Temperature-dependent development of cardiac activity in unrestrained larvae of the minnow Phoxinus phoxinus

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Schönweger, G., T. Schwerte, and B. Pelster. Temperature-dependent development of cardiac activity in unrestrained larvae of the minnow Phoxinus phoxinus. Am J Physiol Regulatory Integrative Comp Physiol 279: R1634–R1640, 2000.—The minnow (Phoxinus phoxinus) was raised up to the stage of swim bladder inflation at temperatures between 10°C and 25°C, and the time of development significantly decreased at higher temperatures. Accordingly, initiation of cardiac activity was observed at day 2 in 25°C animals and at day 4 in 12.5°C animals. Only a minor increase in body mass was observed during the incubation period, and, at the end of the incubation period, animals raised at 25°C did not have a significantly lower body mass compared with animals raised at 15°C. Metabolic activity, determined as the rate of oxygen consumption of a larva, increased from 3.3 to 19.5 nmol/h during development at 15°C and from 5.6 to 47.6 nmol/h during development at 25°C. Heart rate showed a clear correlation to developmental stage as well as to developmental temperature, but at the onset of cardiac activity, diastolic ventricular volume and also stroke volume were higher at the lower temperatures. Furthermore, stroke volume increased with development, except for the group incubated at 12.5°C, in which stroke volume decreased with development. Initial cardiac output showed no correlation to incubation temperature. Although metabolic activity increased severalfold during development from egg to the stage of swim bladder inflation at 15°C and at 25°C, weight-specific cardiac output increased only by ~40% with proceeding development. At 12.5°C, cardiac output remained almost constant until opening of the swim bladder. The data support the notion that oxygen transport is not solely to indicate this fact.

Temperature, and this appears to be especially pronounced in larval stages, where the Q₁₀ for oxygen uptake...
typically is in the range of 3 (18, 25). If, in larvae, the connection between metabolic demand and cardiac performance is not yet established, this temperature-induced increase in oxygen uptake may be met without any increase in cardiac activity. On the other hand, the pacemaker or the contractility of the cardiomyocytes may be subject to a direct influence of temperature, which is independent of a nerve- or hormone-dependent regulated response. The results show, that during the initiation of cardiac activity, cardiac output is almost independent of incubation temperature and thus of metabolic activity, whereas at the time of swim bladder inflation, cardiac output significantly increases with incubation temperature.

MATERIALS AND METHODS

Animals. Eggs of the minnow (Phoxinus phoxinus) were obtained from our own breeding colony. Parent animals were caught from various Alpine lakes near Innsbruck and kept in a freshwater aquarium. To prolong the breeding season, animals were kept at a temperature of 7°C to mimic the cold season. The animals were fed twice per day.

Breeding behavior was induced by increasing the water temperature by ~2–3°C. Within 2 or 3 days after the temperature increase, the parent animals showed the typical breeding colors and behavior. Then the bottom of the aquarium was filled with a gravel substrate that typically induced spawning within 1 or 2 days. Eggs were removed immediately after spawning and incubated in small aquariums (60–80 liters) at a temperature of 10°C, 12.5°C, 15°C, 17.5°C, 20°C, or 25°C. Each of these temperatures was controlled within ±0.2°C.

The imaging system. The imaging system consisted of an inverted microscope (Leica Leitz DMIL) equipped with a [2/3]-in. machine vision charge-coupled device 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 (Imagemation PX-610) with a personal computer (Pentium III 450 MHz). The illumination could be reduced to infrared light with a wavelength of 780 or 913 nm to prevent light-induced stress reactions of the animals. The microscope desk was temperature controlled.

Recording of cardiac activity. Every 24 h after spawning, the eggs or larvae were transferred into the temperature-controlled incubation chamber of the microscope stage. The temperature was set to the incubation temperature of the eggs. First, the anatomical development of the animals was recorded by careful inspection of the eggs and larvae. Animals showing any developmental irregularities were removed. Eggs and 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. Long-term recordings, in which cardiac activity was recorded for up to 1 h after transfer into the observation chamber of the microscope desk demonstrated that the transfer of the eggs or larvae into this chamber did not influence cardiac activity. After initial inflation of the swim bladder, however, this method of observation could not be employed any longer, because swimming activity of the larvae increased significantly at this point, and the animals only rarely settled in a position that allowed for a stable recording of cardiac activity.

Determination of heart rate, stroke volume, and cardiac output. Heart rate was determined by measuring the time interval for 30 heart beats. Determination of stroke volume, using digital image analysis, followed the method described by Hou and Burggren (11). 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 that first calculated the center of mass of the perimeter and subsequently the best-fitting ellipse. The major and minor axes of the ellipse were extracted and directly transferred into a Microsoft Excel worksheet for calculation of the stroke volume using the formula for a prolate spheroid \((4/3) \pi x a \times b^2\) (11). For analysis, five diastoles and systoles were analyzed, and mean stroke volume was calculated as the difference between diastolic and systolic ventricular volume. Cardiac output was calculated as the product of stroke volume times heart rate.

Measurement of oxygen consumption. Oxygen consumption of a group of 10 larvae was measured until day 6 after spawning at 25°C and until day 14 after spawning at 15°C, respectively, in a Cyclobios TwinFlow (Cyclobios, Innsbruck, Austria) (7). Larvae were transferred into the respirometer chamber ~7 h after spawning, and oxygen consumption was continuously measured for 20–22 h every day. Average values obtained for each 60-min period were used to calculate the daily mean oxygen consumption. To correct for possible microbial respiration, background respiration of the respirometer system (including oxygen uptake of the Clark oxygen electrode) was measured once every day after removal of the animals (3). Data were analyzed using a custom-made software package Datgraph (Cyclobios).

Statistical analysis. The acquired data and also the extracted data were automatically exported into an ASCII file for statistical analysis. Statistical analysis was performed using the software package Statistica. Data are presented as means ± SE with \(n = 13\), except when stated otherwise.

RESULTS

For minnows (Phoxinus phoxinus) raised at temperatures between 10°C and 25°C, the development time significantly decreased at higher temperatures. Figure 1 shows the temperature-dependent appearance of some anatomical and physiological landmarks, such as gill development and hatching and of the initiation of cardiac activity and of blood flow. Whereas initiation of cardiac activity was observed at day 2 at 25°C, at 12.5°C it was only observed at day 4. At 25°C, circulation of erythrocytes was established within a few hours after the first heart beat, but at 12.5°C, circulating red cells were observed 1 day after the first heart beat. Similarly, initial inflation of the swim bladder occurred at day 6 in animals raised at 25°C, but at day 21 in animals raised at 12.5°C.

Animals raised at 10°C showed an extremely delayed development. Even at day 14 no hatching was observed, and many animals showed developmental abnormalities such as a malformation of the eye. Therefore, this temperature series was terminated, and no physiological measurements were performed.
Figure 2 summarizes the changes in body weight measured in animals raised at 15°C and at 25°C. The weight of the eggs was the same at 3.34 ± 0.10 and at 3.39 ± 0.10 mg for animals raised at 15°C and at 25°C, respectively. At the time of hatching, the egg mass had increased by ~0.4 mg at 15°C, whereas no change was observed at 25°C. After hatching, the weight of the larvae was ~50% of the egg mass (1.81 ± 0.09 and 1.76 ± 0.14 mg, respectively). During subsequent development until inflation of the swim bladder, body mass increased by ~0.8 mg in both groups so that the weight of the hatchlings as well as the weight of the larvae at the time of swim bladder inflation were not significantly different between animals raised at 15°C or at 25°C.

Oxygen consumption in animals raised at 15°C increased with development (Fig. 3A). Overall, the rate of oxygen consumption increased approximately sixfold within the observation period. Similar results were obtained in animals raised at 25°C. Oxygen consumption in animals raised at 25°C increased from 5.6 to 47.6 nmol/h per larva (Fig. 3B). This resulted in a Q10 of 1.7 for the egg and of 2.4 for the larvae at the time of swim bladder inflation.

Heart rate significantly increased with development and with increasing incubation temperatures (Fig. 4). At 12.5°C, mean heart rate was 92.8 ± 1.38 beats/min at the time of swim bladder filling and 233 ± 1.73 beats/min at 25°C.

![Figure 1](image1.png)

**Fig. 1.** The appearance of circulation-related anatomical and physiological landmarks in minnow (*Phoxinus phoxinus*) raised at 5 different temperatures (*n* = 13).

![Figure 2](image2.png)

**Fig. 2.** Changes in body mass of eggs and larvae of the minnow raised at 15°C and at 25°C (*n* = 10).

![Figure 3](image3.png)

**Fig. 3.** Changes in oxygen consumption of a minnow embryo developing at 15°C (A) and at 25°C (B). Oxygen uptake was measured in a twin-flow respirometer for a group of 10 embryos/larvae for 20–22 h every day between spawning and opening of the swim bladder. Daily means for a single animal were calculated by averaging the oxygen consumption values obtained for each 1-h interval of a day and dividing by the number of animals within the respirometer chamber. Values are means ± SD. ↑, the time of hatching.

![Figure 4](image4.png)

**Fig. 4.** Changes in heart rate with development in the minnow (*Phoxinus phoxinus*) raised at temperatures between 12.5°C and 25°C (*n* = 13).
Q_{10} for heart rate was in the range of 1.7 to 1.9 for incubation temperatures of 15°C and above (Table 1). This was not only true when comparing values of neighboring incubation temperatures (e.g., 15°C and 17.5°C; 17.5°C and 20°C; 20°C and 25°C), but also when comparing the 15°C and the 25°C group. Between 12.5°C and 15°C, however, the temperature sensitivity of heart rate was significantly higher with a Q_{10} of 2.8 (Table 1).

The comparison of diastolic ventricular volumes at the onset of cardiac activity demonstrated that diastolic volumes were higher in fish raised at low temperatures (Fig. 5). With proceeding development, diastolic volume significantly increased in fish raised at 15°C and above, but this increase was less pronounced at 20°C and at 25°C. This was also true for systolic ventricular volume (data not shown), but the increase was much smaller than in diastolic volume. In animals raised at 12.5°C, however, diastolic ventricular volume decreased with development, and systolic volume remained almost unchanged.

At incubation temperatures of 15°C and above, stroke volume increased with development, but at 12.5°C, stroke volume decreased with development (Fig. 6, A and B). Initial stroke volume was significantly higher at low temperatures. Between 42% and 58% of the diastolic volume was ejected (Fig. 6C).

Whereas in animals raised at temperatures of 17.5°C and above, the ratio of stroke volume to diastolic volume remained constant during development, it decreased in animals raised at 12.5°C and at 15°C.

Cardiac output increased with development at incubation temperatures of 15°C and above, whereas at 12.5°C, cardiac output remained almost constant throughout development until initial inflation of the swim bladder (Fig. 7A). A comparison of cardiac output values at the time of the first circulation of blood revealed that at this stage, cardiac output varied be-

### Table 1. Average Q_{10} for heart rate in the temperature range between 12.5°C and 25°C (n = 13)

<table>
<thead>
<tr>
<th>Temperature Range</th>
<th>Q_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5–15°C</td>
<td>2.86</td>
</tr>
<tr>
<td>15–17.5°C</td>
<td>1.64</td>
</tr>
<tr>
<td>17.5–20°C</td>
<td>1.88</td>
</tr>
<tr>
<td>20–25°C</td>
<td>1.89</td>
</tr>
<tr>
<td>15–25°C</td>
<td>1.82</td>
</tr>
</tbody>
</table>

n, No. of eggs.
CARDIAC ACTIVITY IN MINNOW LARVAE

Fig. 7. A: the development of cardiac output in the minnow (*Phoxinus phoxinus*) raised at temperatures between 12.5°C and 25°C. B: change in cardiac output between the first day of cardiac activity and the final measurement at the time of initial swim bladder inflation (n = 13). *Significantly different from initial value (P < 0.05).

between 130 and 210 nl/min, and there was no correlation with incubation temperature (Fig. 7B). At the time of swim bladder inflation, cardiac output was up to twice as high as at the initial stage in animals raised at 15°C and above, whereas in animals raised at 12.5°C, cardiac output remained constant at ~150 nl/min (Fig. 7B). With the use of the average body mass (see Fig. 2) weight-specific cardiac output can be calculated as 72.1 and 95.5 nl·mg⁻¹·min⁻¹ for 15°C hatchlings and larvae at the time of initial swim bladder inflation, respectively. For animals raised at 25°C, weight-specific cardiac output amounted to 104.7 and 149.6 nl·mg⁻¹·min⁻¹ for hatchlings and larvae at the time of initial swim bladder inflation, respectively. This resulted in a Q₁₀ value of 1.45 for hatchlings and of 1.56 for larvae. Calculating Q₁₀ values on the basis of cardiac output determined for individuals, similar values were obtained with 1.41 and 1.48 for hatchlings and larvae, respectively.

**DISCUSSION**

**Critique of methods.** In previous studies, cardiac activity of fish or amphibian larvae has been analyzed using MS-222 (Tricaine) as a water-soluble anesthetic (6, 15, 19, 20). Although there is a bulk of evidence suggesting that in very early stages, MS-222 has no significant effect on cardiac activity, it may still exert some yet unknown effects. Cellulose wadding or nylon meshes have also been used to physically restrict the animals and reduce body movements (2, 16) that may interfere with the gas exchange because of an increase in the thickness of unstirred layers surrounding the body. Our method, using an inverted microscope and infrared illumination, circumvents all the problems associated with these methods, and cardiac performance can be recorded in free-swimming larvae. Because the early stages of the minnow, and also of the zebrafish (unpublished results), typically settle at the bottom of the tank and pigmentation does not yet obscure ventral or lateral view of the ventricle, stable recordings can be obtained in a reasonable time. Only after inflation of the swim bladder does this method become very tedious and inefficient.

**Changes in heart rate.** A Q₁₀ of ~2 indicates complete temperature conformity, and a value ~1 indicates complete compensation (4). In adult fish, chronic temperature changes typically are accompanied by a partial compensation of resting heart rate, and Q₁₀ values, therefore, often are below 2. However, in isolated adult hearts and also after inhibition of adrenergic and cholinergic modulation of cardiac activity, the Q₁₀ value is close to a value of 2. This suggests that the temperature compensation is mainly achieved by extrinsic modulation of cardiac activity and not by the intrinsic properties of the pacemaker itself (4, 8). In minnow larvae, the Q₁₀ was ~1.8 in the temperature range between 15°C and 25°C, which is close to temperature conformity according to Farrell and Jones (4). This suggests that extrinsic control mechanisms, which are responsible for a temperature compensation in adult fish, are not yet functional. Indeed, a number of studies indicate that nervous control of cardiac activity is achieved late in development (14, 17, 24), even though adrenergic and cholinergic receptors are present and the ventricle can respond to external application of these hormones (5, 13, 22). The results of this study thus suggest that minnow larvae at this stage of development do not use the hormonal pathway for temperature compensation.

Over wide temperature ranges, the Q₁₀ may not be uniform, and in contrast to the temperature range between 15°C and 25°C, the Q₁₀ for the temperature range between 12.5°C and 15°C was significantly higher with a value of 2.86. This is even higher than the value of ~2.4 reported for trout larvae in the temperature range 5°C-15°C (15). Thus, in the lower temperature range, a decrease in temperature causes an especially pronounced decrease in cardiac activity, and minnow larvae therefore appear to be very temperature sensitive below 15°C. In this low temperature range, even the proper development of the embryo is endangered, because the experiment at 10°C had to be terminated due to a high rate of malformations.

For rainbow trout larvae, body mass is a major determinant of heart rate during larval development (15). Trout larvae, however, are much larger than minnow larvae, ranging from 10 to 60 mg, whereas the body mass of minnow larvae was ~1.8–2.8 mg in our...
study. We restricted our study to early stages before swim bladder inflation, which typically is the time of first feeding. Thus our animals mainly were dependent on the on-board nutrient supply and, accordingly, showed only a minor increase in body mass. The changes in body mass therefore were only of minor importance for cardiac activity in our study, especially because there was no temperature-dependent change in body mass up to this stage of development.

Changes in stroke volume and cardiac output. Whereas heart rate of minnow larvae increased with temperature and development, initial stroke volume was higher at lower temperatures. In isolated adult trout hearts, stroke volume was about the same at 5°C and at 15°C, whereas ventricle mass at 5°C was higher than at 15°C (8). A comparison of the morphological development at different temperatures indeed revealed that higher temperatures accelerate differentiation, but animals raised at higher temperatures typically are smaller than animals raised at lower temperatures (9, 10, 18, 26). Although no difference in body mass was observed between animals raised at 15°C and at 25°C in our study, diastolic ventricular volume at the onset of cardiac activity was significantly higher in 12.5°C animals compared with animals raised at 20°C or 25°C. If resting diastolic ventricular volume can be accepted as an index of heart size, this suggests that at low temperatures, an increase in heart size compensates for a decrease in heart rate, or vice versa, so that cardiac output remains constant. Initial cardiac output showed no significant correlation to incubation temperature, but between 15°C and 25°C the Q10 was −1.4.

At all experimental temperatures (except 12.5°C), diastolic ventricular volume, stroke volume, and cardiac output increased during development. At the time of initial swim bladder inflation, cardiac output showed a clear increase with incubation temperature, with the Q10 of −1.5. This is much lower than the average Q10 of 3.06 reported for trout larvae at temperatures of 5°C and 15°C (15). A possible explanation for this discrepancy could be the difference in body size. Trout larvae are ~10 times as big as minnow larvae, and the allometric mass exponent for cardiac output in trout larvae was much larger than the mass exponent for oxygen consumption. Accordingly, Mirkovic and Rombough (15) concluded that the cardiovascular system was much less important for delivering oxygen to the tissues in small larvae than in larger larvae. Thus the high allometric mass exponent for cardiac output may suggest that, in these large larvae, the circulatory system has to get ready for the time when diffusive oxygen uptake through the skin can no longer meet the oxygen requirements of the animals, and this is especially the case at higher temperatures with the higher metabolic rate. In minnow embryos, oxygen uptake is ~1.7 times higher at 25°C compared with 15°C, but at the onset of cardiac activity, cardiac output is about the same. This clearly shows that convective oxygen transport in the circulatory system is not important at this stage, and, given the much smaller body mass of minnow larvae, cutaneous respiration and diffusive oxygen transport will be sufficient for a much longer time than in trout larvae. Thus, even at higher temperatures, the circulatory system is not yet needed for oxygen transport in minnow larvae at the time of swim bladder filling. This conclusion is supported by the observation that aerobic metabolism increases by a factor of 6 at 15°C during development and by a factor of 8 at 25°C, but weight-specific cardiac output increases only by ~40% during development at both temperatures.

In contrast to all other acclimation temperatures, cardiac activity did not increase with development at 12.5°C and stroke volume remained constant or even decreased. Similarly, whereas the fraction of blood ejected during a cardiac cycle remained constant with development in animals raised at 17.5°C and above, it decreased in animals raised at 12.5°C. At this temperature, the intrinsic activity of the cardiac muscle is apparently high enough to meet all requirements associated with development, and there is no need for any enhancement. On the other hand, the observations made with the group raised at 10°C may also suggest that this temperature is near the lower (lethal) limit for proper development.

The results of the present study thus clearly support the observation that in these early developmental stages, a coupling of metabolism and cardiac activity has not yet been established, and apparently body mass will be major factor determining the onset of this coupling. But why then is the cardiac muscle the first functional organ even in these small larvae? Convective transport also includes nutrients. The observation that some mutants can survive without a beating heart for quite some time (1) counters the idea that nutrient transport is crucial. A more likely explanation therefore appears to be that other functions, including angiogenesis and proper vascularization of tissues, require the convection of blood and blood pressure.

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