The Journal of Experimental Biology 210, 1435-1445
Published by The Company of Biologists 2007
doi:10.1242/jeb.02754

Parameters influencing the dissolved oxygen in the boundary layer of rainbow trout (*Oncorhynchus mykiss*) embryos and larvae

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Accepted 12 February 2007

Summary

We investigated the influence of oxygen demand (developmental stage) and supply (hypoxia, water flow rate, the chorion and body movements) on the oxygen concentration within the boundary layer next to the chorion of embryos or skin of larvae of rainbow trout (*Oncorhynchus mykiss*). Oxygen microelectrodes were used to measure dissolved oxygen (DO) within the boundary layer of trout embryos and larvae. As the embryos and larvae developed, the DO gradient and the thickness of the boundary layer increased. The DO concentration within the boundary layer next to the chorion or skin surface decreased as the DO concentration in the free-stream water decreased. A decrease in water flow rate increased the magnitude of the gradient and thickness of the boundary layer. In normoxia, the DO in the perivitelline fluid inside the chorion was 16±3.0% saturation at 31 days post fertilization, indicating that the chorion was a significant barrier to oxygen diffusion. The number of body movements did not change when embryos were exposed to hypoxia before hatching, but after hatching, hypoxia resulted in a decrease in body movements of the larvae. Taken together, our data indicate that the oxygen boundary layer around trout embryos and larvae depends on both the oxygen demand and supply. The factors that significantly impacted boundary layer oxygen were developmental stage, free-stream oxygen levels, water flow rate, and the presence of the chorion.

Key words: chorion, development, hypoxia, oxygen microelectrodes, perivitelline fluid.

Introduction

The oxygen requirements for successful development of salmonid embryos and larvae have been estimated to range between 2 and 9 mg l^{-1} (~20–90% saturation) depending on temperature, developmental stage, and flow rate of water around the embryo (Silver et al., 1963; Rombough, 1986; Rombough, 1988a). Although trout prefer to spawn in fast flowing, well oxygenated rivers and streams, the dissolved oxygen (DO) concentration inside redds can vary from 20 to 95% saturation depending on the depth within the gravel bed (Coble, 1961; Youngson et al., 2004). Further, because of a layer of semistagnant water adjacent to the egg known as the boundary layer, the DO concentration at the surface of the egg is depleted as the embryo consumes oxygen, causing the formation of an oxygen gradient from the free-stream to the surface of the chorion. Therefore, the DO concentration experienced by fish embryos depends on the oxygen supply (i.e. DO in the intergravel free-stream and the water flow rate) and demand (i.e. oxygen uptake).

There are very few measurements of the DO concentration in the boundary layer of fish embryos. In loach (*Misgurnis fossilis*) embryos, the decrease in oxygen tensions from free-stream to the chorion surface was small (<5%) (Berezovsky et al., 1979). In frogs, a decrease in the water velocity around the animal results in a lower DO concentration at the skin surface (Pinder and Feder, 1990). As oxygen levels fall in the boundary layer, they are replenished by the diffusion of oxygen and convection of water containing oxygen flowing in the free-stream past the animal (Daykin, 1965; Pinder and Feder, 1990; Vogel, 1994). Intergravel free-stream water velocities near redds are typically about 0.5 mm s^{-1} (Zimmermann and Lapointe, 2005). Therefore, flow rate could limit the amount of oxygen available to rainbow trout embryos inside redds.

Another variable influencing the magnitude of the oxygen boundary layer is the oxygen uptake by the animal, with an increase in oxygen uptake resulting in a decrease in the DO concentration at the surface of the animal, that is, an increase in the gradient from the free-stream to the chorion (Daykin, 1965; Pinder and Feder, 1990). In rainbow trout, the oxygen uptake by embryos is low (<1 μg O_2 h^{-1} per individual) a few days post fertilization (d.p.f.), but increases approximately 15-fold to 16 μg O_2 h^{-1} per individual at the time of hatching (~37 d.p.f. at 9°C), and to ~60 μg O_2 h^{-1} per individual at the time of first feeding (~60 d.p.f. at 9°C) (Rombough, 1986;

Rombough, 1988a). To our knowledge, the influence of developmental time on the oxygen in the boundary layer of aquatic vertebrates has not been studied.

Many aquatic embryos often are exposed to hypoxia in nature, however, to our knowledge, no measurements of the DO in the boundary layer under these conditions have been published. However, Daykin developed a theoretical model to predict the DO concentration at the surface of the chorion based on the properties of the embryo and on water parameters (Daykin, 1965), and predicted that hypoxia would result in a decrease in the DO at the surface of the chorion (and thus a decrease in the gradient from the free-stream to the chorion), but not in the boundary layer thickness.

Another potential parameter that may influence oxygen levels near the embryo is the presence of the chorion (Rombough, 1988b). The rainbow trout chorion is made up of two layers: a thicker internal layer ~24 μm thick and a thinner outer layer ~0.15 μm thick; both penetrated by pore canals 0.5–0.8 μm in diameter (Groot and Alderdice, 1985). The outside of the pore canals are plugged making the chorion a semipermeable membrane, which allows only small molecules (including oxygen) to diffuse through (Groot and Alderdice, 1985; Eddy et al., 1990). We concluded that the chorion was not a barrier to oxygen diffusion based on body mass data of trout embryos with and without an intact chorion (Ciuhandu et al., 2005). We found, however, that intact embryos in normoxia attained the same body mass as dechorionated embryos in hypoxia (50% saturation), suggesting that the chorion may restrict oxygen uptake. Direct measurements of oxygen levels in the perivitelline fluid (pvf) of loach (*Misgurnis fossilis*) embryos were only slightly lower than just outside the chorion (Berezovsky et al., 1979). Thus, the limited data on the impact of the chorion on the resistance to oxygen uptake in fish embryos is ambiguous and requires direct measurements using oxygen microelectrodes.

Some aquatic vertebrate and invertebrate embryos increase the amount of movement when exposed to low oxygen. This is thought to mix the pvf, which in turn will cause an increase in oxygen influx to the embryo (Hunter and Vogel, 1986; Kuang et al., 2002). Similarly, adult bullfrogs (*Rana catesbeiana*) use body movements in order to dissipate the boundary layers that accumulate around the animal while overwintering (Pinder and Feder, 1990). In Atlantic salmon (*Salmo salar*) embryos, spontaneous contractions of the trunk have been shown to begin at 27 d.p.f. (at 6°C hatching occurs at 81 d.p.f.) and rhythmic movements by 32 d.p.f. (Johnston et al., 1999). In rainbow trout embryos, movements in the caudal region have been observed at 20 d.p.f. (at 10°C hatching occurs at 31 d.p.f.) (Vernier, 1969). Therefore, if embryos are exposed to hypoxia it might be beneficial to increase the amount of body movements, which might function to stir the pvf and maintain the oxygen flux to the embryo.

Five hypotheses were tested in the current study. The first hypothesis was that the gradient within the boundary layer of rainbow trout embryos and larvae increases with developmental time. This was tested by measuring oxygen levels in the boundary layer at different developmental times: unfertilized, 11, 16, 23, 30, 33, 43 and 50 d.p.f. The second hypothesis was that exposure to hypoxia will not cause a change in the magnitude of the oxygen gradient and thickness in the boundary layer of rainbow trout embryos. Boundary layer DO was measured in both normoxia (100% saturation) and hypoxia (35% saturation) and after 30 min, 4, and 8 h of exposure to hypoxia (35% saturation). The third hypothesis was that the chorion is not a significant barrier to oxygen diffusion. This hypothesis was tested by taking DO measurements in the boundary layer of trout embryos (31 d.p.f.) with the chorion intact and after the chorion was removed. DO was also measured inside the intact chorion. The fourth hypothesis was that a decrease in water flow rate causes an increase in the gradient and thickness of the boundary layer of rainbow trout embryos. DO in the boundary layer of trout embryos (29 d.p.f.) was measured at three different rates at which water flowed into the chamber: 3, 5 and 7.2 ml min^{-1}. The fifth hypothesis was that movement by the embryos and larvae influences the oxygen gradient in the boundary layer. To test this hypothesis the influence of hypoxia (35 and 50% sat) on the number of movements was measured. It was predicted that if movement had an influence on the boundary layer, then the number of movements of embryos exposed to hypoxia would be higher than the number of movements of embryos exposed to normoxia.

Materials and methods

Experimental animals

Rainbow trout *Oncorhynchus mykiss* Walbaum embryos were purchased from Rainbow Springs (Thamesford, Ontario, Canada) on the day they were fertilized and were held in continuous-flow incubation trays with mesh bottoms supplied with local well water (9°C, pH 7.9; water hardness 411 mg l^{-1} as $CaCO_3$; Ca^{2+}, 2.6 mmol l^{-1}; Cl^-, 1.5 mmol l^{-1}; Mg^{2+}, 1.5 mmol l^{-1}; K^+, 0.06 mmol l^{-1}; Na^+, 1.1 mmol l^{-1}) in the Hagen Aqualab, University of Guelph, Guelph, Ontario, Canada. Incubation trays were protected from the light during the entire experiment. Hatching took place between 33 and 37 days post fertilization (d.p.f.). All batches of embryos were obtained from three females and different females were used for different batches.

Experimental protocols

Measuring the gradient and boundary layer thickness

Oxygen microelectrodes were used to measure dissolved oxygen (DO) profiles in the boundary layer of rainbow trout embryos before and after hatching using a method similar to that described by Rombough (Rombough, 1998). The oxygen microelectrode (Unisense, Clark Style Ox25, 25 μm tip; Aarhus, Denmark) was held with a micromanipulator (Brinkmann, Mississauga, Canada) and connected to a picoammeter (Unisense, PA-2000). Electrodes were calibrated every 2–3 h using 100% saturated water (obtained by aeration) and 0% oxygen solution (2 mol l^{-1} sodium sulphite).

The measurements were taken in a Plexiglas™ chamber (7.5 cm×3.3 cm×2 cm, length×width×depth) supplied with flowing well water (9°C). The water flow rate was controlled using a variable flow mini-pump (Fisher Model 3385, Friendswood, TX, USA). The water flow rate during measurements was 4 ml min^{-1} unless otherwise specified. The gas composition of the flowing water was controlled by bubbling a gas mixture of N_2 and air into a glass mixing tank with a water jacket (9°C) connected to a circulating water pump (Fisher Isotemp 3016, Pittsburgh, PA, USA). The gases were mixed using a Wösthoff pump (Calibrated Instruments Inc., Ardsley, NY, USA). All measurements were taken in a temperature-controlled room (9°C), in order to reduce electrode drift during measurements. Measurements were taken 30 min after the egg, embryo or larva was placed in the chamber to allow adequate time for the boundary layer to form (Green et al., 2006).

The electrode was moved close to the surface of the chorion or skin using a micromanipulator until a slight dimple could be seen on the chorion or skin through the binocular dissecting microscope (magnification 40×). Then the electrode was retracted until the dimple disappeared. The oxygen concentration at this position was then considered to be the dissolved oxygen value at the surface of the chorion or skin. The response time of the oxygen electrode was 90% within 4 s; therefore, the electrode was allowed to stabilize for about 30 s at each measurement. The micromanipulator was used to retract the oxygen electrode in 100 μm increments for unfertilized eggs and embryos, and in 200 μm increments for larvae, until the dissolved oxygen value matched the dissolved oxygen value in the free-stream. The oxygen gradient was calculated as the oxygen concentration in the free-stream minus the oxygen concentration at the surface of the chorion or skin. Boundary layer thickness (BLT) was calculated as the distance from the chorion where the oxygen concentration was 99% of the free-stream concentration. The 99% value is usually used to estimate boundary layer thickness (Vogel, 1996) and was interpolated algebraically from the measured values.

An egg or embryo was placed in the Plexiglas™ chamber on a rubber 'O' ring and two Minuten pins, providing stability. In embryos, measurements were taken in the head forming region or the head area between the eyes. In eggs, measurements were taken in the oil globule region (animal pole). To prevent the larvae from moving, they were anaesthetized (50 mg l^{-1} MS-222, buffered with 1 mol l^{-1} NaOH to pH 8) for 30 min prior to placement in the Plexiglas™ chamber (1 h prior to measurement) and the same level of anaesthetic was used while boundary layer measurements were taken. This concentration of anaesthetic does not affect the rate of O_2 consumption by larvae of chinook salmon, but was sufficient to prevent opercular and pectoral fin movements (Rombough, 1988a).

There were four series of experiments conducted. Series I: oxygen gradient with developmental time and hypoxia; Series II: oxygen gradient and the chorion; Series III: oxygen gradient with changes in water flow rate; Series IV: influence of hypoxia on movement of embryos and larvae. In all experiments, normoxic water (i.e. 100% saturation, sat) refers to water equilibrated with air.

Series Ia: oxygen gradient with developmental time

The oxygen microelectrode was used to measure DO profiles in the boundary layer of rainbow trout embryos before hatching (unfertilized, 11, 16, 23, 30, 33 d.p.f.) and larvae after hatching (43, 50 d.p.f.) in normoxic water. All measurements were on separate animals (N=8 at each developmental time). The effect of developmental times was tested with a one-way ANOVA and differences between days with Tukey's test.

Series Ib: oxygen gradient with acute exposure to hypoxic water

The same developmental stages were used as in Series Ia except that the animals were exposed to hypoxic water (35% sat) for 30 min prior to measuring the oxygen concentration in the boundary layer. Different animals were used to those used in the normoxic trials (N=8 at each developmental time). The effect of acute exposure to hypoxic water was tested with a two-way ANOVA (developmental time, oxygen level, and interaction) and differences with oxygen level tested with Tukey's test at each developmental time.

Series Ic: oxygen gradient with prolonged exposure to hypoxic water

In this set of experiments, the oxygen boundary layer measurements were taken during an 8 h exposure to hypoxic water. At 11, 23, 30 and 50 d.p.f., measurements of the DO in the boundary layer of embryos and larvae were taken after 30 min, 4 and 8 h of exposure to 35% sat. At time zero, the embryo or larva was placed in the chamber and after 30 min oxygen boundary layer measurements were taken. Between measurements, the embryos or larvae were placed in a Heath tray supplied with flowing water (9°C, 35% sat). In this prolonged hypoxia exposure experiment, the same individuals were used to take the measurements at different exposure times within each developmental stage but different animals were used at different developmental times. Because the gradient was measured on the same six embryos, the effect of exposure time was analyzed using ANOVA of exposure time within embryo at each developmental time. The three exposure times were compared with Tukey's tests.

Series IIa: oxygen gradient with and without a chorion

To estimate the influence of the chorion on the oxygen boundary layer, measurements were taken at 31 d.p.f. in normoxic water (N=5). Initially, measurements were taken using intact embryos (embryo with intact chorion). Subsequently, the chorion was removed (embryo with chorion removed) by hand using forceps under a binocular microscope and boundary layer measurements were repeated from the surface of the skin to the free-stream in the same five embryos. Measurements before and

after the removal of the chorion were taken in the presence of a low concentration of anaesthetic (50 mg l^{-1} MS-222) flowing through the chamber to limit movement. The DO at the surface of the chorion in embryos with a chorion was compared with the DO at the surface of the skin in embryos without a chorion using a paired student's *t*-test.

Series IIb: oxygen gradient across the chorion

In a separate batch of embryos, oxygen measurements were taken inside the chorion of intact embryos (31 d.p.f.) in normoxic water (N=6). Embryos were glued to the 'O' ring in the Plexiglas chamber using a tissue adhesive (Vetbond, 3M, St Paul, MN, USA). Measurements were taken in the presence of a low concentration of anaesthetic (50 mg l^{-1} MS-222) flowing through the chamber to limit movement. The DO concentration in the boundary layer was measured after 30 min as described above, before the chorion was pierced, in order to prevent disturbances on the boundary layer by the needle. Then, a syringe and a 28 gauge needle attached to a micromanipulator was used to pierce the chorion. Once the needle penetrated the chorion, a small volume of food colouring (~10 μl) was introduced to mark the location of the puncture. Care was taken not to injure and/or disturb the animal. The needle was removed and replaced with the tip of the oxygen electrode, using the micromanipulator. Once the electrode was inside the chorion and the reading stabilized (~30 s), the dissolved oxygen level inside the chorion was recorded. Because the penetration of the needle could disturb the boundary layer inside the chorion, only one measurement inside the chorion was taken. The DO inside the chorion was compared with that outside the chorion (0 μm value) using a paired Student's *t*-test.

Series III: oxygen gradient with changes is water flow rate

In order to test the influence of water flow rate on the boundary layer oxygen gradient, measurements were taken at 29 d.p.f. in normoxic water on six embryos. Oxygen boundary layer measurements were taken at three different flow rates: 3, 5 and 7.2 ml min^{-1}, starting with the highest flow rate. Over this range of flow rates, there was a linear relation between flow rate and free-stream velocity (velocity=0.141×flow–0.151, where velocity is in cm s^{-1} and flow in ml min^{-1}). Velocity was measured by timing the movement of neutrally buoyant particles in the free-stream near the embryo. Because all three flow levels were tested on the same six embryos, the effect of flow was analyzed using ANOVA of flow within embryo. Particular values of BLT and gradient were compared with Tukey's tests.

Series IV: influence of hypoxia on embryos/larvae movement

To estimate the effect of hypoxia on the number of movements, embryos were divided into three groups (treatments) of nine embryos each and were placed into custom-built chambers. The Plexiglas™ chamber (18 cm×5.7 cm) contained 20 wells (1.9 cm×1.3 cm×1.2 cm, length×width×depth). Three video cameras above each tray (treatment) were used to record movement. Videotapes were analyzed for the total number of movements in each 30-min interval over a 4 h period for each stage (30, 43 and 50 d.p.f.).

The embryos were placed in the chambers 16 h before the initiation of the experiment to reduce potential handling stress. The exception was the post-hatch group (43 d.p.f.), for which embryos were placed inside the experimental trays 1 h before the experiment because some of the embryos escaped from the Plexiglas chamber. Embryos were exposed to three different treatments: normoxia (100% sat), moderate hypoxia (50% sat), and severe hypoxia (35% sat). Different embryos were used for different oxygen treatments and different embryos were used at different developmental stages. Hypoxic water was produced as described above. Oxygen levels were measured with an oxygen meter (Hach, Model HQ20, Loveland, CO, USA) every 30 min over the duration of the video recording. Video recording was started at the same time as the nitrogen was turned on. The gases reached the desired level of saturation over a period of 15–30 min.

Two control experiments were performed. First, in order to test that the effect observed was due to the level of dissolved oxygen (DO) and not simply a tray effect, the experiment was performed as described above except normoxic water was used in all three experimental trays. The number of movements were recorded over a 1-h period and compared between trays. Second, in order to test that the effect of severe hypoxia was reversible, a group of nine 44 d.p.f. larvae were exposed to severe hypoxia (35% sat) as describe above for 4 h and then immediately exposed to normoxia for a further 2 h. The number of movements during 30 min intervals was counted over the 6-h recording period.

A repeated-measures ANOVA was used to test for differences in activity between embryos exposed to normoxia at 43 and 50 d.p.f. over the 4 h period. An ANOVA was used to test for a tray effect. A repeated-measures ANOVA was used to test whether the effect of hypoxia on the number of body movements was reversible. An ANOVA was used to test for differences between the three treatments (35, 50 and 100% sat) during the first 30 min and the last 90 min of the exposure, once the response reached a plateau.

Statistical analysis

Statistical analyses were performed using the Minitab Version 12.1. The results before and after hatching were not compared statistically because the measurements before hatching were taken from the chorion surface, whereas the measurements after hatching were taken from the skin surface. In all cases, where significant differences were found, Tukey's tests were used to identify the differences (P<0.05). Results are presented as means ± s.e.m.

Results

Control measurements with unfertilized eggs showed that less than 5% of the decrease in DO was due to microbes on the surface of the chorion. Because this artefact was small, it was ignored in subsequent analyses. All boundary layer

measurements showed a non-linear decrease in dissolved oxygen (DO) as the surface of the chorion or skin was approached (Fig. 1A-G).

Series I

(a) Oxygen gradient with developmental stage

The gradient or boundary layer thickness (BLT) before hatching cannot be compared statistically with that after hatching because in the former the gradient was measured to the chorion surface, whereas in the latter the gradient was measured right up to the skin of the larvae.

Before hatching, there were significant changes in both the gradient and BLT with developmental time (Table 1): the gradient tended to increase (Fig. 1H) and the BLT tended to decrease (Fig. 1I). However, the only statistically significant differences were that the gradient was greater at 33 d.p.f. relative to 11 and 23 d.p.f. (Tukey's test, P=0.002 and P=0.003, respectively) and the BLT was less at days 23 and 30 relative to day 16 (Tukey's test, P=0.04 and P=0.00 respectively). In general, a larger gradient was associated with a thicker boundary layer even considering only the data before hatching in normoxia, but the relation was not strong (linear regression, P=0.04, data not shown).

After hatching, the change in the gradient was statistically different with developmental time (less at 50 d.p.f. than at 43 d.p.f.) but the BLT was not (Table 1).

(b) Oxygen gradient with acute exposure to hypoxic water

The oxygen gradient was less in hypoxic water than in normoxic water in every trial at every developmental stage, both before and after hatching (Fig. 1; Table 1). Before hatching when embryos were exposed to hypoxic water, the gradient was significantly smaller (Table 1) on each of the 5 days tested (Tukey's test, P<0.001). Similarly, after hatching, when exposed to hypoxic water the gradient was significantly smaller on both days tested (Tukey's test, P=0.001). Both before and after hatching, the interaction term was significant; indicating that although the gradient was smaller when exposed to hypoxic water, the magnitude of the change was not the same on each day.

The BLT was consistently less both before and after hatching when the embryos or larvae were exposed to hypoxic water but this difference was much smaller than the change in gradient (Fig. 1; Table 1).

(c) Oxygen gradient with prolonged (8 h) exposure to hypoxic water

In this separate trial, the six embryos were exposed to hypoxic water for 8 h to see if the gradient would change when measured at 0.5, 4 and 8 h. The gradient increased with developmental time after exposure to hypoxic

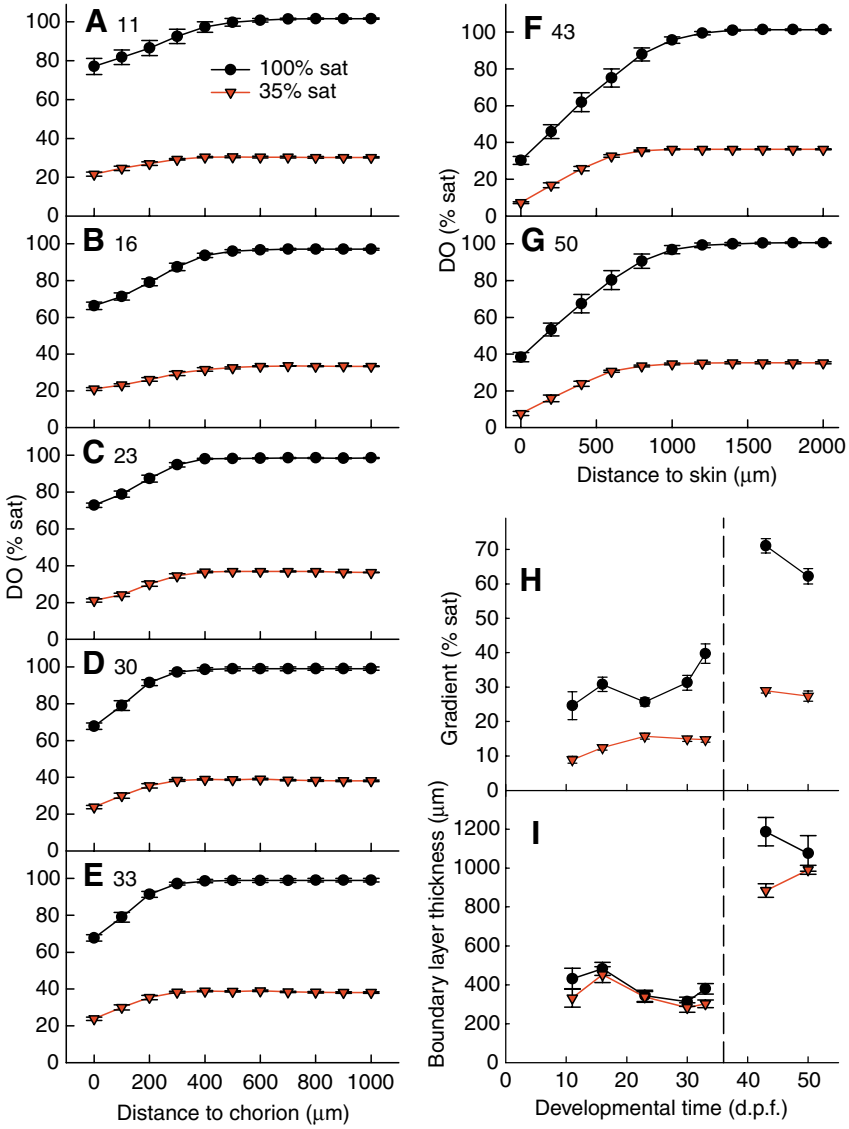

Fig. 1. (A–G) Effect of developmental stage and hypoxia on dissolved oxygen (DO) concentration in the boundary layer of rainbow trout embryos before hatching relative to distance to the chorion (A–E) and in larvae after hatching relative to the distance to the skin (F,G). Boundary layers were measured in normoxic water (100% sat, black circles and black lines) and after 30 min exposure to hypoxic water (35% sat, red triangles and red lines). Numbers beside panel letters indicate developmental time in days post fertilization (d.p.f.). Values are means ± s.e.m., N=8; different animals were used at each developmental stage and different animals were used in normoxia *vs* hypoxia. (H,I) The boundary layer gradient, (the difference between the DO in the free-stream and the DO at the surface; H) and the boundary layer thickness (BLT; I), were calculated from the boundary layer curves in A–G, and are plotted against developmental time (d.p.f.). Vertical broken line indicates approximate time of hatching.

Table 1. *ANOVA tables for results of statistical tests on the effect of various factors on the gradient from the free-stream to the chorion of embryos, or to the skin of larvae, and on the boundary layer thickness in rainbow trout*

Series Ia: effect of developmental stage

		Boundary layer thickness		Gradient	
Before hatching[1]					
Source	d.f.	*F*	*P*	*F*	*P*
d.p.f.	4	4.13	0.008	5.54	0.002
Error	34				
After hatching[2]					
Source	d.f.	*F*	*P*	*F*	*P*
d.p.f.	1	0.90	0.4	8.46	0.011
Error	14				

One-way ANOVA [1](11, 16, 23, 30, 33 d.p.f.); [2](43, 50 d.p.f.).

Series Ib: effect of hypoxia at different developmental stages

		Boundary layer thickness		Gradient	
Before hatching[1]					
Source	d.f.	*F*	*P*	*F*	*P*
d.p.f.	4	7.60	<0.001	7.91	<0.001
Oxy/hypoxy	1	5.24	0.025	204	<0.001
Interaction	4	0.65	0.63	4.23	0.004
Error	68				
After hatching[2]					
d.p.f.	1	0.0	0.97	9.18	0.005
Oxy/hypoxy	1	9.65	0.004	493	<0.001
Interaction	1	3.07	0.09	4.41	0.045
Error	28				

Two-way ANOVA with interaction [1](11, 16, 23, 30, 33 d.p.f.); [2](43, 50 d.p.f.).

Series Ic: effect of prolonged exposure to hypoxic water at 23 and 30 d.p.f.

		Boundary layer thickness		Gradient	
23 d.p.f.					
Source	d.f.	*F*	*P*	*F*	*P*
Time exposed	2	1.93	0.20	3.83	0.06
Embryo	5	0.64	0.68	0.38	0.85
30 d.p.f.					
Source	d.f.	*F*	*P*	*F*	*P*
Time exposed	2	5.25	0.028	13.93	0.001
Embryo	5	0.80	0.57	1.37	0.31

ANOVA (exposure time within embryo).

Series III: effect of flow rate

		Boundary layer thickness		Gradient	
Source	d.f.	*F*	*P*	*F*	*P*
Flow	2	7.91	0.009	65.62	0.001
Embryo	5	1.00	0.46	4.92	0.019

ANOVA (flow within embryo).

d.f., degrees of freedom; d.p.f., days post fertilization; Oxy/hypoxy, tests effect of normoxic *versus* hypoxic water; Interaction, interaction term d.p.f. × Oxy/hypoxy.

water for 30 min, as it did in the previous trials. Inspection of the curves in Fig. 2 reveals that the changes during the 8-h exposure to hypoxic water were trivial at 11 d.p.f. and at 50 d.p.f. At 23 d.p.f., although both the gradient and BLT increased over time, neither change was statistically significant (Table 1). However, at 30 d.p.f. the gradient was smaller and the boundary layer was thinner after 4 h and 8 h exposure than it was at 0.5 h (Tukey's test, $P<0.05$).

Series II

(a) Oxygen gradient with and without a chorion

At 31 d.p.f., the DO concentration in normoxia (100% sat) at the skin surface after the chorion was removed from the embryo was 32% less than the DO concentration at the surface of the chorion (*t*-test, $P=0.0002$; Fig. 3).

(b) Oxygen gradient across the chorion

On a separate batch of embryos at the same developmental stage, the DO concentration inside the chorion was markedly lower relative to that at the surface of the chorion (0 μm) in normoxia (*t*-test, $P<0.0001$; Fig. 4). The DO value inside the chorion of intact embryos was 16±3.0% sat ($N=6$; Fig. 4). Indeed, the gradient across the chorion was approximately the same magnitude as that from the free-stream to the surface of the chorion.

Series III: oxygen gradient with changes in water flow rate

In 29 d.p.f. embryos exposed to normoxia, a decrease in water flow resulted in a steeper oxygen gradient (Fig. 5). Flow had a significant effect on BLT and on the oxygen gradient from the free-stream to the chorion (Table 1). The BLT

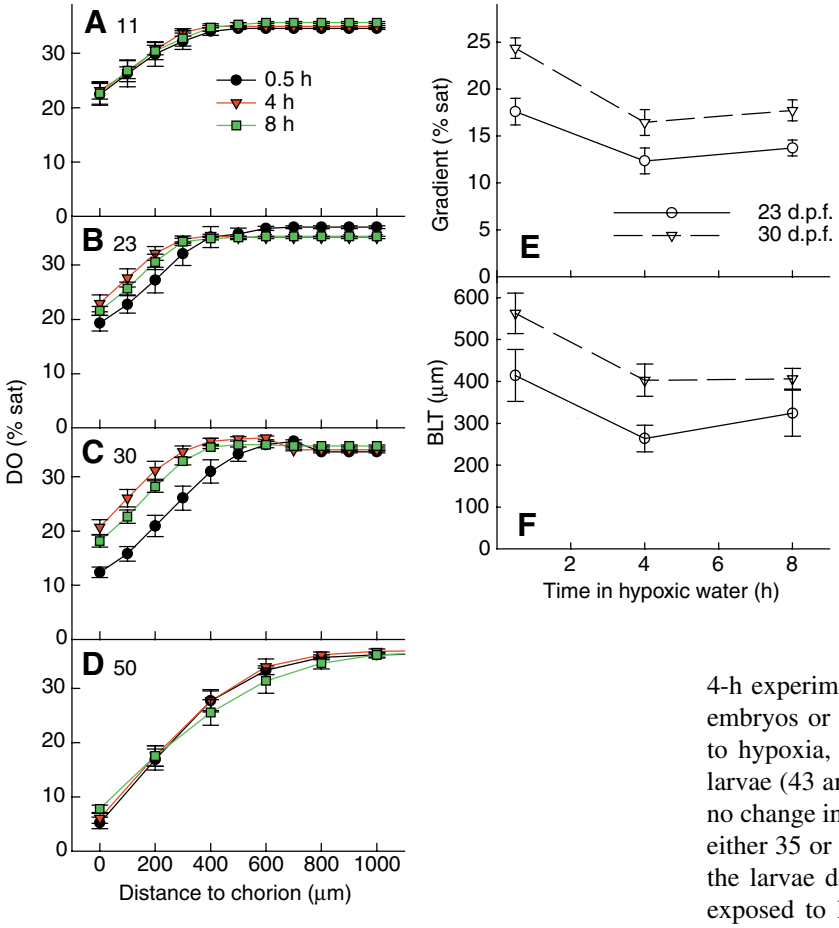

Fig. 2. (A–D) Effect of prolonged exposure to hypoxic water (35% sat) on dissolved oxygen (DO) concentration in the boundary layer of rainbow trout embryos before hatching relative to distance to the chorion (A–C) and in larvae after hatching relative to the distance to the skin (D). Numbers beside panel letters indicate developmental time in days post fertilization (d.p.f.). Values are means ± s.e.m., *N*=8; different animals were used at each developmental stage but the same animals were tested at 0.5, 4 and 8 h exposure to hypoxic water. (E,F) The boundary layer gradient (the difference between the DO in the free-stream and the DO at the surface; E), and the boundary layer thickness (BLT; F), were calculated from the boundary layer curves in B and C, and are plotted against time in hypoxic water (h). Changes during prolonged exposure were trivial at 11 and 50 d.p.f. and are not plotted in E and F.

(Fig. 5C) was significantly greater at a flow of 3 ml min^{-1} than at either 5 or 7.2 ml min^{-1}. The gradient from the free-stream to the chorion (Fig. 5B) was greater at 3 ml min^{-1} than at 5, and was greater at 5 than at 7 ml min^{-1}.

Series IV: influence of hypoxia on embryo and larva movement

A control experiment was performed to test for differences between the trays. Under normoxic conditions, there was no significant difference between the number of movements of embryos between the three trays before hatching at 31 d.p.f. (ANOVA, *P*=0.98, data not shown) and after hatching at 36 d.p.f. (ANOVA, *P*=0.70, data not shown).

At 44 d.p.f., upon exposure to severe hypoxia (35% sat), after a small initial increase, the number of movements gradually decreased over a period of 4 h (Fig. 6). This effect was reversible because upon return to normoxia for 2 h, the number of movements gradually returned to the values during the first 30 min (Tukey's test, *P*>0.05; Fig. 6). Interestingly, the larvae continued to show a low number of body movements for about 30 min after being returned to normoxic water.

The number of movements was extremely low before hatching (2 movements h^{-1} at 33 d.p.f.; Fig. 7A) but it increased orders of magnitude after hatching (950 at 43 d.p.f. and 3000 movements h^{-1} at 50 d.p.f.; Fig. 7B,C). During the 4-h experiment, the amount of activity stayed the same when embryos or larvae were exposed to 100% sat. When exposed to hypoxia, the responses of the embryos (33 d.p.f.) and the larvae (43 and 50 d.p.f.) were different. At 33 d.p.f., there was no change in the number of movements of embryos exposed to either 35 or 50% sat (ANOVA, *P*=0.98; Fig. 7A). By contrast, the larvae decreased their number of body movements when exposed to hypoxia (Fig. 7B,C). At 43 d.p.f., the number of movements did not differ between the first, second, third, or fourth hour in normoxia (ANOVA, *P*=0.091; Fig. 7B). During the last 90 min of exposure, the number of movements of embryos exposed to 35% and 50% sat were 47% less than those exposed in normoxia (Tukey's test, *P*=0.0001 and *P*=0.0011, respectively), but the number of movements in the two hypoxia-exposed groups were not significantly different from each other (Tukey's, *P*=0.49).

At 50 d.p.f., the pattern of activity in normoxia and hypoxia was very similar to the 43 d.p.f. data. The number of movements did not differ between the first, second, third or fourth hour in normoxia (ANOVA, *P*=0.47; Fig. 7C). During the last 90 min of exposure the number of movements of embryos exposed to 35% and 50% sat were 69% less than those exposed in normoxia (Tukey's, *P*<0.0001, and *P*<0.0003 respectively), but the number of movements in the two hypoxia-exposed groups were not significantly different from each other (Tukey's, *P*=0.23). In summary, exposure to hypoxia before hatching did not result in a change in the number of movements, but in post-hatch larvae resulted in a marked decrease in activity that was reversible.

Discussion

The oxygen boundary layer around fish embryos and larvae depends on the supply of oxygen to, and the demand for oxygen by the embryos and larvae. In turn, the supply of oxygen to the

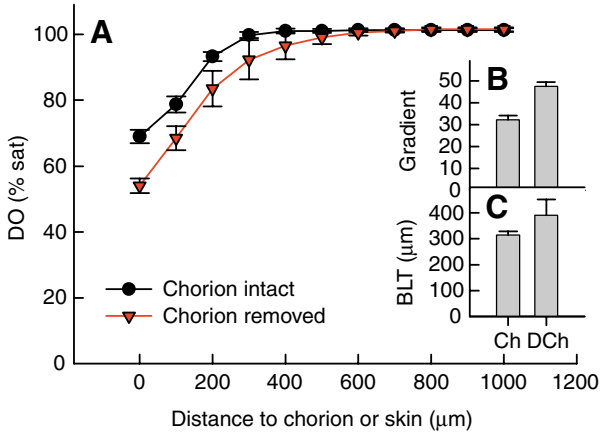

Fig. 3. (A) Effect of the chorion on dissolved oxygen (DO) concentration in the boundary layer in 31 d.p.f. rainbow trout embryos before hatching relative to the distance to the chorion (black circles, black lines) and in the same embryos after manually removing the chorion (red triangles, red lines) relative to the distance to the skin. Values are means ± s.e.m., *N*=5. (B,C) The boundary layer gradient (the difference between the DO in the free-stream and the DO at the surface in % sat; B) and the boundary layer thickness (BLT; C), calculated from the boundary layer curves in A. Ch (chorionated), animals with intact chorion; DCh (dechorionated), animals with chorion manually removed.

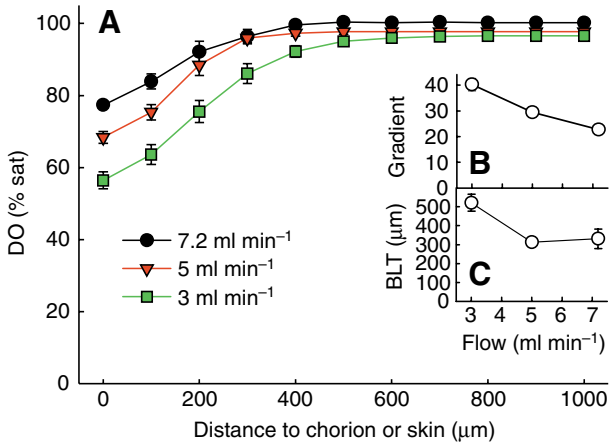

Fig. 5. (A) Effect of the water flow rate on dissolved oxygen (DO) concentration in the boundary layer in 29 d.p.f. rainbow trout embryos before hatching relative to the distance to the chorion. Values are means ± s.e.m., *N*=6. The DO was measured in the same six embryos at the three different flow rates, starting with the highest rate. Flow rate values represent the flow in ml min^{-1} (7.2, 5 and 3) entering the experimental chamber (3.3 cm wide×2 cm deep). (B,C) The boundary layer gradient (% sat, the difference between the DO in the free-stream and the DO at the surface; B) and the boundary layer thickness (BLT; C), were calculated from the boundary layer curves in A and are plotted against flow rate.

embryos and larvae depends on the convection of the water in the free-stream and the diffusion of the oxygen molecules within the boundary layer. The demand for oxygen by the embryos and larvae is a function of the metabolic rate. Therefore, our study focused on the variables that influence oxygen supply, such as water flow rate, the oxygen concentration in the free-stream and movement, and oxygen demand, such as changes in oxygen uptake.

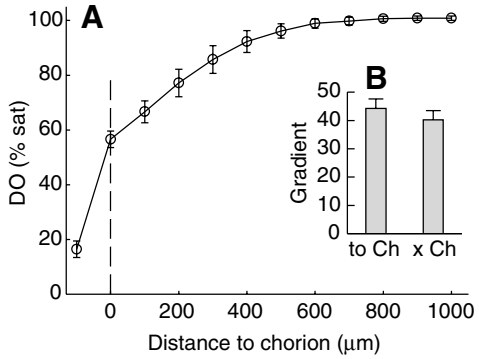

Fig. 4. (A) The dissolved oxygen (DO) concentration ~100 μm inside the chorion in 31 d.p.f. rainbow trout embryos before hatching, and in the same embryos, the DO concentration in the boundary layer relative to the distance to the chorion. Values are means ± s.e.m., *N*=6. (B) The boundary layer gradient (% sat; to Ch, the difference between the DO in the free-stream and the DO at the surface of the chorion) compared with the gradient across the chorion (x Ch, the difference between the DO at the surface of the chorion and the DO inside the chorion).

Series I

(1) Oxygen gradient with developmental time

We hypothesized that the oxygen gradient within the boundary layer of intact embryos would increase over developmental time. This expectation was based on oxygen

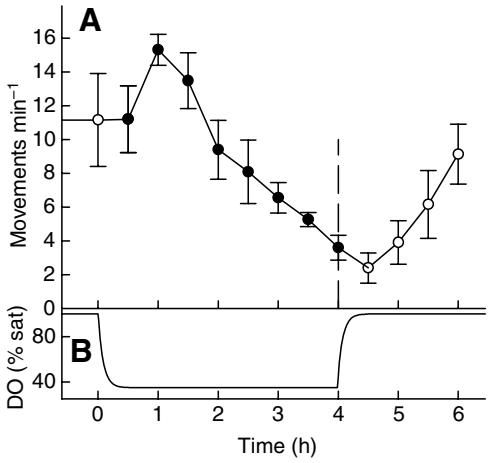

Fig. 6. (A) The number of movements min^{-1} by 44 d.p.f. rainbow trout larvae in normoxic water (first open circle), then exposed to 35% sat for 4 h (closed circles), and then returned (vertical broken line) to normoxic water for a further 2 h (open circles). Values are means ± s.e.m. (*N*=9). The effect of exposure to hypoxia was reversible, but there was a delay of about 30 min and then a gradual return to pre-exposure levels. (B) Time course of the change in DO (% sat) in the free-stream around the larvae while movements were being recorded.

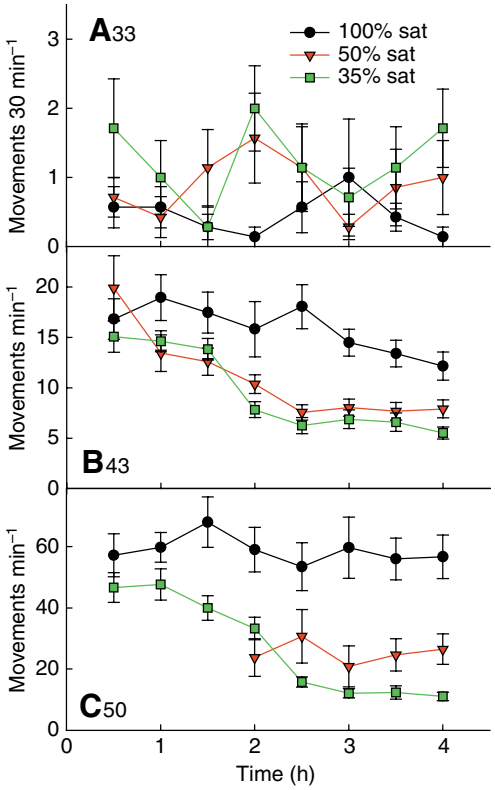

Fig. 7. Number of movements per 30 min (A) or per min (B,C) by rainbow trout embryos exposed to water with 100% (black circles), 50% (red triangles), or 35% sat (green squares) at three different developmental stages; numbers beside panel letters indicate developmental time in days post fertilization (d.p.f.). Values are means ± s.e.m. (*N*=6); note different scale on *y*-axis in A.

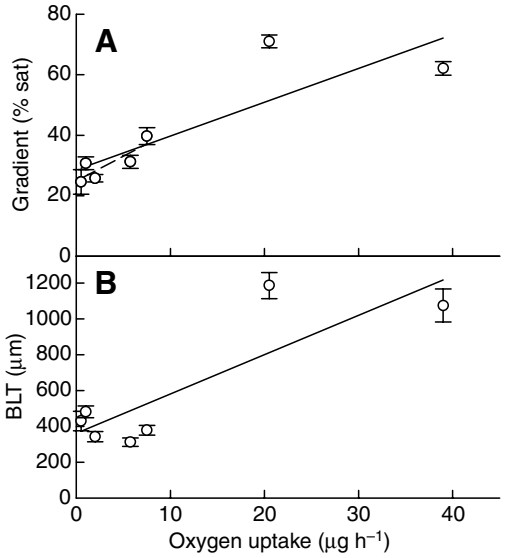

Fig. 8. The relationship between gradient and boundary layer thickness (BLT) with oxygen uptake – both increased with an increase in oxygen demand. Oxygen demand is expressed as oxygen uptake interpolated from Rombough (Rombough, 1988a). Lines are least-square regression lines using the means at each developmental stage in normoxic water.

uptake data for trout embryos. These oxygen uptake data for rainbow trout embryos (Rombough, 1988a) are combined with our oxygen measurements in Fig. 8. Over the broad scale we see that there is a significant positive relation between both the gradient and the BLT and oxygen uptake. Even considering only the data for the embryos before hatching (that is, oxygen uptake values less than 10 μg h^{-1}), the relationship is significant for the gradient. Thus, our results combined with Rombough's oxygen uptake data (Rombough, 1988a) show that the gradient and the BLT are a function of oxygen demand.

(b) Oxygen gradient with acute exposure to hypoxic water

The second hypothesis concerned the relation between the gradient in the boundary layer and oxygen supply. This series, which tested the effect of an acute change in oxygen supply, showed that there was a positive relation between DO at the skin or chorion and the supply (DO in the free-stream). This relationship is illustrated in Fig. 9. Clearly there is a strong positive relationship between DO at the chorion or skin and DO in the free-stream. Most of the values lie below the unity line, reflecting the resistance to oxygen diffusion through the boundary layer. It is also clear that the slope of this relationship is less than 1 for both the embryos and the larvae, suggesting that oxygen demand (i.e. metabolic rate) decreased as the supply decreased (i.e. in hypoxic water). If demand were unchanged (i.e. if oxygen uptake did not change) then the relationship should have a slope the same as the unity line. If, on the other hand, the animals were perfect conformers, then we would expect the relationship to pass through the origin. Our data suggest that in response to acute hypoxia, the embryos are almost perfect conformers and that the larvae are partial conformers.

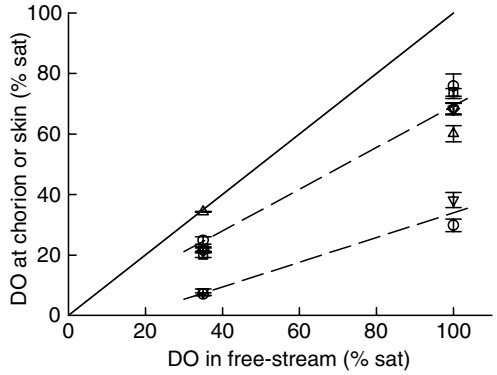

Fig. 9. The relationship between the dissolved oxygen (DO) at the chorion of embryos (top broken line) or at the skin of larvae (bottom broken line) as a function of oxygen supply (DO in the free-stream). Broken lines are least-square regression lines using the mean values at each developmental stage; solid line to the origin is the unity line. Values are means ± s.e.m.

(c) Oxygen gradient with prolonged exposure to hypoxic water

In the present study, when embryos and larvae were exposed to hypoxia over several hours there was a decrease in the DO gradient after 4 and 8 h of exposure to hypoxia at later developmental stages (33 d.p.f.), but not at earlier ones (11 and 23 d.p.f.). Adult fishes have been observed to undergo metabolic depression when exposed to severe hypoxia (Dalla Via et al., 1994) (for a review, see Boutilier, 2001), but the ability of fish embryos and larvae to depress their metabolism has not been well studied. However, zebrafish embryos (8- to 16-cell stage) have been reported to enter a state of suspended animation when exposed to anoxia for 24 h, which indicates that at least some species of fish are capable of metabolic depression at an early developmental stage (Padilla and Roth, 2001). Salmonid embryos and larvae are believed to be oxygen conformers at low oxygen concentration (see above); therefore, their metabolic rate depends on the available oxygen (Rombough, 1988a). The anaerobic capacity of salmonid embryos is very limited and could only play a very minor role at these life stages (Ninness et al., 2006).

Series II: oxygen gradient and the chorion

Our null hypothesis was that the chorion is not a barrier to oxygen diffusion. Our data show that the gradient across the chorion was about the same magnitude as the gradient from the free-stream to the chorion and that the DO of the pvf was about 16% sat. The only previous measurement of DO in the pvf was performed on loach (*Misgurnis fossilis*) embryos (Berezovsky et al., 1979). We conclude that the chorion forms an additional barrier to the diffusion of oxygen from the free-stream to the body of the embryo, at least in 31 d.p.f. trout embryos. The difference between our results in trout and the published value in loach may reflect a combination of factors related both to oxygen supply and demand. Further exploration of these factors in hypoxia-tolerant species (e.g. loach) and intolerant species (e.g. trout) would provide insights into species-specific regulation of embryonic oxygen uptake in fishes adapted to different ecological niches.

Series III: oxygen gradient with changes in flow rate

The third hypothesis was that a decrease in flow causes an increase in the gradient and thickness of the boundary layer of rainbow trout embryos. Our results support this hypothesis. Similar differences in DO in the boundary layer were observed in adult frogs exposed to water velocities between 0 and 5.2 cm s^{-1} (Pinder and Feder, 1990). In newly hatched trout larvae in well-saturated water, skin DO was <10% sat in still water, but increased to ~30% sat when flow was initiated (Rombough, 1992). Therefore, even relatively small changes in water flow rate can produce significant differences in water boundary layer DO. It is difficult to compare directly the flow rates that we used in our chamber (3, 5 and 7.2 ml min^{-1}) with estimates of flow rates through redds in the field because, in redds, each egg is surrounded by other eggs or gravel. Our findings have strong ecological relevance for rainbow trout and other salmonids because intergravel flow velocities are not constant; they vary spatially within streams and between streams and also vary temporally with changes in stream flow rates (Zimmermann and Lapointe, 2005).

Series IV: influence of hypoxia on embryo and larva movement

We hypothesized that hypoxia would increase the number of movements made by embryos, but this was not the case. In gelatinous egg masses of both amphibian and snail embryos, spinning behaviour is thought to increase the amount of mixing of the pvf, thus bringing in more oxygen to the developing embryo (Hunter and Vogel, 1986; Kuang et al., 2002). When exposed to hypoxia, pond snail (*Helisoma trivolvis*) embryos accelerate their spinning behaviour, mixing the pvf (Kuang et al., 2002). Amphibian and snail embryos have a relatively large perivitelline space compared with the trout embryo, where the yolk and body of the embryo occupy over 95% of the space inside the chorion (C. Ciuhandu, personal observation). In the present study, the small space inside the chorion of trout embryos probably limits whole body movements, in agreement with Ninness et al. (Ninness et al., 2006). Pectoral fin flutter has been described in Atlantic salmon as alternatively abducting and adducting the fin (Peterson et al., 1991), therefore a faster moving smaller structure might function well in stirring the pvf when space inside the chorion is limited. Unfortunately, we did not record the effect of hypoxia on fin movements in the present study.

In the present study, the movement of trout embryos increased with developmental time from 2 movements h^{-1} just before hatching to over 3000 movements h^{-1} around the time of first feeding. Previous studies on fish reported similar patterns of body movements throughout development (Peterson and Martin-Robichaud, 1983; Ninness et al., 2006). Therefore, the musculoskeletal system is sufficiently developed for high levels of activity soon after hatching.

After hatching, trout larvae decreased the number of movements by 60% when exposed to hypoxia (Fig. 7B,C). Similar decreases in activity have been reported in other fishes. When exposed to anoxia, adult crucian carp (*Carassius carassius*) reduced activity by 50% relative to that in normoxia (Nilsson et al., 1993), while juvenile white sturgeon (*Acipenser transmontanus*) decreased their activity by 70% when exposed to moderate hypoxia (Crocker and Cech, 1997). In rainbow trout larvae, the number of body movements after exposure to hypoxia decreased gradually and reached a plateau after about 2–3 h. In adult carp, the decrease in activity was similar, with a gradual decrease, and a plateau was reached after 90–120 min of exposure to anoxia (Nilsson et al., 1993). The decrease in the number of movements during exposure to hypoxia is probably a strategy for saving energy during extended exposure to hypoxia (Nilsson et al., 1993). By contrast, other studies have reported an increase in activity with moderate hypoxia exposure in adult sand goby *Pomatoschistus minutus* (Petersen and Petersen, 1990), adult brook trout *Salvelinus fontinalis* (Tang and Boisclair, 1995), and juvenile guppies *Poecilia reticulata* (Weber and Kramer, 1983). These responses are

likely escape responses in fish that often encounter localized hypoxic zones (Petersen and Petersen, 1990).

When embryos were exposed to severe hypoxia for 4 h, the number of movements decreased, and upon re-exposure to normoxia the number of movements returned to initial values. This result demonstrated that the effect of exposure to severe hypoxia was reversible. Interestingly, the number of movements continued to remain low for about 30 min after the return to normoxia. Similar responses have been observed in snail embryos that responded to re-exposure to normoxia by decreasing their number of movements for the initial 10 min (Kuang et al., 2002). There are two possible explanations. First, the reintroduction to normoxia produces a strong inhibition of the oxygen sensor that results in a further inhibition in movement (Kuang et al., 2002). Second, the response could be due to a delay in the time it takes for oxygen to reach the body of the embryo because even though the DO was normoxic in the free-stream, the environment around the embryo was still hypoxic.

In conclusion, the oxygen boundary layer around trout embryos and larvae depends on both the supply of and demand for oxygen. Our results show that increased oxygen demand (due to increased oxygen uptake with development) decreased oxygen levels in the boundary layer. Further, we showed that decreased oxygen supply, as a result of low water flow rates or exposure to hypoxia free-stream water, also decreases oxygen levels in the boundary layer. Finally, the presence of the chorion adds an additional barrier to the diffusion of oxygen.

Thanks to the University of Guelph Aqualab and the University of Alberta Aquatics Facility staff for help with the experimental setup, and to Greg Goss for help during initial experiments. This research was supported by a Natural Sciences and Engineering Research Council of Canada, Discovery and Tools and Equipment grants to P.A.W. and E.D.S., and a Journal of Experimental Biology travelling fellowship to C.S.C.

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