The influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae

Jacob, E., Drexel, M., Schwerte, T. and Pelster, B.

Institut für Zoologie und Limnologie, Universität Innsbruck, Austria

Running head: Hypoxia and hypoxemia in zebrafish larvae

Address for correspondence:
Dr. Bernd Pelster
Institut für Zoologie und Limnologie
Leopold-Franzens-Universität Innsbruck
A-6020 Innsbruck
Austria
Tel.: +43 512 5076180
Fax.: +43 512 5072930
e-mail: Bernd.Pelster@uibk.ac.at

Copyright 2002 by the American Physiological Society.
Abstract

Cardiac activity and anaerobic metabolism were analyzed in zebrafish larvae raised under normoxia (PO$_2$ = 20 kPa) and under chronic hypoxia (PO$_2$ = 10 kPa) at three different temperatures (25°C; 28°C; and 31°C). Heart rate increased with development and with temperature. Under normoxia, cardiac output increased significantly at high temperature (31°C), but not at 28°C or at 25°C. Under chronic hypoxia, however, heart rate as well as cardiac output increased at all temperatures in larvae at about hatching time or shortly thereafter. Cardiac activity of larvae raised for two weeks after fertilization with a reduced hemoglobin oxygen-carrying capacity in their blood (hypoxemia; due to the presence of CO or of phenylhydrazine in the incubation water) was not different from control animals. Whole body lactate content of these animals did not increase. Thus, there was no indication of a stimulated anaerobic energy metabolism. The increase in cardiac activity observed during hypoxia suggests that at about hatching time receptors are present which sense hypoxic conditions, and this information can be used to induce a stimulation of convective oxygen transport in order to compensate for a reduction in bulk oxygen diffusion in the face of a reduced oxygen gradient between environmental water and tissues. Under normoxia, however, the PO$_2$ gradient between environmental water and tissues and diffusional oxygen transport assure sufficient oxygen supply even if hemoglobin oxygen transport in the blood is severely impaired. Thus, under normoxic conditions, and with a normal metabolic rate of the tissues, convective oxygen transport is not required until about two weeks after fertilization.
Introduction

Temperature induced changes in metabolism and in cardiac activity as well as allometric relationships describing the increase in oxygen uptake and the changes in cardiac output with body mass demonstrate that in early larval stages a tight coupling between cardiac activity and metabolic requirements of tissues is not established (18;23;26;29). Furthermore, in early stages convective oxygen transport apparently does not contribute to the oxygen supply to tissues (24;29;30). Instead, oxygen supply is achieved by bulk oxygen diffusion through the body surface. Diffusional oxygen transport is only efficient if the diffusion distance is less than a mm, and a morphometric analysis of *Xenopus* larvae combined with model calculations demonstrated that it therefore is highly likely that small eggs and embryos do not require convectional oxygen transport (29). With increasing body mass, however, convectional oxygen transport will soon become a necessity. In larger embryos and larvae convectional oxygen transport therefore may become of importance much earlier during development than in smaller ones, and the coupling between tissue metabolism and cardiac activity may significantly depend on the size of the species. Based on this assumption one may postulate that the coupling between tissue metabolism and cardiac activity in the small zebrafish larvae is achieved much later than in *Xenopus* larvae.

The linkage between metabolism and cardiac activity also provides the background for the stimulation of cardiac activity typically observed under hypoxic conditions in adult vertebrates. Similar studies performed with embryos and larvae suggested that at least in the earliest stages cardiac activity is not modified under hypoxia. A sometimes observed decrease in cardiac activity was explained as a direct response of cardiomyocytes to the lack of oxygen (7;19). This explanation is in line with a severe metabolic depression, observed in larvae of the Arctic char (*Salvelinus alpinus*) during transient complete lack of oxygen (anoxia; 23), and exposure of early embryonic stages of the zebrafish to anoxia even induces a status of suspended animation (20).
In contrast to anoxia hypoxic conditions provoked a minor but significant tachycardia in salmonid larvae (9;17). If the direct effect of hypoxia on cardiac muscle cells is a reduction in activity, an increase in heart rate under these conditions must be due to an external stimulation, which can only be explained as a coordinated response. Larvae of the African clawed frog *Xenopus leavis*, however, up to Nieuwkoop-Faber (NF) stage 51 do not respond to a reduction in $\text{PO}_2$ of about 50% (28;30). Given the relation of body size and diffusional oxygen transport it can be speculated that these conflicting results may be related to differences in body mass. Salmonid larvae and *Xenopus* larvae are of similar size. Salmonids, however, prefer lower temperatures of about 10-15°C, while *Xenopus* is usually raised at temperatures of about 25°C. It therefore may be possible that the influence of temperature on diffusional gas transport may contribute to these results. Alternatively, control of cardiac activity, which in *Rana temporaria*, *Xenopus* and chicken is only established late during development (6;14;21;25), might be established earlier in fish. This would imply, however, that the onset of the linkage between metabolic demand of tissues and of cardiac activity is not only determined by the increasing body mass. To test this possibility it would be useful to get information on zebrafish larvae, which are several times smaller than *Xenopus* or salmonid larvae.

In order to identify the point in development when cardiac activity becomes responsive to hypoxia and in order to question the importance of diffusional oxygen supply through the skin versus convective oxygen transport in the blood, we raised zebrafish under normoxic and hypoxic conditions, and under conditions with a chronically reduced hemoglobin oxygen transport capacity in the blood (hypoxemia). We used zebrafish larvae because the small body size should facilitate diffusional oxygen transport and render convectional oxygen transport less important even in later developmental stages. Furthermore, zebrafish has become a widely used model to explore the mechanisms of early heart development (16). Thus, the experiments will test the hypothesis that reliance on convectional oxygen transport is mainly
dependent on body mass, and not on the developmental stage. The results reveal that even in the small zebrafish larvae cardiac activity becomes responsive to environmental hypoxia already at about the time of hatching. This is even earlier than in *Xenopus* larvae, which is significantly larger than zebrafish. Nevertheless, convective oxygen transport becomes crucial for aerobic metabolism of zebrafish larvae only at about two weeks after fertilization.

**Materials and Methods**

*Animals*

The experiments were performed using larvae of the zebrafish (*Danio rerio*), which were obtained from our breeding colony. Because of a better transparency poorly pigmented mutants of the zebrafish (Albino, Brass) were used. Parent animals to start the breeding colonies were either obtained from a local supplier or generously provided by Dr. Frohnhöfer from the Max-Planck Institute for Developmental Biology in Tübingen and Mrs. Loos from the University of Konstanz. Breeding colonies and larvae were kept in small aquaria at a temperature either of 25°C, 28°C or 31°C.

*The imaging system*

The imaging system consisted of an inverted microscope (Zeiss Axiovert 25) equipped with a 2/3” machine vision CCD-Camera (Hamamatsu C 2400 without infrared cut-off filter). The camera was connected to the luminance input of a SVHS video recorder (Sony S-9500). The VCR was remote controlled via the RS232 serial communication port. The setting of the video recorder as well as the recorded images were digitized by a monochrome frame grabber card (Imagination PX-610) with a personal computer (PIII 450 MHz). The illumination could be reduced to infrared light with a wavelength of 780 nm or 913 nm in order to prevent light induced stress reactions of the animals. The microscope stage was temperature controlled.
Recording of cardiac activity

Larvae were transferred into the temperature controlled incubation chamber of the microscope stage. The temperature was set to the incubation temperature of the eggs. Early stage larvae typically rested on the bottom of the incubation chamber, so that video recordings of cardiac activity could be taken without any anesthesia and without any physical restriction of swimming activity. At the time of swimbladder inflation larvae raise to the surface, and cardiac activity could only be measured under anesthesia. Starting at 5dpf or 6 dpf (depending on the temperature) larvae therefore were anesthetized with 0.08 mg/ml tricaine (MS222), neutralized with phosphate buffer. A comparison of anesthetized and free swimming larvae revealed that MS222 at these early developmental stages has no influence on cardiac activity. Heart rate was determined by measuring the time interval for 30 heart beats. Determination of stroke volume, using digital image analysis (27), followed the method described by Hou and Burggren (10). Video sequences of the ventricle were saved in computer memory. The perimeter of the ventricle image was outlined manually during end diastole and during end systole using a mouse or a graphic tablet. The perimeter was analyzed with a “fit-to-ellipse” algorithm, which first calculated the center of mass of the perimeter and subsequently the best fitting ellipse (26). The major and minor axis of the ellipse were extracted and directly transferred into a Microsoft Excel worksheet for calculation of stroke volume using the formula for a prolate spheroid \(4/3\pi*a*b^2\) (10). For analysis five diastoles and systoles were analyzed, and mean stroke volume was calculated as the difference between diastolic and systolic ventricular volume.

Contraction speed was calculated by counting the number of video fields (time resolution = 20ms) from the end diastolic to the end systolic stage of the ventricle. Time resolution was enhanced by anti aliasing the changes in endsystolic volume during a period of six subsequent cardiac cycles. Average end diastolic and end systolic volumes determined from six end diastolic and end systolic turning points were used as reference points. The
apparent end diastolic or systolic volumes measured from the video images were corrected to
the real turning point by extrapolation to the reference point. This way the actual time
resolution was improved to about 10 msec.

**Calculation of cardiac output and $Q_{10}$**

Cardiac output was calculated as product of stroke volume times heart rate. $Q_{10}$ of heart
rate, stroke volume and cardiac output was calculated as

$$Q_{10} = \left( \frac{B_{T2}}{B_{T1}} \right)^{10(T2-T1)}$$

where $B$ is the mean of the respective variable (heart rate, stroke volume or cardiac output) at
either high temperature (31°C, = T2) or at low temperature (25°C, = T1). Because
extrapolation from temperature intervals of only 2 or 3°C to a 10°C interval can generate
severe scatter of the data, only values obtained at 25°C and at 31°C have been used for this
analysis.

**Experimental protocol**

In a first set of experiments the influence of hypoxia on the development of the
cardiovascular system was tested. About 20 hours after spawning eggs were transferred to
hypoxic water ($PO_2 = 10kPa$, prepared by equilibrating water with a mixture of air and $N_2$) at
a temperature of 28°C and raised until 5 dpf (days post fertilization). Starting at 2 dpf about
15 to 20 animals were removed from the tank every morning for analysis of cardiac activity.
For comparison a control group was raised under normoxic conditions ($PO_2 = 20kPa$). The
sampling regime was as described before. The experiments were repeated at 25°C and at
31°C. Due to the slower development at 25°C measurements were performed until 6 dpf,
while at 31°C the same developmental stage was already reached at 4 dpf.

In a second set of experiments the influence of a reduced oxygen-carrying capacity in
the blood was tested ($T = 25°C$). About 20 h after spawning eggs were transferred into an
aquarium equilibrated with 2% CO in air (PO$_2$ about 20 kPa, = normoxia) and raised until 15 dpf. At 3, 4, 5, 8, 11 and 15 dpf a group of larvae was removed from the tank for analysis of cardiac activity. A second group of animals was similarly raised in normoxic water (PO$_2$ = 20 kPa) containing 2 mg/l phenylhydrazine, which is known to oxidize hemoglobin, but no additional direct side effects have been described so far (12). A third group was raised as control group in normoxic water. The sampling regime was the same for all three groups.

Measurement of whole body lactate content

For measurement of whole body lactate content larvae were removed from the aquarium and immediately frozen in liquid nitrogen. Tissue of individual larvae was extracted by adding 25 µl of metaphosphoric acid and subsequent homogenization. Lactate concentration of the extract was determined enzymatically in dublicate readings according to Bergmeyer (1). The conversion of NAD$^+$ to NADH induces an increase in light absorbance at 340 nm, but it also increases the fluorescence signal of this substance. The change in fluorescence of NADH was determined using a plate reader (fmax, Molecular Devices, Munich, Germany).

Statistical analysis

The acquired data and also the extracted data from the video recordings were exported into an ASCII file for statistical analysis. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by a multiple-comparison procedure (Bonferroni) (software package Statistica). Significance was accepted when $P<0.05$. Data are presented as mean ± SE.

Results

No significant increase in body mass (including the yolk mass) was observed until complete opening of the swimbladder (Fig. 1), but at all three temperatures tested heart rate
increased with development in larvae of the zebrafish. Heart rate also increased with
temperature.

Fig. 1.
Changes in body mass of eggs and larvae of the zebrafish raised at 25°C, 28°C and 31°C until
complete opening of the swimbladder (Mean ± SE; n = 10).

At 25°C, hypoxic conditions caused a significant increase in heart rate at 5 and 6 dpf,
but not between 2 and 4 dpf (Fig. 2A). In animals raised under hypoxia cardiac output was
significantly elevated at 4, 5 and 6 dpf (Fig. 2B). A comparison of systolic and end-diastolic
ventricular volume suggested that an increase in diastolic volume in hypoxic animals caused
an increase in stroke volume at 4, 5 and 6 dpf (Fig. 2C), which contributed to the increase in
cardiac output observed at this point of development. Similarly, in animals raised at 28°C
heart rate started to increase significantly compared to control animals at 4 dpf (Fig. 2D), and
cardiac output was significantly elevated at 3, 4, and 5 dpf (Fig. 2E). The comparison of
systolic and end-diastolic ventricular volume again revealed a significantly increased diastolic
volume at 3 and 4 dpf, but this increase did not persist on 5dpf (Fig. 2F). A slightly different
picture emerged in animals raised at 31°C under normoxic and hypoxic conditions. Heart rate
was elevated at 2 dpf, but not at 3 and 4 dpf (Fig. 2G). Cardiac output was elevated at 3 and 4
Fig. 2.

Cardiac activity [heart rate, (panels A, D, G); cardiac output (panels B, E, H); systolic and diastolic ventricular volume (panels C, F, I)] in zebrafish larvae raised under normoxia (PO$_2$ = 20 kPa) and under chronic hypoxia (PO$_2$ = 10 kPa) at a temperature of 25$^\circ$C (panels A, B, C), 28$^\circ$C (panels D, E, F) or 31$^\circ$C (panels G, H, I). Experiments performed at 25$^\circ$C, n = 10; at 28$^\circ$C, n = 12 for 2 and 5 dpf; 3 dpf, n = 18; 4 dpf, n = 16; experiments performed at 31$^\circ$C, n = 12. *, significantly different from controls (p < 0.05)

dpf, but this difference was only significant at 3 dpf (Fig. 2H). Differences in systolic and diastolic volume were only observed at 4 dpf, where systolic as well as diastolic volume was significantly elevated in hypoxic animals (Fig. 2I). In consequence, stroke volume was not different at 4 dpf in hypoxic and normoxic animals.
An important information included in the data presented in figure 2 is the change in cardiac output induced by development between the first heart beat and functioning of the swimbladder, and the effect of hypoxia on these changes. Under normoxic conditions, a significant increase in cardiac output was observed only at 31°C, but at 25°C and at 28°C, development of the larvae from the first heart beat to the inflation of the swimbladder was not accompanied by an increase in cardiac output. In animals raised under hypoxic conditions (PO₂ = 10 kPa), however, cardiac output was significantly elevated at 25°C and at 28°C. At 31°C, hypoxia did not enhance the increase in cardiac output, which was already observed under normoxia.

The speed of cardiac contraction can be used as an indicator of myocardial contractility. In control animals (28°C) at the time of swimbladder opening 210 ± 13.2 msec (mean ± s.d.; n = 7) elapsed between the onset of contraction and end systole, and a similar value was recorded for hypoxic animals (200 ± 15.9 msec). Similarly, no difference in myocardial contractility was observed at the first day of cardiac contraction (2 dpf; 300 ± 15.9 msec and 300 ± 18.5 msec, respectively). The same was true at 25°C and 31°C, no difference in myocardial contractility was observed between control animals and animals raised under hypoxia.

The data presented in figure 2 can also be used to calculate Q₁₀ values. While the Q₁₀ for stroke volume was mostly in the range of 1.0 to 1.2, the Q₁₀ for heart rate as well as for cardiac output was much higher with values around 1.8 to 2.2.

In a second set of experiments larvae were raised with chronically reduced oxygen-carrying capacity in their blood, either by incubation with a CO containing atmosphere or by incubation with phenylhydrazine. A comparison of heart rate in animals of these two groups
Fig. 3

The development of heart rate (A) and of stroke volume (B) in zebrafish larvae raised under normoxia and with a significantly reduced hemoglobin oxygen-carrying capacity.

Hemoglobin oxygen-carrying capacity was reduced by raising the animals in the presence of CO or of phenylhydrazine in the incubation water. T = 25°C; n values are listed in brackets; *, significantly different from controls (P<0.05).

8 dpf, heart rate of animals raised with a reduced oxygen-carrying capacity in their blood was about 20 beats/min lower than in control animals (p<0.05). At 11 dpf this difference disappeared again. At 15 dpf animals raised in the presence of phenylhydrazine again had a reduced heart rate, while heart rate of animals raised in the presence of CO was not different.
from controls (Fig. 3A). Stroke volume showed almost no differences in all three groups. Only in animals raised in the presence of phenylhydrazine a reduced stroke volume was observed at 15 dpf (Fig. 3B).

Because the reduction in hemoglobin oxygen-carrying capacity had very little influence on heart rate and on stroke volume, it was not surprising that cardiac output was hardly influenced. In all three groups no difference in cardiac output was observed until 11 dpf (Fig. 4). Only in phenylhydrazine animals a 30% reduction in cardiac output was observed at 15 dpf. Animals raised in the presence of CO had the same cardiac output as control animals until 15 dpf. The experiment was terminated at 15 dpf because the viability of animals raised in the presence of CO or of phenylhydrazine was reduced and the first casualties occurred.

Fig. 4
Changes in cardiac output in zebrafish larvae raised under normoxia and with a significantly reduced hemoglobin oxygen-carrying capacity. Hemoglobin oxygen-carrying capacity was reduced by raising the animals in the presence of CO or of phenylhydrazine in the incubation water. T = 25°C; see Fig. 3B for n-values; *, significantly different from controls (P<0.05).
To test for a possible stimulation of lactate production in animals raised with a reduced hemoglobin oxygen-carrying capacity, whole body lactate content was measured. With only one single exception whole body lactate content of animals raised with a reduced oxygen-carrying capacity did not show an elevated lactate content in their body (Fig. 5). Only in animals raised in the presence of phenylhydrazine at 8 dpf a 40% increase in body lactate was found, but this was again alleviated at 11 dpf.

Fig. 5
Whole body lactate content in zebrafish larvae raised under normoxia and with a significantly reduced hemoglobin oxygen-carrying capacity. Hemoglobin oxygen-carrying capacity was reduced by raising the animals in the presence of CO or of phenylhydrazine in the incubation water. T = 25°C; n = 6; *, significantly different from controls (P<0.05).

Discussion

Morphological changes

Chronic hypoxia during embryonic and larval development may significantly modify differentiation and growth (see (22) for review). Several studies reported retarded growth rates in fish larvae under hypoxia. On the other hand, chorioallantoic membrane capillarization of chicken embryos is increased during hypoxia (5;13). In larval amphibians,
aquatic hypoxia stimulates growth of respiratory surfaces and enhances the transition from gill respiration to lung respiration (3;4;8). Compared to the development of amphibians like *Xenopus* or *Rana* or the development of salmonid larvae, zebrafish development is very rapid and even at the lowest temperature our study with hypoxic animals did not exceed 6 days of development. During this time we did not observe any morphological differences (including gill development) or differences in body weight between hypoxic animals and control animals. Hypoxia-induced morphological adaptations therefore did not contribute to the results of our study.

*Control of cardiac activity*

Previous studies, especially with *Xenopus laevis*, demonstrated that early stages do not show a regulated response on exposure to hypoxia. At low PO$_2$ cardiac activity eventually decreases, which is interpreted as a direct affect of hypoxia on cardiomyocytes (7;19). The results of our study, in which zebrafish larvae were raised under chronically hypoxic conditions, confirm that very early stages do not respond to hypoxia. At all three temperatures the first 24 h of hypoxia did not elicit any change in cardiac output. But already at 3 dpf (28°C), which is around hatching or shortly after hatching, chronically hypoxic larvae show a significantly elevated cardiac output. If the direct effect of hypoxia on cardiomyocytes is a decrease in activity this increase in activity must be caused by external stimulation of the heart, and thus clearly is a regulated response. Previous studies demonstrated the early presence and functioning of oxygen sensors, because environmental hypoxia stimulates ventilation in very early stages of *Salvelinus alpinus* and *Rana catesbeiana*, for example (2;17;22). At least a mild tachycardia has also been observed in hypoxic larvae of salmonids at 1 day after hatching (9;17), which in terms of development is a slightly later stage then the first response observed in zebrafish larvae in the present study. These results are in contrast to data reported for *Xenopus* larvae raised under chronic hypoxia. *Xenopus* raised under normoxia (PO$_2$ = 21 kPa), hypoxia (PO$_2$ = 11 kPa) or under hyperoxia (PO$_2$ = 35 kPa) until
Nieuwkoop-Faber (NF) stage 51 showed no difference in oxygen uptake and in cardiac activity (28;30). Thus, fish larvae may respond to environmental hypoxia at an earlier stage than amphibian larvae. Alternatively, a PO\(_2\) of 11 kPa may not have been low enough to elicit a coordinated response in *Xenopus* larvae. The latter explanation is not very likely, because for our experiments we used a similar PO\(_2\) of 10 kPa, and this was very close to the lower threshold which permitted a complete development of zebrafish larvae without significantly increasing the rate of mortality.

*The influence of body mass and of basic cardiac activity*

This difference in the responsiveness to hypoxia was unexpected because body mass of *Xenopus* larvae in the early developmental stages is about ten times as high as body mass of zebrafish larvae. The larger *Xenopus* larvae therefore should have been more sensitive to a decrease in PO\(_2\) gradient between environment and tissues. Oxygen diffusion and oxygen capacity of fluids varies with temperature, but Territo and Altimiras (28) performed their study on *Xenopus* at a temperature of 24\(^\circ\)C, which is very close to our low value of 25\(^\circ\)C. The observed difference in the response to hypoxia therefore cannot be attributed to temperature dependent differences in diffusional oxygen transport.

A comparison of the three temperatures selected for our experiments resulted in obvious differences in the magnitude of the response. The smallest increase by far in cardiac activity was observed in animals raised at 31\(^\circ\)C. Given the increase in metabolic rate with increasing temperature metabolic activity in these animals is higher than in animals raised at lower temperature, and it may be expected that these animals are most sensitive to hypoxia. The comparatively small increase in cardiac activity may be due to the fact that cardiac activity of zebrafish larvae is already very high at 31\(^\circ\)C. Under normoxia heart rate reaches values of about 250 to 300 beats-min\(^{-1}\). This is a level that was reached by larvae raised at 28\(^\circ\)C under
hypoxic conditions. It may be that this is about the highest heart rate the larvae can reach, and therefore a further stimulation is hardly possible. The results of our experiments showed that 31°C is about the upper thermal limit for the proper development of zebrafish.

_Hypoxia versus hypoxemia_

Hypoxic conditions may not only arise from environmental hypoxia, they may also arise in normoxia if the oxygen demand of the tissue exceeds oxygen delivery to the tissue. This can be caused by a stimulation of the metabolic demand (e.g. exercise), or by a reduction in the oxygen-carrying capacity in the blood (hypoxemia). In most vertebrates the first red cells are observed very early during development, and hematopoiesis is characterized by successive waves of development, including a primitive and a definitive phase of hematopoiesis, and including various anatomical sites at which hematopoiesis is observed (15). Consequently, experiments have been performed to induce hypoxic conditions by decreasing the hemoglobin oxygen transport capacity. Hypoxemia has been induced using two different methods, by blocking the oxygen binding site of the hemoglobin with CO, and by chemical ablation of the hemoglobin by incubating the animals with phenylhydrazine. Both methods have successfully been used for adult fish and fish or amphibian larvae (12;24;29;30), and none of these studies provided any indication for sides effects which may have influenced the results, even in long term studies. After inducing anemia by an incubation with phenylhydrazine goldfish have been observed for several weeks (11).

In hypoxemic animals no significant modification of cardiac activity during the first two weeks of development was observed. This result provides additional support for the conclusion drawn in previous studies that convective oxygen transport is not essential to assure oxygen supply to the tissues of larvae in early stages (18;23;24;28-30). In early developmental stages oxygen supply to tissues is achieved by bulk oxygen diffusion through the body wall, and oxygen consumption in _Xenopus laevis_ (28;30) or in zebrafish (24) is not
reduced if hemoglobin oxygen transport is impaired. Our present results also reveal that anaerobic metabolism, i.e. lactate production, is not stimulated under these conditions suggesting that aerobic energy production can be sustained even if hemoglobin oxygen transport is largely reduced. Somewhat unexplained remains the transient increase in whole body lactate content at 8 dpf in hypoxemic animals. We could not identify any developmental landmark that might explain this stimulation of anaerobic metabolism at 8 dpf.

Thus, hypoxemia does not stimulate cardiac activity in zebrafish larvae, but hypoxia does. Under hypoxemia the $\text{PO}_2$ gradient between the environmental water and the tissues is not different from normoxic conditions, and thus bulk oxygen diffusion is sufficient to meet the oxygen requirements of the tissues. Under hypoxia, however, the diffusion gradient of oxygen is reduced, and even in the small zebrafish larvae (body weight less than 1 mg at this stage) this induces an increase in convective oxygen transport in order to compensate for the reduction in oxygen diffusion. This also demonstrates that in zebrafish larvae at hatching time the afferent nervous system is able to sense hypoxic conditions. Furthermore, central control units are active and bring about a coordinated response to environmental perturbations (like oxygen deficiency), either by direct stimulation of the heart, or by stimulation of hormone secreting cells (like chromaffin cells). The secreted hormone in turn could then activate cardiac activity.

Interesting and not yet completely explained is the observation of Territo and Altimiras (28) that stroke volume and cardiac output are significantly enhanced in *Xenopus laevis* under chronic exposure to CO, but not under hypoxia. This is in clear contrast to our present observations on zebrafish larvae, and the increase in cardiac output was only observed in very early stages of *Xenopus*, not in later stages with a significantly larger body mass. The authors conclude that the increase in cardiac output observed in embryonic stages was intrinsic in nature and did not significantly improve oxygen uptake. In fact, aerobic metabolism was similar in all experimental groups.
**Perspectives:** The observation that the circulatory system of embryonic and early larval stages is not working in order to supply the tissue with oxygen and nutrients was accepted given the small size of the larvae. It was assumed that the physical limitations of diffusion through tissues eventually would induce the linkage between cardiac activity and metabolic requirements of tissues, as it is well established for adults. Our results show that intrinsic developmental aspects appear to play a role as well. Although the zebrafish larvae are about ten times smaller than *Xenopus* larvae, external stimulation of cardiac activity in response to environmental hypoxia becomes possible in zebrafish earlier than in *Xenopus* larvae. It has been proposed that the proper vascularization of tissues may require early blood flow and blood pressure, but to our knowledge there is no report as yet demonstrating that mutants with a reduced cardiac performance have a modified vascular system compared to wildtype animals. Thus, although the heart typically is the first organ functioning, we still have no convincing explanation as to the physiological function of the circulatory system in the earliest stages. Further studies may reveal that the early functioning of the heart is related to a variety of functions, ranging from ion regulation or hormonal communication to angiogenesis, and finally to nutrient transport.

**Acknowledgements:** The study was financially supported by the Fonds zur Förderung der wissenschaftlichen Forschung (FWF, P12571-BIO).
Reference List


