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Growth and metabolism of larval zebrafish: effects of swim training

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Summary

Larval zebrafish (*Danio rerio*) of three different age classes ('yolk-sac' larvae, 96 h; 'swim-up' larvae, 9 days old; and 'free-swimming' larvae, 21 days old) were trained for 2, 6 and 11 days, respectively, to swim at 0 body lengths per second (0 *BL* s^{-1}), 2 *BL* s^{-1} and 5 *BL* s^{-1}. Survival was significantly diminished in larvae trained at 5 *BL* s^{-1} compared to controls (0 *BL* s^{-1}). Although training produced no significant differences in mass and length, the youngest larvae absorbed their yolk at a faster rate during training. Routine oxygen consumption ($\dot{M}_{O_2r}$) and mass-specific routine oxygen consumption ($\dot{M}_{O_2r,m}$) were not significantly affected by chronic training in the yolk-sac larvae and swim-up larvae. However, trained free-swimming larvae had a significantly higher $\dot{M}_{O_2r}$ (after 11 days of training) and $\dot{M}_{O_2r,m}$ (after 8 and 11 days of training) compared to control larvae. Trained free-swimming larvae consumed significantly less oxygen during swimming compared to control larvae, as measured by closed-system respirometry. Trained yolk-sac larvae exposed to increasing hypoxia levels were more effective O_2 regulators. Additionally, training enhanced survival during exposure to extreme hypoxia in all age groups. Thus, physiological acclimation to chronic swimming occurs in the earliest stages of life in the zebrafish.

Key words: development, oxygen consumption, larvae, swimming, hypoxia, zebrafish, *Danio rerio*.

Introduction

Swimming is an integral part of existence in most fish and has been recognized as one of the most important factors influencing energy turnover (Brett and Groves, 1979). Since many variables are involved in measuring overall swimming performance, it is difficult to determine the extent to which swimming performance is genetically inherited. However, given that most fish will instinctively swim against a current (positive rheotaxy) and can easily be manipulated in training experiments, they provide an ideal system for studying the influence of chronic exercise by swimming ('training') on metabolism.

Training leads to increases in growth rate and food-conversion efficiency in many adult salmonids (Davison and Goldspink, 1977; Davison, 1997; East and Magnan, 1987; Houlihan and Laurent, 1987; Totland et al., 1987; Christiansen and Jobling, 1990; Farrell et al., 1990; Christiansen et al., 1991; Jorgensen and Jobling, 1993). While most studies have focused on salmonids, studies on other fish species can offer insight as to how non-migratory species respond to training. Previous work on training non-salmonid species has produced both positive (Hinterleitner et al., 1992; Hammer, 1994; Young and Cech, 1994) and negative (Davison and Goldspink, 1978; Hinterleitner et al., 1992; Sanger, 1992; Davison, 1994) effects on growth rate.

Larval fish have a unique physiology and ecology and, therefore, are subjected to different selective pressures than adult fish. Larval fish experience more pronounced changes in supply–demand relationships for O_2 and CO_2 during the course of embryonic and larval development than they do as adults (Rombough, 1988). During early larval development, thermal tolerances are at their narrowest and lethal concentrations (LC_{50} values) of toxic chemicals are at their lowest (Brett, 1964; Rombough, 1996). Size-dependent processes such as diffusion of respiratory gases, and the relative importance of viscous, as opposed to inertial, forces, are likely to be much more important during early life (Rombough and Moroz, 1997). As such, hypoxic environments would have a more detrimental affect on larval fish as larvae have fewer internal mechanisms for compensation. In addition, large amounts of energy are required for their extremely fast growth. Therefore, physiological differences between larval and adult fish may ultimately result in changes in growth rates and overall metabolism when exposed to swim training.

The objective of this study is to determine the effects of swim training in the zebrafish *Danio rerio*. It is hypothesized that swim training during development will significantly increase growth rate, routine oxygen consumption and acute hypoxia tolerance. Additionally, trained zebrafish larvae

should consume less oxygen while swimming compared to controls. Since larval fish apportion relatively large amounts of resources to growth, chronically training them during this time may alter this resource allocation pattern such that growth and metabolic rate are ultimately affected.

Materials and methods

Animals

Wild-type adult zebrafish *Danio rerio* (Hamilton, 1822), obtained from Scientific Hatcheries Inc. (Huntington Beach, CA, USA) were reared by crossing true wild-type zebrafish from India with a Florida-strain wild type. Adult zebrafish were maintained in 38 l aquaria and fed a diet of brine shrimp and a custom dry food (Scientific Hatcheries Inc.). To control for random breeding, we developed a breeding system using an open breeding box containing artificial grass. The box was placed into the aquarium the evening before eggs were desired for an experiment. Zebrafish routinely bred in this box, allowing a more predictable and efficient means of collecting eggs.

Based on pilot experiments using physiological, behavioural and morphological data, the zebrafish larvae were categorized into three different age groups. (1) 'Yolk-sac' larvae were 96 h of age post-fertilization. Yolk-sac larvae describe the nutritional state of the larvae during this developmental period. Larvae at this stage did not need to be removed from the training tubes for feeding. To remain within this particular developmental nutritional state, training did not extend beyond 48 h. (2) 'Swim-up' larvae were 9 days post-fertilization. The swim-up larvae were so named because by 9 days post-fertilization, all larvae that successfully make the transition from yolk-sac utilization to external feeding swim up to the surface of the water to feed. Because the training tubes were closed, the swim-up larvae could not be fed during active training. Therefore, a regime of night training (15 h) and daytime feeding was employed. The training regime for this age group was 6 days in length to encompass this nutritional state and avoid the significant increase in mass that begins at approximately 17 days post-fertilization. (3) 'Free-swimming' larvae were 21 days post-fertilization. This age group was chosen to encompass a period of significant growth. Again, continuous training was not possible because of the design of the training apparatus, so the same regime of night training (15 h) and day feeding was used. This regime was terminated at 11 days due to the increasing size of the fish compromising the training system.

Apparatus

Eggs were harvested from breeding tanks and raised in 4 l jars. The water in the jars was kept at normoxic levels using gentle aeration (one small air bubble per second) and water was replaced once daily. Depending on their size, larvae in jars were fed four times daily with *Paramecium multinucleatum* or newly hatched *Artemia*. Once a group of larvae attained the appropriate age, they were then transferred to the training apparatus, which was used to maintain an environment of known water velocity and a set volume. The apparatus is a gravity-fed system consisting of 8 separate treatment tubes, each allowing for fine adjustment of water velocity. The treatment tubes, in which the larvae were placed, had an inside diameter of 1.27 cm and a length of 23 cm, for a total volume of 30 ml of water. Fine nylon mesh on each end of the tube retained the larvae within the body of the tube. A filtering apparatus was placed in the top water reservoir to ensure that large particulate matter did not enter the gravity-fed water-flow system. A small bolus of dye injected upstream from the first nylon mesh confirmed that water flow was laminar through the tube. A desired water velocity was set for each tube by measuring and adjusting the flow ($l\,min^{-1}$) out of each tube and using the equation:

$$\Delta m/\Delta t = \rho A V, \quad (1)$$

where $\Delta m/\Delta t$ is the flow ($g\,min^{-1}$), ρ is the density of the fluid ($g\,cm^{-3}$), A is the cross-sectional area of the tube (cm^2), and V is the velocity of the fluid ($cm\,min^{-1}$). Velocities calculated in this manner were initially confirmed by timing the movement of dye along a given distance in the tube. Once inside the training tubes, the larvae were subjected either to a treatment of constantly flowing water, or to a control tube with insignificant water velocity ($<0.1\,BL\,s^{-1}$). P_{O_2} was maintained at normoxic levels by vigorous aeration in the reservoir tanks.

A maximum of 30 larvae were placed in each treatment tube, creating the maximum density suggested by Scientific Hatcheries Inc. as compatible with optimal larval growth. The ratio of total larval cross-sectional area to tube cross-sectional area ranged from 0.1 % in yolk-sac larvae to 1.0 % in free-swimming larvae. Thus, solid blocking was not an issue in any age group, based on behavioural observations. Behavioural observations were recorded randomly using a VHS-C video camera.

Training protocol

Larvae were placed into the training tubes once they reached one of the three defined age groups. 20–30 larvae of the same age were placed in each of 12 tubes with water velocities of $0\,BL\,s^{-1}$ (body lengths per second) $2\,BL\,s^{-1}$ or $5\,BL\,s^{-1}$. Larvae were trained for 48 h continuously and measurements began 24 h following the onset of training (i.e. at 120 h post-fertilization). Larvae in the older age groups were fed during the day and trained at night. Preliminary data show that $\dot{M}_{O_2r}$ (defined as the amount of oxygen used during routine, unrestrained, unforced activity, measured in $nmol\,O_2\,fish^{-1}\,h^{-1}$) increases by about 15 % from feeding, but this has not been isolated from effects of handling or circadian rhythm. This increase disappears within 4 h, therefore measuring control and trained fish before feeding in the morning should have eliminated any such SDA effects.

Each day, during the course of the experiments, the larvae in each tube were counted to assess survival rate. Additionally, a subsample of larvae from each tube was removed and length, wet mass, dry mass and yolk volume (yolk-sac larvae only) were measured. Individual body lengths in swim-up and free-

swimming larvae were measured optically under a dissecting microscope using a micrometer. Lengths of yolk-sac larvae were obtained by capturing images *via* a camera where they were analyzed using Image-Pro® (Version 4.1) software. Yolk-sac volumes were obtained in a similar manner; larvae were manipulated until all three dimensions (anterio–caudal length, dorso–ventral height and lateral width) of the yolk were recorded. All masses were obtained in individual larvae using a Cahn C-31 microbalance. Wet masses were obtained by lightly dabbing the larvae with a Kimwipe®. Care was taken to ensure consistent timing during this process. To obtain the dry mass, the larvae were desiccated at 50 °C for 24 h and then reweighed.

Routine oxygen consumption $\dot{M}_{O_2r}$ was measured using closed system respirometry in larvae in the morning, 1 h after removal from the training apparatus (no feeding that morning), at given intervals during the training process in all three age groups. This technique in zebrafish is described elsewhere (Barrionuevo and Burggren, 1999; Pelster and Burggren, 1996). Briefly, the larvae were transferred to 2 ml glass syringes sealed with a 3-way stopcock (5 larvae per syringe in the youngest age group, 1 larva per syringe in the older two age groups). The partial pressure of oxygen P_{O_2} of the water in the syringe was measured every 30 min for 2 h and the mean P_{O_2} calculated. Measurements were corrected for background oxygen consumption, which was found to be minimal. The P_{O_2} from water in the syringe respirometers was measured with a BGM 2000 Blood Gas Meter and associated P_{O_2} electrode (Cameron Instruments). Oxygen consumption is presented both as values per fish and per mg wet tissue. The merits of each presentation have been previously discussed (McNab, 1999); however, both formats reveal valuable information and thus are given here.

Active oxygen consumption $\dot{M}_{O_2a,m}$ (defined as the amount of oxygen required to swim at a set speed) was also determined using the same P_{O_2} system. Following the 11-day training period in the oldest group, larvae were placed individually into swim respirometers. Each swim respirometer was constructed of glass tubing (5 mm inside diameter) shaped into a rectangle (mean volume of micro-swim respirometers = 32.3 ml) with two sample ports, a glass 2-way stopcock, and a small magnetic pump, all in series. Once the larvae were transferred to the micro-swim tunnel, a water velocity between 0 $BL\,s^{-1}$ and 5 $BL\,s^{-1}$ was set and the P_{O_2} decline in a water sample was measured after 1 h of swimming.

Critical P_{O_2} (P_{crit}) was calculated by using a series of closed system oxygen consumption measurements. Larvae were transferred to a 5 ml glass syringe (8 larvae per syringe) and the P_{O_2} in the syringe was measured at predetermined intervals until the O_2 consumption by the larvae themselves eventually caused the water to become extremely hypoxic (<20 mmHg) (Barrionuevo and Burggren, 1999). The resulting data were plotted with MathCad® 4.0 using a modified version of the program (Yeager and Ultsch, 1989) to determine the P_{crit}. Critical debilitation P_{O_2} (P_d) was concurrently measured during the P_{crit} experiments. When each individual larva lost its equilibrium, the P_{O_2} was recorded. Calculating the P_d was then performed in the same manner as above.

Statistics

For survival, control and trained groups were compared independently within each age group by performing a repeated measures ANOVA. Wet mass, dry mass, water content, length, yolk-sac volume and routine oxygen consumption, were compared independently for control and trained larvae within each age group by performing a two-way ANOVA. Where significant treatment effects were observed, *post-hoc* pairwise comparisons were performed using Tukey's multiple-comparisons procedure. Percentage data that were non-normal were arcsine-transformed, which normalized the data (Zar, 1984). Active metabolic rate and P_{crit} measurements were analyzed by comparing the regression slopes and elevations between the two lines (Zar, 1984). All analyses were performed using SigmaStat® and the level of significance was set at $P<0.05$. All values presented are mean ± 1 S.E.M.

Results

Behavioural observations

Even though trained larvae were observed to be swimming only 9.3±0.6 % of the time at 5 $BL\,s^{-1}$, this was significantly greater ($P<0.001$) than the control larvae, which were rarely observed to be actually swimming (less than 0.2 % of the time).

Although control swim-up larvae were more active (52.4±5.5 % of the time) compared to yolk-sac larvae, there was still significantly less activity compared to yolk-sac swim-up larvae training at 5 $BL\,s^{-1}$ (78.6±6.3 % of the time) ($P=0.011$). The small size of the larvae and their low density in the training tube probably contributed to the lack of observation of blocking by larvae during swimming.

Free-swimming larvae maintained their position in the training tube by swimming in short bursts, but these bursts appeared longer in duration and appeared to produce more force, both of which provided more time for drifting and searching behaviour compared to the swim-up age group. Because of increased drifting and searching behaviour, free-swimming larvae training spent less time (59.0±5.2 %) actually swimming against the current compared to swim-up larvae. However, the total amount of time spent swimming (71.9±8.1 %) was significantly greater than by control free-swimming larvae (57±4.8 %) ($P=0.03$).

Survival

Survival was most affected by the training regime in yolk-sac (day 4) zebrafish larvae (Fig. 1). There was a significant training ($P<0.001$) and age ($P<0.001$) effect on survival within this age group. Specifically, there was a significantly reduced survival at 5 $BL\,s^{-1}$ ($P<0.05$) after the first day of treatment. The training velocity of 2 $BL\,s^{-1}$ did not produce a significant reduction in survival until after the second day of treatment, when the reduction became significant ($P<0.001$). Approximately 30 % of

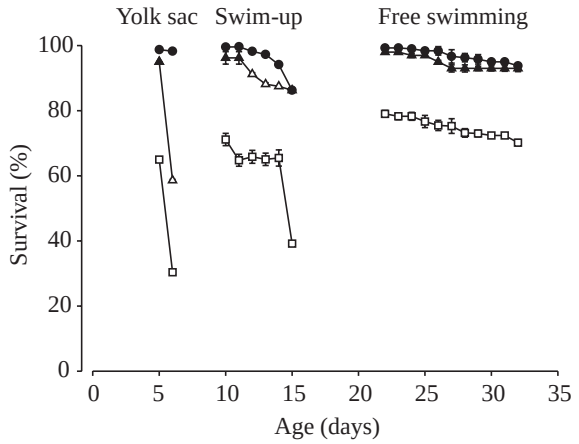

Fig. 1. Survival of zebrafish yolk-sac larvae (day 4), swim-up larvae (day 9), and free-swimming larvae (day 21), at the conclusion of a training period. Circles, control group; triangles, larvae training at 2 *BL* s^{-1}; squares, larvae training at 5 *BL* s^{-1}. Open symbols indicate a value significantly different from the corresponding control value. Values are means ± 1 S.E.M.; *N*=120 for each treatment group at the beginning of the treatment.

the yolk sac zebrafish larvae survived the second day of treatment at 5 *BL* s^{-1}.

Swim-up (day 9) larvae also showed a significant training (P=0.036) and age (P<0.001) effect (Fig. 1). Swim-up larvae were more active swimmers compared to the younger group, tolerating the 2 *BL* s^{-1} training velocity for 2 days with no significant mortality. However, under the 5 *BL* s^{-1} treatment, there was a significant reduction in survival after the first day of training, and a further significant reduction after the sixth day of treatment.

In free-swimming (day 21) zebrafish larvae, there was a significant training (P<0.001) effect but no age (P=0.65) effect (Fig. 1). There was no significant mortality during training at 2 *BL* s^{-1}; however, as with the swim-up larvae, the free-swimming larvae showed a significant mortality after the first day of training at 5 *BL* s^{-1}.

Morphometrics

During the first 32 days of development, mean larval zebrafish wet and dry mass increased 13-fold, and length increased twofold (Table 1). The bulk of these increases occurred during the free-swimming stage.

There was no significant training effect on wet mass, dry mass, length or water content in yolk sac zebrafish larvae (Table 1). However, there was a significant effect of training (P<0.001) observed in yolk size, in that trained larvae lost their yolk sac at a significantly higher rate than control larvae (Fig. 2). Yolk-sac larvae changed over the duration of the experiment showing a significant decrease in dry mass, increase in length and decrease in water content (P<0.05), but no change in wet mass.

As in the yolk-sac larvae, the swim-up larvae showed no significant effects of training (P>0.1) in wet mass, dry mass, length or water content (Table 1). During the 6-day experimental period, swim-up larvae increased significantly in wet mass, dry mass, length and water content (P>0.05).

A similar trend occurred in the free-swimming zebrafish larvae, with this group showing no significant effects of training but significant effects of age (P<0.001) in all morphometric variables measured (Table 1).

Oxygen consumption

Only the free-swimming larvae age group displayed an effect of training on both routine oxygen consumption ($\dot{M}_{O_2r}$)

Table 1. *Wet mass, dry mass, length and water content of zebrafish yolk-sac larvae (day 4), swim-up larvae (day 9), and free-swimming larvae (day 21), during exposure to training*

		Group							
	Swim	Yolk-sac		Swim-up			Free-swimming		
Variable	training speed	Day 1	Day 2	Day 2	Day 4	Day 6	Day 5	Day 8	Day 11
Wet mass (mg)	Control	0.331±0.010	0.338±0.011	0.397±0.019	0.440±0.025	0.488±0.035	1.38±0.33	2.16±0.32	3.69±0.38
	2 *BL* s^{-1}	0.346±0.019	0.353±0.010	0.389±0.013	0.454±0.017	0.489±0.037			
	5 *BL* s^{-1}	0.341±0.009	0.348±0.011	0.380±0.015	0.489±0.033	0.454±0.045	1.24±0.42	2.06±0.40	3.46±0.48
Dry mass (mg)	Control	0.061±0.002	0.055±0.002	0.064±0.003	0.073±0.005	0.075±0.007	0.180±0.043	0.350±0.049	0.610±0.064
	2 *BL* s^{-1}	0.061±0.002	0.055±0.001	0.061±0.002	0.067±0.003	0.073±0.006			
	5 *BL* s^{-1}	0.057±0.003	0.060±0.002	0.059±0.002	0.068±0.004	0.074±0.007	0.170±0.029	0.320±0.052	0.595±0.087
Length (mm)	Control	3.9±0.03	4.0±0.05	4.2±0.06	4.4±0.06	4.6±0.08	6.4±0.15	6.9±0.20	8.2±0.22
	2 *BL* s^{-1}	4.1±0.05	4.0±0.05	4.2±0.05	4.4±0.07	4.5±0.06			
	5 *BL* s^{-1}	4.1±0.07	4.1±0.05	4.3±0.06	4.4±0.07	4.6±0.08	6.2±0.16	6.7±0.17	8.3±0.39
Water content (%)	Control	83.8±0.6	80.2±0.5	84.0±0.4	84.6±0.5	85.6±0.3	84.9±0.6	85.6±0.7	83.9±0.3
	2 *BL* s^{-1}	84.3±0.6	81.2±0.7	85.1±0.6	85.4±0.4	85.0±0.3			
	5 *BL* s^{-1}	82.5±0.5	82.9±0.7	84.7±0.7	85.1±0.3	85.0±0.3	85.9±0.5	86.1±0.7	84.5±0.4

N=40 for each group of masses and water content; *N*=20 for each group of lengths. Values are mean ± S.E.M

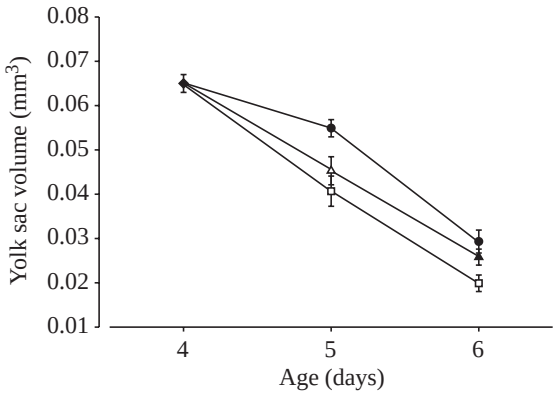

Fig. 2. Yolk-sac volume in day-4 zebrafish larvae exposed to training at $2\,BL\,s^{-1}$ and $5\,BL\,s^{-1}$. Diamond, starting point; circles, control group; triangles, larvae training at $2\,BL\,s^{-1}$; squares, larvae training at $5\,BL\,s^{-1}$. An open symbol indicates a value significantly different from the corresponding control value. Values are means ± 1 S.E.M.; N=10 for each group.

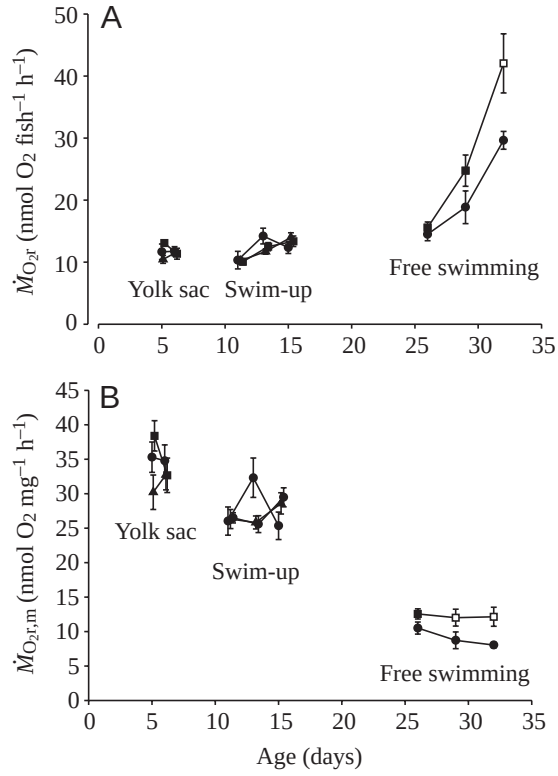

Fig. 3. Routine oxygen consumption ($\dot{M}_{O_2r}$) (A) and mass-specific routine oxygen consumption ($\dot{M}_{O_2r,m}$) (B) of zebrafish yolk-sac larvae (day 4), swim-up larvae (day 9), and free-swimming larvae (day 21), exposed to training. Circles, control group; triangles, training at $2\,BL\,s^{-1}$; squares, larvae training at $5\,BL\,s^{-1}$. An open symbol indicates a value significantly different from the corresponding control value. Values are means ± 1 S.E.M.; N=8 for each group.

(P=0.004) and mass-specific routine oxygen consumption ($\dot{M}_{O_2r,m}$) (P<0.001) (Fig. 3). A significant age effect in $\dot{M}_{O_2r}$ was found in both swim-up and free-swimming larvae

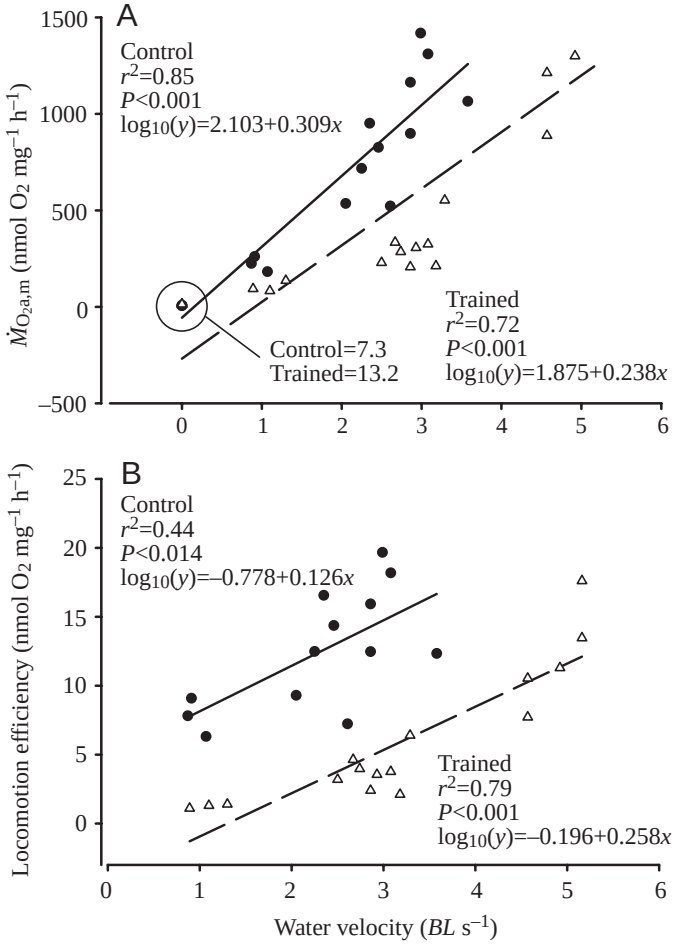

Fig. 4. Active mass-specific oxygen consumption ($\dot{M}_{O_2a,m}$) (A) and locomotion efficiency (nmol $O_2\,mg^{-1}\,m^{-1}$) (B) in free-swimming zebrafish (day 21) as a function of water velocity. Larvae were measured after the 11-day experimental period of either no training or training at $5\,BL\,s^{-1}$. Open symbols indicate that the regression line is significantly different from the control (filled circles).

(P<0.001), but there were no significant differences after standardizing for body mass. At the end of the training period, the free-swimming larvae increased $\dot{M}_{O_2r}$ in both the control (2.5-fold) and trained (3.6-fold) groups compared to the yolk-sac larvae. Mass-specific $\dot{M}_{O_2r,m}$ decreased with growth, as anticipated. $\dot{M}_{O_2r,m}$ at the end of the experimental period in free-swimming larvae was almost 25% that of the yolk-sac larvae. Unlike the other age groups, trained free-swimming larvae maintained a significantly higher $\dot{M}_{O_2r,m}$ than controls on days 8 and 11 of training.

$\dot{M}_{O_2a,m}$ at a particular water velocity was plotted in control free-swimming zebrafish larvae and those trained for 11 days at 5 BL s–1 (Fig. 4A). A polynomial regression analysis showed the control group having a significant first-order regression (r^2=0.85; P<0.001). Although polynomial regression analysis showed the trained group having a more significant second-order regression (r^2=0.89; P<0.001), the significant first-order regression (r^2=0.72; P<0.01) was used to facilitate comparison. When larvae swam against a current (i.e.

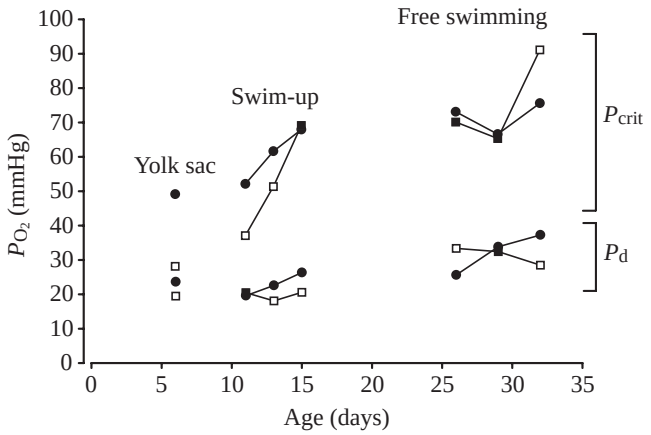

Fig. 5. Critical P_{O_2} (P_{crit}) and critical debilitation P_{O_2} (P_d) of zebrafish yolk-sac larvae (day 4), swim-up larvae (day 9), and free-swimming larvae (day 21), exposed to training. Circles, control groups; squares, larvae training at 5 *BL* s^{-1}. An open symbol indicates a value significantly different from the corresponding control value. Values are means ± 1 S.E.M.; *N*=8 for each group.

when they were in non-routine conditions) $\dot{M}_{O_2a,m}$ at all swimming speeds was significantly lower in larvae that were trained. Trained larvae also consumed significantly less oxygen to travel a given distance, at a given water velocity (Fig. 4B).

Yolk-sac larvae trained at 5 *BL* s^{-1} were able to maintain oxygen consumption in the face of progressive hypoxia at levels close to resting values down to a P_{crit} of just 27 mmHg (Fig. 5A). This was not the case for the control yolk-sac larvae, which had a P_{crit} of 48 mmHg, and which had reduced their $\dot{M}_{O_2}$ by about 50 % at a P_{O_2} of 25 mmHg. At this extremely low P_{O_2}, the majority of the trained larvae were able to maintain righting ability, which was not the case for the majority of the control larvae (Fig. 5B).

Swim-up larvae exhibited a similar trend (Fig. 5A). Trained larvae had a P_{crit} of 36 mmHg after the second day of training compared to 51 mmHg for controls. This effect disappeared in larvae that were trained for 6 days (trained P_{crit}=68 mmHg; control P_{crit}=67 mmHg). The percentage of larvae that were able to maintain righting ability was significantly greater (P<0.001) in those larvae that were trained. This trend began at day 4 of training and persisted throughout the sixth day of training.

Critical P_{O_2} values for free-swimming larvae were significantly higher in the trained larvae only on the eleventh day of training (P=0.023; trained P_{crit}=90 mmHg; control P_{crit}=75 mmHg) (Fig. 5A). However, the P_{O_2} at which the animals became debilitated was significantly lower in the trained larvae on the eleventh day (P<0.001; trained P_d=29 mmHg; control P_d=37 mmHg) (Fig. 5B).

Discussion

The aim of this study was to determine the effects of swim training in the zebrafish. It was hypothesized that swim training during development would significantly increase growth rate, routine oxygen consumption and acute hypoxia tolerance. Additionally, we predicted that trained zebrafish larvae should consume less oxygen while swimming compared to controls. It was found that while training had no effect on the growth rate of zebrafish larvae, it significantly increased routine oxygen consumption in older larvae and decreased active oxygen consumption. Training also improved hypoxia tolerance in all larval age groups.

Consideration of experimental design

According to Westerfield (1993) and many other zebrafish geneticists, the preferred temperature of the adult zebrafish is 28 °C. However, Scientific Hatcheries Inc. (Huntington Beach, CA, USA; personal communication) and B. Bagatto (unpublished) have shown that survival and fecundity are markedly reduced at 28 °C compared to 25 °C, at least in populations reared at and purchased from this company. Therefore, all zebrafish in this study were maintained at 25 °C, ensuring maximum survivability and egg production. As a result, staging and hatching were slightly delayed (B. Bagatto, unpublished) relative to tables in Westerfield (1993) devised for 28.5 °C.

Since this is an investigation not only of training effects, but also of developmental physiology, the developmental time factor must be examined very carefully. Although it would be ideal to impose a standard ‘adult’ training protocol on a group of larvae as they develop, we must take into account the qualitative and quantitative changes that occur during the developmental time frame studied. This complicates the study, requiring changes to be made in the protocol to accommodate changes in physiology and morphology during development. For example, different feeding and swimming behaviors were evident at different stages of development. Yolk-sac larvae tended to cling to the wall of the training tube using their mouth. This alone was not sufficient to maintain their position in the training tube, so they swam sporadically, but often, to maintain their position in the training tube. In contrast, the actively feeding swim-up larvae maintained their position in the training tube by swimming in short bursts. Short burst swimming was nearly constant, with very little drifting or searching behaviour (defined as swimming in any direction except parallel to the current) observed. These differences in swimming and feeding behavior necessitated changes in the protocol during the course of larval development. Nonetheless, there were considerable similarities in protocol, allowing comparison of physiological performance during development.

Growth and metabolism in control populations

Zebrafish used in this study maintained survival, growth and metabolic characteristics that were consistent with other studies on similarly aged zebrafish. All control populations maintained survival rates above 80 % (Fig. 1). Goolish et al. (1999) reported significantly reduced survival rates (as low as 15 %) when newly hatched zebrafish were maintained on processed diets. However, their preferred diet of *Paramecium* and brine shrimp could produce survival rates above 80 % in

some cases, especially with the right combination of water turnover. Zebrafish in this study were maintained on *Paramecium* larvae and brine shrimp with aquarium water that was changed daily. Growth rates at day 18 post-fertilization in this study (mean dry mass=0.13 mg, mean standard length=5.1 mm) were slightly lower compared to zebrafish in the study of Goolish et al. (1999) (mean dry mass=0.18 mg, mean standard length=5.9 mm). However, these differences were probably temperature related, as our study was completed at 25 °C compared to 28 °C for animals described by Goolish et al. (1999) (Table 1). In one other study (Barrionuevo and Burggren, 1999) involving growth in zebrafish at 25 °C, wet masses during similar developmental stages were quite similar to those in our study, though body lengths in our study were 30–40 % longer. This difference may be attributed to a more rigorous feeding regime and/or a different standard length measurement technique used in this study.

Oxygen consumption in zebrafish increased approximately threefold over the initial 32 days of development assessed in this study, which was very similar to the results obtained by Barrionuevo and Burggren (1999) over the same developmental period (Fig. 3). Pelster and Burggren (1996) measured $\dot{M}_{O_2}$ in zebrafish, but they concentrated on the first few days of development. Although their metabolic rates (mean $\dot{M}_{O_2}$=17 nmol O_2 $fish^{-1}$ h^{-1}) were slightly higher on day 4 post-fertilization compared to this study (mean $\dot{M}_{O_2}$=13 nmol O_2 $fish^{-1}$ h^{-1}), development and measurement at 26 °C may account for this difference. Overall, the general aspects of our control animals were in reasonable agreement with previous studies.

Effects of chronic swim training on growth

Chronic training significantly reduced zebrafish survival in all age groups (Fig. 1). There appear to be three potential factors that affect mortality: the age of the larvae at the start of training, the magnitude of the swim velocity used to train the fish, and the time spent in the training regime. Natural variation results in fish with different swimming abilities (Brett, 1964), especially at early stages in development. Thus, there are some zebrafish larvae that were physiologically unequipped to deal with training, especially at a younger age and at higher water velocities. A question arising from using a training regime that produces mortality in populations of fish larvae is whether the results obtained describe a selection effect (those larvae that survive the training have an inherent specific characteristic) or a true training effect (the measured characteristic is the direct result of chronic training). In examining this feeding regime and a different regime adopted by B. Bagatto and W. W. Burggren (unpublished), it appears that the mortality in the swim-up and free-swimming larvae was primarily due to the widely used feeding procedure suggested for zebrafish (Westerfield, 1993). Problems with swallowing *Paramecia* or *Artemia*, coupled with possible competition for food with a comparatively low nutritional value, may have contributed to the initial mortality observed in this series of experiments. One must clearly demonstrate that nutrition, and animal husbandry in general, have minimal effects on survival. Later experiments measuring cardiovascular variables (B. Bagatto and W. W. Burggren, unpublished) resulted in no significant mortality, even after the first day of training, when larvae were fed a newly developed specialty larval powder obtained from Scientific Hatcheries Inc. This leads us to believe that the training-induced mortality in the swim-up and free-swimming larvae was primarily the result of the commonly recommended feeding regime. It is likely that those larvae that were unsuccessful in acquiring ample *Paramecia* or *Artemia* during rearing died after the first day of training. This leaves the yolk-sac larvae, which were not fed during the experiments. Since similar survival results in this age group were obtained in our later experiments, it is likely that the results obtained during training included some combination of selection and training effects.

The resource allocation most relevant to larval zebrafish training is the metabolic fuel for swimming and for growing. The problem of energy partitioning in larval cyprinids characterized by high levels, but low scopes, for aerobic metabolism has been addressed (Wieser et al., 1988). The conclusion was that the feeding level determined the rate of growth but not the rate of routine activity. Since there were no significant treatment effects in mass or length in our study (Table 1), the allocation of resources for growth were not altered by training. Stated differently, training did not improve growth rate in zebrafish larvae as it seems to do in most adult fish tested (Davison, 1997). Wieser et al. (1988) postulated a 'switching strategy' of energy allocation, since high rates of growth and high levels of swimming activity appear to be mutually exclusive within the energy constraints beyond maintenance. This suggests that those zebrafish larvae that survived the training process could not allocate additional resources to growth. Again, while this may have been due to inadequacies in the diet, later studies using the improved diet similarly show no mass differences between treatments. This indicates that training fish allocate any extra energy obtained into swimming. Indeed, trained yolk-sac larvae lost their yolk sac significantly faster than the control group, indicating that there was an overall increase in energy use during training (Fig. 2). However, given that there was such a significant mortality in this age group, it is possible that those larvae with initially bigger yolk sacs survived the training simply because of the initial quantity of resources. This would explain the lack of a training effect on larval mass in this age group. Without measuring energy budgets in the older two larval groups, it is difficult to determine whether trained fish consumed more food to compensate for the increased fuel expenditure during training or whether the larvae became more efficient at energy conversion.

Effects of chronic swim training on oxygen consumption

Routine oxygen consumption was significantly different between trained and control groups only in free-swimming larvae following 11 days of training (Fig. 3A). This trained group of larvae had a significantly higher $\dot{M}_{O_2r}$ than controls,

and when oxygen consumption was calculated on a per mg basis, the trained group of free-swimming larvae had a significantly higher $\dot{M}_{O_2r,m}$ at 8 and 11 days of training compared with controls (Fig. 3B). Since Wieser et al. (1988) documented that feeding level had no effect on the $\dot{M}_{O_2r}$ but positively affected growth rate in *Rutilus rutilus* (L.) and *Chalcalburnus chalcoides mento* (Agassiz), it was unlikely that differences would be observed in $\dot{M}_{O_2r}$ during our study, given that there were no mass differences between the trained and control groups. However, since $\dot{M}_{O_2r,m}$ was significantly higher in trained free-swimming larvae, the tissues themselves are routinely more active compared to controls. This might be an actual training effect that causes an elevation in resting oxygen consumption, or could just simply be the result of an increased routine activity level. Behavioral observations in other experiments suggest that significantly increased routine activity levels in trained larvae contribute to the increase in $\dot{M}_{O_2r,m}$.

Free-swimming larvae that were trained consumed less oxygen than control larvae at any given swimming speed over a range of water velocities, although there was no change in the maximum $\dot{M}_{O_2}$ (Fig. 4). This suggests that chronic training during growth and development enhanced any one or all of muscle physiology, energy conversion, and/or locomotion efficiency. In general, maintaining fish at chronic, sustainable swimming speeds leads to an increase in red muscle (expressed as a percentage of total myotomal muscle) attributable to both hypertrophic (increase in muscle cell size) and hyperplastic (increase in number of muscle cells) growth (Johnston et al., 1977). White muscle also appears to be affected by training, inducing hypertrophic growth, even at low speeds when contraction of the white muscle would involve essentially no physical load (Johnston et al., 1977). Muscle enzyme activities also increase with training, particularly those that convey lipids into the Krebs' cycle, indicating that fish under training stress use lipids as the major energy source (Farrell et al., 1990; Farrell et al., 1991), which may account for the increase in routine $\dot{M}_{O_2r,m}$ documented in our study. Specifically, training induces an increase in succinic dehydrogenase activity in red muscle of *Channa punctata* (Urfi and Talesara, 1989) and in all muscle types in zebrafish *Danio rerio* (de Graaf et al., 1990). This seems to be the likely mechanism for the significant increase in swimming efficiency, but the specific answer remains uncertain without a detailed biochemical muscle analysis.

If increased swimming efficiency results in part from enhanced oxygen delivery to muscles, then trained fish should be better able to cope with hypoxic exposure. Indeed, trained groups of the yolk-sac larvae had a lower P_{crit} (Fig. 5A). This suggests that training altered yolk sac larval physiology such that $\dot{M}_{O_2}$ could be maintained under extreme hypoxia. Why is this benefit observed only in the younger trained larvae? During the early embryonic phases of all vertebrates, gas exchange and distribution strictly *via* diffusion is gradually replaced by internal convective transport using a respiratory hemoglobin distributed by action of the heart (Burggren and Territo, 1995). Pelster and Burggren (1996) documented that zebrafish larvae could survive well into the fifth day post-fertilization without functional red blood cells. Perhaps the greater role of cutaneous diffusion (Rombough, 1988), in conjunction with training, plays a greater role in the smaller larvae, allowing them to maintain cardiorespiratory function in severe hypoxia.

A decrease in ambient P_{O_2} to the level of the P_{crit} does not necessarily signify the eventual death of the animal. By observing a loss of equilibrium (or debilitation) in larvae under increasing hypoxia, the impending death of the animal was more apparent. Overall, P_d values were much lower than the corresponding P_{crit} (Fig. 5). The effects of training clearly displayed a benefit as trained yolk-sac larvae became debilitated at significantly lower P_{O_2} values than control larvae (Fig. 5B). In trained swim-up larvae, the lower P_d did not appear until day 4 of training and in free-swimming larvae, it was not significantly lower until day 11 of training. Therefore, this benefit took longer to be established during training in older larval groups. Perhaps this result relates to the process of gill formation in the early larva. Newly hatched fish undergo a transition from cutaneous respiration to gill respiration. It is between the time of hatching and yolk sac absorption that the skin thickens and the fish must develop the gills structurally and functionally to the extent that they can assume the role of primary gas exchange (Holeton, 1971; Rombough, 1988). Training could induce a number of factors (greater affinity of hemoglobin to oxygen or greater hematocrit) to aid the larvae in surviving severe external hypoxia.

Conclusions

In summary, chronic training benefits zebrafish larvae even though a small amount of training mortality (selection) may have occurred in the younger larvae. Trained zebrafish not only swim more efficiently, but they are able to better cope with severe external hypoxia. Therefore, it is clear that acclimation can quickly occur at the earliest stages of development. However, further experiments are needed to determine the exact mechanisms by which these changes are manifested, and the permanence of these changes. It is likely that some part of the oxygen transport system is the limiting factor, especially in older larvae. Whether it is changes mitochondria, oxidative capacity, cardiac output, oxygen transport, or diffusion at the gills, the exact mechanism remains unknown at present.

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