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PARTITIONING OF OXYGEN UPTAKE BETWEEN THE GILLS AND SKIN IN FISH LARVAE: A NOVEL METHOD FOR ESTIMATING CUTANEOUS OXYGEN UPTAKE

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Summary

The goal of this study was to develop an alternative to the traditional rubber dam method for measuring cutaneous oxygen uptake in bimodally respiring (skin + gills) fish larvae. The method tested involved using microelectrodes to measure the P_{O_2} gradient in the diffusive boundary layer adjacent to seven positions on the skin surface (one on the head, two on the yolk sac, two on the trunk, one at the base of the dorsal fin-fold and one on the proximal portion of the caudal fin-fold) of rainbow trout (*Oncorhynchus mykiss*) larvae in still water. The P_{O_2} gradient ($\Delta P_{O_2}/\Delta x$, where x is the distance from the skin surface) was then used to calculate area-specific rate of O_2 uptake ($\dot{M}_{O_2}/A$) according to the Fick equation, $\dot{M}_{O_2}/A = D\beta(\Delta P_{O_2}/\Delta x)$, where A is the cross-sectional area of the boundary layer, D is the diffusion coefficient and β is the capacitance coefficient for O_2 in water. The accuracy of the method was assessed by comparing it with the rubber dam method. After correcting for differences in body mass, the two methods gave essentially identical results. According to the boundary layer method, the mean (±95 % CI) rate of O_2 uptake across the skin of newly hatched rainbow trout at 10 °C is 3.13±0.18 $\mu g\, O_2\, cm^{-2}\, h^{-1}$ (N=265). The corresponding value obtained using the rubber dam method was 3.36±0.35 $\mu g\, O_2\, cm^{-2}\, h^{-1}$ (N=27). The advantages of the boundary layer method are that it can be used with smaller, more delicate larvae and that variables, such as flow rate, that can affect the efficiency of gas exchange can be regulated more precisely. The boundary layer method also permits examination of regional differences in exchange efficiency, although in still water such differences do not appear to be significant in trout larvae. The mean steepness of the P_{O_2} gradient in the boundary layer and, hence, the mean rate of area-specific O_2 uptake were essentially the same (P>0.05) at all seven locations tested in this study. The boundary layer method can potentially be used to study the transcutaneous flux, not only of O_2 but of virtually any diffusible substance that can be measured with microelectrodes and that is consumed (e.g. Na^+, Ca^{2+}) or excreted (e.g. CO_2, NH_3) by fish larvae or other small organisms.

Key words: cutaneous respiration, oxygen partitioning, fish, larva, boundary layer, skin, method, rainbow trout, *Oncorhynchus mykiss*.

Introduction

The skin is an important site of respiratory gas exchange in fish, particularly during embryonic and larval development. It is the sole site of gas exchange during embryonic development in oviparous species. The relative importance of the skin begins to decline once the gills become functional. Many species, however, do not form gills until fairly late in larval development, and in these species the skin persists as the only site of gas exchange well into the larval stage (Rombough, 1988*a*). The skin continues to play a significant role in gas exchange even after the gills have formed. Salmonid larvae, which hatch with relatively well-developed gills compared with most species, still obtain approximately 40 % of their oxygen *via* their skin at the end of the larval stage (Rombough and Ure, 1991; Wells and Pinder, 1996). Adults of water-breathing fish typically obtain 10–20 % of their oxygen across the skin, but values as high as 30 % have been reported (Feder and Burggren, 1985).

Although the importance of cutaneous gas exchange during larval development has long been recognized, little is known about the mechanisms involved. Indeed, it is only recently that attempts have been made even to measure the rate of cutaneous oxygen uptake in fish larvae. Part of the problem in studying cutaneous gas exchange in larvae has been the difficulty in distinguishing cutaneous oxygen uptake from that taking place across the gills. With adult fish, the solution has been to use some kind of membrane (usually a rubber dam) to isolate water surrounding the skin from that flowing over the gills (e.g. Kirsch and Nonotte, 1977). This approach has been used successfully with larvae of chinook salmon *Oncorhynchus tshawytscha* (Rombough and Ure, 1991) and Atlantic salmon *Salmo salar* (Wells and Pinder, 1996). However, the larvae of both salmon species are considerably larger (30–100 mg wet tissue mass at hatching) than those of most fish and it is

unlikely that the technique can be applied to species such as plaice or zebrafish where larvae weigh less than 1 mg at hatching. The rubber dam method is not well suited for looking at regional differences in oxygen uptake and, because of the way the dam is placed, the pattern of water flow over the skin is not representative of what occurs in nature. Flow pattern may be very important to the efficiency of cutaneous uptake. Many larvae orient themselves so that they face into the current. Liem (1981) reported that *Monopterus albus* larvae were able to extract a significantly greater fraction of oxygen from the water if the flow was in an anterior-to-posterior direction (countercurrent to the blood flow in the vitelline veins) than if the flow was in a posterior-to-anterior direction. With the rubber dam method, it is virtually impossible to control the direction or velocity of water flow. The rubber dam method has the additional disadvantage that it is relatively stressful. Even when large, robust larvae such as those of salmon are used, not all individuals survive. This raises the possibility that results obtained using this method might not be fully representative of what takes place in unrestrained animals.

The goal of this study was to develop a less restrictive method for estimating cutaneous oxygen uptake that could be used with smaller, more delicate larvae. The technique that was tested involved measuring the oxygen partial pressure gradient in the diffusive boundary layer adjacent to the outer surface of the skin. A diffusive gas exchanger such as the skin can be considered to consist of a series of resistances, each of which contributes to the total resistance. Because the resistances are in series, the rate of flux through any single element is equal to that across the gas exchanger as a whole. The element that provides the greatest resistance to oxygen uptake across the skin in fish larvae is the diffusional boundary layer (≈95 % of total resistance in still water; Rombough, 1992). The rate at which oxygen diffuses ($\dot{M}_{O_2}$) through a unit cross-sectional area (A) of the boundary layer can be described by rearranging the Fick equation:

$$\dot{M}_{O_2}/A = D\beta(\Delta P_{O_2}/\Delta x)\,, \qquad (1)$$

where D is the diffusion coefficient for O_2 in water, β is the capacitance coefficient for O_2 in water, P_{O_2} is the partial pressure of O_2 and x is the thickness of the boundary layer. Values for the physical constants D and β are available in the literature. The oxygen partial pressure gradient ($\Delta P_{O_2}/\Delta x$) can be measured using oxygen microelectrodes (Pinder and Feder, 1990; Feder and Booth, 1992). Once area-specific uptake ($\dot{M}_{O_2}/A$) is known, total cutaneous uptake can be estimated simply by multiplying area-specific uptake by skin surface area. A similar procedure has been used to estimate flux rates for various inorganic ions (Smith *et al.* 1994; Collier and O'Donnell, 1997) but, to my knowledge, the technique has not previously been used to measure O_2 flux in whole organisms.

The accuracy of the boundary layer method was evaluated by comparing it with the rubber dam method. The rubber dam method only works with fairly large larvae, so rainbow trout *Oncorhynchus mykiss* was used as a test species.

Materials and methods

Experimental animals

Rainbow trout *Oncorhynchus mykiss* (Walbaum) were obtained as eyed embryos from the Rockwood Fish Culture Centre operated by the Canadian Department of Fisheries and Oceans at Gunton, Manitoba. Eggs were transferred to Brandon University, where they were reared in fresh water (pH 7.9; hardness 136 mg l^{-1} as $CaCO_3$) in the dark until hatching. Eggs initially were kept at 2–3 °C to slow development and delay hatching. Eggs were transferred to 10 °C to speed up development 5–15 days before they were needed for experiments. This allowed a sequential series of experiments to be carried out using the same group of animals. Once transferred to 10 °C, eggs and larvae were kept at that temperature (±0.1 °C) until experiments were completed.

Two groups of newly hatched larvae (1–5 days posthatch) from the same brood stock were used in this study; one in the rubber dam study and one for the boundary layer experiments. The larvae used in the rubber dam experiments were significantly larger (P>0.05) than those used in the boundary layer experiments (Table 1). In terms of the relative amount of yolk consumed, however, the two groups of larvae were at almost exactly the same stage of development (43.3 % *versus* 46.7 % tissue; Table 1).

Respirometry

Cutaneous oxygen uptake was estimated for the larger trout larvae (the 50 mg tissue mass group) at 10 °C using the rubber dam method. Equipment and procedures were similar to those employed previously to measure cutaneous oxygen uptake in chinook salmon larvae (*Oncorhynchus tshawytscha*; see Rombough and Ure, 1991, for details). The major difference was an improvement in respirometer design. The respirometer in the present study consisted of two identical 6.46 ml glass chambers held together by pinch clamps. A 0.25 mm thick rubber dental dam was used to separate the chambers. A miniature (2 mm tip diameter) polarographic oxygen electrode (model MI 730, Microelectrodes Inc., Londonderry, NH, USA) was inserted into a small opening at the far end of each chamber. Stopcock grease and O rings gave a water-tight fit. The electrodes were connected to an OM4 oxygen meter (Microelectrodes Inc., Londonderry, NH, USA), and the output was recorded on a flat-bed chart recorder. Electrodes were calibrated to air and to 100 % N_2. Each chamber contained a small magnetic stirrer to ensure that the water was well mixed. The assembled respirometer was immersed in an insulated 45 l water bath held at 10.0±0.1 °C by a refrigerating circulator.

Larvae were anaesthetized using 50–75 mg l^{-1} tricaine methanesulphonate (MS222). A small hole was cut in the rubber dam and the larva inserted so that the membrane gripped the body immediately posterior of the pectoral fins. This isolated the gills on one side of the membrane and approximately 85 % of total skin area including the yolk sac on the other side (Table 1). The rubber dam was mounted between the two chambers of the respirometer, and the

Table 1. *Summary of the morphological variables of the newly hatched rainbow trout larvae used in the rubber dam and boundary layer experiments*

Characteristic	Rubber dam (*N*=19)	Boundary layer (*N*=14)
Total wet mass of larva plus yolk sac (mg)	115.7±15.7	67.4±19.0
Larval tissue wet mass (mg)	50±7.3	31.6±9.4
Proportion tissue (% total)	43.3±4.7	46.7±9.3
Total skin area (mm^2)	206.4±19.8	
Head area (mm^2)	24.22±3.51	
Pectoral fin area (mm^2)	7.39±1.42	
Yolk sac area (mm^2)	77.29±10.10	
Trunk area (mm^2)	97.49±11.44	
Total skin area posterior of pectoral fins (mm^2)	174.8±18.3	
Proportion skin area posterior of pectoral fins (% total)	84.6±1.8	
Proportion skin area on yolk sac (% total)	37.4±3.4	

Values are means ± S.D.

respirometer was closed. Tests were terminated after approximately 2 h or if oxygen levels in one of the chambers dropped below approximately 50 % of air saturation. Oxygen uptake was calculated from the rate at which P_{O_2} in the chambers declined using the longest linear portion of the polarographic record exclusive of the first 15 min of the recording. Uptake rates were adjusted to compensate for bacterial oxygen consumption. In addition, a series of tests was carried out on unrestrained fish to determine whether the stress of being placed in the rubber dam had a significant effect on their overall rate of oxygen consumption. Larvae were removed from the respirometer at the end of the test and placed in a recovery tank without anaesthetic. Test results were not used if the larvae did not recover within 30 min.

Morphometry

Viable larvae from the respirometry study were killed at the end of the recovery period with an overdose of MS222 and preserved in 10 % neutral buffered formalin. Larvae were kept in the preservative for a minimum of 30 days to ensure that shrinkage was complete before any morphometric measurements were made. At the end of the shrinkage period, larvae were weighed to the nearest 0.1 mg. Each larva was dissected into tissue and yolk components, and the two components were weighed separately.

For the purposes of this study, total cutaneous surface area was subdivided into head area, pectoral fin area, yolk sac area and trunk area. Head area was defined by the placement of the rubber dam in the respirometry experiments. Trunk area included the area of the trunk without the yolk sac and the area of the fin folds. Head plus pectoral fin area represented the fraction of total cutaneous surface area in the anterior compartment of the respirometer, while yolk sac plus trunk area was the fraction in the posterior compartment.

Methods for estimating the surface area of the various components were similar to those used by Rombough and Moroz (1990) to measure the cutaneous surface area of chinook salmon larvae. The major difference was that calibrated microscope images of the various components were transferred directly to the computer through a digital camera instead of first being traced onto a digitizing tablet. The microscopic images were obtained from temporary mounts of the various components. Temporary mounts of the yolk sac epithelium were produced by carefully dissecting the yolk sac from the trunk, cutting it into small pieces and flattening the pieces with a coverslip. Iridectomy scissors were used to remove the pectoral fins from the body. The head was then cut from the body immediately posterior of where the pectoral fins had been. A midsagittal cut was made dividing the head into left and right halves. The two halves were mounted medial side down on a glass slide and gently flattened using a coverslip. The trunk was cut transversely into three roughly equal-sized pieces. The two anteriormost pieces were sectioned with a midsagittal cut and mounted medial side down. The posteriormost trunk section consisted mostly of the caudal fin and was thin enough that it did not require midsagittal sectioning. Still images of the various components were taken using a digital camera (model 100C; Pixera Corp.) attached to a Wilde M1 dissecting microscope. The images were stored on computer and analyzed using the SigmaScan for Windows 2.0 computer program (Jandel Scientific).

Boundary layer measurements

Oxygen microelectrodes were used to measure P_{O_2} profiles in the diffusional boundary layer at seven locations (one on the head, two on the yolk sac, two on the trunk, one at the base of the dorsal fin-fold and one on the proximal region of the caudal fin-fold) on the body of recently hatched (less than 5 days posthatch) rainbow trout (see Fig. 1). Equipment and procedures were similar to those described in Rombough (1992). P_{O_2} was measured using Diamond General 737 Clark-type electrodes with tip diameters ranging from 50 to 95 μm mounted in a micromanipulator and connected to a Diamond General Chemical Microsensor II. Current output was recorded on a flat-bed chart recorder. Electrodes were calibrated before

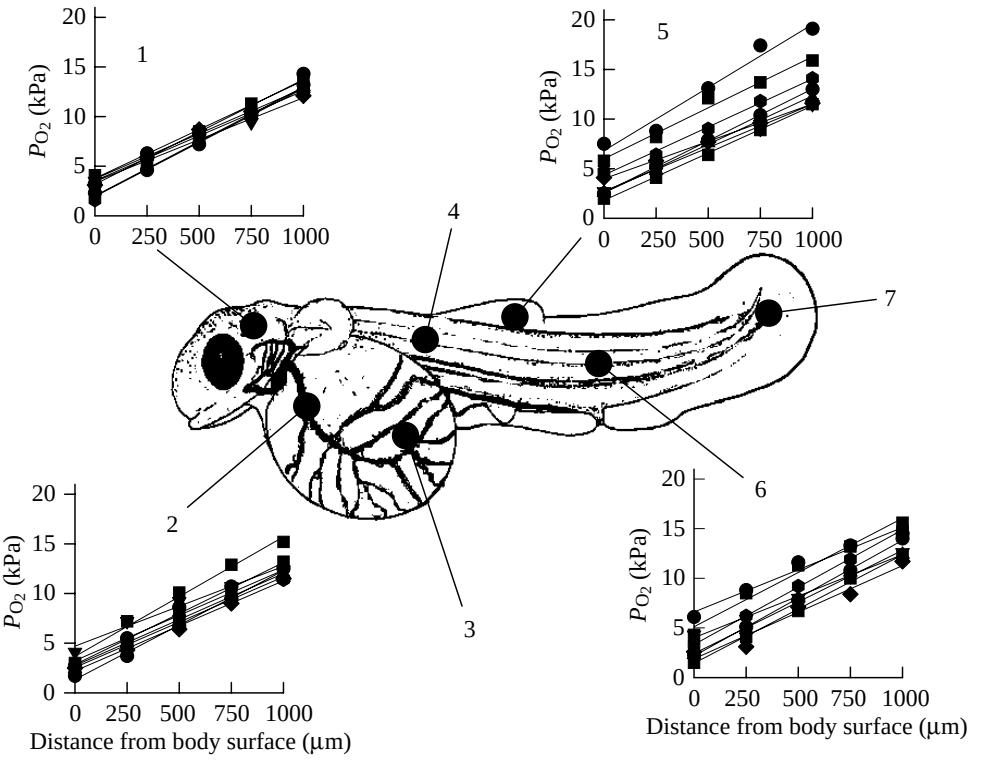

Fig. 1. Locations on the skin of rainbow trout larvae where boundary layer P_{O_2} values were measured. Boundary layer P_{O_2} as a function of distance from the skin surface is shown for positions 1, 2, 5 and 6 (results at the other positions were similar). The various symbols indicate results obtained using different individuals.

and after each experiment to 100 % air saturation and 100 % N_2. Any drift during the course of the experiment was assumed to be linear. Additional calibrations using a 5.16 % O_2 certified gas mixture were carried out periodically to test for linearity of response.

Larvae were anaesthetized using 50 mg l^{-1} MS222 and placed in fresh water (+MS222) on a cork board mounted on the floor of a double-jacketed flow chamber. Previous studies found that this level of anaesthetic did not affect the rate of O_2 consumption by larvae of chinook salmon but was sufficient to halt opercular and pectoral fin movements (P. J. Rombough, unpublished results). The temperature of the water in the flow chamber was held at 10.0±0.2 °C by circulating chilled water between the walls of the jacket. Larvae were held in place by a thin rubber band (<1 mm wide) passing across the trunk approximately half way between the vent and the tail. The band was held in position with two fine insect pins 1–2 cm from the body surface. Another fine insect pin was pushed into the cork board between the open jaws of the larva (the pin did not pierce tissue) to prevent it from moving forward. Once the larva had been immobilized, the water in the flow chamber was aerated vigorously for at least 20 min to ensure that the ambient P_{O_2} of the bulk water was close to 100 % air saturation. Aeration was halted approximately 5 min before the first measurements were taken.

Boundary layer P_{O_2} values were measured at positions 2, 3, 5 and 6 (see Fig. 1) for eight individuals and at positions 1, 4 and 7 for seven individuals. The order of position measurements was assigned randomly for each individual. At each position, the electrode was advanced using the micromanipulator until a slight dimpling of the skin could be observed through a dissecting microscope (50× magnification). The electrode was then slowly pulled back until the dimpling just disappeared. This location was assumed to be representative of the P_{O_2} at the skin surface. The electrode was kept at this location until the reading stabilized (readings were assumed to have stabilized if there was no change in the level of the trace on the chart recorder for at least 2 min; stabilization typically took 5–10 min). The electrode was pulled back by 250 μm using the micromanipulator and the reading again allowed to stabilize. This procedure was repeated at 500, 750 and 1000 μm from the skin surface. After all the positions had been measured, the larva was removed from the flow chamber and placed in anaesthetic-free water in a recovery tank. After it was verified that the larva was undamaged, the larva was killed with an overdose of MS222 and preserved in neutral buffered formalin.

Statistical analyses

The boundary layer data were analyzed using regression analysis and analysis of covariance (ANCOVA). The relationships between boundary layer P_{O_2} and distance from the skin surface for each individual and body position were tested for linearity of response using the SigmaStat (SPSS Inc.) linear regression program. One-way ANCOVA (Systat; SPSS Inc.) was used to test for significant differences in slopes and elevations due to body position. In this test, P_{O_2} was the dependent variable and body position the independent variable. Distance from the skin surface was treated as a covariate. Values for different individuals at the same body position were considered to be replicates. Mean regression lines were calculated using the Sigmastat linear regression program for

each of the various body positions and compared pairwise for significant differences in slopes and elevations following the procedure of Snedecor and Cochran (1980). Two-way ANCOVA, in which P_{O_2} was assumed to be dependent on the individual as well as on body position (distance remained the covariate), was used to test whether variation due to different individuals masked significant differences with respect to body position. The slope of the combined regression equation averaged over all body positions was used in the Fick equation ($\Delta P_{O_2}/\Delta x$, equation 1) to estimate average area-specific rate of O_2 uptake. Values of $1.60\times10^{-5}\,cm^2\,s^{-1}$ (Wickett, 1975) and $2.2399\,\mu mol\,l^{-1}\,mmHg^{-1}$ ($2.2399\,\mu mol\,l^{-1}\,kPa^{-1}$) (Dejours, 1981), respectively, were used for D and β at 10 °C.

Results

Rubber dam method

While the rubber dam presumably caused larvae some distress (approximately 10 % of the restrained larvae died before the end of the experiment), it did not appear to have had much of an impact on their overall rate of oxygen consumption. The mean $\dot{M}_{O_2}$ of larvae restrained by the rubber dam during the first couple of hours was not significantly different from that of unrestrained larvae (Table 2). In the restrained larvae, 62.1 % of total O_2 uptake, on average, took place in the posterior compartment of the respirometer where the skin was the only site of gas exchange (Table 2). Assuming that the efficiency of transcutaneous uptake was approximately the same in the anterior compartment as it was in the posterior compartment, this means that the skin as a whole accounted for approximately 73 % of total O_2 uptake in 1- to 5-day-old larvae (Table 2). On the basis of individual rates of O_2 uptake and the corresponding cutaneous surface areas in the posterior compartment of the respirometer, a mean value (±S.D.) of 4.05±1.08 $\mu g\,O_2\,cm^{-2}\,h^{-1}$ was calculated for the area-specific rate of uptake of O_2 across the skin (Table 2). A mean mass-specific rate of O_2 uptake of $236\,\mu g\,O_2\,g^{-1}\,h^{-1}$ (Table 2) was estimated on the basis of mean total $\dot{M}_{O_2}$ (restrained + unrestrained larvae; Table 2) and mean total tissue mass (Table 1).

Boundary layer measurements

In still water, P_{O_2} levels at the skin surface (3.08 kPa, Table 3) were, on average, only approximately 14.5 % of the free-stream value. P_{O_2} levels in the boundary layer increased fairly slowly with distance and typically did not approach free-stream values until 3–5 mm from the body surface. The decision to use a linear model (equation 1) to estimate O_2 flux was based on the fact that, for the first 1000 μm, the rate of increase was linear or close to linear (Fig. 1). Simple linear regressions based on measurements taken at distance of 1000 μm or less from the skin surface were all highly significant (P<0.001). In most cases, a simple linear regression provided as good a fit, or a better fit, to the data as a hyperbolic relationship (Fig. 1). Both one-way ANCOVA (Table 4) and the paired comparisons indicated that there were no significant differences (P>0.05) in terms of either slope or elevation of the mean regression equations for the various body positions. Two-way ANCOVA confirmed that body position had no significant effect on slope (F=1.11; P=0.358; d.f.=6, 237). There were significant differences in slope among individuals (F=3.94; P<0.001; d.f.=7, 237) but, given that individual variability at the same body position tended to be relatively small (Fig. 1; Table 3), they are probably not important in the context of this study. Because slope did not vary significantly with body position, mean area-specific O_2 uptake could be estimated relatively simply using the value for the combined slope averaged over all body positions. Calculating a mean value for $\dot{M}_{O_2}/A$ would have been more difficult if the slopes had varied with body position since it would have required estimating the fraction of total surface area to which each of the various slope values applied. On the basis of a combined slope of $10.1\,kPa\,mm^{-1}$ (Table 3) and values of $1.60\times10^{-5}\,cm^2\,s^{-1}$ and $2.2399\,\mu mol\,l^{-1}\,mmHg^{-1}$, respectively, for D and β, the mean (±S.D.) area-specific rate of O_2 uptake across the skin at 10 °C was estimated to be 3.13±1.41 $\mu g\,O_2\,cm^{-2}\,h^{-1}$ (note that the actual S.D. is probably somewhat greater than ±1.41 $\mu g\,O_2\,cm^{-2}\,h^{-1}$ since uncertainties associated with the values for D and β were not taken into account).

Table 2. *Partitioning of O_2 uptake in newly hatched rainbow trout larvae*

$\dot{M}_{O_2}$ total of unrestrained larvae ($\mu g\,O_2\,h^{-1}$)	11.99±1.57 (26)
$\dot{M}_{O_2}$ total of restrained larvae ($\mu g\,O_2\,h^{-1}$)	11.58±2.75 (27)
$\dot{M}_{O_2}$ in anterior chamber ($\mu g\,O_2\,h^{-1}$)	4.48±2.08 (27)
$\dot{M}_{O_2}$ in posterior chamber ($\mu g\,O_2\,h^{-1}$)	7.10±1.74 (27)
Proportion of uptake in posterior chamber (% total)	62.1±9.4 (27)
Cutaneous uptake (% total)	73.3±11.1 (27)
Area-specific skin uptake ($\mu g\,O_2\,cm^{-2}\,h^{-1}$)	4.05±1.08 (27)
Total mass-specific uptake, combined ($\mu g\,O_2\,g^{-1}\,h^{-1}$)	236±46.8 (53)

A rubber dam positioned immediately posterior of the pectoral fins was used to restrain larvae and to partition the respirometer into anterior and posterior chambers.

Values are means ± S.D. (*N*).

Discussion

The two methods of estimating area-specific O_2 uptake gave essentially identical results when the difference in the size of the fish is taken into account. The raw value (mean ± 95 % CI) obtained using the boundary layer method (3.13±0.18 $\mu g\,O_2\,cm^{-2}\,h^{-1}$) was slightly less than that estimated using the rubber dam method (4.05±0.42 $\mu g\,O_2\,cm^{-2}\,h^{-1}$). The difference, although small, considering the potential sources for error (e.g. different flow regimes), was significant (P<0.05). This appears to be due, at least in part, to the fact that the larvae used in the boundary layer experiments were smaller than those used in the rubber dam tests. Mass-specific O_2 uptake in rainbow trout

Table 3. *Values for the intercept (*a*) and slope (*b*) of simple linear regression equations relating* P_{O_2} *levels in the boundary layer to distance from the skin surface of newly hatched rainbow trout larvae measured at seven different positions on the skin surface*

Flow ($cm\,s^{-1}$)	Position	*a* (kPa)	*b* ($kPa\,mm^{-1}$)	r^2	*N*	*P*
0	1	3.09±0.20	9.83±0.32	0.965	35	<0.0001
	2	2.91±0.31	9.74±0.51	0.904	40	<0.0001
	3	2.42±0.44	11.1±0.72	0.858	40	<0.0001
	4	2.87±0.55	10.5±0.90	0.802	35	<0.0001
	5	3.87±0.62	9.84±1.02	0.703	40	<0.0001
	6	3.37±0.45	10.2±0.73	0.832	40	<0.0001
	7	2.99±0.46	9.80±0.76	0.831	35	<0.0001
	Combined	3.08±0.17	10.1±0.28	0.830	265	<0.0001

The locations of the various positions are shown in Fig. 1.
Values are means ± S.E.M.

(Rombough, 1988*b*) and most other fish (reviewed in Giguere *et al.* 1988; Rombough, 1988*a*; Post and Lee, 1996) is independent of body mass during most of larval development. The relative surface area of the skin, in contrast, declines with increasing body mass (reviewed in Rombough and Moroz, 1997). The net result is that smaller larvae effectively have larger surface areas over which to take up the same amount of O_2. This is reflected in lower area-specific uptake rates in small larvae both within and among species (Rombough and Moroz, 1997). On the basis of geometric similarity (i.e. a scaling exponent for skin surface area of 0.67), one would expect $\dot{M}_{O_2}/A$ for the 32 mg larvae used in the boundary layer experiments to be approximately 17 % less than in the 50 mg larvae used in the rubber dam experiments. If one makes this correction, the mean (±95 % CI) values estimated using the two methods are not significantly different ($3.13\pm0.18\,\mu g\,O_2\,cm^{-2}\,h^{-1}$ and $3.36\pm0.35\,\mu g\,O_2\,cm^{-2}\,h^{-1}$ respectively, for the boundary layer and rubber dam methods at a common tissue mass of 32 mg).

The efficiency of cutaneous uptake and the overall importance of the skin in early larval O_2 exchange appear to be fairly similar in the three species of salmonid fishes examined to date. The efficiency of cutaneous gas exchange expressed in terms of area-specific uptake is approximately the same in rainbow trout ($3.13\,\mu g\,O_2\,cm^{-2}\,h^{-1}$ at 32 mg; this study) as it is in Atlantic salmon *Salmo salar* ($3.2\,\mu g\,O_2\,cm^{-2}\,h^{-1}$ at 33 mg tissue mass; Wells and Pinder, 1996). Area-specific uptake rates appear to be considerably higher in chinook *Oncorhynchus tshawytscha* larvae ($7.5\,\mu g\,O_2\,cm^{-2}\,h^{-1}$; Rombough and Ure, 1991) but the chinook larvae were somewhat larger (≈100 mg tissue mass at hatching) than the larvae of the other two species so some of the difference is probably due to a smaller surface-to-mass ratio in the chinook. The major cause of the difference, however, seems to be a much higher mass-specific rate of O_2 consumption in chinook ($354\,\mu g\,O_2\,cm^{-2}\,h^{-1}$; Rombough and Ure, 1991) than in rainbow trout ($236\,\mu g\,O_2\,cm^{-2}\,h^{-1}$; this study) and Atlantic salmon ($245\,\mu g\,O_2\,cm^{-2}\,h^{-1}$; Wells and Pinder, 1996). $\dot{M}_{O_2}$ was measured at 10 °C using similar methods in all three species, so why chinook should have such a high metabolic rate is not clear. During the first few days posthatch, approximately the same fraction of total O_2 uptake takes place across the skin in all three species. In rainbow trout, approximately 73 % of total O_2 uptake occurs across the skin (this study). The corresponding mean values for chinook and Atlantic salmon are 74 % (Rombough and Ure, 1991) and 72 % (Wells and Pinder, 1996), respectively.

There do not appear to be any significant regional differences in the efficiency of cutaneous O_2 uptake in still water. The mean slopes of the lines relating P_{O_2} and distance from the skin surface (Table 3) used to calculate area-specific uptake rates at the various positions on the skin surface were not significantly different (Table 4). If there were regional differences in efficiency, the slopes should have been significantly different. The extent to which different regions of the skin are vascularized varies considerably (Rombough, 1988*a*). This has led to speculation that the better-vascularized regions such as the yolk sac may play a particularly important role in larval gas exchange (e.g. Balon, 1975; Lanzing, 1976;

Table 4. *Results of ANCOVA tests for equality of slopes and elevations of regression equations relating* P_{O_2} *levels in the boundary layer to distance from the skin surface at seven different positions on the skin of rainbow trout larvae*

Homogeneity of slopes		Equality of elevations	
F	*P*	*F*	*P*
0.459 (6, 251)	0.838	1.95 (6, 257)	0.073

Tests were conducted in still water.
The dependent variable was P_{O_2}. Position was treated as an independent variable and distance from the skin surface as a covariate. Measurements involving different individuals were considered to be replicates.
Degrees of freedom are given in parentheses.
Slopes and elevations were considered significantly different if $P<0.05$.

Liem, 1981). This does not appear to be the case in rainbow trout. The rate of area-specific O_2 uptake across the yolk sac (positions 2 and 3, Fig. 1) of the trout was not significantly greater than elsewhere on the body surface (Table 3). The situation is similar in Atlantic salmon. Wells and Pinder (1996) were able to modify the rubber dam method so that they could measure O_2 uptake across just the yolk sac epithelium of newly hatched Atlantic salmon. The area-specific uptake rate for the yolk sac ($2.7\,\mu g\,O_2\,cm^{-2}\,h^{-1}$) turned out to be approximately the same as that for the rest of the body ($3.2\,\mu g\,O_2\,cm^{-2}\,h^{-1}$). The reason there is so little regional variation in uptake efficiency appears to lie with the diffusive boundary layer. In still water, the boundary layer is several millimetres thick and accounts for approximately 95 % of the diffusive resistance to O_2 flux (Rombough, 1992). According to the 'pipes in a wall' model of Malvin (1988), differences in capillary density should have little impact on uptake efficiency if, as is the case for trout larvae in still water, the total diffusion distance is long compared with the distance between capillaries.

The boundary layer method is potentially a very powerful technique for studying exchange processes during development. One of the major problems for those interested in developmental physiology has been the difficulty in measuring flux rates on a microscale. The boundary layer method is a flexible and relatively simple way to do this. Previous studies have shown that the technique works well with cells (e.g. Smith *et al.* 1994) and isolated tissues (e.g. Collier and O'Donnell, 1997). The present study shows that it also can be used with whole organisms. It is important to recognize that the boundary layer method need not be restricted to the study of O_2 uptake or trout larvae. With appropriate modifications, it can be used with many other organisms (not only fish) to estimate flux rates for virtually any diffusible substance consumed (e.g. Na^+, Ca^{2+}) or excreted (e.g. CO_2, NH_3) during development that can be measured with microelectrodes.

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