

RESEARCH ARTICLE

Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (*Danio rerio*)

Dao H. Ho^{1,2} and Warren W. Burggren^{1,*}

¹Developmental Integrative Biology Research Cluster, Department of Biological Sciences, University of North Texas, 1155 Union Circle #305220, Denton, TX 76203-5017, USA and ²Section of Experimental Medicine, Department of Medicine, Georgia Health Sciences University, 1459 Laney Walker Boulevard, CB 2200, Augusta, GA 30912, USA

*Author for correspondence (burggren@unt.edu)

SUMMARY

Parental influences are a potentially important component of transgenerational transfer of phenotype in vertebrates. This study examined how chronic hypoxic exposure on adult zebrafish (*Danio rerio*) affected the phenotype of their offspring. Separate adult populations were exposed to hypoxia (13.1 kPa O₂) or normoxia (21.1 kPa O₂) for periods ranging from 1 to 12 weeks. Adults were then returned to normoxia and bred within experimental groups. Adult fecundity and egg characteristics (volume of egg, yolk and perivitelline fluid) were assessed. Subsequently, larval body length, time to loss of equilibrium in severe hypoxia (~4 kPa O₂), and critical thermal minima (CT_{min}) and maxima (CT_{max}) were measured at 6, 9, 12, 15, 18, 21 and 60 days post-fertilization (d.p.f.). Adult fecundity was depressed by hypoxic exposure. Egg component volumes were also depressed in adults exposed to 1–2 weeks of hypoxia, but returned to control levels following longer hypoxic exposure. Adult hypoxic exposures of >1 week resulted in longer body lengths in their larval offspring. Time to loss of equilibrium in severe hypoxia (i.e. hypoxic resistance) in control larvae decreased from 6 to 12 d.p.f., remaining constant thereafter. Notably, hypoxic resistance from 6 to 18 d.p.f. was ~15% lower in larvae whose parents were exposed to just 1 week of chronic hypoxia, but resistance was significantly increased by ~24–30% in 6–18 d.p.f. larvae from adults exposed to 2, 3 or 4 weeks of hypoxia. CT_{min} (~10–12°C) and CT_{max} (~39.5°C) were unchanged by parental hypoxic exposure. This study demonstrates that parental hypoxic exposure in adult zebrafish has profound epigenetic effects on the morphological and physiological phenotype of their offspring.

Key words: parental effect, critical thermal maxima, epigenetics.

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INTRODUCTION

Many freshwater aquatic vertebrates may be subjected to hypoxic stress at more frequent and variable schedules than marine or terrestrial species (Nikinmaa, 2002; Pelster, 2002; Farrell, 2007; Tattersall and Ultsch, 2008; Porteus et al., 2011; Richards, 2011; Sandblom and Axelsson, 2011). In addition to the immediate cardio-respiratory physiological responses, which have been well characterized, freshwater fish can develop acclimatory phenotypes in response to hypoxic conditions, presumably affording greater hypoxic resistance during that hypoxic episode and perhaps any subsequent hypoxia challenge (Pelster, 2002; Richards, 2011). For example, many species show branchial remodeling, including hypertrophy, in response to aquatic hypoxia (see Jonz et al., 2004; Negreiros et al., 2011; Tzaneva et al., 2011). Such changes in gill structure are typically viewed as a beneficial acclimation response.

In addition to physiological and morphological responses to environmental stressors that affect their own survival, adult fishes respond in ways that may influence the survival of their offspring in such environments. Such changes can include alterations of the egg environment through inclusion of hormonal and other signals in the yolk (e.g. Lindeman and Pelegri, 2010; Segers et al., 2012). Such changes constitute a so-called ‘maternal’ or ‘parental’ effect (sometimes referred to as a ‘indirect genetic effect’ or even ‘transgenerational plasticity’). Not all of these effects are beneficial. Hypoxic exposure in adult freshwater fish can reduce fitness due

to low fecundity, decreased mating rates and low survivorship of offspring (Wu et al., 2003). When exposed to even moderate levels of hypoxia, adult carp, for example, suffer disruption of the endocrine system that lowers offspring hatchability as well as induces lethal mutation phenotypes in offspring (Wu et al., 2003). In contrast, resistance to a stressor may be conferred upon the offspring. For example, killifish and feral white suckerfish from recently polluted environments produce offspring that are more resistant to the specific pollutant (Munkittrick and Dixon, 1988; Meyer et al., 2003), likely through parental influences on the egg environment in which early development occurs.

Collectively, then, there is evidence among fishes indicating a link between the stressors experienced by the parents and the increased fitness of their offspring to survive these same stressors. While such enhanced offspring fitness in the past has often been interpreted in the traditional context of heritable changes in gene sequence leading to altered phenotype across generations, there has been a burgeoning of epigenetic studies of stressors in environments and how they are specifically affecting transgenerational transfer of phenotype without alteration in gene sequence [for discussion, see Lacey (Lacey, 1998) and Ho and Burggren (Ho and Burggren, 2010)].

Most studies of transgenerational epigenetics in fishes as they relate to enhanced offspring resistance have focused on environmental contaminants (e.g. heavy metals and other toxicants).

Whether parental hypoxic exposure specifically heightens hypoxia resistance in offspring through epigenetic mechanisms is only beginning to be explored. A few examples of a possibly advantageous 'parental effect' following hypoxic exposure in adults exist in invertebrates. In the fruit fly *Drosophila melanogaster*, offspring of hypoxia-reared parents raised in normoxia developed larger tracheal systems (Henry and Harrison, 2004). This presumably increases respiratory surface area and conveys increased hypoxia resistance, but an actual enhanced resistance to hypoxia in those with larger tracheal systems was not directly tested. In another invertebrate example, adult females of the water flea *Daphnia magna* exposed to hypoxia subsequently produce broods whose individuals have higher metabolic rate than control neonates from non-exposed adult females (S. Andrewartha and W.W.B., unpublished), though whether this is an adaptive response also remains to be demonstrated. However, the evidence for such epigenetic, transgenerational transfer of hypoxic resistance in fishes is scant.

In this study, we hypothesized that adult zebrafish express parentally mediated hypoxia-induced effects that, through epigenetic mechanisms, increase subsequent resistance to hypoxia in the larvae of the subsequent generation. Zebrafish provide an excellent model for testing this hypothesis because both mature and developing zebrafish are sensitive to acute and chronic hypoxia, and exhibit behavioral, physiological, biochemical and molecular level responses (Pelster and Burggren, 1996; Barrionuevo and Burggren, 1999; Padilla and Roth, 2001; Rees et al., 2001; Jacob et al., 2002; Ton et al., 2003; Ngan and Wang, 2009; Barrionuevo et al., 2010; Yaqoob and Schwerte, 2010; Kamei et al., 2011; Lo et al., 2011). In our experiments we have assessed clutch size (i.e. number of eggs laid) and egg components of hypoxia-exposed adult zebrafish. Also measured was body length and hypoxic resistance of the progeny of the adults, to better understand how hypoxia-induced changes of maternally derived factors may impact offspring phenotype. Finally, depressed oxygen levels and elevated ambient temperature are often co-stressors in freshwater aquatic habitats. Indeed, thermal stress elicits molecular and biochemical changes similar to hypoxic stress (Iwama et al., 2004), so the dual study of hypoxic and thermal stressors allows for the assessment of the potential mechanisms of hypoxia-induced parental effects. Thus, we additionally assessed thermal tolerances of the larval progeny of adult zebrafish exposed to hypoxic conditions.

MATERIALS AND METHODS

Fish care and maintenance

Wild-type adult zebrafish [*Danio rerio* (Hamilton 1822); 4 to 6 months old at the start of the study] obtained from EkkWill Farms (Ruskin, FL, USA) were housed in 13 liter glass aquariums, at a density of 10 fish per aquarium, for 2 weeks prior to experimentation. They were fed brine shrimp and dry flake food twice a day during non-breeding periods, and three times per day beginning 5 days prior to breeding. Care was taken to feed equal amounts of food to each population held in separate aquaria. Temperature inside the aquaria was maintained at $27\pm 0.5^{\circ}\text{C}$ by a submersible water heater. The light cycle was set at 14h:10h light:dark.

Newly hatched embryos were housed in 95-mm Petri dishes (~70 embryos per dish) filled with clean, normoxic aquarium water treated with 0.05% Methylene Blue (14h:10h light:dark cycle). After 5 days post-fertilization (d.p.f.), larvae were transferred to 1 liter containers at a density of ~ 150 larvae l^{-1} . Air saturation of the water in each container was maintained by bubbling ambient air into the water. Beginning at 6 d.p.f., larvae were fed dry powder food (TetraMin, Baby Fish Food 'E' for egglayers, Blacksburg, VA, USA) three

times daily. Brine shrimp were added to feedings beginning at 16 d.p.f. All embryos and larvae were housed in a temperature-controlled incubation room maintained at $27\pm 0.5^{\circ}\text{C}$.

Normoxia in control populations was maintained by constantly running room air through a sponge filter located at the bottom of the aquarium. Hypoxia was maintained in the experimental populations by constantly running a gas mixture of 13% oxygen and 87% nitrogen through a sponge filter located at the bottom of the aquarium. Aquaria were fitted with a gas-tight lid equipped with a circular opening small enough to create a very slight positive air pressure inside the aquarium that permitted mixed gas from the airstone to be vented to the atmosphere.

Breeding

During the holding period of 2–6 weeks, fish were bred weekly to increase the likelihood of successful breeding during the actual experiments. Three males and three females were placed in breeding containers filled with clean, normoxic aquarium water the evening prior to the morning of egg collection. Breeding containers consisted of a 1-liter rectangular container that held a smaller rectangular container with a mesh bottom. The bottom of the inner container rested ~1 cm above the bottom of the outer container to allow expelled eggs to fall to the aquarium bottom to prevent them from being eaten by the adult fish. Aquaria for routine breeding were maintained in a $27\pm 0.5^{\circ}\text{C}$ incubator room during the breeding period. During routine breeding, males and females were placed together to breed 4 h after the lights came on the next morning, and were then returned to aquatic holding aquaria.

Experimental design

Breeding pairs that produced >100 eggs during routine breeding were assigned to one of two experimental conditions: hypoxia exposed or normoxia exposed. Prior to placement into experimental aquaria containing hypoxic or normoxic water, pairs were bred, and these were eggs discarded to ensure that pre-existing eggs were eliminated before experiments began. Twelve breeding pairs were assigned to each experimental group.

The evening prior to the start of an experiment, breeding adult pairs were placed overnight in individual breeding aquaria containing normoxic water. Breeding in normoxia ensured that newly laid eggs of both hypoxic and normoxic groups were exposed to identical, normoxic conditions. An aquarium divider was used to keep the female and male separated until the start of the light cycle to control for spontaneous breeding during the dark cycle. At the start of the light cycle, the divider was removed, and pairs were allowed to breed for ~3 h before eggs were collected and rinsed in clean aquarium water.

Both morphological and physiological parameters were assessed in zebrafish larvae whose parents (P) were exposed to 1, 2, 3 or 4 weeks of normoxia (NP-1, NP-2, NP-3 or NP-4) or hypoxia (HP-1, HP-2, HP-3 or HP-4). The hypoxic resistance of these larvae was assessed at 6, 9, 12, 15 and 18 d.p.f. Each clutch, for every period of parental exposure to experimental conditions (1, 2, 3 or 4 weeks), came from an entirely different breeding pair to avoid pseudoreplication bias. Each experimental group included larvae from at least two separate clutches.

In a separate experiment designed to assess the effects of hypoxia exposure on the fecundity and egg characteristics of fish exposed to hypoxia or normoxia, cohorts of five females and five males were exposed to experimental conditions for 1, 2, 6 and 12 weeks to assess fecundity (number of eggs per clutch), and whole egg, yolk and perivitelline volume of collected eggs. At each point during the

course of exposure to experimental conditions, breeding pairs were randomly selected from each experimental group and returned to their assigned aquaria for continuing exposure. After 12 weeks of parental hypoxic exposure in this experiment, the critical thermal minima (CT_{min}) and maxima (CT_{max}) of NP and HP larvae were recorded at 6, 9, 12, 15, 18, 21 and 60 d.p.f. to assess the thermal tolerance of these two groups (see below).

Larval body length measurements

Standard body length of larvae was defined as the distance from the tip of the snout to the rear center of the tail. This variable was assessed in larval zebrafish using an imaging system consisting of a compound microscope with an attached digital camera and ImagePro Plus data analysis software (v.4.1, Media Cybernetics, Bethesda, MD, USA).

Fecundity and egg, yolk and perivitelline volume

Immediately after a 3 h breeding period, eggs were collected from breeding aquaria and counted. Images of the eggs were captured using a high-speed camera (Model JE3010, Javelin Electronics, Torrance, CA, USA). Egg, yolk and perivitelline volume were assessed from these images by making radius measurements of egg and yolk diameter of the circular eggs, using light microscopy images of $20\times$ magnification (Image Pro Plus v.4.1). Egg and yolk volumes were calculated from the equation $V=4/3\pi r^3$. Perivitelline volume was calculated by subtracting yolk volume from egg volume.

Time to loss of equilibrium to hypoxia in larvae

The time that it takes for larval or adult fish to lose equilibrium when exposed to certain stressors is an indication of its resistance to that stress. Loss of equilibrium has been considered a reliable and valid measure of 'ecological death' in many fish species, and has been used in studies as an alternative to physiological death (Beitinger et al., 2000).

Larval zebrafish (6, 9, 12, 15 and 18 d.p.f.) were placed individually in an airtight acrylic box ($10\times 10\times 5$ cm) filled with extremely hypoxic aquarium water (~ 4 kPa O_2). Timing to loss of equilibrium started immediately upon hypoxic exposure, and ended upon the larva's inability to recover from loss of equilibrium (failure to right itself) for at least a 3 s period. Three seconds were subtracted from the total time to account for the time taken to determine loss of equilibrium. Embryos younger than 6 d.p.f. are not free swimming and do not display a clearly observable loss of equilibrium, and thus were excluded from these experiments.

CT_{max} and CT_{min}

The critical thermal method (CT_{max} and CT_{min}) is frequently used to estimate the scope of thermal tolerance (also known as thermal scope) of an organism (Diaz-Herrera et al., 1998; Ford and Beitinger, 2005). This estimation directly quantifies the temperatures at which an aquatic organism loses equilibrium.

Adult males and females were held in either normoxia or hypoxia (13.1 kPa O_2) for 12 weeks. The hypoxic adults were then returned to normoxia, and both populations were separately bred as described above. Zebrafish larvae from these two populations were assessed for CT_{min} and CT_{max} at 6, 9, 12, 15, 18 and 21 d.p.f. Juveniles at 60 d.p.f. were also assessed for thermal tolerance to determine the validity of the protocol by comparison with previously published studies on the thermal scope of juvenile/adult zebrafish.

All fish were placed in 100 ml glass beakers filled with fresh normoxic water. The beakers were then placed in a temperature-

controlled water bath (Fisher Isotemp 1028P, Fisher Scientific, Pittsburg, PA, USA). Fish were kept in the beakers ≤ 5 min before the temperature of the water was increased (CT_{max}) or decreased (CT_{min}) at a rate of $0.3\pm 0.1^\circ C\ min^{-1}$. This rate of temperature change is recommended for small fish as it allows for an insignificant time lag between water temperature and body temperature (Beitinger et al., 2000). The temperature at which a larva was unable to recover from loss of equilibrium for at least 3 s was recorded as the CT_{max} or the CT_{min} . Prior to 18 d.p.f., CT_{min} values of larvae were not obtainable because larvae did not lose equilibrium at extreme cold temperatures, and no other suitable behavioral assay can be readily substituted. Therefore, CT_{min} was assessed in larvae at 18, 21 and 60 d.p.f.

Statistical analyses

Time to loss of equilibrium in hypoxia and body length were subjected to factorial three-way ANOVAs with respect to treatment group (NP or HP), parental exposure period (1, 2, 3 and 4 weeks) and developmental age (6, 9, 12, 15 and 18 d.p.f.). Subsequent separate two-way analyses with respect to treatment group and development measured as d.p.f. were performed for each parental exposure period. Subsequent Tukey's *post hoc* analyses were used to detect differences between treatment groups with respect to treatment exposure times. Time to loss of equilibrium was also expressed as composite hypoxia tolerance for each population, which indicated how tolerance was affected over the course of larval development that was monitored. Composite hypoxia tolerance was determined by first generating the sum of the times to loss of equilibrium at all days (6, 9, 12, 15 and 18) for each population. The difference from the control population was then calculated for each of the experimental populations, and finally expressed as a percent change from the control population.

Variables of fecundity, whole egg volume and egg component volumes were analyzed in a similar fashion using two-way ANOVAs on factors of treatment group (normoxia or hypoxia-exposed) and treatment exposure times (1, 2, 6 or 12 weeks). A two-way ANOVA with respect to treatment group (NP and HP) and developmental time (6, 9, 12, 15, 18, 21 and 60 d.p.f.) was performed on variables of CT_{max} and CT_{min} . Subsequent Tukey's *post hoc* analyses were used to detect differences between treatment groups.

For all analyses, if normality and/or equality of variances were not achieved, data were ranked prior to statistical analyses. Statistical significance was assigned a value of $P\leq 0.05$. Data are expressed as means \pm s.e.m.

RESULTS

Fecundity

The fecundity of adult zebrafish exposed to 1, 2, 6 or 12 weeks of hypoxia or normoxia is depicted in Fig. 1. After 1 week of exposure to experimental conditions, the mean fecundity of hypoxia-exposed fish was not significantly different from that of normoxia-exposed fish. However, after 2, 6 and 12 weeks of exposure, hypoxia-exposed fish laid significantly fewer eggs per clutch than normoxia-exposed fish ($q=2.96$, $P=0.048$; $q=3.06$, $P=0.042$; $q=2.99$, $P=0.046$, respectively).

Egg component volume

The egg, yolk and perivitelline volumes of the eggs of control and hypoxia-exposed zebrafish are depicted in Fig. 2. After 1 and 2 weeks of hypoxic exposure, adults produced eggs with significantly smaller egg volume and perivitelline volume than normoxia-exposed fish (both $q\geq 4.62$, $P\leq 0.001$). However, this reduced egg size

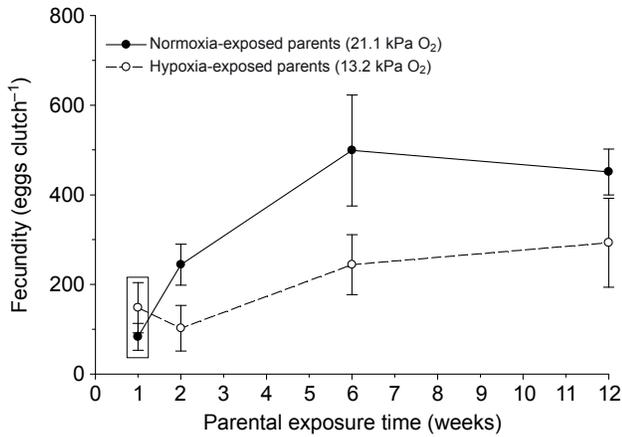


Fig. 1. Fecundity of adult zebrafish held in normoxia (solid circles and lines) or hypoxia (open circles and dashed lines) for 1, 2, 6 and 12 weeks. $N=3-6$ per group. Mean values \pm 1 s.e.m. are presented. Mean values grouped within boxes are not significantly different from each other ($P>0.05$).

disappeared after 6 and 12 weeks of exposure. Mean yolk volume was not significantly different between the two treatment groups at any length of exposure.

Larval body length

Mean larval body length, as anticipated, significantly increased as a function developmental time for both NP and HP larvae (all $P<0.05$; Fig. 3). However, the increase in mean body length differed in magnitude between NP and HP larvae, with HP larvae displaying significantly longer mean body length than NP larvae, at variable points during larval development, over the course of the four parental exposure periods (1, 2, 3 and 4 weeks). After 1 week of exposure to experimental conditions, HP larvae were longer than NP larvae at 6, 15 and 18 d.p.f. ($q=2.147$, $P=0.03$; $q=3.62$, $P<0.001$; $q=4.70$, $P<0.001$, respectively; Fig. 3A). As parental exposure period increased to 2 weeks, the difference in mean body length between NP and HP larvae was lost at 6 d.p.f., but remained significant at 15 d.p.f. ($q=3.90$, $P=0.006$) and 18 d.p.f. ($q=6.58$, $P<0.001$; Fig. 3B). Exposure periods of 3 and 4 weeks exhibited further dampening of mean body length differences between NP and HP larvae, with HP larvae significantly longer than NP larvae only at 18 d.p.f. (3 week exposure, $q=3.24$, $P=0.002$; Fig. 3C) and 15 d.p.f. (4 week exposure, $q=2.57$, $P=0.011$; Fig. 3D).

Hypoxia and loss of equilibrium

Generally, the susceptibility of control zebrafish larvae to extreme hypoxia (~ 4 kPa O_2) increased over the course of early development. Larvae showed a nearly twofold highly significant ($P<0.01$) decrease in time to loss of equilibrium from 6 to 15 d.p.f. in larval populations never previously exposed to hypoxia (Fig. 4). However, the greatest reductions in hypoxia resistance were evident in early development, with no significant difference in time to loss of equilibrium to severe hypoxia between 15 and 18 d.p.f.

There was a significant effect of both time and treatment at each parental hypoxic exposure period (two-way ANOVA, $P<0.05$), but the pattern of change over developmental time was complex (Fig. 5). In particular, after 1 week of parental exposure to either hypoxic or normoxic conditions, 9 and 12-day-old HP larvae displayed significantly shorter mean time to loss of equilibrium in hypoxia (that is, lower hypoxia resistance) than NP larvae at comparable

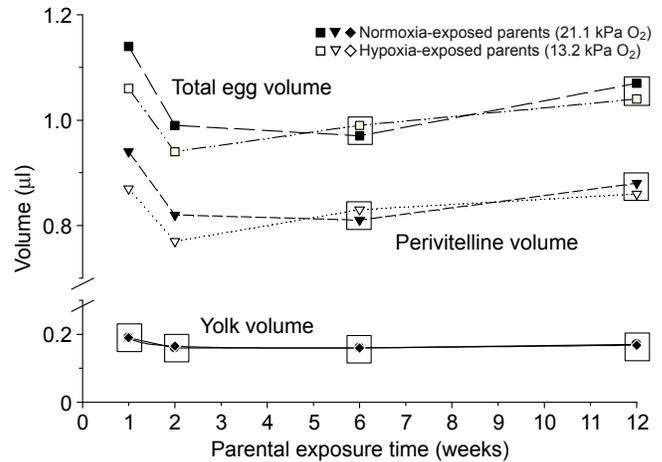


Fig. 2. Egg volume, perivitelline volume and yolk volume of newly laid eggs of adult zebrafish held in normoxia (solid symbols) or hypoxia (open symbols) for 1, 2, 6 and 12 weeks. Each volume was separately analyzed via two-way ANOVA with Tukey's *post hoc* analyses. Mean values grouped within boxes are not significantly different from each other ($P>0.05$). $N=90-179$ for each group at each time point. Mean values \pm 1 s.e.m. are presented (note: s.e.m. values are smaller than the symbol size).

ages (Tukey's, $q=2.93$, $P=0.038$; $q=2.85$, $P=0.04$, respectively; Fig. 5A). However, after longer 'doses' of hypoxia (i.e. 2, 3 or 4 weeks of parental hypoxia exposure), time to loss of equilibrium in hypoxia increased significantly ($P\leq 0.04$) on at least one day for each of the three hypoxic populations, reflecting a greater hypoxia resistance. Larvae from parents exposed to 2 weeks of hypoxia were significantly more capable of coping with hypoxic stress than the offspring of normoxia-exposed fish at 9 ($q=3.90$, $P=0.006$) and 18 d.p.f. ($q=3.25$, $P=0.022$). After 3 and 4 weeks of parental hypoxia exposure, HP larvae were more resistant to hypoxia stress than NP larvae at days 12 and 18 ($q=5.47$, $P<0.001$; $q=3.03$, $P=0.034$).

The composite patterns of change in larval hypoxia resistance are illustrated in Fig. 6 as percentage changes from control larvae, grouped by weeks of parental exposure to hypoxia. When averaged over all developmental time points (6 to 18 d.p.f.), the composite hypoxic tolerance of HP larvae is actually lowered by just 1 week of parental hypoxic exposure. However, this pattern reverses, as 2, 3 or 4 weeks of hypoxic exposure actually increases larval hypoxia tolerance.

Thermal tolerances – CT_{min} and CT_{max}

The thermal tolerance in normoxia of larval offspring (6–21 d.p.f.) of hypoxia- or normoxia-exposed adult zebrafish was assessed using the temperature at which equilibrium was lost in the face of increasing or decreasing ambient temperature. CT_{min} ranged from 9.5 to 12°C, while CT_{max} ranged from 39.5 to 40.5°C over the range of 6 to 60 days, with the total range of thermal tolerance increasing slightly with development.

There were no significant differences ($P>0.05$) in thermal tolerance, as measured by CT_{min} and CT_{max} , between the larvae from parents exposed to hypoxia for 12 weeks and the larvae from normoxic parents at any time in their development (Fig. 7).

DISCUSSION

Hypoxia tolerance during larval development

As embryos, zebrafish display increased sensitivity to environmental oxygen depletion as development progresses (Padilla and Roth,

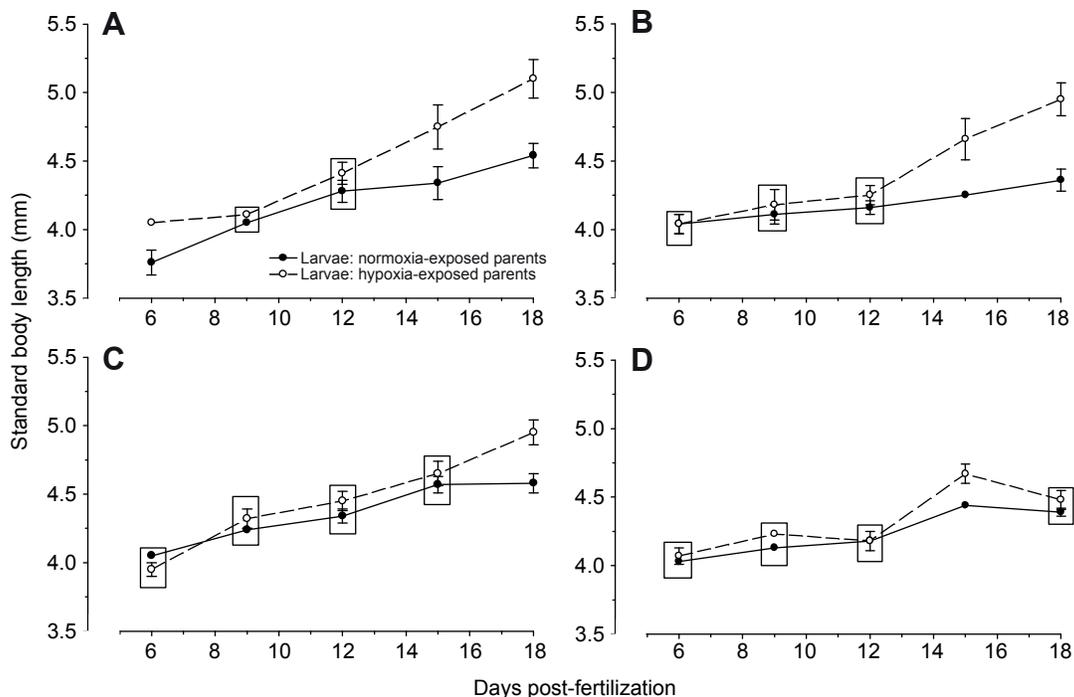


Fig. 3. Mean body length of larval offspring of adult zebrafish exposed to hypoxia or normoxia for 1 (A; $n=7-21$ per group), 2 (B; $n=9-14$ per group), 3 (C; $n=12-15$ per group) or 4 weeks (D; $n=22-26$ per group). A box indicates no significant difference among the groups. Mean values \pm 1 s.e.m. are presented.

2001; Ton et al., 2003). At the two-cell stage, zebrafish embryos tolerate anoxic conditions by entering into a state of 'suspended animation', with this anoxia tolerance gradually disappearing by the time of hatching (Padilla and Roth, 2001). Moreover, after 24 h post-fertilization, zebrafish embryos become less tolerant to environments of 5% oxygen (Ton et al., 2003). The present study extends these findings in zebrafish larvae by demonstrating that resistance to severe, acute hypoxia (~ 4 kPa O_2) gradually decreases as development progresses from 6 to 18 d.p.f. (Fig. 4). During this same period of larval development, zebrafish larvae undergo both qualitative and quantitative changes in mode of oxygen transport to tissues and oxygen demand of tissues (Pelster and Burggren, 1996; Jacob et al., 2002; Rombough, 2002; Rombough and Drader, 2009). Prior to 12 d.p.f., oxygen supply to the tissues occurs *via* diffusion across the body surface, but as the larvae increase in size (~ 14 d.p.f.), convective circulation becomes necessary to maintain adequate oxygen supply to tissues (Jacob et al., 2002). It is during this time that cardio-ventilatory mechanisms begin to be evoked in response to hypoxia (Jacob et al., 2002; Pelster, 2002; Schwerte et al., 2003; Jonz and Nurse, 2005; Vulesevic et al., 2006a; Vulesevic and Perry, 2006b; Barrionuevo et al., 2010).

Fecundity

Teleost fish exposed to hypoxia typically have reduced fecundity (e.g. Landry et al., 2007), a phenomenon attributed to the disruption of normal testosterone and estradiol levels in the female fish after chronic exposure to hypoxic conditions (Wu et al., 2003; Landry et al., 2007). Fecundity, as well the characteristics of egg components, are largely under maternal influence, and so are susceptible to alteration when gravid females are exposed to environmental disturbances such as hypoxia. The ability to alter fecundity and egg composition has been implicated in acclimatory responses to environmental conditions as a mechanism for increasing

fitness of the individual and/or its offspring (Dzimirski and Alford, 2005; Einum and Fleming, 2007; Landry et al., 2007; Olofsson et al., 2009).

In the present study, control females showed an increase in fecundity in the first 6 weeks of the 12-week observation period (Fig. 1). This likely represents a continuing maturation of the reproductive system of these young females, and a phenomenon much reported on for many teleost fishes (e.g. Blaxter, 1988; Tyler and Sumpter, 1996). Hypoxia-exposed adult female zebrafish produced significantly fewer eggs (reduced fecundity) than normoxic controls after just 2 weeks of hypoxia exposure (Fig. 1).

Egg volumes

Egg size and egg composition (e.g. proportion of yolk to total egg volume) varies greatly in teleost fishes (Kamler, 2005). In zebrafish, egg size is related to subsequent condition and survivability of juveniles (Uusi-Heikkilä et al., 2010). Zebrafish egg volume is $\sim 20\%$ yolk (Fig. 2), with the remainder being perivitelline fluid, a fairly typical composition for small freshwater fishes. Generally for teleosts, as fecundity goes up, total egg size decreases (Blaxter, 1988), and this inverse relationship is evident in the eggs laid by control zebrafish females (Figs 1, 2) (Uusi-Heikkilä et al., 2010).

In the present experiments, egg composition was affected by maternal hypoxic exposure, but the length of hypoxic exposure affected the response. Brief hypoxic exposure (<6 weeks) resulted in females producing significantly smaller eggs (Fig. 2), but after 6 and 12 weeks of hypoxia exposure, fish egg size had returned to control values. These findings suggest that, unlike for fecundity, females were becoming acclimated to the longer-term (≥ 6 weeks) hypoxic conditions. Egg size in teleosts is influenced by parental size, spawning group, season and diet (Blaxter, 1988). However, we know of no other study that has drawn a correlation between chronic hypoxic exposure and egg size.

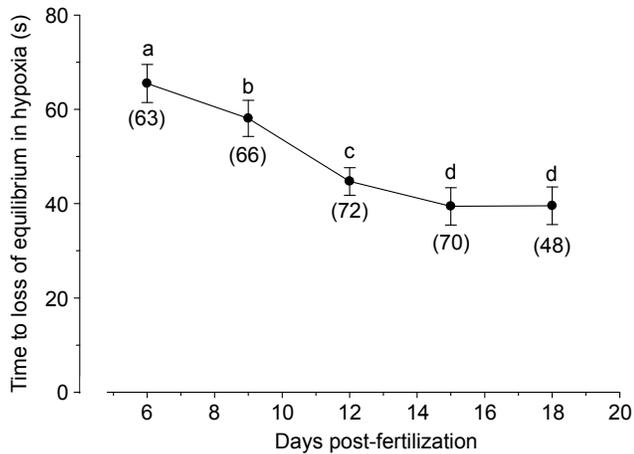


Fig. 4. Time to loss of equilibrium in hypoxia (~ 4 kPa O_2) of zebrafish larvae from 5 to 18 days. Neither the larvae nor their parents had previously experienced hypoxia at any level. Mean values ± 1 s.e.m. are presented. *N*-values are in parentheses. Letters distinguish statistically significant groups.

Interestingly, the relative decrease in whole egg volume produced by hypoxia-exposed adult female zebrafish in response to <6 weeks of hypoxic exposure was not accompanied by a significant reduction in yolk volume, rather remaining constant in both populations at $\sim 0.2 \mu\text{l}$ in both populations across the measurement period (Fig. 2). Indeed, the decreased egg volume in the 1 and 2 week populations was primarily the result of a significantly smaller perivitelline volume. The function of the perivitelline space – the cavity between the outer surface of the yolk and the inner surface of the chorion – includes protection of the embryo, nutritive supply and, arguably, the regulation of embryonic metabolism and gas exchange (Laale, 1977; Burggren, 1985; Seymour and Loveridge, 1994; Mueller and

Seymour, 2011). However, the direct impact of the volume of the perivitelline space (and thus the diffusion distances for respiratory gases) to development of the fish embryo *in ovo* remains largely unexplored.

Larval body length

Body lengths of larvae from hypoxic parents were larger than that of larvae from normoxic parents at variable times in development. The effect appeared, disappeared and reappeared at significant levels as a function of length of hypoxic exposure period (Fig. 3). The majority of differences between the two groups were observed at 15 and 18 d.p.f., with HP-1 diverging from the normal developmental trajectory as early as 6 d.p.f. Although the relationship between body size and hypoxia resistance is controversial, the effect of body size has clear implications in the regulation of oxygen consumption (see Feder, 1983b; Feder, 1983a; Burleson et al., 2001; Robb and Abrahams, 2003; Ospina and Mora, 2004; Nilsson and Ostlund-Nilsson, 2008). Some larger-bodied fishes are better able to survive severe hypoxia stress due to increased levels of glycogen available for anaerobic adenosine-triphosphate production (Nilsson and Ostlund-Nilsson, 2008). In contrast, Robb and Abrahams (Robb and Abrahams, 2003) found that large yellow perch (~ 34 g) were significantly less tolerant to extreme hypoxia stress than small individuals of the same species (~ 2 g). However, these contrasting reports were based on adult fish, and such assumptions should not be automatically interpolated to the very different situation for developing fish (Burggren, 2005). In the present study, longer body length induced by specific lengths of parental hypoxic exposure did not specifically correlate with increased hypoxia resistance during larval development (compare Fig. 3 with Fig. 5). Although changes in these two parameters both result from parental hypoxia exposure, there does not appear to be a strong causal relationship. In support of this conclusion, independence of regulation of oxygen consumption and body size has been also been observed in amphibian larvae (Feder, 1983b; Feder, 1983a).

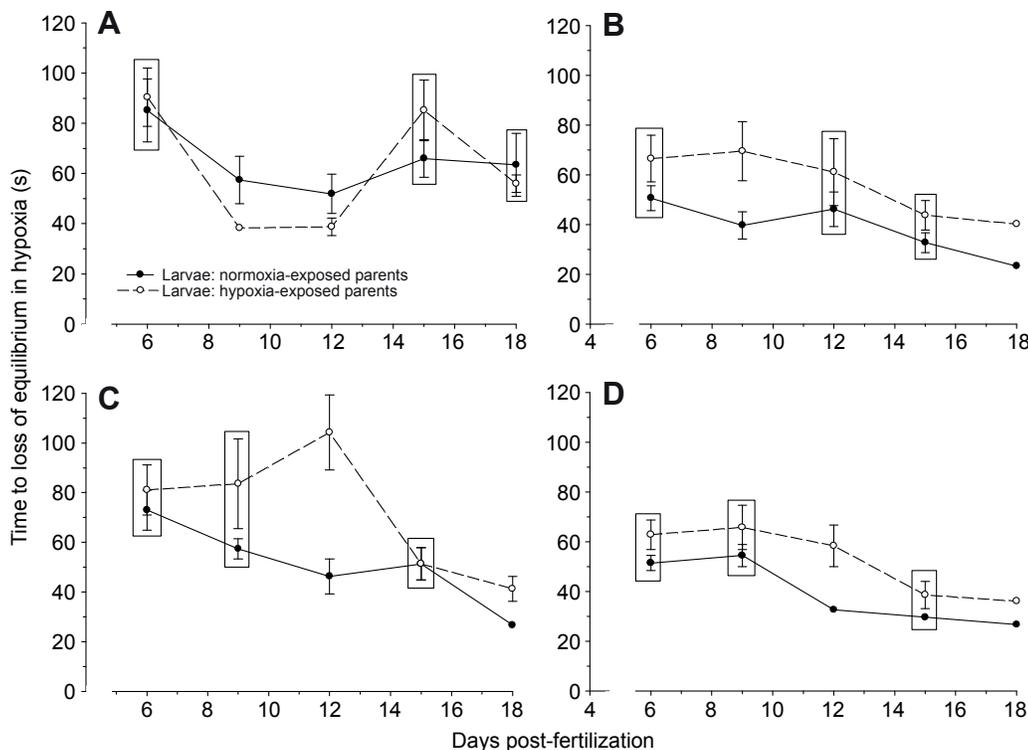


Fig. 5. Time to loss of equilibrium in hypoxia (~ 4 kPa O_2) of zebrafish of parents reared in normoxia or hypoxia for 1 (A; $n=10$ –23 per group), 2 (B; $n=10$ –14 per group), 3 (C; $n=9$ –14 per group) or 4 weeks (D; $n=18$ –29 per group). Points grouped within boxes are not significantly different from each other (two-way ANOVA with Tukey's *post hoc* analyses between treatment groups, $P>0.05$). Mean values ± 1 s.e.m. are presented.

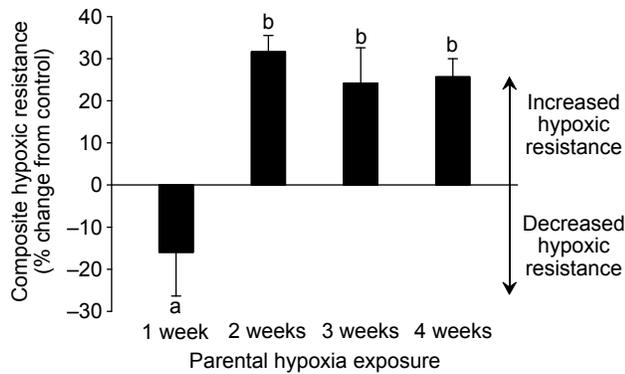


Fig. 6. Patterns of change in larval hypoxic resistance induced by varying lengths of parental hypoxic exposure, expressed as percentage changes from control. Each plotted bar is the mean \pm 1 s.e.m. calculated as an average of all hypoxic resistance measurements from day 6 to 18 for each parentally derived larval population. See Materials and methods for details. Different letters indicate significant differences ($P < 0.05$) between populations.

Thermal tolerance

Hypoxia exposure induces gene expression profiles very similar to those induced by extreme temperatures, suggesting an intimate relationship between hypoxic resistance and thermal tolerance (Airaksinen et al., 1998; Sørensen and Loeschke, 2001). Regulatory proteins such as heat-shock proteins and hypoxia-inducible factor-1 (HIF-1) have been shown to be upregulated in a similar fashion as a result of either hypoxic or thermal stress (Rissanen et al., 2006; Horowitz, 2007; Tetievsky et al., 2008). Moreover, resistance (or exposure) to one stress has been shown to confer resistance to a wide range of other stresses (Katschinski and Glueck, 2003; Burlinson and Silva, 2011).

In this study, the upper thermal tolerances of 6 to 21 d.p.f. larvae were comparable to those reported for adult zebrafish (Cortemeglia and Beiting, 2006). The thermal tolerances of larval offspring of hypoxia-exposed adult zebrafish and that of offspring of normoxia-exposed adult zebrafish were not significantly different (Fig. 6). This suggests that in our model, the regulation of parentally conferred hypoxic resistance does not necessarily confer resistance to other stressors. In support of this, although stress responses of coral reef fish brought on by a variety of stressors (including hypoxic and cold and heat shock) induced changes in common functional gene groups, hypoxic stress induced widely different changes in expression of individual genes within these functional groups when compared with cold or heat shock stress (Kassahn et al., 2007). A limitation of the present study is that hypoxia resistance was assessed in offspring of parents exposed to 1–4 weeks hypoxia exposure, while thermal tolerance was assessed in offspring of parents exposed to 12 weeks of hypoxia exposure. Further studies are necessary to elucidate whether molecular mechanisms that underlie heightened hypoxia resistance in HP larvae in the present study are hypoxia-specific.

Larval hypoxia resistance and parental influences

Our study is the first to demonstrate for any vertebrate that hypoxia resistance in the F1 offspring is altered by parental hypoxic exposure. It has been previously reported that hypoxia exposure in the adult fruit fly *D. melanogaster* results in increased tracheal size, which could contribute to increased hypoxic resistance (Henry and Harrison, 2004). However, physiological measurements were not

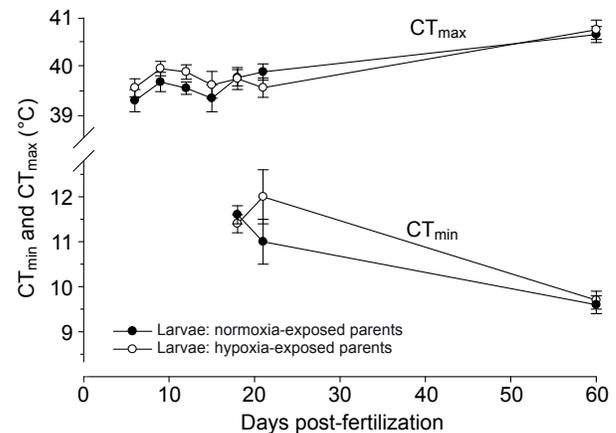


Fig. 7. Critical minimum thermal limit (CT_{min}) and maximum thermal limit (CT_{max}) of zebrafish larvae from parents reared in normoxia or hypoxia for 12 weeks. CT_{min} and CT_{max} were not significantly different between treatment groups (two-way ANOVA with Tukey's *post hoc* analyses, $P > 0.05$). $N = 8$ –12 for each group at each time point. Mean values \pm 1 s.e.m. are presented.

made to determine whether there was an actual increase in hypoxic resistance in the F1 generation. In the water flea *D. magna*, exposure of clonal females to chronic hypoxia produced broods whose individuals have higher metabolic rate than those from non-exposed adult females (S. Andrewartha and W.W.B., unpublished). Whether such a metabolic rate shift is adaptive is unclear but, interestingly, this effect 'washed out' as the females produced subsequent broods.

The pattern of epigenetic, transgenerational influences on hypoxic resistance in zebrafish in the present study, as actually measured by time to loss of equilibrium in severe hypoxia, is complex (Fig. 5). For example, a single week of parental exposure to hypoxia results in larval zebrafish less resistant to a first bout of severe hypoxia (Fig. 5A). In contrast, longer periods of chronic hypoxic exposure in parents (2, 3 or 4 weeks) create larvae with significantly greater resistance to a first, acute bout of severe hypoxic stress than did the larvae from normoxia-exposed zebrafish. Specifically, the age at which the increased hypoxic resistance occurred, as well as the magnitude of the effect, varied with the length of parental hypoxic exposure (Fig. 5C,D). Nonetheless, consistently across parental chronic hypoxic exposures of 2 to 4 weeks, larvae from these adults on day 18 post fertilization invariably showed a significantly elevated hypoxic resistance compared with control larval populations. Future experiments to see whether these differences between experimental and control populations further diverged with additional maturation of the juveniles would be very productive.

Enhanced hypoxia resistance in zebrafish larvae does not manifest itself until after 1 week of parental hypoxia exposure (Fig. 5), suggesting that at an exposure level of 13.1 kPa O₂, greater than 1 week of exposure is required to effect changes in the adult zebrafish that lead to the transference of increased hypoxic resistance upon their offspring. The time course of hypoxia exposure on gene expression and potentially physiological function is evidenced in the estuarine fish *Gillichthys mirabilis*, where relatively few genes are upregulated just 8 h after the onset of hypoxia exposure, but upregulation increases dramatically after 6 days of hypoxia exposure (Gracey et al., 2001). Future experiments to determine whether there is a dosing effect of O₂ level are warranted.

Epigenetic mechanisms for transgenerational hypoxic effects

Are the effects on larvae produced by parental chronic hypoxic exposure truly epigenetic, transgenerational effects? This is not a trivial question, nor is it trivial to provide an unequivocal answer. The most parsimonious explanation for these phenomena suggests that as-yet-unidentified epigenetic mechanisms resulting in modified gene expression in larvae are responsible for our observation of this transgenerational transfer of increased hypoxia resistance in the offspring. Abundant evidence for such mechanisms exists in vertebrates, including fishes, and the most common include chromatin remodeling (DNA methylation, histone modification) and RNA signaling (non-coding and micro-RNAs). For an entry into the extensive literature on this topic, the reader is directed to recent reviews by Ho and Burggren (Ho and Burggren, 2010), Nelson and Nadeau (Nelson and Nadeau, 2010) and Skinner (Skinner, 2011). However, to our knowledge, upregulation or downregulation of genes through maternal/paternal factors that elicit one or more of these epigenetic mechanisms has not been specifically linked to hypoxic exposure.

Finally, in discussing mechanisms, it is important to emphasize that studies of epigenetic, transgenerational transfer of phenotypes rarely control for direct exposure of germ lines, and our study is no exception. Not only were the adult zebrafish exposed to chronic hypoxia, but their germ cells – the unfertilized egg cell of the female and sperm of the male – were also chronically exposed to hypoxia along with all other tissues of the adult males and females. While generally viewed as unlikely in such studies, it remains a possibility that all of the larval phenomena we observed – changes in body length and complex changes in hypoxic resistance – could have resulted from direct changes to these germ cells. If this were the case, it would certainly not negate the findings or their impact on hypoxic survival in natural environments, but it would shift the emphasis from ‘maternal’ effects transferred *via* the egg environment to equally interesting ‘direct’ effects on the germ cell line. In any event, only by rearing animals through several generations can this direct effect of exposure be eliminated as a component of the response.

Conclusions

Collectively, our findings suggest that parental exposure to chronic hypoxia can, in a time-dependent fashion, induce changes in fecundity and egg size. Additionally, there are direct effects on the larvae themselves, including changes in body length as well as hypoxia resistance. The present study extends and expands upon the current literature describing potentially advantageous, environmentally induced ‘parental effects’. That hypoxia-induced parental effects modulate the larval hypoxic stress response, but not the thermal stress response, highlights the need for better understanding of the mechanisms that underlying these phenomena. Future studies on zebrafish should be directed at revealing the specific mechanism(s) by which gene regulation is altered epigenetically, as well as identify which genes in larvae have altered expression in a manner that affects hypoxic resistance.

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