The Journal of Experimental Biology 212, 778-784
Published by The Company of Biologists 2009
doi:10.1242/jeb.026575

Hemoglobin enhances oxygen uptake in larval zebrafish (*Danio rerio*) but only under conditions of extreme hypoxia

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Accepted 16 December 2008

SUMMARY

The role of hemoglobin (Hb) in O_2 uptake by zebrafish larvae ranging in age from 5 to 42 days postfertilization was assessed under conditions of normoxia, moderate hypoxia and extreme hypoxia. This was achieved by exposing larvae with and without functional Hb to continuously declining oxygen levels (P_{O_2}) in closed-system respirometers. Exposure to 5% CO for 2–4 h was used to render Hb effectively non-functional in terms of its ability to transport O_2. Routine metabolic rate ($r\dot{M}_{O_2}$), critical dissolved oxygen level (P_c) and residual oxygen level (P_r) were determined and used, respectively, as indicators of response in normoxia, moderate hypoxia and extreme hypoxia. $r\dot{M}_{O_2}$ was defined as the average rate of O_2 uptake before O_2 became limiting (i.e. at high P_{O_2}s). P_c is the P_{O_2} at which $r\dot{M}_{O_2}$ first becomes O_2-limited and P_r is the P_{O_2} below which larvae are no longer able to extract O_2 from the ambient medium. CO poisoning had no significant impact on $r\dot{M}_{O_2}$ or P_c at any age, indicating that the lack of functional Hb does not impair routine O_2 usage in normoxia or at moderate levels of hypoxia [down to at least 25–50 torr (1 torr≈0.133 kPa), depending on age]. P_r, however, was significantly lower overall for control larvae (6.7±1.1 torr; mean ± 95%CI) than for CO-poisoned larvae (11.2±2.1 torr). It would appear that the presence of functional Hb allows zebrafish larvae to extract O_2 from water down to lower P_{O_2}s under conditions of extreme hypoxia. This is the first documented (as opposed to inferred) benefit of Hb in developing zebrafish. However, given the relatively small magnitude of the effect it is unclear if this benefit on its own is sufficient to balance the costs associated with Hb production and maintenance.

Key words: zebrafish, *Danio rerio*, hemoglobin, larva, O_2, aerobic metabolism, cost–benefit analysis.

INTRODUCTION

Hemoglobin (Hb) has a variety of functions in vertebrates including oxygen transport, CO_2 transport, acid–base balance (Brittain, 2002), nitric oxide transport (Allen and Piantadosi, 2006), oxygen storage and buffering (Baumann and Dragon, 2005) as well as a number of indirect functions arising from the metabolic machinery associated with the red blood cell in which the hemoglobin resides (Hume et al., 1996). Of these functions, blood O_2 transport generally is consider the most critical (Burggren, 2004). This perception is based on observations that poisoning of hemoglobin in higher vertebrates (birds and mammals) rapidly leads to death from asphyxiation. In mammals Hb, depending on its concentration, can increase the O_2 carrying capacity of the blood >100-fold compared with what can be transported dissolved in the blood plasma (Burns et al., 2007). Without functional Hb, there is simply not enough O_2 dissolved in the plasma to meet metabolic demand. Adult fishes typically have lower Hb concentrations so the increase in blood transport capacity is not as great as in mammals, but is still usually in the range of 15- to 30-fold (Gallauger and Farrell, 1998). To date, the only adult vertebrates found not to use Hb to supplement plasma O_2 transport are Antarctic fishes of the family Channichthyidae. It is believed that they are able to get by without Hb because of the special circumstances of their habitat and life history, namely the fact that they inhabit very cold water (–1.8°C to +1.5°C) and have a sedentary life style (Sidell and O'Brien, 2006). Cold and a sedentary life styles reduce the metabolic demand for O_2, and cold water (and cold blood plasma) is able to hold more dissolved O_2 than warm water (the dissolved oxygen content of water at 0°C is about 1.9× that at 28°C, the temperature at which the zebrafish used in this study were reared).

Zebrafish, *Danio rerio* is a small, active tropical fish that is widely used as a model of vertebrate development, including the ontogeny of the blood and circulation (Zon, 1995). Zebrafish begin to produce hemoglobin at a very early age. Erythrogenesis is initiated at about 15 h postfertilization (hpf) and red blood cells containing hemoglobin are present in the circulation by 24 hpf, 24–48 h prior to hatching (Brownlie et al., 2003). Given the large metabolic costs associated with hemoglobin production (Paul et al., 2004), one would suspect that the hemoglobin produced by zebrafish embryos was performing some critical function.

This supposition led Pelster and Buggren (Pelster and Buggren, 1996) to examine what effect blocking hemoglobin function would have on zebrafish embryos. Somewhat surprisingly, functional ablation of hemoglobin using either carbon monoxide (which competes with O_2 for the O_2 binding site on Hb) or phenylhydrazine (which blocks the production of erythrocytes and hence Hb synthesis) had no significant effect on heart rate, blood pressure or, most significantly, whole body metabolic rate at any age up to 4.5 days postfertilization (dpf), the last stage tested. Jacob et al. (Jacob et al., 2002) extended these observations to older larvae, up to 15 dpf. They found that functional ablation of hemoglobin had no effect on heart rate, stroke volume and cardiac output or, presumably, on metabolic rate since larvae without functional hemoglobin did not appear to have to resort to increased anaerobic metabolism to make up for any deficiency in aerobic metabolism (i.e. there was no increase in whole body lactic concentration). Both

studies looked at the response to functional ablation of the O_2 carrying capacity of hemoglobin under normoxic condition. Based on these experiments, it would appear that under normoxic conditions direct diffusion of O_2 from the environment combined with any O_2 transported in the blood plasma is sufficient to meet the metabolic demands of zebrafish larvae up to at least 15 dpf. This, however, does not necessarily mean that there is no selective advantage to using hemoglobin to increase the O_2 transport capacity of the blood during embryonic and larval development.

Zebrafish are a tropical flood plain species that lay their eggs on the bottom of shallow ephemeral ponds and in slow moving streams where O_2 levels are likely to be low (Spence et al., 2008). The advantage of Hb in terms of increasing the O_2 transport capacity of the blood is greater at moderate levels of hypoxia than in normoxia. For example, the blood of adult rainbow trout at 20°C transports about 15 times as much O_2 in the form of oxyhemoglobin as is dissolved in the plasma at a partial pressure of oxygen (P_{O_2}) of 150 torr (Burggren et al., 1992). The fractional increase in carrying capacity at 35 torr, however, is >30-fold. It is not until P_{O_2} drops below about 15 torr that the fractional increase in O_2 carrying capacity falls below the 15-fold value seen at 150 torr. It could be that developing zebrafish are able get by without Hb when P_{O_2} levels are relatively high but that when P_{O_2}s are lower, the higher O_2 transport capacity engendered by the presence of Hb becomes a distinct advantage. The aim of this study was to test this hypothesis.

Routine metabolic rate ($r\dot{M}_{O_2}$), critical oxygen level (P_c), total body conductance (G) and residual O_2 levels (P_r) were determined using closed-system respirometry for zebrafish larvae aged between 5 dpf and 42 dpf after exposure to 5% carbon monoxide (CO) for 2–4 h. Values for these indicators were compared with those of control larvae. Acute exposures were chosen to exclude the possibility of developmental adaptations that might mask the direct effects of carbon monoxide on blood O_2 transport. For example, increases in heart size and blood volume have been shown to compensate for a lack of Hb in Antarctic ice-fishes (Sidell and O'Brien, 2006). A minimum of 2 h exposure was deemed sufficient. The hemoglobin of the red-blooded Antarctic fish *Pagoathenia bernacchii* was 100% CO-saturated within 3 min of exposure to 7% CO (DiPrisco et al., 1992). The blood of adult rainbow trout *Oncorhynchus mykiss* exposed to 5% CO was 93–95% CO-saturated when tested after 3 h (Holeton, 1971a). Larvae of rainbow trout displayed increases in both heart rate and ventilation rate within 10–15 min of being exposed to 5% CO (Holeton, 1971b). Routine metabolic rate ($r\dot{M}_{O_2}$) was defined as the average rate of O_2 consumption in the respirometer before O_2 became limiting in closed-system, 'rundown' tests. The critical oxygen level (P_c) is the P_{O_2} at which metabolic rate first becomes O_2 limited on exposure to graded hypoxia. It was assumed that if Hb played a role in O_2 transport, P_c would be lower for larvae with functional Hb than for those in which the O_2 binding capacity of Hb was blocked (i.e. CO-exposed larvae would not be able to satisfy routine O_2 demand down to as low a P_{O_2} as control fish). Total conductance (G) is a measure of the total resistance (R) to O_2 flux from the body surface to the mitochondria ($G=1/R$). It can be calculated from the general transfer equation $\dot{M}_{O_2}=G\Delta P_{O_2}$, where ΔP_{O_2} is the P_{O_2} at the body surface (P_o) minus the P_{O_2} at the level of the mitochondria (P_i) (Dejours, 1981). When $P_o=P_c$, P_i by definition is zero (or very close to it). Total conductance can therefore be estimated as $G=r\dot{M}_{O_2}(P_c)^{-1}$. Total conductance includes that component associated with convective O_2 transport. If Hb facilitates convective O_2 transport, the value of G should be higher for larvae with functional Hb than for those without. When fish are placed in a closed-system respirometer, they gradually deplete the O_2 that was originally in the respirometer. Consumption of O_2, however, does not continue until all the O_2 is consumed. At some point P_{O_2} becomes so low that there is no longer sufficient driving force to move O_2 into the body and O_2 consumption stops even though some O_2 is still present. The P_{O_2} at which this occurs is termed the residual oxygen level, P_r (Grigg, 1969). If Hb facilitates O_2 uptake at very low P_{O_2} values, one would expect residual O_2 levels to be higher for CO-poisoned larvae.

MATERIALS AND METHODS

Animals and rearing conditions

Freshly fertilized eggs were obtained from adult wild-type zebrafish reared under a 14 h:10 h light-dark photoperiod at 28°C in dechlorinated freshwater (total hardness <180 mg l^{-1} as $CaCO_3$). Batches of eggs from the same breeding stock were incubated at 28±0.1°C in aerated 250 ml glass containers for the first 6 days postfertilization (dpf). Larvae hatched between 2 dpf and 3 dpf under these conditions. Swimbladder inflation occurred at about 5 dpf. Larvae were introduced to artificial food (finely ground Tetramin flakes; Tetra Werke, Melle, Germany) at 6 dpf. At 7 dpf, larvae were transferred to well-aerated 5 l glass aquaria containing aged water with an abundance of natural protozoans. Larvae continued to be fed artificial food but were able to supplement their diet with the protozoans during the critical first few days of the transition to exogenous feeding. Larvae were kept in the 5 l aquaria for the remainder of the experiment (up to 42 dpf) during which time they received the bulk of their food in the form of ground Tetramin flakes. Water temperature in the 5 l aquaria was maintained at 28±1.0°C. Larval survival was >90% after transfer to the 5 l containers.

Respirometry

Routine metabolic rates, critical dissolved oxygen levels and residual oxygen tensions were determined at 28±0.1°C for zebrafish larvae at 5, 7, 14, 21, 30 and 40–42 dpf using two closed-cell Stathkelvin RC300 glass respirometers and SI 130 microcathode oxygen electrodes (Stathkelvin Instruments, North Lanarkshire, UK). Groups of larvae were placed in a respirometer cell and the oxygen level allowed to decline naturally as a result of consumption by the fish. Group size ranged from 15 to two individuals depending on age (fewer older individuals) and was chosen so that it took about the same length of time (40–60 min) for the oxygen in the respirometer to become depleted. Test larvae were lightly sedated using 30 mg l^{-1} neutral buffered MS-222 (Sigma-Aldrich, St Louis, MO, USA) to reduce stress and limit activity. Larvae of all ages remained responsive to external stimuli at this level of sedation and were able to swim normally. Preliminary tests with 7 dpf larvae indicated exposure to 30 mg l^{-1} MS-222 resulted in about a 90% reduction in the frequency of spontaneous swimming activity (P.R., unpublished data). Oxygen levels in the respirometer were recorded as a function of time both digitally and using a chart recorder. Respirometers were reopened 3–5 min after O_2 levels in the respirometer ceased falling as a result of larval O_2 uptake. This typically occurred at a P_{O_2} of 5 to 10 torr. Larvae tolerated of this relatively brief period of extreme hypoxia and recovered if placed back in air-saturated water. Normally, however, larvae were removed from the respirometer and placed directly in an air-saturated solution of 100 mg l^{-1} MS-222. Once they were fully anaesthetized, larvae were transferred to 5% Bouin's fixative and kept there for at least 7 days to allow their preserved mass to stabilize. Larvae were

individually weighed to the nearest 10 µg using a semi-micro balance. Preserved samples of the oldest group of larvae tested (42 dpf) were examined under a microscope at 10× magnification and their total body length, maximum body depth and maximum body width determined.

Blank chambers (i.e. no fish) were assayed at the beginning and end of each daily set of experiments to account for bacterial and background oxygen uptake. The chart recordings of dissolved oxygen level as a function time were used to estimate routine metabolic rates, critical oxygen levels and residual O_2 tensions using the graphical technique outlined in Rombough (Rombough, 2007). With this technique the initial approximately linear portion of the curve relating dissolved oxygen and time, excluding the first 5–10 min, is taken as representative of routine metabolic rate. The point at which this linear portion of the curve begins to deviate from a straight line is use to estimate P_c. P_r was taken as the point at which the curve flat-lined near the end of the test. Two regression methods for estimating P_c were tested [asymptotic curve fitting using the SPSS TableCurve 2.0 program and complex linear regression analysis using the program provided by Yeager and Ultsch (Yeager and Ultsch, 1989)] but the graphical method was found to be generally superior.

Experimental procedure

Experimental animals were exposed to 5% CO (balance air) for a minimum of 2 h and a maximum of about 4 h prior to being tested. The requisite number of larvae for the age being tested along with water saturated with 5% CO and containing 30 mg l^{-1} neutral buffered MS-222 were transferred to one of the two respirometers. A simultaneous trial was conducted in the other respirometer using control animals of the same age. Controls were treated identically to experimental animals in terms of the acclimation procedure except for exposure to CO After loading, the respirometers were closed and oxygen levels allowed to run down as described previously. A typical test series consisted of three or four parallel tests of control and CO-exposed fish. Respirometers used for control and experimental animals were alternated to avoid potential confounding affects arising from any slight differences in the two respirometers. Two different batches (eggs from the same stock but fertilized on different days) of larvae were used for all ages except 30 dpf, for which only a single batch was used.

Statistics

Two-way ANOVA (SigmaStat, SPSS) followed by Holm–Sidak pairwise comparisons was used to test for age and treatment effects for $r\dot{M}_{O_2}$, P_c and G. Allometric relationships between metabolic rate and tissue mass were calculated using a log–log regression model. Slope comparisons were conducted using ANCOVA. A sign rank test was used to test for overall differences in P_r. Paired t-tests were used to test for differences in P_r at the various ages. Differences were considered significant if $P<0.05$.

RESULTS

Mean fish mass increased approximately 20-fold, from 160 µg at 7 dpf to 3.5 mg at 40–42 dpf (Fig. 1). Estimated mean total length, maximum body depth and maximum body width at a body mass of 3.5 mg (the mean mass at 40–42 dpf) were 14.1 mm, 1.4 mm and 1.0 mm, respectively.

$r\dot{M}_{O_2}$ increased about 14-fold over the period from 7 dpf to 40–42 dpf, from ≈9 nmol h^{-1} at 7 dpf to ≈125 nmol h^{-1} at 40–42 dpf (Fig. 2A). There were no significant differences between the $r\dot{M}_{O_2}$ of control larvae and larvae exposed to CO overall or at any of the ages tested (Fig. 2A). There, similarly, were no significant differences between control and CO-treated fish in terms of P_c (Fig. 2B) or total conductance (Fig. 2C).

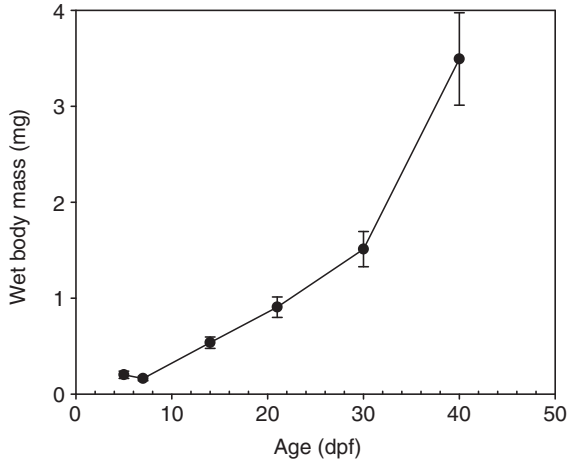

Fig. 1. Changes in body mass with age of the zebrafish larvae used in this experiment. Values are means ± 95% confidence interval (CI). dpf, days postfertilization.

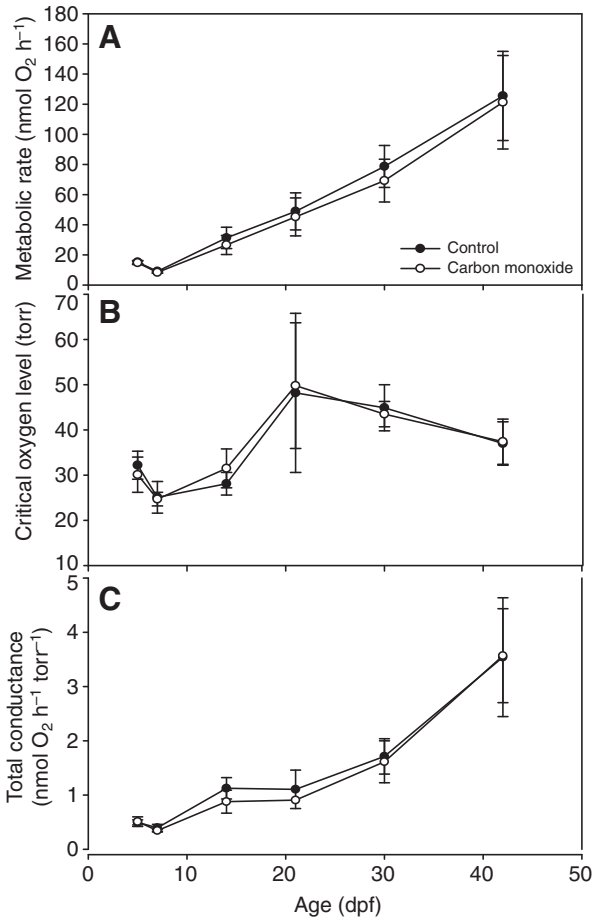

Fig. 2. Effect of CO exposure on the routine metabolic rate (A), critical oxygen level (B) and total conductance (C) of zebrafish larvae. Values are means ± 95% CI.

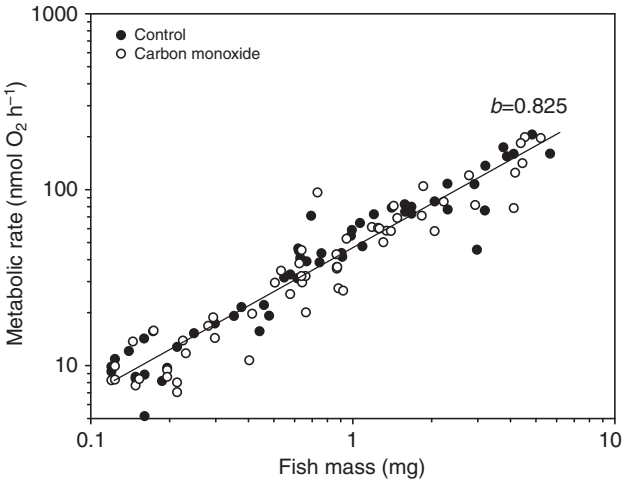

Fig. 3. Allometric scaling for routine metabolic rate of CO-exposed and control zebrafish larvae. *b* is the mass exponent for the line of best fit for all trials combined.

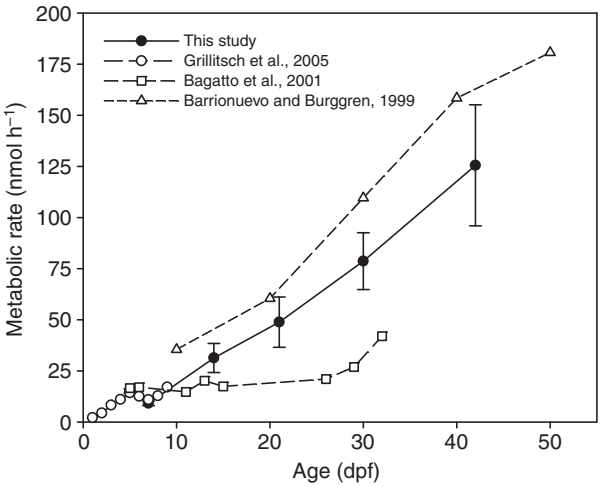

Fig. 5. Comparison of routine metabolic rates (nmol O_2 h^{-1}) for control larvae in this experiment with literature reports of routine metabolic rates for zebrafish larvae.

The allometric relationship between $r\dot{M}_{O_2}$ and fish mass (M; mg) was not significantly different for control and CO-treated fish (Fig. 3). The equation of best fit for the two groups combined (R^2=0.909; N=109) was:

$$r\dot{M}_{O_2} = 46.1M^{0.825} . \quad (1)$$

Residual O_2 levels were significantly higher overall (Sign rank test, P<0.001, N=52) for CO-treated larvae than for control larvae (Fig. 4) Overall mean (±95% CI) values were 11.2±2.0 and 6.7±1.1 torr, respectively. Paired *t*-tests indicated that differences at specific ages were significant only for 7 dpf and 14 dpf larvae (P<0.001 for both).

DISCUSSION

The first task in trying to make sense of these results was to check their reliability by comparing them with what has been reported in the literature. In terms of routine metabolic rate, the results obtained in this study are in line with those reported by other investigators (Fig. 5) suggesting that the basic methodology used in the current study was sound.

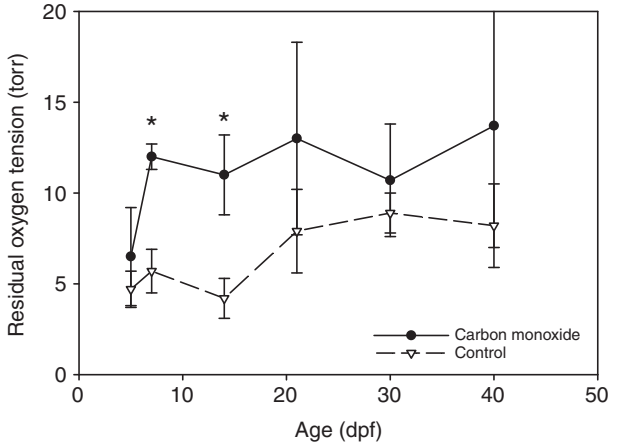

Fig. 4. Residual oxygen tension for zebrafish larvae at various ages. Values are means ± 95% CI. The asterisks indicate a significant difference from same age controls (P<0.05).

The estimates obtained for P_c in this study (range 25–50 torr) are generally lower than those reported for similar age larvae by Barrionuevo and Burggren (Barrionuevo and Burggren, 1999) (range 40–75 torr) and Bagatto et al. (Bagatto et al., 2001) (range 75–50 torr). However, this does not necessarily mean they are unreliable as each of the three studies to date that have looked at how P_c varies with age (this study) (Barrionuevo and Burggren, 1999; Bagatto et al., 2001) have reported very different ontogenetic patterns. Barrionuevo and Burggren (Barrionuevo and Burggren, 1999) indicated that P_c declined at a fairly constant rate with age from hatch to 100 dpf. Bagatto et al. (Bagatto et al., 2001), however, reported a more-or-less continuous increase with age up to 35 dpf, the last age tested. This study reveals a more complex pattern (Fig. 2B). There was a modest decline in P_c between 5 dpf and 7 dpf. This coincides with a similar modest decrease in $r\dot{M}_{O_2}$ over the same period and is probably a reflection of reduced energy availability during the transition from endogenous to exogenous feeding. Once the transition to exogenous feeding was complete, P_c levels began to rise and reached a maximum at around 21 dpf. Beyond 21 dpf, there was a gradual decrease in P_c value with age. It is tempting to link the rise followed by a decline in P_c centered on 21 dpf to changes in the efficiency of branchial gas exchange. Gill lamellae begin to form at about 12–14 dpf in zebrafish (Rombough, 2002) and proliferate rapidly until about 21 dpf to 28 dpf at which time the gill assumes its definitive adult morphology and the rate of lamellar expansion levels off (Tara Klassen, personal communication). Which of the three ontogenetic patterns reported for P_c is 'correct' is difficult to predict at this time. P_c values in adult fish are dependent on metabolic rate (Hoar, 1983). This probably holds true for larvae. Therefore, the different patterns that have been reported could simply be a reflection of variations in activity levels. In this regard, the current study is the only one to have tested sedated larvae which probably explains the generally lower P_c values. One of the major reasons larvae were sedated was to try to standardize activity levels for different aged fish. Hopefully this aim was achieved and the current study has captured the underlying response to ontogenetic changes in

the structure and function of the respiratory system missed by the other two studies.

On reflection, it is not particularly surprising that CO had no significant effect on $r\dot{M}_{O_2}$. Larvae, even at 42 dpf, are still small enough that tissue demand should be able to be met by direct diffusion across body surfaces. Harvey's formula (Dejours, 1981) can be used to calculate the maximum radius of a theoretical spherical animal without circulation that could be supported solely by simple diffusion if it had the same metabolic intensity of a 42 dpf zebrafish (35 nmol O_2 h^{-1} mg^{-1}). Using the value reported by Kranenbarg et al. (Kranenbarg et al., 2003) for Krogh's constant of diffusion for zebrafish embryonic tissue (3.4×10^{-5} nmol O_2 s^{-1} cm^{-1} $torr^{-1}$) and a partial pressure gradient of 150 torr, the maximum distance works out to be about 0.6 mm. The maximum distance O_2 would actually have to diffuse in a 42 dpf larvae is only 0.5 mm (i.e. half the maximum body width of 1.0 mm), so theoretically a spherical larva should be able to fully satisfy its metabolic demand for oxygen in air-saturated water using only simple diffusion. The situation in terms of supplying tissues with oxygen in carbon monoxide poisoned zebrafish larvae is actually considerably more favorable than this simple model suggests. Larvae are not spheres so mean diffusion distances in reality are considerably less than maximum diffusion distances. The model also assumes no internal circulation, which is obviously not the case. Circulation of blood, even without functional Hb, tends to transport O_2 from the periphery of the body towards deeper tissues where it can be used in lieu of O_2 that would otherwise have to reach these tissues by simple diffusion. It was estimated, based on the solubility of O_2 in plasma and the value reported by Jacob et al. (Jacob et al., 2002) for cardiac output, that plasma O_2 could supply between 13 and 21% of metabolic oxygen demand at 15 dpf. This might seem like a relatively small fraction of total O_2 consumption, but it should be remembered that O_2 transported in the plasma would be largely reserved for central tissues since peripheral tissues would continue to rely on direct diffusion (Territo and Burggren, 1998). The net result is that even a relatively simple circulation significantly increases the maximum size a larva can attain before it requires some kind of specific O_2 transporter. Although a number of models have been developed to predict maximum size in the absence of internal circulation (e.g. Territo and Altimiras, 2001; Kranenbarg et al., 2000), no one, to our knowledge, has yet published a predictive model for the maximum size of vertebrate embryos or larvae with an internal circulation but no specific O_2 transporter.

It is more difficult to explain why the lack of functional Hb did not affect P_c. The concentration of O_2 in blood plasma at 28°C would be expected to decline at a rate of about 0.005 vol% $torr^{-1}$ based on Territo and Burggren's (Territo and Burggren, 1998) value for the O_2 content of the plasma of *Xenopus* larvae. At P_{O_2}s corresponding to the range of P_cs observed in this study (25–45 torr), the blood plasma of zebrafish larvae would only hold between 17% and 30% as much O_2 as it would at normoxia (≈150 torr). The amount of O_2 bound to Hb depends on the Hb concentration ([Hb]) which, unfortunately is not known for zebrafish larvae. However, even a relatively low [Hb] would increase the [O_2] in the blood to several times that dissolved in the plasma in normoxia and, because of the shape of the Hb–O_2 dissociation curve, by an even greater relative amount at P_{O_2}s near P_c (see Introduction). One, therefore, would have expected larvae with intact Hb to be able to supply tissues with enough O_2 to meet routine demand down to lower P_{O_2}s than could CO-poisoned larvae forced to rely solely on plasma O_2. There are several possibilities why this did not occur. The most obvious is that the methods used in the current experiment were somehow deficient. For example, the concentration and duration of the CO exposure could have been insufficient to fully block O_2 transport by Hb. However, as pointed out in the Introduction, this appears unlikely based on the literature. Rainbow trout larvae exposed to the same [CO] used in this study responded by increasing heart and ventilation rates within 10–15 min of being exposed (Holeton, 1971b). Even partial blockage of O_2 transport capacity should have resulted in some degree of impairment if Hb played a major role in O_2 delivery. The possibility of methodological error, however, was of enough concern that a follow-up study was initiated to test whether the methods used here could detect an effect of CO-poisoning on P_c in adult zebrafish. That study found that P_c values are indeed significantly higher for CO-poisoned adults than for controls just as one would expect (S. Dorn and P.R., in preparation). This finding strongly suggests that the lack of effect on P_c in the current study is a reflection of differences in larval and adult physiology rather than an artefact of the methodology.

One possible reason why CO-poisoning had no significant effect on P_c could be the high affinity of larval Hb. The O_2 binding affinity of the Hb of zebrafish larvae has not been determined but larval Hbs in general have higher O_2 affinities than adult Hbs (reviewed in Rombough, 1997; Baumann and Dragon, 2005). In some cases the differences can be considerable. For example, the P_{50} of bullfrog (*Rana catesbeiana*) larvae is only 9–10 torr whereas that of adults is about 35 torr (Pinder and Burggren, 1983). P_{50}s for larval and adult Hb are 0.9 torr and 10.3 torr, respectively, for the southern hemisphere lamprey *Geotria australis* (Macey and Potter, 1982). The P_{50} of larval Hb was only 53% of that of adult Hb in rainbow trout (Iuchi, 1973). Larval Hbs also tend to display little or no Bohr effect (Rombough, 1997). The result of a reduced Bohr effect and high affinity means that larval Hb is not able to unload significant amounts of O_2 until P_{O_2} falls to relatively low levels corresponding to the steep part of the O_2–Hb dissociation curve. If the affinity of the Hb of zebrafish larvae is similar to that of larvae of other lower vertebrates, the P_{O_2} at which unloading begins is probably well below the P_c for zebrafish larvae (25–40 torr). This would mean that even though Hb may transport a lot of O_2 at higher ambient P_{O_2}s, that O_2 is effectively unavailable to the tissues, at least at normal levels of physical activity (O_2 might become available during intense exercise but that is a subject for future studies).

The high affinity of larval Hb could also explain why residual oxygen levels (P_r) are lower for control larvae than for CO-exposed larvae (Fig. 4). Grigg (Grigg, 1969) noted that in the adult bullhead, *Ictalurus nebulosus*, arterial P_{O_2} was essentially zero at P_r in all fish but that P_r was lower for fish with higher affinity Hb. Why higher affinity Hb should reduce the partial pressure gradient needed for O_2 uptake across the gill is not clear but a similar phenomenon could be at work in larval zebrafish. If this were the case, CO-poisoned larvae without functional Hb would require a larger partial pressure gradient for net O_2 uptake than would larvae with Hb – the higher the affinity of the Hb, the greater the disadvantage of the CO-poisoned larvae.

One would expect that larvae that produce hemoglobin would have a selective advantage because of the ability to continue to extract O_2 from the environment down to lower ambient P_{O_2} values. Residual O_2 levels averaged about 4.5 torr lower for control than for CO-poisoned zebrafish larvae at 28°C (the temperature of the current study). The difference would probably be somewhat greater at higher temperatures (the upper lethal temperature for zebrafish

embryonic development is about 34°C) because of higher metabolic rates and lower O_2 solubility. However, even at high temperatures a reduction in residual O_2 levels is unlikely to be the whole story behind why zebrafish larvae produce hemoglobin.

Larvae that produce hemoglobin incur costs as well as benefits. Hemoglobin is an energetically expensive molecule to produce. In addition to the direct cost of synthesis, there are costs associated with the metabolic machinery necessary to produce and maintain it (e.g. methemoglobin reductase). There are also costs associated with producing the red cell in which it is packaged. Paul et al. (Paul et al., 2004) have shown that in the small, pelagic crustacean *Daphnia* there is a complex tradeoff between the costs and benefits of producing hemoglobin. *Daphnia* balance costs and benefits by upregulating or downregulating hemoglobin synthesis depending on O_2 availability and predator presence. There is some suggestion that zebrafish larvae might be able to upregulate Hb concentration in response to chronic hypoxia (Schwerte et al., 2005) although this capacity, if it in fact exists, appears to be much less than that exhibited by *Daphnia* (<35% *vs* <1600%). The situation in developing zebrafish is complicated in terms of benefits by the fact that their hemoglobin is probably involved in more physiological functions than just simple O_2 transport. The high O_2 affinity of larval hemoglobin means that, like myoglobin, it probably provides the larvae with some reserve O_2 capacity at very low P_{O_2} values. In addition, hemoglobin has been implicated in nitric oxide transport (Allen and Piantodosi, 2006). The vasculature of zebrafish larvae has been shown to be responsive to nitric oxide (Fritsche et al., 2000) suggesting a possible indirect role for Hb in vascular function. Hb may also play an important role in larval acid–base balance. These other functions need to be taken into account in any overall cost-benefit analysis. It could turn out that the benefits associated with any one function on its own is not sufficient to cover the cost of Hb production. It may be that it is only when several benefits are combined that the balance is tipped in favor of Hb production.

Trade-offs between cost and benefits probably explain why the larvae of some marine fish species do not produce Hb until very late in development. For example, larvae of halibut *Hippoglossus hippoglossus* (Pittman et al., 1990), dolphinfish *Coryphaena hippurus* (Benetti and Martinez, 1993) and spot *Leiostomus xanthurus* (Govoni et al., 2005) do not begin to produce hemoglobin until near metamorphosis. In spot, measurable amounts of Hb are not observed until about 48 days post hatch (Govoni et al., 2005). The larvae of these species are all pelagic and, thus, are unlikely to experience acute episodes of extreme hypoxia (or if they do the magnitude and duration of such events are likely to be so large that Hb would be of little practical use). Pelagic larvae also need to take into account that hemoglobin makes them more visible to predators. Limited benefits and high costs provide a plausible explanation for why larvae of these species delay Hb production. The fact that larvae of some species can thrive without Hb indicates that none of the services provided by Hb during development are essential in an absolute sense. It also reinforces the importance of environmental context when it comes to trying to understand the ontogeny of vertebrate physiological processes.

LIST OF ABBREVIATIONS

G	conductance
P_c	critical oxygen level
P_{O_2}	partial pressure of oxygen
P_r	residual O_2 level
R	resistance
$r\dot{M}_{O_2}$	routine metabolic rate

Funding for this study was provided by the Brandon University Research Fund. Protocols involved in rearing and testing larvae were approved by the Brandon University Animal Care Committee.

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