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The effects of hypoxia and temperature on metabolic aspects of embryonic development in the annual killifish *Austrofundulus limnaeus*

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Abstract

Embryos of *Austrofundulus limnaeus* are exceptional in their ability to tolerate prolonged bouts of complete anoxia. Hypoxia and anoxia are a normal part of their developmental environment. Here, we exposed embryos to a range of PO₂ levels at two different temperatures (25 C and 30 C) to study the combined effects of reduced oxygen and increased temperature on developmental rate, heart rate, and metabolic enzyme capacity. Hypoxia decreased overall developmental rate and caused a stage-specific decline in heart rate. However, the rate of early development prior to the onset of organogenesis is insensitive to PO₂. Increased incubation temperature caused an increase in the developmental rate at high PO₂s, but hindered developmental progression under severe hypoxia. Embryonic DNA content in pre-hatching embryos was positively correlated with PO₂. Citrate synthase, lactate dehydrogenase, and phosphoenolpyruvate carboxykinase capacity were all reduced in embryos developing under hypoxic conditions. Embryos of *A. limnaeus* are able to develop normally across a wide range of PO₂s and contrary to most other vertebrates severe hypoxia is not a teratogen. Embryos of *A. limnaeus* do not respond to hypoxia through an increase in the capacity for enzymatic activity of the metabolic enzymes lactate dehydrogenase, citrate synthase, or phosphoenolpyruvate carboxykinase. Instead they appear to adjust whole-embryo metabolic capacity to match oxygen availability. However, decreased DNA content in hypoxia-reared embryos suggests that cellular enzymatic capacity may remain unchanged in response to hypoxia, and the reduced capacity may rather indicate reduced cell number in hypoxic embryos.

Keywords

hypoxia; development; metabolic compensation; heart rate

Introduction

Understanding the effects of hypoxia on vertebrate development is crucial to understanding how the developmental environment affects organismal form and function. As a rule vertebrate tissues require oxygen to support aerobic metabolism, and exposure to hypoxia has consequential effects such as cardiovascular impairment and disease, neuropathies, inflammatory disease, diabetes, and cancer (Brahimi-Horn and Pouyssegur 2007). Proper

and complete development of vertebrate embryos requires oxygen, and even brief exposures to hypoxia or anoxia during development can have severe detrimental effects (Sloper et al. 1980; Podrabsky et al. 2012b). Most vertebrate embryos have minimal capacity to deal with the detrimental effects of hypoxia, and thus hypoxic exposures result in a plethora of negative outcomes such as impaired early stage development, decreased survival rate, delayed development, developmental anomalies, and early hatching, and even death (Altimiras and Phu 2000; Bradford and Seymour 1988; Grabowski 1961; Podrabsky et al. 2012b). In contrast to most vertebrates, embryos of the annual killifish *Austrofundulus limnaeus* have an exceptional tolerance to prolonged anoxia (Podrabsky et al. 2012b; Podrabsky et al. 2007). However, the oxygen-dependency of embryonic development has not been explored in this species. In this paper we explore the effects of hypoxia on development in embryos of *A. limnaeus* developing along two alternate developmental trajectories regulated by incubation temperature.

The annual killifish *Austrofundulus limnaeus* is endemic to South America and is predominately found in the Maracaibo basin of Venezuela (Hrbek et al. 2005). This species inhabits ephemeral ponds in areas that experience pronounced dry and rainy seasons. While adult and juvenile fish flourish throughout the rainy season (May-August) they cannot survive the drying of the ponds and thus the adult and juvenile population typically dies on an annual basis (Myers 1952). In some parts of their range, climate is very harsh and unpredictable, and ponds may go several years between inundations. Even during the rainy season, rain events are often periodic and a single pond may be inundated and dried several times. Populations survive the dry periods in a given location as drought-tolerant fertilized embryos deposited by spawning adults into the muddy pond substrate.

A. limnaeus embryos survive the prolonged desiccation of their ponds by entering diapause, a period of suspended embryonic development that increases resistance to unfavorable environmental conditions (Podrabsky et al. 2010b; Podrabsky et al. 2012b; Podrabsky et al. 2007; Machado and Podrabsky 2007; Podrabsky et al. 2001; Podrabsky and Hand 1999; Wourms 1972a, b). Annual killifish embryos may enter diapause at three developmental stages named diapause I, II and III (Wourms 1972b). In the stock of *A. limnaeus* used in our laboratory, embryos routinely enter diapause II and III (Podrabsky and Hand 1999). Diapause II occurs about midway through development after completion of somitogenesis, but prior to organogenesis (Wourms 1972b, a). Diapause III occurs in the late pre-hatching embryo, after embryonic development and yolk depletion are nearly complete (Wourms 1972b, a).

Diapause II appears to be an inducible phenotype in *A. limnaeus* as evidenced by laboratory studies illustrating that about 10–15 % of embryos incubated at 25 C follow a separate developmental trajectory that bypasses diapause II and leads to direct development to diapause III (Podrabsky et al. 2010a; Wourms 1972b). These embryos are termed “escape” embryos because they escape dormancy in diapause II (Wourms 1972b). An increase in incubation temperature of just 5 C from 25 to 30 C increases the percentage of escape embryos to 100% under laboratory conditions (Podrabsky et al. 2010a). Importantly, the escape embryos follow a different developmental trajectory compared to embryos that enter diapause II that leads to different aerobic and anaerobic capacities at the completion of

development (Chennault and Podrabsky 2010). Embryonic diapause is presumably an adaptive mechanism of annual killifish to sustain populations in their naturally variable and seasonal environment.

During both the dry and rainy season, embryos of *A. limnaeus* are buried in the pond sediment and are likely routinely exposed to hypoxia (low partial pressures of oxygen, PO_2) and anoxia ($PO_2 = 0$) as part of their normal development (Podrabsky et al., 1998). During the rainy season, PO_2 in the inundated soils is drastically decreased by microbial activity and limited diffusion through the soil matrix. During the dry season, although soil PO_2 is likely high, the physiological mechanisms employed by the embryos to retain water and avoid dehydration very likely impose severe hypoxia or anoxia on the embryos (Podrabsky et al. 2001). Extreme tolerance to anoxia is gained during early development in *A. limnaeus* and peaks during diapause II (Podrabsky et al. 2012b; Podrabsky et al. 2007). Importantly, extreme tolerance to anoxia is retained for at least 4 days of post-diapause II development. In addition, escape embryos at roughly equivalent developmental stages to embryos at 4 days post-diapause II also exhibit extreme tolerance to anoxia, suggesting that anoxia tolerance may not be associated entirely with entrance into diapause II, but may rather be a stage-specific trait (Podrabsky et al. 2012b). Embryos of all stages respond to anoxia by entering into a profound state of metabolic and developmental arrest that is accompanied by a cessation of neural and cardiac activity (Podrabsky et al. 2010b; Podrabsky et al. 2012a; Podrabsky et al. 2007; Fergusson-Kolmes and Podrabsky 2007). While diapause II embryos of *A. limnaeus* are the most anoxia tolerant vertebrates, this exceptional tolerance is lost during late development and by the time of hatching they share a sensitivity to hypoxia similar to other ectothermic vertebrates (Podrabsky et al. 2012b). In fact, when the rainy season returns and ponds refill with water, the subsequent return to hypoxic conditions is thought to be a major cue that induces hatching of killifish embryos (Podrabsky and Hand 1999; DiMichele and Powers 1984; DiMichele and Taylor 1980, 1981; Wourms 1972b). Thus, oxygen availability is not only a potential limiting factor determining developmental rates, but it is also an environmental cue potentially regulating critical life history transitions.

Despite the fact that embryos of *A. limnaeus* are likely exposed to hypoxia as a regular part of their embryonic development, very little is known about the effects of hypoxia on development in this species. It is not clear when or if oxygen limitation will alter the rate of embryonic development, or if hypoxia will alter the ability of embryos to develop along the escape trajectory. It is also not known if the embryos will respond to oxygen limitation by altering their metabolic capacity for either aerobic or anaerobic metabolism as has been illustrated for many adult organisms in response to hypoxia. This study focuses on the effects of chronic exposure to a range of oxygen partial pressures on developmental progression, cardiac function, and metabolic enzyme capacities in embryos of *A. limnaeus* incubated at 25 and 30 °C. The use of these two temperatures allows for the effects of hypoxia to be investigated in two alternate developmental trajectories, embryos that enter or escape from diapause II. Importantly, these two trajectories are known to result in embryos with differing capacities for activity of both aerobic and anaerobic metabolic enzymes when reared under normal atmospheric levels of PO_2 (Chennault and Podrabsky 2010). We report that even severe hypoxia is not teratogenic in this species. Although severe hypoxia slows developmental progression in late-stage embryos, there is little or no effect of PO_2 on

developmental progression in embryos until post-diapause II developmental stages. Embryos at 30 °C develop along the escape trajectory independent of PO₂, suggesting that oxygen limitation will not affect alteration of developmental trajectory by increased temperatures. Metabolic capacity is not altered even during long-term exposures to hypoxia, suggesting that metabolic plasticity is limited during development in *A. limnaeus*.

Materials and Methods

Husbandry of adult fish and collection of embryos

Adult fish were housed and cared for as previously described in high-density rack systems (21, 9.5 l tanks per rack connected to a common sump, total volume 190 l) equipped with biological, chemical and physical filtration systems (Machado and Podrabsky 2007; Podrabsky 1999). The adult fish used for embryo production in this study were raised as embryos at 25 ± 1°C, and as larvae and adults at 26 ± 1°C. Photoperiod was set at 14h of light and 10h of dark, representing the beginning of the rainy season in Venezuela. Fish were fed frozen bloodworms (chironomid larvae, Hikari) or chopped earthworms twice daily on Monday through Friday, and once daily on Saturday and Sunday. Ten percent water changes were performed on the same schedule as described for feeding. Embryos were collected from two independent rack systems (each with 21 spawning pairs of fish, 42 pairs total) twice weekly by natural spawning activity into plastic trays filled with 500 µm glass beads (Thomas Scientific). Typical spawning activity produces 500–1000 embryos per system each day. Embryos were separated from the glass beads and transferred into plastic culture dishes containing embryo medium (10 mM NaCl, 0.14 mM KCl, 2.2 mM MgCl₂, 0.0013 mM MgSO₄, 0.8 mM CaCl₂, 28 mOsm or ~1 ppt salinity), supplemented for the first 4 d of development with methylene blue to discourage fungal and bacterial growth (Podrabsky 1999). Embryos were then transferred to embryo medium containing 10 µg/l gentamicin at 4 days post-fertilization (dpf)

The effects of hypoxia on developmental progression and heart rate

Embryos were exposed continuously to seven different PO₂s spanning moderate to severe hypoxia: 21.2, 15.6, 10.8, 8.4, 6.1, 3.6, and 2.2 kPa, at two different temperatures, 25C and 30C, for the entire duration of embryonic development. Within 2–4 h following fertilization (2–4 cell-stage embryos) embryos from a complete spawning of two rack systems (each representing 21 spawning pairs of fish) were combined, randomly divided into 14 culture dishes and placed into one of 14 different PO₂ and temperature combinations. At the time of their first observation in hypoxia (24 h later) 12–20 embryos were randomly separated into 24-well tissue culture plates (one embryo per well) so that individual embryos could be followed through development. This was the maximum number of embryos (over 300 embryos) that could be observed and manipulated in a day given the number of treatments in the experimental design. The actual number of embryos observed for the duration of development in the 25°C diapause and 30°C escape treatments varied from 9–19.

Exposure to hypoxia was accomplished using a Bactrox hypoxic chamber (Sheldon Laboratories, Cornelius, OR) that controls oxygen partial pressure by mixing atmospheric air with industrial grade high-pressure nitrogen gas (Matheson Inc.). The chamber was

initially set at atmospheric levels of oxygen (21.2 kPa) and at the initiation of the experiment the partial pressure was decreased in a step-wise fashion allowing at least 30 min for the PO₂ to equilibrate within the chamber once the set point was reached. The chamber moved between set points within 10–20 min, with lower PO₂s taking longer to achieve. Embryo medium (Podrabsky 1999) was brought to equilibrium with the chamber atmosphere by bubbling the hypoxic chamber air through the medium with an aquarium pump and scintered glass diffuser for at least 30 min after the chamber had reached the appropriate set point. The embryo medium was changed with appropriately equilibrated medium each time the embryos were observed. The culture dishes or well-plates containing the embryos were then sealed in 550 ml polypropylene plastic containers (Lock-n-Lock) fitted with air-tight (rubber gasket sealed) lids at their respective oxygen level and temperature prior to moving the chamber to the next PO₂ set point. Preliminary experiments confirmed that the PO₂ was constant in the containers for at least 24 h. At the end of the first day the chamber was left at a PO₂ of 2.2 kPa. The following day the first embryos observed were those sealed the first day into conditions of 2.2 kPa PO₂. Chamber PO₂ was then increased step-wise and embryos were removed from their plastic containers, observed under the appropriate PO₂ conditions, and re-sealed into the plastic containers before moving to the next PO₂ set point. At the end of the second day the last embryos observed were those exposed to a PO₂ of 21.2 kPa. The chamber was always left at the final oxygen partial pressure achieved each day. Thus, every other day the chamber was at either 2.2 or 21.2 kPa for the start of observations. This schedule eliminated any systematic effects in developmental staging and progression due to the time of day the embryos were observed across the different oxygen treatments. The temperature of the embryo medium was controlled at 30°C by placing it in the built-in incubator set to 30°C contained within the Bactrox chamber. A temperature of 25°C was achieved by using the chamber working area which remained at 25 ± 1°C.

Embryos were observed within the hypoxic chamber at the appropriate PO₂ and temperature using an inverted compound microscope (Leica, DMIL) outfitted with a constant temperature stage (20/20 Technology, Inc., Wilmington, NC) set at either 25 or 30°C. Observations were made daily for the first 85 d of the experiment, or until the embryos had completed development and been in stage 43 (diapause III) for 11 days. For those embryos that required greater than 85 days to complete development, they were observed every other day from 85 days until they were at stage 43 for 11 days. The maximum time for developmental observations was 104 days. Embryonic stages were determined according to definitions outlined by Wourms and are described in Wourms' stages (WS) (Wourms 1972a). After the initiation of heart function, heart rate was quantified at the appropriate temperature and oxygen conditions. For determination of heart rate, heart beats were counted for 1–2 min and expressed in beats per min (BPM). At the end of the experiment, individual embryos were blotted dry using a mesh screen over a stack of paper towels, weighed, and flash-frozen in 0.65 ml microcentrifuge tubes by submersion in liquid nitrogen. Embryos were stored at –80°C until determination of enzyme activity.

When incubated at 25°C, the great majority of embryos enter diapause II. In order to complete the observations in a reasonable amount of time, diapause II was experimentally broken in these embryos by placing the sealed containers into an incubator at 30°C with a long-day photoperiod (14 h light, 10 h dark). The embryos were returned to the Bactrox

chamber daily to inspect for resumption of development. As soon as development resumed the embryos were returned to 25°C to complete development. Most embryos break diapause II within 2–3 d of this treatment, however a few embryos took as long as a week before resuming development.

The effects of temperature on developmental progression and heart rate

To evaluate the effect of temperature on developmental rates for different periods of development, temperature quotients (Q_{10} values) were calculated from rates of development (stages/day) for several major periods using the equation: $Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$. For a large range of biological rate processes a 10°C increase in temperature leads to a doubling in rate, and thus a Q_{10} value of 2 would indicate typical temperature sensitivity of development (Withers 1992). Development was separated into 6 different developmental periods for this analysis: cleavage through completion of epiboly (Cl/Ep, WS 1–19), dispersion and reaggregation of blastomeres (Ds/Re, WS 20–27), formation of the embryonic axis and neurogenesis through completion of somitogenesis (Ne/So, WS 28–31), early organogenesis (EO, WS 34–36), mid-organogenesis (MO, WS 37–40), and late organogenesis (LO, WS 41–43). Rates of development for each period were estimated by linear regression analysis of developmental progression at either 25°C or 30°C.

For heart rate, temperature sensitivity was assessed by calculating Q_{10} values for heart rates determined for embryos incubated continuously at 25°C and 30°C at each developmental stage during post-diapause II development. Because these embryos were incubated continuously at either temperature, this analysis represents the cumulative effects of temperature on heart rate when development proceeds at a constant temperature.

The effects of hypoxia on the capacity for metabolic enzyme activity

Embryo homogenates (10x) of single embryos were prepared by adding 9 volumes (by mass) of ice-cold 10 mM tris (pH= 7.2) directly to the microcentrifuge tube containing the frozen embryo, followed by homogenization with an acrylic pellet pestle in the microcentrifuge tube (Thomas Scientific). The homogenate was always kept on ice. The homogenate was used directly for determination of phosphoenolpyruvate carboxykinase enzymatic capacity. For determination of citrate synthase and lactate dehydrogenase activity a 20x homogenate was prepared by dilution of the original homogenate with additional tris buffer. All enzyme assays were conducted at a temperature of 25°C in water-jacketed cell using a UV/VIS spectrophotometer (Shimadzu PharmaSpec 1700). Data were recorded digitally using UVProbe software (Version 2.01, Shimadzu), and were exported into Prism software (Version 5.0, GraphPad Software) for determination of slopes and enzymatic activities. Due to the small amount of sample available from a single embryo (~ 2.5 mg tissue/embryo), single enzyme assays were run for each enzyme for each embryo. Biological replication was achieved by performing assays on 4 individual embryos from each treatment group in the 25°C diapause and the 30°C escape treatments.

Citrate synthase—Citrate synthase (CS) enzymatic capacity was determined as described previously (Chennault and Podrabsky 2010). Each assay consisted of 1 ml of 50 mM imidazole buffer (pH 8 @ 25°C), 1.5 mM KCl, 0.1 mM DTNB, and 0.1 mM acetyl-

coenzyme A. Background activity was measured for 5 min after the addition of 10 μ l of 20x diluted embryo homogenate. The CS reaction was then initiated by the addition of 12.5 μ l of 0.7 mM oxaloacetic acid. An increase in absorbance at 412 nm was monitored for 10 min and the last 30 sec used to calculate a slope for CS activity. Data are expressed in International Units (IU, μ mol substrate min^{-1})

Lactate dehydrogenase—Lactate dehydrogenase (LDH) enzymatic capacity was determined as described in previously (Chennault and Podrabsky 2010). Each assay consisted of 1 ml of assay buffer containing 80 mM imidazole (pH = 7.2 @ 25°C), 100 mM KCl, 0.15 mM NADH, and 4 mM pyruvic acid prepared in reagent grade water. The reaction was initiated by the addition of 5 μ l of 20x diluted homogenate. Disappearance of NADH was monitored at a wavelength of 340 nm. Initial slopes were determined on 30 sec of data starting at 10 sec after addition of the embryo homogenate. Data are expressed in International Units (IU, μ mol substrate min^{-1}).

Phosphoenolpyruvate carboxykinase—Phosphoenolpyruvate carboxykinase (PEPCK) enzymatic activity was determined on individual embryos based on the methods outlined in (Lockwood and Somero 2012). Each assay consisted of 1 ml of assay buffer containing 100 mM imidazole-HCl (pH = 6.9 @ 20°C), 1 mM MnCl_2 , 1 mM MgCl_2 , 20 mM NaHCO_3 , 1 mM GDP, 0.15 mM NADH, and 5 U/ml MDH prepared in reagent grade water. Background activity was measured for 5 min after the addition of 0.01 ml of 10x diluted embryo homogenate. The PEPCK reaction was then initiated by the addition of 40 μ l of 25 mM phosphoenolpyruvate. Disappearance of NADH was monitored at a wavelength of 340 nm and the first 60 sec of data starting 10 sec after addition of the embryo homogenate was used to calculate a slope for PEPCK activity.

DNA quantification

The amount of total DNA per embryo was determined using a Quant-iT dsDNA broad range assay kit (Invitrogen, Eugene, OR). Working reagent and standard curves were generated according to the manufacture's instructions using 200 μ l of working reagent and 10 μ l of each standard in a flat-bottom black 96-well plate (Costar). One μ l of 10x diluted embryo homogenate and 9 μ l of water were added for each experimental sample. Total fluorescence was measured using an excitation of wavelength of 480 nm and an emission wavelength of 530 nm (Tecan Infinite M200 Pro microplate reader).

Statistics

Statistical analyses were performed using Prism 5.0 (Graphpad Software Inc., 1994–2010) and Microsoft Office Excel (2010). Two-way ANOVA was performed on the 25°C and 30°C data to test for differences in developmental progression, heart rate, DNA content, and enzymatic capacity at the various levels of PO_2 . Bonferroni corrected post-hoc tests were then used to determine which PO_2 treatments were significantly different at the two experimental temperatures. One-way ANOVA was used to evaluate differences in mean DNA content and enzymatic capacity within each temperature treatment relative to PO_2 . Student-Newman-Keuls *post-hoc* tests were used to compare individual means where appropriate.

Results

The effect of hypoxia on developmental trajectory

All embryos incubated at 30 °C, even those exposed to severe hypoxia, bypassed diapause II and followed the escape trajectory (data not shown). However, embryos incubated at 30 °C at PO₂ levels less than 6.1 kPa were unable to complete embryonic development (see below). The cessation of development associated with severe hypoxia should not be confused with entrance into diapause because the embryos were clearly past the stage of development when embryos may enter diapause II. The intent of this study was not to investigate the effects of hypoxia on escape embryos at 25 °C, but by chance enough escape embryos (n = 3–7) were produced to allow evaluation of the effects of hypoxia on escape embryo development at 25 °C for PO₂ levels of 8.4 kPa and above. However, it must be noted that the sample size is only one embryo for the 2.2 and 6.1 kPa treatments and no escape embryos were observed in the 3.6 kPa treatment. The escape embryo data below 6.1 kPa at 25 °C are presented because they are interesting and enrich the data set, however they should be interpreted with great caution due to low sample size. In addition, only developmental data are presented, and no biochemical analyses were performed on embryos that followed the escape trajectory at 25 °C due to the low sample size.

The effect of hypoxia on developmental progression

Survival of embryos ranged from 30–50% across all treatments groups and no relationship was found between survival and hypoxia or temperature treatments (data not shown). Nearly all of the mortality occurred in the first 3 days of incubation, with survival after 4 d at or near 100% for the duration of development. This pattern and level of mortality is typical for embryos for *A. limnaeus* and has been described previously (Podrabsky 1999).

The time required for each embryo to reach each developmental stage is presented in Figure 1. Developmental progression was delayed significantly at low PO₂s in embryos incubated at both experimental temperatures. For example, embryos incubated at 25 °C and a PO₂ of 21.2 kPa reached completion of development (stage 43, diapause III) after an average of 43 days of active development (not counting days in diapause II) whereas embryos raised at 2.2 kPa took an average of 85 days. To compare developmental progression, benchmarks of development (Table 1) were established and the mean time required for embryos to reach these developmental stages were compared using two-way analysis of variance (Online Resource 1). In early developmental stages, PO₂ did not have an affect on developmental progression at either temperature. Interestingly, hypoxia did not delay development until post-diapause II stages (WS 34 and greater) in embryos entering diapause II, or equivalent embryonic stages in escape embryos incubated at 25 °C (Online Resource 1). At an incubation temperature of 30 °C, only the most severe hypoxic treatment affected developmental progression during early development (Online Resource 1). Thus the rate of development during nearly the entire duration of early development through somitogenesis is independent of PO₂. For post-diapause II development and equivalent stages in escape embryos, there is a general pattern of slower developmental rates at lower PO₂s (Fig. 1). While all embryos incubated at 25 °C were able to complete development at even the lowest

PO₂ treatments, incubation at 30 °C was arrested prior to the completion of development at PO₂s lower than 6.1 kPa.

The effect of temperature on developmental progression

Incubation of embryos at 30 °C accelerated the rate of development compared to embryos incubated at 25 °C resulting in the embryos reaching developmental benchmarks in a shorter amount of time for all PO₂ levels examined (Fig. 1, Online Resource 2). The one exception to this rule is the time taken to reach completion of epiboly (WS 19) at PO₂ levels below 21.2 kPa. However, at lower PO₂ levels the effect of temperature is much less pronounced and is largely offset by the slowing of development by hypoxia, especially during late development (Fig. 1, Online Resource 2). Rate of development for major periods of development were modeled by linear regression analysis with r^2 values ranging from 0.78–0.99. For the 30 °C data, r^2 values were 0.94 ± 0.06 (mean $\pm$ s.d.) while those for the 25 °C data were 0.91 ± 0.05 . These rates of development were used to calculate Q₁₀ values (Fig. 2). Two-way ANOVA revealed no relationship between developmental trajectory and Q₁₀ values at any of the PO₂ levels ($p > 0.2$). Thus, one-way ANOVA was used to compare mean Q₁₀ values across different developmental periods within each treatment level of PO₂. For all levels of PO₂, temperature sensitivity of developmental rate increased from typical Q₁₀ values around 2 during cleavage and epiboly to values between 4 and 10 during the dispersion/reaggregation and neurulation/somitogenesis periods of development. Q₁₀ values then decrease during mid to late organogenesis to levels that approach 1, suggesting temperature insensitivity during late development. Importantly, at lower PO₂s, Q₁₀ values during mid and late organogenesis are below 1, indicating that hypoxia has slowed development significantly at 30 °C enough to offset the expected increase in developmental rate at increased temperature.

Effects of hypoxia on heart rate

Heart rates were compared at each development stage starting just after formation of the heart (WS 31) through completion of embryonic development (WS 43) for each treatment group (Fig. 3). The pattern of heart rate is different in embryos developing along the diapause and escape trajectories. For embryos that will enter diapause, heart rate reaches a pre-diapause II peak shortly after heart formation and then decreases to rates at or below 10 beats per minute during diapause II at all levels of PO₂ (Fig. 3). In contrast, heart rate of escape embryos increases continuously during early development (Fig. 3). While there is a general trend for decreased heart rate with decreased PO₂, this effect is relatively mild and does not become pronounced until PO₂ is reduced to 6.1 kPa and below at 30 °C and 3.6 kPa and below at 25 °C. Two-way ANOVA reveals that heart rate is significantly depressed during mid to late organogenesis at PO₂ levels below 15.6 kPa in embryos incubated at both 25 and 30 °C (Online Resource 3). One interesting pattern in heart rate is that stage 43 embryos converge on a common heart rate independent of PO₂ when incubated at 30 °C, while PO₂ appears to affect heart rate in a graded manner in stage 43 embryos at 25 °C.

The effect of temperature on heart rate

Heart rate was higher in embryos incubated at 30 °C compared to those at 25 °C (Online Resource 4). Q₁₀ values suggest an effect of temperature on heart rate that is typical of

temperature effects on biological rates at PO₂s of 15.6 kPa and above (Fig. 4). However, at PO₂ levels of 8.4 kPa and lower, Q₁₀ values drop below 2, indicating an increasing effect of hypoxia on the suppression of heart rate that overcomes the expected increase associated with increased incubation temperature.

The effect of hypoxia on total DNA content in stage 43 embryos

There is a significant relationship between reduced PO₂ and reduced DNA content at in stage 43 embryos at both temperatures (Fig. 5; Two-way ANOVA, $p < 0.049$ for PO₂; $p < 0.0001$ for temperature; $p = 0.77$ for interaction). While one-way ANOVA of the 25 °C data failed to identify a relationship between PO₂ and DNA content ($p = 0.56$), there was a significant decline in DNA content in embryos reared at 30 °C and 6.1 kPa ($p = 0.048$, see Fig. 5 for *post hoc* comparisons). This decline in total DNA content of embryos incubated under hypoxic conditions was a surprising and unexpected result.

The effect of temperature on total DNA content in stage 43 embryos

Increasing the incubation temperature of embryos from 25 °C to 30 °C caused a significant decrease in DNA content of embryos at the completion of development (Fig. 5; for statistical information see results of the two-way ANOVA in the preceding paragraph). This effect was especially apparent in the lower PO₂ treatments.

The effect of hypoxia on metabolic enzyme activity in stage 43 embryos

Citrate synthase—Development under hypoxic conditions caused a decrease in the whole embryo capacity for citrate synthase (CS) activity (Fig. 6; two-way ANOVA, $p < 0.0001$ for PO₂; $p = 0.37$ for temperature; $p = 0.96$ for interaction). This relationship was confirmed at each temperature by one-way ANOVA ($p < 0.001$ for both 25 and 30 °C treatments, see Fig. 6 for *post hoc* comparisons). When CS activity was expressed per unit of DNA (a proxy for cell number) the significant relationship between PO₂ and CS capacity was retained, although with greatly reduced significance (Fig. 6; two-way ANOVA, $p = 0.05$ for PO₂; $p = 0.09$ for temperature; $p = 0.71$ for interaction). One-way ANOVA failed to find a significant affect of PO₂ on the capacity for CS activity expressed per unit of DNA at either incubation temperature ($p = 0.23$ for 25°C and $p = 0.16$ for 30°C).

Lactate dehydrogenase—Whole embryo capacity for lactate dehydrogenase (LDH) activity was reduced in stage 43 embryos as a result of reduced PO₂ (Fig. 7; two-way ANOVA, $p < 0.0001$ for PO₂; $p = 0.118$ for temperature; $p = 0.76$ for interaction). Significant declines were observed in both temperature treatments under even mild hypoxia, with more severe hypoxia causing a graded response in embryos incubated at 30 °C (one-way ANOVA $p < 0.0007$ for both 25 and 30 °C, see Fig. 7 for *post hoc* comparisons). No significant relationships were observed when the capacity for LDH activity was expressed per unit of DNA (Fig. 7; two-way ANOVA, $p = 0.30$ for PO₂; $p = 0.12$ for temperature; $p = 0.56$ for interaction).

Phosphoenolpyruvate carboxykinase—A significant relationship was found between whole embryo capacity for phosphoenolpyruvate carboxykinase (PEPCK) activity and reduced PO₂ (Fig. 8; two-way ANOVA, $p = 0.029$ for PO₂; $p = 0.024$ for temperature; $p =$

0.82 for interaction). This relationship was supported by one-way ANOVA in the 30 °C but not the 25°C treatments ($p = 0.11$ for 25°C and $p = 0.047$ for 30°C, see Fig. 8 for *post hoc* comparisons). No significant relationship was identified between PEPCK capacity expressed per unit of DNA and PO₂ (two-way ANOVA, $p = 0.12$ for PO₂; $p = 0.48$ for temperature; $p = 0.86$ for interaction).

The effect of temperature on metabolic enzyme activity in stage 43 embryos

Temperature had no significant effect on the capacity for activity of CS or LDH when expressed per whole embryo or per unit DNA (Figs. 6, 7; see above paragraphs for results of two-way ANOVA analyses). In contrast, whole embryo capacity for PEPCK activity was reduced in the 30 °C compared to the 25 °C treatment (Fig. 8; see above for statistical analyses). This effect is likely due to the reduced PEPCK activity in 30 °C embryos incubated at 6.1 and 8.4 kPa.

Discussion

The effect of temperature on developmental progression and heart rate

The general increase in the rate of development and heart rate associated with incubation at 30 °C compared to those at 25 °C is consistent with previous investigations and would be predicted based on basic principles of physiology. However, more detailed analysis reveals a pattern for increasing temperature sensitivity during early development that peaks during somitogenesis or early organogenesis depending on the level of hypoxia (Fig. 2). Late development appears to be much less temperature-sensitive than would be predicted by theory as judged by Q_{10} values below 2 and approaching 1. This change in temperature sensitivity cannot be attributed to hypoxic delays in development because they occur even at the relatively high partial pressures of oxygen that do not result in significant developmental delay. Interestingly, the peak of temperature sensitivity shifts to earlier developmental stages as hypoxia becomes more severe. The causes and consequences of this pattern are currently unknown, but point towards a shift in the expression of temperature-sensitive physiological systems.

Similar to the data for developmental progression, heart rate appears to become less sensitive to incubation temperature during late development (Fig 4). In fact, during late development Q_{10} values for heart rate approach 1, indicating temperature insensitivity. Thus, heart activity appears to be more temperature sensitive during the early portions of cardiac development compared to later stages. Hypoxia tends to decrease temperature sensitivity of heart activity, even at levels of hypoxia that do not impede development. The lower temperature sensitivity of heart rate in late embryos is somewhat unexpected, and may suggest metabolic limitations to supporting heart activity in late embryos.

The effects of hypoxia on developmental progression

Vertebrate embryos exhibit a general intolerance to hypoxia. Delayed development and early hatching are often observed in both frog and fish embryos at low partial pressures of oxygen (Mueller et al. 2011; Bradford and Seymour 1988). Even short bouts of hypoxia are often enough to impair early stage development and decrease survival rates in chicken embryos

(Altimiras and Phu 2000). Hypoxia is often teratogenic in fish embryos (Alderdice et al. 1958; Shang and Wu 2004) and in fact for most vertebrate embryos (Grabowski 1961; Grabowski and Paar 1958; Grabowski and Schroeder 1968; Parer 1998).

While there are many studies that have evaluated the effects of short-term hypoxia on development, there are relatively few that have documented the chronic PO₂ level that is required to complete embryonic development through first feeding, which is the most comparable stage of development to a pre-hatching *A. limnaeus* that begin feeding immediately upon hatching. When these studies are compared, embryos of *A. limnaeus* exhibit a tolerance of hypoxia for the completion of embryonic development that is higher than most other species of fish, especially when incubation temperature is taken into consideration (Fig 9). Thus, we conclude that embryos of *A. limnaeus* are extraordinarily tolerant of hypoxia and are uniquely adapted for development under oxygen limitation. This ability would clearly be an advantage in the severely hypoxic or anoxic sediments in which these embryos must develop.

Progression through early development up to stage 33 (diapause II) is independent of PO₂ for embryos incubated at 25 °C, and only the most severe level of hypoxia slowed development at 30 °C. This pattern is consistent with other species of fish, with early development being able to proceed even under extreme hypoxia and sometimes even anoxia (Crawford and Wilde 1966). Oxygen-independence of developmental rate up to the point of diapause II may reflect the metabolic poise of the early *A. limnaeus* embryos to rely more heavily on anaerobic pathways (Chennault and Podrabsky 2010; Podrabsky and Hand 1999), or it may indicate that diapause II occurs at a critical point in development just prior to when oxygen demands dramatically increase. Hypoxia delayed developmental progression only during post-diapause II development (early organogenesis) at both temperatures. This pattern is consistent with reports in the literature for other fish species (Alderdice et al. 1958; Carlson and Siefert 1974; Kaur and Toor 1978; Siefert et al. 1973; Spoor 1977; Czerkies et al. 2002; Kinne and Kinne 1962; Mueller et al. 2011). This is also consistent with the few studies that have explored aerobic versus anaerobic metabolism during fish development through combined calorimetry and respirometry. These studies all concluded that development was supported though almost exclusively aerobic metabolism during mid to late development (Podrabsky and Hand 1999; Finn et al. 1995; Paynter et al. 1991). The oxygen-dependent delay in development is exacerbated at the higher incubation temperature of 30 °C to the extent that embryos exposed to severe hypoxia stalled at early to mid organogenesis developmental stages. From these data we would predict that the critical oxygen tension would increase as the embryos develop, as has been shown in other fish species during development (Mueller and Seymour 2011). It is curious that hypoxia-dependent developmental delay begins in this species at the developmental stage when hemoglobin expression is first observed (Stage 34). While at this point it is only a correlation, these data suggest that convective oxygen transport may facilitate organ growth and development through more efficient gas, nutrient, and waste exchange as embryonic structures reach greater sizes. This would be a very interesting and important conclusion because there is some evidence that hemoglobin function does not impact oxygen-dependent physiological functions in late zebrafish embryos and larvae (Pelster and Burggren 1996).

Impressively, despite the slowing of developmental rate, embryos of *A. limnaeus* appear to be resistant to developmental abnormalities that are commonly induced by severe hypoxia during development. In almost every study examining the effects of hypoxia on fish and amphibian development, abnormal development is described for at least a portion of the embryos that survive severe hypoxia (Seymour et al. 2000; Strecker et al. 2011; Garside 1959; Keckeis et al. 1996; Shang and Wu 2004; Barry et al. 1995). Thus, we conclude that embryos of *A. limnaeus* are uniquely adapted to developing under severe hypoxia, and are able to alter developmental rate and metabolism to match oxygen availability and avoid the detrimental effects associated with a mismatch in metabolic demands and oxygen availability that often leads to cell death or damage in other vertebrates. Perhaps the arrested development associated with extreme tolerance of anoxia in this species (Podrabsky et al. 2012b; Podrabsky et al. 2007) represents the ultimate extension of hypoxia tolerance as the embryos continue to match oxygen supply and metabolic demands down to levels where anaerobiosis is the only option.

The effect of hypoxia on heart rate during embryological development

Hypoxia caused a stage-specific decrease in heart rate at both incubation temperatures, with the most pronounced effects observed at 30 °C. This is consistent with what little information is available for the effects of hypoxia on heart rate in fish embryos (Bagatto 2005; Barrionuevo and Burggren 1999). However, in embryos incubated at 25 °C this effect was not observed until post-diapause II development and only under severe hypoxia. These data support the earlier conclusion that early development up to diapause II is relatively unaffected by oxygen partial pressure in embryos of *A. limnaeus*.

No obvious developmental defects were observed in heart development as a consequence of hypoxia in embryos of *A. limnaeus*. In contrast, embryos from other vertebrates suffer greater consequences under conditions similar to those used in this study. For example, chick embryos raised at a PO₂ of 5.1 kPa exhibited a range of heart defects such as an increased number of ventricles, septum defects, hemorrhaging, necrosis, and vessel misplacement (Jaffee 1974), while those raised at a PO₂ of 6.1 kPa had an increase in heart size and blood vessel size (Grabowski and Schroeder 1968). In zebrafish embryos, heart defects such as pericardial edema and the inability to establish blood circulation were two of the main abnormalities reported under severe hypoxia (Strecker et al. 2011). While it is possible that subtle changes in heart development are associated with developmental hypoxia in embryos of *A. limnaeus*, overall it appears that normal heart development is possible across a wide range of oxygen availability.

The effect of hypoxia on total DNA content of stage 43 embryos

The decline in total DNA content of embryos incubated under hypoxic conditions was an unexpected result of this study because the embryos were all measured at the same developmental stage. There are three possible ways to explain this pattern. First, embryos raised under hypoxia may hatch with fewer cells. Second, because many vertebrate embryos are thought to contain large amounts of mitochondrial DNA (Dawid 1966, 1965), it is possible that hypoxia causes a reduction in mitochondrial volume or at least mitochondrial DNA content. This hypothesis is consistent with the lower levels of CS activity (a

mitochondrial enzyme) in hypoxia treated embryos (see below). Third, if the yolk DNA is indeed “cytoplasmic DNA” as described by (Baltus et al. 1968), then differential degradation or utilization of this yolk DNA could explain the patterns observed. While unfertilized embryos of *A. limnaeus* have a significant amount of total DNA (Podrabsky and Hand 1999), much greater than can be accounted for by the nuclear genome, the nature of the DNA has not been explored. The true nature and significance of this decrease in embryonic DNA content will have to await more detailed studies on the nature of the yolk DNA in this species, and the changes in the various “types” of DNA during development at various levels of hypoxia.

The effects of hypoxia on metabolic enzyme activity

Hypoxia has profound effects on the physiology of most organisms. In adults and juveniles, hypoxia typically induces biochemical changes that support anaerobic metabolic pathways, sometimes at the expense of aerobic capacity (Papandreou et al. 2006). For example, juveniles of the common estuarine fish *Leiostomus xanthurus* show a significant increase in LDH activity and no significant change in citrate synthase activity in gill, liver, and muscle tissue when exposed to 10% oxygen saturation (~2.1 kPa) for 12 hours (Cooper et al. 2002). In contrast, evidence from the work presented here for embryos of *A. limnaeus* and those from the literature for other species such as Atlantic salmon (Matschak et al. 1998) suggest not only a lack of compensatory acclimation capacity in fish embryos, but rather a decrease in capacity for both aerobic and anaerobic pathways. This could be an adaptive mechanism to match rates of yolk consumption with available external resources such as oxygen, leading to more efficient usage of the finite yolk resources available for development. However, this also suggests that development can be supported across a wide range of metabolic and developmental rates, and there must presumably be mechanisms that maintain appropriate developmental sequences across organ systems such that the ontogeny of development is not greatly perturbed. In adults, there are many tissue-specific responses to hypoxia, but perhaps the need to maintain developmental sequences within a rather narrow range of values, has selected for a single response of all organ systems to a reduction in oxygen availability in embryos.

At the heart of the adult vertebrate response to hypoxia is a signaling pathway that depends on the oxygen-dependent activity of a transcription factor known as hypoxia-inducible factor-1, or HIF-1 (Papandreou et al. 2006). Activation of this transcription factor by reduced levels of oxygen typically leads to upregulation of the expression of genes involved in anaerobic energy production including most glycolytic enzymes and LDH and PEPCK, and often an active down-regulation of aerobic mitochondrial metabolism (Papandreou et al. 2006). The absence of an increase in LDH or PEPCK activity in hypoxic *A. limnaeus* embryos suggests either a lack of a canonical HIF-1 response or a dramatically altered response that does not result in a functional increase in these enzymes as is observed in other species. The activity of HIF-1 is currently not known in embryos of *A. limnaeus*, and future studies should focus on the possible role, (or lack thereof) for this highly conserved transcription factor in mediating survival of hypoxia in this species.

Significance

Most vertebrate species require relatively high oxygen partial pressures to develop functionally and completely; therefore, the ability of *A. limnaeus* embryos to complete development in the face of severe hypoxia is significant. The lack of a teratogenic effect of chronic severe hypoxia is also noteworthy. Embryos of *A. limnaeus* appear to adjust their metabolic rate to match oxygen availability and thus avoid a mismatch between oxygen supply and metabolic demand. However, this strategy appears to come at the cost of reduced total DNA content and enzymatic capacity in response to severe hypoxia. Thus, oxygen availability has the potential to generate different physiological capacities in embryos may translate into differences in individual performance and fitness. Understanding how hypoxia may alter developmental outcomes is a critical step towards revealing how environmental influences can alter ecological interactions and evolutionary trajectories in vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The effect of hypoxia on developmental progression.

Minimum number of days to reach each developmental stage for **a** embryos incubated at 25 °C that enter diapause II, **b** escape embryos incubated at 25 °C, and **c** escape embryos incubated at 30 °C. Development is delayed starting at early organogenesis at low partial pressures of oxygen at both incubation temperatures. The x-axis starts at stage 28 (solid neural keel) in the large panels because developmental rate of early development was unaffected by PO_2 . Insets display developmental progression as a function of time in days post-fertilization (dpf) for all of development. Note that embryos incubated at 30C and PO_2

levels of 2.2 and 3.6 kPa did not complete development by the end of the experiment. Symbols are means $\pm$ sem (n = 9–13 embryos for 25 °C diapause; n = 3–7 for 25 °C escape at 8.4 kPa and above, and n = 1 for 2.2 and 6.1 kPa; n = 12–15 embryos for 30 °C escape embryos).

Figure 2. The effect of temperature on developmental progression.

The rates of development during the dispersion/reaggregation and neurulation-somitogenesis periods of development are the most sensitive to incubation temperature. Developmental trajectory did not affect the temperature sensitivity of developmental rate (two-way ANOVA, $p > 0.19$). Bars are means $\pm$ sem ($n = 8-10$ embryos). Bars with different letters indicate mean Q_{10} values that are statistically different during different developmental periods within each level of PO_2 for embryos incubated at 25 °C on the diapause trajectory compared to those incubated at 30 °C on the escape trajectory (Kruskal-Wallis test, with Dunnett's *post-*

hoc comparisons, $p < 0.05$). Cl/Ep = cleavage and epiboly; Ds/Re = dispersion and reaggregation; Ne/So = neurulation and somitogenesis; EO = early organogenesis; MO = mid organogenesis; LO = late organogenesis.

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Figure 3. The effect of hypoxia on heart rate at each developmental stage.

Heart rate (bpm) relative to developmental stage (WS) in embryos developing across a range of PO₂ levels at **a** 25 °C on the diapause trajectory, **b** at 25 °C on the escape trajectory, and **c** at 30 °C on the escape trajectory. Hypoxia causes a decrease in heart rate during development (See Online Resource 3 for results of two-way ANOVA). Note that embryos incubated at 30 °C and PO₂ levels of 2.2 and 3.6 kPa did not complete development by the end of the experiment. Symbols are means $\pm$ sem. (n = 9–13 for 25 °C diapause trajectory

and 30 °C escape trajectory embryos; n = 3–7 embryos for the 25 °C escape trajectory at 8.4 kPa and above, and n = 1 for 2.2 and 6.1 kPa).

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Figure 4. The effect of temperature on heart rate during development.

Heart rate exhibits typical temperature sensitivity during development at PO_2 levels of 10.8 kPa and above. At lower levels of PO_2 , hypoxia appears to overcome the expected effects of temperature resulting in Q_{10} values near or below 1. Bars are means $\pm$ sem (see Figure 3 for information on experimental replication). Bars with different letters indicate mean Q_{10} values that are statistically at different developmental stages within each level of PO_2 in embryos developing at 25 °C on the diapause trajectory compared to those developing at

30 °C on the escape trajectory (Kruskal-Wallis test, with Dunnett's *post-hoc* comparisons, $p < 0.05$).

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Figure 5. Whole embryo DNA content at the completion of development (Stage 43, diapause III). Hypoxia caused a reduction in total DNA content of embryos incubated at 30 °C (see text for details of statistical comparisons). Symbols and bars represent the mean $\pm$ sem ($n = 8$ embryos). Symbols with an asterisk denote means that are statistically different between the two temperatures within an oxygen level (two-way ANOVA with Bonferroni corrected t -tests, $p < 0.05$). Bar graphs depict statistical differences within each temperature treatment as a function of PO₂ (one-way ANOVA, SNK post hoc tests). Bars with different letters are statistically different ($p < 0.05$). Note that embryos incubated at 2.2 and 3.6 kPa did not complete development at 30 °C and thus were not compared statistically to the rest of the data. However, the data are presented in the bar graphs with the most advanced stage of development reached listed above the bars.

Figure 6. The effect of hypoxia and temperature on enzymatic capacity for citrate synthase activity in stage 43 embryos.

Enzyme activity is expressed in international units (IU) per gram of embryonic tissue (upper panels) or per gram of total DNA (lower panels). Symbols and bars represent means $\pm$ sem ($n=4$ embryos). Bar graphs depict statistical differences within each temperature treatment as a function of PO₂ (one-way ANOVA, SNK post hoc tests). Bars with different letters are statistically different ($p < 0.05$). Note that embryos incubated at 2.2 and 3.6 kPa at 30 °C did not complete development and thus were not compared statistically to the rest of the data. However, the enzyme capacity of these embryos is presented in the bar graphs and the most advanced stage of development reached by those embryos is listed above the bars.

Figure 7. The effect of hypoxia and temperature on enzymatic capacity for lactate dehydrogenase activity in stage 43 embryos.

Enzyme activity is expressed in international units (IU) per gram of embryonic tissue (upper panels) and per gram of total DNA (lower panels). Symbols and bars represent means $\pm$ sem ($n=4$ embryos). Bar graphs depict statistical differences within each temperature treatment as a function of PO₂ (one-way ANOVA, SNK post hoc tests). Bars with different letters are statistically different ($p < 0.05$). Note that embryos incubated at 2.2 and 3.6 kPa did not complete development and thus were not compared statistically to the rest of the data. However, the enzyme capacity of these embryos is presented in the bar graphs and the most advanced stage of development reached by those embryos is listed above the bars.

Figure 8. The enzymatic capacity for phosphoenolpyruvate carboxykinase activity in stage 43 embryos.

Enzyme activity is expressed in international units (IU) per gram of embryonic tissue (upper panels) and per gram of total DNA (lower panels). Symbols and bars represent means $\pm$ SEM (n=4 embryos). Bar graphs depict statistical differences within each temperature treatment as a function of PO_2 (one-way ANOVA, SNK post hoc tests). Bars with different letters are statistically different ($p < 0.05$). Note that embryos incubated at 2.2 and 3.6 kPa did not complete development and thus were not compared statistically to the rest of the data. However, the enzyme capacity of these embryos is presented in the bar graphs and the most advanced stage of development reached by those embryos is listed above the bars.

Figure 9. The minimum PO_2 required to complete normal development in several species of fishes.

Data from the literature for several species of coldwater and warmwater fish species that were incubated under chronic hypoxia for the duration of embryonic and larval development to the stage of first feeding. First feeding was used to functionally define completion of development because this is the stage that is most similar to the pre-hatching/diapause III stage in *A. limnaeus* that hatch with most of their yolk absorbed and begin feeding immediately. Data are for: Australian lungfish (Mueller et al. 2011), black bream (Hassell et al. 2008), channel catfish (Carlson et al. 1974), lake trout (Carlson and Siefert 1974; Duodoroff and Warren 1965), largemouth bass (Carlson and Siefert 1974; Dudley and Eipper 1975), mountain whitefish (Siefert et al. 1974), northern pike (Siefert et al. 1973), scale carp (Kaur and Toor 1978), smallmouth bass (Siefert et al. 1974), and zebrafish (Strecker et al. 2011).

Table 1.

Benchmarks of development chosen to compare the effects of PO₂ on developmental progression.

WS ^a	Description ^b
19	<i>Completion of epiboly</i> ; beginning of dispersion/reaggregation phases
28	<i>Solid neural keel</i> ; initiation of neurogenesis
31	<i>Twenty-five somite embryo</i> ; optics lobes present, major subdivisions of brain established, tubular heart formed and functional
32	<i>Diapause II</i> ; 38–40 pairs of somites, neural subdivisions apparent, optic lobes present, otic, olfactory and lens placodes present, functional tubular heart
34	Early post-diapause II, major brain regions enlarged, heart starting to loop, gut tube present
36	Retinal pigmentation light, brain regions considerably enlarged, hemoglobin expressed, otoliths present, melanocytes scattered on dorsal surface, gut tube prominent and starting to loop, embryo has grown about half way around yolk
40	Retinal pigmentation dark, brain even larger, many melanocytes, embryo reaches completely around yolk, gut is fully looped
43	<i>Late pre-hatching</i> ; nearly fully formed larvae, embryos enter diapause III here

^aWS = Wourms' Stage (Wourms, 1972)

^bstages with names are given in italics