

Fish embryo and juvenile size under hypoxia in the mouth-brooding African cichlid *Pseudocrenilabrus multicolor*

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Abstract We used a field survey and a laboratory rearing experiment to (a) examine response (size and survival) to life-long hypoxia in offspring of the African maternal mouth-brooding cichlid *Pseudocrenilabrus multicolor victoricae* (Seegers) and (b) explore the degree to which developmental response can be environmentally-induced. Embryo size metrics were quantified in 9 field populations across a range of dissolved oxygen (DO) concentrations. In the laboratory, first generation (F1) broods of low-DO origin were reared under high or low DO. Brooding period was quantified for the mothers; and egg size, egg metabolic rate and juvenile size-at-release were quantified in their (F2) offspring. The F2 offspring were split and grown for 3 months post-release under high or low DO, and juvenile size and survival were quantified. In the field survey, across stages, embryos from low-DO field populations were shorter and weighed less than embryos from high-DO populations. In the laboratory experiment, F2 eggs and juveniles-at-release from mother's mouth did not differ in mass, length, survival regardless of development DO environment. However, juveniles diverged in size after leaving mother's mouth, exhibiting smaller size when grown under low DO. Size differences in embryo size across field populations and divergence in embryo size after release from the mother's mouth support predictions for smaller body size under hypoxia. There was no evidence for negative effects on survival of juveniles after 3 months. Brooding period was 16% shorter in females reared under low DO suggesting that hypoxia may accelerate embryo development. This work provides insights into how bearer fishes respond to hypoxic stress relative to fishes with no post-spawning parental care; a shorter brooding interval and smaller body size may provide an optimal solution to parent and embryo survival under hypoxia in brooding fishes [*Current Zoology* 58 (3): 401–412, 2012].

Keywords African cichlid, Lake Victoria, Life-history, Parental care, Dissolved oxygen

Hypoxia, or low dissolved oxygen (DO), can operate as a significant environmental stressor for water-breathing organisms (Pollock et al., 2007; Wu, 2009) given the high costs of acquiring oxygen from water relative to air (Lindsey, 1978; Wootton, 1990). Aquatic hypoxia occurs naturally, but it is becoming widespread as a consequence of eutrophication and pollution of water bodies and is now recognized as a large-scale threat to fresh and coastal waters. The increasing frequency of hypoxic events has resulted in changes in biodiversity, population decline, and production of extensive “dead zones” that affect commercially and recreationally important fisheries (Pollock et al., 2007; Diaz and Breitburg, 2009; Wu, 2009). These negative impacts of hypoxia on wild fish populations have been associated with reproductive and developmental impairment (e.g. Wu, 2009). Several aspects of reproduction in fishes and invertebrates have shown impairment due to hypoxia, including gametogenesis, the number and quality of

gametes, reproductive behavior, fertilization success, impaired respiration, hatching success, and survivorship of the young (e.g. Garside, 1959; Hamor and Garside, 1976; Rombough, 1988; 2007; reviewed in Wu, 2009 and Mueller et al., 2011). However, the majority of the evidence for the impacts of hypoxia on reproduction and early-life stage development comes from short-term (hours to weeks) laboratory experiments. We understand far less about life-long effects of hypoxia on reproduction and early life-history traits.

Several reviews of fishes and early life-history traits suggest that large egg/embryo size is positively associated with fitness correlates such as higher survival, faster growth, and enhanced swim performance (Reznick, 1991; Heath and Blouw, 1998). However, much of the early life-history and maternal effects literature for fishes supports the idea that eggs, embryos, and/or juveniles should actually be smaller under low DO, because they will have higher survival, and thus

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higher fitness, than larger individuals at the same stage (e.g., Sargent et al., 1987; Hendry et al., 2001). The benefits of small size under hypoxia can be explained physiologically in terms of the surface area to volume ratio; where surface area limits the amount of oxygen that can be taken up, and volume (theoretically) represents the oxygen demand (Krogh, 1959; Pauly, 1981). At small sizes the surface area to volume ratio is higher, which means that there is a larger surface area to meet the metabolic demand. In fish eggs, oxygen uptake occurs across the egg membrane. In hatched embryos and small juvenile fish, oxygen uptake occurs primarily across the skin, while the gills at early life stages function predominately for ion exchange (Moyle and Cech, 1996). Thus, optimal egg and juvenile size under hypoxia suggest a negative size-fitness function i.e., under hypoxia, smaller should be better in terms of fitness. However, there is some argument as to whether the whole egg is metabolically active (e.g., Einum et al., 2002; Kolm and Anesjo, 2005; Rombough, 2007), which could alter the above prediction.

In addition to the physiological advantages of small egg size under hypoxia, it is possible that small size facilitates faster development. While widely supported in the theoretical literature, empirical support for this prediction is contradictory (Marshall and Bolton, 2007). Several empirical studies of marine invertebrates, marine fish, and amphibians do support the longer development time in large eggs (Pauly and Pullin, 1988; Bradford, 1990; Marshall and Keough, 2008). However, others provide support for the opposite or no relationship between egg size and development within and across taxa (Marshall and Keough, 2008; Przeslawski and Webb, 2009; Ruis et al., 2010). Clearly, there is a need for additional empirical work that explores the effects of environmental stressors on egg size and development.

In this study, we used a field survey and a laboratory rearing experiment to (a) examine response (size and survival) to life-long hypoxia in embryos and juveniles of the African maternal mouth-brooding cichlid *Pseudocrenilabrus multicolor victoriae* (Seegers) and (b) explore the degree to which developmental response can be environmentally-induced. Much of the work-to-date has been done predominately on batch spawning fishes and nest guarders (reviewed in Wu, 2009 and Mueller et al., 2011). However, bearer species (those that carry their offspring externally or internally) may have evolved different strategies to mitigate costs associated with reproduction and/or development under hypoxia

given that they carry their offspring throughout embryonic development. *P. multicolor* was used to explore these questions because it is found across a wide range of oxygen habitats throughout the Lake Victoria basin of East Africa and exhibits strong interdemec variation in morphology, physiology, and life-history traits (e.g., Chapman et al., 2000; Chapman et al., 2002a,b; Chapman et al., 2008; Martinez et al., 2009; Reardon and Chapman, 2009). In *P. multicolor*, it is unclear whether fertilization occurs on the substrate, in the mouth, or some combination of both (Wickler, 1962; Fryer and Iles, 1972; Hellweg, 2008). As with other bearer cichlid species, embryos undergo direct development (no larval stage; Balon, 1986; Noakes, 1991). Offspring are released from the mouth as juveniles, when their yolk sacs are completely absorbed and they are ready to feed exogenously (Noakes, 1991).

We compared embryo size of 4 developmental stages of *P. multicolor* among 9 field populations. These populations were selected from sites that differed in aquatic oxygen availability. In the laboratory, we reared first generation (F1) broods of low-DO origin under either high or low DO. Once the F1 generation reproduced, we quantified the brooding period (i.e. the length of time the mother held the young in her mouth from fertilization to release) and the F2 egg size, egg metabolic rate, and juvenile size at release (see Fig. 1 for experimental design schematic). The second generation (F2) juveniles from the laboratory experiment were then split and grown for 3 months post-release under either high or low DO. We predicted the following:

- Across field populations, embryo size will be positively correlated with DO.
- Egg size and juvenile size at release will be smaller when mothers are reared and embryos brooded (embryo development environment) under low DO compared to high DO.
- If egg and embryo size are smaller under low DO, then brooding period will be reduced because smaller embryos should develop faster. Juvenile size after 3 months of post-release growth will be smaller in juveniles grown under low DO compared to high DO.

1 Methods and Materials

1.1 Field survey

We collected brooding *P. multicolor* females in Uganda during the May-June dry season (2004–2007). Fish were sampled from 9 field populations across a range of DO concentrations (range: 0.24 to 8.7 mg L⁻¹).

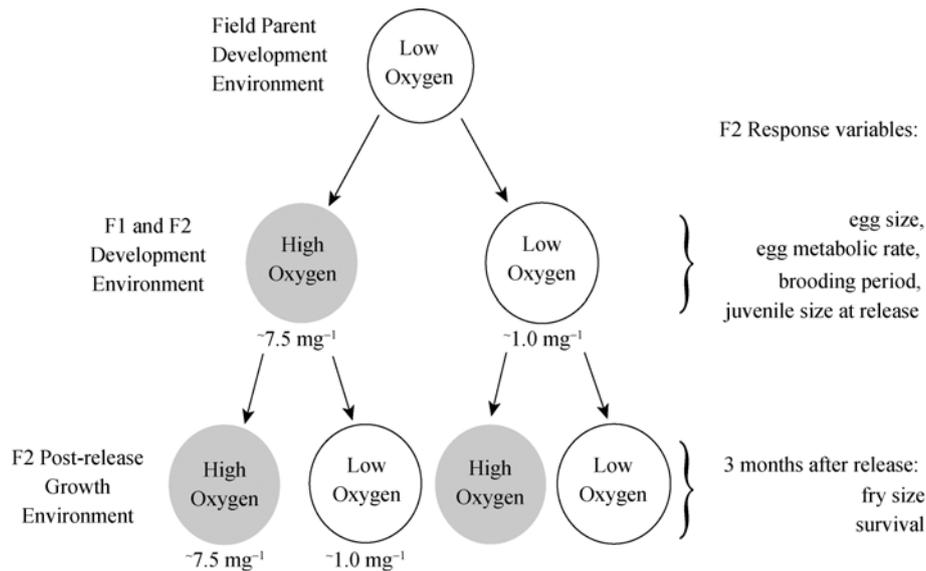


Fig. 1 Schematic of the experimental design for development and growth environments of *Pseudocrenilabrus multicolor* in the laboratory rearing experiment

First generation (F1) broods of low-DO (Lwamunda Swamp) origin were reared under either high or low DO. Once the F1 generation reproduced, we quantified the brooding period and the F2 egg size, egg metabolic rate, and juvenile size. The second generation (F2) juveniles from the laboratory experiment were then split and grown for 3 months post-release under either high or low DO after which fry size and survival were measured.

We chose sites representative of the range of DO conditions experienced by this species (see Fig. 2 map, adapted from Reardon and Chapman, 2009 and Crispo and Chapman, 2010). These included: Lake Saka and four sites in the Mpanga River drainage of western Uganda, and four sites in the Nabugabo region of the Lake Victoria basin. The Mpanga River is located >150 km west of the Nabugabo system and joins the westward flowing section of the Katonga River feeding into Lake George. Environmental data were collected from multiple micro-sites within each location in the afternoon on days when fish were collected. Environmental variables included: DO, water temperature, conductivity, pH, and transparency (Table 1; see Reardon and Chapman 2009 for full description). Aquatic dissolved oxygen concentration was converted from mg L^{-1} to partial pressure (mmHg), correcting for temperature and altitudinal differences across sites. These readings were then averaged to produce a mean DO level for each field population. Although *P. multicolor* breed throughout the year, there are peaks in reproductive activity associated with peaks in rainfall (Welcomme, 1969; Reardon and Chapman, 2008). Expeditions were carried out in the late May - early June period to standardize for potential seasonal effects on reproductive traits. We live-captured an average of 48 females per population per year sampled (range: 22-74) using metal minnow traps as part of a larger study estimating size at maturity and other

Table 1 Mean dissolved oxygen (DO) values for each of the field sampling sites where *Pseudocrenilabrus multicolor* were sampled (adapted from Reardon and Chapman, 2009)

Field Population	Region	Mean DO (mg L^{-1})
Bunoga	Mpanga	7.32
Kahunge	Mpanga	4.20
Kamwenge	Mpanga	6.92
Kantembwe	Mpanga	0.24
Laka Saka	Mpanga	8.73
Lake Kayanja	Nabugabo	6.30
Kaz Lagoon	Nabugabo	1.63
Lwamunda Swamp	Nabugabo	0.95
Lake Nabugabo	Nabugabo	2.76

life-history traits in this species (reported in Reardon and Chapman, 2009). Brooding females and their broods were used to quantify egg traits (Reardon and Chapman, 2009) and size and number of developing embryos (this study). Among the females collected, offspring were in all stages of pre-release development (unhatched eggs to fully developed juveniles). However, females are very secretive while brooding, and we were not always able to capture all stages of development at every site. Thus, embryo size data were combined across years to increase power. We were unable to test for an effect of year on brood traits; but as stated above,

we controlled for season sampled to minimize seasonal effects. When a brooding female was live-captured, the brood was gently removed from the mouth. Each brood was counted for offspring number, euthanized in tricaine methanesulfate (MS222), and preserved in 4% paraformaldehyde buffered with PBS. Egg size and number, female size at maturity, and environmental data were previously reported in Reardon and Chapman (2009).

Total length and mass were measured for each embryo and macroscopically classified into one of the following five developmental stages: Stage 1 is the fertilized unhatched egg (reported in Reardon and Chapman, 2009); Stage 2 is the embryo hatched out of the egg with a very large yolk sac, on a small translucent body, with a small eye; Stage 3 is similar to Stage 2 with a reduced yolk sac, a larger eye but still a relatively small body; Stage 4, the yolk sac is almost completely absorbed; the eye fills a larger portion of the head; Stage 5, the yolk sac is completely gone (see Fig. 4). Between 474 and 1,009 individual hatched embryos were measured for each these five developmental stages. Two analytical approaches were used to detect relationships between DO and embryo size. First, analysis of covariance (ANCOVA) was used to test for an effect of development stage and region (Mpanga versus Nabugabo) on mean embryo size per population with mean DO per site included as a covariate. In a second analysis, the four sample sites where the mean DO was equal to or less than 3.0 mg l^{-1} were classified as low DO, and the other five populations were classified as high DO (high DO $\sim 6.0 \text{ mg l}^{-1}$; mean DO values reported in Reardon and Chapman, 2009). We used an analysis of variance (ANOVA) to test for an effect of DO (high, low) and region on mean embryo size (mass and length) across developmental stages with individual population included as a random factor (nested within oxygen regime and region). We used the mean mass and length per brood.

1.2 Rearing experiment

Adult live specimens were wild-caught from the hypoxic Lwamunda Swamp that surrounds Lake Nabugabo, Uganda and transferred to our aquatic facility at McGill University. In the swamp, DO levels average 1.85 mg l^{-1} throughout the year (Reardon and Chapman, 2008). This population exhibits larger gills, higher hematocrit, and a smaller size at maturity than conspecifics that inhabit well-oxygenated lakes and rivers, traits that may represent an adaptive response to hypoxic stress (Chapman et al., 2000; Chapman et al., 2002a,b; Chapman et al., 2008; Martinez et al., 2009;

Reardon and Chapman, 2009). Four families of the Lwamunda Swamp population were bred under normoxic conditions (Fig. 1). The F1 broods were then split into two groups, and full siblings were raised under high or low DO. One family was raised per aquarium per treatment. We reduced broods randomly to 8 juveniles per aquarium after 3 months. This density allows fish to grow to a size reflective of field populations. The DO in the experimental growth tanks was controlled by Point Four System's PT4 Monitor by automatically pulsing nitrogen through a diffuser in each aquarium to reduce DO levels. Mean ($\pm SE$) DO was $7.98 \pm 0.02 \text{ mg l}^{-1}$ in the normoxic aquarium and $1.31 \pm 0.00 \text{ mg l}^{-1}$ ($n = 537$) in the hypoxic aquaria, representative of field DO levels (Reardon and Chapman, 2009). Aquaria were held at $24.9 \pm 0.1 \text{ }^\circ\text{C}$ (recorded once daily) and exposed to a 12/12 photoperiod. Underwater filtration was achieved with Hagan Fluval 1+ Underwater pumps with sponge biofiltration. pH, ammonia, nitrate, and conductivity were monitored biweekly. Juvenile fish were fed fry bites daily for the first two weeks post-release from the mouth then switched to Tetramin flakes once per day.

F1 females were observed daily for evidence of brooding (not feeding, dropped jaw, secretive). Ten females produced multiple broods representing three out of the four families grown in each treatment. The fourth family produced no brooders in either DO treatment. The first brood from each mother was collected within the first 24 hr after fertilization; egg metabolic rate was measured individually on 5 eggs per brood. Egg metabolic rates were measured using LoligoSystems micro-glass respirometer (volume: 0.7 ml) and AutoResp software using intermittent closed respirometry and a LoliTemp temperature controller to maintain target temperature to $\pm 0.1 \text{ }^\circ\text{C}$. Metabolic rates were measured at mean ($\pm SE$) temperature of $24.61 \pm 0.06 \text{ }^\circ\text{C}$ ($\pm SE$; $n = 32$ eggs; temperature logged once per second throughout trials) in nearly saturated water (8.2 mg l^{-1}). All eggs were held for 1 hr under high DO before respirometry measurements. Each egg experienced a 20-minute acclimation period in the respirometry chamber before measurements began. Metabolic rate was calculated based on the average metabolic rate across seven 10-minute measurement periods and was expressed as $\text{ugO}_2 \text{ hr}^{-1}$. Levels of background respiration were estimated from controls run in the empty respirometer. All eggs were euthanized in MS222 and then preserved in 4% paraformaldehyde buffered with PBS for estimates of egg size (mass and volume). Each egg was placed in a drying oven for 24 hr at $60 \text{ }^\circ\text{C}$ to

achieve dry mass. Individual egg wet and dry mass were measured to 0.001 mg using a Sartorius Microbalance. Percentage of water was calculated as: $(\text{Wet mass} - \text{Dry mass}) / \text{Wet mass}$. Individual egg volume was calculated using the equation for a spheroid $[V = 4/3 * \pi * (D_1/2) * (D_2/2) * ((D_1 + D_2)/4)]$ (Niciu, 1995). D_1 and D_2 (two perpendicular diameters of each egg) were measured using digital photographs and the imaging software, Motic Images 2000 1.3[©]. When the second brood of each female was released from the mother's mouth, fry were collected, euthanized in MS222, and preserved for estimates of juvenile size at release (total length, mass). When the third brood was released from each mother's mouth, they were split and grown for an additional three months under either low $1.527 \pm 0.003 \text{ mg l}^{-1}$ ($\pm SE$) or high ($7.84 \pm 0.06 \text{ mg l}^{-1}$) DO (Fig. 1). Mean ($\pm SE$) temperature was $25.98 \text{ }^\circ\text{C} \pm 0.08$ in the low-DO growth tanks and $25.85 \text{ }^\circ\text{C} \pm 0.09$ in the high-DO growth tanks. Three months post-release, juveniles were collected and preserved for estimates of size at time and survival. Individual juvenile mass was recorded on a Mettler Bal-

ance, AC-100. Total length was measured using digital photographs and the imaging software, Motic Images 2000 1.3[©]. Brooding period (from egg fertilization to release from mother's mouth) was calculated using the second and third broods of each female.

ANOVA was used to test for an effect of the treatment under which the F2 young were brooded (development DO: high or low) on mean egg size (wet mass, dry mass, volume, % water) and mean F2 juvenile size at release (total length, mass) with family and female (nested within family and development DO) included as random factors. Female is defined as the mother of each brood, and family is defined as groups of individuals originating from the same wild-caught parents. ANOVA was also used to test for an effect of treatment on brooding period (time in which the F2 offspring were held in the mouth of the females from fertilization to release) with family included as a random factor. For F2 juveniles grown for 3 months post-release, ANOVA was used to test for an effect of development treatment (high or low DO), post-release growth treatment (high or

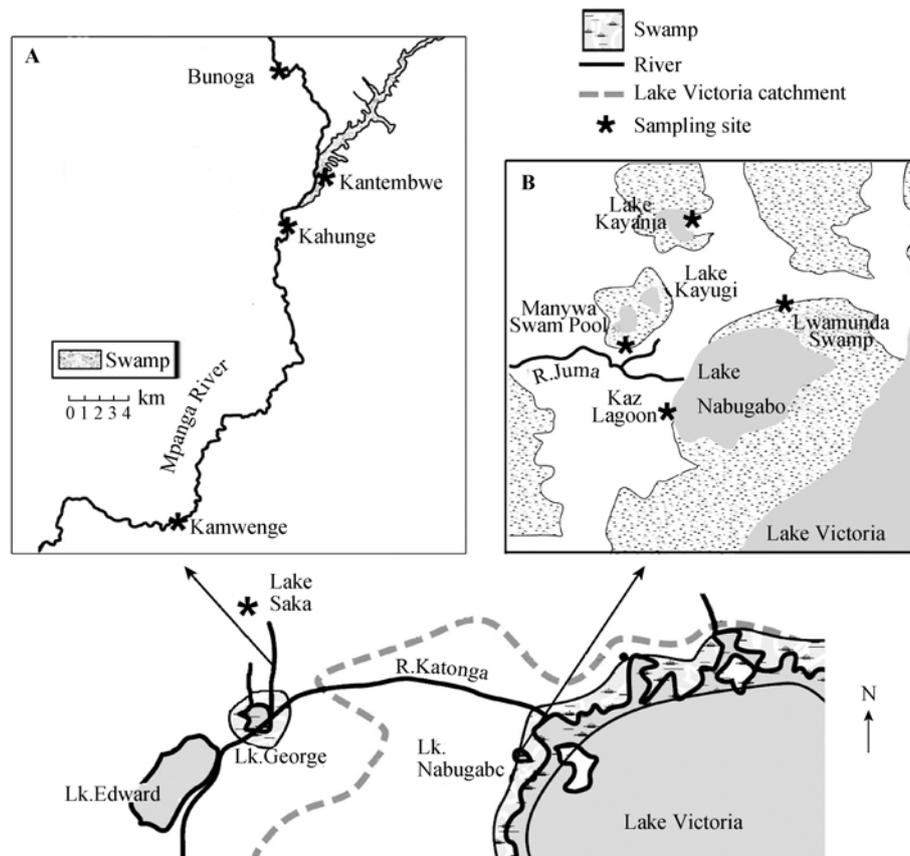


Fig. 2 Map of Katonga River system in Uganda, a river that flows both east and west due to the historical rift uplifting (adapted from Reardon and Chapman 2009 and Crispo and Chapman 2010)

One sampling (Lake Saka) is indicated here. Inset A illustrates the location of our four sampling sites (asterisks) on or near the Mpanga River that flows into the westward-flowing Katonga River and eventually into Lake George. Inset B illustrates the four sampling sites in the Lake Nabugabo region where the Katonga River meets lowlands and flows into Lake Victoria.

low DO), and their interaction on individual mass and total length, with female and family included as random factors. Similarly, ANOVA was also used to test for an effect of development treatment (high or low DO), post-release growth treatment (high or low DO), and their interaction on brood survival with family included as a random factor. ANCOVA was used to test for an effect of DO treatment and family on individual egg metabolic rate with individual egg mass as a covariate. No egg/juvenile size traits were related to female standard length, although our range in female length was small.

2 Results

2.1 Field survey

In total, 113 brooding females were collected across populations and years. We measured each individual embryo from every brood collected, ranging between 25–37 embryos per female. The interaction between DO and developmental stage was not significant (mass $P = 0.752$; length $P = 0.508$), nor between DO and region (mass $P = 0.708$; length $P = 0.710$), indicating homogeneous slopes. There was a positive relationship between mean embryo size and mean DO across developmental stages (ANCOVA for embryo mass: DO $F_{1,19} = 5.258$, $P = 0.033$, stage $F_{3,19} = 2.987$, $P = 0.057$, region $F_{1,19} = 3.121$, $P = 0.093$; ANCOVA for embryo length: DO $F_{1,19} = 5.711$, $P = 0.027$, stage $F_{3,19} = 39.556$, $P < 0.001$; region $F_{1,19} = 25.121$, $P < 0.001$; Fig. 3). Embryos from low-DO field populations were smaller (mass and length) than embryos from high-DO field populations across all stages of development (ANOVA mass: DO $F_{1,10.5} = 6.668$, $P = 0.026$; stage $F_{3,101} = 16.928$, $P < 0.001$; pop

$F_{6,101} = 1.611$, $P = 0.152$, region $F_{1,9.5} = 0.450$, $P = 0.519$; length: DO $F_{1,12.8} = 5.937$, $P = 0.030$; stage $F_{3,101} = 83.938$, $P < 0.001$; pop $F_{6,101} = 1.122$, $P = 0.355$; region $F_{1,11.6} = 15.214$, $P = 0.002$; Fig. 4). In both analyses, across developmental stages, embryos from sites in the Mpanga region were longer than embryos from sites in the Nabugabo region, but there was no difference in embryo mass between regions.

2.2 Rearing experiment

The (F2) egg size traits of eggs produced by F1 mothers did not differ significantly between DO treatments (Table 2, $n = 150$ eggs). There was a significant interaction between family and DO treatment for egg % water content (Table 2). However, within each DO treatment, there was no difference in egg % water content among families (Sidak post hoc: High DO $P = 0.119$; Low DO $P = 0.098$). Metabolic rate was higher in the eggs of females reared under low DO (mean $\pm SE$, $1.00 \pm 0.053 \mu\text{gO}_2 \text{ hr}^{-1}$) compared to eggs from females reared under high DO (mean $\pm SE$, $0.51 \pm 0.054 \mu\text{gO}_2 \text{ hr}^{-1}$; $n = 32$ eggs; ANCOVA: treatment $F_{1,5} = 24.366$, $P = 0.016$). Slopes were homogenous ($P = 0.313$) between treatment groups. There was no effect of egg mass on egg metabolic rate ($P = 0.656$).

The total length and wet mass of F2 juveniles at the time of release from the mother's mouth did not differ between DO treatments (Table 2; $n = 82$ juveniles). However, brooding period was 16% shorter in broods produced in the low-DO treatment compared to broods produced under high DO (Table 2). Offspring from both DO treatments were at the same macroscopic developmental stage (juveniles) when released from the mouth of females.

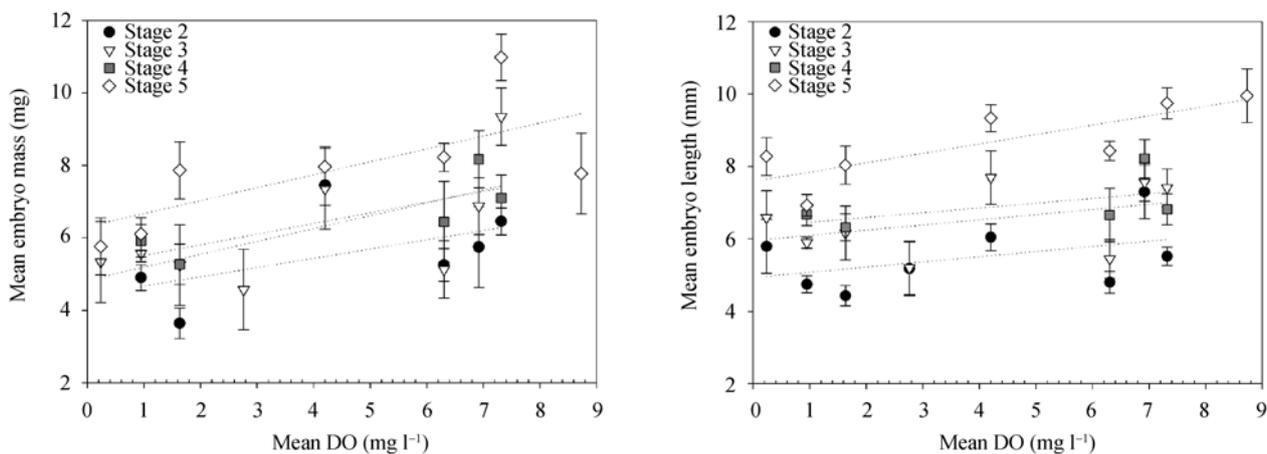


Fig. 3 Relationship between (a) mean embryo mass, (b) mean embryo length and DO across field populations of *Pseudocrenilabrus multicolor*

DO data were adapted from Reardon and Chapman (2009).

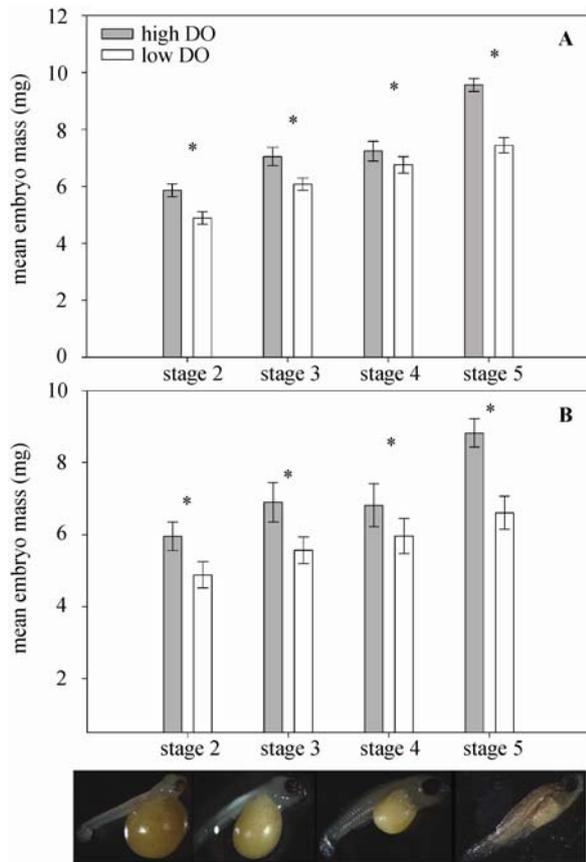


Fig. 4 Mean length (panel A) and mass (panel B) of *Pseudocrenilabrus multicolor* embryos of four developmental stages from high and low-DO field populations in Uganda

Means were produced by ANOVA testing for an effect of DO (high, low), controlling for potential region effects across developmental stages with individual population included as a random factor (nested within oxygen regime and region). Error bars represent standard errors. Stars (*) represent a significant difference in embryo mass or length between high and low DO populations within each stage. As reported in the text, within each stage, trends were similar for mean total length between high- and low-DO populations.

Regardless of the DO environment in which they were brooded, F2 juveniles grown for 3 months under low DO were shorter than full siblings grown under high DO (Fig. 5, Table 3; $n = 71$ juveniles). There was no significant difference in mass between the growth DO treatments or developmental DO treatments, although the trend was similar to length (Fig. 5, Table 3). Female had a significant effect on embryo mass and length (Table 3) indicating some variation among females in their offspring size. There was no effect of embryo development DO, juvenile growth DO, or their interaction on survival (Table 2). After 3 months of post-release juvenile growth, mean survival was 66.5% (± 7.7 SE) under high DO and 77.7% (± 7.6 SE) under low DO.

3 Discussion

3.1 Development in the mouth

In field populations of *P. multicolor*, embryos from low-DO field populations were smaller (mass and length) than embryos from high-DO field populations across all stages of development. A significant region effect was detected, driven by longer embryos in sites of the Mpanga region. However, there was no difference in embryo mass between regions, suggesting a potential difference in embryo shape/morphology between these two regions. Regardless of region, our results support the positive relationship between egg size and DO reported in Reardon and Chapman (2009), suggesting that in wild populations, smaller eggs are indeed producing smaller embryos. The observed interpopulational and regional level variation could be genetically determined and/or environmentally induced.

Because of the difficulties of quantifying development, growth, and survival in wild populations, we used a laboratory rearing experiment to quantify potential environmental influences of DO for one low-DO field population. The results indicated that while in the protection of the mother's mouth, DO did not affect egg and embryo size; however, size had diverged three months after the juveniles were released from the mother's mouth. The lack of treatment effect on egg and juvenile size-at-release in the lab suggests that the differences in egg and embryo size across field populations may be genetically-determined. In an earlier study, Reardon and Chapman (2009) reported larger egg size in F1's of high-DO origin than in F1s of swamp origin supporting a population effect on egg size. The difference in egg volume between DO treatments detected by Reardon and Chapman (2009) was due to a difference in water content of the eggs—a difference we were not able to replicate in this study. Experimental studies on the African cichlid *Simochromis pleurospilus* found that mothers prepare their offspring for the growth environment they experience as juveniles, rather than the environment they experience during adulthood and/or spawning (Taborsky, 2005; 2006). If true in *P. multicolor*, we should have been able to induce a treatment response in this study. Our data suggest that this is not the case. The lack of treatment response could suggest that a mother's preparation of offspring for their juvenile environment may arise due to long-term genetic selection, rather than environmental response. Future studies that rear offspring from multiple populations are needed to address these possibilities.

Table 2 Mean trait values for *Pseudocrenilabrus multicolor* broods mouth brooded by mothers reared under high or low DO and results for ANOVA testing for an effect of embryo developmental DO environment on size traits with family and female (nested within family and developmental DO environment) included as random factors

Egg/Juvenile at release Trait	Development DO	Mean Trait Value ($\pm SE$)	Factor	F	df	P
Egg wet mass (mg)	High DO	5.73 (± 0.03)	DO Treatment	3.844	1,1.8	0.203
			Family	2.111	2,1.6	0.356
	Low DO	5.29 (± 0.05)	Female	24.272	7,136	<0.001*
			DO*Family	0.852	2, 6.9	0.467
Egg dry mass (mg)	High DO	2.81 (± 0.02)	DO Treatment	37.344	1,1.2	0.081
			Family	44.575	2, 0.5	0.251
	Low DO	2.59 (± 0.03)	Female	16.549	7, 135	<0.001*
			DO*Family	0.192	2, 6.9	0.830
% Water	High DO	0.51 (± 0.003)	DO Treatment	0.024	1, 1.9	0.891
			Family	0.202	2, 1.9	0.832
	Low DO	0.51 (± 0.002)	Female	3.342	7, 135	0.003*
			DO*Family	22.115	2, 6.6	<0.001*
Volume (μ L)	High DO	6.82 (± 0.14)	DO Treatment	0.569	1, 1.9	0.535
			Family	0.088	2, 1.7	0.920
	Low DO	6.24 (± 0.09)	Female	10.929	7, 136	<0.001*
			DO*Family	1.212	2, 6.9	0.354
Juvenile at release wet mass (mg)	High DO	10.42 (± 0.12)	DO Treatment	1,1.9	0.126	0.758
			Family	2,1.5	5.050	0.219
	Low DO	9.97 (± 0.11)	Female	3,63	44.483	<0.001*
			DO*Family	2,3	0.303	0.759
Juvenile at release total length (mm)	High DO	8.92 (± 0.08)	DO Treatment	0.033	1, 1.9	0.874
			Family	1.276	2, 1.9	0.443
	Low DO	8.77 (± 0.08)	Female	8.491	4, 71	<0.001*
			DO*Family	2,4.1	0.880	0.481
Brooding period	High DO	19.22 (± 1.22)	DO Treatment	10.904	1, 2.9	0.047*
	Low DO	16.22 (± 1.28)	Family	4.333	2, 2	0.187
			DO*Family	0.222	2, 7	0.801

The stars (*) represent significant *P* values.

Despite the lack of difference in egg or juvenile size-at-release between treatments, the brooding period data suggest the possibility that development may be faster under low DO. Embryos brooded by mothers that were reared under low DO left the mouth 16% earlier than embryos brooded by mothers reared under high DO, but they were at the same stage of development (yolk sac fully absorbed) when released from the mouth. A similar finding was reported in wild-caught *P. multicolor* acclimated to high and low DO (Reardon and Chapman, 2010). Although future studies are clearly necessary to examine development at a finer scale (e.g., histological studies), to our knowledge, this is

among the first evidence for faster development of fish in response to development under hypoxia and is in contrast to other reports of delayed development under hypoxia in fishes (Garside, 1959; Hamor and Garside, 1976; Rombough, 1988; reviewed in Wu, 2009; Mueller et al., 2011). However, our study of *P. multicolor* is also the first to explore these issues in a bearer fish species that has lived and evolved under low-DO for many generations in the wild; it may be that parental care plays a role in developmental response to hypoxia. In their study of the costs of mouth brooding in *P. multicolor*, Reardon and Chapman (2010) found that the standard metabolic rates were ~48% higher in brooding females

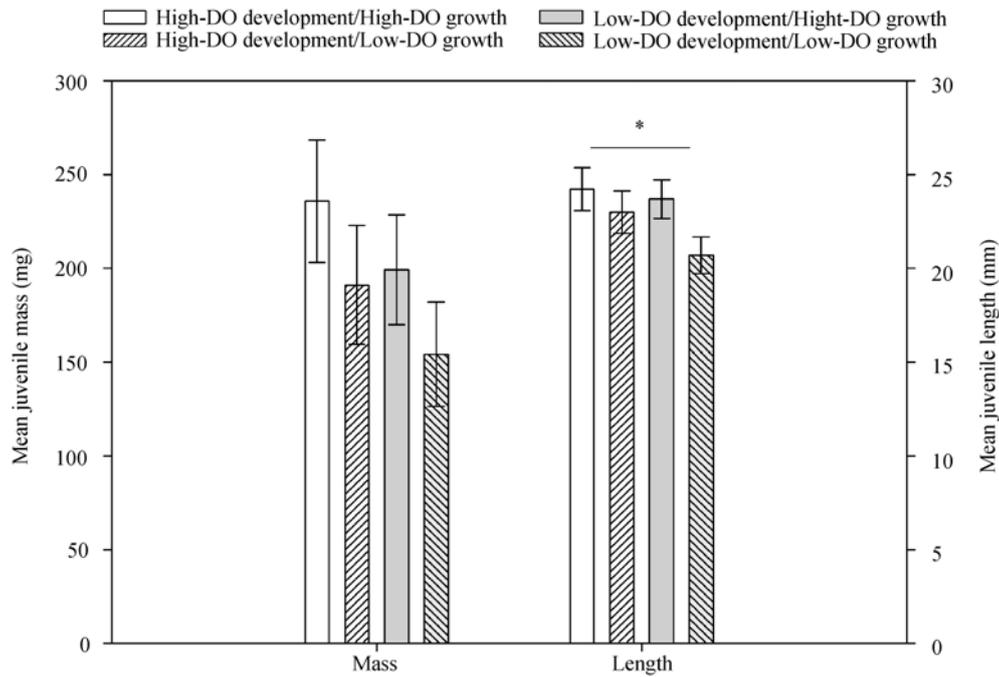


Fig. 5 Mean mass and length of F2 *Pseudocrenilabrus multicolor* juveniles grown for 3 months post-release from the mouth under either high or low DO

The star (*) represent a significant difference between high- and low-DO post-release juvenile growth environments within each size trait. Error bars represent standard errors.

Table 3 Results for ANOVA testing for an effect of DO environment (high or low DO) during embryo development in the mother's mouth (development DO), three months of growth after release from the mother's mouth (growth DO), and their interaction on total length and mass for F2 *P. multicolor* with family and female (nested within family and developmental DO) included as random factors

Size Trait	Factor	df	F	P
Total length (mm)	Development DO	1, 3.8	0.101	0.768
	Growth DO	1, 61	4.129	0.047*
	Development*Growth DO	1, 61	0.722	0.399
	Family	2, 4.4	0.377	0.706
	Female	4, 61	2.575	0.046*
Mass (mg)	Development DO	1, 3.8	0.121	0.746
	Growth DO	1, 61	2.311	0.134
	Development*Growth DO	1, 61	0.000	0.998
	Family	2, 4.3	0.129	0.883
	Female	4, 61	3.232	0.018*
Survival	Development DO	1, 10	0.862	0.311
	Growth DO	1, 10	1.139	0.375
	Development*Growth DO	1, 10	0.066	0.803
	Family	2, 10	9.954	0.004*

The stars (*) represent significant *P* values.

compared to post-brooding females, suggesting a high energetic cost to the female during brooding. It is possible that *P. multicolor* females compensate for this cost

with a reduced brooding period (Reardon and Chapman, 2010). There may also be an advantage to offspring of moving quickly through this developmental stage in a

hypoxic environment. Clearly more work is necessary to tease apart whether the reduced brooding period/embryo development time is a parental, offspring, or mutual adaptation to hypoxia. The mechanisms underlying the shorter brooding period also remain unknown. In a study exploring the developmental plasticity of the cardiovascular system in zebrafish embryos grown under high or low DO, Pelster (2003) found that four days post-fertilization, embryos developing under low DO had enhanced blood vessel formation and increased cardiac output compared to those under high DO. Given the positive link between cardiac output and metabolism in the literature, Pelster's results are supportive of our increased metabolic rate in low DO eggs and the potential link to faster development rate. A study on brine shrimp *Artemia franciscana* also reported faster development in shrimp cultured under chronic hypoxia relative to high DO due to earlier respiratory regulation in the hypoxia-cultured individuals. The improved respiratory regulation in the hypoxia-reared brine shrimp took place before development of the heart and gills suggesting that observed shifts in haemoglobin concentration may be the mechanism driving the faster development under chronic hypoxia (Spicer and El-Gamal, 1999). If respiratory regulation is conserved across taxa, perhaps a similar shift in haemoglobin concentration is occurring in *P. multicolor*. In adult *P. multicolor*, Martínez et al. (2009) showed that fish from the same population as this study had elevated haematocrit and LDH activities when compared to high-DO populations of the same species. More work is needed to explore the possibilities of the hypoxia tolerance mechanisms acting in developing embryos.

3.2 Development outside of the mouth

F2s brooded by low-DO reared F1 mothers exhibited a divergence in their growth measured 3 months after they were released; juveniles grown under high DO were, on average, ~10% longer than juveniles grown under low DO. These findings are supportive of the predictions for smaller size under low DO and consistent with the positive relationship with embryo size and DO across field populations. Mass did not diverge significantly between juvenile growth environments; but the trend was in the same direction as length. Although we were unable to measure juveniles in a comparable post-release stage in field populations, the trends for egg and embryo size support a smaller juvenile size in hypoxic field populations. Low-DO field populations had smaller eggs and embryos relative to high-DO populations whereas, in the lab rearing experiment, egg and

embryo size was the same between high- and low-DO development environments. Thus, differences in post-release juvenile size in the field may be more extreme than differences reported in this lab rearing study. To explore this possibility, juvenile size will need to be quantified across field populations after growth outside the mouth of the mother. Trade-offs between high- and low-DO environments may contribute to variation in embryo and juvenile size among field populations. Large juveniles from high-DO habitats may suffer respiratory distress if they moved into hypoxic waters, while smaller sized juveniles from low DO may perform sub-optimally if they moved to high-DO waters associated with other ecological factors. For example, there is a growing body of literature suggesting that predation pressure of fish from piscine predators is reduced under low DO (Robb and Abrahams, 2003, Chapman and McKenzie, 2009). For the Lwamunda Swamp population, predation pressure from piscine predators is likely to be very low (Chapman, Chapman and Chandler, 1996a; Chapman et al., 1996b; 2002a). The main predation pressure in this population is aerially from kingfishers (Randle and Chapman, 2004), which do not target the juveniles, thus reducing negative consequences of small size under low DO. *P. multicolor* from high-DO river and lake sites live in sympatry with other cichlids that consume juveniles (Binning and Chapman, 2008), and thus bigger should be better in the high-DO habitats (Reznick, 1991) and smaller better in low-DO habitats (Reardon and Thibert-Plante, 2010). It would be interesting to explore these questions in a high-DO population of *P. multicolor* and in other bearer species of fish to see if the *P. multicolor*'s response to development and growth under low DO is widespread across populations and in other bearer species from a diversity of lineages.

It is important to note that some studies report slower growth and/or smaller size under hypoxia as evidence of negative fitness effects (e.g. Wu, 2009; Mueller et al., 2011) i.e. there is no advantage to being small under hypoxia, rather small size is a limitation of the growth environment. Future studies that quantify metabolic rates of juveniles from both high- and low-DO populations under high and low DO may be useful in informing our understanding of factors driving small size under hypoxia in eggs and embryos. Our findings support the predictions in the hypoxia and development literature, providing evidence for smaller body size across embryo stages under hypoxia in both the laboratory and field. In contrast to the literature, we found no evidence for slower development or lower survival under hypoxia.

Many of the studies reporting reduced fitness under hypoxia were conducted on species with no post-spawning parental care. Yet, a high proportion of parental care species occur in low DO habitats suggesting that parental care may offset negative impacts of hypoxia on their offspring (Chapman and McKenzie, 2009). This work provides insights into how bearer fishes may respond to increases in global hypoxia and suggest that compared to species with no parental care, bearer fishes may be more able to adapt to long-term hypoxia in their environment.

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