CARDIO-RESPIRATORY ONTOGENY DURING CHRONIC CARBON MONOXIDE EXPOSURE IN THE CLAWED FROG XENOPUS LAEVIS

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Summary

The present study investigates the ontogeny of cardiorespiratory physiology in Xenopus laevis where O₂ transport is obstructed. Animals were raised from eggs (NF stage 1) to metamorphic climax (NF stage 63), while maintained either in air or in chronic 2 kPa CO, which functionally abolates O₂ transport by hemoglobin (Hb). Whole-animal rate of oxygen consumption (ṀO₂), whole-body lactate concentration, individual mass, heart rate (fH) and stroke volume (VS) were measured. Additionally, cardiac output (Qt) and the ratio of the rate of oxygen consumption to the total rate at which oxygen is transported in the blood (ṀO₂/Qt) were calculated to determine limitations imparted when O₂ transport is impaired.

Our data on early development suggest that the onset of convective blood flow occurs prior to the absolute need for convection to supplement diffusive transport. Values for ṀO₂, whole-body lactate concentration, mass and fH did not differ significantly between controls and CO-exposed animals. However, CO-exposed animals showed a significant (P<0.05) increase in VS, ṀO₂/Qt, and Qt compared with controls. These results indicate that limiting blood O₂ transport is not deleterious to metabolism and development as a whole and that convective oxygen transport via Hb is not essential for normal cardiovascular or respiratory function during larval development.

Key words: ontogeny, development, Xenopus laevis, carbon monoxide, oxygen consumption, lactate, heart rate, stroke volume, cardiac output, O₂ consumption/transport quotient.

Introduction

The heart is the first organ to function in the vertebrate embryo (Burrgren and Keller, 1997; Gilbert, 1990). Concomitant with heart development is the formation of blood elements and hemoglobin (Hb). The early convection of these newly formed elements has led to speculation on their importance to gas exchange (Adolph, 1979; Boell et al., 1963; Burrgren and Just, 1992; Burrgren and Pinder, 1991; Burrgren and Territo, 1995). The assumption that blood convection is critical to gas exchange has gained circumstantial support from morphological observations that gill differentiation, and blood flow in both the gills and the caudal arteries, occurs immediately after the heart begins to beat (Ballard, 1968; Medvedev, 1937; Nieuwkoop and Faber, 1967; Taylor and Kollros, 1946). Furthermore, it has been suggested that these changes in circulation may contribute to the sharp initial rise in ṀO₂ during early development (Romanoff, 1960).

The synchronous appearance of blood convection and the need for convection to supplant diffusive O₂ delivery between the environment and the tissues has been termed 'synchronotropy' (Burrgren and Territo, 1995). Although the link between Hb convection and early embryonic/larval O₂ consumption has become dogma, there exist no data that have explicitly tested the hypothesis of synchronotropy. However, we have put forward an opposing hypothesis that specifically questions these assumptions of synchronotropy (Burrgren and Territo, 1995). This alternative hypothesis, termed 'prosynchronotropy', argues that the cardiovascular system begins to generate convective blood flow well before there is an absolute need for internal convection of oxygenated blood. Early results have begun to clarify our understanding of when convective O₂ transport becomes necessary in embryos and larvae (Mellish et al., 1998; Pelster and Burrgren, 1996), but these studies do not include complete metabolic and cardiovascular profiles throughout development and the interplay that occurs between these two systems.

The purpose of this study was to evaluate the validity of 'prosynchronotropy' utilizing embryos of the clawed frog Xenopus laevis. This particular species is well suited to test this hypothesis because it has a well-studied developmental sequence, lays large numbers of eggs in a laboratory setting, has embryos that are free-living and transparent and because considerable descriptive physiological and morphological
information exists that can serve as a basis for formulating more detailed mechanistic questions. Our approach has been to elucidate the dependence of the rate of O₂ uptake on convective blood flow in larval Xenopus laevis. First, we have evaluated the relative contributions of aerobic and anaerobic metabolism. Second, we have measured cardiac performance (heart rate, stroke volume and cardiac output) to determine the role of blood flow in supporting aerobic metabolism. Third, we have determined the relative coupling of cardiac function to oxygen uptake. This was achieved in larvae by functionally ablating Hb through chronic exposure to 2 kPa carbon monoxide (CO). Disruption of Hb O₂-binding with CO has been used by Holeton (1971b) and Pelster and Burggren (1996) in larval fish and in embryonic chickens (Cirootto and Arangi, 1989). By rearing larvae in the presence of CO, we can determine what role the cardiovascular system plays in oxygen uptake and its distribution. Furthermore, we can assess the inevitable and changing role of Hb in bulk O₂ transport as development progresses. These studies enable us to determine whether the cardiovascular system forms prior to the requirement for convective O₂ transport.

Materials and methods

Experimental animals

Fertilized eggs were obtained in our laboratory from the breeding of four adult pairs of Xenopus laevis according to Thompson and Franks (1978). Equal numbers of newly laid eggs were placed into two 251 holding tanks, where they were maintained in dechlorinated water at 24±0.2°C.

Experimental conditions

Holding tanks for rearing larvae were aerated with one of two gas mixtures: 21 kPa O₂/79 kPa N₂ (control) or 2 kPa CO/21 kPa O₂/77 kPa N₂. Carbon monoxide (CO) gas mixtures were generated with a Cameron GF-4 gas-mixing flowmeter. Larvae in these tanks were fed Nasco frog brittle (Nasco Inc.) ad libitum during the course of development and fasted for 24 h prior to measurements. All animals were maintained on a 14 h:10 h light:dark cycle throughout development.

Grouping of developmental stages

Animals were staged according to the NF staging system (Nieuwkoop and Faber, 1967). Experiments were conducted on X. laevis ranging from recently laid eggs (NF 1) to metamorphic climax (NF 63). Animals were grouped into 11 different developmental categories according to major morphological and physiological landmarks. Developmental groupings were as follows: NF 1–21, 22–30, 31–33/34, 35–36, 37–41, 42–45, 46–47, 48–49, 50–51, 52–54 and 55–63. For readers unfamiliar with characteristics at these developmental stages, see Nieuwkoop and Faber (1967) and Burggren and Just (1992).

Experimental procedures

Animals drawn at random from each experimental condition were sampled for rate of oxygen consumption (M₀₂), whole-body lactate concentration, wet mass, heart rate, total ventricular volume at end diastole and end systole, and stroke volume. Cardiac output and the ratio of the rate of O₂ consumption to O₂ transport by the blood (see below for calculations of M₀₂/O₂) were also calculated. An additional group of recently metamorphosed juveniles was sampled for whole-blood O₂-carrying capacity and for plasma O₂ content during CO exposure.

Total rate of oxygen consumption (M₀₂)

Aerial and aquatic O₂ consumption in larval Xenopus laevis were measured by modified closed-system respirometry, as described previously (Hastings and Burggren, 1995). Briefly, the respirometer consisted of a 20 ml vial filled with dechlorinated tap water and sealed with an aluminum cap and double Viton O-rings. Air (5 ml) was introduced into the vial, displacing water. Each respirometer was attached to a reservoir and pumping apparatus and allowed to equilibrate with the gas mixture for 2 h prior to closure of the system (Fig. 1A). During this period, the chambers were continually bubbled with the gas mixture corresponding to that used during rearing. All

![Diagram](https://via.placeholder.com/150)

Fig. 1. A schematic representation of the respirometer (A) and oxygen analyzer with modified bypass tube (B) used to measure M₀₂ in aquatic Xenopus laevis (for a detailed description, refer to Materials and methods).
experiments were carried out with additional blank respirometers (minus animals) to account for any bacterial O₂ consumption in the respirometers. The total gas volume of the system was 7 ml. Each respirometer was thermostatted at 24°C. All chambers were placed in a dark enclosure to minimize visual disturbances to the animals. The length of the measurement period varied depending on the NF stage and on the number of animals required to achieve an approximately 3 kPa drop in O₂. In most cases, animals were placed into respirometers at approximately 10:00h (PST) and were sampled for approximately 24 h. Upon closure of the system, and at the conclusion of the experimental period, a 1 ml sample of gas was analyzed for O₂ with an Ametek S3A/I oxygen analyzer. Gas samples were injected into a three-way stopcock fitted on the analyzer inlet, which was also fitted with a 1 m length of polyethylene tubing (PE-90, Beckton-Dickinson Inc.). The injected gas sample was first diverted into the PE tubing and then drawn at atmospheric pressure back into the Ametek pump through a micro Drierite column at a rate of 20 ml min⁻¹ (Fig. 1B). Results were compared with a standard curve (data not shown), which was established for known O₂ content at a given flow rate. Here, mass-specific \( M_{O2} \) has the units of nmol O₂ g⁻¹ h⁻¹ and was calculated as follows:

\[
M_{O2} = \frac{\%O_{2,1} - \%O_{2,2}}{\Delta P - P_{H2O}}B_bV_g, \tag{1}
\]

where \( \%O_{2,1} \) and \( \%O_{2,2} \) are the initial and final percentages of O₂ in the respirometer, as read by the Ametek oxygen analyzer, \( P_b \) is the barometric pressure in kPa, \( P_{H2O} \) is the water vapor pressure at a given temperature, \( B_b \) is the capacitance of O₂ in air in nmol L⁻¹ kPa⁻¹, \( V_g \) is the volume of gas in the manometric chamber in liters, \( M_a \) is the animal wet mass in milligrams and \( t \) is the time in hours.

**Whole-body lactate analysis**

Whole-body lactate concentration was determined on each animal sampled for \( M_{O2} \). Upon completion of the experiment, larvae were removed from their respirometers and promptly (<2 min) flash-frozen in liquid nitrogen. The frozen larvae were then transferred to a storage vial and kept at -70°C. All samples were assayed for lactate within 2 months of cold storage. Animals were ground in glass tissue grinders with a ratio of 5 ml of 8% (v/v) perchloric acid to 1 g animal wet mass. Assays for whole-body lactate concentration were performed using kit number 826-B (Sigma Chemical Co., USA) and were read on a Sequoia-Turner Spectrophotometer at 340 nm.

**Mass determination**

Individual wet mass for \( M_{O2} \) and whole-body lactate calculations was determined by first wick-drying animals with a Kimwippe and then weighing them to the nearest 0.1 mg on a Denver Instrument analytical micro balance, model AB-300. In most cases, several animals were pooled for \( M_{O2} \) and lactate analysis. The mass of an individual mass was determined by averaging over the total mass.

**Heart rate (fH), stroke volumes (VS) and cardiac output (Q)**

Animals were sampled randomly at each developmental stage. Each animal was video-taped for 30 s (120 frames s⁻¹) using a Zeiss M3Z microscope fitted with a video camera. Output from the camera was recorded on a Panasonic sVHS video tape recorder and stored for later analysis (for a review, see Burggren and Friswold, 1995). A value for fH was obtained from the number of beats in a given time, and VS values were determined from the difference between total ventricular volumes at end diastole and end systole and were calculated using Optimas software (BioScan Inc.). These volumes were estimated by calculating the rotational volumes (V) of a delimited planar area, which is based on a modified formula (Burggren and Friswold, 1995; Hou and Burggren, 1995a) for a prolate spheroid (v = \( \pi^{-1.5} A^{1.5} \)), where \( A \) is the area of the delineated heart. Individual cardiac output was determined by the product of fH and VS.

**O₂ consumption/transport quotient**

An index of the relationship between aerobic metabolism (\( M_{O2} \)) and blood \( O_2 \) transport (\( \dot{Q}_{O2} \)), a unitless term we call the \( O_2 \) consumption/transport quotient, was calculated as follows:

\[
\frac{M_{O2}}{\dot{Q}_{O2}} = \frac{M_{O2}}{Q \times C_{O2}}, \tag{2}
\]

where \( M_{O2} \) is the rate of oxygen consumption in nmol O₂ g⁻¹ h⁻¹, \( C_{O2} \) is the content of blood at complete saturation (\( P_{O2} = 21 \) kPa) expressed in nmol O₂ µl⁻¹ blood (see Hematology) and \( \dot{Q} \) is total cardiac output in µl blood g⁻¹ h⁻¹.

These estimations of oxygen flow are highly conservative on the basis of the following arguments. (1) 100% oxygen extraction by the tissues is assumed. An arterio-venous (A–V) difference of 33% has been reported by Hillman (1978) for adult Xenopus laevis; however, to our knowledge there are no data about oxygen extraction or arterio-venous differences available for embryos of any amphibian species. (2) Blood oxygen content as calculated from mature Rana catesbeiana larvae (1.7 pmol O₂ µl⁻¹ blood; Pinder and Burggren, 1983) is expected to be larger than the oxygen content of embryonic blood. Again, to our knowledge, there are no data available on oxygen content or blood hemoglobin concentration in early embryos. However, the hemoglobin (Hb) concentration in the blood of the chicken embryo is known to rise exponentially with development (Romanoff, 1967). (3) Direct measurements of \( P_{O2} \) in the vitelline vein (3.4 kPa) in newly hatched trout larvae (Rombough, 1992) suggest that embryonic blood might not be fully oxygen-saturated. This decreased saturation could be partly compensated for by the low \( P_{Sat} \) of embryonic blood. In addition, \( P_{Sat} \) is known to increase with development (Pinder and Burggren, 1983) and the degree of amniotic teratostyly (Boutilier et al. 1992); therefore, it is likely that embryonic Hb will have a higher affinity for O₂, although a lower O₂ content, than post-metamorphic juvenile blood.

In combination, these factors would give lower values for the defined quotient, thus overestimating the contribution of
convective oxygen transport, and this would make our arguments on the relevance of diffusion highly conservative.

Hematology

Ventricular blood was obtained from anesthetized juveniles using methods described by Burggren et al. (1987). Total sampling time was less than 3 min. Samples were taken from 31 animals ranging from 5 to 30 g in mass and from 3 to 11 months in age. Each animal yielded 400–700 µl of whole blood, which was immediately heparinized (1000 i.u. ml⁻¹). Whole-blood oxygen content was determined according to the methods described in Tucker (1967). Plasma oxygen content (CPO₂) was determined in 15 of the 31 animals sampled and was obtained by centrifugation of whole blood at 10,000 revs min⁻¹ (6700g) for 15 min. Plasma samples were bubbled with air (P₀₂=21 kPa) and the subsequent oxygen content (nmol O₂ 100 ml⁻¹ blood) was determined as follows:

\[
CPO₂ = \left( \frac{P₀₂}{P₀} \right) \beta_p.
\]

(3)

where \( P₀ \) is the barometric pressure in kPa, \( P₀₂ \) is the partial pressure of oxygen for a given temperature and \( \beta_p \) is the capacitance of O₂ in plasma in nmol L⁻¹ kPa⁻¹.

The Haldane affinity coefficient (H; Haldane and Smith, 1897) was calculated to determine the affinity of Xenopus laevis blood for O₂ relative to O₂ using the following formula:

\[
H = \left[ \frac{[CO]HbP₀₂}{[O₂]HbPCO} \right].
\]

(4)

where \([CO]Hb\) is the concentration of CO bound to Hb (µmol CO 100 µl⁻¹ blood), \( P₀₂ \) is the partial pressure of O₂ in the blood (kPa), \([O₂]Hb\) is the concentration of O₂ bound to Hb (µmol O₂ 100 µl⁻¹ blood) and \( PCO \) is the partial pressure of CO in the blood (kPa).

Hemoglobin (Hb) concentration was measured on 15µl blood samples using a spectrophotometric hemocytometer (model OSM 2, Radiometer). Although this hemocytometer used wavelengths appropriate for human Hb, Wood (1971) has demonstrated that amphibian Hb can be assayed accurately with this instrument. Hematocrit (Hct) was determined on 15µl blood samples centrifuged at 10,000 revs min⁻¹ (6700g) for 4 min.

Oxygen and carbon monoxide equilibration was achieved by bubbling gas into a 1.5 ml centrifuge tube containing 100 µl of whole blood and spinning the tube gently on low speed in a mixer for 3 min periods for a total of 20 min at 25°C. All whole-blood samples were exposed in sequence to 0, 0.5, 1.0, 1.5 and 2 kPa (approximately 2%) CO, balance air. Plasma samples were exposed to air only. In all cases, juvenile blood and plasma O₂ content were used for calculations of \( QO₂ \).

Statistical analyses

The effects of experimental treatment upon \( M₀₂ \) and [lactate] were compared for each variable’s covariance with wet mass. To meet the assumptions of the multiple analysis of covariance (MANCOVA), all variables were log₁₀-transformed. MANCOVA (1993 BMDP-VAX-mainframe) determines (1) the correlation coefficient, (2) the equation of the line describing the relationship and (3) the probability that the slope of the line is not significantly different from zero. Heart rate, \( V_s \), \( Q \) and \( M₀₂/Q₀₂ \) were all tested for significant differences with development and between treatments utilizing a multiple analysis of variance (MANOVA) (Brown–Forsyth procedure, BMDP). If significant differences occurred, a Tukey’s (HSD) multiple-range test was used to determine which points were different within, and between, the experimental groups. All values are reported as mean ± 1 standard error of the mean (S.E.M.). The fiduciary level of significance was taken at \( P<0.05 \).

Oxygen content data were analyzed by a first-order least-squares linear regression (Statistica v5.0 on a PC) against Hb concentration. This analysis determines (1) the regression coefficient, (2) the equation of the line describing the relationship and (3) the probability that the slope of the line is not significantly different from zero. In addition, a one-sample \( t \)-test was run to determine whether the slope of the experimental line was significantly different from the normoxic equilibrated line. In all cases, the values are means ± 1 S.E.M.

Results

Wet mass

Wet mass increased significantly (\( P<0.05 \)) in an exponential fashion with progressive development in both normoxic and CO-exposed animals. All stages except NF stages 1–21 and 35–36 in normoxic and NF 1–21 and 22–30 in CO-exposed animals increased incrementally in wet mass with development (Table 1). Interestingly, there was no significant (\( P=0.10 \)) difference between normoxic and CO-treated animals at any stage.

Hematology

In the absence of CO (\( P₀₂=21 \) kPa), the blood of Xenopus laevis showed a significant relationship between Hb concentration and oxygen content (\( r^2=0.94, P<0.05 \), Fig. 2). Details of the regression analysis are given in Table 2. Addition of 0.5 kPa CO reduced whole-blood (Hct 34±2 %) oxygen content to the same level as that of plasma with an average value of 0.8±0.14 µmol O₂ 100 µl⁻¹ blood. Higher levels of CO (1.0, 1.5 and 2.0 kPa) also reduced the whole-blood O₂ content to that of the plasma. There were no significant differences between blood O₂ contents at the various CO levels. Calculation of the Haldane affinity coefficient revealed that whole blood from recently metamorphosed Xenopus laevis froglets had an affinity 168 times greater for CO than for O₂.

Developmental changes in whole-animal oxygen consumption (\( M₀₂ \))

In both normoxic and CO-exposed animals, \( M₀₂ \) covaried with wet mass (normoxia, \( r^2=0.85, P<0.05 \); CO-exposed,
Fig. 2. Blood oxygen content during exposure to carbon monoxide in *Xenopus laevis*. The oxygen content of whole blood is plotted against blood hemoglobin content. Values for exposure to normoxic air (filled symbols) were fitted with a first-order linear regression and the results are presented in Table 2. Values for exposure to increasing 
$P_{CO}$ (open symbols) are plotted against their respective hemoglobin concentration.

$r^2=0.79, P=0.05$ and developmental stage (normoxia, $r^2=0.67, P=0.05$; CO-exposed, $r^2=0.61, P=0.05$). However, $M_{O_2}$ of eggs (NF 1–13) and very young embryos (NF 14–30) showed a non-linear relationship with wet mass. During these early stages, $M_{O_2}$ increased 50-fold with just a 1.5-fold increase in mass from 4.4 to 6.8 mg (Fig. 3, inset). Conversely, larvae from NF 31 to NF 63 showed clear allometric relationships for both groups described by the equations $M_{O_2}=2.59M^{0.71}$ for normoxia and $M_{O_2}=3.17M^{0.61}$ for CO-exposed animals. The increase in $M_{O_2}$ over development in CO-exposed animals was not significantly different from that in normoxic animals (Fig. 3).

**Whole-body lactate concentration**

Whole-body lactate concentration and wet mass were highly correlated in normoxic larvae ($r^2=0.81, P=0.05$), but only moderately correlated in CO-exposed animals ($r^2=0.57, P=0.05$; Table 2). Despite the correlations between lactate concentrations and wet mass for both groups, [lactate] increased episodically with respect to development for both normoxic and CO-exposed animals (Table 1). Whole-body lactate concentration was variable through development, but *Xenopus laevis* larvae showed a trend towards an increase in lactate concentration as animals approached metamorphosis. Further analysis of normoxic data on a stage-by-stage basis revealed that, in all cases except NF 1–21 and 48–49, whole-body [lactate] was significantly different from that for the preceding stage. Similarly, stage-specific responses for CO-exposed larvae indicated that, between NF stages 37 and 54, all groups were different from the preceding stage range (Table 1). Whole-body [lactate] in CO-exposed animals was, overall, not significantly different ($P=0.09$) from that for control (normoxic) animals.

**Heart rate**

Heart rate during development followed a curvilinear relationship for both control and CO-exposed larvae. This is illustrated by the progressive rise from the initial rates of $59+2$ beats min$^{-1}$ for control and $85+5$ beats min$^{-1}$ for CO-exposed animals at NF 31–33/34 to a maximum of $183+5$ beats min$^{-1}$ for control and $167+1$ beats min$^{-1}$ for CO-exposed animals at NF 48–49. Rates obtained between NF stage 52 and 54 declined steadily in both populations to final values of $88+12$ beats min$^{-1}$ (normoxia) and $92+11$ beats min$^{-1}$ (CO-exposed). Overall, heart rates for normoxic larvae were significantly different ($P=0.05$) with progressive development in all stages ranges except NF 37–41 and 50–51 (Table 3). As

<table>
<thead>
<tr>
<th>Developmental stage range</th>
<th>Normoxia [Lactate] (μmol g$^{-1}$)</th>
<th>Wet mass (mg)</th>
<th>CO-treated [Lactate] (μmol g$^{-1}$)</th>
<th>Wet mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–21</td>
<td>1.2±0.2</td>
<td>5.0±0.3</td>
<td>1.2±0.4</td>
<td>5.1±0.2</td>
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<tr>
<td>22–30</td>
<td>0.2±0.1†</td>
<td>4.6±0.2†</td>
<td>1.1±0.3</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>31–34</td>
<td>1.0±0.1†</td>
<td>2.4±0.2†</td>
<td>1.2±0.5</td>
<td>2.2±0.1†</td>
</tr>
<tr>
<td>35/36</td>
<td>0.8±0.2†</td>
<td>2.6±0.3</td>
<td>0.7±0.1</td>
<td>2.8±0.2†</td>
</tr>
<tr>
<td>37–41</td>
<td>2.2±0.7†</td>
<td>3.3±0.3†</td>
<td>1.1±0.2†</td>
<td>3.4±0.3†</td>
</tr>
<tr>
<td>42–45</td>
<td>4.5±0.4†</td>
<td>5.1±0.3†</td>
<td>0.7±0.3†</td>
<td>6.7±0.9†</td>
</tr>
<tr>
<td>46–47</td>
<td>0.4±0.2†</td>
<td>8.5±0.1†</td>
<td>0.2±0.2†</td>
<td>8.3±0.4†</td>
</tr>
<tr>
<td>48–49</td>
<td>0.2±0.2</td>
<td>34.0±2.4†</td>
<td>0.8±0.3†</td>
<td>32.8±4.3†</td>
</tr>
<tr>
<td>50–51</td>
<td>3.6±1.0†</td>
<td>71.2±10.6†</td>
<td>6.2±1.7†</td>
<td>123.8±13.4†</td>
</tr>
<tr>
<td>52–54</td>
<td>5.5±1.2†</td>
<td>190.5±25.1†</td>
<td>12.1±2.6†</td>
<td>224.7±38.3†</td>
</tr>
<tr>
<td>55–63</td>
<td>8.2±1.9†</td>
<td>685.7±107.8†</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m., $N=6$ at each stage.

†Significantly different from the value for the preceding stage ($P=0.05$).
Normoxic stroke volumes ranged from a minimum of 1.1±0.2 nl at NF stage 31–33/4 to 554.9±149.7 nl at NF stage 52–54. Similarly, V_s in CO-exposed larvae increased by the same amount between NF 31 and 54. Significant stage-specific differences occurred both during the course of development and as a result of treatment in both control and CO-exposed larvae (refer to Table 3). Although exposure to CO had a significant effect on V_s overall, interestingly, only stages 35/36, 46–47 and 50–51 showed a significant effect of treatment.

**Cardiac output**

Cardiac output displayed a significant (P ≤ 0.05) exponential rise with progressive development in both CO-exposed and normoxic larvae. This stage-dependent increase in Q showed a near doubling in flow at each successive stage over NF stages 33–41 in both populations of larvae (Fig. 4). Cardiac output between NF stages 37 and 45, however, increased sixfold (from 219.3±3.0 to 1326.9±3.6 nl min⁻¹) in normoxic animals, while in CO-exposed animals it only increased 4.5-fold (from 310.6±1.3 to 1361.9±4.9 nl min⁻¹) over the same stage range. Curiously, Q was not maintained between NF stages 47 and 49, decreasing 1.5-fold for control larvae and 1.2-fold for CO-exposed larvae. Total cardiac output, however, increased by nearly an order of magnitude for both populations of animals over the subsequent staging intervals NF 50–54. In all cases, each stage was significantly different from the preceding stage and, overall, CO exposure had a significant effect (P ≤ 0.05) on Q (Fig. 4).

**O₂ consumption/transport quotient**

The M_o₂/Q_o₂ ratio is a quantitative measure of the relative contributions of perfusive conductance to total O₂ consumption. This is achieved by expressing both M_o₂ and Q_o₂ in nmol O₂ g⁻¹ h⁻¹, thereby making this ratio dimensionless. Although similar relationships exist (Piiper and Scheid, 1975), our ratio allows a non-invasive evaluation of the potential cardiovascular contribution to total oxygen consumption and can be measured in the context of development, where the

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**Table 2. Regression analysis of whole-blood oxygen content, rate of oxygen consumption and lactate concentration in developing Xenopus laevis**

<table>
<thead>
<tr>
<th>y</th>
<th>Condition</th>
<th>N</th>
<th>a</th>
<th>b</th>
<th>r²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_o₂</td>
<td>Normoxia</td>
<td>31</td>
<td>0.6897</td>
<td>0.9861</td>
<td>0.94</td>
<td>≤0.05</td>
</tr>
<tr>
<td>M_o₂</td>
<td>Normoxia</td>
<td>61</td>
<td>2.585</td>
<td>0.7064</td>
<td>0.85</td>
<td>≤0.05</td>
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<tr>
<td>M_o₂</td>
<td>CO</td>
<td>59</td>
<td>3.173</td>
<td>0.6091</td>
<td>0.79</td>
<td>≤0.05</td>
</tr>
<tr>
<td>[Lactate]</td>
<td>Normoxia</td>
<td>55</td>
<td>0.0034</td>
<td>1.0590</td>
<td>0.81</td>
<td>≤0.05</td>
</tr>
<tr>
<td>[Lactate]</td>
<td>CO</td>
<td>55</td>
<td>0.0032</td>
<td>0.9902</td>
<td>0.57</td>
<td>≤0.05</td>
</tr>
</tbody>
</table>

Relationships for M_o₂ and whole-body [lactate] are expressed as log(y)=log₁₀a+blog₁₀M, where y is M_o₂ (nmol h⁻¹) or whole-body [lactate] (µmol g⁻¹) and M is wet mass.

Whole-blood O₂-carrying capacity is a linear function of blood O₂ content and [Hb] and is described by the equation y=α+bx; where α is the plasma O₂ content (C_o₂) and b is the slope associated with HbO₂-loading.

N, r² and P are sample size, regression coefficient and fiduciary level of significance, respectively; Hb, hemoglobin.
ability to instrument animals may be limiting. A value of 1 indicates absolute coupling between the respiratory and cardiovascular system, provided that all of the O₂ consumed is being transported in the blood. A value greater than 1 indicates that the rate of O₂ consumption is not matched by the amount of O₂ transported in the blood in a given unit of time. Lastly, values below 1 indicate that the potential for O₂ transport in the blood is greater than the rate of O₂ consumption.

The O₂ consumption/transport quotient in both normoxic and CO-exposed animals showed a significant (P<0.05) decline with progressive development, although the magnitude of the decline in CO-exposed animals between NF stages 33 and 47 was greater (1.76) than the value of 0.36 seen in normoxic animals. This reduction in M₀/Q₀₂ ratio indicates that, by stage NF 52–54, Q₀₂ exceeds M₀ by 127-fold in normoxic animals and ninefold in CO-exposed animals (calculated from Table 4). All normoxic stages from NF 33 to 54 had M₀/Q₀₂ ratios below the theoretical coupling threshold of 1, while CO-exposed larvae between NF stages 42–47 and 50–54 were the only stages to show values below 1. Normoxic animals between NF 37 and 54 had M₀/Q₀₂ values that were significantly different (P<0.05) at each stage (Table 4; Fig. 5). In contrast, only CO-exposed larvae at NF 37–41, 42–45 and 48–54 were significantly different from the preceding stage. Treatment with CO significantly (P<0.05) affected the M₀/Q₀₂ ratio (Table 4; Fig. 5).

**Discussion**

**Critique of method**

A potential criticism of this study could be the use of CO to eliminate the oxygen-carrying capacity of Hb and thus eliminate its contribution to total O₂ transport. However, it is clear from the O₂ content data that the addition of low levels of CO seriously impairs the O₂-carrying capacity of the blood. Circulating carboxyhemoglobin provides no O₂ transport and, therefore, animals exposed to CO are more reliant upon direct diffusion to tissues and upon O₂ physically dissolved in plasma.

Another potential criticism may be that our study used the blood of juvenile Xenopus laevis to determine the effect of CO on the O₂-carrying capacity of blood. It is clear from work in amphibians that P₅₀ increases with ontogeny and the degree of terrestriality (Boutinier et al. 1992; Pinder and Burggren, 1983). In addition, Xenopus laevis blood has a higher affinity for O₂ than that of terrestrial amphibians. It is therefore unlikely that embryonic or larval stages of Xenopus laevis will have lower blood affinities than post-metamorphic animals. In addition, since juvenile blood is likely to have a higher Hct than larval blood, its use in calculations during development would affect the results.

![Fig. 4. The relationship between blood flow and developmental stage for normoxic animals (filled symbols) and chronically CO-exposed animals (open symbols). Cardiac output \( \dot{Q} \) is plotted on a logarithmic scale against the linear dimension of development. Values are shown as means ± s.e.m., N=6 at each stage. In some cases, the error bars are smaller than the symbols. A dagger indicates a significant (P<0.05) difference from the previous stage, and an asterisk indicates a significant difference from the value for controls (normoxia).](image-url)
Table 4. The ontogeny of O₂ consumption/transport coordination with normoxia and CO exposure

<table>
<thead>
<tr>
<th>Stage range</th>
<th>Normoxia ( \frac{M_{O_2}}{Q_{O_2}} )</th>
<th>CO-treated ( \frac{M_{O_2}}{Q_{O_2}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–21</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>22–30</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>33/34</td>
<td>0.38±0.01</td>
<td>2.29±0.04*</td>
</tr>
<tr>
<td>35/36</td>
<td>0.41±0.12</td>
<td>2.98±0.19*</td>
</tr>
<tr>
<td>37–42</td>
<td>0.16±0.05†</td>
<td>1.55±0.58†,*</td>
</tr>
<tr>
<td>43–45</td>
<td>0.05±0.01†</td>
<td>0.72±0.02†,*</td>
</tr>
<tr>
<td>46–47</td>
<td>0.02±0.01†</td>
<td>0.53±0.10*</td>
</tr>
<tr>
<td>48–49</td>
<td>0.09±0.01†</td>
<td>1.99±0.29†,*</td>
</tr>
<tr>
<td>50–51</td>
<td>0.05±0.01†</td>
<td>0.45±0.03†,*</td>
</tr>
<tr>
<td>52–54</td>
<td>0.01±0.01†</td>
<td>0.11±0.01†,*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.; \( N=6 \) at each stage.

*Significantly different from the control value \( (P<0.05) \); †significantly different from the value for preceding stage \( (P<0.05) \).

would tend to lead to an over-estimate rather than an under-estimate of the O₂-carrying capacity.

An additional concern is the possibility of CO toxicity because of the interactions between CO and hemoproteins other than Hb. A \( P_{CO}/P_{O_2} \) ratio of 5:1 is required to inhibit oxidative metabolism by 50% in mammals (Ewer, 1942). The present study used a \( P_{CO}/P_{O_2} \) ratio of 0.1:1, which is far below the level affecting cytochrome oxidase reducing potentials. In addition, CO has been shown to be specific to Hb and does not interfere with carbonic anhydrase activity, which is important in \( CO_2 \) transport and buffering (Ewer, 1942). These facts indicate the suitability of CO to evaluate the role of convective \( O_2 \) transport, in general, and the role of Hb, more specifically, in total \( O_2 \) uptake.

**Hematology**

There is a high correlation between Hb concentration and the O₂-carrying capacity of the blood of *Xenopus laevis*, and the majority of O₂ transport in the blood occurs via Hb-dependent processes. Carbon monoxide at levels as low as 0.5 kPa reduced the functional capacity of the blood to levels nearing that of plasma. This is consistent with the findings of Brody and Coburn (1969), who demonstrated that inspiration of small quantities of CO in humans was capable of reducing total blood O₂ content by 75%. Moreover, CO interacts with Hb in a pressure-dependent fashion and is capable of forming equilibrium curves at pressures an order of magnitude below those required for \( O_2 \) (Root, 1965; Roughton, 1954, 1970). *Xenopus laevis* exhibits a value for \( H \), the Haldane affinity coefficient (Haldane and Smith, 1897), that clearly shows that CO combines with Hb with an affinity 168 times higher than that for \( O_2 \). This explains the fact that, despite a 75% reduction in \( P_{CO} \), blood O₂-carrying capacity was unaffected and remained at levels close to the plasma values.

**Metabolism**

Although there has been speculation for some time that the transport of oxyhemoglobin by the cardiovascular system may play a significant role in total \( M_{O_2} \) in pre-metamorphic amphibians (Adolph, 1979; Boell et al. 1963; Burggren and Just, 1992; Burggren and Pinder, 1991), these claims have yet to be validated for early developmental stages. If metabolism were solely dependent on the \( O_2 \) convection by the cardiovascular system, the elimination of Hb-dependent \( O_2 \) transport would disrupt it. To investigate this possibility, \( M_{O_2} \) and whole-body [lactate] were assessed in *Xenopus laevis* from eggs to metamorphic climax to determine the extent to which the elimination of convective O₂ transport affected total substrate turnover.

Our results demonstrate that aerobic metabolism during early life (NF 1–30) increased in an exponential fashion regardless of the state of Hb O₂-transport. The fact that \( M_{O_2} \) increased 100-fold despite the absence of functional Hb indicates a minimal role for Hb in bulk O₂ transport during early development.

A clear rise in \( M_{O_2} \) during development in spite of CO exposure indicates that the role of Hb in bulk O₂ transport, and the role of the circulatory system in general, is quite minimal in the maintenance of aerobic metabolism. These interpretations gain support from several quarters. Mellish et al. (1998) showed that values for \( M_{O_2} \), in cardiac lethal mutants and in CO-exposed, cardio-ableted and control embryonic *Ambystoma mexicanum* were the same over a wide range of
ambient $P_O_2$ values. This observation is further supported by the existence of mutant strains of adult *Xenopus laevis* whose blood lacks formed elements and which were, therefore, presumed to be living on dissolved $O_2$ in the blood (de Graaf, 1957; Ewer, 1959). Moreover, work by Flores and Frieden (1969) demonstrated that the functional oxygen-carrying capacity of the blood can be impaired with phenylhydrazine with few ill effects on survivorship in tadpoles of *Rana catesbeiana*. Hillman (1980) has shown that phenylhydrazine-induced anemia in exercising *Rana pipiens* impairs performance, but that treated animals functionally retaining little Hb are still capable of sustaining moderate levels of activity. This combined evidence suggests that amphibians are capable of surviving with a minimal contribution of Hb to $O_2$ transport and uptake.

Although the aerobic component of total metabolism was unaffected in our study, amphibians are capable of dealing with sustained periods of stress by supplementing aerobic pathways with anaerobic metabolism (for a review, see Gatten *et al.*, 1992). The level of lactate, the major end-product of anaerobic metabolism in amphibians, is an excellent indicator of metabolic stress. The animals in our study were assayed for whole-body [lactate] to evaluate the extent of CO-induced stress on metabolism with development. Whole-body [lactate] was not significantly affected by exposure to CO. Our measured values of [lactate] are consistent with the finding of Hastings and Burggren (1995) and are lower than those reported by Feder and Wassersug (1984) when converted to similar units at 25°C. Normoxic animals approaching metamorphic climax (NF 55–63) had lactate concentrations (8.2 μmol·g$^{-1}$) that were slightly higher than those of *Xenopus laevis* tadpoles at comparable stages (Hastings and Burggren, 1995). The highly episodic nature of the increase in whole-animal [lactate] and the consistently low levels that persisted until late in development suggest that anaerobic metabolism contributed little to the total energy turnover during most of larval development. Moreover, these data suggest that lactate formation did not result from a supplementation of aerobic metabolism, but represents the baseline levels produced by normal maintenance metabolism during development. Additionally, these data suggest that cytochrome activity was not impaired by exposure to CO, which would have led to levels of lactate much higher than those observed.

Animals between NF 1 and 30 did not follow an allometric pattern of change predicted from interspecific data. In fact, the dramatic rise seen in $M_{O_2}$ over this stage range was accompanied by only a modest change in wet mass. However, $M_{O_2}$ between stages 31 and 63 did show a clear allometric rise ($b=0.71$, normoxic; and $b=0.61$, CO-exposed) when metabolic rate increased by two orders of magnitude over an increase in mass of 2.5 orders of magnitude ranging from 2 to 1100 mg (Fig. 3). Although both Hb and the circulatory system were fully functional over this range of mass, there was no significant effect of CO exposure on $M_{O_2}$. This allometric rise in $M_{O_2}$ is consistent with the findings of Feder (1981), although the slopes ($b$) we report are slightly lower than those published ($b=0.85$). A possible reason for the difference between the two studies could be that fact that Feder (1981) restricted analysis to only a few stages, whereas our study covers development from early embryos to metamorphic tadpoles.

**Cardiovascular function**

Although aerobic and anaerobic metabolism were unchanged upon exposure to CO, it is reasonable to assume that $Q$ was augmented to meet metabolic demands in the face of a lowered blood $O_2$ content. Support for this premise is found in work on larval and adult rainbow trout (*Oncorhynchus mykiss*) exposed to 5% CO, which elicited a 1.5-fold increase in heart rate, and possibly in $Q$, thereby facilitating a greater $O_2$ delivery at a lower blood oxygen-carrying capacity (Holeton, 1971a,b).

Icefish (*Chaenocephalus aceratus*) living at 1.5°C, which lack both erythrocytes and Hb, use a large $Q$ to maintain $M_{O_2}$, at levels comparable to those of amphibians at 25°C (Holeton, 1970; Ruud, 1954, 1958, 1965) and provide a useful model for understanding how *Xenopus laevis* can survive without Hb $O_2$-transport. Maintenance of high metabolic rates is icefish in the absence of Hb $O_2$-transport can be explained by two phenomena. First, the capacitance for $O_2$ in water at 1.5°C is approximately twice as high as at 25°C, and it is therefore reasonable to assume that plasma capacitance will also increase by this same factor to provide greater plasma $O_2$ delivery. Second, all out-flow vessels from the heart in fish empty into the gills, where the arrangement of vessels and hydrodynamic flow allows for counter-current exchange, resulting in greatly reduced diffusion limitations and, therefore, higher $P_O_2$ gradients than in the infinite pool exchangers (i.e. skin and buccal cavity) in amphibians (Piiper, 1981). Although these mechanisms work well for icefish at 1.5°C, they are not applicable to amphibians, which live at much higher temperatures and have both parallel and series circulation. On the basis of these arguments, it is unlikely that $O_2$ transport via plasma alone can meet total oxygen demands in *Xenopus laevis* other than at rest, although further measurements are needed to validate these assertions.

In the present study, $Q$ was the only factor that was modified by exposure to CO. Total blood flow in both groups of animals, from NF stage 33/34 to the first plateau at NF stage 42–45, increased more than 10-fold within 2 days of the initiation of a heart beat. These finding are consistent with previously published work (Hou and Burggren, 1995b; Orlando and Pinder, 1995). Interestingly, this dramatic increase in $Q$ occurred while wet mass only doubled. Control larvae showed a significant increase in $Q$ between NF stages 42 and 47, while $Q$ of CO-exposed larvae hardly changed. Over the next two stage ranges (NF 46–49), $Q$ fell for both normoxic and CO-exposed larvae in spite of a near doubling of body mass and advancement of 1 week of development. Total peripheral resistance ($R_{TP}$) decreases precipitously from 701 $R_T$ units to values approaching zero (0.9 $R_T$ units) over a range of wet mass from 3 to 20 mg in larval *Xenopus laevis* (Hou and Burggren, 1995b), who speculated that this fall in $R_T$ was
concomitant with an increase in the total cross-sectional area of the vasculature. Indirect supporting evidence comes from morphological studies showing that *Xenopus laevis* form gills, and a simple lung, and start using the alimentary tract over this stage range (NF 42–47), each structure adding in parallel to the total vasculature (Nieuwkoop and Faber, 1967). In addition, Hou and Burggren (1995a) have demonstrated little, if any, rise in mean truncus pressure over a range of masses corresponding to NF 42–47. The formation of these complex structures and their vascularization could explain the fall in $R_{TP}$. However, the net effects on important physiological cardiac variables such as preload and $V_S$ are difficult to predict without additional data.

$Q$ increased in both normal and CO-exposed animals between stages 48 and 54 in proportion to body mass, as seen in early development. Interestingly, $Q$ was higher for CO-exposed animals up to NF stage 42–45, where they reached an almost identical value to controls. Between NF 46 and 49, the trend was reversed, with higher $Q$ in controls than in CO-exposed animals. This reversal could be explained by the complex changes in total vascular area in CO-exposed animals. Anecdotally, we have observed that animals raised in the presence of CO show an increase in the area of vascular beds in the skin and buccal cavity. Total flow increased in controls between NF 50 and 52, in contrast to larvae between NF 46 and 49. Fritsche and Burggren (1996) have shown that cardiovascular regulation during hypoxia begins between NF stages 49 and 53 and that these changes are suggestive of a Frank–Starling mechanism. Moreover, Nieuwkoop and Faber (1967) have shown that anatomical innervation of the ventricle exists at NF 48–49, suggesting that active regulation of cardiac function may exist over this stage range. Interestingly, changes in $Q$ in CO-exposed animals over this range (NF 48–51) are due to an eightfold increase in $V_S$. The increase in $Q$ seen between NF 50 and 54, when taken in the context of both anatomical and physiological responses, strongly suggests that the changes observed were due to intrinsic cardiac regulation in CO-exposed animals.

Heart rate increased in a curvilinear fashion with progressive development, and there was no significant effect of CO treatment. The general trend of heart rate is similar to that reported previously (Fritsche and Burggren, 1996; Hou and Burggren, 1995a; Orlando and Pinder, 1995).

The complex changes seen in $Q$ in response to CO exposure, and the fact that $f_H$ was not significantly affected by CO exposure, suggest that $V_S$ was a major contributor to the resultant changes seen in $Q$. In adult fish and reptiles, bouts of hypoxia result in increases in $Q$ largely as a result of an increase in stroke volume (Fritsche and Nilsson, 1990; Holeton, 1971b; Millard and Johansen, 1974; Wood and Shelton, 1980). Increased stroke volume results from increases in preload and/or decreases in downstream resistance, which reduce afterload. Pelster and Burggren (1991) have shown that preload increases with development which, in turn, would increase $V_S$. It is therefore probable that CO exposure would induce increased $V_S$ not only from the perspective of development but also via induced physiological changes resulting from the hypoxic stress itself.

On the basis of this rationale, we evaluated the effects of chronic CO exposure on stroke volume during development. Stroke volume increased in an exponential fashion with ontogeny and body mass, consistent with observations reported previously (Fritsche and Burggren, 1996; Hou and Burggren, 1995b; Orlando and Pinder, 1995; Tang and Rovianen, 1996). However, unlike $f_H$, $V_S$ was significantly different overall in animals exposed to CO, although further analysis revealed that only a few stages contributed to the difference when compared with controls at a similar stages. The variability observed in $V_S$ when compared with $f_H$ and $Q$ is consistent with the argument that cardiac regulation occurs late in development, while early regulation seems to be largely intrinsic.

We conclude that reductions in arterial O$_2$-carrying capacity result in an augmentation of blood flow largely based on changes in stroke volume. Interestingly, Farrell (1991) speculates that adult amphibians regulate cardiac function via rate-dependent processes, suggesting that metamorphosis must be a period of transition from stroke-dependent regulation to rate-dependent regulation of cardiac output.

**Matching of *O₂* consumption and *O₂* transport**

The regulation and coordination of cardiac and respiratory function has been assessed in several anuran larvae (Burggren and Feder, 1986; Wassersug et al., 1981; West and Burggren, 1982). The coordination of both mechanisms has important consequences for regulating blood gas levels and $pH$ (Boutilier and Shelton, 1986a,b; Weintraub and MacKay, 1975). In the case of a developing *Xenopus laevis*, this regulation must take into account three distinct respiratory surfaces (gills, lungs and skin). Morphometric analysis of these respective exchange surface areas relative to the flow which supplies them (e.g. Mulvin, 1994) is beyond the scope of this paper. However, the coordination of tissue O$_2$ demand relative to total convective delivery of O$_2$, as defined by $M_{O_2}/Q_{O_2}$, gives an indication of the relative amount of cardiac work involved in transporting a quantity of gas, provided that all respiratory gases are transported in the blood. If gas transport were to occur via parallel diffusion, then cardiac work would be uncoupled from O$_2$ transport, resulting in values above the coupling threshold value of 1.

In anoxic animals between NF stages 33 and 54, there was a significant reduction in $M_{O_2}/Q_{O_2}$, suggesting either that $M_{O_2}$ was falling relative to $Q$ or that cardiac output was increasing during development. Our data suggest that $Q$ increased rapidly, with only relatively small changes in $M_{O_2}$. The $M_{O_2}/Q_{O_2}$ ratio in CO-exposed animals showed a similar pattern to that in anoxic animals, but the changes occurred at a level six times higher than that for control animals. Clearly, the fact that $M_{O_2}/Q_{O_2}$ was elevated in CO-exposed animals suggests that the cardiovascular system was contributing little to total O$_2$ transport and that diffusion is more important in total gas exchange. Values for $M_{O_2}$ in cardiac-ablated and cardiac mutant *Ambystoma* are similar to control levels,
suggesting that the major contribution to the total rate of O₂ uptake is from simple O₂ diffusion across the body wall (Mellish et al. 1998).

The role of transcutaneous diffusion in embryonic gas exchange

The reduction of whole-blood oxygen-carrying capacity by CO treatment did not impede resting aerobic metabolism, implying that oxygen uptake occurs via diffusion alone in early development. Although these data are not surprising for eggs and very small embryos, it is unexpected that large (≥3 mg) larvae deprived of significant convective O₂ transport are capable of surviving through metamorphic climax. Since the ablation of the O₂-carrying capacity of the blood resulted in only modest changes in \( \dot{Q} \), it appears that the cardiovascular system plays only a small role in total oxygen uptake in larval development. This contention is in general agreement with studies of early development in other lower vertebrates (Mellish et al. 1998; Pelster and Burggren, 1996).

Taken together, the available data suggest that the initiation of cardiac function is not for the sole purpose of transporting oxygen, but that the heart begins to function as part of a developmental (genetic) program prior to the absolute need for convective O₂ transport, a hypothesis we call "prosynchronotropy" (Burggren and Territo, 1995). Thus, the role of cardiac function in supplying oxygen to the tissues may be redundant during periods of low environmental P O₂ (Burggren and Territo, 1995). Moreover, it has been suggested that the role of the cardiovascular system in early development may serve several purposes other than O₂ distribution. Burggren and Warburton (1994) have suggested that early heart beat and pressure generation may serve to distend newly developing vascular beds. Alternatively, the role of early convective transport may be for nutrient distribution (Burggren and Territo, 1995). Whatever the purpose of early convection, it is clear from the present study that the bulk of O₂ transport is not dependent on Hb in the early developmental stages.

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