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Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*

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Summary

A variation on the classic ablation method was used to determine whether O_2 uptake or ionoregulation is the first to shift from the skin to the gills in developing zebrafish, *Danio rerio*. Zebrafish larvae, ranging in age from 3 to 21 days postfertilization, were prevented from ventilating their gills and forced to rely on cutaneous processes by exposing them to one of two anaesthetics (tricaine methanesulphonate or phenoxyethanol) or by embedding their gills in agar. They were then placed in solutions designed to compensate selectively for impaired O_2 uptake (42 % O_2), impaired ionoregulatory capacity (50 % physiological saline) or impairment of both functions (42 % O_2+50 % physiological saline). Survival under these conditions was compared with that in normoxic (21 % O_2) fresh water. Neither hyperoxia nor 50 % physiological saline had any significant effect on the survival of newly hatched larvae (3 days postfertilization), suggesting that at this stage cutaneous exchange was sufficient to satisfy both ionoregulatory and respiratory requirements. At 7 days postfertilization, the skin still appeared capable of satisfying the O_2 requirements of larvae but not their ionoregulatory requirements. Physiological saline significantly improved survival at 7 days postfertilization; hyperoxia did not. At 14 days postfertilization, both hyperoxia and 50 % saline significantly improved survival, indicating that at this stage gills were required for O_2 uptake as well as for ionoregulation. At 21 days postfertilization, only hyperoxia significantly improved survival. By this stage, larvae apparently are so dependent on gills for O_2 uptake that they suffocate before the effects of ionoregulatory impairment become apparent. Thus, it would appear that in zebrafish it is the ionoregulatory capacity of the skin not its ability to take up O_2 that first becomes limiting. This raises the possibility that ionoregulatory pressures may play a more important role in gill development than is generally appreciated.

Key words: fish, zebrafish, *Danio rerio*, gill, development, ontogeny, respiration, ionoregulation, oxygen, natural selection, evolution.

Introduction

Gills perform a variety of physiological functions in adult fish including respiratory gas exchange, ion and water balance, excretion of nitrogenous wastes and the maintenance of acid–base balance. Gills, however, do not form until relatively late in development and until then these vital exchange processes must take place elsewhere, presumably somewhere on the surface of the skin. When the gills do begin to develop, the dominant site of these activities eventually shifts from the skin to the gills. What is not well known is when and in what sequence these shifts occur.

Until recently, it was generally accepted that the first critical physiological function to shift to the developing gill was oxygen uptake. Indeed, oxygen uptake is widely assumed to be the *raison d'être* for gill development in fish (Krogh, 1941; Hughes, 1984; Blaxter, 1988). The results of several recent morphological studies of gill development, however, cast doubt on this assumption. In at least four species, tilapia *Oreochromis mossambicus* (Li et al., 1995: van der Heijden et al., 1999), Japanese flounder *Paralixhthys olivaceous* (Hiroi et al., 1998), rainbow trout *Oncorhynchus mykiss* (Gonzalez et al., 1996; Rombough, 1999) and brown trout *Salmo trutta* (Rojo et al., 1997; Pisam et al., 2000), large numbers of mitochondria-rich cells (ionocytes) appear on the gill well before there is any indication of secondary lamellae, the definitive adult gas-exchange structure. In rainbow trout, for example, ionocytes were first observed on the gills 3 days before hatch, while secondary lamellae did not begin to differentiate until 8 days after hatch (Rombough, 1999). The histological appearance (van der Heijden et al., 1999; Pisam et al., 2000) and biochemical characteristics (Li et al., 1995) of these larval ionocytes are virtually identical to those found in the gill of adult fish, suggesting that they probably perform much the same functions. In adult fish, gill ionocytes are involved in a variety of interrelated processes including ion exchange, acid–base balance (Perry, 1997) and, in some species, ammonia excretion (Wilson et al., 2000). The appearance of large numbers of branchial ionocytes so far in advance of secondary lamellae has led to speculation that the

first critical physiological function to shift to the developing gill may be ion balance (or some correlate) and not O_2 uptake as previously believed (Li et al., 1995; Hiroi et al., 1998; Rombough, 1999). At present, however, the evidence supporting this view is indirect, based on morphological indicators which, unfortunately, do not always accurately reflect physiological realities. The goal of the present study was to try to obtain direct physiological evidence concerning which function, O_2 uptake or ion balance, is the first to shift to the gills in developing zebrafish, *Danio rerio*.

The method used to determine the order in which functions shift to the gill was a variation on the classic ablation study. Zebrafish larvae at various stages of development were prevented from ventilating their gills and then placed in media designed to compensate selectively for impaired oxygen uptake (42 % O_2), impaired ionoregulation (50 % physiological saline) or impairment of both functions (42 % O_2+50 % saline). Survival in these media was compared with survival in normoxic (21 % O_2) fresh water. It was hypothesized: (i) that if gills were required for O_2 uptake, hyperoxia would improve survival, (ii) that if gills were needed for ionoregulation, physiological saline would improve survival, and (iii) that if gills were necessary for O_2 uptake as well as ionoregulation, both hyperoxia and physiological saline would improve survival.

Materials and methods

Animals

Adult zebrafish *Danio rerio* (Hamilton, 1822) were purchased from a local supplier. The fish were transferred to Brandon University, where they were raised at a nominal temperature of 28 °C in dechlorinated fresh water (total hardness 136 mg l^{-1} as $CaCO_3$, pH 7.9) under a 14 h:10 h light:dark photoperiod. Mature adults were selected for breeding the day before eggs were required. The fish (typically eight females and four males) were placed in a 20 l breeding tank, the bottom of which was covered with several layers of glass marbles, in the evening 2–4 h before the lights went out. Eggs were collected the next morning 2 h after the lights came back on. Eggs were assumed to have been fertilized 1 h after the lights came on (zebrafish normally breed shortly after 'dawn').

Fertilized eggs (typically 100–200) were transferred to 250 ml aerated glass finger bowls, which were then placed in a constant-temperature water bath held at 28±0.3 °C. On average, larvae hatched after 2.5 days under these conditions. Larvae that were to be tested at ages younger than 8 days postfertilization were not fed (at 28 °C, larvae can survive until approximately 10–11 days postfertilization on their endogenous yolk reserves). Larvae that were to be tested at older ages were fed high concentrations of live *Paramecium* sp. starting 7 days postfertilization. At 9 days postfertilization, these larvae were transferred to larger 20 l aquaria containing vigorously growing mixed protozoan populations. Larvae were gradually introduced to finely ground dry commercial fish food (Tetramin). Beginning approximately 18 days postfertilization, the dry food was supplemented with live *Artemia* sp. This regime resulted in good survivorship with very little mortality before 9 days postfertilization or beyond 11 days postfertilization. When mortality did occur, it was usually approximately 10 days postfertilization and appeared to be the result of a failure to learn to feed.

Overview

Larvae were tested 3, 7, 14 and 21 days postfertilization. Three methods were used to block gill ventilation: (i) deep anaesthesia using MS222 (tricaine methanesulphonate), (ii) deep anaesthesia using phenoxyethanol and (iii) agar-embedding. All tests were carried out at 28.0±0.3 °C following general procedures outlined in Sprague (1973). In brief, this involved placing groups of 15 larvae in fine mesh baskets suspended in glass containers containing 100 ml of (i) normoxic (21 % O_2 or 100 % air saturation) fresh water (FW), (ii) hyperoxic (42 % O_2 or 200 % air-saturation) fresh water (FW+O_2), (iii) normoxic 50 % physiological saline (50%PS) or (iv) hyperoxic 50 % physiological saline (50%PS+O_2). Reconstituted soft, fresh water (total hardness 44 mg l^{-1} as $CaCO_3$, pH 7.4) (APHA, 1985) was used for the 'fresh water' tests. Hickman's freshwater teleost saline (Hoar and Hickman, 1983) diluted 1:1 with distilled water (final osmolarity 117 mosmol l^{-1}, pH 7.7) was used for the 50 % physiological saline tests. Containers were aerated continuously with either air (21 % O_2) or a mixture of air and medical-grade O_2 (42 % O_2). Polarographic O_2 electrodes were used to monitor O_2 levels continuously. Responses to treatment, for the most part, were assessed on the basis of mortality, but in a few tests behavioural responses were also examined. The absence of a heartbeat was used as the indicator of mortality. The behavioural responses that were assessed were the ability swim, to ventilate the gills and to respond to mechanical stimulation.

MS222 experiments

Four series of experiments were conducted using MS222 to block gill ventilation. All MS222 solutions were made up freshly and adjusted to pH 7.3–7.7 using 0.1 mol l^{-1} NaOH.

The first series of experiments involved exposing groups of 7 days postfertilization larvae to a logarithmic series of MS222 concentrations ranging from 10 to 1000 mg l^{-1} in normoxic fresh water. Larvae were observed periodically over 72 h; individual times of death were recorded and median survival times (ET_{50}) calculated for each concentration.

The second and most extensive series of experiments involved placing groups of 3, 7, 14 and 21 days postfertilization larvae in the each of the four experimental solutions (FW, FW+O_2, 50%PS, 50%PS+O_2) containing the minimum MS222 concentration necessary to block gill ventilation. Larvae were observed periodically over 72 h, individual times of death were recorded and median survival times (ET_{50}) for each experimental condition calculated. The minimum MS222 concentrations necessary to block gill ventilation (100 mg l^{-1}

for 3 and 7 days postfertilization larvae, 130 mg l^{-1} for 14 days postfertilization larvae and 150 mg l^{-1} for 21 days postfertilization larvae) were arrived at on the basis of preliminary tests in which larvae were exposed to a graded series of anaesthetic concentrations and observed under a dissecting microscope to see whether they ventilated their gills.

A third series of experiments was conducted in which 24 h median lethal concentrations (LC_{50}) were determined for 3 and 7 days postfertilization larvae. In these tests, groups of larvae were exposed to a graded series of MS222 concentrations in FW, FW+O_2 and 50%PS for 24 h. Mortality was assessed at the end of the experimental period and 24 h LC_{50} values calculated.

A fourth series of experiments were conducted in which 7 days postfertilization larvae were exposed to a graded series of non-lethal MS222 concentrations ranging from 30 to 150 mg l^{-1} for 1 h in FW and in 50%PS. At the end of the hour, the larvae were gently prodded with a fine camelhair brush and their response noted. The proportion responding to this mechanical stimulation at each dose level was used to estimate 1 h median effective concentrations (EC_{50}).

Phenoxyethanol experiments

Bioassays were conducted to determine 24 h LC_{50} values for 3 and 7 days postfertilization larvae exposed to phenoxyethanol in FW, FW+O_2 and 50%PS. Procedures were essentially the same as those used determine 24 h LC_{50} values for MS222, with the obvious difference that phenoxyethanol rather than MS222 was used to block gill ventilation.

Agar-embedding

Agar-embedding was used to prevent 7 days postfertilization larvae from ventilating their gills. The embedding procedure involved anaesthetizing the larvae in 100 mg l^{-1} MS222 and then placing them in a drop of 1 % liquid agar on a glass slide just as the agar was about solidify. The agar was allowed to harden and then trimmed, leaving a thin (1–2 mm) layer of agar around the head but none around the rest of the body. The larvae were transferred to normoxic FW containing 10 mg l^{-1} buffered MS222 and allowed to recover for 1–2 h. Addition of a low concentration of MS222 to both the recovery and test solutions was found to be necessary to prevent larvae from struggling to escape from the agar coating. Preliminary experiments indicated that a concentration of 10 mg l^{-1} (≈1/10 the 24 h LC_{50}) effectively eliminated struggling behaviour but did not appear to have any significant effect on larval survival.

At the end of the recovery period, groups of agar-embedded larvae were placed in FW, FW+O_2, 50%PS or 50%PS+O_2 containing 10 mg l^{-1} MS222. Only larvae that displayed normal behaviour when gently prodded with a fine camelhair brush were used. Larvae were examined at intervals, individual times of death recorded and median survival times (ET_{50}) for each experimental condition calculated. ET_{50} was also determined for a 'sham-operated' group of larvae that were freed from their agar coating just before they were placed in the FW solution.

Statistical analyses

Individual response (mortality or behaviour) was recorded as a function of either time or dose, depending on the experiment. Probit analysis (Finney, 1971) using the NCSS computer program (NCSS Inc.) was then performed to obtain estimates of median survival time (ET50), median effective concentration (EC_{50}) or median lethal concentration (LC_{50}). Significant differences among treatments for these indicators were evaluated using repeated-measures analysis of variance (ANOVA; Sigmastat; SPSS Inc.). Pair-wise comparisons were carried out using the Bonferroni *post-hoc* ANOVA method and/or paired *t*-tests. Linear regression analysis (Sigmastat; SPSS Inc.) was used to test for significant correlations between median survival times and anaesthetic concentrations. Unless specified otherwise, a significance level of $P<0.05$ was employed throughout.

Results

MS222 experiments

In the first series of MS222 experiments, concentrations of 50 mg l^{-1} and above completely blocked gill ventilation (Fig. 1). Median survival times were independent of dose between 50 and 200 mg l^{-1}, but were dose-dependent at concentrations above 200 mg l^{-1} (Fig. 1).

Exposing 3 days postfertilization larvae to sufficient MS222 to block gill ventilation (100 mg l^{-1}) in the second series of MS222 experiments did not result in significant excess mortality compared with unanaesthetized larvae in normoxic fresh water within the 72 h experimental period under any of the four experimental conditions (FW, FW+O_2, 50%PS, 50%PS+O_2). Using equivalent levels of MS222 to block gill ventilation at 7, 14 and 21 days postfertilization, in contrast, resulted in significant excess mortality within the 72 h experimental period under all four conditions. The rate and extent of the excess mortality varied depending on the experimental condition.

At 7 days postfertilization, the mean (±95 % CI) median survival time for larvae exposed to 100 mg l^{-1} MS222 in FW was 25.5±3.7 h. The ET_{50} for unanaesthetized larvae under the same condition was more than 72 h. Exposing the larvae to the anaesthetic in either 50%PS or 50%PS+O_2 as opposed to FW resulted in significant increases in median survival times (Fig. 2). Exposure to MS222 in FW+O_2, in contrast, did not significantly improve survival beyond that observed in FW (Fig. 2). Similarly, exposure in 50%PS+O_2 did not significantly improve survival beyond that observed in 50%PS.

The mean (±95 % CI) median survival time for 14 days postfertilization larvae exposed to 130 mg l^{-1} MS222 in FW was 5.3±2.0 h *versus* more than 72 h for unanaesthetized larvae. Median survival times for anaesthetized larvae were significantly greater under all three compensating conditions (FW+O_2, 50%PS, 50%PS+O_2) than in FW (Fig. 3). The difference between median survival times in FW+O_2 and 50%PS+O_2 was significant; that between 50%PS and 50%PS+O_2 was not.

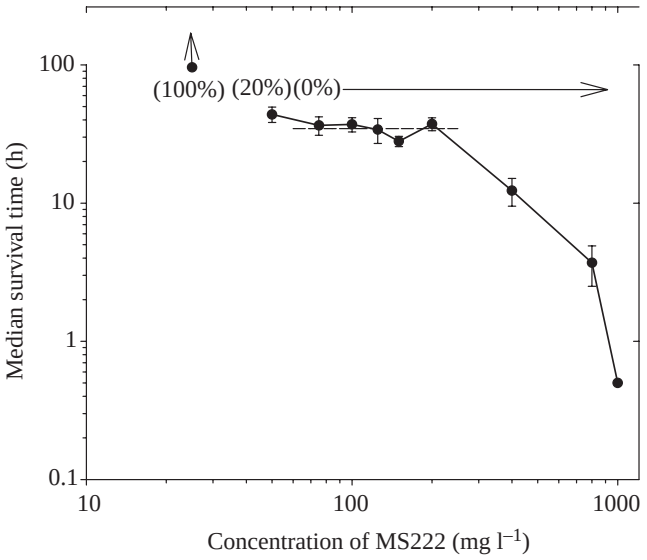

Fig. 1. Median survival times (±95 % CI) for 7 days postfertilization zebrafish exposed to various concentrations of MS222 in normoxic fresh water. The dashed line indicates the regression equation based on median survival times (ET_{50}) between 50 and 200 mg l^{-1}. The slope of this line was not significantly different from zero (P>0.05). Values in parentheses indicate the percentage of larvae ventilating their gills at the various MS222 concentrations. 0 % gill ventilation continues as indicated by the horizontal arrow. The vertical arrow indicates <50 % mortality.

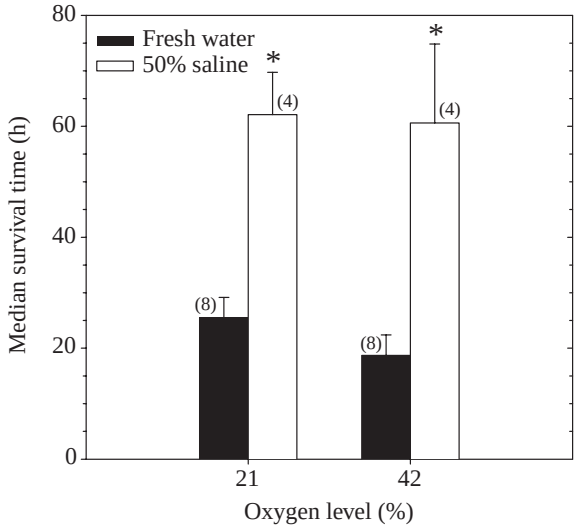

Fig. 2. Median survival times (+95 % CI) for 7 days postfertilization zebrafish prevented from ventilating their gills because of exposure to 100 mg l^{-1} MS222 in normoxic fresh water, normoxic 50 % physiological saline, hyperoxic fresh water and hyperoxic 50 % physiological saline. Asterisks indicate significant differences in median survival times compared with normoxic fresh water. Numbers in parentheses indicate the number of trials.

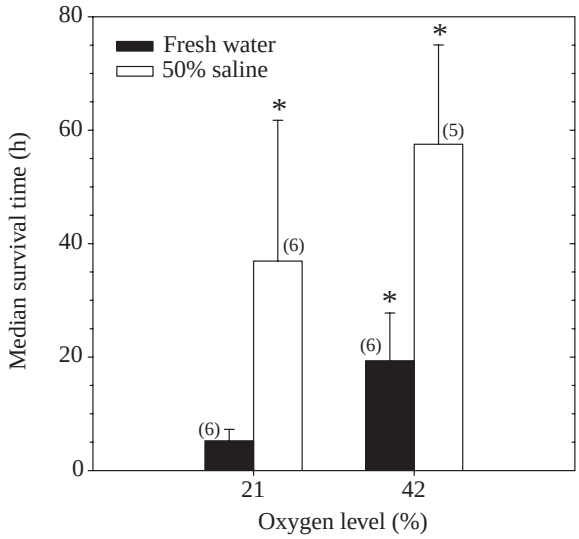

Fig. 3. Median survival times (+95 % CI) for 14 days postfertilization zebrafish prevented from ventilating their gills because of exposure to 130 mg l^{-1} MS222 in normoxic fresh water, normoxic 50 % physiological saline, hyperoxic fresh water and hyperoxic 50 % physiological saline. Asterisks indicate significant differences in median survival times compared with normoxic fresh water. Numbers in parentheses indicate the number of trials.

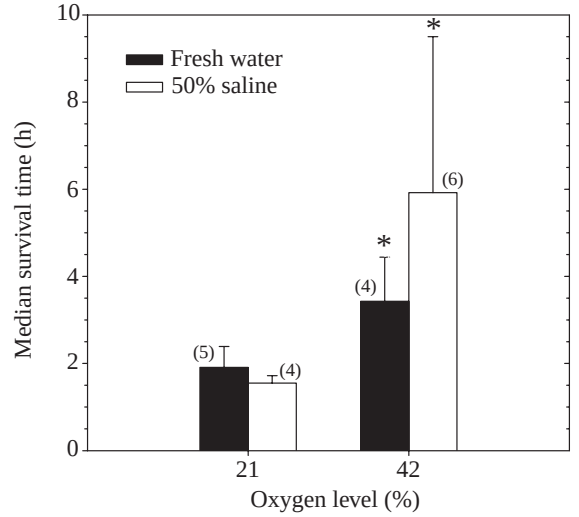

Fig. 4. Median survival times (+95 % CI) for 21 days postfertilization zebrafish prevented from ventilating their gills because of exposure to 150 mg l^{-1} MS222 in normoxic fresh water, normoxic 50 % physiological saline, hyperoxic fresh water and hyperoxic 50 % physiological saline. Asterisks indicate significant differences in median survival times compared with normoxic fresh water. Numbers in parentheses indicate the number of trials.

The mean (±95 % CI) median survival time for 21 days postfertilization larvae exposed to 150 mg l^{-1} in FW was only 1.9±0.5 h as opposed to more than 72 h for unanaesthetized larvae under the same conditions. Survival times were significantly greater both in FW+O_2 and in 50%PS+O_2 than in FW (Fig. 4). Differences between survival times in FW and 50%PS and between survival times in FW+O_2 and 50%PS+O_2 were not significant.

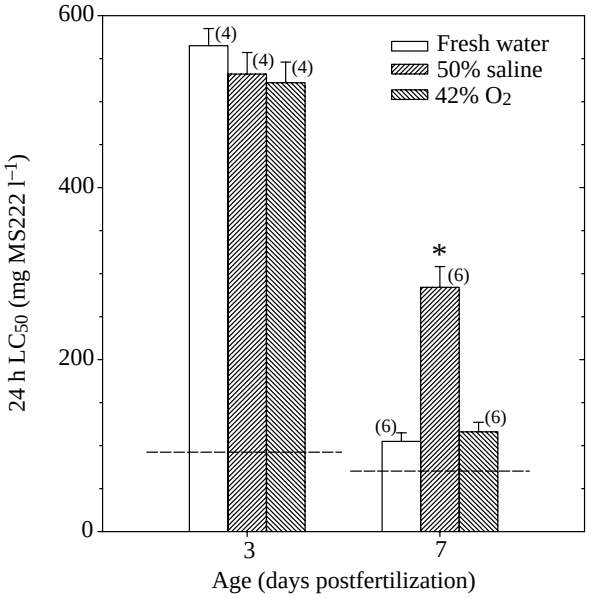

Fig. 5. 24 h median lethal concentrations (LC_{50}) (+95 % CI) for 3 and 7 days postfertilization zebrafish larvae exposed to MS222 in normoxic fresh water, normoxic 50 % physiological saline and hyperoxic fresh water. Dashed lines indicate approximate median survival times (ET_{50}) for suppression of ventilatory movements. The asterisk indicates a significant difference in median survival time compared with normoxic fresh water within an age class. Numbers in parentheses indicate the number of trials.

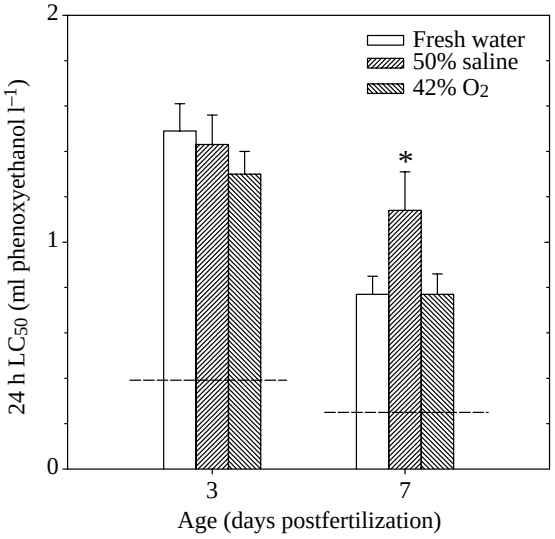

Fig. 6. 24 h median lethal concentrations (LC_{50}) (+95 % CI, N=4) for 3 and 7 days postfertilization zebrafish larvae exposed to phenoxyethanol in normoxic fresh water, normoxic 50 % physiological saline and hyperoxic fresh water. Dashed lines indicate approximate median survival times (ET_{50}) for suppression of ventilatory movements. The asterisk indicates a significant difference in median survival time compared with normoxic fresh water within an age class.

Neither hyperoxia nor 50 % physiological saline had any significant effect on the 24 h LC_{50} for MS222 at 3 days postfertilization (Fig. 5). At 7 days postfertilization, 24 h LC_{50} values were significantly greater in 50%PS than in FW or in FW+O_2 (Fig. 5); LC_{50} values in FW+O_2 were not significantly different from those in FW.

The anaesthetic effectiveness of MS222 in 7 days postfertilization larvae assessed on the basis of their response to touch was not significantly different in 50%PS from that in FW. The mean (±95 % CI) 1 h EC_{50} in 50%PS was 75±12 mg l^{-1}; the corresponding value in FW was 82±13 mg l^{-1}.

Phenoxyethanol experiments

At 3 days postfertilization, 24 h LC_{50} values for phenoxyethanol were not significantly different in FW, 50%PS and FW+O_2 (Fig. 6). At 7 days postfertilization, 24 h LC_{50} values were significantly greater in 50%PS than in either FW or FW+O_2 (Fig. 6); LC_{50} values in FW+O_2 were not significantly different from those in FW.

Agar-embedding

The mean (±95 % CI) median survival time for agar-embedded 7 days postfertilization larvae in FW was 9.9±5.3 h. In contrast, the mean median survival time of the sham-operated group in FW was more than 72 h (Fig. 7). Survival times were significantly greater both in 50%PS and in 50%PS+O_2 than in FW. Survival times in FW+O_2 were not significantly different from survival times in FW. Similarly, survival times in 50%PS+O_2 were not significantly different from those in 50%PS.

Discussion

The results of this study indicate that developing zebrafish need gills for ionoregulatory purposes (or some correlate) before they need them for oxygen uptake. Beginning at 7 days postfertilization, 50 % physiological saline significantly improved the survival of larvae that were unable to ventilate their gills. Hyperoxia (42 % O_2), in contrast, did not significantly improve survival until 14 days postfertilization. This sequence, ionoregulation before respiration, and the apparent timing of the shifts are consistent with what little is known about the morphology of gill development in zebrafish. Dimethylaminostyrylethylpyridiniumiodine (DASPEI) staining for mitochondria-rich cells indicates that large numbers of ionocytes begin to appear on the gill of zebrafish larvae around 5–7 days postfertilization (P. Rombough, unpublished data). Lamellar buds, however, are not evident on microscopic examination of the gill until 12–14 days postfertilization (Knopek and Hintze-Podufal, 1989; P. Rombough, unpublished data). As mentioned in the Introduction, the same morphological sequence (ionocytes appearing on the gill before secondary lamellae) has been documented for at least four other unrelated species. This suggests that the functional sequence observed in the present

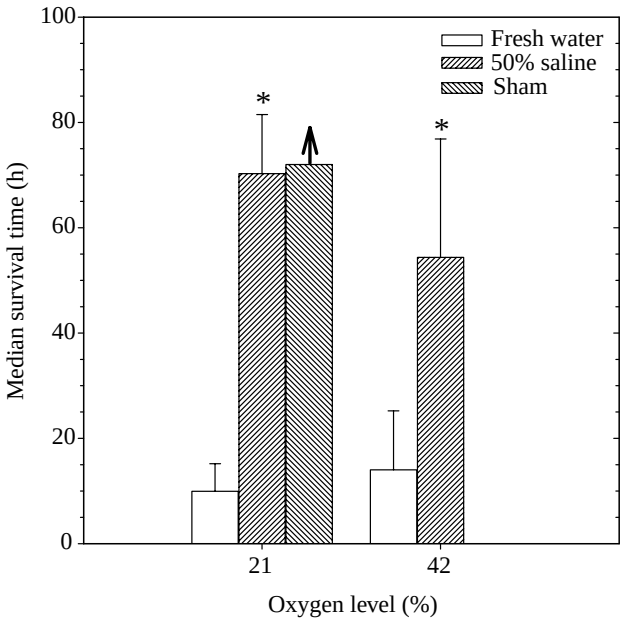

Fig. 7. Median survival times (±95 % CI, *N*=4) for 7 days postfertilization agar-embedded zebrafish in normoxic fresh water, normoxic 50 % physiological saline, hyperoxic fresh water and hyperoxic 50 % physiological saline. Asterisks indicate significant differences in median survival times compared with normoxic fresh water. Sham indicates the median survival time for sham-operated larvae in normoxic fresh water. The vertical arrow indicates <50 % mortality.

study, in which ionoregulatory activity shifted to the gill before respiratory activity, may apply to more species than just zebrafish. There are theoretical reasons to suspect that this is the case.

Both ion exchange and O_2 uptake are surface phenomena. The effective surface area on the larval skin across which ion exchange can take place, however, is much smaller than that available for O_2 uptake. O_2 uptake occurs by simple diffusion and, as a result, is not restricted to a particular cell type. Blood vessels can be a considerable distance from the skin surface and still participate in O_2 uptake (Malvin, 1988). Ion exchange, in contrast, requires specific cell types with their apical membrane opening onto a free body surface and their basolateral membrane in close contact with a blood vessel (Alderdice, 1988). Only a small fraction of the skin is underlain by blood vessels close enough to the surface to satisfy this condition. The problem is compounded as larvae grow. In many fish species, the region of the skin with the highest density of ionocytes is the yolk sac (Hwang, 1989; Ayson et al., 1994; Rombough, 1999). As larvae grow, they consume the yolk in the yolk sac, and the vitelline circulation associated with it regresses. In addition, the skin tends to thicken, leaving many blood vessels too deep to participate in ion exchange. The net effect is a reduction in the number of ionocytes on the skin often in absolute as well as in relative terms (Rombough, 1999). The relative (but not absolute) surface area available for O_2 uptake also decreases with growth (Rombough, 1999) but, since O_2 uptake is not as constrained by blood vessel density or depth as ion exchange, it does so at a slower rate. One would, therefore, predict, simply on the basis of differences in scaling, that ion balance should shift to the gills before O_2 uptake.

Recently hatched zebrafish larvae (3 days postfertilization) do not appear to need gills to obtain sufficient oxygen for survival or to maintain ionic balance in soft water under normoxic conditions at 28 °C. In this study, neither hyperoxia nor 50 % physiological saline had any significant effect on ET_{50} or LC_{50} for 3 days postfertilization larvae exposed to either MS222 or phenoxyethanol. That 3 days postfertilization larvae could obtain sufficient O_2 without using their gills is not particularly surprising given their small size. Preliminary measurements indicate that the mass-specific surface area of the skin of newly hatched zebrafish larvae (300 $cm^2\,g^{-1}$; P. Rombough, unpublished data) is approximately seven times that of a newly hatched trout or salmon larva (40 $cm^2\,g^{-1}$; Wells and Pinder 1996a). Partitioning studies indicate that newly hatched trout and salmon obtain approximately 85 % of their oxygen across their skin (Rombough and Ure, 1991; Wells and Pinder, 1996b; Rombough, 1998). On the basis of the relative surface area of their skin and their metabolic rate, one would expect newly hatched zebrafish to be able to obtain at least as much O_2 in relative terms through their skin. Blocking gill ventilation at this stage is unlikely, therefore, to have much impact on O_2 delivery to the tissues and, by extension, on mortality. The situation is probably similar with respect to ionoregulatory activity but, unfortunately, there is no information available on the relative numbers of chloride cells on the skin and gills of newly hatched zebrafish.

In functional ablation studies, the assurance with which one can assign cause and effect depends in large part on the specificity of the method used to block the function under consideration. In this study, it was considered particularly important that the effects of ablation effectively be restricted to blocking gill ventilation. It was for this reason that three different methods were used; the rationale being that any unintended effects would be evident as discrepancies when the results obtained using the different methods were compared. The consistency of the observed results, especially for the 7 days postfertilization experiments in which all three methods were used, suggests that concerns about unintended effects were unwarranted. At 7 days postfertilization, exposure to 50 % physiological saline but not hyperoxia significantly improved the survival of larvae prevented from ventilating their gills irrespective of whether this was accomplished by exposing them to MS222, phenoxyethanol or embedding their gills in agar (MS222 and phenoxyethanol, while both anaesthetics, have different modes of action; Burka et al., 1997). In the case of the larvae embedded in agar and those anaesthetized using MS222, the similarity extended to actual survival times. Median survival times for the agar-embedded and the MS222-anaesthetized larvae were not significantly different from each other under three ($FW+O_2$, PS, $PS+O_2$) of the four experimental conditions. The conclusion that mortality at

moderate anaesthetic levels is primarily a consequence of impaired gill ventilation is supported by the absence of a normal dose/response relationship for MS222 between 50 mg l^{-1} (the minimum MS222 concentration required to block gill ventilation) and 200 mg l^{-1}. If MS222 were directly toxic at the concentrations used to block gill ventilation (100–150 mg l^{-1}), one would have expected ET_{50} to decline with increasing dose at concentrations between 50 and 200 mg l^{-1} just as they did at higher concentrations.

One explanation for the enhanced survival in 50%PS compared with FW of 7 days postfertilization larvae exposed to MS222 that needed to be discounted before ionoregulatory activity could be firmly ascribed to the gill at that stage was the possibility of chemical or depositional antagonism. While it has not been reported for MS222, salt solutions are known to reduce the solubility and/or limit the biological availability of some chemicals. However, if chemical or depositional antagonism were responsible for the enhanced survival in 50%PS at 7 days postfertilization, one would have expected a similar effect on EC_{50} as on LC_{50}. This was not the case. At 7 days postfertilization, 1 h EC_{50} values were virtually identical in 50%PS and FW. In contrast, 24 h LC_{50} values in 50%PS was almost three times those in FW. The observation that 50%PS did not mitigate anaesthetic toxicity at 3 and at 21 days postfertilization but did so at 7 days postfertilization is also inconsistent with chemical or depositional antagonism. Finally, one would have trouble explaining how either type of antagonism could be responsible for the improved survival of agar-embedded larvae in 50%PS.

Although 50%PS enhanced the survival of zebrafish larvae at 7 and 14 days postfertilization, it did not do so at 21 days postfertilization. The reason for this is unlikely to be that the gills are no longer needed for ionoregulation. A more likely explanation is that by 21 days postfertilization the larvae were so dependent on their gills for oxygen uptake that they died from lack of oxygen before the effects of ionoregulatory impairment could become evident. The mean ET_{50} of MS222-anaesthetized larvae in FW at 21 days postfertilization was only 1.9 h. Mean ET_{50} values under the same conditions at 14 and 7 days postfertilization were 5.3 h and 25.5 h, respectively.

Knowing the sequence in which physiological functions shift to the gills during ontogeny is important for several reasons, not the least of which is that it influences how biologists view problems. For example, catastrophic events in larval life, such as critical-stage mortality, have been linked to gill insufficiency (Iwai and Hughes, 1977). It has been assumed that an inability to obtain sufficient O_2 was the problem but, depending on when mortality occurs, the problem well could be ionoregulatory rather than respiratory. The order in which the gill assumes critical functions undoubtedly also has structural implications. The design characteristics of a multifunctional organ such as the adult fish gill are the consequence of complex interactions among what have been termed external (environmental) and internal (developmental and functional) constraints (Wiebel, 1998). One internal constraint is that the addition of new functions to the gill must not compromise pre-existing functions. This obviously limits the ways the gill can be designed. Appreciating the nature of this constraint should lead to a more holistic understanding of structural and functional relationships in the gill of adult as well larval fish.

Finally, the order in which functions shift to the gills may have evolutionary significance. It generally is assumed that the gill of vertebrates is homologous with the branchial basket of filter-feeding protochordates. The evolutionary pathway leading from the branchial basket to the gill, however, is not clear. The scenario with the most support among biologists has the increase in size and mineralization of the skin that presumably took place in early vertebrates driving gill development by making cutaneous O_2 uptake progressively less effective (Schmitz et al., 2000). These pressures (increases in size and mineralization) are essentially the same as those faced by zebrafish during larval development. In zebrafish, however, the skin function that first becomes limiting is ion balance not O_2 uptake. This raises the possibility that ionoregulatory requirements may have played a more important role in gill evolution than is generally believed.

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