O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2

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Barrionuevo, W. R., and W. W. Burggren. O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. *Am. J. Physiol.* 276 (*Regulatory Integrative Comp. Physiol.* 45): R505–R513, 1999.—Body mass, length, oxygen consumption ($\dot{M}O_2$) and heart rate (f_H) were measured in "embryos" (prior to hatching), "larvae" (*days 10–20*), "juveniles" (*days 30–70* in 10-day intervals), and "adults" (*day 100*) of the zebrafish *Danio rerio*. Fish were chronically reared at either 25, 28, or 31°C and then acutely exposed to hypoxia at different developmental stages. We hypothesized that at any given rearing and measurement temperature, *D. rerio* would maintain $\dot{M}O_2$ at lower ambient PO_2 [i.e., have a lower critical partial pressure (P_{crit})] as development progressed and that at any given developmental stage individuals reared and measured at higher temperatures would show a more pronounced hypoxic bradycardia. $\dot{M}O_2$ in normoxic fish at 28°C peaked at ~40 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at *day 10*, thereafter falling to 4–5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at *day 100*. The Q_{10} for $\dot{M}O_2$ was 4–5 in embryos, falling to 2–3 from *day 10* to *day 60* and rising again to 4–5 at *day 100*. P_{crit} at 28°C was ~80 mmHg in embryos but decreased sharply to 20 mmHg at 100 days, supporting the hypothesis that more mature fish would be better able to oxygen regulate to lower ambient PO_2 levels. P_{crit} increased sharply with measurement temperature. Heart rate (f_H) at 28°C increased from about 125 beats/min in embryos to a peak of ~175 beats/min at *days 10–30* and then fell to ~130 beats/min by *day 100*. Unlike for $\dot{M}O_2$, the Q_{10} for f_H was more constant at 1.2–2.5 throughout development. Hypoxic exposure at any temperature had no effect on f_H until ~*day 30*, after which time a hypoxic bradycardia was evident. As evident for $\dot{M}O_2$, the bradycardia in older larvae was more profound at higher temperatures. On the assumption that bradycardia is indicative of hypoxic stress, the increasing prevalence of a hypoxic bradycardia in older, warmer individuals supports the hypothesis that increasing hypoxic susceptibility with development would be exacerbated by increasing temperature. Collectively, these data indicate that the ability to regulate $\dot{M}O_2$ and f_H in response to the compounding demands of increased temperature and/or decreased oxygen availability first develops after ~20 days in *D. rerio* and, thereafter, the ability to maintain $\dot{M}O_2$ in the face of ambient hypoxia progressively builds through to adulthood. Additionally, the temperature responses of metabolism and heart rate differ substantially at different phases of development, suggesting a loose coupling between the respiratory and cardiovascular systems, at least early in development.

development; hypoxia; embryos

THE ZEBRAFISH, *Danio rerio* (Brachydanio), is a tropical Cyprinid teleost fish that recently has been the focus of increasing numbers of developmental studies. Physiological interest in this species has been spurred, in part, by the relative ease with which cardiovascular and other mutants can be induced by chemomutagenesis (see Ref. 8). However, our understanding of the basic physiology of *D. rerio*, in particular the normal processes that occur during development, lags far behind that of other vertebrate models for development (see Ref. 2). This paucity of information for *Danio*, combined with a fragmentary knowledge of developmental physiology in fishes generally (28, 29) argues for basic studies of developmental physiology in this species.

Temperature and hypoxia, known to fluctuate in the environments in which lower vertebrates develop, can profoundly affect the physiology and morphology of lower vertebrate embryos (for review, see Refs. 5, 23, 28). Moreover, the effects of chronic exposure to environmental challenge may be quite different from acute effects, given the considerable developmental plasticity of embryonic and larval organ systems (see numerous chapters in Ref. 2). *D. rerio* presents an excellent model for investigating environmental influences on physiological development in lower vertebrate embryos and larvae. Because zebrafish reach adulthood in under 100 days (37), the impact of environmental perturbation can be assessed over relatively short experimental periods.

The purpose of this study was to investigate oxygen consumption ($\dot{M}O_2$) and heart rate (f_H) as a function of acute hypoxic exposure throughout development in *D. rerio* chronically reared at 25, 28 (the preferred temperature), and 31°C. Our experiments were designed to test the hypotheses that at any given rearing and measurement temperature, older *D. rerio* would be able to maintain $\dot{M}O_2$ at lower ambient PO_2 [i.e., have a lower critical partial pressure (P_{crit})] and that, at any given stage, individuals reared and measured at higher temperatures would show a more pronounced hypoxic bradycardia. These experiments have additionally provided new data on the changes in the temperature responses of body mass, length, metabolism, and f_H over a very wide developmental span in *D. rerio*.

MATERIAL AND METHODS

Animals

Newly laid eggs (<24 h) of *D. rerio* obtained from commercial suppliers (Scientific Hatcheries, Huntington Beach, CA) were reared in the laboratory under 14:10-h light-dark conditions during the period January–July of 1996. Three separate groups were maintained: one reared at 28°C, the preferred temperature for this species (37); one group reared at 25°C; and a final group reared at 31°C. Larvae,[1] juveniles, and

[1] In this paper we use the terms "embryo" to describe an individual within an unhatched egg (generally <72 h) and "larva" for an individual after hatching up to *day 20*. "Juvenile" is reserved for a fish with a markedly adult appearance at 30–70 days of age, while "adult" is reserved for a sexually mature fish of 100 days.

adults from all groups were fed twice daily, except for the 24-h period preceding measurement, when fish were fasting.

$\dot{M}O_2$ and f_H were measured in unhatched embryos (<72 h) and at ages 10, 20, 30, 40, 50, 60, 70, and 100 days. Body mass (wet) and length (snout to tail) was measured immediately after each series of metabolic measurements and was additionally measured in each group at 150 and 200 days of development. Accurately measuring the extremely small body mass of embryos presented particular difficulties. Embryos were first removed from the egg capsule, leaving the yolk sac intact. The collective weight of 10 individual embryos was then determined using a microbalance, and the average weight of that group was calculated. This procedure was repeated 10 times, providing 10 separate body mass measures from which was calculated an average and SE for embryos.

Body length measurements were made to the nearest 0.1 mm using a dissecting microscope fitted with a calibrated scale in one eyepiece. Body length was not measured in the embryos, which are normally in a highly curled natural posture within the egg.

$\dot{M}O_2$

$\dot{M}O_2$ determinations were made at the rearing temperature of each group (25, 28, and 31°C) over the range from unhatched eggs to *day 100*. To determine $\dot{M}O_2$, animals were placed in closed respirometers created from glass syringes containing aerated water. Respirometers were covered with opaque material to minimize visual disturbances to the fish and were placed in a temperature-regulated water bath. Syringe water volume was matched to fish biomass within the syringe to regulate the rate of oxygen depletion during the experiments (see below). Because of the extremely small size of the embryos and, to a lesser extent, *day 10* larvae, measurements of $\dot{M}O_2$ in individuals were not practical. Consequently, for these two early stages a known total mass comprising several individuals of the same developmental stage was placed in each respirometer, with the overall calculated $\dot{M}O_2$ within the syringe divided by the number of individuals to generate a single point for mass-specific $\dot{M}O_2$ for an individual fish at that developmental stage. Thus each $\dot{M}O_2$ measurement derived from one respirometer run was considered one data point for that developmental stage, regardless of the number of animals within the syringe that had contributed to the calculation of that datum. For subsequent stages of *days 20–100*, only individual fish were placed in each respirometer and, again, each $\dot{M}O_2$ measurement was considered one data point.

All fish were allowed a 2-h acclimation period in the respirometers before measurements. Although this period is unlikely to have alleviated all stress from handling in this very active species of fish, preliminary experiments indicated that there were no significant differences in $\dot{M}O_2$ measured after 1, 2, or 3 h of acclimation, so at least the fish were in a relative steady state if not completely acclimated. At the end of the acclimation period, one-half of the water in each respirometer was very gently and slowly exchanged with air-saturated water (normoxia), taking great care not to disturb the fish within. Before commencement of the first $\dot{M}O_2$ measurement period, the initial water P_{O_2} (in mmHg) of each respirometer was measured in a 100-µl water sample injected directly from the respirometer into a water-jacketed O_2 electrode (Microelectrodes) connected to a Radiometer pHM71 gas meter. At this time, the initial water volume was also recorded. Water P_{O_2}, which then began to decline over time due to the $\dot{M}O_2$ of the fish, was measured in each respirometer over seven or eight successive 20-min intervals. On the basis of $\dot{M}O_2$ data from preliminary experiments on each developmental stage, different-sized syringes and/or different initial volumes of water were employed to ensure that the $\dot{M}O_2$ by the fish in each respirometer volume produced a P_{O_2} drop of no more than 15 mmHg during the 20-min measurement period. This ensured that each animal contributed one point to the 15-mmHg-wide P_{O_2} "bins" used in subsequent analysis (see below).

Mass-specific $\dot{M}O_2$ for individual fishes was calculated from the rate of decrease in water P_{O_2} in the respirometer, volume of the respirometer before water sample, elapsed time between successive measurements, mass of the fish in each respirometer, and O_2 capacitance of water at measurement temperature. $\dot{M}O_2$ data, including the P_{crit} for each developmental group, were plotted and analyzed as described in Ref. 11. Despite matching of respirometer volume to fish biomass, subtle differences in metabolism between identically aged fish might produce, say, a water P_{O_2} of 130 mmHg in one respirometer at the end of the 20-min measurement period but a water P_{O_2} of 124 mmHg in another respirometer. Thus, to allow for simplification of calculation as well as greatly enhanced clarity in graphical presentation, each $\dot{M}O_2$ value successively generated as hypoxia developed during a respirometer run was assigned to one of eight 15-mmHg-wide bins described by a mean P_{O_2} of 130, 115, 100, 85, 70, 55, 40, or 25 mmHg. This process resulted in an $\dot{M}O_2$ value being generated for each animal for each P_{O_2} bin. Mean $\dot{M}O_2$ values ±SE for a given stage at a given temperature were then calculated by averaging each of the 10 individual $\dot{M}O_2$ values for each fish in each bin. In unusual cases, a particularly low rate of metabolism by an individual fish would result in two $\dot{M}O_2$ values calculated from two successive 20-min measurements falling within a single P_{O_2} bin. In this instance, the two $\dot{M}O_2$ values were averaged to produce a single estimator for that fish in that bin.

The P_{crit} for a specific temperature group at a specific developmental stage was calculated from the intersection of two lines (determined by least-squares linear regression) passing through the mean $\dot{M}O_2$ values representing each P_{O_2} bin (11). Initially, one line was plotted through the obviously decreasing values of mean $\dot{M}O_2$ at the lower P_{O_2} values, and the other line was plotted through the obviously unchanging values of mean $\dot{M}O_2$ at high P_{O_2} values just slightly below air saturation. Then the mean values at the intermediate P_{O_2} values were alternately included in the data for the upper line and then lower line until the combined values of the r^2 for each line were minimized, indicating the best overall fit of the two lines through all mean values for $\dot{M}O_2$. The intersection of the two best-fitting lines was determined to be the P_{crit}. The margin of error for this method of P_{crit} calculation is estimated to be ±4 mmHg, which is, for example, just 5% of the P_{crit} difference calculated between 25- and 31°C-acclimated embryos.

f_H

f_H was measured in a range of developmental stages of *D. rerio* reared at 25, 28, or 31°C. f_H measurements in embryos and *day 10* larvae were made on individuals placed in water-filled holding chambers constructed from petri dishes. Water flow was maintained through the chambers, with both water temperature and P_{O_2} constantly regulated and monitored. Because embryos and early larvae are translucent, f_H was measured visually through the body wall. Video recordings of the in vivo beating heart were made using a Javelin JE3010 color camera and were subsequently analyzed (after Ref. 24). During the acclimation period, water P_{O_2} was maintained at 150 mmHg and f_H was measured at normoxic

P_{O_2}. Water P_{O_2} was then lowered over a 5-min period to 105 mmHg and held for 30 min before f_H was again measured. This process was repeated in sequence for P_{O_2} values of 60, 30, and 10 mmHg. Water P_{O_2} flowing into the holding chamber was controlled by equilibration with gas produced by a GF-3 gas mixing flowmeter (Cameron Instruments).

Individual older larvae (≥20 days), juveniles, and adults were placed in a 15-mm-diameter glass tube irrigated at a rate of 30 ml/min with water of appropriate temperature and P_{O_2} (regulated as described above for embryos and young larvae). Although fish from 20 days to adult were no longer translucent, appropriate obliquely directed fiber-optic lighting clearly revealed cardiac-induced pulsations of the ventral body wall, which were recorded with a Sony AF charge-coupled device video camera mounted vertically under the glass tube. Each individual was allowed to acclimate overnight (~14 h) before measurements, and the same protocol of hypoxic exposure and f_H measurement described above for embryos and young larvae was employed. In all experiments, a 1-min-long videotape segment was analyzed, which, for the highest possible f_H recorded (~200 beats/min), would result in a margin of error of less than ±4 beats/min.

Differences in $\dot{M}O_2$, f_H, and P_{crit} as a function of development and differences in $\dot{M}O_2$ and f_H at the same developmental stage as a function of P_{O_2}, were individually assessed using one-way ANOVA performed by Sigmastat statistical software (Jandel; San Rafael, CA). A significance level of 0.05 was used for all tests.

RESULTS

Body Mass and Length

Changes in wet body mass and body length as a function of chronological age (0–200 days) and rearing temperature (25, 28, or 31°C) are presented in Table 1. Not surprisingly, there are significant disparities in both mass and length induced by rearing temperature. At any given chronological age up to ~60 days, both body mass and length were lowest at 25°C and highest at 31°C. The exception was embryos, in which rearing temperature had presumably not had time to create an effect. Interestingly, from about *day 70* on, the 31°C-acclimated fish actually showed leaner body mass than 28°C-acclimated fish, although the warmer fish were longer in length. This may reflect the greater cost of maintenance at the highest rearing temperature.

Interpretation of these mass and length data is highly complex (see DISCUSSION), and we made no attempt in this study to correct the measured chronological age in days to the developmental age, which is properly assessed using a variety of morphological, physiological, and biochemical markers. Consequently, the metabolic and heart rate data reported below compare fish of identical chronological age at the three different rearing temperatures.

Influences of Development and Rearing Temperature

$\dot{M}O_2$. $\dot{M}O_2$ in unhatched embryos at 28°C, ~3.6 μl·g^{-1}·h^{-1}, increased >10-fold with hatching and initial growth, reaching a peak of 39.4 μl·g^{-1}·h^{-1} at *day 10* (Fig. 1). $\dot{M}O_2$ then decreased progressively to ~5.8 μl·g^{-1}·h^{-1} at *day 60* and showed little additional change during the next 40 days of development. Zebrafish groups reared at 25 and 31°C showed this same general pattern of change in $\dot{M}O_2$ with development (Fig. 1). The effect of development on $\dot{M}O_2$ was highly significant ($P < 0.0001$) in all three groups, with the single exception of a nonsignificant change between *days 20* and *30* at 25°C.

As anticipated, at any given developmental stage, $\dot{M}O_2$ at a rearing and measurement temperature of 25°C was significantly lower than at 28°C, which in turn was significantly lower than at 31°C ($P < 0.05$). Q_{10} for $\dot{M}O_2$ was calculated over the intervals 25–28°C and 28–31°C (Fig. 2, *top*). Q_{10} for $\dot{M}O_2$ over the range 25–28°C in all developmental stages was equal to or greater than values measured over the range 28–31°C. Q_{10} for $\dot{M}O_2$ showed a highly distinctive, U-shaped pattern of change with development over both temperature ranges. Q_{10} in embryos was relatively high at ~4–5, but the temperature sensitivity of metabolism fell sharply with continued development to a Q_{10} of 2–3 in larvae from ~10–60 days. However, Q_{10} increased once again to values of 4–5 in *day 100* adults.

Table 1. *Body mass and length as a function of development and rearing temperature in Danio rerio*

Chronological Age, Days Postfertilization	Rearing Temperature					
	25°C		28°C		31°C	
	Mass, mg	Length, mm	Mass, mg	Length, mm	Mass, mg	Length, mm
0	0.2 ± 0.1*		0.2 ± 0.1		0.2 ± 0.1	
5	0.4 ± 0.1	1.9 ± 0.3	0.5 ± 0.1	3.1 ± 0.5	1.0 ± 0.1	3.4 ± .6
10	0.6 ± 0.1	2.7 ± 0.5	0.9 ± 0.1	3.6 ± 0.5	1.4 ± 0.1	3.4 ± .7
20	1 ± 0.1	3.3 ± 0.5	2 ± 0.3	3.5 ± 0.7	2 ± 0.2	4.5 ± .7
30	2 ± 1	4.1 ± 0.3	4 ± 1.0	7.2 ± 1.4	5 ± 1	8.7 ± 1.2
40	2 ± 1	5.0 ± 0.4	9 ± 2.0	9.8 ± 1.4	11 ± 2	10.9 ± 1.6
50	5 ± 1	6.4 ± 1.1	13 ± 4.0	11.1 ± 2.5	18 ± 4	13.6 ± 3.5
60	10 ± 1	8.0 ± 1.4	27 ± 5.0	15.2 ± 2.6	59 ± 7	14.8 ± 2.3
70	14 ± 3	9.8 ± 2.2	214 ± 12.6	17.2 ± 4.1	102 ± 14	16.5 ± 3.5
100	92 ± 2	16.0 ± 1.0	481 ± 13.4	25.0 ± 3.4	260 ± 23	20.4 ± 3.3
150	172 ± 4	22.2 ± 1.2	681 ± 135	27.3 ± 3.4	520 ± 50	30.4 ± 3.3
200	367 ± 98	24.3 ± 1.2	831 ± 132	29.8 ± 3.4	739 ± 134	32.3 ± 3.0

Values are means ± SE ($n = 10$ for all measurements). *Body mass of embryos excludes egg capsule and extraembryonic fluid but includes yolk sac.

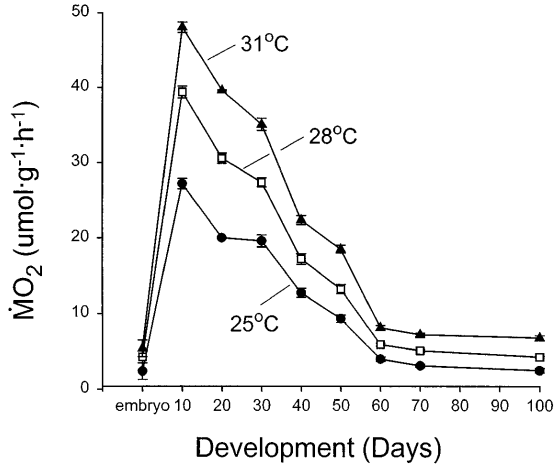

Fig. 1. Oxygen consumption ($\dot{M}O_2$) as a function of development at 3 rearing temperatures in the zebrafish *Danio rerio*. Mean values ± SE are plotted. Values of *n* for each plotted developmental stage, beginning with embryos, are 30, 20, 10, 10, 10, 10, 10, 10, and 10 for each rearing temperature. Effect of development on $\dot{M}O_2$ was highly significant ($P < 0.0001$) at all 3 rearing temperatures.

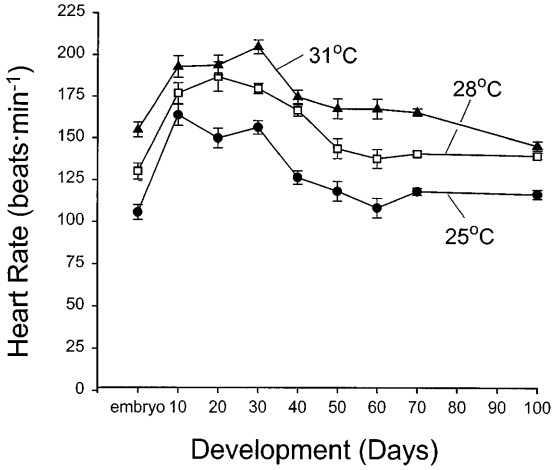

Fig. 3. f_H as a function of development at 3 rearing temperatures in *D. rerio*. Mean values ± SE are plotted. $n = 10$ for each plotted developmental stage at all 3 temperatures. Effect of development on f_H was significant at all 3 rearing temperatures ($P < 0.03$ at 25°C, $P < 0.05$ at 28°C, and $P < 0.0001$ at 31°C).

f_H. f_H in embryos at 28°C was ~125 beats/min but climbed to ~175 beats/min by *day 10* (Fig. 3). From a peak at *day 20*, f_H at 28°C declined to ~130 beats/min at *day 50* and older. f_H at 25°C was significantly lower than, and at 31°C significantly higher than, f_H values at 28°C at all development stages. The effect of development on f_H was significant in all three groups ($P < 0.03$ at 25°C, $P < 0.05$ at 28°C, and $P < 0.0001$ at 31°C). However, no significant ($P > 0.05$) change in f_H occurred after 60 days with the exception of *day 70* at 25°C, which was significantly, but only slightly, higher than at *day 60*.

Q_{10} for f_H was relatively constant at ~1.5–2.5 over the entire span of development (Fig. 2, *bottom*).

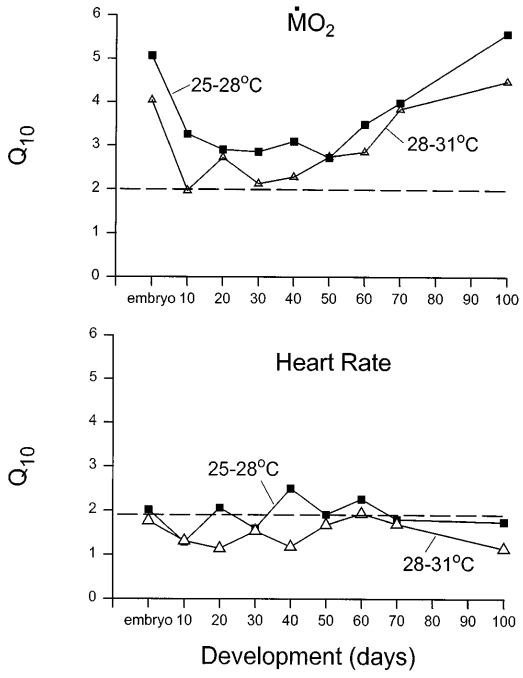

Fig. 2. Q_{10} values for $\dot{M}O_2$ (*top*) and heart rate (f_H; *bottom*) as a function of development in *D. rerio*. Q_{10} values are shown for 2 temperature ranges: 25–28°C and 28–31°C. Each plotted point is a single value calculated from the mean values of $\dot{M}O_2$ and f_H at each rearing temperature.

Influence of Acute Hypoxia

$\dot{M}O_2$ in embryos and young larvae (*days 10–30*) at all three rearing temperatures declined significantly ($P < 0.001$) from normoxic (control) values as acute hypoxic exposure became progressively more severe (Fig. 4). Only $\dot{M}O_2$ at *days 20* and *30* at 25°C showed no significant ($P > 0.10$) effect of hypoxia. Figure 5 illustrates P_{crit} as a function of development and measurement temperature. Older larvae, juveniles, and adults were capable of regulating $\dot{M}O_2$ at normoxic levels, even in the face of severe hypoxia. These findings supported our hypothesis that the ability to regulate oxygen consumption increases with developmental stage. Although the patterns of decline in P_{crit} showed similar patterns at all three temperatures, the absolute value of P_{crit} at any given developmental stage was highly temperature dependent, especially early in development. *Day 10* larvae at 25°C, for example, could maintain $\dot{M}O_2$ to a P_{crit} of ~50 mmHg, but this value increased to ~65 mmHg at 28°C and to >90 mmHg at 31°C.

The influence of acute, graded hypoxia on f_H as a function of development is shown in Figs. 6–8. Hypoxic exposure had no significant effect on f_H in embryos and *days 10* and *20* larvae at any rearing temperature (Fig. 6). In *day 30* juveniles there was no hypoxic bradycardia at 25°C, but at the two higher rearing temperatures a bradycardia began to develop below a P_{O_2} of 30 (28°C) or 100 mmHg (31°C) (Fig. 7). In both cases, the bradycardia at the lowest tested P_{O_2} was severe, with a drop in f_H of as much as 100 beats/min from normoxic values. This pattern, in which a hypoxia-induced bradycardia became more pronounced as temperature increased, similarly occurred in all groups from *day 40* to *day 100* (Figs. 7 and 8). Interestingly, in

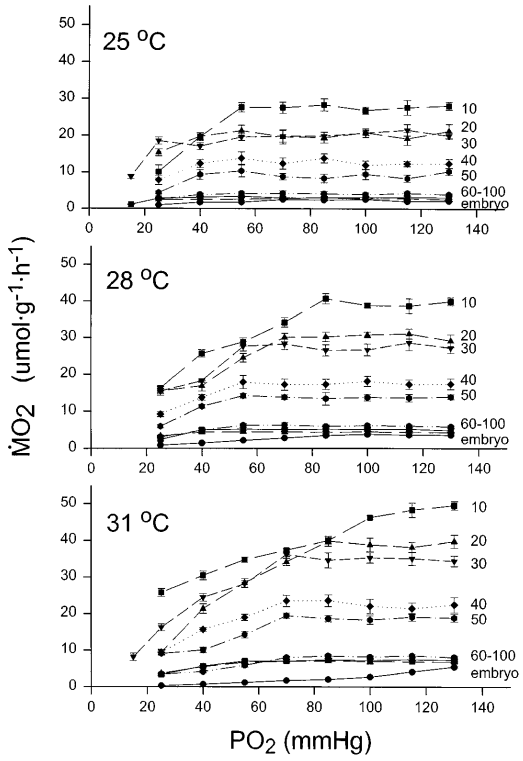

Fig. 4. Influence of acute hypoxic exposure on $\dot{M}_{O_2}$ as a function of development and temperature in *D. rerio*. Developmental stage is indicated at *right*. $n = 10$ for each plotted developmental stage at all 3 temperatures. See RESULTS for statistical assessment.

the most mature *day 100* fish, a more profound level of hypoxia was required to elicit bradycardia than at any of the juvenile stages.

On the assumption that the development of severe bradycardia indicates hypoxic stress, these data collectively support our hypothesis that at any given stage heart rate is more susceptible to hypoxic-induced change at warmer rearing temperatures.

DISCUSSION

Body Mass and Length

Attempts to interpret development change as influenced by body temperature in poikilotherms will be

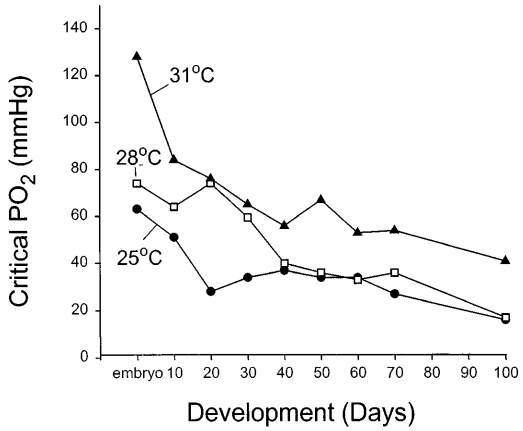

Fig. 5. Critical values of P_{O_2} (P_{crit}) as a function of development and temperature in the zebrafish *D. rerio*.

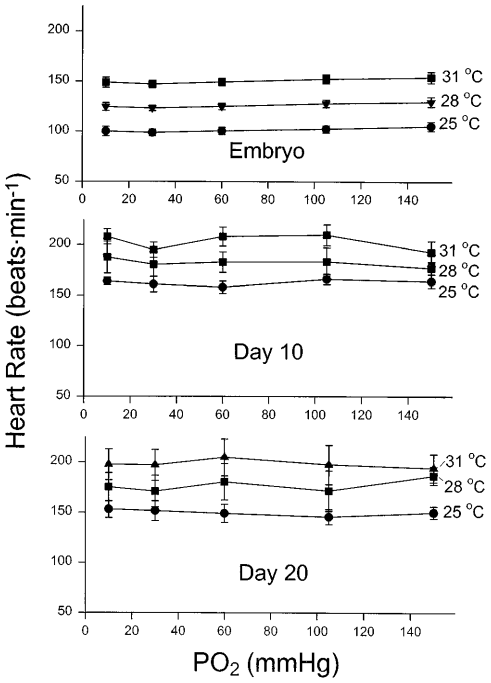

Fig. 6. Influence of acute hypoxic exposure on heart rate as a function of temperature in embryos and larvae (*days 10, 20*) of the zebrafish *D. rerio*. Mean values ± SE are plotted; $n = 10$ for each plotted developmental stage at all 3 temperatures. See RESULTS for statistical assessment.

fraught with pitfalls that stem from the deviation in chronological from actual developmental age. Clearly, for a given chronological age, zebrafish reared at 25°C are not as developmentally mature as zebrafish reared

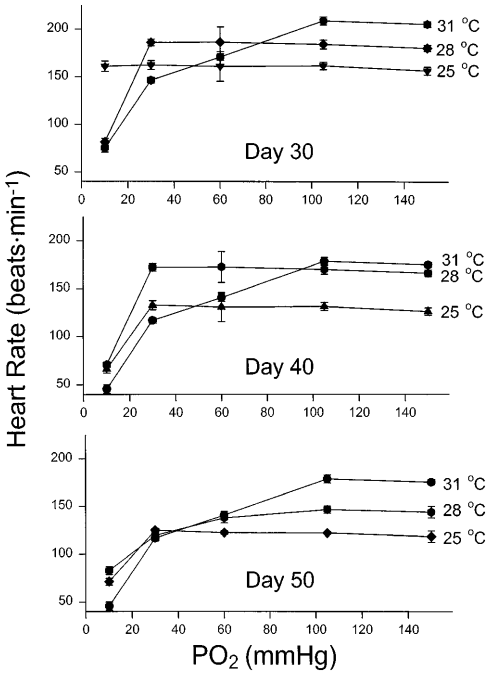

Fig. 7. Influence of acute hypoxic exposure on heart rate as a function of temperature in juveniles (*days 30, 40,* and *50*) of the zebrafish *D. rerio*. Mean values ± SE are plotted; $n = 10$ for each plotted developmental stage at all 3 temperatures. See RESULTS for statistical assessment.

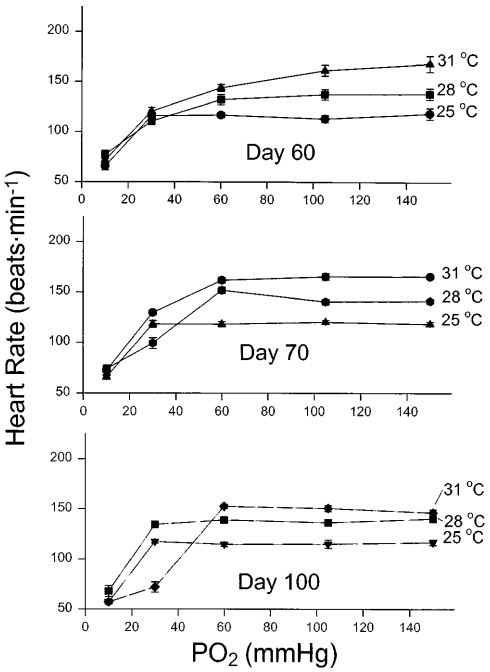

Fig. 8. Influence of acute hypoxic exposure on heart rate as a function of temperature in juveniles (*days 60, 70*) and adults (*day 100*) of the zebrafish *D. rerio*. Mean values ± SE are plotted; $n = 10$ for each plotted developmental stage at all 3 temperatures. See RESULTS for statistical assessment.

at 31°C, at least early in development. That is, there is a Q_{10} for development in larval fishes that impacts everything from body mass to energy assimilation, a fact that is widely recognized (see Ref. 27) but difficult to measure and even more difficult to extrapolate to experimental findings in growing animals reared at different temperatures. Rombough and others (22, 27) and Wells and Pinder (35) have attempted to circumvent this problem by reporting development as a function of "accumulated thermal units," or ACUs, which are calculated from the mean temperature multiplied by the number of days posthatch. Although this creative approach recognizes the problem inherent in measurements at different temperatures, it by itself cannot overcome the fact that the Q_{10} for development may be different over different temperature intervals (just as we found Q_{10} for $\dot{M}O_2$ to differ between 25 and 28°C and between 28 and 31°C), making less useful the simple arithmetic approach of multiplying time by temperature, regardless of actual temperature. Moreover, calculating ACUs cannot by itself correct for the fact that certain morphological, physiological, and biochemical variables may each have different Q_{10}s, resulting in a larval or juvenile animal that may have various organ systems at different stages of development depending on rearing temperature! Our data for zebrafish (Table 1) indicate that there is not a simple relationship between temperature, body mass, and length, with, for example, older and warmer fish being longer but also leaner. Similarly, Rombough's data on energy assimilation, metabolic rate, and body mass in steelhead (27) suggest that a fish larva at 6°C differs from one at 15°C by more than a simple "van't Hoff equation" form of correction that would require that all measured variables increase equivalently for the 9°C temperature difference.

We have elected in the present study not to introduce any corrections to our chronological ages for the zebrafish, on the assumption that no correction (i.e., presentation of the raw data) is at this point more appropriate than creating a correction for zebrafish that is grounded in the currently poorly understood thermal biology of this species. There will definitely be a difference in developmental age when comparing our data from different rearing temperatures at the same chronological age, in which, for example, f_H at *day 60* in a 25°C-reared fish may be more strictly comparable to f_H at *day 50* or even *day 40* in a 31°C-reared fish. Yet the absolute differences between groups across time are so large that we feel our arguments and conclusions are not qualitatively affected. Obviously, understanding the full implications of temperature on development is an enormously complex problem that is beyond the scope of this study but which deserves additional comprehensive investigation in studies designed expressly for that purpose.

Metabolic and Heart Rate Patterns During Development

Mass-specific $\dot{M}O_2$ in developing zebrafish showed an early peak followed by a sharp, steady decline as development progressed toward adulthood. Direct comparison of this pattern with published metabolic data for other fish species during development is problematic, because prior studies have typically focused extensively on the period of development equivalent to just the first development interval (embryo to *day 10*) of the eight intervals that we measured. Consequently, very few data from a single study exist for the later larval, juvenile, and adult periods equivalent to our 10-day intervals through *day 100*. Nonetheless, sharply rising mass-specific metabolism in the days after hatching similar to what we observed in zebrafish appears typical of salmonids, which have probably been the most intensively studied (28, 31). In the Atlantic salmon, *Salmo salar*, for example, $\dot{M}O_2$ at 10°C increases >10-fold from hatching (body wt ~30 mg) to age 3 mo (body wt ~0.5 g). A similar early peak in metabolism occurs in the steelhead, *Salmo gairdneri* (27). This pattern of metabolic apex early in development persists over a temperature range of 6–15°C but occurs about five times later in chronological age in steelhead at the lower temperature (27). An early peaking of mass-specific metabolism early in overall development followed by a sharp decline has also been demonstrated for anuran amphibians (see Refs. 5, 11) and reptiles (34, 38) and can be calculated for the chick embryo using data from Refs. 15 and 26, suggesting that this pattern of metabolic change may be a general vertebrate pattern.

The initial rise in metabolic rate that occurs in zebrafish during the first 10 days is likely associated with organogensis and the conversion of egg yolk into new metabolizing biomass. Soon, however, the onset of

free swimming in zebrafish at about *day 4* and of feeding a few days later could begin to influence metabolic rate. The subsequent fall in $\dot{M}O_2$ in zebrafish after *day 10* until *day 100* may be attributed in part to allometry, as body mass rises nearly 400-fold during this period.

Developmental changes in f_H in *D. rerio* approximately paralleled those for $\dot{M}O_2$, with f_H peaking at *days 10–30* at all measurement temperatures and then falling with additional development. This particular developmental pattern in f_H differs somewhat from some of the other teleost species that have been observed (e.g., walleye, rainbow trout, Arctic char), where maximum f_H typically peaks much earlier before or around hatching and then decreases with further development (12, 16–20, 29). Although different vertebrate species show considerable variation in f_H patterns during development, there is a general tendency in lower vertebrates for f_H to rise sharply during early development to an apex and then subsequently decline (7). Qualitatively, then, f_H change during development in the zebrafish does reflect that observed in other lower vertebrates, although the timing of the apex is somewhat delayed. The cause of these general changes in resting f_H during development remains unknown, but, as for $\dot{M}O_2$, may involve a complex interplay of early heart maturation followed by the increasing influences of body mass that is increasing rapidly with additional growth and development. Clearly, more detailed studies of the relationship between body mass, metabolic rate, and f_H during development in vertebrates are required to delineate the influence of development per se from those resulting from the ubiquitous effects of scaling on biological processes.

Influence of Temperature on Metabolism and Heart Rate

Typically, physiological processes show Q_{10} values of ~2, with temperature insensitivity revealed by values approaching 1, and enhanced temperature sensitivity revealed by Q_{10} values substantially >2 (25). As anticipated, rearing temperature had a profound effect on both $\dot{M}O_2$ and f_H in *D. rerio*. However, the analysis of the Q_{10} relationship describing temperature effects on these physiological processes revealed intriguing patterns of change during development (Fig. 2). The temperature sensitivity of $\dot{M}O_2$ declined sharply in early development, but increased again as the animals approached maturity. Q_{10} values for metabolic rate in larval fish vary enormously, but of 21 species surveyed by Rombough (28), only 6 had values >4 while 9 had values <2. Temperature sensitivity reflected in a high Q_{10} is common in stenothermic animals at measurement temperatures outside of their narrow range of preferred temperature. The high values of Q_{10} of the present study suggest that adult zebrafish are relatively stenothermal animals that live and breed at rather constant temperatures in their natural tropical habitat. Indeed, captive zebrafish rarely breed >31 or <25°C in laboratory settings. Mortality at these temperatures is high and surviving embryos show increased incidence of abnormal development (32, 37). The biochemical basis for these complex changes in $\dot{M}O_2$ responses to temperature during development in *D. rerio* remain unknown. However, almost all temperature-related characteristics of metabolism ultimately derive from the properties of key metabolic enzymes. Thus the changing temperature sensitivity of metabolism during development in *D. rerio* suggests complex and interesting development-associated changes in isozyme populations and warrants additional biochemical study.

The relationship describing Q_{10} for f_H with development in zebrafish does not resemble the U-shaped curve relating Q_{10} for metabolism with development. Indeed, Q_{10} for f_H ranged from ~1.2 to 2.5 over the temperature range of 25–31°C, a temperature sensitivity for f_H that compares favorably with very recent data on trout larvae (10–60 mg body mass), which showed a Q_{10} of ~2.4 over the range 5–15°C (22). Why does this discrepancy exist between Q_{10}s for $\dot{M}O_2$ and f_H in zebrafish? Cardiac function in adult vertebrates is typically viewed as tightly coupled to metabolism, which it supports through the transport of nutrients, respiratory gases, and wastes. Accordingly, it might be anticipated that temperature sensitivity of f_H would generally reflect that of $\dot{M}O_2$ throughout development, yet such was clearly not the case for zebrafish. Early in development teleost fishes rely heavily on cutaneous gas exchange in support of $\dot{M}O_2$ (22, 24, 27–31, 35, 36). Because cutaneous gas exchange in very small organisms does not rely on convective oxygen transport by blood, but rather on direct diffusive supply of oxygen to metabolically active tissues, there is no reason to assume that cardiovascular system performance would be tightly linked to metabolism early in development. Indeed, the tight "supply-and-demand" relationship between cardiac output and metabolic demand for O_2 typical of mature vertebrates is largely lacking in early trout larvae on the basis of the lack of coupling of growth-induced increases in cardiac output and $\dot{M}O_2$ as these larvae increase in body mass (22). The apparent dissociation of cardiac and metabolic patterns of change in early development in both zebrafish and trout is additionally supported by recent studies in embryonic vertebrates showing that blood circulation and/or convective blood oxygen transport is not required to maintain normal metabolic rates in zebrafish embryos younger than *hour 108* (24), as well as in embryonic and larval amphibians (6, 21, 33) and 3–5 day chicken embryos (W. Burggren, S. Warburton, and M. Slivkoff, unpublished). Thus it would appear that metabolic and cardiovascular performance become more tightly linked as development progresses, a sequence of events that begs further description in any lower vertebrate.

Although a dissociation of cardiac and metabolic functions due to cutaneous gas exchange occurs early in development, the lack of correlation of Q_{10} in $\dot{M}O_2$ and f_H in older, maturing zebrafish remains intriguing, especially because there is a tight link between cardiovascular performance and metabolism in adult teleosts (3, 9). In the absence of data on the interplay between heart rate and stroke volume, an explanation for the muted

temperature sensitivity of heart rate relative to $\dot{M}O_2$ in maturing zebrafish awaits further exploration of the comprehensive cardiovascular response to temperature change.

Influence of Hypoxia on $\dot{M}O_2$

The influence of hypoxia on $\dot{M}O_2$ in *D. rerio* was highly dependent on both developmental stage and rearing temperature, as evident from calculations of P_{crit}. As would be expected due to the significant O_2 diffusion barrier presented by the chorion and the unstirred or poorly stirred perivitelline fluid (27), unhatched embryos showed the highest P_{crit} and embryos at 31°C become oxygen conformers at just slightly below normoxic O_2 levels. In support of our initial hypothesis, P_{crit} declined (i.e., the animals were able to regulate oxygen consumption at lower PO_2 values) as development progressed. Rombough (27) has presented one of the very few other studies to examine P_{crit} in larval fish. He reported that in larval steelhead (which develop much more slowly at much lower temperatures than zebrafish) P_{crit} at 15°C similarly declines from a peak of ~140 mmHg (just under air saturation) at hatching at 20 days postfertilization to a value of ~75 mmHg (1:2 air saturation) after ~30 days postfertilization. A fall in P_{crit} with development has also been documented in larvae of the anuran amphibian *Xenopus laevis*, where P_{crit} at 20°C falls from near air saturation in water breathing larvae to ~75 mmHg once air breathing begins, terminating at a value of just 30 mmHg in adult frogs (11).

As was evident for steelhead larvae (27), at any given developmental stage in zebrafish P_{crit} was temperature sensitive, being highest at 31°C and lowest at 25°C. This reflects the higher tissue O_2 demand and lower water O_2 content at higher measurement temperatures. Nonetheless, the ability of zebrafish to obtain O_2 from a hypoxic environment, particularly as older larvae, juveniles, and adults, is impressive, and the P_{crit} of ~20 mmHg for *day 100* zebrafish at 28°C indicates the potential for acute survival in very hypoxic waters.

It is tempting to declare one developmental stage of zebrafish as a "better" or "more competent" O_2 regulator than another stage in accordance with the contemporary trend of assuming that the lower the P_{crit}, the more capable is the animal at the overall maintenance of its oxygen consumption in hypoxia. However, a comparison of P_{crit} values across development is compounded by the profound differences in mass-specific $\dot{M}O_2$ between younger and older fishes. For example, the $\dot{M}O_2$ in *day 10* larvae is ~15–30 times higher (depending on temperature) than in adults at *100 days*, this despite the fact that the early larvae are relatively lethargic compared with the normally actively swimming adults. Thus the demonstrated ability for adult fish to oxygen regulate to far lower levels of ambient PO_2 may as much reflect the "advantage" of their much lower unit metabolic rate than any inherent superiority of the oxygen-delivery mechanisms of adults compared with larvae.

Influence of Hypoxia on f_H

Hypoxic exposure caused complex changes in f_H in *D. rerio*, the exact nature of which depended on developmental stage and, to a lesser extent, rearing temperature. Interestingly, embryos and early larvae showed virtually no hypoxic response in f_H over the entire range of ambient PO_2. The lack of a hypoxic bradycardia in early larval stages has also been noted for rainbow trout (12) and Arctic char (18). Similarly, very early larvae of anuran amphibians show little or no hypoxic bradycardia (1, 5, 10), although this capability appears with additional development. A hypoxic bradycardia developed only after ~20–30 days of development, suggesting that both cardiac control mechanisms and chemoreceptors for detecting oxygen have developed and are functioning.

The severity of the bradycardia in zebrafish and the ambient PO_2 at which it developed were related to measurement temperature, first appearing at higher PO_2 values and being most severe at 31°C. It is unknown whether stroke volume changes in the same or opposite direction as f_H during severe hypoxic exposure in zebrafish. In adult teleost fishes, bradycardia does not necessarily signify reduced cardiac output, because many fishes control cardiac output primarily through stroke volume (3, 9). However, larval trout regulate cardiac output primarily through changes in heart rate (22). Whether heart rate is the major modifier of cardiac output in most larval teleosts will require further investigation.

In conclusion, we initially hypothesized that *D. rerio* would be more capable of maintaining oxygen consumption as development progressed and that at any given developmental stage, individuals reared and measured at higher temperatures would be more susceptible (as evidenced by hypoxic bradycardia) to a given level of acute hypoxia. These hypotheses have been borne out by the present experiments. Our findings additionally suggest that the direction and magnitude of f_H changes only generally reflect those of $\dot{M}O_2$. The lack of coupling of cardiac and metabolic patterns early in development most likely reflects the heavy dependence on cutaneous gas exchange by the larvae. However, the similar disparity in temperature-induced heart rate and metabolic changes in older, maturing fishes serves to highlight the need for more comprehensive cardiovascular and metabolic data over a wide range of development if we are to determine the changing interrelationships between cardiac output, blood oxygen, and metabolism during ontogeny.

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