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The influence of environmental P_{O_2} on hemoglobin oxygen saturation in developing zebrafish *Danio rerio*

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Summary

Several studies suggest that during early larval development of lower vertebrates convective blood flow is not essential to supply oxygen to the tissues, but information about the oxygenation status of larvae during the time of cutaneous respiration is still missing. If convective oxygen transport contributes to the oxygen supply to tissues, venous blood in the central circulatory system should be partly deoxygenated, and hyperoxia should increase the oxygen saturation of the hemoglobin. To analyze the changes in hemoglobin oxygen saturation induced by hyperoxic incubation, zebrafish larvae were incubated in a tiny chamber between polytetrafluoroethylene membranes (Teflon), so that the oxygen supply could be rapidly modified. Hemoglobin oxygen saturation was measured *in vivo* by combining video imaging techniques with a spectrophotometrical analysis of hemoglobin light absorption at specific wavelengths for maximal absorption of oxygenated and deoxygenated blood (413 nm and 431 nm, respectively) under normoxic conditions and after a 10 min period of hyperoxia (P_{O_2}=100 kPa), assuming that at a P_{O_2} of 100 kPa the hemoglobin is fully saturated. The results demonstrated that red blood cell oxygenation of zebrafish larvae at 4 days post fertilization (d.p.f.), 5 d.p.f. and 12 d.p.f. could be increased by hyperoxia. The data suggest that at the time of yolk sac degradation (i.e. 4 d.p.f. and 5 d.p.f.), when the total surface area of the animal is reduced, bulk diffusion of oxygen may not be sufficient to prevent a partial deoxygenation of the hemoglobin. The decrease in hemoglobin oxygenation observed at 12 d.p.f. confirms earlier studies indicating that at 12–14 d.p.f., convective oxygen transport becomes necessary to ensure oxygen supply to the growing tissues.

Key words: ontogeny, oxygen exchange, circulatory system, heart, zebrafish, *Danio rerio*.

Introduction

Within recent years several studies have shown that during early larval development of lower vertebrates such as *Danio rerio* and *Xenopus laevis*, convective blood flow is not essential to supply oxygen to the tissues (Pelster and Burggren, 1996; Chen and Fishman, 1997; Jacob et al., 2002; Territo and Burggren, 1998; Territo and Altimiras, 1998). Pelster and Burggren (1996) documented that disruption of hemoglobin oxygen transport had no impact on oxygen-dependent processes in early zebrafish larvae. In addition, mutant zebrafish lacking erythrocytes survive almost completely until about 2 weeks after fertilization (Weinstein, 1996). Accordingly, cardiac activity of zebrafish larvae raised under hypoxemic conditions was not significantly different from that of control animals, raised with normal oxygen carrying capacity in their blood (Jacob et al., 2002). Analysis of gas exchange of larval *Xenopus laevis* suggested that for normal cardiovascular or respiratory function convective oxygen transport *via* hemoglobin was not essential, and *Xenopus* larvae are much larger than zebrafish larvae (Territo and Burggren, 1998; Territo and Altimiras, 1998). The ability to maintain normal aerobic metabolism in *Xenopus* larvae despite an impaired convective oxygen transport in the blood clearly indicated that in early developmental stages direct diffusion of O_2 through the skin (= bulk diffusion) plays a vital role in supplying oxygen to the tissues. This appears to be especially true for the zebrafish, because their gills become completely functional relatively late in development (12 days post fertilization, d.p.f.) and initially appear to be involved in ion regulation, rather than in the uptake of oxygen (Rombough, 2002). Cutaneous gas exchange is facilitated by well developed vascular networks such as an extensive capillary network on the large yolk sac. In addition, the skin of young larvae is only two cell layers thick over most of the body surface (Lasker, 1962; Jones et al., 1966; Roberts et al., 1973) and thus appears to be a highly efficient organ for gas exchange due to (i) its large surface area, (ii) the relatively short diffusive pathway, and (iii) the partial pressure gradient between the environmental water and the tissues (Rombough, 1988).

Collectively, these results indicate that up to a certain body size of the larvae the oxygen supply of the tissues can be met

by bulk diffusion (Territo and Altimiras, 2001). Nevertheless, it is quite obvious that beyond a certain body mass convective oxygen transport must come into play, but data on the oxygenation status of the larvae during the time of cutaneous respiration is still missing. If convective oxygen transport contributes to the oxygen supply to tissues, loading and unloading of the blood must be detectable, i.e. partly deoxygenated blood and hemoglobin must be found in central parts of the body. Blood vessels underneath the skin, in turn, should carry mostly oxygenated hemoglobin. Based on these considerations we hypothesized that the oxygenation state of hemoglobin in central blood vessels would be an indication of loading and unloading of hemoglobin, and thus reflect the contribution of convective oxygen transport to the oxygen supply of the tissues. The presence of partly deoxygenated hemoglobin in central parts of the body, however, could indicate hypoxic areas (tissues) in the body. In this case, incubation under hyperoxic conditions should improve oxygenation of the hemoglobin and thus of the tissues. The aim of the present study was therefore to test whether tissue oxygenation of zebrafish larvae can be improved by hyperoxic exposure. Tissue oxygenation was assessed from hemoglobin oxygen saturation *in vivo* by combining video imaging techniques with spectrophotometrical analysis of hemoglobin light absorption.

Materials and methods

Animals

For this study we used larvae of the poorly pigmented zebrafish mutant 'brass' (see Schwerte et al., 2003). Soon after spawning developmental stages of the eggs were determined to ensure a maximum deviation in age of no more than 3 h within each clutch. For each experiment eggs from at least 3 or 4 different clutches were used to account for possible interclutch differences. Eggs were then raised in normoxic freshwater at a constant temperature of 28°C. To prevent an infestation with fungi or bacteria, sanitized beakers were used and water and beakers were exchanged on a daily basis. Larvae were fed with an *Artemia nauplii* replacement (Cyclop-Eeze; Argent Laboratories USA, Redmond, USA) when they reached the developmental stage of buoying upwards, representing the filling of the swim bladder, which took place at about 4 or 5 d.p.f.

Rearing

The experimental design of the present study required culturing zebrafish larvae under constant normoxic conditions. Therefore, a new rearing system was developed, which guaranteed an equivalent oxygen supply for all animals investigated. Before water was introduced into the system, it was vigorously aerated. Fresh water at a temperature of 28°C was pumped into a water reservoir above the rearing system. In a gravity-fed system water dropped from the reservoir into the rearing beakers, which had a bottom of fine plastic meshwork, ensuring a permanent normoxic, downward water flow. To reduce the danger of infection, a UV-light (Selzle UV-C Keimfilter, Typ UV500, TC 5 Watt, Selzle Technik GmbH, 63110 Rodgau, Germany) was included into the water circuit.

Respirometry

For measuring oxygen consumption of zebrafish larvae, fertilized eggs of 'brass' mutants were placed into the examination chamber of a 'Twin-flow' respirometer (Cyclobios, Innsbruck, Austria), equipped with Clark oxygen electrodes at the inflow and outflow of the chamber (Gnaiger, 1983). The oxygen consumption of seven groups of eggs could be continuously measured from the egg (1 d.p.f.) until day 9 of development, when the experiment had to be terminated because too many larvae died in the respirometer. Microbial respiration and background respiration of the respirometer system (including oxygen uptake of the Clark oxygen electrode) were measured daily (Dalla Via, 1983) and subtracted from fish oxygen consumption values. For determination of background respiration, recalibration and cleaning of the system, measurements were interrupted for about 3 h every day.

Preparation of the animals for video recordings

Preliminary experiments revealed that the oxygenation of larvae embedded into low melting agarose (see Schwerte and Pelster, 2000) was not always reproducible. Therefore a modified incubation chamber was developed, in which the slightly anesthetized fish larvae (0.1 g l^{-1} MS222; Sigma Aldrich Chemie GmbH, 89552 Steinheim, Germany) were kept between polytetrafluoroethylene (PTFE; Teflon) membranes (YSI Membrane Kit STANDARD; PTFE membranes for oxygen electrodes, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA). The larva was placed in a drop of anesthetic (0.1 g l^{-1} MS222) onto the lower PTFE membrane (Fig. 1) and a cylindrical top containing a second PTFE membrane lowered on top, enclosing the zebrafish larva between two membranes in a sandwich-like position. Aeration of the larva was carried out by directing temperature controlled and humidified air (or gas) from a gas-mixing device directly onto the two PTFE membranes. The high oxygen permeability of PTFE membranes ensured that the oxygen reached the surface of the animal virtually without delay.

Measurement of hemoglobin oxygen saturation

Maximum absorption peaks of fully oxygenated blood (413 nm), and fully deoxygenated blood (431 nm), and the position of the isosbestic point (421 nm), were ascertained by spectrophotometrical analysis using zebrafish whole blood. The absorption of zebrafish blood in the ventricle was visualized by successively irradiating the embedded larva with light of the three specified wavelengths (413 nm, 421 nm and 431 nm), provided by a monochromator (Polychrome IV, Till Photonics, 82152 Martinsried, Germany) connected to an inverted microscope (Axiovert 200M, Carl Zeiss GmbH, 37030 Göttingen, Germany) *via* a light guide. With an imaging system (Optimas 6.5, Media Cybernetics, Inc. Silver Spring,

MD, USA) 100 consecutive pictures of the ventricle at each wavelength, beginning at 431nm, were recorded. Wavelengths were switched manually. The acquisition of 100 absorption images and their storage onto the computer's hard drive required approximately 30 s at each wavelength.

By using Optimas 6.5, two templates were drawn into a single recorded image of a larva's diastolic ventricle. One template confined the boundaries of the diastolic blood volume, while the second was used for standardization and therefore set into the nearby background. By using a macro, the imaging system calculated the template's mean pixel values for each of the 100 images and exported them for further examination into an Excel-file. Images in which absorption peaked were taken to represent the end diastolic stage of the ventricle (i.e. maximum filling with blood). Only those images were used for subsequent calculations. The changing dimensions of the ventricle caused a changing path length of light and resulted in a changing light absorption. It was assumed that end-diastolic volume was similar between subsequent contractions of the ventricle and therefore path length was constant. By using only values recorded during end-diastole, the path length was thus eliminated as a possible source of error. The background-template always had very high pixel values due to the low absorption of PTFE membranes and of the fluid film surrounding the larva.

Ventricular blood absorption values (as % of light emitted from the light source) were determined using the equation: % absorption = 100 – (mean of the five lowest pixel values of 100 images / mean background pixel values of 100 images)×100.

Finally, graphs were made for the three absorption values obtained under the varying oxygen saturation conditions. Absorption values of the isosbestic points differed slightly between the three graphs due to a varying amount of blood within the template during end diastolic stage. Standardization was therefore carried out using a method that normalized the absorption values at 413 nm and 431 nm to the mean isosbestic point of all three measurements (Tateishi et al., 1992). The wavelength for deoxygenated blood, 431 nm, turned out to be the most sensitive value for visualizing differences in blood oxygen saturation.

Measurement of blood absorption values under varied oxygen partial pressures

The oxygenation status of larval zebrafish blood was analyzed by comparing the absorption pixel values of the blood at different levels of oxygenation. Absorption values obtained from larvae under hyperoxic conditions were set to 100% oxygenation. It was assumed that data obtained under normoxic conditions revealed 'complete oxygenation' if the values were within the standard deviation of those obtained under hyperoxic conditions. 'Normoxic' data, significantly exceeding the absorption ratios obtained from the same animal under hyperoxic conditions, would be due to a greater

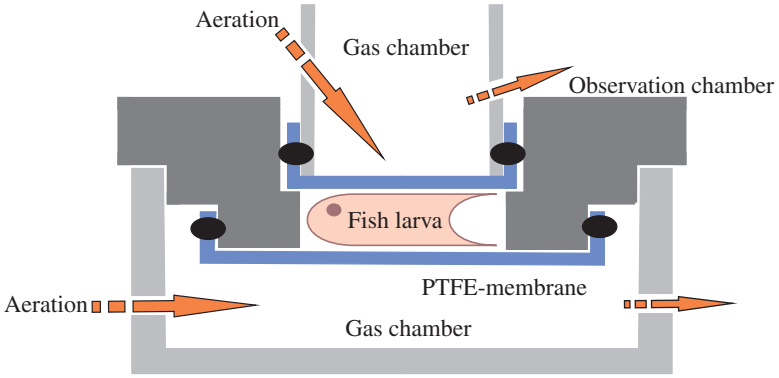

Fig. 1. Schematic drawing of the experimental chamber with an embedded zebrafish larva.

absorption at 431 nm, the specific wavelength for deoxygenated hemoglobin. Accordingly, these values indicated a partial deoxygenation of the hemoglobin under normoxic conditions.

In order to relate the measured absorption values of the blood to oxygen partial pressure, red blood cell (RBC) absorption values were determined in relation to the oxygen partial pressure in the fluid. After connecting the embedding chamber with the aeration device, a zebrafish larva was superfused with varying oxygen partial pressures. The experimental series was started with air (P_{O_2}=20 kPa). By mixing of air and nitrogen the oxygen partial pressure was then successively lowered to 16 kPa, 12 kPa, 8 kPa, 4 kPa, 0 kPa and in a final step again increased to 20 kPa. Incubation of the larvae at 0 kPa oxygen also provided the absorption value for complete deoxygenation of the hemoglobin. To verify the gas partial pressures in the embedding device an oxygen electrode (FOXY-18G probe with FOXY-AF-MG overcoat; Ocean Optics, 6921 RK Duiven, The Netherlands) was inserted into the lower gas chamber during the experiments. With the high gas permeability of the PTFE membranes and the relatively small tissue barrier of a fish larva in early developmental stages a stable oxygenation signal of blood in the ventricle was observed within a few minutes. No difference in oxygenation of the larvae's blood between 10 and 20 min after changing gas partial pressure of the incubation medium was detected. Therefore, for each step an equilibration period of 10 min was used.

Hyperoxygenation of larval zebrafish blood and tissue was achieved by superfusing larvae with pure oxygen (100 kPa) for a period of 10 min. Under these conditions ventricular blood was completely oxygenated, and hemoglobin oxygen saturation was 100%.

In a final step the internal calibration for the experiment was determined by measuring the maximum difference in absorption values between full saturation and deoxygenation of the hemoglobin for each individual larva. This step was necessary because optical conditions for spectrophotometric recording and analysis of blood oxygen saturation differed, depending on the developmental status of the zebrafish larvae

(tissue thickness and blood cell content). Because of a decrease in transparency the change in light absorption observed between full oxygenation and deoxygenation significantly decreased from 11.25±0.61% at 4 d.p.f. to 4.06±1.47% at 12 d.p.f. (Fig. 2). Accordingly, in later developmental stages our method for measuring hemoglobin oxygen saturation *in vivo* was not applicable. Actual absorption values of blood were then expressed as percentage of maximum RBC saturation.

Recording of the absorption images and determination of heart rate was performed 10 min after adjusting the appropriate oxygen partial pressure. The mean value of three consecutive measurements at each applied gas tension was used.

Heart rate

Heart rate was obtained from zebrafish larvae from 2 to 12 d.p.f. Heart rate was determined by measuring the time interval for 30 heart beats. The average value obtained from triplicate measurements was extrapolated to get the number of beats per minute for each individual fish.

Statistics

For comparison of two means, statistical significance was evaluated by unpaired Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test was used. Differences were considered significant at $P<0.05$.

Results

Hemoglobin oxygen saturation level

The method developed to measure hemoglobin oxygen saturation *in vivo* would allow for repeated sampling of the same animals, but we decided not to measure animals twice in order to avoid any possible effects of adaptation. A comparison of hemoglobin oxygen saturation under normoxic conditions with values obtained under hyperoxic conditions revealed periods of partially deoxygenated venous return, especially at 4 d.p.f., 5 d.p.f. and 12 d.p.f. (Fig. 3A). At 2 d.p.f., 4 out of 12 zebrafish larvae showed partial deoxygenation of ventricular RBCs. Comparison with hyperoxia revealed an average oxygen saturation of 91.7±1.4% at this stage, but the absorption under normoxic conditions was not significantly different from that under hyperoxic conditions (Fig. 3B). At 4 d.p.f., 15 out of 16 animals analyzed showed a partial deoxygenation of ventricular RBC, and the hemoglobin oxygen saturation was 64.2±4.1%. At 5 d.p.f., the ventricular RBC of 13 of the 18 assayed zebrafish larvae were partially deoxygenated. Under hyperoxic conditions blood oxygen saturation at this stage increased by 25.0±5.0%. At 8 d.p.f. only 2 out of 8 zebrafish larvae showed a partial deoxygenation of the blood, but on average hyperoxygenation did not significantly increase hemoglobin oxygen saturation. At 12 d.p.f. all of the tested animals (N=6) had partially deoxygenated blood in their ventricle, and hyperoxia increased the level of oxygen saturation by 36.6±6.8%. The number animals that could successfully be analyzed at 8 and 12 d.p.f. was lower than at earlier stages, because the transparency of the larvae was so low (see Fig. 2).

As to be expected, a reduction in P_{O_2} of the incubation water (progressive hypoxia) resulted in a progressive reduction in hemoglobin saturation in the ventricle of animals

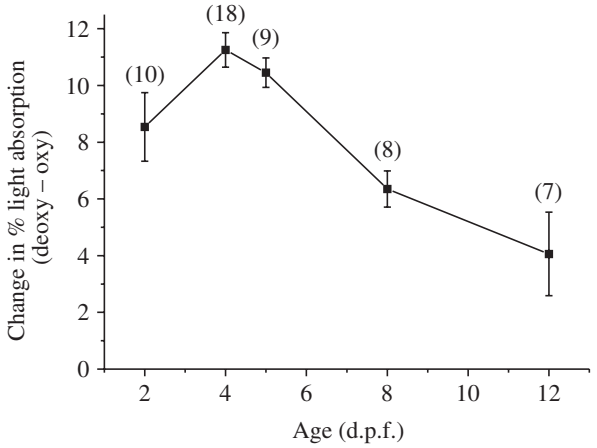

Fig. 2. Maximum differences in light absorption of oxygenated and deoxygenated zebrafish blood between 2 d.p.f. and 12 d.p.f. Values are means ± S.E.M.; *N* values are given in parentheses above each symbol.

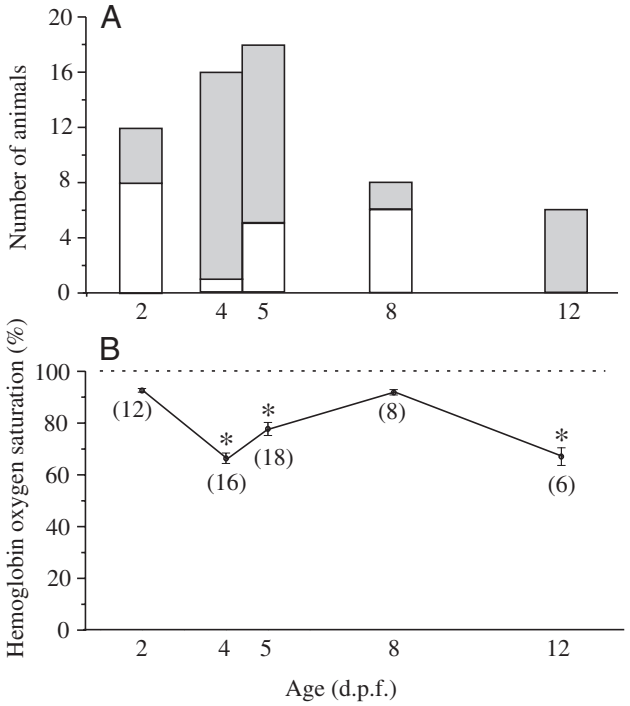

Fig. 3. (A) Number of zebrafish larvae showing partial deoxygenation of the blood under normoxic conditions (filled bar) and the number of animals, showing complete oxygen saturation (open bar). (B) Oxygen saturation of the blood in the ventricle of normoxic zebrafish larvae (P_{O_2}=20 kPa). Values are means ± S.E.M. *Significant differences from full saturation. *N* values are given in parentheses below each symbol.

at all stages. As an example, Fig. 4 presents the data for 2 and 8 d.p.f. animals, in which a complete oxygen saturation of the hemoglobin was observed under normoxic conditions. At 2 d.p.f. oxygen saturation decreased under hypoxia and at a P_{O_2} of 4 kPa oxygen saturation was significantly reduced compared to normoxic conditions. After switching back to normoxia hemoglobin oxygen saturation was restored. At 8 d.p.f. a significant deoxygenation of the hemoglobin was first observed at a P_{O_2} of 8 kPa. In all other stages the blood in the ventricle was already hypoxic under normoxic conditions (see Fig. 3), and with progressive hypoxia the oxygen saturation of the blood continuously decreased down to zero at a P_{O_2} of 0 kPa.

Heart rate under hyperoxic and hypoxic conditions

Heart rate was measured in order to test whether the changing oxygen partial pressure not only affected hemoglobin oxygen saturation, but also cardiac activity. At 2 d.p.f. and 3 d.p.f., heart rate under normoxic and hyperoxic conditions did not differ significantly (Fig. 5). Beginning at 4 d.p.f. hyperoxia significantly lowered heart rate in all stages until 12 d.p.f. At 9 d.p.f. and 12 d.p.f., however, this difference was not significant.

Heart rate at 4 d.p.f. and 5 d.p.f. was also modified in response to acute hypoxia, but the actual oxygen partial pressure at which this response was observed was dependent on the developmental stage (Fig. 6). At 4 d.p.f. a significant increase in heart rate was observed only at a P_{O_2} of 8 and 4 kPa, 5 d.p.f. larvae responded at 8, 4 and 0 kPa.

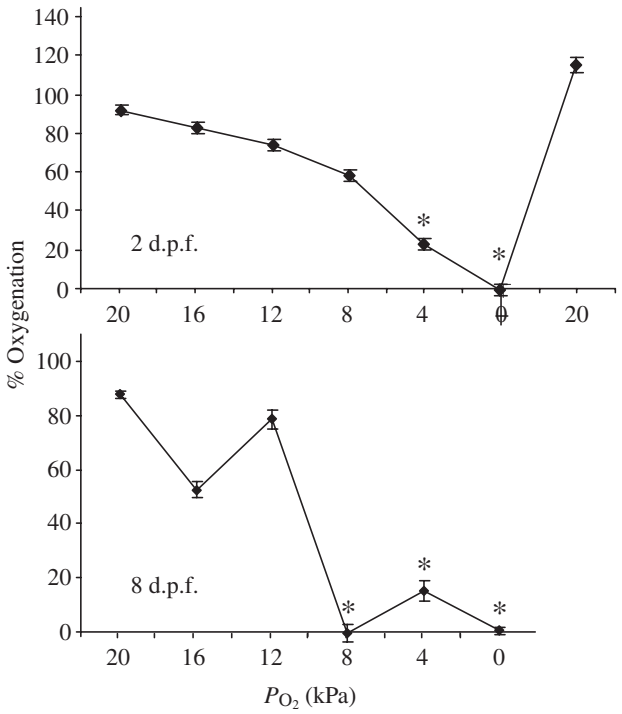

Fig. 4. Oxygenation of blood in the ventricle under progressive hypoxia in 2 d.p.f. animals (N=12) and at 8 d.p.f. (N=8); *Significant differences from control values recorded at normoxia (P_{O_2}=20 kPa).

At 8 d.p.f., zebrafish larvae showed no significant response to hypoxia, although at 8 and 12 d.p.f. a minor decrease in heart rate was observed at the onset of hypoxia, but this bradycardia was not significant. Under more pronounced hypoxia, however, heart rate increased at 12 d.p.f., as was seen in earlier stages.

Oxygen consumption

Oxygen consumption rates of developing zebrafish significantly increased from day 1 (egg) until day 5 (swim-up larvae, digesting external food). Starting at 3.8±0.135 nmol h^{-1} per individual at 1 d.p.f., oxygen consumption increased more or less continuously to 15±0.46 nmol h^{-1} per individual at 5 d.p.f.. No significant difference was found between 5 d.p.f. and 6 d.p.f., whereas a significant decrease in oxygen consumption rate was detected from 6 d.p.f. to 7 d.p.f., followed by another significant elevation of oxygen consumption rate from 7 d.p.f. to 8 d.p.f. and 9 d.p.f., respectively (Fig. 7). Oxygen consumption of zebrafish larvae in later stages has been measured in several studies (Barrionuevo and Burggren, 1999; Bagatto et al., 2001), and these values are in line with our data. Adjusting the data of Bagatto et al. (2001) to the conditions of our study revealed that the increase in oxygen consumption as development progressed continued as expected due to the increase in body mass (Fig. 7).

Discussion

Rearing and embedding

Agar embedding is a technique commonly used for studies on developmental physiology of fish and amphibian larvae, and has occasionally also been used for chick embryos (Battiato et al., 1996; Rombough, 2002; Schwerte and Fritsche, 2003; Yamamoto and Jeffery, 2002). We decided against the

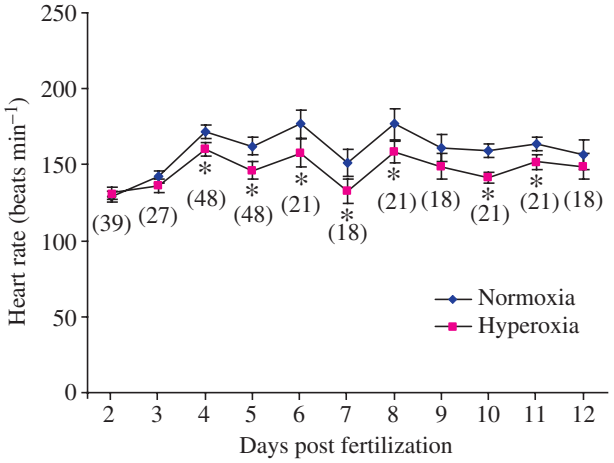

Fig. 5. Heart rate of zebrafish larvae between 2 d.p.f. and 12 d.p.f. under normoxic (P_{O_2}=20 kPa) and hyperoxic (P_{O_2}=100 kPa) conditions. Values are means ± S.E.M.; *Significant differences between normoxia and hyperoxia (P<0.05). N values are given in parentheses below each symbol.

agar-embedding method, because measurements of oxygen saturation in the medium surrounding the larvae using an oximeter showed low reproducibility. An explanation for this observation could be differences in environmental temperature, encountered during the agar-embedding procedure. The embedding method used in the present work resulted in highly reproducible P_{O_2} values in the bathing medium of the larvae, and the anesthetics added to the solution around the larva during PTFE-embedding reduced movements and prevented desiccation.

Immobilization of the larvae between the two PTFE membranes was mainly achieved by regulating the volume of the liquid surrounding the larvae. The aeration chamber itself provided an excellent device for experiments using different gas mixtures, as the gas flow along the PTFE membranes above and below the zebrafish larvae guaranteed constant gas tensions.

Data acquisition and analysis

The templates drawn within the diastolic ventricle of the zebrafish for the determination of the mean pixel values were identical throughout the measurements in an individual larva. However, in some cases the larva moved slightly between the normoxic and hyperoxic or hypoxic measurements. If this happened, another template was drawn at about the same position, ensuring comparable values. Particularly in later developmental stages some graphs, obtained under normoxic and hyperoxic conditions, showed too little contrast in gray values. This appeared to be due to an increase of ventricular and body cell mass of the zebrafish larvae, which reduced the visibility of the erythrocytes in the vascular system (see Fig. 2). These data were not used for further analysis. Beyond 12 d.p.f. our method for the determination of hemoglobin oxygen saturation *in vivo* was no longer applicable, because of decreased transparency.

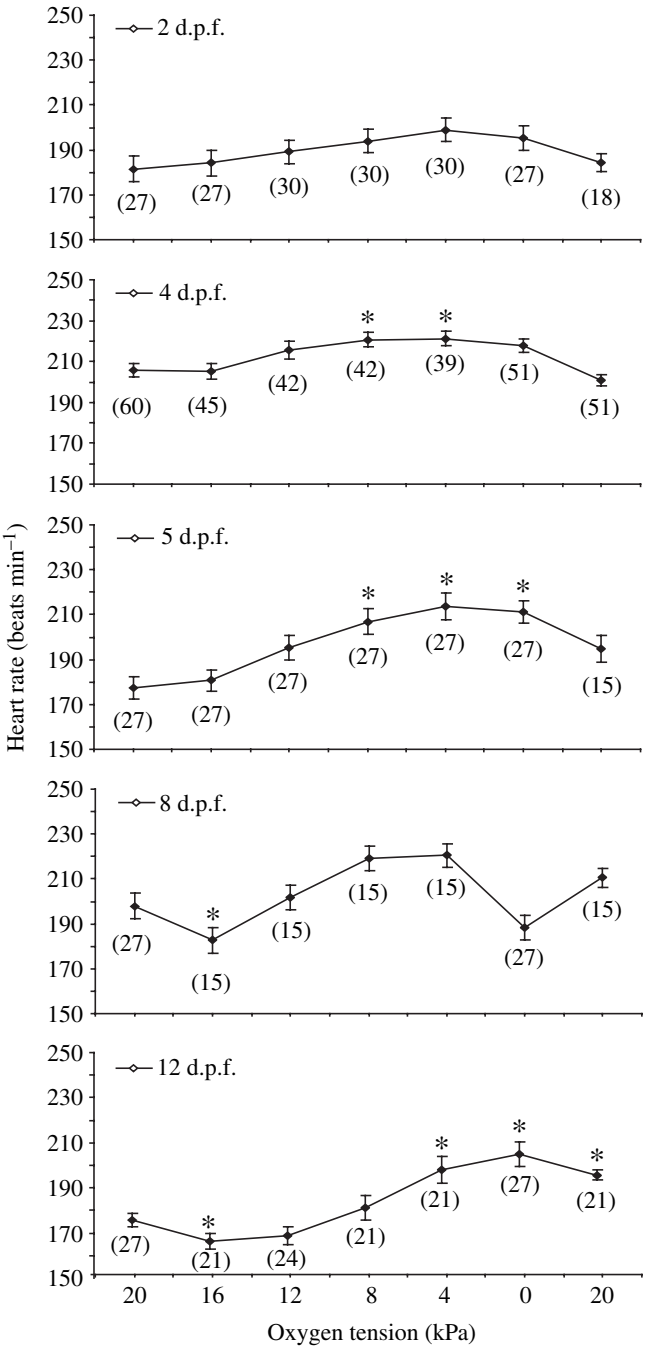

Fig. 6. Heart rate of zebrafish larvae between 2 d.p.f. and 12 d.p.f. under normoxic conditions (P_{O_2}=20 kPa) and progressive hypoxia down to anoxic conditions. Values are means ± S.E.M. *Significant differences between normoxia and hypoxic conditions (P<0.05). N values are given in parentheses below each symbol.

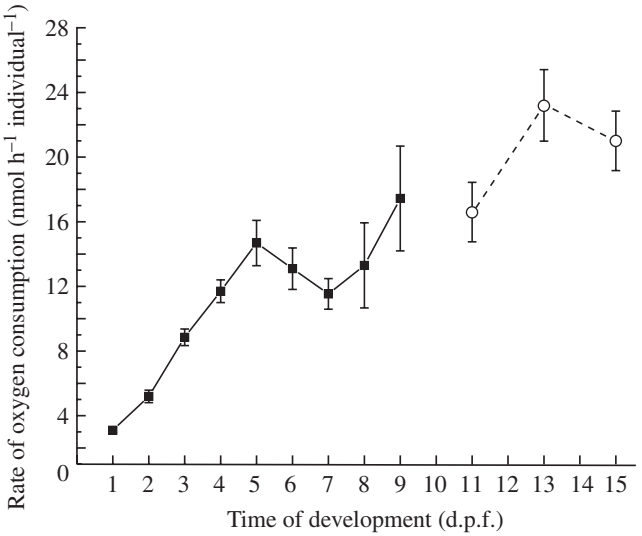

Fig. 7. Oxygen consumption of developing zebrafish larvae between 2 d.p.f. and 15 d.p.f., Values for animals between 1 and 9 d.p.f. were measured in a twin flow respirometer. Values are means ± S.E.M. from seven experiments. Within each experiment 10–15 eggs were pooled. Values for 11, 13 and 15 d.p.f. were taken from Bagatto et al. (2001), originally measured at 25°C. Data were recalculated to 28°C using a Q_{10} of 3.2, as determined for 10 d.p.f. animals for the temperatures between 25°C and 28°C (Barrionuevo and Burggren, 1999). *Significant differences relative to the previous day (P<0.05).

Oxygenation status of venous blood

Several studies have shown that bulk diffusion appears to be sufficient to fuel aerobic metabolism during early development of the zebrafish. The present results indicate, however, that this does not prevent partial deoxygenation of the blood in the ventricle, which contains venous blood. At 4 d.p.f., 5 d.p.f. and also at 12 d.p.f. the significantly different blood absorption values observed under normoxic and hyperoxic conditions clearly showed that venous return into the ventricle was partially deoxygenated. During these periods, acute hyperoxia significantly increased oxygen saturation. Nevertheless, measurements of oxygen consumption at this time do not provide any indication of a switch to anaerobic metabolism (Pelster and Burggren, 1996; Barrionuevo and Burggren, 1999; Bagatto et al., 2001).

At 4–5 d.p.f. we observed a nearly complete reduction of the yolk extensions and the yolk sac itself (see also Kimmel et al., 1995). Rombough (1998), investigating the impact of the yolk sac as a possible site of gas exchange in rainbow trout larvae, concluded that the blood vessel density in the richly vascularized yolk sac had no significant effect on gas exchange. In zebrafish we observed the lowest blood oxygen saturation at 4 d.p.f. and 5 d.p.f., a stage when yolk sac degradation was largely completed. A reduction of the gas exchange area would probably affect the balance between oxygen supply and oxygen consumption and thus could be responsible for a partial deoxygenation of the blood. This coincidence therefore suggested that in zebrafish larvae the yolk sac does contribute to oxygen uptake and thus to gas exchange. *Danio rerio* is a tropical fish that typically experiences higher temperatures (25–30°C) than trout larvae, which are typically exposed to temperatures of 5–15°C. Given the effect of temperature on metabolism, zebrafish larvae should thus have a significantly higher metabolic rate. Metabolic rate typically increases by a factor of two for a temperature increase of 10°C (Q_{10}=2), and in fish larvae Q_{10} values of up to 5 and 6 have been reported (Pelster, 1999; Barrionuevo and Burggren, 1999). It thus appears possible that the higher metabolic rate in zebrafish larvae requires additional sites of oxygen uptake, whereas in trout bulk diffusion through the body surface without the support of the yolk sac is sufficient. Besides, protuberances on the surface of the zebrafish yolk sac were observed, which may increase the surface area in order to support oxygen uptake (T. Schwerte, unpublished observation). Accordingly, the yolk sac of zebrafish larvae could substantially contribute to the diffusive O_2 uptake.

Similar to the putative role of the zebrafish yolk sac in gas exchange, the highly vascularized chorioallantoic membrane (CAM) of chick embryos contributes to gas exchange and even adapts to different environmental oxygen pressures. Richards et al. (1991) reported that chorioallantoic membrane wet mass increased under hypoxic conditions, and was reduced under hyperoxic conditions.

The improved oxygenation of the blood observed in our study beyond day 5 coincided with a decrease in oxygen consumption. It was not obvious why oxygen uptake decreased between day 5 and 7, but with this lower rate of oxygen uptake bulk diffusion apparently again ensured a better oxygenation of the whole animal. In spite of the continuing development of the animal, oxygen consumption at 8 d.p.f. was slightly lower than at 5 d.p.f., and at 8 d.p.f. venous blood in the ventricle was almost completely oxygenated.

A second period in which partially deoxygenated blood was detected in the ventricle of the zebrafish occurred at 12 d.p.f. At this relatively late stage in larval development the incomplete oxygen saturation of the tissues could be attributed to an increase in diffusional resistance, to an increased internal oxygen consumption, or perhaps to both factors. Between 6 d.p.f. and 13 d.p.f., zebrafish wet and dry mass increased from 0.35±0.01 mg to 0.46±0.02 mg, and from 0.057±0.002 mg to 0.069±0.007 mg, respectively (Bagatto et al., 2001). Respiration data of zebrafish larvae reveal a significant increase in oxygen consumption from day 7 to day 15. An increase in oxygen demand requires an increase in oxygen supply, and the secondary lamellae, the actual site of gas exchange in fish, start to form between days 12 and 14 (Rombough, 2002). Our data clearly show a partial deoxygenation of blood at 12 d.p.f. It can therefore be assumed that this is mainly caused by an increase in the rate of oxygen consumption, while the secondary lamellae, mainly responsible for the gas exchange, are only just starting to develop (Rombough, 2002).

Thus, the data available so far fit together nicely. Early developmental stages of the zebrafish up to 12–14 d.p.f. do not need a circulatory system for oxygen supply to tissues under normal ‘resting’ conditions. At about 12 d.p.f. the partial deoxygenation of hemoglobin in the central circulation indicates that oxygen is not only taken up by bulk diffusion, but also removed from the blood. This is also the time (12–14 d.p.f.) where chronic hypoxemia is no longer compatible with proper development (Jacob et al., 2002) and confirms the conclusion that at this point in development hemoglobin becomes necessary for oxygen transport. Thus, at about 12–4 d.p.f. the cardiovascular system of the zebrafish takes over responsibility for the oxygen supply of tissues.

This may also be the time when cardiac activity and metabolic demand of the tissues become coupled, as in adult vertebrates. The control system is necessary to achieve such a coupling is established much earlier. Hypoxic incubations (Jacob et al., 2002; Schwerte et al., 2003) and studies in which metabolic activity was enhanced by swimming movements (Pelster et al., 2003) revealed that oxygen receptors sensing hypoxic conditions are present at 3 or 4 d.p.f., and the control loop modifying cardiac activity in response to the afferent information of oxygen receptors is also operating at this time in development.

Implications of hyperoxia and acute hypoxia on heart rate

In a previous study it was demonstrated that heart rate represents a sensitive parameter for identifying cardiovascular

responses to chronic hypoxia. Therefore it appeared interesting to see whether the presence of partially deoxygenated hemoglobin would somehow correlate with cardiac activity. Partially deoxygenated blood was present at a P_{O_2} of 20 kPa at 4 and 5 d.p.f.; nevertheless, a significant elevation of heart rate during progressive hypoxia was not observed down to a P_{O_2} of 8 kPa at any of the stages analyzed. Thus, the presence of partially deoxygenated blood in the ventricle did not provoke significant changes in heart rate. This result is in line with the data of Jacob et al. (2002), who reported that hypoxemia (i.e. a reduced oxygen-carrying capacity of the blood) does not modify cardiac activity in early developmental stages.

Acute hyperoxia had no effect on larval zebrafish's heart rate at 2 d.p.f. and 3 d.p.f., but led to a significant bradycardia in all stages from 4 d.p.f. until 16 d.p.f.. Zebrafish raised under chronic hypoxia have an increased heart rate at about hatching time or shortly thereafter (Jacob et al., 2002), which indicates that at this time oxygen receptors are present, sensing hypoxic conditions and using the information for a suitable response, i.e. to increase cardiac activity in order to stimulate convective oxygen transport. Our study complements the work of Jacob et al. (2002). Oxygen sensors in the larval zebrafish are working in both directions. Hypoxia induces a stimulation of cardiac activity, while hyperoxia reduces cardiac activity, and these responses are observed at a stage when the circulatory system is apparently not yet essential to ensure oxygen supply to the tissues.

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