The Journal of Experimental Biology 206, 1299-1307

doi:10.1242/jeb.00249

Non-invasive imaging of blood cell concentration and blood distribution in zebrafish *Danio rerio* incubated in hypoxic conditions *in vivo*

Thorsten Schwerte, Dietmar Überbacher and Bernd Pelster

Institute for Zoology and Limnology, University of Innsbruck, Austria

Author for correspondence (e-mail: thorsten.schwerte@uibk.ac.at)

Accepted 16 January 2003

Summary

This is the first study to use a combination of digital imaging techniques and vital video microscopy to study hypoxia-induced changes in blood cell concentration, angiogenesis and blood redistribution in entire animals. Zebrafish *Danio rerio*, which are known to be independent of convective oxygen transport until about 2 weeks post-fertilization, were raised under chronic hypoxia (P_{O_2}=8.7 kPa) starting at 1 day after fertilization (d.p.f.) until 15 d.p.f. In control animals, the concentration of red cells (i.e. the number of red cells per nl blood) remained constant until 7 d.p.f., and than decreased by approximately 70% until 15 d.p.f. In hypoxic animals, however, the concentration of red cells remained significantly elevated compared to control animals at 12 and 15 d.p.f. Assuming that the hemoglobin content of the red cells is similar, hypoxic animals have a higher oxygen carrying capacity in their blood. Red cell distribution within the various parts of the circulatory system, taken as an indicator for blood distribution, revealed a significant modification in the number of blood cells perfusing the organs in hypoxic animals. At 12 d.p.f., gut perfusion was reduced by almost 50% in hypoxic animals, while perfusion of the segmental muscle tissue was increased to 350% of control values. No significant changes in brain perfusion were observed under these conditions. At 15 d.p.f., the reduction in gut perfusion was abolished, although muscle perfusion was still significantly elevated. At this time, growth of hypoxic animals was less compared to control animals, revealing that hypoxia had become deleterious for further development. The vascular bed of various organs was not obviously different in hypoxic animals compared to normoxic animals.

Key words: ontogeny, erythropoiesis, angiogenesis, hypoxia, digital video imaging, zebrafish, *Danio rerio*.

Introduction

The zebrafish *Danio rerio* has become an important model organism for the study of vertebrate biology, being well suited for developmental and genetic studies. Large-scale genetic screens have identified hundreds of mutant phenotypes, many of which may serve as models of human disease (Warren and Fishman, 1998; Barut and Zon, 2000). However, studies focussing on the physiology of the developing zebrafish embryo or larvae are scarce, and our understanding of the basic physiology of *D. rerio* lags far behind our knowledge of the genetics (Burggren and Keller, 1997). Blood circulation in all vertebrates starts early in development, and the first heart beat is typically observed before the heart is completely differentiated (Pelster and Bemis, 1991). Despite this early onset of cardiac activity and blood circulation, the physiological function of blood convection has been questioned (Pelster and Burggren, 1996; Pelster, 1999). In small larvae such as zebrafish, diffusion of oxygen through the body surface alone appears to be sufficient to meet the metabolic needs of the animal (Territo and Burggren, 1998; Territo and Altimiras, 1998; Pelster, 1999; Gielen and Kranenbarg, 2002). This demonstrates that coupling between metabolism and convective oxygen transport is not yet established in the early larval stages, and Rombough (2002) suggests that ion- and osmoregulatory functions may require blood flow much earlier in development than metabolism.

Hypoxic conditions are observed in the flowing and stagnant waters that are the natural environment of the tropical zebrafish. The coupling of convective oxygen transport and metabolic activity ensures sufficient oxygen supply to the cells and prevents oxygen shortages at the organ level. Accordingly, in adult animals hypoxia itself acts a stimulus and induces profound changes in cardiac activity and peripheral resistance, and even stimulates erythropoiesis. If this coupling is not yet established in early developmental stages, it could mean that hypoxia does not act as a stimulus in early developmental stages. In a recent study in zebrafish we were able to demonstrate that long before coupling between metabolic requirements and blood flow is established, environmental hypoxia can be sensed and induces stimulation of cardiac activity (Jacob et al., 2002). A reduction in the oxygen-carrying capacity of the blood, however, had no effect on cardiac activity. Thus, hypoxia does exert a signaling effect,

even in early larval stages. The aim of the present study was to investigate whether hypoxia, in addition to the modification of cardiac activity shown in an earlier study, would also induce a redistribution of blood and/or stimulate the production of red blood cells in the zebrafish. In addition, the vascular bed of various organs was compared in animals raised under normoxic and hypoxic conditions in order to test the hypothesis that hypoxia stimulates the formation of blood vessels even at early developmental stages. To answer these questions for millimetre-sized zebrafish, we used the recently developed method of digital motion analysis (Schwerte and Pelster, 2000) and extended it so that we could determine the concentration of red bloods cells in a defined volume of blood and also visualize blood distribution within the animal.

Materials and methods

Animals

Zebrafish *Danio rerio* Hamilton 1822 larvae were obtained from our own breeding colony. Because of their greater transparency, poorly pigmented mutants of the zebrafish (Albino, Brass) were used. Parent animals used to start the breeding colonies were either obtained from a local supplier or generously provided by Dr Frohnhöfer (Max-Planck Institute for Developmental Biology, Tübingen, Germany) and Ms Loos (University of Konstanz, Germany). Breeding colonies and larvae were kept in small aquaria at 28°C. Animals were fed after swimbladder filling (4–6 d.p.f.) with micro powder food (Zebrafish Management; www.zmsystems.demon.co.uk) and *Artemia* (from 10 d.p.f.).

The imaging system

An inverted microscope (Zeiss Axiovert 25 CF) was placed on a solid, heavy-weight steel plate to reduce vibration and the illumination set to infrared light (913 nm wavelength) to prevent any light-induced stress reactions in the animals. The microscope was equipped with a 2/3″ CCD camera (Hamamatsu C-2400 without infrared cut-off filter) which, in turn, was connected to the luminance input of a SVHS video recorder (Sony S-9500), remote-controlled *via* the RS232 serial communication port. Recorded images were digitized by a monochrome frame-grabber card (Imagenation PX-610) with a personal computer (PIII 450 MHz). The depth of view was adjusted to provide images that visualized most of the erythrocytes in one field.

Visualization of the vascular bed

A cast of the vascular bed was obtained by accumulation of the shifting vectors of moving erythrocytes from a number of subsequent difference pictures, as described in a previous study (Schwerte and Pelster, 2000). Briefly, by subtracting the two fields of a video frame, any movement that occurred within the 20 ms necessary for the acquisition of one field was visualized. The length of the shifting vectors, generated by this subtraction, represent a direct measurement of the velocity of a moving particle, i.e. an erythrocyte in the vascular system. By accumulation of shifting vectors generated from several consecutive video frames, a complete trace of the routes moved by the erythrocytes was obtained (Fig. 1). Vascular beds of the entire animal can be visualized non-invasively using this method (Schwerte and Pelster, 2000). Typically the difference pictures of about 30 consecutive images were accumulated to obtain a complete cast of the vascular bed.

As shown in Fig. 1, an image showing a complete cast of the vasculature of a body section or an organ permits measurement of the total area covered by these vessels relative to the size of the total area covered by the organ. This value can be used as an indicator for the vascularization of a tissue. Values for the whole tail and the gut were calculated.

Blood distribution using digital motion analysis

Developing this method further, it is also possible to obtain data about the concentration of erythrocytes in a given section of a blood vessel. The grey-scale value of any given pixel, or of a defined number of pixels, in the image generated by digital motion analysis increases linearly from 0 to 255, depending on the number of erythrocytes passing it. Although the depth of the grey scale display on the screen is limited to 8 bit, the actual range for the calculations was extended to 24 bit. Thus the erythrocyte distribution could automatically be recorded in defined blood vessels or in the whole animal. Calibration of the signal was done by correlating the grey scale values of a defined area with the number of erythrocytes passing this area, as counted by the conventional frame-to-frame technique (Schwerte and Pelster, 2000). These recordings were made using a 20× objective.

To evaluate blood redirection, mean values for the number of blood cells passing a specific tissue were compared and taken as an indicator of relative blood flow. A comparison of the concentration of red cells in various sections of the vascular system did not reveal the existence of any significant organ-specific differences in the hematocrit in zebrafish larvae, and our studies were focused on the brain, gut and tail musculature. The mean grey levels of the specific tissues obtained by this method were used to determine relative changes in tissue blood flow.

Red blood cell count

Using a 40× objective, 4–8 image frames had to be patched in order to obtain a picture from the whole animal. After accumulating sufficient difference pictures in order to obtain a cast of the vascular bed, the blood vessel diameter of defined sections of the vessels was measured and the volume of a defined blood vessel section calculated, assuming a circular cross-section of the vessels.

Subsequently, erythrocytes within the defined section of the vessel were tracked on individual images and a series of images showing individual blood cells was stored to the computer hard drive. Blood cells were automatically detected by their characteristic grey scale value and motion, marked with a red cross and counted. The results were controlled by optical inspection of an image series and misinterpretations were eliminated. This determination was repeated five times for each

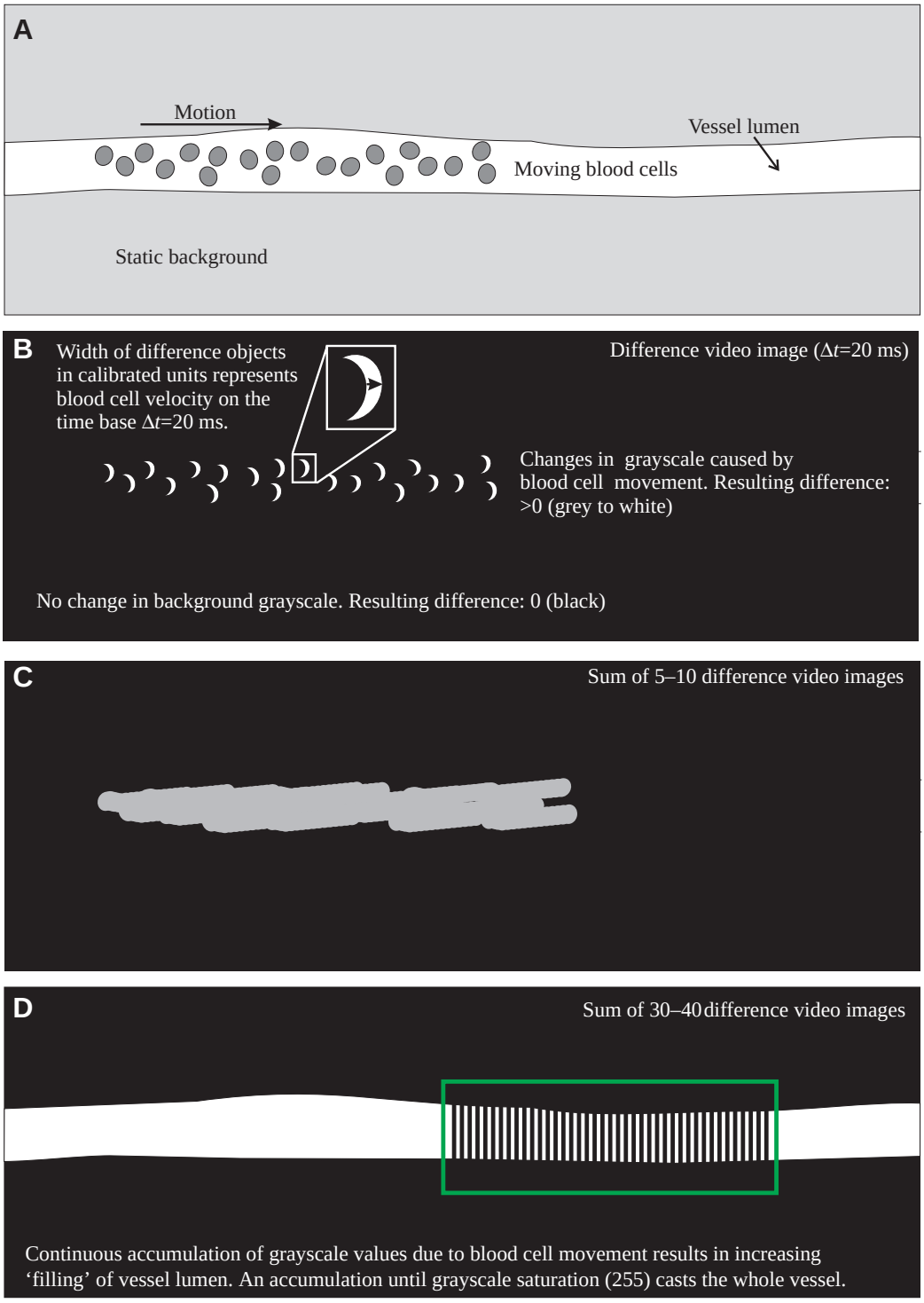

Fig. 1. The basic principle of digital motion analysis and measurement of blood cell concentration. (A) Blood cells moving through a vessel can easily be detected by their motion. (B) A difference image obtained by subtracting the two fields of one video frame (the odd and the even frames), showing several moving erythrocytes. Insert: enlargement of one erythrocyte image showing the direction of movement (arrow) (C,D) A schematic drawing showing moving erythrocytes and the subsequent summation of these differences, ending up with a complete cast of the vasculature. In the region of interest all diameters along the vessel were measured in 0.3 μm steps. The volume of a single erythrocyte cross section is calculated as: $\pi r^2 0.3$ (μm^3). The sum of all these sub-volumes along the vessel (white and black lines in D) gives in the vascular volume. The number of detected cells in B divided by this volume gives the red blood cell concentration in the area of interest (green box). See Materials and methods for further explanation.

animal to take possible clustering of blood cells into account. The process of counting red blood cells and the visualization of red blood cell distribution is described in Fig. 2.

Hypoxic incubation

Albino zebrafish eggs were incubated under hypoxic conditions (P_{O_2} 8.7 kPa) at 28°C. Oxygen tension was adjusted using a gas flow meter, which prepared a gas mixture of air and nitrogen. The gas was infused into the water (28°C) of sealed 16 liter aquaria through a fine-pored tube. Oxygen tension in the water was controlled twice a day with a calibrated Clark oxygen electrode (Radiometer Copenhagen, Willich, Germany) to ensure stable values.

Experimental protocol

Measurements were made at 3, 5, 7, 12 and 15 d.p.f. For

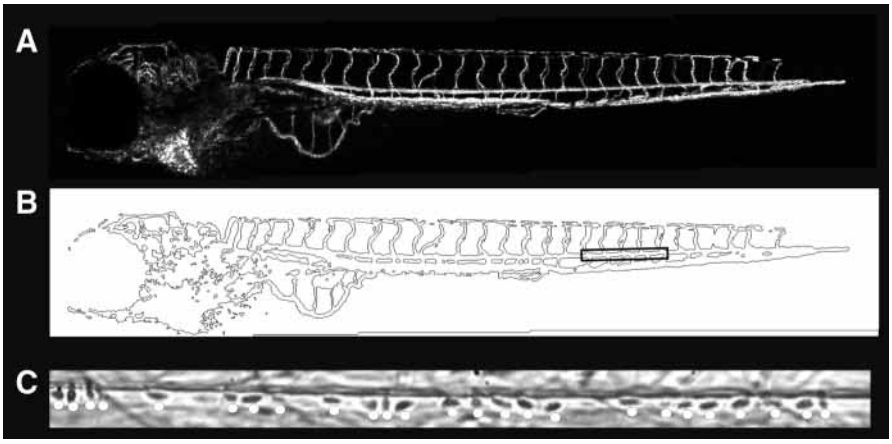

Fig. 2. Method for determination of red blood cell concentration. (A) Vascular cast of a 4 d.p.f. zebrafish larva obtained by digital vascular contrasting method. (B) Edge detection using colour thresholding method. The frame in the tail indicates the region of interest (ROI). (C) Close up of the ROI. White circles indicate motion detected single blood cells. The vascular volume in the ROI is calculated from the geometric data of the vessel shown in B.

measurement the animals were anaesthetized with a neutralized tricaine solution (100 mg l^{-1}) at the adjusted P_{O_2}, and embedded in low-melting-point agarose (containing 100 mg l^{-1} tricaine). For animals older than 11 d.p.f., the gills were sculpted free from the agarose to allow gill ventilation (Rombough, 2002). The animals were covered with a thin layer of water (100–200 μm, containing 100 mg l^{-1} tricaine). In the hypoxic groups all media were adjusted to the desired P_{O_2} and the animal chamber was sealed tight. The sealed animal chamber had a reservoir of 0.7 ml gas with the desired P_{O_2}, enough to maintain the P_{O_2} during the measurement. This was checked using a miniature oxygen electrode. Oxygen deprivation has been shown to cause general developmental retardation, so the staging was carefully done using the following morphological criteria: yolk sac, animal length, diameter at the position of the heart and vascular bed (Isogai et al., 2001) (Fig. 1). The animals were not fed prior to experiments. To avoid artefacts from differently fed animals (older than 6 d.p.f.), the gut filling was microscopically inspected and found to be similar in all animals prior to data acquisition. The experimental groups were pooled from 2–3 clutches obtained from a group of 15 female with 5 male 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

Animal size

Hypoxic and normoxic animals were not different in size from 3 to 12 d.p.f. By 15 d.p.f. the body length of animals of the hypoxic group was reduced by 0.2±0.03 mm (N=6) compared to normoxic animals (not shown).

Red blood cell count

Between 3 and 7 d.p.f. the concentration of red cells in the blood remained quite stable and no differences between normoxic and hypoxic animals were observed (Fig. 3). At 12 and 15 d.p.f., however, the concentration of red blood cells decreased significantly, and at 15 d.p.f. it was reduced to approximately 30% of the value recorded at 7 d.p.f. in control animals. In hypoxic animals the concentration of red blood cells also decreased at 12 and 15 d.p.f., but this decrease was significantly smaller than in normoxic animals.

Vascularization

There were no significant changes in vascularization of the tail musculature and the gut. The basic pattern of the vascular bed was similar in normoxic and hypoxic animals (see Fig. 4). Nevertheless, in hypoxic animals the number of intersegmental anastomosis was not significantly higher in hypoxic animals, and the number of animals showing a caudal vascular tree was higher in hypoxic than in normoxic animals. A statistical analysis of the morphometric data obtained from sections

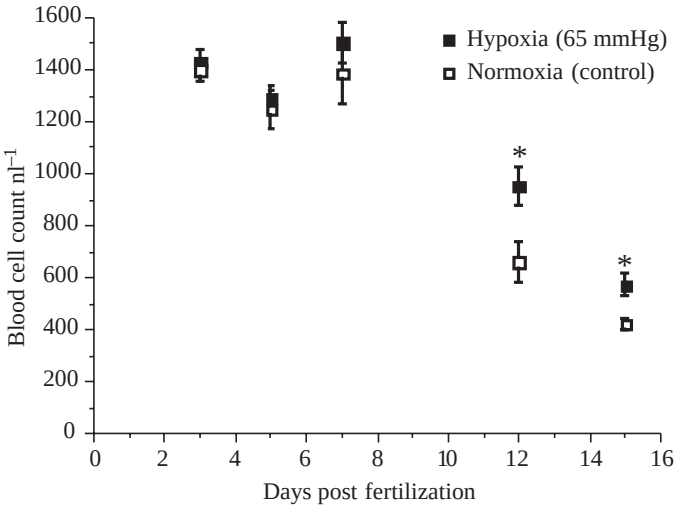

Fig. 3. Developmental changes in red blood cell concentration in zebrafish under chronic hypoxic and normoxic conditions. Values are means ± S.E.M., N=15; *significant difference ($P<0.05$). 1 mmHg = 1.333 Pa.

Table 1. *Developmental changes in tissue vascularization (% coverage) during chronic normoxic and hypoxic incubation*

Region	Embyonic age (d.p.f.)				
	3	5	7	12	15
Tail					
Normoxia	41.3±0.9	39.4±0.7	35.7±0.4	24.1±0.6	22.9±0.6
Hypoxia	40.9±0.2	39.1±0.3	34.9±0.1	24.4±0.2	23.9±0.4
Gut					
Normoxia	19.2±0.7	24.4±0.6	41.5±0.2	49.7±0.3	48.9±0.8
Hypoxia	19.5±0.6	25.1±0.4	41.3±0.2	49.4±0.4	49.3±0.3

through normoxic and hypoxic incubated animals (Table 1), however, showed no significant differences in the area covered by blood vessels.

Blood flow distribution

Fig. 4 shows typical false-colour-coded images of the vascular cast obtained from 12 and 15 d.p.f. animals raised under either normoxic or hypoxic conditions. It was obvious that in 12 and in 15 d.p.f. animals, blood perfusion was higher in the muscle tissue of hypoxic animals. By contrast, perfusion of the gut was significantly lower only in 12 d.p.f. animals, but not in 15 d.p.f. animals. Brain perfusion was not affected by hypoxia. Fig. 5 summarizes these differences as percentage of control value (100%).

Discussion

Critique of methods

The methods described in the present study are very sensitive to animal movements, so to restrict their movement the animals were mounted in low-melting-point agarose. The gelling temperature of the agarose (26–30°C) is within the physiological temperature range for zebrafish. High viscosity solutions reduce water convection and therefore increase the magnitude of unstirred layers, which may significantly impair gas exchange (Pinder and Feder, 1990; Feder and Booth, 1992). Measurements of oxygen equilibration in a 15.0 mm layer of 1% agarose compared with a layer of stirred water confirmed that the time necessary to equilibrate the agarose was approximately twice as long.

The quality of the automated red blood cell count was ensured by careful visual inspection of detected blood cells. Double labelling as well as underestimation because of blood cell clusters could easily be detected by visual inspection of the image series. Repeated measurements in the same animal were within an error range of 2%.

Visualization of blood vessels for morphometric analysis is based on the movement of red blood cells, and therefore plasma skimming (Schmid-Schönbein, 1988) or layers of erythrocyte-free plasma may cause an underestimation of the vessel diameter. On the basis of model calculations of red cell movements in a capillary system, this plasma layer is estimated to have a thickness of approximately 1 μm or less (Schmid-Schönbein, 1988). To evaluate the accuracy of our method, we compared the blood vessel thickness determined from the accumulated difference images with the diameter measured from a complete video image of the same site assessed by microscopical inspection. The results differed by no more than 2–3%. We therefore conclude that our volumetric analysis based on the movement detection and the red blood cell count provides very accurate and highly reproducible results.

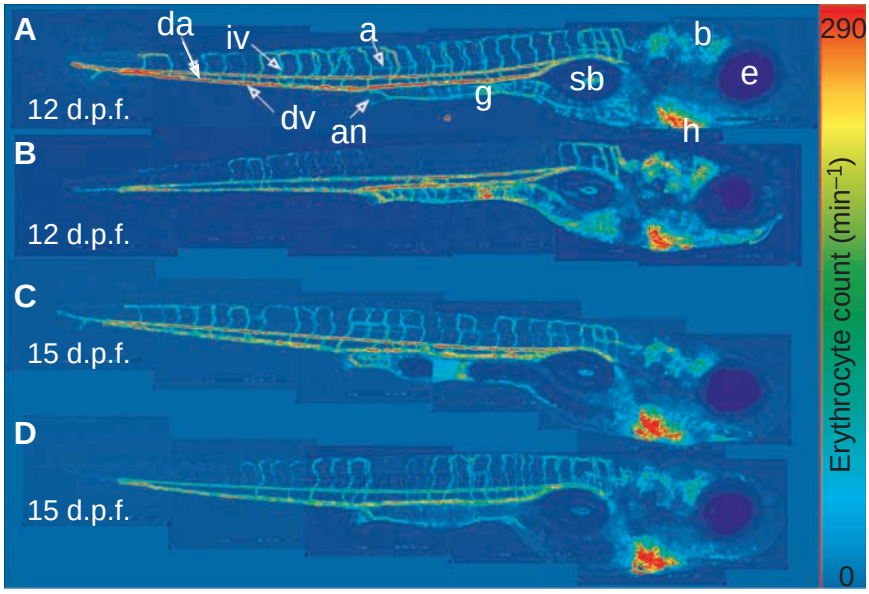

Fig. 4. Typical changes in red blood cell perfusion in zebrafish under chronic hypoxic (A,C) and normoxic (B,D) conditions. Red blood cells were counted per minute at every position of the tissue, and represented in colour according to the calibration bar on the right. d.p.f., days post fertilization; a, anastomosis; an, anus; b, brain; da, dorsal artery; dv, dorsal vein; e, eye; g, gut; h, heart; iv, intersegmental vessel; sb, swimbladder.

The influence of hypoxia

In adult vertebrates, cardiac activity and blood flow to tissues are both mainly determined by metabolic demand. Adult

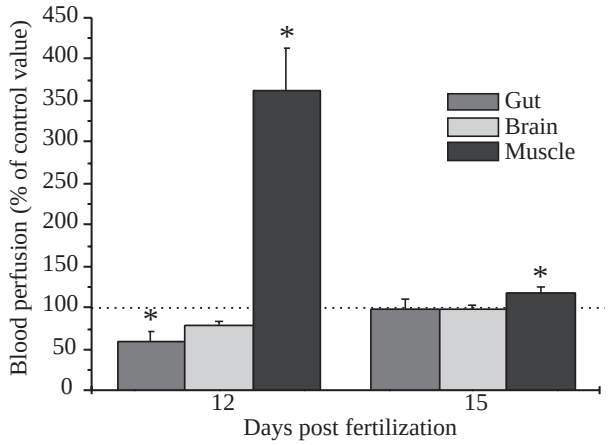

Fig. 5. Blood perfusion changes in zebrafish tissues under chronic hypoxic and normoxic conditions. Values are means ± S.E.M., N=15; *significant difference (P<0.05).

fish and amphibians are typically oxy-regulators, but in the earliest embryonic and larval stages of these vertebrates oxygen uptake appears to decrease with decreasing environmental P_{O_2}, i.e. they are oxyconformers (Hastings and Burggren, 1995). In fact, the first embryonic stages of the zebrafish can survive complete anoxia in a state of suspended animation (Padilla and Roth, 2001), and the Arctic charr can survive several hours of anoxia, with severe metabolic depression and a significantly reduced cardiac activity (Pelster, 1999). Anoxia certainly is an extreme situation for a vertebrate embryo, but mild or chronic hypoxia has also been shown to provoke significant physiological adaptations, ranging from metabolic effects to ventilatory and circulatory adjustments (for a review, see Pelster, 1997), and in zebrafish larvae a stimulation of cardiac activity in response to hypoxia was observed as early as 3 d.p.f. at 28°C (Jacob et al., 2002). Chronic hypoxia during embryonic and larval development may also significantly modify differentiation and growth. In larval amphibians, for example, aquatic hypoxia stimulates growth of respiratory surfaces and enhances the transition from gill to lung respiration (Guimond and Hutchison, 1976; Burggren and Mwalukoma, 1983; Burggren and Just, 1992), but often hypoxia results in retardation of development (Pelster, 1997). In our experiments the first signs of retarded development were observed at 15 d.p.f., and therefore at this age the experiment was terminated in order to prevent a situation where physiological effects induced by hypoxia are mixed up with effects induced by developmental retardation.

Hypoxia and red cell concentration

In mammals hypoxia usually causes an increase in the oxygen transporting capacity of the blood, i.e. an increase in the number of circulating erythrocytes and in hemoglobin concentration. Similar observations have been reported for embryos of the turtle *Pseudemys nelsoni* (Kam, 1993; Jacob et al., 2002), whereas in chicken embryos during early development no stimulation of red cell production was observed (Baumann and Meuer, 1992). The results of our study clearly show that hypoxia increases red blood cell concentration in zebrafish larvae during the second week after fertilization, but no effect was observed until 7 d.p.f. Up to 7 d.p.f., the concentration of red cells remained fairly constant in normoxic as well as in hypoxic animals. Weinstein et al. (1996) showed that the first presumptive proerythroblast-like cells can be detected in zebrafish by the end of the first day (20 h post-fertilization), and this primitive cohort of cells originates in the intermediate cell mass. By transfusing fluorescently labelled blood cells into 1.5-day-old host embryos, recording the fraction of these labelled cells and comparing the histology of the red cells, these authors concluded that this first cohort of primitive red cells provides the embryo with all, or nearly all, its red blood cells for at least 4 days. A population of new, larger and more adult-appearing erythrocytes became predominant by 10 d.p.f.. The constant concentration of red cells observed until 7 d.p.f. in our experiments is in line with these results, but the severe decrease in red cell count at 12 d.p.f. was unexpected. A possible explanation would be that the growing volume of the vascular bed is accompanied by an increase in plasma volume, but not by an equivalent production of erythrocytes. Accordingly, hematocrit and red cell concentration would reduce. An increased cell volume of the newly produced erythrocytes at constant hematocrit would also cause a decrease in red cell concentration, but this latter explanation does not appear very likely.

In hypoxic animals this decrease in the red cell concentration was significantly less than in normoxic animals. If the decrease in red cell concentration observed between 7 d.p.f. and 12 d.p.f. is caused by an expansion of plasma volume, in hypoxic animals the increase in plasma volume would be smaller than in normoxic animals. Information about the total blood volume, however, is necessary to test this idea.

Our method provides a two-dimensional projection of the complete cast of the vascular system. A comparison of projection areas revealed no significant differences between the experimental and the control groups. Assuming a circular cross-section and similar size of vessels, this result means that there was no significant difference in the total volume of the vascular bed in the two groups. However, the acquisition of images for the visualization of the blood redistribution as compared to the acquisition of images to create a complete vascular cast required a compromise between acquisition speed and spatial resolution. Although the resolution of our images was sufficient to show changes in blood distribution, small changes in the diameter of blood vessels, and therefore changes in total blood volume, may not be detectable. For the exact determination of the total blood volume, further studies using a more refined method with higher spatial resolution are needed.

Another explanation for this observation would be that hypoxia stimulates erythropoiesis in zebrafish larvae, as it does in fetal mammals (Richardson and Bocking, 1998) or in embryos of the turtle *Pseudemys nelsoni* (Kam, 1993; Jacob et al., 2002). Recent molecular studies revealed that

hematopoiesis in zebrafish larvae is stimulated by VEGF, and VEGF is also known to drive angiogenesis (Liang et al., 2001). The differential regulation of these processes appears to include a regulatory loop by which VEGF controls survival of hematopoietic stem cells in mice (Gerber et al., 2002). Erythropoietin (EPO), also well known to be involved in hypoxia-induced erythropoiesis, has been shown to mediate hypoxia-induced VEGF expression in rats (Liu et al., 1995).

Blood distribution

A redistribution of blood in response to different metabolic demands is a well-established physiological adaptation in many species. This is the first study to visualize a 'source and sink' pattern of blood distribution in zebrafish larvae in response to oxygen deprivation. It is obvious that under hypoxic conditions blood is driven from the gut (source) to the muscles in the tail (sink), but until 7 d.p.f. no changes in blood distribution were observed. In a previous study we observed a hypoxic stimulation of cardiac activity as early as 3 d.p.f., which demonstrates that at about 1 day after hatching oxygen receptors are present that respond to hypoxia, and this information can be translated into a signal generating an increase in heart rate and in cardiac output (Jacob et al., 2002). Thus, the lack of oxygen can be identified by the larvae, but until 7 d.p.f. this information is not used to induce a change in blood distribution and in erythrocyte production. At 12 d.p.f., however, hypoxia induces a significant increase in the perfusion of the muscle tissue, and the so-called red-layer of muscle has been implicated in the uptake of oxygen in early larvae and at about the time of hatching (El-Fiky and Wieser, 1988). Possibly blood is redistributed towards the muscle tissue in order to enhance oxygen uptake through the body surface under hypoxic conditions. Under normoxic conditions, the oxygen requirements of zebrafish larvae apparently are met by bulk diffusion until 12–14 d.p.f. (Jacob et al., 2002), and this is the time when the gills with secondary lamellae are developed (Rombough, 2002). Also at this time the site of oxygen uptake is shifted towards the gills, and convective oxygen transport becomes a necessity for transporting oxygen from the gills to the tissues. This implies that blood flow is not necessary to sustain aerobic metabolism in normoxic larvae until about 12 d.p.f., but under hypoxic conditions zebrafish larvae are obviously able to stimulate cardiac activity and to redirect blood flow in order to increase perfusion of the muscle tissue. Following the hypothesis that the red layer of muscle may be implicated in the uptake of oxygen in early larvae (El-Fiky and Wieser, 1988), this would suggest that oxygen uptake through the red layer may be enhanced, which, given the small cross-sectional area of the larvae, is essentially cutaneous respiration in the area of the red muscle tissue. To test this idea, we are currently developing a method to visualize changes in hemoglobin oxygenation *in vivo* by recording changes in the absorption spectrum of the hemoglobin.

On the other hand, the redirection of blood may not be related to cutaneous respiration, but simply reflect a maturation of control systems (e.g. hypoxic vasodilation and hypoxic vasoconstriction). The presence of α-adrenergically controlled precapillary sphincters in the intersegmental muscle tissue of zebrafish larvae at 8 d.p.f. has already been shown (Schwerte and Pelster, 2000). Furthermore, a general vasodilation in that tissue may be caused by nitric oxide, as demonstrated by Fritsche et al. (2000) to already occur by 5 d.p.f. Thus, hormonal control mechanisms contributing to a redistribution of blood flow are certainly established at this time of development.

The increase in muscle tissue perfusion could also be a consequence of an increased cardiac output, but this would not explain the decrease in gut perfusion simultaneously recorded in our experiments. In a previous study, Jacob et al. (2002) observed an increase in cardiac output by 20–30% under hypoxic conditions, but in our experiments the effect on cardiac activity was even smaller, so that a change in cardiac activity alone cannot explain the redistribution of blood measured in our experiments.

Another possibility is that the vascular volume in muscle tissue increased during hypoxia. As already mentioned, however, the two-dimensional projections of the complete cast of the vascular system did not reveal an increased vascular volume.

Hypoxia and tissue vascularization

Yue and Tomanek (1999) demonstrated that coronary vessels from cultured 6-day-old quail embryo grow faster under hypoxic conditions, while hyperoxia induced a delayed angiogenesis. By contrast, chorioallantoic membrane capillarization of chicken embryos has been shown to increase during hypoxia (Dusseau and Hutchins, 1988; Hudlicka et al., 1992). In larval amphibians, aquatic hypoxia stimulates growth of respiratory surfaces and enhances the transition from gill respiration to lung respiration (Mwalukoma and Burggren, 1983).

In our study, however, no significant changes in the vascular bed were observed. There appeared to be quite a high interindividual variation in the expression of small blood vessels like the intersegmental anastomsis or the caudal vascular tree, and we cannot exclude the possibility that minor changes in the expression of these vessels did occur in hypoxic animals. The overall morphometric analysis, however, did not show any significant differences between control and hypoxic animals. Compared with the development of amphibians such as *Xenopus* or *Rana*, or the development of salmonid larvae, zebrafish development is very rapid, and it may be possible that a rearrangement of the vascular bed can only occur in later developmental stages. On the other hand, molecular signals involved in the formation of blood vessels have been identified in early embryonic stages. The molecular mechanisms that lead to the extremely regular pattern in the zebrafish trunk have been shown by Childs et al. (2002), and VEGF up- and down-regulation seems to be involved in this process. Whole-mount *in situ* hybridization of zebrafish embryos indicated that strong expression of VEGF had already occurred at 18 h post fertilization (Weinstein et al., 1996; Liang et al., 1998; Tan et al., 2001).

Physiological significance

Under normoxic conditions, oxygen supply *via* diffusion seems to be sufficient to meet the metabolic demand up to 12–14 d.p.f. This changes during hypoxia, however, because the reduced oxygen gradient cuts down diffusion of oxygen to the tissues. The data available so far show that in this situation convective transport can be enhanced by increasing blood flow (Jacob et al., 2002), and the oxygen carrying capacity of the blood can be increased. In addition, blood flow can be redirected towards a potential site of oxygen uptake. Thus, although under normoxic conditions convective transport is not necessary, the larvae can use the circulatory system as a back-up system to augment oxygen distribution and oxygen supply to the tissues, which is strong evidence that the cardiovascular system can operate as a convective transport system for oxygen much earlier then required under normal circumstances. This situation was called 'prosynchronotropy' by Burggren and Fritsche (1995). A prosynchronotropic development of convective oxygen transport appears to be very useful, because it creates a safety belt for a situation where bulk oxygen diffusion alone would not be sufficient to ensure oxygen supply to all tissues, and thus widens the range of environmental conditions in which the larvae can survive. Angiogenesis, however, seems not to play a key role in facilitating oxygen uptake. It could be that in the small zebrafish larvae the diffusion distances between tissue capillaries and the cells are small enough to permit an optimal supply with oxygen, so that additional blood vessels would not yet enhance oxygen transport to the cells.

The present study was supported by the Fonds zur Förderung der wissenschaftlichen Forschung (FWF P14976-BIO and FWF P16272-B06).

References

Barut, B. A. and Zon, L. I. (2000). Realizing the potential of zebrafish as a model for human disease. *Physiol. Genomics* **2**, 49-51.

Baumann, R. and Meuer, H.-J. (1992). Blood oxygen transport in early avian embryo. *Physiol. Rev.* **72**, 941-965.

Burggren, W. and Fritsche, R. (1995). Cardiovascular measurements in animals in the milligram range. *Braz. J. Med. Biol. Res.* **28**, 1291-1305.

Burggren, W. and Mwalukoma, A. (1983). Respiration during chronic hypoxia and hyperoxia in larval and adult bullfrogs (*Rana catesbeiana*) I. Morphological responses of lungs, skin and gills. *J. Exp. Biol.* **105**, 191-203.

Burggren, W. W. and Just, J. J. (1992). Developmental changes in physiological systems. In *Environmental Physiology of the Amphibians* (ed. M. E. Feder and W. W. Burggren), pp. 467-530. Chicago: The University of Chicago Press.

Burggren, W. W. and Keller, B. B. (1997). *Development of Cardiovascular Systems: Molecules to Organisms.* Cambridge: Cambridge University Press.

Childs, S., Chen, J. N., Garrity, D. M. and Fishman, M. C. (2002). Patterning of angiogenesis in the zebrafish embryo. *Development* **129**, 973-982.

Dusseau, J. W. and Hutchins, P. M. (1988). Hypoxia-induced angiogenesis in chick chorioallantoic membranes: a role for adenosine. *Respir. Physiol.* **71**, 33-44.

El-Fiky, N. and Wieser, W. (1988). Life styles and patterns of development of gills and muscles in larval cyprinids (Cyprinidae; Teleostei). *J. Fish Biol.* **33**, 135-145.

Feder, M. E. and Booth, D. T. (1992). Hypoxic boundary layers surrounding skin-breathing aquatic amphibians: Occurrence, consequences and organismal responses. *J. Exp. Biol.* **166**, 237-251.

Fritsche, R., Schwerte, T. and Pelster, B. (2000). Nitric oxide and vascular reactivity in developing zebrafish, *Danio rerio*. *Am. J. Physiol Regul. Integr. Comp. Physiol.* **29**, R2200-R2207.

Gerber, H. P., Malik, A. K., Solar, G. P., Sherman, D., Liang, X. H., Meng, G., Hong, K., Marsters, J. C. and Ferrara, N. (2002). VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. *Nature* **417**, 954-958.

Gielen, J. L. and Kranenbarg, S. (2002). Oxygen balance for small organisms: an analytical model. *Bull. Math. Biol.* **64**, 175-207.

Guimond, R. W. and Hutchison, V. H. (1976). Gas exchange of the giant salamanders of North America. In *Respiration of Amphibious Vertebrates* (ed. G. M. Hughes), pp. 313-338. London, New York, San Francisco: Academic Press.

Hastings, D. and Burggren, W. (1995). Developmental changes in oxygen consumption regulation in larvae of the South African clawed frog *Xenopus laevis*. *J. Exp. Biol.* **198**, 2465-2475.

Hudlicka, O., Brown, M. and Egginton, S. (1992). Angiogenesis in skeletal and cardiac muscle. *Physiol. Rev.* **72**, 369-417.

Isogai, S., Horiguchi, M. and Weinstein, B. M. (2001). The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev. Biol.* **230**, 278-301.

Jacob, E., Drexel, M., Schwerte, T. and Pelster, B. (2002). Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R911-R917.

Kam, Y.-C. (1993). Physiological effects of hypoxia on metabolism and growth of turtle embryos. *Respir. Physiol.* **92**, 127-138.

Liang, D., Chang, J. R., Chin, A. J., Smith, A., Kelly, C., Weinberg, E. S. and Ge, R. (2001). The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. *Mech. Dev.* **108**, 29-43.

Liang, D., Xu, X., Chin, A. J., Balasubramaniyan, N. V., Teo, M. A., Lam, T. J., Weinberg, E. S. and Ge, R. (1998). Cloning and characterization of vascular endothelial growth factor (VEGF) from zebrafish, *Danio rerio*. *Biochim. Biophys. Acta* **1397**, 14-20.

Liu, Y. X., Cox, S. R., Morita, T. and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells: identification of a 5′ enhancer. *Circ. Res.* **7**, 638-643.

Mwalukoma, A. and Burggren, W. W. (1983). Respiration during chronic hypoxia and hyperoxia in larval and adult bullfrogs (*Rana catesbeiana*). I. Morphological responses of lungs, skin and gills. *J. Exp. Biol.* **105**, 191-203.

Padilla, P. A. and Roth, M. B. (2001). Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proc. Natl. Acad. Sci. USA* **98**, 7331-7335.

Pelster, B. (1997). Oxygen, temperature, and pH influences on the development of nonmammalian embryos and larvae. In *Development of Cardiovascular Systems* (ed. W. W. Burggren and B. B. Keller), pp. 227-239. Cambridge: Cambridge University Press.

Pelster, B. (1999). Environmental influences on the development of the cardiac system in fish and amphibians. *Comp. Biochem. Physiol. A* **124**, 407-412.

Pelster, B. and Bemis, W. E. (1991). Ontogeny of heart function in the little skate *Raja erinacea*. *J. Exp. Biol.* **156**, 387-398.

Pelster, B. and Burggren, W. W. (1996). Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (*Danio rerio*). *Circ. Res.* **79**, 358-362.

Pinder, A. W. and Feder, M. E. (1990). Effect of boundary layers on cutaneous gas exchange. *J. Exp. Biol.* **154**, 67-80.

Richardson, B. S. and Bocking, A. D. (1998). Metabolic and circulatory adaptations to chronic hypoxia in the fetus. *Comp. Biochem. Physiol. A* **119**, 717-723.

Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. *J. Exp. Biol.* **205**, 1787-1794.

Schmid-Schönbein, H. (1988). Fluid dynamcis and hemorheology in vivo: The interactions of hemodynamic parameters and hemorheological 'properties' in determining the flow behavior of blood in microvascular networks. In *Clinical Blood Rheology*, Vol. 1 (ed. G. D. O. Lowe), pp. 129-219. Boca Raton: CRC Press.

Schwerte, T. and Pelster, B. (2000). Digital motion analysis as a tool for analysing the shape and performance of the circulatory system in transparent animals. *J. Exp. Biol.* **203**, 1659-1669.

Tan, D. C., Kini, R. M., Jois, S. D., Lim, D. K., Xin, L. and Ge, R. (2001). A small peptide derived from Flt-1 (VEGFR-1) functions as an angiogenic inhibitor. *FEBS Lett.* **494**, 150-156.

Territo, P. R. and Altimiras, J. (1998). The ontogeny of cardio-respiratory function under chronically altered gas compositions in *Xenopus laevis*. *Respir. Physiol.* **111**, 311-323.

Territo, P. R. and Burggren, W. W. (1998). Cardio-respiratory ontogeny during chronic carbon monoxide exposure in the clawed frog *Xenopus laevis*. *J. Exp. Biol.* **201**, 1461-1472.

Warren, K. S. and Fishman, M. C. (1998). 'Physiological genomics': mutant screens in zebrafish. *Am. J. Physiol.* **275**, H1-H7.

Weinstein, B. M., Schier, A. F., Abdelilah, S., Malicki, J., Solnica-Krezel, L., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Driever, W. and Fishman, M. C. (1996). Hematopoietic mutations in the zebrafish. *Development* **123**, 303-309.

Yue, X. and Tomanek, R. J. (1999). Stimulation of coronary vasculogenesis/angiogenesis by hypoxia in cultured embryonic hearts. *Dev. Dyn.* **216**, 28-36.