PARENTAL HYPOXIC EXPOSURE CONFERS OFFSPRING HYPOXIA RESISTANCE
IN ZEBRAFISH (DANIO RERIO)

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SUMMARY

Parental influences are a potentially important component of transgenerational transfer of phenotype in vertebrates. This study on zebrafish (*Danio rerio*) examined how chronic hypoxic exposure on adults affected the phenotype of their offspring. Separate adult populations were exposed to hypoxia (13.1 kPa O$_2$) or normoxia (21.1 kPa O$_2$) 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 O$_2$), and critical thermal minima (CT$_{Min}$) and maxima (CT$_{Max}$) were measured at 6, 9, 12, 15, 18, 21 and 60 days post fertilization (dpf). 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 dpf, remaining constant thereafter. Notably, hypoxic resistance from 6-18 dpf 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 dpf in larvae from adults exposed to 2, 3 or 4 weeks of hypoxia. CT$_{Min}$ (~39.5°C) and CT$_{Max}$ (~10-12 °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.
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 for example 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” (sometimes referred to as a “indirect genetic effect” or even “transgenerational plasticity”). Not all of these changes 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 lower offspring hatchability as well as induce lethal mutation phenotypes in offspring (Wu et al., 2003). On the other hand, 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,
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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). Only beginning to be explored is whether parental hypoxic exposure specifically heightens hypoxic resistance in their offspring through epigenetic mechanisms. A few examples of a possibly advantageous “parental effect” following hypoxic exposure in adults exist in invertebrates. In the fruit fly *D. 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. Burggren, 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 larval progeny of adult zebrafish exposed to hypoxic conditions.
MATERIALS AND METHODS

Fish Care and Maintenance

Wild-type adult zebrafish (*Danio rerio*; 4 to 6 months old at the start of the study) obtained from Ekkwill Farms, FL, were housed in 13 liter glass aquariums, at a density of 10 fish/aquarium, for 2 weeks prior to experimentation. They were fed brine shrimp and dry flake food twice a day during non-breeding periods, and 3 times per day beginning 5 days prior to breeding. Care was taken to feed equal amounts of food to each population held in separate tanks. Temperature inside the tanks was maintained at 27 ± 0.5°C by a submersible water heater. Light cycle was set at LD 14 h:10 h.

Newly hatched embryos were housed in 95-mm Petri dishes (~70 embryos per dish) filled with clean, normoxic tank water treated with 0.05% methylene blue (14:10 h light:dark cycle). After 5 days post-fertilization (dpf), larvae were transferred to 1 L containers at a density of ~150 larvae/L. Air saturation of the water in each container was maintained by bubbling ambient air into the water. Beginning at 6 days post fertilization (dpf), larvae were fed dry powder food (TetraMin, Baby Fish Food “E” for egglayers) 3 times daily. Brine shrimp were added to feedings beginning at 16 dpf. All embryos and larvae were housed in a temperature-controlled incubation room maintained at 27 ± 0.5°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. Aquariums were fitted with a gas-tight lid equipped with a circular opening small enough to create a very slight positive air pressure inside the tank that only allowed permitted mixed gas from the air-stone 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 about 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. Breeding aquaria for routine breeding were maintained in a 27 °C (± 0.5 °C) incubator room during the breeding period. During routine breeding, males and females were placed together to breed 4 hours after the lights came on the next morning, and were then returned to
Experimental Design

Breeding pairs that produced > 100 eggs during routine breeding were assigned to one of two experimental conditions: hypoxic exposed or normoxic exposed. Prior to placement into experimental aquaria containing hypoxic or normoxic water, pairs were bred, and these 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 wks of normoxia (NP-1, NP-2, NP-3 or NP-4) or hypoxia (HP-1, HP-2, HP-3 or HP-4). Hypoxic resistance of these larvae was assessed at 6, 9, 12, 15 and 18 dpf. Each clutch, for every period of parental exposure to experimental conditions (1, 2, 3 or 4 wks), came from an entirely different breeding pair to avoid pseudoreplication bias. Each experimental group included larvae from at least 2 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 5 females and 5 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 assigned aquaria for continuing exposure. After 12 weeks of 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 dpf 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 attached digital camera and ImagePro Plus data analysis software (v. 4.1, Media Cybernetics, Bethesda, MD).

**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). 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 20X magnification (Image Pro Plux v 4.1). Egg and yolk volumes were calculated from the equation \( V = \frac{4}{3} \pi r^3 \). 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 dpf) were placed individually in an airtight acrylic box (10cm x 10cm x 5cm) 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 sec period. Three seconds were subtracted from the total time to account for the time taken to determine loss of equilibrium. Embryos younger than 6 dpf are not free swimming, and do not display clearly observable loss of equilibrium, and thus were excluded from these experiments.

**Critical Thermal Maxima and Minima (CT\(_{\text{Max}}\) and CT\(_{\text{Min}}\)).**

The critical thermal method (CT\(_{\text{Max}}\) and CT\(_{\text{Min}}\)) is frequently used to estimate the scope of thermal tolerance (also known as thermal scope) of an organism (Herrera et al., 1998; Ford and Beitinger, 2004). 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 oxygen) for 12 weeks. The hypoxic adults were then returned to normoxia, and both populations separately bred as described above. Zebrafish larvae from these two populations were assessed for CT\(_{\text{Min}}\) and CT\(_{\text{Max}}\) at 6, 9, 12, 15, 18 and 21 dpf. Juveniles at 60 dpf were also assessed for thermal tolerance to determine the
validity of the protocol by comparison to 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). Fish were kept in the beakers ≤ 5 min before the temperature of the water was increased (CTMax) or decreased (CTMin) at a rate of 0.3 ± 0.1°C min⁻¹. This rate of temperature change is recommended for small fish since 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 sec was recorded as the CTMax or the CTMin. Prior to 12 dpf, CTMin 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, CTMin was assessed in larvae at 12, 15, 18, 21 and 60 dpf.

**Statistical Analyses**

Time to loss of equilibrium in hypoxia and body length were subjected to factorial three-way analyses of variance (ANOVA) 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 dpf). Subsequent separate two-way analyses with respect to treatment group and development measured as dpf 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 % 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 dpf) were performed on variables of CTMax and CTMin. 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 were 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, 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 \( (q's > 4.62, p's < 0.001) \). However, this reduced egg size disappeared after 6 and 12 wks 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 \( (p's < 0.05; \) Fig. 3A, B, C and D). 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 dpf \( (q = 2.147, p = 0.03; q = 3.62, p < 0.001; t = 4.70, p < 0.001, \) 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 dpf, but remained significant at 15 dpf \( (q = 3.90, p = 0.006) \) and 18 dpf \( (q = 6.58, p < 0.001; \) 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 dpf \( (3 \) week exposure \( (Fig. 3C) q = 3.24, p = 0.002), \) and 15 dpf \( (4\) wk exposure \( (Fig. 3D); q = 2.57, p =0.011).\)

#### 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 2-fold highly significant (p < 0.01) decrease in time to loss of equilibrium from 6 to 15 dpf 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 dpf.

There was a significant effect of both time and treatment at each parental hypoxic exposure period (p’s < 0.05, two-way ANOVA), 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 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 < 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 treatment were significantly more capable of coping with hypoxia stress than the offspring of normoxia-exposed fish, at 9 (q = 3.90, p = 0.006) and 18 dpf (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 day (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 dpf), the composite hypoxic tolerance of HP larvae is actually lowered by just one 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<sub>Min</sub> and CT<sub>Max</sub>**

Thermal tolerance in normoxia of larval offspring (6 to 21 dpf) of hypoxic- or normoxic-exposed adult zebrafish were assessed for temperature at which equilibrium was lost in the face of increasing or decreasing ambient temperature. CT<sub>Min</sub> ranged from 9.5 to 12°C, while CT<sub>Max</sub> was in the range of 39.5-40.5°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’s > 0.05) in thermal tolerance, as measured by CT<sub>Min</sub> and CT<sub>Max</sub>, 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, 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 decreased as development progressed from 6 to 18 (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 days post-fertilization (dpf), oxygen supply to the tissues occurs via diffusion across the body surface, but as the larva increases in size (~14 dpf), 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 (Dziminski 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 approximately 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 (Baxter, 1988), and this inverse relationship is evident in the eggs laid by control zebrafish females (Fig.s 1 and 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 (Baxter, 1988). However, we know of no other study that has drawn a correlation between chronic hypoxic exposure and egg size.

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 about 0.2 µl 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. 3A-D). The majority of differences
between the two groups were observed at 15 and 18 dpf, with HP-1 diverging from the normal
developmental trajectory as early as 6 dpf. Although the relationship between body size and hypoxia
resistance is controversial, body size has clear implications in the regulation of oxygen consumption (see
for example Feder, 1983b; Feder, 1983a; Burleson et al., 2001; Robb and Abrahams, 2003; Ospina and
Mora, 2004; Nilsson and Oslund-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 Oslund-Nilsson, 2008). In contrast, 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. 3A-D with Fig. 5A-D). 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).

**Thermal Tolerance**

Hypoxia exposure induces very similar gene expression profiles to that of extreme temperatures,
suggesting an intimate relationship between hypoxic resistance and thermal tolerance (Airaksinen et al.,
1998; Sørensen and Loeschcke, 2001). Regulatory proteins such as heat-shock proteins and hypoxia-
inducible factor-1 (HIF-1) have been shown to be up-regulated in a similar fashion to 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; Burleson and Silva, 2011).

In this study, the upper thermal tolerances of 6 – 21 dpf larvae were comparable to those reported
for adult zebrafish (Cortemeglia and Beitinger, 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 to cold or heat shock stress (Kassahn et al., 2007). A limitation of the current study is that hypoxia resistance was assessed in offspring of parents exposed to 1 to 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 (*Drosophila melanogaster*) results in increased tracheal size, which could contribute to increased hypoxic resistance (Henry and Harrison, 2004). However, physiological measurements were not made to determine if there was an actual increase in hypoxic resistance in the F1 generation. In the water flea (*Daphnia 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. Burggren, 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 normoxic-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. 5 C,D). Nonetheless, consistently across parental chronic hypoxic exposures of 2-4 weeks, larvae from these adults on day 18 post fertilization invariably showed a significantly elevated hypoxic resistance compared to control larval populations. Future experiments to see if 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 leads 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 up-regulated just 8 h after the onset of hypoxia exposure, but up-regulation increases dramatically after 6 days of hypoxia exposure (Gracey et al., 2001). Future experiments to determine if 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, (2010), Nelson and Nadeau (2010), and Skinner (2011). However, up- or down-regulation of genes through maternal/paternal factors that elicit one or more of these epigenetic mechanisms has not been specifically linked to hypoxic exposure to our knowledge.

Finally, in discussing mechanism, 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 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.
Conclusion

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. These studies extend and expand 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 underly 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.

LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA analysis of variance
CTMin critical thermal minima
CTMax critical thermal maxima
Dpf days post fertilization
HIF-1 hypoxia-inducible factor-1
kPa kilopascal

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REFERENCES


FIGURE LEGENDS

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 ± 1 S.E.M. are presented.

Fig. 2. Egg volume, perivitelline volume and yolk volume of newly laid eggs of adult zebrafish held in normoxia (unfilled symbols) or hypoxia (filled 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 (two-way ANOVA with Tukey’s post hoc analyses between treatment groups, p > 0.05). N = 90-179 for each group at each time point. Mean values ± 1 S.E.M. are given. (note: S.E.M. values are smaller than the symbol size).

Fig. 3. Mean body length of larval offspring of zebrafish exposed to hypoxia or normoxia (triangle, solid line) for 1 (A, n=7-21/group), 2 (B, n=9-14/group), 3 (C, n=12-15/group) or 4 (D, n=22-26/group) weeks. A box indicates no significant difference among the groups. N’s > 16 for each group at each time points. Mean values ± 1 S.E.M. are presented.

Fig. 4. Time to loss of equilibrium in hypoxia (4 kPa O2) of zebrafish larvae from 5-18 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.

Fig. 5. Time to loss of equilibrium in hypoxia (4 kPa O2) of zebrafish of parents reared in normoxia or hypoxia for 1 (A, n=10-23/group), 2 (B, n=10-14/group), 3 (C, n=9-14/group), or 4 (C, n=18-29/group) weeks. 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 ± 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 text for details. Different letters indicate significant differences (P<0.05) between populations.

Fig. 7. Critical minimum thermal limit (CTMin) and maximum thermal limit (CTMax) of zebrafish larvae from parents reared in normoxia or hypoxia for 12 weeks. CTMin and CTMax 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 ± 1 S.E.M. are presented.
FIGURE 2

Filled Symbols = Normoxia Parents (21.1 kPa O$_2$)
Open Symbols = Hypoxia Parents (13.2 kPa O$_2$)

Egg Parameter Volumes (µl)

- Yolk Volume
- Perivitelline Volume
- Total Egg Volume

Parental Exposure Time (weeks)
FIGURE 3

A. 1 week parental exposure

B. 2 week parental exposure

C. 3 week parental exposure

D. 4 week parental exposure

Days Post Fertilization

Standard body length (mm)
FIGURE 5

A. 1 week parental exposure

B. 2 week parental exposure

C. 3 week parental exposure

D. 4 week parental exposure

Time to Loss of Equilibrium in Hypoxia (sec)

Days Post Fertilization

- Normoxia
- Hypoxia
FIGURE 6

Composite Hypoxic Resistance
(% Change From Control)

-30 -20 -10 0 10 20 30 40

Parental Hypoxic Exposure

1 Week

2 Weeks

3 Weeks

4 Weeks

Decreased Hypoxic Resistance

Increased Hypoxic Resistance

Increased Hypoxic Resistance

Decreased Hypoxic Resistance

a

b

b

b