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Extreme anoxia tolerance in embryos of the annual killifish *Austrofundulus limnaeus*: insights from a metabolomics analysis

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Summary

The annual killifish *Austrofundulus limnaeus* survives in ephemeral pond habitats by producing drought-tolerant diapausing embryos. These embryos probably experience oxygen deprivation as part of their normal developmental environment. We assessed the anoxia tolerance of *A. limnaeus* embryos across the duration of embryonic development. Embryos develop a substantial tolerance to anoxia during early development, which peaks during diapause II. This extreme tolerance of anoxia is retained during the first 4 days of post-diapause II development and is then lost. Metabolism during anoxia appears to be supported mainly by production of lactate, with alanine and succinate production contributing to a lesser degree. Anoxic embryos also accumulate large quantities of γ-aminobutyrate (GABA), a potential protector of neural function. It appears that the suite of characters associated with normal development and entry into diapause II in this species prepares the embryos for long-term survival in anoxia even while the embryos are exposed to aerobic conditions. This is the first report of such extreme anoxia tolerance in a vertebrate embryo, and introduces a new model for the study of anoxia tolerance in vertebrates.

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Key words: anaerobiosis, anoxia, development, fish, GABA, lactate, metabolic depression.

Introduction

Tolerance of anoxia is the exception rather than the rule for vertebrates (Lutz et al., 1996). The physiology of anoxia-tolerance has been examined in relatively few species of vertebrates, notably the painted turtle *Chrysemys picta* and the crucian carp *Carassius carassius*. Anaerobiosis in these organisms is supported by a complex suite of traits that includes the storage of large amounts of glycogen to support glycolytic production of lactate (turtles) or ethanol (crucian carp), and elevated levels of γ-aminobutyrate (GABA) in neural tissues (Lutz et al., 1996). In both species, the single most critical factor for survival of anoxia appears to be a severe reduction in metabolism (ATP turnover), a state that is achieved *via* a coordinated downregulation of energy producing and energy consuming processes (Hand and Hardewig, 1996; Jackson, 2000; Krumschnabel, 2000; Lutz and Nilsson, 2004).

In this study we investigated the anoxia tolerance of diapausing embryos of the annual killifish *Austrofundulus limnaeus*. This species occurs in ephemeral ponds in the Maracaibo Basin of Venezuela, which are a harsh and highly variable habitat in which few aquatic vertebrates can survive. Conditions in these ponds change radically during transitions from the rainy season to the dry season. Rainy season ponds may remain inundated for several weeks to several months with some ponds being semi-permanent (Thomerson and Taphorn, 1992). During the dry season, ponds may remain dry for several months and perhaps years. *A. limnaeus* has evolved a life history strategy similar to many aquatic invertebrate species, which includes the production of drought-tolerant diapausing embryos (Wourms, 1972b; Podrabsky et al., 2001) that are deposited into the muddy pond substrate (Myers, 1952). Oxygen limitation is often imposed by microbial activity in inundated soils, and the habitat of *A. limnaeus* is no exception (Podrabsky et al., 1998). Consequently, embryos of *A. limnaeus* are likely to experience long bouts of severe hypoxia or anoxia as a normal part of their developmental environment.

Diapausing embryos of *A. limnaeus* share many metabolic characteristics with other vertebrate facultative anaerobes such as freshwater turtles and crucian carp. A significant metabolic depression (80–90% reduction compared to

developing embryos) accompanies diapause (Podrabsky and Hand, 1999). This metabolic depression is associated with a severe reduction in the rate of protein synthesis (Podrabsky and Hand, 2000). Thus, these embryos are already in a state of metabolic depression very similar to anoxia-induced quiescence even when incubated under aerobic conditions. In fact, during aerobic development from fertilization through entry into diapause II, there is a significant contribution of anaerobic pathways to the overall metabolism of these embryos, as assessed by calorimetry and respirometry (Podrabsky and Hand, 1999). The present study was performed to further characterize the predicted extreme anoxia tolerance of diapause II embryos of *A. limnaeus* and identify the metabolic pathways that may provide this species with its remarkable abilities to survive under extreme environmental conditions.

Materials and methods

Collection and incubation of embryos

Adult *Austrofundulus limnaeus* (Schultz 1949) were housed in 20 l aquaria as previously described (Podrabsky, 1999). Temperature in the aquaria was regulated at 26°C. Embryos were collected twice weekly to keep the spawning interval between 3 and 4 days, which has been shown to produce the highest number of healthy embryos under laboratory conditions (Podrabsky, 1999). All embryos were incubated in the dark at 25°C in embryo medium containing 10 mg l^{-1} gentamycin sulfate (Podrabsky, 1999) unless otherwise noted. These conditions are known to support entry of embryos into diapause II, and exposure to a long-day photoperiod has been shown to break diapause II (Podrabsky and Hand, 1999). On the day of exposure to anoxia, the embryos were treated with two 5 min washes in 0.001% (w/v) hypochlorite bleach to reduce the chances of bacterial and yeast growth during the long-term anoxia exposures (Podrabsky, 1999).

Anoxia tolerance

Embryo medium containing 10 mg l^{-1} gentamycin sulfate was made anoxic by purging the medium with high purity nitrogen gas for 20–30 min. Groups of 20 embryos were sealed into 20 ml glass screw-cap vials (caps contained a silicon and Teflon septum) containing anoxic embryo medium. Just prior to sealing the vials, sodium sulfite crystals were added to a final concentration of 1.0 mg ml^{-1} to remove any residual oxygen and to help protect against the reintroduction of oxygen. Vials were visually inspected to ensure the absence of air bubbles. Calculations suggest that the amount of sulfite added to each vial would be ten times the amount needed to produce anoxic conditions from fully oxygenated medium. These levels were non-toxic (see below). Anoxic vials were then sealed in a glass bell jar, which was subsequently purged with nitrogen gas for 20 min. Anoxic conditions were confirmed using a polarographic oxygen electrode (Model 1302; Strathkelvin Instruments, Motherwell, UK) when each vial was opened for processing. At each sampling time, a single vial of embryos from each replicate was opened and the embryos were washed briefly in embryo medium, and then placed in fresh, oxygenated embryo medium to recover from the anoxic exposure. Survival of the embryos was assessed immediately and after 1 week of aerobic recovery, using an inverted compound microscope. Embryos were scored as alive if they were structurally intact and morphologically normal. Survival after 1 week was assessed by the same criteria as the initial assessment with the additional requirements of successful resumption of development (in non-diapausing embryos) or normal cardiac function (in developing and diapausing embryos). Mean lethal time (to 50% mortality; LT_{50}) estimates were calculated based on the number of embryos that were able to survive and resume normal development or physiological function after 1 week of aerobic recovery.

Anoxia tolerance was assessed in early developing and diapause II embryos at 1, 2, 4, 8, 12, 16, 24 and 32 days post-fertilization (d.p.f.) and in post-diapause II embryos at 4, 8 and 12 days post-diapause II (d.p.d.). Embryos normally enter diapause II at 24 d.p.f. when incubated at 25°C. Embryos were induced to break diapause synchronously by exposure to a long-day photoperiod (14 h:10 h L:D) (Podrabsky and Hand, 1999) at an incubation temperature of 30°C for 2 days. After the embryos were observed to break diapause they were immediately returned to conditions of 25°C in the dark. For all developmental stages, three different spawning events were used to produce three replicates for statistical analysis at all time points.

Toxicity of sodium sulfite

The potential toxicity of sodium sulfite during anoxic exposure was assessed in 4 and 8 d.p.f. embryos. Embryos for these studies were treated as described above, except that sodium sulfite was added to a final concentration that ranged from 0.25 to 2.5 mg ml^{-1}. For each developmental stage a single batch of embryos was used to produce three replicates of 20 embryos for each time point. At the concentrations used in this study, sodium sulfite appears to have no toxic effect on the survival of embryos exposed to anoxia (Figs S1 and S2 in supplementary material).

Preparation of metabolite extracts for gas chromatography–mass spectrometry analysis

During normoxic development embryos at 0, 4, 8, 12, 16, 20, 24 and 32 d.p.f. were flash frozen in liquid nitrogen and stored at –80°C prior to extraction of metabolites. Embryos at 1, 2, 4, 8, 16, and 32 d.p.f. were exposed to anoxia and sampled at times consistent with their anoxia tolerance: 0.5 and 1 day of anoxia for 1 and 2 d.p.f. embryos, 7 and 21 days for 4 d.p.f. embryos, and 21 and 60 days for 8, 16 and 32 d.p.f. embryos. Embryos were removed from anoxia and the surviving embryos were quickly counted and their mass determined prior to flash freezing. Embryos were lyophilized in pre-tared microcentrifuge tubes and then pulverized using a Teflon pellet pestle. The pulverized powder was then homogenized with 20 volumes of ice-cold 10% (w/v)

trichloroacetic acid (TCA). The TCA homogenate was then centrifuged at 14 000 ***g*** for 30 min at 4°C to pellet the protein and other TCA-insoluble materials. The supernatant was carefully removed and retained. The pellet was then washed once with 20 volumes of ice-cold 10% TCA and centrifuged as described above. The two supernatants were then pooled and lyophilized overnight to remove the TCA and dehydrate the extract. The dried extract was then resuspended in water (0.2–0.4 ml) and a small fraction of the rehydrated extract (0.04–0.06 ml) was dispensed into a glass gas chromatography vial and again lyophilized to dehydrate the sample [modified from Gradwell et al. (Gradwell et al., 1998)].

Silylation gas chromatography–mass spectrometry analysis

The lyophilized TCA extract powder was derivatized with 0.025 ml of a 1:1 ratio mix of acetonitrile: *N*-methyl-*N*-[*tert*-butyldimethylsily]trifluoroacetamide (MTBSTFA). The vials were then sealed and sonicated at room temperature for 3 h. Following sonication, 0.5 μl of the derivatized extract was injected into a Varian 3400 gas chromatograph (Varian Instruments, Palo Alto, CA, USA) coupled with a Finnigan ITD 806 mass spectrometer (Finnegan MAT, San Jose, CA, USA) according to the methods of Fan et al. (Fan et al., 1993). A mixture of 37 compounds was similarly silylated and analyzed by gas chromatography–mass spectrometry (GC–MS) to serve as calibration standards.

^{1}H nuclear magnetic resonance spectroscopy

Perchloric acid extracts were prepared for three samples for analysis using nuclear magnetic resonance (NMR) spectroscopy: diapause II, diapause II embryos exposed to 75.5% relative humidity for 3 days, and 8 d.p.f. embryos. These embryos were extracted twice in 20 vol of ice-cold 10% perchloric acid (PCA). PCA was removed by precipitation with KOH (pH adjusted to between 6 and 7) and centrifugation of the precipitated materials at 14 000 ***g*** for 15 min (Fan et al., 1986). The neutralized PCA extracts were then passed over a Chelex-100 column (Bio-Rad, Hercules, CA, USA) to remove paramagnetic ions, lyophilized and dissolved in D_2O. One-dimensional and two-dimensional total correlation spectroscopy (TOCSY) ^{1}H NMR spectra were acquired at 25°C using a Varian Unity Plus NMR spectrometer operating at 11.75 Tesla.

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS Inc., v.14.0.1, Chicago, IL, USA). LT_{50} was calculated for each time series of anoxic exposures using probit regression analysis (Chapman et al., 1995). LT_{50} values determined for each developmental stage were compared using analysis of variance (ANOVA, general linear models procedure). Comparison of means for all data sets was accomplished using ANOVA (as above). Unless noted otherwise, the Student–Neuman–Keul's *post-hoc* test was applied to determine the differences among means. Statistical significance was set for $P<0.05$ in all comparisons.

Results

Anoxia tolerance

Embryos of *A. limnaeus* developed a substantial tolerance to anoxia as they progress towards and enter diapause II (Fig. 1). Anoxia tolerance increased during early development from LT_{50} values around 1 day of anoxia (1 and 2 d.p.f.) to a maximum of 62±8 days (mean ± s.e.m.) in diapause II embryos. This high degree of anoxia tolerance was retained for at least 4 days of post-diapause II development, but is then lost by 8 d.p.d.

Recovery from anoxia

Embryos that are exposed to anoxia for 30 days or less are highly likely to fully recover from anoxia, as determined by the ability to resume normal development (Fig. 2A,B). However, after 60 days of anoxia, a duration that is near or past the LT_{50} for most stages, a significant number of the embryos that survive the initial exposure failed to resume normal development and died (Fig. 2C; paired *t*-test, one-tailed $P<0.05$). For the 90 days exposure, the sample size was too small to make meaningful statistical inferences (data not shown). Embryos that survived 1 week of aerobic recovery appeared to complete development normally and eventually hatched or entered diapause III.

Anoxia-induced quiescence

Morphological observations indicate that exposure to anoxia at any stage of development causes embryos to enter a state of anoxia-induced quiescence. Cessation of development in these embryos is most clearly illustrated by counting the number of somite pairs present in an embryo after long-term exposure to anoxia (Fig. 3). No somite development was observed even after 90 days in anoxia (ANOVA, $P=0.417$). By comparison,

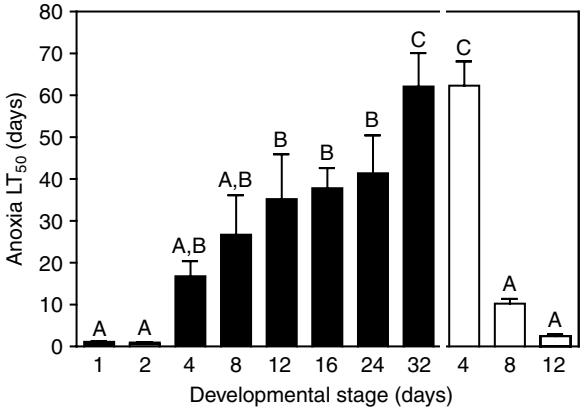

Fig. 1. Lethal time to 50% mortality (LT_{50}) for embryos of *A. limnaeus* exposed to anoxic conditions at 25°C. Filled bars represent early development through diapause II (days post-fertilization; d.p.f.). Open bars represent post-diapause II development (days post-diapause II; d.p.d.). Values are means ± s.e.m. ($N=3$). Bars with different letters are statistically different (Student–Neuman–Keul's *post-hoc* test, $P<0.05$).

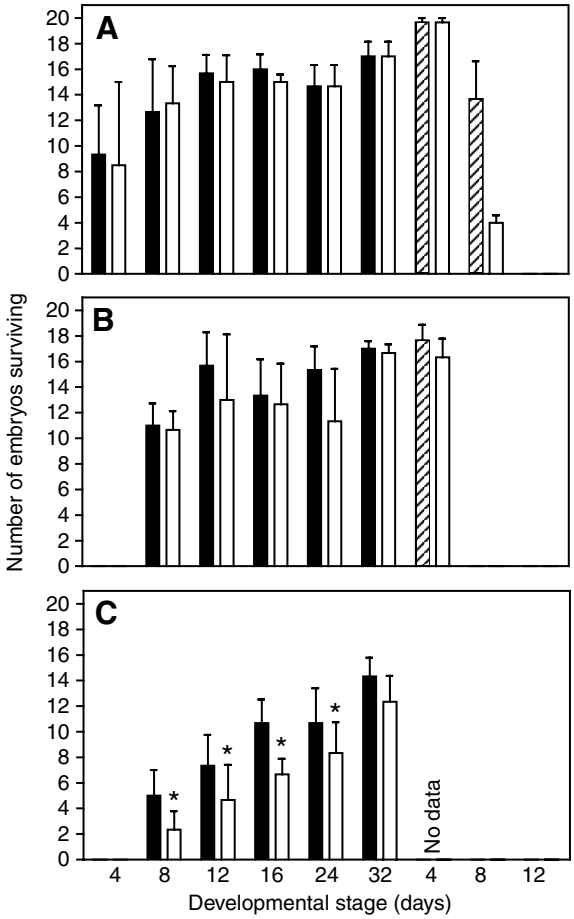

Fig. 2. Aerobic recovery from anoxia in embryos of *A. limnaeus* exposed to anoxia for (A) 7, (B) 30 and (C) 60 days. Filled and open pairs of bars represent the number of embryos (out of a total of 20) initially surviving anoxia (filled) and those that resumed normal development after 1 week of aerobic recovery (open) from early development through diapause II (age of embryos given in days post-fertilization). Hatched and open pairs of bars represent the number of embryos initially surviving anoxia (hatched) and those that resume normal development (open) after 1 week of aerobic recovery as post-diapause II embryos. The stages of these embryos are given as days post diapause II. Bars are means ± s.e.m. (N=3). Asterisks over the open bars indicate a significant difference between initial survival of anoxia and survival after 1 week of aerobic recovery (paired t-test, P<0.05, one-tailed).

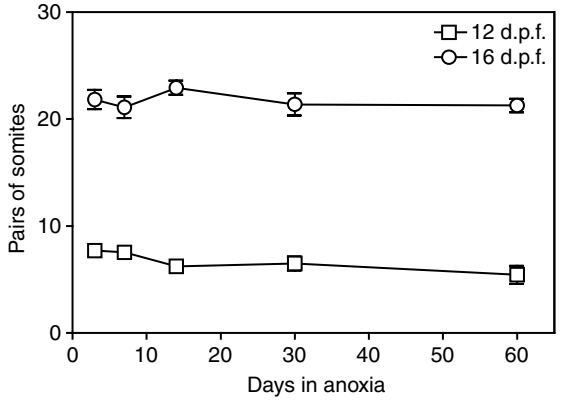

Fig. 3. Anoxia induces quiescence in embryos of *A. limnaeus*. The number of somite pairs does not increase during long-term exposure (ANOVA, $P \ll 0.05$), indicating a state of anoxia-induced quiescence. Values are means ± s.e.m. (N=3).

many of the control embryos (those that broke diapause II spontaneously) had completed development and hatched prior to the 90 days anoxia time point (unpublished data).

One-dimensional and two-dimensional TOCSY ^{1}H-NMR

The NMR analysis (Figs S3, S4 in supplementary material) corroborates the GC–MS data presented below, by confirming the identity of a number of metabolites present in the embryo extracts. In addition, the NMR data revealed major metabolites not observed by GC–MS, including glycinebetaine (GB), phosphorylcholine (PC), and polysaccharides (broad resonances between 3.5 to 5.2 p.p.m. (Fig. S3 in supplementary material). These metabolites appeared to accumulate in diapause II embryos, but their response to anoxia was not investigated. GB is known to be a compatible solute involved in osmoregulation whereas PC is important to phospholipid metabolism. The accumulation of polysaccharides may reflect development of an energy reserve in the diapause II embryos in preparation for anoxia exposure or dehydration. The roles of these metabolites during diapause and exposure to anoxia warrants further investigation.

Glycolytic metabolites

Only two glycolytic intermediates, glyceraldehyde 3-phosphate (G3P) and lactate, were detected in this study, despite the ability of these methods to identify several other potentially important metabolites such as pyruvate. The concentration of G3P increased from around 0.5–2.0 mmol kg^{-1} embryo H_2O during early development through diapause II in embryos incubated aerobically (Fig. 4), however this trend is not statistically significant (ANOVA, P=0.065). During exposure to anoxia, G3P concentration increased significantly in 1, 16, and 32 d.p.f. (ANOVA, P<0.014) but not in 2, 4 and 8 d.p.f. embryos. During normoxic development lactate increased significantly (ANOVA, $P \ll 0.000001$) until 8 d.p.f. and then declined as embryos continued to develop and enter diapause II (Fig. 4). This pattern agrees both qualitatively and quantitatively with lactate levels previously reported for normoxic embryos (Podrabsky and Hand, 1999). During exposure to anoxia, lactate was the most abundant metabolic end-product accumulated and reached values between 30 and 40 mmol kg^{-1} embryo H_2O (Fig. 4). The rate of lactate accumulation during anoxia is inversely correlated (r=–0.97, $P \ll 0.000001$) with the anoxia tolerance of the embryos as estimated by the LT_{50} (Fig. 5). This pattern of slower accumulation of metabolites in diapausing embryos is apparent in most of the compounds analyzed in this data set and is likely correlated with the metabolic rate of these embryos under anoxia.

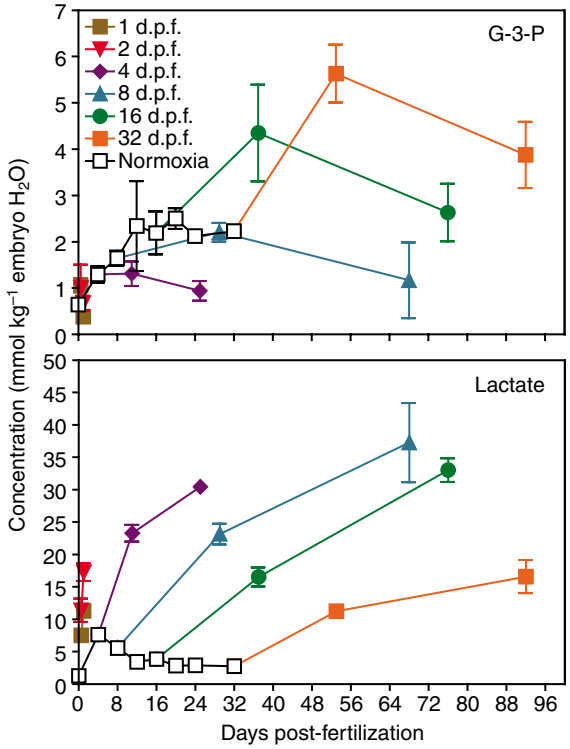

Fig. 4. Concentration of glyceraldehyde 3-phosphate and lactate in embryos during normoxic development and after exposure to anoxia in developing and diapause II embryos. Open squares represent the normal pattern of metabolite concentration during normoxic development. Colored symbols represent embryos exposed to anoxia. 1 and 2 d.p.f. embryos were sampled at 0.5 and 1 day of anoxia. 4 d.p.f. embryos were sampled after 7, and 21 days of anoxia. 8, 16 and 32 d.p.f. embryos were sampled after 21 and 60 days of anoxia. Values are means ± s.e.m. (*N*=3).

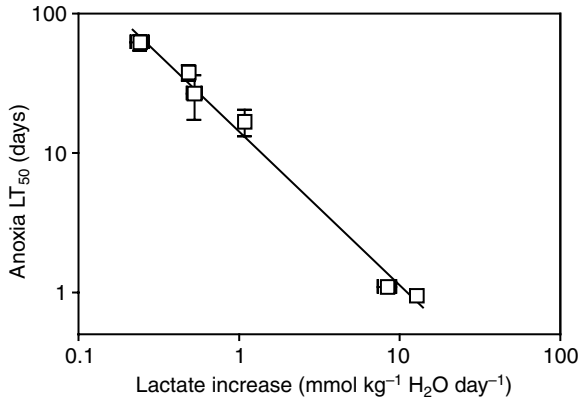

Fig. 5. The rate of lactate accumulation during anoxia is highly correlated with survival in embryos of *A. limnaeus*. (r=–0.97, $P \ll 0.000001$). Values are means ± s.e.m. (*N*=3) for both rate of lactate accumulation and LT_{50} values. Each symbol represents a single developmental stage.

Citric acid cycle metabolites

Three citric acid cycle intermediates were identified in these samples: succinate, malate and citrate (Fig. 6). Fumarate, α-ketoglutarate, β-hydroxybutyrate and t-aconitate are all potentially quantifiable using the methods employed in this study but were not detected. Succinate levels were undetectable during normoxic development, but increased significantly (ANOVA, P<0.002) to concentrations between 0.5–1.0 mmol kg^{-1} embryo H_2O during exposure to anoxia (Fig. 6). Malate concentrations increased by a small but statistically significant amount during normal development (ANOVA, P=0.023), and tended to decrease during anoxia, although this decrease is only significant (ANOVA, P=0.034) for 8 d.p.f. embryos (Fig. 6). Citrate levels increased during normoxic development (ANOVA, P=0.042) and declined significantly (ANOVA, P<0.042) in 1, 2 and 8 d.p.f. embryos exposed to anoxia (Fig. 6). Overall, there appears to be an accumulation of succinate, a small increase in malate, and little change in citrate concentrations in embryos exposed to anoxia that possess a substantial anoxia tolerance.

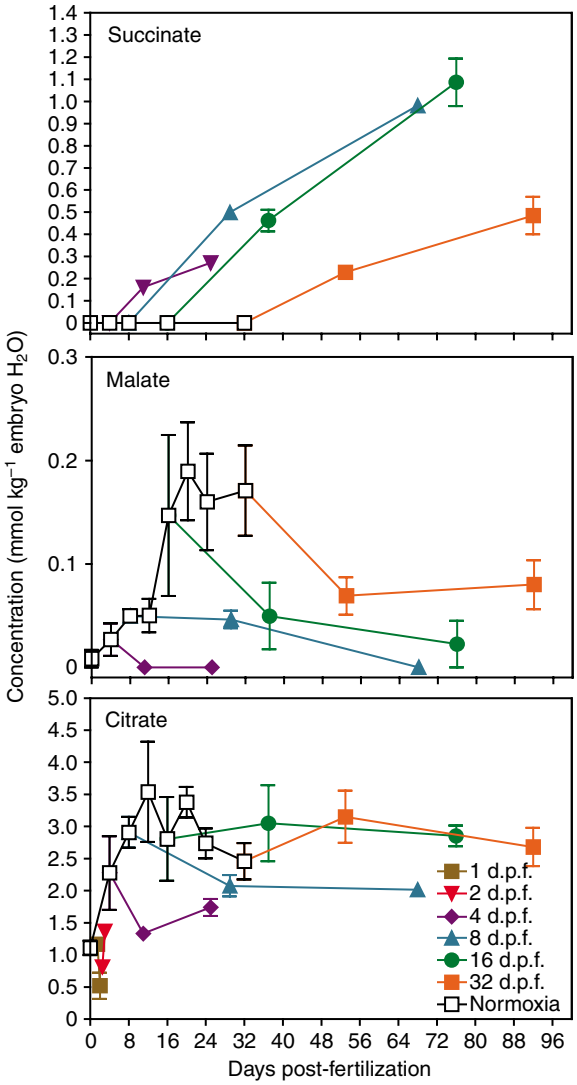

Fig. 6. Concentration of succinate, malate and citrate in embryos of *A. limnaeus* during normoxic development and after exposure to anoxia. Symbols are the same as in Fig. 4; values are means ± s.e.m. (*N*=3).

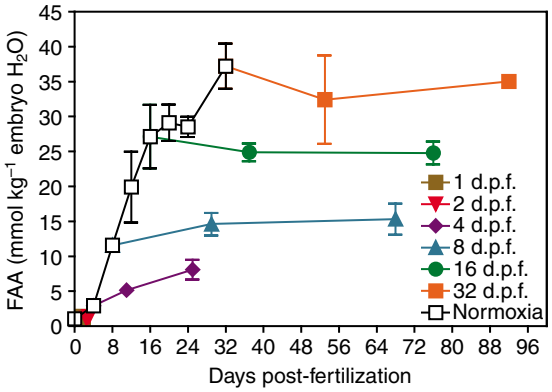

Fig. 7. Concentration of total free amino acids (FAA) in embryos of *A. limnaeus* during normoxic development and after exposure to anoxia. Total free amino acids increase substantially during normoxic development, but do not change during exposure to anoxia in embryos with extreme anoxia tolerance. Symbols are the same as in Fig. 4; values are means ± s.e.m., *N*=3.

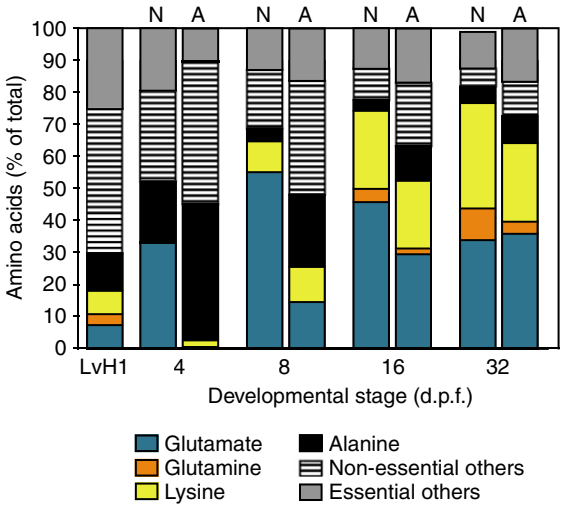

Fig. 8. The composition of the free amino acid pool changes as a result of normoxic development and exposure to anoxia in embryos of *A. limnaeus*. N=normoxic, A=21 days of anoxia for all developmental stages. The composition of amino acids in lipovitellin heavy chain I (LvH1) was deduced from amino acid sequences (see text for details).

Free amino acids

The free amino acid (FAA) pool (as estimated by combining the amounts of all amino acids detected using GC–MS analysis) increased significantly (ANOVA, P=0.000001) as a function of aerobic development in embryos of *A. limnaeus* from near 0 at fertilization to about 35 mmol kg^{-1} embryo H_2O during diapause II (Fig. 7). Embryos with a very high tolerance of anoxia (8 d.p.f. and older) showed no significant increase (ANOVA, P>0.22) in total free amino acids even after 60 days in anoxia (Fig. 7). However, this must be interpreted with caution because the composition of the amino acid pool changes substantially during exposure to anoxia (see below). It should be noted that the total amino acid pool presented here does not include cysteine and tryptophan, which were not detected by the GC–MS method employed. However, these two trace amino acids represent only about 1 and 0.5% of the total amino acids found in the yolk protein precursor vitellogenin, respectively (data not shown). In addition, histidine and arginine were not detected in our samples, even though they can be quantified by the methods used in this study.

During normoxic development and during exposure to anoxia, the pool of free amino acids appears to be dominated (50–70%) by glutamate, alanine and lysine in most cases (Fig. 8). There is a significant increase in the concentration of all amino acids during normoxic development except for aspartate and methionine, which are found in very low concentrations (Figs 9–12). Exposure to anoxia is accompanied by an increase in the total levels of essential free amino acids (Fig. 8). Changes in the concentration of individual amino acids during exposure to anoxia can be categorized into three major patterns. (1) A statistically significant increase in the concentration of nine amino acids (ANOVA P<0.05) occurs for at least one developmental stage after 60 days of anoxia; six of these are considered essential amino acids in fish (Fig. 9) and three of them are classified as non-essential (Fig. 10). These amino acids include, in order of abundance, alanine, valine, glycine, leucine, isoleucine, proline, phenylalanine, methionine and threonine. (2) A significant decrease in the concentration (ANOVA, P<0.05) of four amino acids: aspartate, asparagine, glutamate, glutamine, occurs after 60 days of exposure to anoxia in all treatments except for embryos at 32 d.p.f. (Fig. 11). (3) No significant change occurred in three amino acids during exposure to anoxia: serine and tyrosine (non-essential) and lysine (essential, Fig. 12). One trend that is obvious for all of the amino acids is an overall lower rate of accumulation, and often smaller total amount accumulated in embryos diapausing at 32 d.p.f. compared with those at 8 and 16 d.p.f.

γ-aminobutryate

Concentrations of the inhibitory neurotransmitter γ-aminobutyrate (GABA) rise to levels between 7 and 12 mmol kg^{-1} embryo H_2O during exposure to anoxia (Fig. 13). This compound is not found in embryos during normoxic development, and is only accumulated to significant levels in embryos that have substantial anoxia tolerance.

Discussion

Anoxia tolerance

Embryos of *A. limnaeus* appear to be the most anoxia tolerant life stage of any vertebrate yet described. The anoxia tolerance of embryos in diapause II and during early post-diapause II development (Fig. 1) is comparable with that of a number of invertebrates, and is 30- to 60-fold higher than the tolerance of freshwater turtles (*Chrysemys* and *Trachemys* species) and goldfish (*Carassius auratus*) when compared at similar temperatures (Hand, 1998; Jackson, 2000).

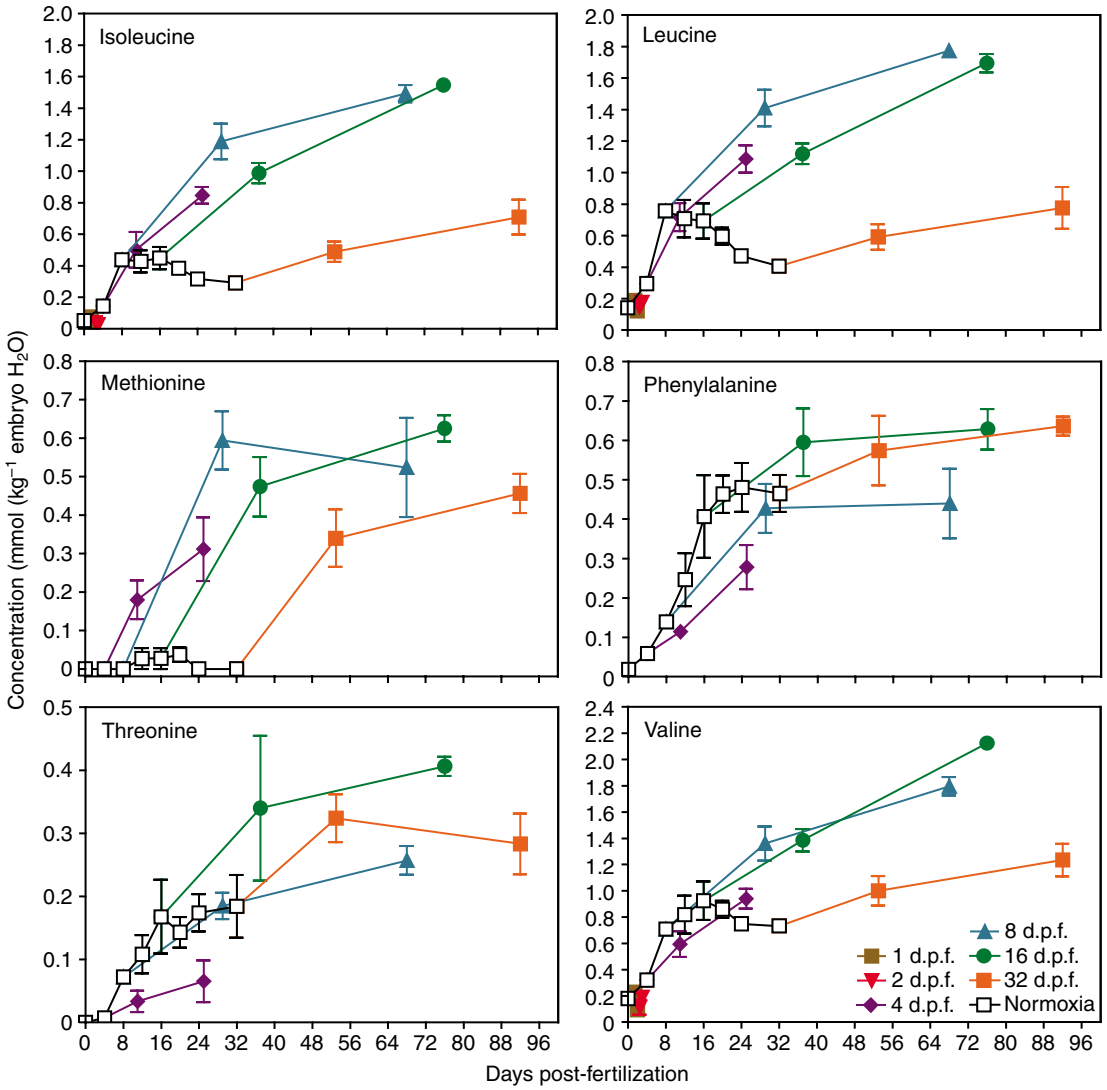

Fig. 9. Essential amino acids (isoleucine, leucine, methionine, phenylalanine, threonine and valine) that increase during exposure to anoxia in embryos of *A. limnaeus*. Symbols are the same as in Fig. 4; values are means ± s.e.m., *N*=3.

In vertebrates, the tissues most sensitive to oxygen deprivation are the brain and heart (Lutz et al., 1996; Lutz and Nilsson, 2004; Wasser, 1996; Giordano, 2005). Interestingly, the embryos of *A. limnaeus* that exhibit the highest degree of anoxia tolerance are composed mainly of neural tissues, and they contain a functional heart (Wourms, 1972b; Podrabsky and Hand, 1999). Diapause II embryos can be observed 'flexing' spontaneously and in response to light. They also respond physiologically to light cues (Podrabsky and Hand, 1999). Many of the embryos establish a closed circulation with immature red blood cells prior to entry into diapause II. During early post-diapause II development, the differentiation and maturation of the neural and cardiac tissues progresses, and includes the pigmentation of the retina, increased volume of the brain, circulating red blood cells that express hemoglobin, as well as the beginnings of the endoderm-derived internal organs such as the liver and gut (Wourms, 1972a). Thus, anoxia tolerance in these embryos is not simply a situation of 'undifferentiated' cells surviving anoxia. Rather, these embryos contain functional neurons and cardiomyocytes that are incredibly resistant to anoxia.

Anoxia-induced quiescence

When developing embryos of *A. limnaeus* are exposed to anoxia, they stop developing and enter a state of anoxia-induced quiescence (Fig. 3). Direct observation of embryos during post-diapause II development indicates that these embryos arrest development and experience a severe bradycardia or a complete cessation of cardiac activity under anoxia (Fergusson-Kolmes and Podrabsky, in press). Developmental arrest has been reported for embryos of other species of teleost fish in response to environmental anoxia and chemical anoxia induced by mitochondrial inhibitors (Crawford and Wilde, 1966; Padilla and Roth, 2001). However, in these instances survival is relatively short (1–2 days) compared to the survival times reported here for embryos of *A. limnaeus*.

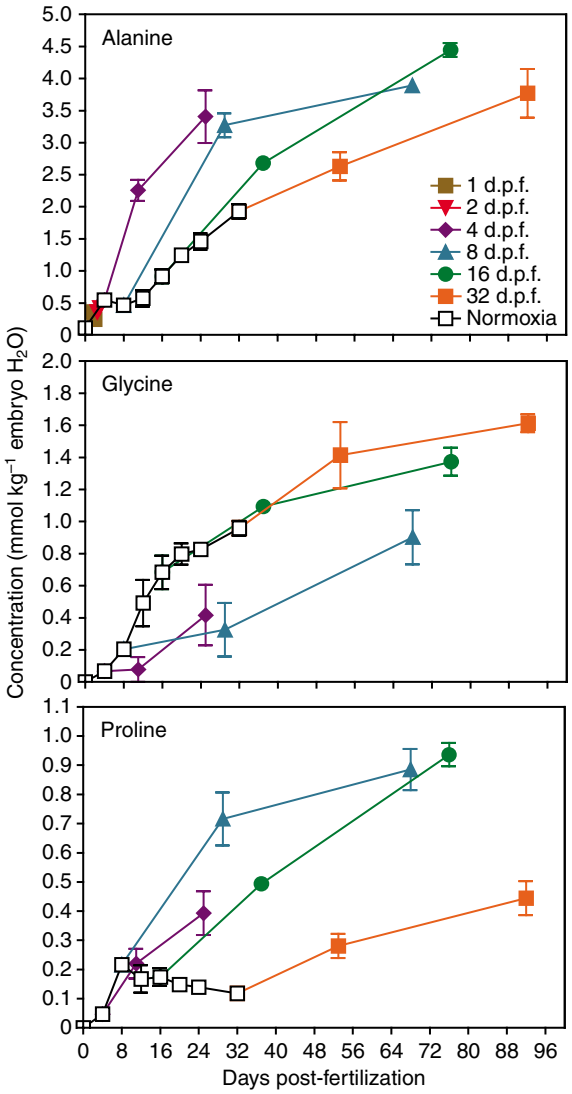

Fig. 10. Non-essential amino acids (alanine, glycine and proline) that increase during exposure to anoxia in embryos of *A. limnaeus*. Symbols are the same as in Fig. 4; values are means ± s.e.m., *N*=3.

Early post-diapause II embryos (4 d.p.d.) exhibit a tolerance of anoxia that is equal to that of diapause II embryos (Fig. 1). This result is impressive, considering the increased metabolism and complexity associated with further development in these embryos (see above). It appears that there is a window of time during early post-diapause II development when the embryos retain the ability to enter a profound state of anoxia-induced quiescence. This is similar to the situation for anoxia-induced quiescence in post-diapause embryos of the brine shrimp *Artemia franciscana* (e.g. Hand and Gnaiger, 1988; Clegg, 1997). Perhaps this extended tolerance of anoxia reflects the ephemeral nature of these species' habitats, where a return to low oxygen levels might occur after developmental arrest is over. Thus, the evolution of diapause and anoxia-induced quiescence appear to be linked in both of these distantly related species. This possible example of convergent evolution is probably the result of high selection pressures generated in harsh and highly unpredictable ephemeral habitats.

Recovery from anoxia

Embryos exposed to anoxia for 30–40 days are able to fully recover from the anoxic insult (Fig. 2). However, after 60 days of anoxia, many of the embryos fail to successfully resume development and eventually die. This failure to recover may be due to metabolic failure associated with build-up of toxic amounts of lactate (Fig. 4). An alternative explanation is that the embryos suffer from damage caused by the reintroduction of oxygen after long-term anoxia in a manner similar to reperfusion injury associated with recovery from ischemia in mammalian tissues (e.g. Gross and Auchampach, 2007). If a 'reperfusion' of oxygen into the embryos is indeed a source of damage after long-term anoxia, then comparing the metabolic changes and gene expression patterns in embryos recovering from short-term and long-term anoxia may lend insight into the mechanisms that induce reperfusion injury.

General considerations for metabolite analysis in embryos

The levels of metabolites reported in this paper are from whole embryos. These embryos contain several compartments that must be considered in the interpretation of these data: the perivitelline space, the embryonic tissues, and the yolk. The perivitelline space is filled with perivitelline fluid (PVF) and contains about 50% of the total embryo water (Podrabsky et al., 2001). This compartment surrounds the embryonic tissues and yolk. The PVF is derived partially from the contents of the cortical vesicles and is known in many species of teleost fish to contain mostly large macromolecules that attract water and cause osmotic swelling of the egg immediately following fertilization (Eddy, 1974; Shephard, 1987). If we assume that the perivitelline space is devoid of the metabolites examined in this study, then their effective concentrations in the embryo + yolk compartments may be twice that reported, based on total embryo water content. The embryonic tissues surround the central yolk mass, and for most of the embryonic stages observed in this study constitute only 10–20% of the total embryo mass. The embryonic tissues are separated from the yolk by the yolk syncytial layer, which is a known barrier to diffusion of water and small organic molecules, such as cryoprotectants, in freshwater fish embryos (e.g. Hagedorn et al., 1997). In addition, enzymes have been demonstrated to be distributed in a non-uniform manner between the yolk and embryonic tissues in embryos of the loach *Misgurnus fossilis* (Klyachko et al., 1982). Thus, compartmentalization of metabolites and metabolic function is a distinct possibility and without specific information on where in the embryo/yolk/PVF system these compounds may be located we are limited in our ability to interpret the results. However, this study does identify the major metabolic pathways that contribute to anoxia tolerance in these embryos, and our findings represent a first critical step in mechanistically understanding the metabolic underpinnings of anoxia tolerance in this species.

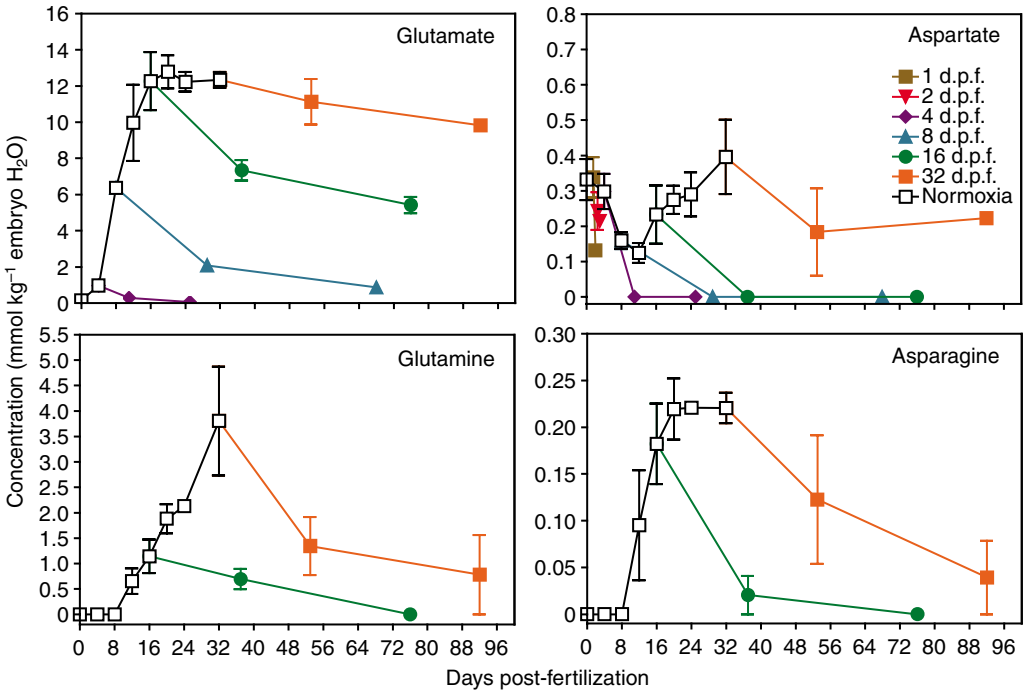

Fig. 11. Four amino acids (glutamate, glutamine, aspartate and asparagine) decrease in concentration during exposure to anoxia in embryos of *A. limnaeus*. Symbols are the same as in Fig. 4; values are means ± s.e.m. (*N*=3).

Anaerobic end-product accumulation

In general, animals that can survive long bouts of anoxia rely on utilization of carbohydrates to fuel metabolism (Hochachka and Somero, 2002). This is true of both the crucian carp and freshwater turtle species, which have large glycogen reserves used to fuel anaerobic metabolism (Jackson, 2000; Lutz and Nilsson, 2004). Metabolism appears to be dominated by glycolytic production of lactate during exposure of *A. limnaeus* embryos to anoxia (Fig. 4B). Levels of glycogen and glucose that are presumably available to support anaerobic metabolism during early development and diapause II, have been previously reported for *A. limnaeus* (Podrabsky and Hand, 1999). About 80–90% of the available glycosyl units are stored as glycogen, and after 2 d.p.f. the average amount of glycosyl units available for the production of lactate during early development and diapause II is 36.2±1.0 nmol embryo^{-1} (mean ± s.e.m.), which would yield about 72.4 nmol of lactate per embryo. This glycogen reserve is similar to that of other fish species with similar size embryos such as loach and medaka (Yurowitzky and Milman, 1973; Hishida and Nakano, 1954), and an order of magnitude lower than that of trout embryos (Terner, 1968). Therefore, it does not appear that accumulation of large glycogen stores can explain the exceptional anoxia tolerance of this species.

The exact metabolic pathways that are used to support metabolism during anoxia in embryos of *A. limnaeus* have not yet been confirmed with biochemical studies. However, a simple analysis of end-products accumulated suggests that available glycogen stores can support most, if not all, of the accumulated compounds. The amounts of lactate, succinate and alanine produced after 21 (4 d.p.f.) or 60 days (8, 16, 32 d.p.f.) of anoxia are presented in nmol embryo^{-1} in Table 1. When these data are compared with the theoretical amount of three to four carbon compounds that could be produced from glycogen and glucose, it is apparent that exhaustion of carbohydrate stores may be a limiting factor in the survival of embryos 4 d.p.f. and older. In contrast, embryos at 1 and 2 d.p.f. die long before they run out of fuel to support glycolytic metabolism (data not shown, but see Fig. 4) perhaps due to an inability to buffer the acidosis that likely accompanies anoxic metabolism [2 moles of H^+ per mole of glycosyl unit (Hochachka and Mommsen, 1983)], but no data are available for intracellular pH changes associated with anoxia in this species. Rough calculations based on the mean rate of lactate production in diapausing embryos (0.48±0.08 nmol day^{-1}) at 32 d.p.f. would suggest an exhaustion of available glycosyl units after about 151 days of anoxia. When the survival experiments were terminated at 90 days of anoxia, about 20% of the 32 d.p.f. embryos survived the treatment, and thus it is possible that available glycogen stores will ultimately limit survival of anoxia in these embryos as well. The strong negative correlation between the rate of lactate production during anoxia and survival of anoxia (Fig. 5) reinforces the importance of metabolic rate depression to long-term survival of anoxia in embryos of *A. limnaeus*.

Significant increases in succinate and alanine are also observed in embryos of *A. limnaeus* that are exposed to anoxia after 4 d.p.f., although the magnitudes of these increases are dwarfed by lactate production (Figs 6, 10). It is important to note that in 4, 8 and 16 d.p.f. embryos alanine increases five-

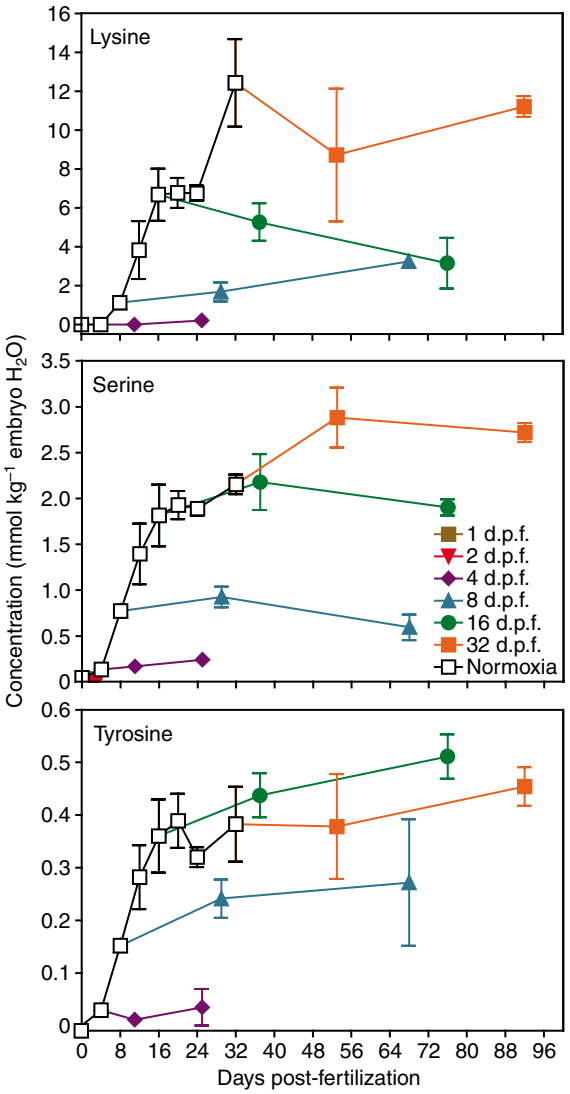

Fig. 12. Three amino acids (lysine, serine and tryosine) exhibit no change in concentration during exposure to anoxia in embryos of *A. limnaeus*. Symbols are the same as in Fig. 4; values are means ± s.e.m., *N*=3.

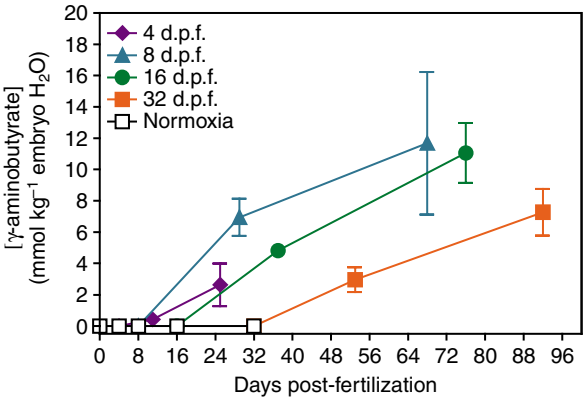

Fig. 13. There is a substantial increase in the concentration of γ-aminobutyrate in embryos that can survive long-term anoxia. Symbols are the same as in Fig. 4; values are means ± s.e.m., *N*=3.

to ninefold after long-term exposure to anoxia whereas the essential amino acids such as leucine increase by only two- to threefold on average during this same time, despite their roughly equal representation in the yolk proteins (Fig. 8). For diapause II embryos (32 d.p.f.) the proportional increase in alanine and leucine are both around twofold after a long-term exposure to anoxia. These data suggest that, with the exception of diapause II embryos, the increase in alanine is at least partially due to de novo production during anoxia, and not due simply to protein degradation. The anaerobic production of succinate and alanine has been documented in a variety of facultative anaerobes (Hochachka and Somero, 2002). The role of these metabolites during anaerobiosis in *A. limnaeus* is not yet understood, but a limited maintenance of Krebs cycle activity to provide intermediates for other metabolic conversions (such as the production of GABA) is a distinct possibility and has been reported in other vertebrate anaerobes (Van der Boon et al., 1992; van Raaij et al., 1994). High levels of succinate relative to available α-ketoglutarate can also inhibit prolyl hydroxylase, which may lead to stabilization and activation of hypoxia-inducible factor 1α, thereby enhancing glycolytic activity and oxygen-dependent gene expression (Gottlieb and Tomlinson, 2005).

One additional metabolite that warrants discussion is citrate (Fig. 6). Citrate increases in concentration during early development and reaches levels around 3 mmol kg^{-1} embryo H_2O during diapause II, but these concentrations do not tend to change during exposure to anoxia. This amount of citrate is an order of magnitude higher than that found in embryos of loach (Milman and Yurowitzky, 1967). Citrate is known to be a potent inhibitor of phosphofructokinase with inhibition occurring in the micromolar range (e.g. MacDonald and Storey, 2004). Given the above information on the rate of lactate production, we must conclude that whole embryo levels of citrate probably do not reflect cytoplasmic levels of citrate in these embryos. It is highly likely that a large percentage of this citrate is partitioned in the yolk, but it may also be sequestered in the mitochondria. Citrate has the potential to bind metal ions of various types including calcium, magnesium and iron (Salovaara et al., 2003) and perhaps may be functioning as a chelating agent for the storage of metals in the yolk. Citrate accumulation has also been implicated in redox balance and may play a role in antioxidant defenses in some cells (Mallet and Sun, 2003). Moreover, citrate is the source of acetyl CoA in fatty acid biosynthesis, and thus can serve as a reserve for new membrane synthesis during development. The unique biochemistry of citrate and the high levels accumulated in diapausing embryos of *A. limnaeus* suggest that this molecule may play a critical role in the biochemistry of diapause.

Free amino acids

The free amino acid pool in teleost fish embryos is thought to be generated by degradation of yolk proteins (Monroy et al.,

Table 1. *Accumulation of lactate, succinate and alanine and a comparison to available carbohydrate stores*

d.p.f.	Lactate	Succinate	Alanine	Total	%Stores*	%Stores†
4	42.0±3.1	0.51±0.06	5.4±1.3	47.9	66.2	70.1
8	62.2±6.6	1.9±0.02	6.7±0.07	70.9	97.9	114.5
16	56.6±3.6	2.1±0.2	6.8±0.1	65.5	90.5	95.2
32	28.7±4.8	1.0±0.2	4.0±0.6	33.7	46.5	52.2

Values are in nmol embryo^{-1} (mean ± s.e.m., N=3). Values were calculated by subtracting the amount of each metabolite at time=0 (normoxia) from the amount of metabolite present at terminal sampling, which was 21 days of anoxia for 4 days embryos and 60 days of anoxia for 8, 16 and 32 d.p.f. embryos. The percentage of carbohydrate stores was calculated using 36.2 nmol embryo^{-1} as the average glycosyl units available for anoxic end-product production [see text for details, glycogen and glucose data from Podrabsky and Hand (Podrabsky and Hand, 1999)].

*The percentage of the glycosyl units accounted for by lactate, succinate and alanine.

†The percentage of glycosyl units accounted for by lactate, succinate, alanine and GABA, after taking into consideration the possible contribution of the amino acids in Table 2 to the production of GABA.

Table 2. *Accumulation of GABA and the percent of GABA accumulation that can be accounted for by reduction in the amount of four amino acids*

d.p.f.	Glutamate	Glutamine	Aspartate	Asparagine	Total	GABA	%
4	–1.8±0.4	0	–0.6±0.1	0	–2.4	5.3±3.0	45.3
8	–10.6±0.2	0	–0.3±0.05	0	–10.9	22.9±7.3	47.6
16	–14.9±3.6	–2.4±0.7	–0.5±0.2	–0.4±0.09	–8.2	21.5±3.4	84.2
32	–4.0±0.5	–5.9±0.5	–0.6±0.1	–0.3±0.08	–10.8	15.0±3.1	72.5

Values are in nmol embryo^{-1} (mean ± s.e.m., N=3). Values were calculated by subtracting the amount of each amino acid at time=0 (normoxia) from the amount present at terminal sampling, which was 21 days of anoxia for 4 d.p.f. embryos and 60 days of anoxia for 8, 16 and 32 d.p.f. embryos.

1961; Love, 1970; Srivastava et al., 1995; Gunasekera et al., 1999; Finn et al., 2002). In embryos of *A. limnaeus*, the total protein content of the embryos decreases significantly during early development through diapause II (Podrabsky and Hand, 1999), which suggests liberation of amino acids from yolk protein stores to fuel metabolism and biosynthesis during development, as observed in other teleost embryos (Rønnestad and Fyhn, 1993). Previous evaluation of the distribution of amino acids between the embryonic tissues and non-embryonic compartments (perivitelline space and yolk) indicates that a very large percentage of the free amino acids (93–99%) are not found in the embryonic cells (Podrabsky and Hand, 2000). Thus, the most likely source for the increase in the essential amino acids observed during normoxic development and after exposure to anoxia is protein degradation of yolk proteins. However, we cannot exclude the possibilities that degradation of proteins in the embryonic tissues, or reduced consumption of amino acids due to arrest of protein synthesis do not contribute as well. Although there is a dramatic ontogenetic increase in the total free amino acid pool during early development, this still probably represents an insignificant fraction of the osmotic pool at fertilization and only about 10–15% of the total osmolality of the egg if we assume an internal osmolality of 290 mOsmol kg^{-1} embryo H_2O (Machado and Podrabsky, 2007).

The composition of the free amino acid pool in normoxic and anoxic embryos of *A. limnaeus* is quite divergent from their average representation in lipovitellin heavy chain I (LvH1) or in the precursor of this major yolk protein vitellogenin [Fig. 8; mean percentage of amino acids in LvH1 from three species as listed in GenBank: *Krytolebias marmoratus* (accession number AY279214), *Fundulus heteroclitus* (accession number Q90508) and *Oncorhynchus mykiss* (CAA63421.1)]. Similar percentages of amino acids are obtained if the whole vitellogenin protein is used, except for serine which increases due to its high representation in the phosvitin domain of the vitellogenin (data not shown). This altered profile of amino acids is likely the result of de novo synthesis of a few select amino acids such as glutamate and alanine, and selective sparing of others such as lysine. In fact, lysine is the only essential amino acid that increases substantially more than the others during diapause II in embryos under normoxic conditions, and can account for most of the additional increase in FAA observed in ‘steady-state’ diapausing embryos at 32 d.p.f. compared with those just entering diapause II at 24 d.p.f. (Fig. 7). The role of elevated lysine during diapause II is currently unknown, but it is doubtful that it plays a major role during exposure to anoxia.

Glutamate, glutamine, aspartate and asparagine are all non-essential amino acids of the ‘citric acid cycle superfamily’ and can be transaminated into citric acid cycle intermediates with relative ease within most cells (Mathews and van Holde, 1990). Glutamate and glutamine accumulate in large quantities in diapause II embryos of *A. limnaeus* during normoxic

development. These amino acids are consumed during exposure to anoxia (Fig. 11), and thus their accumulation during early development and diapause may be an important preparation for the metabolic changes associated with exposure to anoxia. Similarly, decreases in brain tissue glutamate concentrations have been documented for turtles and crucian carp (reviewed by Lutz et al., 1996). The amino acids are probably used as substrates for the production of GABA (see below). However, even if 100% of these amino acids are shunted to the production of GABA, there is still a substantial proportion of GABA which cannot be accounted for (Table 2) that could be produced from glycogen stores (Table 1).

γ-aminobutyrate

The metabolic pathways that support γ-aminobutyrate (GABA) production and degradation are well characterized in vertebrate nervous tissues. GABA is typically synthesized from glutamate by the enzyme glutamate decarboxylase and is degraded in post-synaptic neurons to succinate (Turner and Whittle, 1983). The large decreases in glutamate and glutamine observed in embryos exposed to anoxia are probably associated with synthesis of GABA. The production of GABA at the expense of glutamate would seem to be beneficial due to the decrease in glutamate (an excitatory neurotransmitter), a consumption of protons, and the increase in GABA as discussed above. The degradation pathways for GABA lead to succinate, and thus perhaps the accumulation of succinate during anoxia may be partially due to post-synaptic degradation of GABA.

The evidence available on the role of GABA during oxygen limitation suggests that GABA is acting to limit excitotoxic neuronal cell death in embryos of *A. limnaeus* exposed to anoxia. GABA is the most abundant inhibitory neurotransmitter in the adult vertebrate central nervous system (Turner and Whittle, 1983; Martyniuk et al., 2005) and is generally thought to protect the brain of a variety of organisms from excitotoxic cell death during oxygen limitation (Lutz and Milton, 2004; Cheung et al., 2006). The best studied receptors for GABA are the $GABA_A$ receptors, which are ligand-gated chloride channels (Stephenson, 1995). The action of GABA *via* these receptors as excitatory or inhibitory is dependent on the concentration of intracellular chloride ions ($[Cl^-_i]$), with low $[Cl^-_i]$ resulting in inhibition (Cheung et al., 2006; Tyzio et al., 2006). Recent reports indicate that regulation of $[Cl^-_i]$ *via* expression of the NKCC1 electroneutral cation–chloride cotransporter may play a critical role in protecting neurons of a variety of organisms during exposure to hypoxia or ischemia (Cheung et al., 2006; Tyzio et al., 2006).

GABA has been shown to play important roles in the development of the vertebrate central nervous system (CNS). During development and maturation of the CNS, GABA is known to act as an excitatory neurotransmitter (Tyzio et al., 2006). The excitatory actions of GABA are due to a high $[Cl^-_i]$ early in development (Cherubini et al., 1991) prior to the insertion of Cl transporters (see above) into the neuronal membrane. Insertion of the Cl^- transporters causes a decrease in $[Cl^-_i]$ and GABA then assumes an inhibitory role. The accumulation of high levels of GABA in embryos of *A. limnaeus* suggests that it is likely to be inhibitory at this stage of development. Future studies might examine at what stage in development the Cl^- transporter appears in brain tissue.

In embryos of the teleost fish *Gasterosteus aculeatus*, GABA is the first neurotransmitter to be expressed in the central nervous system and is thought to play a role in nervous system patterning and in the establishment of the early axonal scaffold (Ekström and Ohlin, 1995). Presently, there is no information on the development of GABAergic neurons in embryos of *A. limnaeus*. The lack of detectable GABA in normoxic embryos may indicate that these neurons have not yet developed or are very few in number in *A. limnaeus* embryos entering diapause II. However, the large increases observed in GABA concentration during exposure to anoxia could act as a signal to activate the expression of genes involved in GABA-mediated signaling, as has been shown in a variety of vertebrates including goldfish (Martyniuk et al., 2005). Indeed, in anoxic turtle brains a rise in GABA is accompanied by an increase in $GABA_A$ receptors (Lutz and Milton, 2004), which is thought to strengthen the inhibitory effects of GABA. The complex interaction between GABA production and the expression of GABA-related genes, and the pivotal role that GABA plays in the development of the vertebrate CNS suggests that production of GABA during anoxia may alter gene expression patterns that could have lasting effects on the development of the CNS in embryos of *A. limnaeus*.

In addition to its role in the CNS, GABA is known to play a role in the physiology of a number of organ systems, including the heart. $GABA_B$ receptors are known to be expressed in mammalian cardiomyocytes, and activation of these channels is thought to result in post-synaptic inhibition and subsequent protection of cardiac tissue from ischemia (Lorente et al., 2000). Thus, GABA may have a role in supporting survival of cells during anoxia outside of the CNS in embryos of *A. limnaeus*. A critical next step in elucidating the role of GABA in the tolerance of anoxia in *A. limnaeus* will be to establish the compartmentalization of GABA within the embryo and establish which receptors (if any) are expressed in the various tissues of the embryo.

Diapause embryos are 'primed' for anoxia tolerance

Metabolic depression is supported in most organisms by a coordinated downregulation of energy consuming and energy producing pathways (Hand and Hardewig, 1996; Hand, 1998; Hochachka and Somero, 2002). In general, protein synthesis and ion pumping are the two most energetically expensive processes contributing to the basal metabolic rate of a cell (Buttgereit and Brand, 1995; Rolfe and Brown, 1997). A common response in all animals that survive long-term anoxia is a downregulation of protein synthesis, and a reduction in ion leakage, which results in reduced ion motive ATPase activities (Hand and Hardewig, 1996). Entry into diapause II in embryos of *A. limnaeus* is associated with a substantial decrease in metabolic rate as estimated by both oxygen consumption and

heat dissipation (Podrabsky and Hand, 1999). In addition, anaerobic metabolic pathways are thought to be making a significant contribution to overall metabolism in diapause II embryos, even though they are incubated under aerobic conditions (Podrabsky and Hand, 1999). Rates of protein synthesis are severely reduced in diapause II embryos compared to developing embryos, which can account for about 30% of the overall depression in metabolic rate (Podrabsky and Hand, 1999). Recently, we have found that embryos of *A. limnaeus* have very low Na^+-K^+-ATPase activity during all of early development, and levels of this enzyme do not increase substantially until about 8 d.p.d. (Machado and Podrabsky, 2007). Therefore, it is logical to hypothesize that ion homeostasis with the environment may not be a significant metabolic demand in these embryos. Thus, the two most important biochemical adjustments associated with metabolic depression and survival of anoxia, reduction of rates of protein synthesis and reduced ion pumping, have already been made in aerobically incubated diapausing embryos. In addition, diapausing embryos of *A. limnaeus* accumulate large stores of glutamate and glutamine during normal development, which can be easily converted into GABA for protection of neural tissues. Based on these observations, it appears that diapause II embryos of *A. limnaeus* have already made the adjustments necessary to survive anoxia, prior to any anoxic exposure, and are thus 'primed' for survival of anoxia as part of their normal developmental program. Importantly, this high tolerance of anoxia is retained during early post-diapause II development and thus embryos may break diapause and still survive long bouts of anoxia. The ecological and evolutionary implications of 'preparing' for anoxia are intriguing and further exploration of this system seems likely to yield interesting insights into the ecology and evolution of anoxia tolerance in vertebrates.

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