfish) are found only in isolated desert holes characterized by extremes of salinity, pH, and temperature (Brown and Feldmeth 1971). Lastly, several species within the Order have asexual or semi-sexual reproduction, which is extremely rare in vertebrates (Avise 2008; Vrijenhoek 1994).

Figure 3.1. Phylogenetic tree showing the taxonomic distribution of diapause and habitat type in killifish (redrawn after Chapter 1).

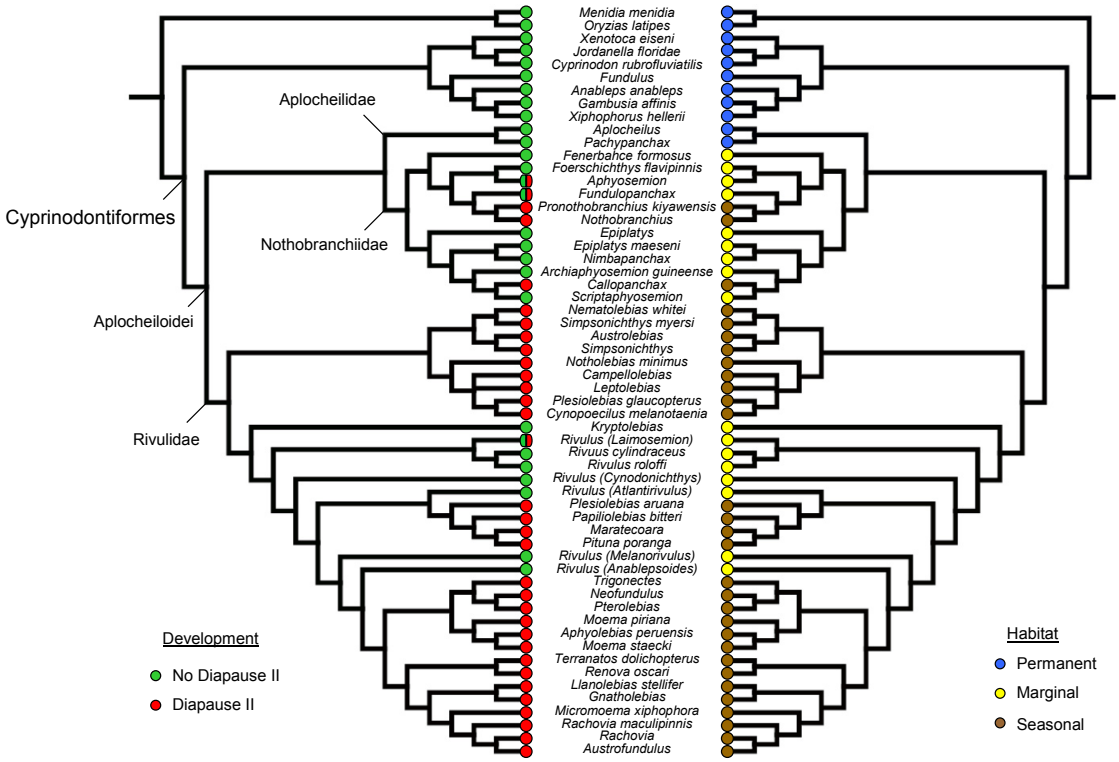

Figure 3.2. Drawing of five progressively more ephemeral aquatic habitat types in which killifish are found.

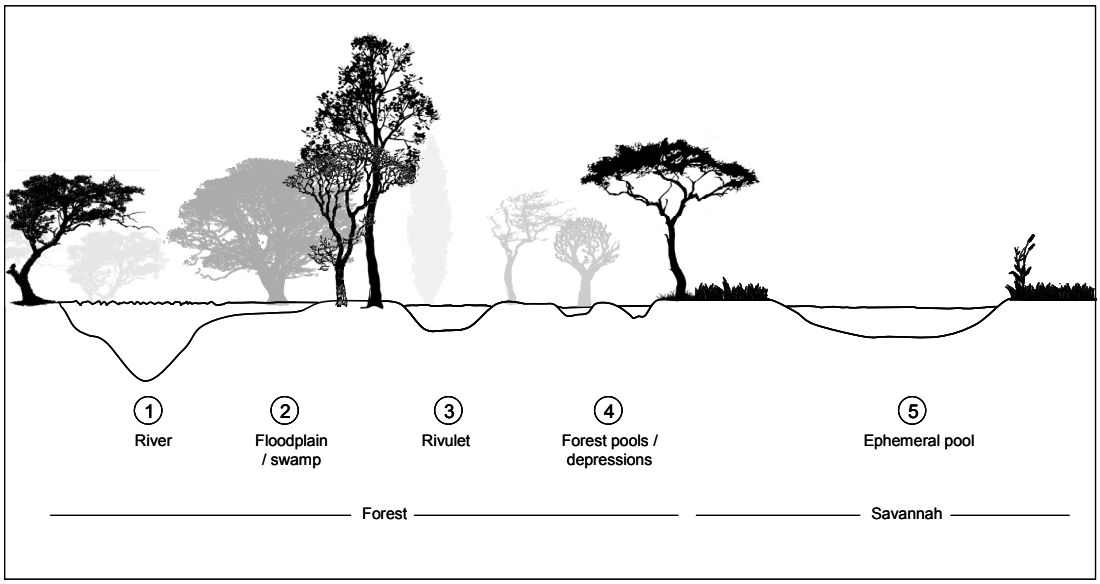

Figure 3.3. (A, B) Ephemeral pools in savannah habitat in Mozambique that contained the annual killifish *Nothobranchius furzeri* (C). (D) Fully developed (Diapause III) *Nothobranchius furzeri* embryos on peat moss.

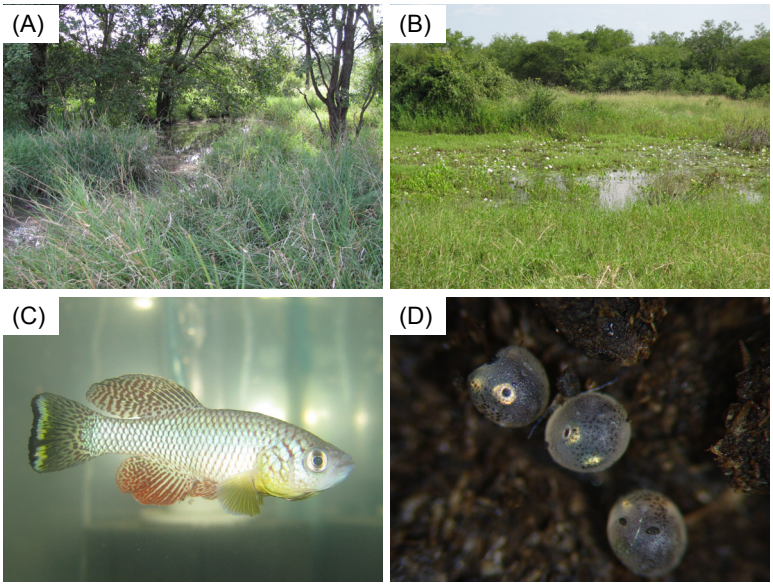

Figure 3.4. Different habitat of *Rivulus hartii* on the island of Trinidad. (A) Wide, swiftly flowing river. Here, *Rivulus hartii* tend to be found in shallow edge regions that lack current. (B) Low flow, tributary to larger river. This tributary contained predatory species and *Rivulus* were found at high density in a very shallow side channel covered by over-hanging vegetation. (C) Pitch lake in Southern Trinidad. This unique habitat resembles an expanse of naturally occurring asphalt. During the rainy season it is covered in water, during the dry season (pictured) water recedes to narrow, sometimes deep cracks. (D) Small rivulet that lacks predatory species. *Rivulus* were found throughout the channel. (E) Isolated forest pool that during the wet season is part of a small rivulet. (F) *Rivulus hartii* in a five-gallon bucket captured and released unharmed as part of an assessment of density. Note the individuals that have flipped and adhered to the sides. *Rivulus* are adept escape artists and by making several leaps in succession could get out of the bucket. In nature, they are capable of crossing land via these sustained flipping movements and surviving in moist environments such as leaf litter.

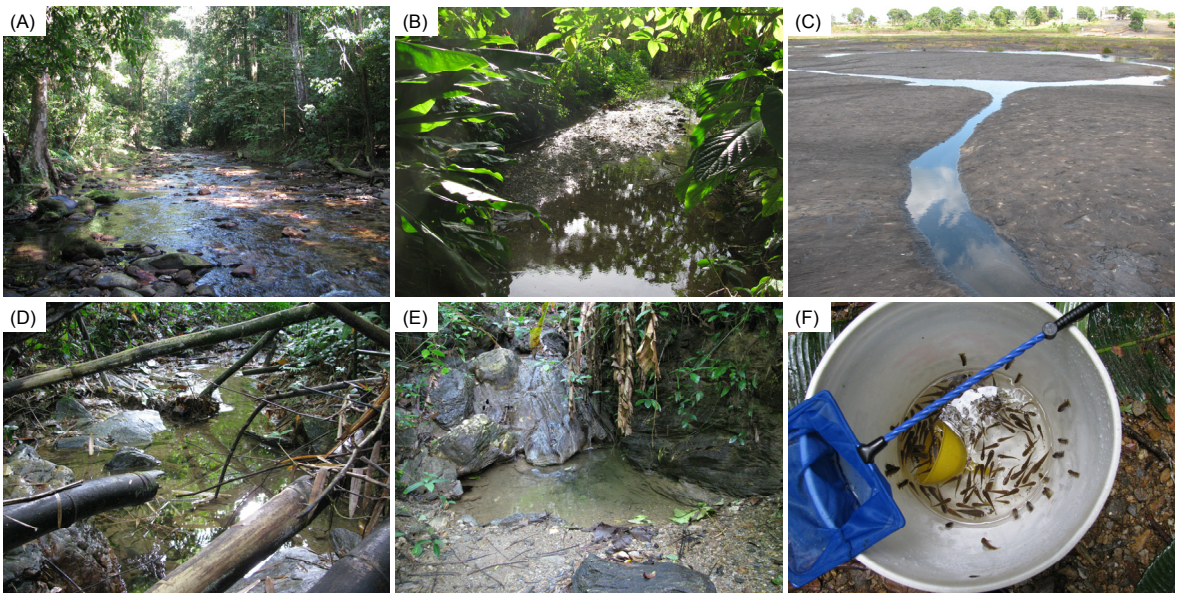

Figure 3.5. Frequency histograms depicting the distribution of hatching times (days from fertilization until hatching) in (A) *Pachypanchax playfairii*, (B) *Oryzias latipes*, (C) *Fundulopanchax scheeli*, and (D) *Fundulopanchax gardneri*. Adult fish of each species were maintained in stock tanks and provided with floating plastic plants as spawning substrate. Eggs were collected daily (such that I knew the twenty four hour window in which they were fertilized) checked for viability, rinsed in distilled water, and incubated one egg per well in 24-well tissue culture plates (3.5 mL per well) containing clean aquarium water with the same parameters at which the adult fishes were kept. Eggs were incubated at 25 C and water in each well was changed approximately once weekly. Eggs were monitored at least once daily and the date at which embryos went bad or hatched was recorded. Only embryos that successfully hatched into viable fry were used to generate hatching distributions. Hatching distributions were constructed by plotting the frequency of 'days until hatch' for each species.

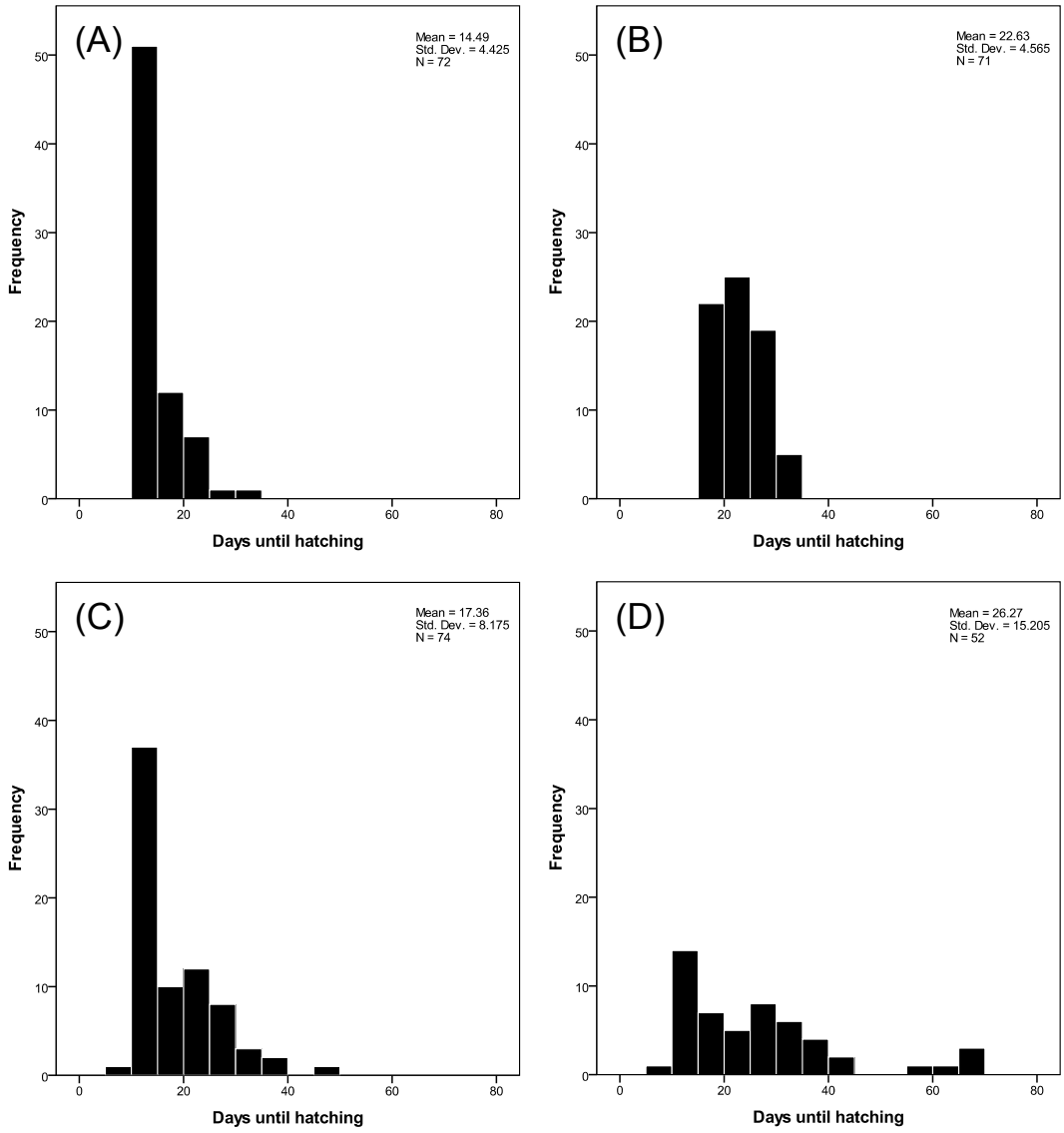

Figure 3.6. (A) Frequency histograms depicting the distribution of hatching times (days from fertilization until hatching) in *Rivulus hartii* embryos that were incubated individually in aquarium water. (B) Embryonic heart rate as a function of days post fertilization in *Rivulus hartii* embryos. (C) Cumulative survival of *Rivulus hartii* embryos incubated on slightly moist peat moss (n=76). Pictured are an embryo at 2 days post-fertilization and approximately 14 days post-fertilization, when development is complete. (D) *Rivulus hartii* embryo on peat moss. Note the indentation in the egg surface due to water loss.

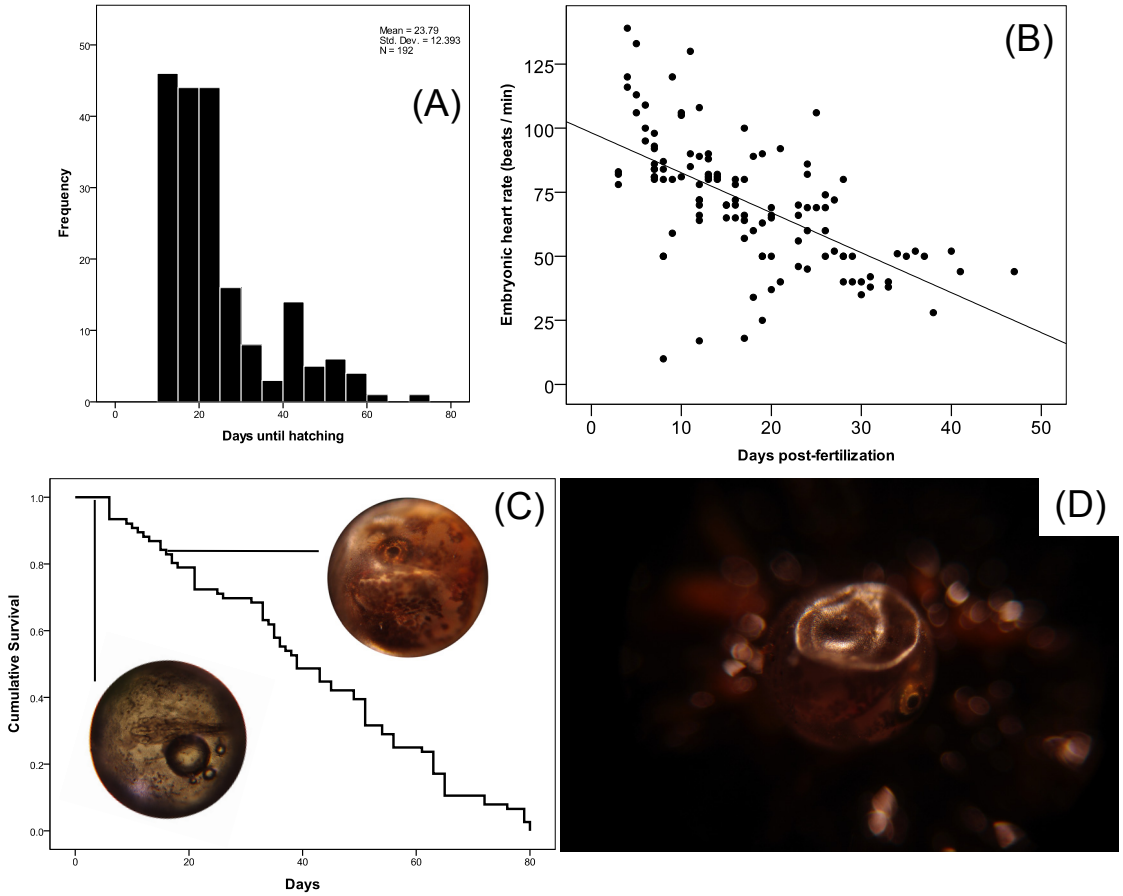

Table 3.1. Glossary.

Annual killifish. Killifish that inhabit ephemeral aquatic environments that dry seasonally and are able to persist through the production of desiccation resistant embryos capable of undergoing diapause at one or more stages of development.

Annual life cycle. A life cycle in which growth, maturity, and reproduction take place within a single calendar year. Typically, associated with the production of embryos (seeds, cysts, eggs) capable of surviving harsh seasonal conditions.

Delayed hatching. Phenomenon in non-annual killifish in which embryos that have completed development, either spontaneously or because conditions are not appropriate, delay hatching for a period of days, weeks, or months. In annual killifish this is referred to as entering diapause III and in non-annual killifish it appears functionally equivalent.

Diapause. Developmental arrest with an accompanying reduction in metabolic rate. In killifish adapted to life in ephemeral aquatic environments diapause can occur during the embryo stage at any of three points during development (Diapause I, II, and III).

Diapause I. The earliest stage in which annual killifish embryos are capable of arresting development. Occurs before the embryonic axis has formed in what has been termed the dispersed cell phase (Wourms 1972c). Embryos have been induced to enter this diapause through exposure to low temperatures, hypoxia, or chemical factors produced by adult fish (Wourms 1972c, Inglima et al. 1981, Denuce 1989).

Diapause II. Stage mid-way through embryogenesis in which annual killifish embryos can arrest; occurs after the formation of the body axis, in embryos possessing approximately 38 pairs of somites. Embryos of annual species spontaneously enter this state under a wide range of environmental conditions, embryos are capable of arresting at this stage for many months, and it is at this stage that they are most resistant to temperature extremes, desiccation, and oxygen deprivation (Matias 1982, Matias and Markofsky 1978, Podrabsky and Hand 1999, Podrabsky et al. 2010).

Diapause III. Stage in which annual killifish embryos are fully developed and awaiting appropriate environmental hatching cues. Embryos at this stage exhibit a slowing of heart and metabolic rate (Podrabsky and Hand 1999).

Ephemeral aquatic environment. Any aquatic environment that periodically or regularly dries out. Examples include isolated pools found in grassland or savannah habitat that are formed during the rainy season, small forest brooks or streams that only flow during the rainy season, swamps or floodplains that expand during the rain season and recede during the dry, and pools, depressions, or puddles directly adjacent to permanent water bodies.

Killifish. A general term for oviparous (egg-laying) fishes of the Order Cyprinodontiformes. This includes all species in the families Aplocheilidae, Cyprinodontidae, Fundulidae, Nothobranchiidae, Profundulidae, Rivulidae, and Valenciidae. Killifish are generally small-bodied, sexually dimorphic, and inhabit mainly fresh or brackish waters across much of North and South America, Africa, Southern Europe, the Middle East, Southern Asia, and several islands in the Indian Ocean. The name killifish is derived from the Dutch work "kilde", which means small puddle.

Non-annual killifish. Killifish that inhabit aquatic environments that do not dry on a regular, seasonal basis. Such species have embryos which are incapable of entering diapause I or II.

Cyprinodontiformes. An order of ray-finned fishes that includes the egg-laying killifish and live-bearers (families Goodeidae, Anablepidae, and Poeciliidae).

Aplocheiloidei. A sub-order of Cyprinodontiformes that includes the families Aplocheilidae, Nothobranchiidae, and Rivulidae.

Aplocheilidae. A, family of killifish within the sub-order Aplocheiloidei, that contains 14 recognized species found on Madagascar, India, the Seychelles Islands and Southeast Asia. Diapause II has not evolved in this family.

Nothobranchiidae. A family of killifish, within the sub-order Aplocheiloidei, that contains 261 recognized species found on the continent of Africa. Diapause II (roughly equivalent to an annual life cycle) has evolved independently at least three times within this family.

Rivulidae. A family of killifish, within the sub-order Aplocheiloidei, that contains 392 recognized species found in Central and South America and Caribbean islands. Diapause II (roughly equivalent to an annual life cycle) has evolved independently at least three times within this family.

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Concluding Remarks

An annual life cycle allows for the colonization of otherwise uninhabitable terrain and thus opens new niche space for whole groups of organisms. This life cycle has evolved independently in many different groups across the tree of life - plants, insects, crustacea, and rarely, vertebrates. In this dissertation I examined the evolution of an annual life cycle in killifish and how it allows for adaptation to seasonal and variable aquatic habitat.

Chapter one demonstrated convergent evolution of alternative developmental pathways associated with diapause in both African and South American annual killifish. The comparative methods suggest diapause II, the most prominent stage of developmental arrest that occurs mid-embryogenesis, evolved independently multiple times in killifish. Annual killifish embryos destined to enter diapause II become conspicuously different in appearance from embryos that skip this diapause stage. This morphological and physiological divergence occurs prior to and during the phylotypic period in which vertebrate development is supposedly highly conserved owing to an increase in developmental and genetic interactions during organogenesis. The observed divergence along alternative developmental pathways apparently functions to reduce investment in organ systems for embryos entering diapause II, which has the effect of reducing embryonic metabolic rate and increasing long-term survival. Taken together these results emphasize the primary role of natural selection in generating this prominent intraspecific divergence during early and mid-development. This phenomenon of divergence early in development in association with alternative developmental pathways may be more widespread than is currently recognized. 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 and this variation could prove useful in providing a fuller picture of the role of selection and constraint during embryology.

In chapter two I performed a series of experiments to test two methods (phenotypic plasticity and bet-hedging) by which killifish eggs may have adapted to survive in variable environments. I found that the embryos of annual killifish exhibit a combination of both bet-hedging (intrinsic variability in whether and for how long diapause is entered) and phenotypic plasticity (the proportion of eggs that enter diapause is sensitive to environmental cues - light and temperature - that are correlated with seasonality). This result is consistent with recent theory that considers the intermediate reliability of cues indicating future conditions (Wong and Ackerly 2005). One can imagine phenotypic plasticity and bet hedging as being on opposite ends of a continuum. In a completely unpredictable environment it is advantageous to produce eggs with a scattershot of different development times such that no matter what the environment turns out to be some eggs will be able to capitalize. Alternatively if the environment is perfectly predictable one would expect eggs of the appropriate type to be produced. However in nature, the environment is likely neither perfectly predictable or unpredictable, there will be a large parameter zone where environmental cues are somewhat correlated with seasonality, but not perfectly so, such that it may be advantageous to have a combination of both bet-hedging and plasticity. In this area there is scope for modeling effort and eventually testing quantitative predictions perhaps using different populations that vary in the degree of environmental predictability.

Chapter three reviewed adaptations for life in ephemeral aquatic environments in killifish and synthesized data on habitat, life historical, and embryonic diversity in order to better understand how an annual life cycle could have evolved through intermediate steps. Different species of killifish are found in a variety of aquatic habitats that span a continuum from permanent (never dry out) to seasonal (dry out regularly on an annual basis). This provides the variation necessary to perform comparative experiments and make inferences regarding the

evolution of this life cycle in a phylogenetic context. Non-annual killifish that inhabit marginal aquatic habitat exhibit a number of features, particularly the embryo properties of desiccation resistance and delayed hatching, that likely make them pre-adapted toward the colonization of ephemeral waters and the evolution of a true annual life cycle. The natural replication and variation in development among living species provide future research opportunities to look at the genetics of diapause and gain a better understanding of convergence at the molecular level.

Some universal themes in the annual plant literature include the evolution of seed dormancy, bet-hedging and plasticity in the timing of germination, rapid growth and development, tradeoffs between size and fecundity, and rapid senescence. Many of these same phenomenon apply to annual killifish adapted to life in ephemeral aquatic environments. The existence of this abbreviated life cycle within a vertebrate, its independent origins, and natural variation among extant species provide a multitude of future research opportunities pertaining to adaptation to environmental extremes, vertebrate embryology, and aging. Further study of the evolutionary history, ecology, and natural history of annual fish may prove of great importance.