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Alternative developmental pathways associated with diapause regulated by temperature and maternal influences in embryos of the annual killifish *Austrofundulus limnaeus*

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SUMMARY

Embryos of the annual killifish *Austrofundulus limnaeus* enter a state of developmental arrest termed diapause as part of their normal developmental program. Diapause can occur at two distinct developmental stages in this species, termed diapause II and III. When incubated at 25°C, most embryos enter diapause II, whereas a small percentage of 'escape' embryos develop continuously past diapause II and enter diapause III. Control of entry into diapause II can be altered by maternal influences and the incubation environment experienced by the embryos. Young females produce a higher proportion of escape embryos than do older females. In addition, increasing the incubation temperature from 25 to 30°C induces all embryos to escape from diapause. Surprisingly, escape embryos follow a different morphological and physiological developmental trajectory than do embryos that enter diapause II. Development of anterior structures is advanced compared with that of posterior structures in escape embryos when compared with embryos that will enter diapause II. The difference in timing of development for these two trajectories is consistent with changes observed between two species but is very atypical of variation observed within a species. Importantly, the two developmental pathways diverge early in development, during the segmentation period, when, according to evolutionary theory, constraint on developmental pathways should be relatively high. The possession of alternative developmental pathways in a vertebrate embryo is a novel finding, the ecological and evolutionary importance of which is still unknown, but potentially significant in terms of life-history evolution.

Key words: development, heart rate, morphology, temperature, maternal effect, diapause.

INTRODUCTION

Annual killifish are found in areas of Africa and South America that experience pronounced dry and rainy seasons. There are several hundred species of fish that exhibit embryonic diapause in this group that is composed of two separate families, the African Aplocheilidae and the South American Rivulidae (Murphy and Collier, 1997). Although the evolutionary history of this group is not clearly understood, it is likely that diapause has arisen more than once in South America (Hrbek and Larson, 1999), and there is some contention that diapause evolved separately on the two continents. Regardless of evolutionary history, there is a remarkable similarity between the development of all annual killifish (Wourms, 1972a; Wourms, 1972b; Wourms, 1972c). There are three possible developmental stages in annual killifish when embryos can enter a reversible metabolic and developmental arrest termed diapause I, II and III (Wourms, 1972a; Wourms, 1972c). Diapause I is often described as facultative and occurs early in development before the establishment of the embryonic axis (Wourms, 1972c). Diapause II occurs about midway through development just before the major stages of organogenesis. Diapause II embryos possess 38 pairs of somites, the foundations of the central nervous system, including optic cups and otic vesicles and olfactory placodes, and a functional tubular heart (Wourms, 1972a; Wourms, 1972c). This stage of diapause represents the stage of greatest resistance to environmental stresses such as anoxia and dehydration (Podrabsky et al., 2001; Machado and Podrabsky, 2007; Podrabsky et al., 2007). Diapause III embryos have completed embryonic development and are awaiting the appropriate cues to hatch (Wourms, 1972c). Because there are several stages of development when embryos can arrest, and the sojourn of an embryo in diapause is highly variable, a single batch of embryos can produce a wide range of developmental stages when observed at a single point in time (Wourms, 1972c).

Austrofundulus limnaeus is an annual killifish that survives in ephemeral ponds in regions of northern South America, especially Venezuela (Hrbek and Larson, 1999). The ponds that these fish inhabit are especially harsh, unpredictable and variable (Podrabsky et al., 1998). In the laboratory, under our standard rearing conditions and an incubation temperature of 25°C, most embryos enter diapause II midway through development and then later enter diapause III just before hatching (Podrabsky and Hand, 1999). A small percentage of the embryos do not enter diapause II but instead develop continuously from fertilization to diapause III. Wourms (Wourms, 1972c) first described these 'escape embryos' in a closely related Colombian species, *Austrofundulus myersi*. Because diapause II embryos are very tolerant of dehydration and other environmental stresses, skipping this stage of diapause might have profound effects on the likelihood of survival through the dry season.

Animal development is thought to be buffered against genetic and environmental perturbations to ensure accurate recapitulation of form and function, a concept termed 'canalization' by Waddington (Waddington, 1942). Canalization and developmental stability both contribute to the generation of robust developmental programs that are highly constrained in time and space (Willmore et al., 2007), especially during early development and the establishment and patterning of the primary body axes (Duboule, 1995). Thus,

developmental plasticity (West-Eberhard, 2005; Gomez-Mestre and Buchholz, 2006) and heterokairy (inter-individual variation in developmental sequence or timing) (Spicer and Burggren, 2003; Spicer and Rundle, 2007) are minimized in most developmental systems, especially among the vertebrate taxa. In most cases where alternative phenotypes have been documented in vertebrates, they tend to occur late in the developmental sequence, and typically as rather minor alterations in morphology or physiology (Spicer and Burggren, 2003; Gomez-Mestre and Buchholz, 2006; Spicer and Rundle, 2007; Storz and Travis, 2007). Here, we describe two alternative developmental pathways that diverge during formation of the primary embryonic axis and are associated with entry into diapause in embryos of the annual killifish *Austrofundulus limnaeus*. Development through the alternative pathways is part of the normal developmental program in this species and can be regulated by maternal effects and by the incubation temperature experienced by the embryo.

MATERIALS AND METHODS

Husbandry of adults and collection and treatment of embryos

Adult *Austrofundulus limnaeus* (Schultz 1949) were housed in the aquatic vertebrate facility at Portland State University according to methods outlined previously (Podrabsky, 1999). Embryos were collected from 42 spawning pairs of fish. For the first four days of development, the embryos were incubated in embryo medium formulated to mimic the ionic composition of their native ponds (10 mmol l^{-1} NaCl, 2.14 mmol l^{-1} $MgCl_2$, 0.8 mmol l^{-1} $CaCl_2$, 0.14 mmol l^{-1} KCl, 0.0013 mmol l^{-1} $MgSO_4$) (Podrabsky et al., 1998; Podrabsky, 1999). At four days post-fertilization (d.p.f.) embryos were treated with 0.01% sodium hypochlorite (Clorox bleach) to prevent fungal infections (Podrabsky, 1999). Following the bleaching treatment, the embryos were incubated in embryo medium supplemented with 10 mg l^{-1} gentamycin sulfate (Podrabsky, 1999). In all experiments, embryo medium was changed daily. All embryos were incubated for the first 1 d.p.f. at 25°C. Embryos were then either left at 25°C or transferred to 20 or 30°C, depending on the experiment. For some experiments, the embryos from each spawning pair were kept separate, whereas in other experiments the embryos were combined into one large sample. In both cases, each experiment was replicated using at least four different spawning events.

Effect of maternal age on the proportion of escape embryos

Embryos were collected from six spawning pairs of fish selected randomly from the larger breeding stock of the 12th laboratory generation. These fish were allowed to spawn twice a week for the entire reproductive lifespan of the fish. Embryos were incubated at 25°C in the dark and were kept separated by spawning pair for the duration of the experiment. At 20 d.p.f., the embryos were inspected with an inverted compound microscope (Leica DMIRB) and scored as embryos that were on track to enter diapause II or escape embryos. Embryos enter diapause II at 24–26 d.p.f. when incubated at 25°C, and thus scoring the embryos at 20 d.p.f. allowed for a clear distinction between diapausing and escape embryos, while ensuring that embryos were not able to enter diapause and then immediately exit. There is little ambiguity between embryos that will enter or escape from diapause II; escape embryos are morphologically and physiologically distinct from diapausing embryos (see morphology results below). The number of embryos that entered or escaped diapause was recorded and used to calculate the percentage of escape embryos for each clutch produced by a female. In most cases, the spawning pairs of fish were kept the same. However, to test for possible male-specific effects on the production of escape embryos, the male fish were swapped for six spawning events starting when the fish were approximately 200 days old.

The effect of incubation temperature on the proportion of escape embryos

Embryos were collected from a stock of 42 pairs of fish from the 14th laboratory generation of our stock of *A. limnaeus*. All the embryos from each spawning event (always over 1000 embryos) were grouped together, and embryos were sampled from this group randomly. Each spawning event is considered a single replicate, and each experiment was replicated with at least four different spawning events. Groups of 24 embryos were housed in plastic 24-well plates (1 embryo per well). Each well contained 5 ml of embryo medium supplemented with 10 μg l^{-1} gentamycin sulfate. Embryos were observed periodically during development using an inverted compound microscope (Leica DMIRB) and were scored as entering diapause II or escaping diapause at 13 d.p.f. when incubated at 30°C and 20 d.p.f. when incubated at 25°C. Embryos incubated at 20°C were observed for over 2 months, and all embryos entered diapause II.

Temperature transfer experiments were performed to test when during development temperature might have an effect on the proportion of embryos that escape diapause II. For these experiments, the embryos from 42 spawning pairs of older fish (over 200 days old) were combined as described above. All the embryos were incubated at 25°C until 1 d.p.f. At this time, most of the embryos (typically over 1000 embryos) were transferred to 30°C and allowed to continue to develop. Embryos were incubated at 30°C from 1 d.p.f. until transfer back to 25°C in groups of 30–50 embryos in 100 mm × 15 mm plastic Petri dishes. On the day of transfer, the embryos were staged according to the number of pairs of somites and then placed into a 24-well plate at 25°C (1 embryo per well, as described above). The embryos were then observed a week later (well before entry into diapause) and were scored as escaping or entering diapause II. One hundred embryos from each spawning date were left at 25°C for the duration of the experiment to measure the background proportion of escape embryos produced on that spawning date.

Morphology of escape and diapausing embryos

Embryos were incubated as described above in groups of 30–50 embryos in 100 mm × 15 mm Petri dishes at either 25 or 30°C in the dark. On the day of measurement, the embryos were separated into a 96-well plate at a density of one embryo per well. Embryos were transferred to a depression slide containing 3% methyl cellulose and staged by counting the number of pairs of somites. The embryos were then manipulated with dental picks until a clear and flat image of the head region could be captured using a Leica DC480 digital camera mounted on an inverted microscope. The width of the head at the optic cups and the length of the head from the snout to the rear of the otic vesicles were determined using ImagePro Plus software previously calibrated using images of a stage micrometer at the appropriate magnification. Following observation, the embryos were returned to the 96-well plate and observed for several days to confirm their developmental trajectory as escape or diapausing embryos. Each embryo was only measured a single time.

Determination of heart rate

A pilot experiment was performed to assess the stability of heart rate measurements and the time-course of shifts in heart rate in response to shifts in temperature using our experimental setup. For this experiment, heart beats were counted for 30 s and multiplied by two

to produce measurements in beats min^{-1}. Escape embryos (24–30 pairs of somites, N=6) incubated at 25°C were placed into a temperature-controlled microscopy chamber (20/20 Technology, Wilmington, NC, USA) pre-equilibrated to 25°C. The temperature was then increased to 30°C and the heart rate was determined as soon as the temperature in the chamber reached the new set-point, a time of about 3 min. The heart rate was determined again 10 min later. The temperature set-point was then changed to 25°C, and the heart rate was determined at 3, 13, 23 and 60 min following set-point change. The set-point was then changed back to 30°C, and the heart rate was measured at 5, 10 and 20 min following the change in set-point.

To determine the heart rates of embryos developing along the two developmental trajectories, embryos were collected and pooled as described above for the morphological studies. On the day of observation, the embryos were separated into 24-well plates at a density of one embryo per well. The embryos were staged by counting pairs of somites. The heart rate was determined by observing embryos for 2 min intervals under an inverted compound microscope. Temperature was controlled as described above at either 25 or 30°C during the observation period. Embryos were placed in the temperature-controlled observation chamber 10–15 min before determining the heart rate. Embryos were always measured first at their experimental incubation temperature and then switched while in the observation chamber to the alternative temperature. The observation chamber took less than 2 min to reach the new temperature. Embryos were allowed 10–15 min at the new temperature before the second determination of heart rate. The microscope light was kept at a very low level for the duration of the heart-rate-determination studies in order to minimize the effects of changes in light intensity and radiant heating on the embryos. Following determination of heart rate, the embryos were placed in the 24-well plates and returned to their experimental temperature. Several days later, the embryos were scored as escape or diapausing embryos based on their morphology.

Statistics and data analysis

Graphical and statistical analyses of the data were performed using Prizm 5.0 software (GraphPad). Where appropriate, analysis of variance (ANOVA), *t*-tests or linear regression analysis were used. Tukey's honest significant difference (HSD) or Dunnett's test were used for *post hoc* comparisons where appropriate. Statistical significance was always determined at a level of P<0.05. For percentage data, the statistics were applied to the arcsine transformation of the proportions (Zar, 1996).

Table 1. A summary of experiments performed to investigate the biology of escape embryos in *Austrofundulus limnaeus*

		Temperature (°C)		
	Treatment	Incubation	Measurement	Figure
Female age				
Young		25	25	1, 2
Old		25	25	
Temperature				
Old	Constant	20	20	3
	Constant	25	25	
	Constant	30	30	
	Transfer	30 → 25	25	
Morphology				
Old	Diapause II	25		4, 5
	Escape	25		
	Escape	30		
Heart rate				
Old	Diapause II	25	25, 30	6, 7
	Escape	25	25, 30	
	Escape	30	25, 30	

RESULTS

The results of several experiments are presented below concerning many aspects of the biology of escape embryos in *A. limnaeus*. In order to help organize the presentation of the data, Table 1 is provided to summarize the experiments conducted and direct the reader to the figure where the results of those experiments are presented.

Young females produce more escape embryos

When assessed as an entire group, the proportion of *A. limnaeus* embryos that escape diapause II decreases as the age of the fish increases (Fig. 1A). However, a single female can produce embryos that enter or bypass diapause II within a single clutch of embryos, and there is a great deal of intra- and inter-individual variation observed in the production of escape embryos (Fig. 1B–G). This temporal variation in the production of escape embryos results in a wide range of phenotypes in terms of the proportion of total embryos produced by a female that escape diapause II in the laboratory (Fig. 2). This phenomenon appears to be female specific because switching of males between different females had no effect on the proportion of escape embryos produced (J.E.P., I.D.F.G. and Z.F.K., unpublished observations).

Increased incubation temperature increases the proportion of escape embryos

Increasing the incubation temperature of embryos induces an increase in the proportion of escape embryos observed. As expected, when incubated at 20 or 25°C, only a small percentage of embryos produced by older females are escape embryos, while incubation at 30°C results in 100% escape embryos (Fig. 3A). Thus, incubation temperature can directly alter the developmental trajectory of an embryo, transforming any embryo into an escape embryo. To determine when during early development at 30°C embryos are committed to escape diapause II, embryos were incubated at 30°C and transferred to 25°C as a function of developmental stage. The probability that an embryo will bypass diapause II when incubated at 30°C despite transfer to 25°C begins to increase above background rates in embryos with 10 pairs of somites or greater (Fig. 3B). About 85% of all embryos with 18 pairs of somites were committed to escaping diapause II, and 100% of the embryos with 23 pairs of somites or more are escape embryos when incubated at 30°C and then transferred to 25°C (Fig. 3B). Thus, the window of temperature sensitivity occurs across a rather wide range of stages during the segmentation period of development. It is important to note that the developmental trajectory of embryos with fewer than 18–20 pairs of somites is very difficult to distinguish without careful measurement (see below).

The timing of morphological development is different in escape embryos

Close examination of escape embryos at either 25 or 30°C revealed major differences in the timing of morphological and physiological development compared with those that enter diapause II at 25°C. Head morphology is very similar in escape and diapausing embryos until the embryos reach the stage of approximately 16–18 pairs of somites (Fig. 4). After this point, the two developmental pathways diverge significantly. By the time the embryos reach 24 pairs of somites, the difference between the two developmental trajectories

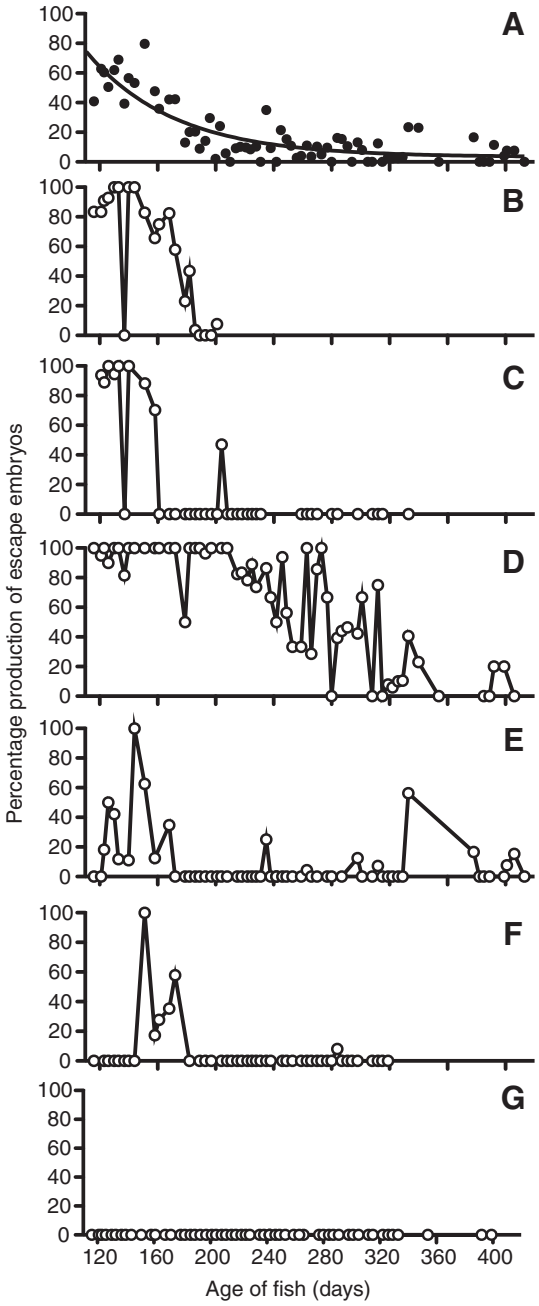

Fig. 1. Production of escape embryos in *A. limnaeus*. (A) The proportion of escape embryos is higher in young females compared with older females when the reproductive output of all females is combined. Each point represents the total percentage of escape embryos produced by six females on a single spawning event. The line is a one-phase exponential decay regression (R^2=0.73). (B–G) The proportion of escape embryos produced for six different females illustrating the variety of patterns in escape embryo production.

is obvious (Fig. 5). Regression analysis confirms that escape embryos that develop at either 25 or 30°C share similar morphological development in the head region (*t*-test comparing slopes, $P>0.3$ for both length and width). However, the slope of the combined regression equation for escape embryos is statistically distinct from that of the embryos entering diapause II at 25°C for both head length and width (*t*-test comparing slopes, $P<0.0001$).

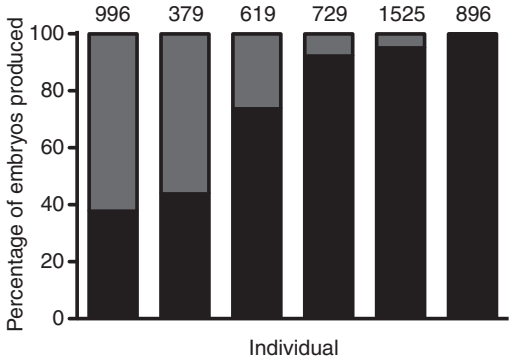

Fig. 2. Lifetime total production of embryos by six females illustrating the diversity in escape embryo production in our laboratory stock of *A. limnaeus*. Gray bars represent escape embryos, whereas black bars represent diapausing embryos. Numbers above the bars are the total number of fertile embryos collected for that female. There is a significant difference in the proportion of escape embryos produced by each female (ANOVA, $P<0.0001$).

The timing of physiological development is different in escape embryos

Heart rates for individual embryos of *A. limnaeus* are very stable for at least an hour when observed using our experimental setup (Fig. 6). The effect of temperature shifts on heart rate appears to be almost instantaneous using our methods because new and stable

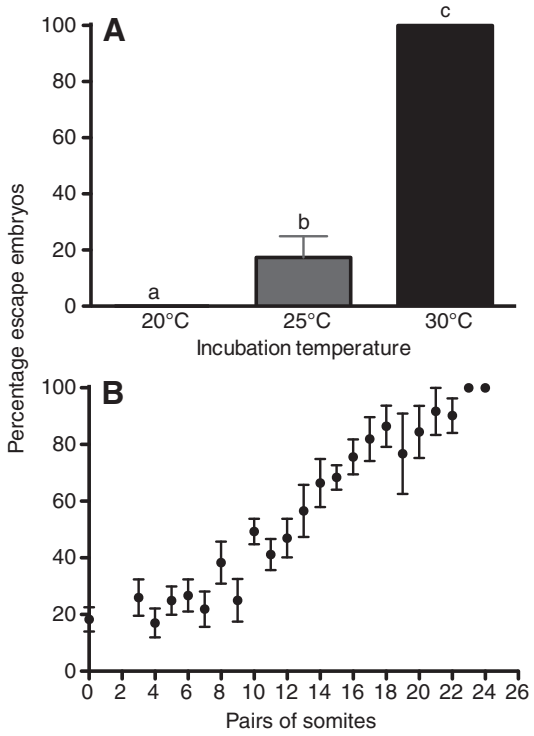

Fig. 3. (A) Increased incubation temperature significantly increases the proportion of embryos that escape diapause II (ANOVA, $P<0.0001$). Bars are means ± s.e.m. (N=4). Bars with different letters are significantly different (Tukey's HSD, $P<0.05$). (B) Temperature transfer experiments indicate that embryos are committed to escaping diapause II if transferred from 30°C to 25°C when they obtain between 10 and 18 pairs of somites. Symbols are means ± s.e.m. (N=4–11).

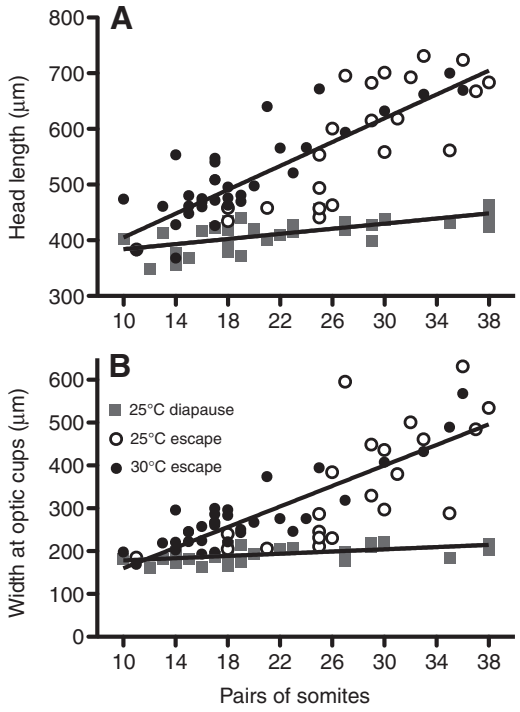

Fig. 4. Escape embryos reared at 25°C and 30°C have head morphologies that are different to those of embryos that will enter diapause II. (A) Head length increases at a faster rate (*t*-test comparing slopes, $P<0.0001$) in escape embryos (black line, $R^2=0.68$, $P<0.0001$) compared to embryos that will enter diapause II (gray line, $R^2=0.41$, $P=0.0001$). The data for escape embryos reared at both 25 and 30°C were combined because regression analysis indicated no significant differences in the relationship between somite number and head length for these groups (*t*-test comparing slopes, $P=0.33$). (B) Head width increases at a faster rate in escape embryos compared with those that enter diapause II (*t*-test comparing slopes, $P<0.0001$). Regression analysis indicates a similar relationship between somite number and head width in all escape embryos (*t*-test comparing slopes, $P=0.35$) and thus a single regression line was fit to the data for escape embryos ($R^2=0.64$, $P<0.0001$). Embryos that will enter diapause have a low but significant increase in head width as pairs of somites are added to the embryo ($R^2=0.32$, $P<0.001$).

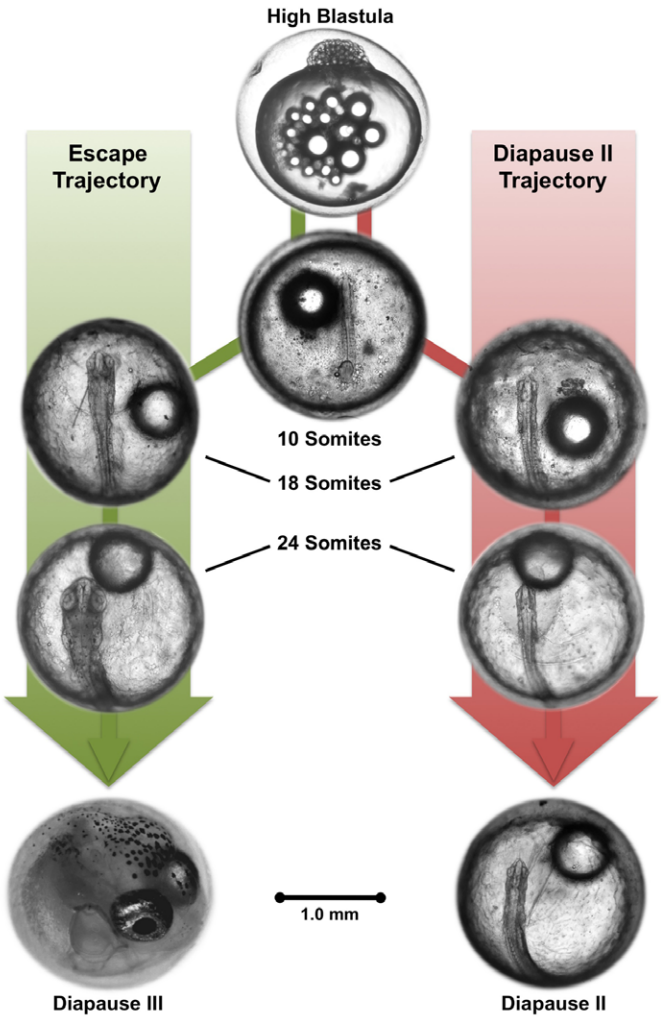

Fig. 5. A schematic representation of the two developmental trajectories observed in embryos of *A. limnaeus*. Early development through about the 10-somite stage is indistinguishable for the two trajectories using the morphological characters. By the 18-somite stage, the embryos are distinguishable by both morphological and physiological characters. Both of these trajectories occur naturally at temperatures of 25°C and above, but increasing the incubation temperature to 30°C can force all the embryos onto the escape trajectory.

steady-state heart rates are reached at about the same time that the new temperature is reached within the experimental chamber (Fig. 6).

The morphological and physiological development of the heart and circulatory system is advanced in escape embryos compared with those entering diapause II (Fig. 7). At a stage of approximately 16–18 pairs of somites, the heart rate of escape embryos is slightly elevated compared with embryos that will enter diapause II. In embryos older than 18 pairs of somites, the heart rate is significantly higher in escape embryos compared with those that will enter diapause II, especially when determined at 30°C. The relationship between developmental stage and heart rate is statistically different for embryos entering diapause II compared with those that escape this stage of diapause independent of the temperature at which the heart rate is measured (*t*-test comparing slopes, $P<0.0001$ for both temperatures). When measured at 30°C (Fig. 7A), escape embryos reared at both temperatures have statistically indistinguishable slopes when assessed with regression analysis (*t*-test comparing slopes of regression lines, $P=0.5635$). However, when measured at 25°C (Fig. 7B), the slopes of the regression lines for the two groups of escape embryos are different (*t*-test comparing slopes, $P=0.005$). This difference in slopes is due to reduced temperature sensitivity in escape embryos incubated at 30°C. In fact, the calculated Q_{10} for heart rate (Fig. 8) averages around 2.3±0.04 (mean ± s.e.m.) for escape embryos reared at 25°C and is only 1.9±0.14 for embryos entering diapause II and 1.4±0.04 for escape embryos reared at 30°C. Interestingly, the larger variation in the diapausing embryos is the result of very high Q_{10} values for embryos in diapause II.

DISCUSSION

Different developmental trajectories

The annual killifish *Austrofundulus limnaeus* inhabits ephemeral pond habitats that dry on a seasonal basis. This harsh and often unpredictable environment has presumably driven the evolution of embryonic diapause as an adaptation for survival. The ability to arrest development and tolerate extreme environmental stress is not just a pause in development but an alternative developmental

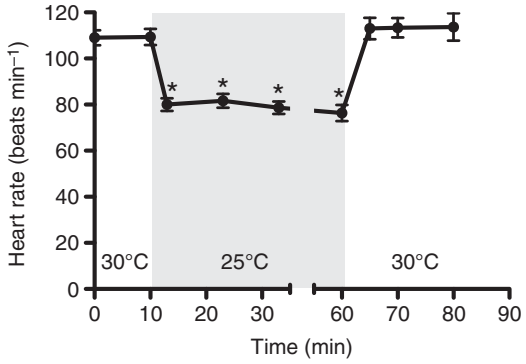

Fig. 6. Heart rate changes rapidly in response to temperature in escape embryos of *A. limnaeus*. Embryos were spontaneous escape embryos incubated at 25°C with between 24 and 30 pairs of somites. Stable heart rates were observed within 5 min of shifting the temperature in the chamber regardless of the direction of temperature change. The chamber reaches the new temperature approximately 2–3 min after the set-point is changed. *Heart rate was significantly lower in embryos at 25°C compared with 30°C (Dunnett's, $P<0.001$).

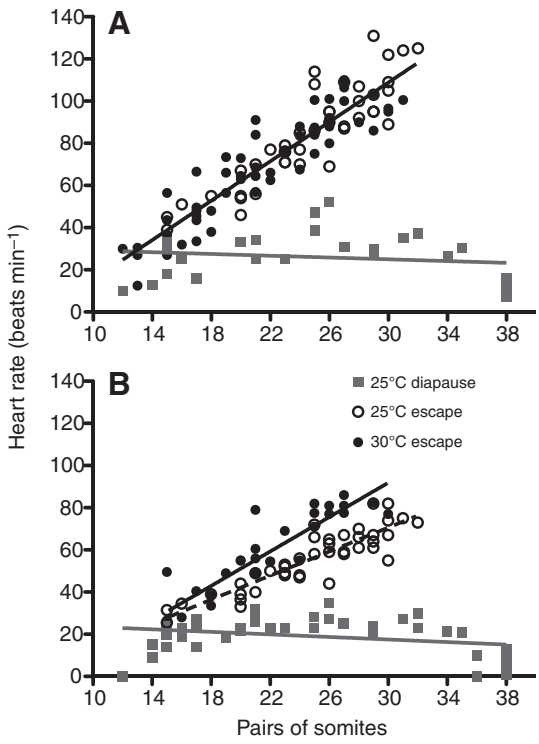

Fig. 7. Heart rate increases at a faster rate in escape embryos when compared with embryos that will enter diapause II. (A) Heart rates measured at 30°C are similar for escape embryos reared at both temperatures (*t*-test comparing slopes, $P=0.56$). Heart rate increases significantly faster (*t*-test comparing slopes, $P<0.0001$) in escape embryos (combined regression $R^2=0.84$, $P<0.0001$) than in embryos that enter diapause II ($R^2=0.024$, $P=0.45$). (B) When measured at 25°C, the three groups of embryos all exhibit statistically different relationships between heart rate and somite number (*t*-test comparing slopes, $P=0.005$). Embryos that will enter diapause II have a low but significant slope ($R^2=-0.09$, $P=0.042$). Escape embryos reared at 25°C have a slope ($R^2=0.79$, $P<0.0001$) that is slightly lower than those reared at 30°C ($R^2=0.81$, $P<0.0001$).

pathway that differs morphologically (Figs 4 and 5), physiologically (Figs 7 and 8) and biochemically (Chennault and Podrabsky, 2010) from development in the absence of diapause II.

In escape embryos, anterior structures such as the brain and heart are developmentally advanced relative to posterior elements such as axial and endodermal structures when compared with embryos that will enter diapause II. For example, when embryos are staged according to the normal addition of somites to the segmental plate of the paraxial mesoderm (part of the axial skeleton) (Dubrulle and Pourquié, 2004), the morphological growth and development of the brain is advanced in escape embryos compared with those entering diapause II. In fact, many of the features present in an escape embryo with 24 pairs of somites [e.g. melanocytes, hemoglobin expression, gut primordium (Fig. 5)] will not develop until several days of post-diapause II development in an embryo with well over 42 pairs of somites. The two developmental trajectories appear to diverge, based on morphological and physiological data, at about 16–18 pairs of somites. This type of difference in the timing of early developmental events is a rather extreme form of developmental plasticity that is not often observed in vertebrate embryos, especially not embryos from the same species or even the same clutch of embryos incubated under the same conditions.

Few studies have reported significant intraspecific plasticity in the timing of events during vertebrate development, and, in most cases, these changes are associated with an environmental cue or manipulation. For example, with respect to the skeletal system there appears to be a small (but likely evolutionarily significant) amount of intraspecific variation in the sequence of skull bone development in some species of fish (Mabee et al., 2000) and perhaps significant variation in the timing of bone ossification in larval fire-bellied toads (Hanken and Hall, 1984). Muscle development is known to be plastic and responsive to a number of environmental variables in several species of fish (e.g. Johnston, 2006). Body shape and trophic morphology are also affected by environmental variables in fishes and amphibians (e.g. Meyer, 1987; Wimberger, 1992; Storz and Travis, 2007). In addition, meristic counts have been shown to vary with temperature in some species of fish (e.g. Harrington and Crossman, 1976). However, most of these examples of plasticity describe a continuous change in shape or timing for structures appearing rather late in embryological development or during the larval period. In fact, developmental plasticity is believed to be rather restricted during early development (Smith-Gill, 1983; Moran, 1992) presumably because formation of the primary body axes is highly conserved among vertebrates, and changes during this period of development would more likely lead to serious deviations in the developmental program that would not be beneficial. For studies focused on larval individuals, an additional complication must be taken into account. Because the individuals are active and can exhibit behavioral differences, the variation observed in skeletal and muscle development might be affected by activity and feeding levels that could complicate interpretations of developmental timing *per se*. Thus, the occurrence of alternative developmental trajectories that diverge very early in the development of *A. limnaeus* appears to be a novel phenomenon among vertebrate embryos.

Maternal influences

Maternal influences are one set of mechanisms that appear to regulate developmental trajectory in this system. Younger females are more likely to produce escape embryos than older females (Fig. 1). However, other factors are also at play because there is significant variation in the proportion of escape embryos produced

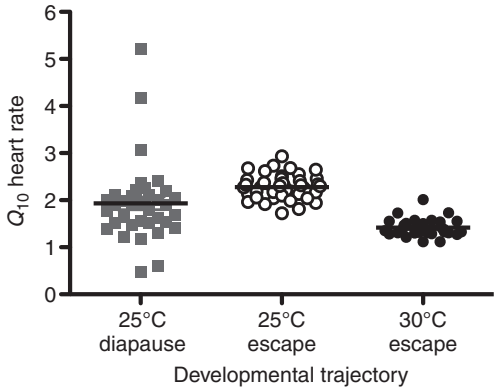

Fig. 8. Q_{10} analysis of heart rate in embryos that enter or escape diapause II. Embryos that follow the escape trajectory at 25°C appear to be more sensitive to temperature shifts than do those that escape at 30°C. All three means are statistically different (ANOVA, $P<0.001$, Tukey's HSD, $P<0.05$). Symbols are individual data points, whereas the lines in the center are means ± s.e.m.

by a given female. Because these experiments were performed in a laboratory environment under relatively controlled conditions, there are a number of variables that are very unlikely to cause this variation. For example, temperature, photoperiod, light intensity and salinity are all tightly controlled and thus not likely to explain the variation observed. By contrast, food quality or quantity, intraspecific interactions and, perhaps, handling stress are all possible environmental variables that might contribute to the observed variation. The mechanism responsible for maternal influence on escape embryo production is currently unknown. However, patterns in the data suggest some likely avenues for future research. Steroid hormone levels have been suggested as a possible regulator of diapause in an African species of annual killifish (Levels, 1988) and are known to cause changes in embryo quality in other species of fish (McCormick, 1998) and other vertebrates (Dufty et al., 2002). In addition, Berkeley and colleagues (Berkeley et al., 2004) report that maternal age can have a significant affect on larval survival in black rockfish. Thus age-related changes in maternal hormone levels are very likely an avenue worth pursuing in *A. limnaeus*, especially considering the importance that hormones play in the regulation of diapause in insects (Mousseau and Dingle, 1991). Maternal provisioning or programming is another possible route for controlling the switch between the two alternative trajectories, as has been shown in insects (Mousseau and Dingle, 1991). Many substances are packaged into fish oocytes, and these factors affect a myriad of processes during early development in fish (Pelegri, 2003). In this scenario, differential rates of degradation and/or different initial quantities of a maternally provisioned gene product (RNA or protein) could hypothetically underlie the observed variation. However, this conjecture has yet to be evaluated experimentally, and future studies that detail the mRNA and protein complement, as well as patterns of DNA methylation in the maternally derived genome, might shed light on a mechanism for this epigenetic programming of development.

Temperature effects

The fact that the incubation temperature of the embryo can override maternal influences on development is significant. In this system, regulation of entry into diapause is not vested in input from only one life-stage, but from at least two. This plasticity in the regulation of the alternative pathways is probably important for survival of the species in an unpredictable environment (Moran, 1992). The wide window of development where temperature appears to exert its effect has interesting implications. We interpret this observation to support a model for degradation or synthesis of a maternally provisioned gene product that is packaged into embryos at different initial amounts (see above). Thus, it takes a slightly different amount of time at 30°C for each embryo to reach the threshold that causes a shift in developmental trajectory. This hypothesis is completely unsupported by mechanistic data, but it forms a very reasonable framework for future studies of the mechanisms that regulate this switch between two alternative developmental pathways.

The observation that heart rate is differentially affected by temperature in escape embryos reared at 25 and 30°C is an interesting result. While embryos reared at 25°C have a rather typical response to temperature (Q_{10} near 2) regardless of developmental trajectory, embryos reared at 30°C have a rather low Q_{10} that is just slightly above 1. The reason for this lack of temperature sensitivity is currently unknown but might have to do with the biochemical or physiological adjustments necessary to maintain heart activity at the elevated temperature. Barrioneuvo and Burggren (Barrioneuvo and Burggren, 1999) report Q_{10} values near 1 for some stages of larval development in zebrafish. In addition, these authors discuss the lack of a clear relationship between metabolic rate and cardiovascular performance in early life stages of zebrafish. Thus, there is no reason to assume an *a priori* relationship between heart rate and temperature in fish embryos. However, the results reported in Fig. 7 suggest that a 30°C escape embryo would be relatively 'over-perfused' at 25°C owing to the elevated heart rate, assuming that cardiac output remained constant as well. This seems to imply a 'waste' of energy maintaining a higher heart rate than would be dictated by temperature as defined by the typical responses exhibited by embryos incubated at 25°C. Alternatively, it could be argued that there is some need for an elevated heart rate in embryos incubated at 30°C that is not alleviated instantaneously (or perhaps at all) by reduced temperature. One possibility is an increased need for transport of nutrients or waste products through the circulatory system. If this hypothesis is correct, we would expect heart rate to decline in these embryos after several hours at the reduced temperature – a time-frame consistent with changes in gene expression and physiology that accompany changes in metabolic organization. The relationship between cardiac physiology, metabolic rate and temperature during development is an interesting and complicated topic that certainly deserves future attention in this system as well as others.

Ecological and evolutionary implications

The production of escape embryos by young females is probably a form of 'bet hedging' (Moran, 1992). When a pond is young, the probability that it will remain inundated for long enough to support a second generation of fish in a single rainy season is likely to be substantially higher than only a few weeks in the future. Escape embryos produced early in the season might be able to complete development and reproduce in the same year as their parents. However, the older a female (or pond) becomes, the less likely it is that there will be sufficient time for completion of the whole life-cycle within a given rainy season. Thus, it makes sense to produce mostly diapausing embryos late in the rainy season (when the female is older). However, very few field studies have been conducted on the ecology and behavior of these fish, and only a well-planned and -executed ecological study can shed light on the validity of this conjecture.

The embryonic development of *A. limnaeus* is highly variable compared with that of other teleosts such as zebrafish. This high degree of variation is reflected in the morphological (Fig. 4) and physiological (Fig. 7) data presented in this study. We routinely observe differences in developmental rate, egg size and embryo size in our laboratory stock of *A. limnaeus* (J.E.P., I.D.F.G. and Z.F.K., unpublished observations). Much of the variation we observe is among females, whereas variation within a female is relatively low. Thus, some (if not most) of the variation in the data presented here is likely due to variation (genetic or epigenetic) present within our stock of *A. limnaeus*. However, some of the variation is due to differences in the strength of the environmental affect on these embryos. Interestingly, the variation in embryo morphology reported in this study is not correlated with differences in egg size (J.E.P., I.D.F.G. and Z.F.K., unpublished observations). One reason for this variation might be the unique dispersion and reaggregation of blastomeres that is a normal part of development in this species (Wourms, 1972b). In annual killifish, at the end of epiboly, the embryonic blastomeres are randomly distributed across the yolk (Wourms, 1972b). These blastomeres then continue to divide and migrate until they reaggregate into a mass of cells several days later. Embryogenesis occurs in the reaggregated cells. Thus, variation in the number of cells that reaggregate could be translated into different-sized embryos. An experimental evaluation of this hypothesis could lead to very interesting implications for the canalization of development in this species. Variation in heart rate is more difficult to understand in this species because so little is known about the physiological development of the cardiovascular system in general. However, if maternal effects can control the decision to enter or escape from diapause, then it is certainly reasonable to hypothesize that heart rate during development could vary owing to genetic, environmental and epigenetic mechanisms.

In general, this species is poised to generate variation during development at many levels of organization. The occurrence of two and possibly three different stages of diapause creates what Wourms (Wourms, 1972c) referred to as the multiplier effect, which is a mechanism that ensures a lack of developmental synchrony within a single clutch of embryos. In addition, the time for the completion of dispersion and reaggregation of blastomeres to occur is variable, and thus synchrony in a single clutch of eggs from a single female is often variable. Based on our data on production of escape embryos, maternal provisioning or maternal effects can also contribute to developmental variation in this species. Lack of developmental synchrony then sets the stage for differential developmental programming by environmental cues. Because the embryos are not all in the same stage at the same time, environmental variation might have differential effects on different embryos from the same clutch of eggs. It is reasonable to argue that increased variation would be adaptive in this species given its highly variable and unpredictable environment. It is our opinion that developmental variation in *A. limnaeus* is probably adaptive and that at least part of this variation is epigenetic. Exploration of the mechanisms that generate developmental variants in this species and their evolutionary implications is a subject worthy of future investigations.

Conclusions

Our data document the occurrence of two distinct developmental trajectories in *A. limnaeus*. These trajectories are naturally occurring in the population, but the expression of the phenotypes is altered by maternal age and incubation temperature in the laboratory. The differences in the timing of developmental events observed in embryos of *A. limnaeus* would be typical of differences for species that have diverged over millions of years of evolution, a process known as heterochrony (Gould, 1977; Mitgutsch et al., 2008). However, we document these differences within a single species and from individual clutches of embryos produced by a single female. Importantly, these trajectories diverge relatively early in development during the primary events of embryogenesis, which is an unusual pattern in animal development and raises many questions about how the gene-regulatory networks that control development (Davidson and Erwin, 2006) might be altered to allow such differences in developmental timing and the suspension of development in the embryos that enter embryonic diapause. Escape embryos reared at 25°C have been shown to complete development with significantly less aerobic and anaerobic enzymatic capacity than embryos that enter diapause or those that escape diapause II owing to incubation at 30°C (Chennault and Podrabsky, 2010). Thus, if these biochemical differences translate into performance differences, it is possible that developing along these two alternative trajectories might influence the performance and perhaps even fitness of the fish for its entire life. The developmental, physiological and evolutionary outcomes of these two trajectories remain to be characterized but hold promise for better understanding the possibilities for plasticity and evolution in vertebrate development.

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