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THE RESPIRATORY DEVELOPMENT OF ATLANTIC SALMON

I. MORPHOMETRY OF GILLS, YOLK SAC AND BODY SURFACE

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Summary

During development from larva to juvenile in Atlantic salmon, *Salmo salar*, there is a change in the anatomical potential for gas exchange among gills, body skin and yolk sac as the larvae resorb yolk, grow and develop gills. Newly hatched Atlantic salmon have poorly developed gills but do have a high skin area to mass ratio and a large well-vascularized yolk sac. Cutaneous surfaces accounted for over 95 % of the total area available for respiration in newly hatched Atlantic salmon (body mass 0.032–0.060 g). The branchial contribution to total area increased rapidly, however, so that by the end of yolk absorption (body mass 0.19–0.23 g) it constituted 22 % of the total area and overtook cutaneous surface area between 5 and 6 g wet body mass. Harmonic mean diffusion distance across the skin increased through development from 20 μm at hatch (14 μm across the yolk sac) to 70 μm in an 11 g fish. Diffusion distances across both the filaments and lamellae of the gills decreased through development, from 3.7 to 2.4 μm for lamellae and from 14.5 to 10.8 μm for filaments. The total anatomical diffusion factor (ADF, mass-specific surface area per unit diffusion distance) remained constant over early development and appeared to be higher than in adult fish. The distribution of ADF changed over early development from 50 % yolk sac, 42 % body surface and 8 % branchial in newly hatched fish to 68 % branchial and 32 % cutaneous at the end of yolk resorption. Generally, early post-hatch development of gills, ADF and some cutaneous surfaces showed high mass exponents. After yolk resorption (body mass 0.2 g), however, these coefficients were lower and closer to unity. The change in scaling at the end of yolk resorption in this study may reflect the completion of larva to juvenile metamorphosis in Atlantic salmon. Comparison between our data and values in the literature suggests that the timing of gill development is related more to developmental stage than to body size.

Key words: morphometry, fish, development, respiration, Atlantic salmon, *Salmo salar*, gills, yolk sac.

Introduction

In fish, the development from larva to juvenile includes many morphological and physiological transformations (Blaxter, 1988; Rombough, 1988*a*; Burggren and Pinder, 1991). One of the most important transformations is the transfer of the major site of O_2 uptake from the body surface to the gills (Rombough, 1988*a*; Burggren and Pinder, 1991). At hatch, larvae of most species of fish have poorly developed or non-functional gills (Rombough, 1988*a*); thus, it is generally accepted that O_2 uptake in larval fishes initially takes place across cutaneous surfaces (Blaxter, 1988; Rombough, 1988*a*; Rombough and Ure, 1991; Burggren and Pinder, 1991). The only direct measurements of O_2 uptake partitioning are those of Rombough and Ure (1991), who showed that, shortly after hatch, approximately 80 % of the total oxygen consumed by larvae was taken up through cutaneous surfaces. Several authors have suggested that there is a critical size at which the gills must become functional as a result of the decrease in body surface area to mass ratio during growth (DeSilva, 1974; Blaxter, 1986; Osse, 1989); to date, there are few physiological data available to test this hypothesis. Very large fish larvae, such as the salmonids, have circulating red cells at hatch, while smaller larvae often do not (Blaxter, 1988), also suggesting a size-dependence of oxygen uptake and transport. The present study of developing Atlantic salmon correlates changes in surface area and diffusion distances to physiological measurements of oxygen uptake partitioning across the yolk sac, body skin and gills.

According to Fick's law of diffusion, surface area and blood–water barrier thickness determine the morphological potential for gas exchange across skin and gills:

$$O_2 \text{ uptake} = (A/D) \times K \times \Delta P_{O_2}, \quad (1)$$

where A is surface area, D is the thickness of the water-to-blood barrier, K is Krogh's diffusion coefficient and ΔP_{O_2} is the difference in O_2 partial pressures (P_{O_2}) between blood and respired water. Diffusion capacity (D_{O_2}) is the conductance of

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the gas exchange organ for gases (Hughes, 1984*a*) and can be calculated morphometrically or physiologically:

$$D_{O_2} = O_2 \text{ uptake}/\Delta P_{O_2} = (A/D) \times K. \quad (2)$$

Physiological and morphological estimates of D_{O_2} often differ by a factor of three or four, however (Piiper *et al.* 1986); thus, we have chosen to use 'anatomical diffusion factor' (ADF), defined as mass-specific surface area/harmonic mean diffusion distance (Perry, 1990), to compare the morphological potential for gas exchange across various surfaces. This more clearly isolates the morphological determinants of diffusion capacity and avoids the confusion between morphological and physiological diffusion capacity. Using ADF also emphasizes that diffusion distance is as important as surface area in determining gas exchange capacity.

Cutaneous diffusion distances, surface areas and physiological diffusion capacities have been measured in analyses of cutaneous exchange in amphibians (Feder and Burggren, 1985), although seldom in larval fish. Cutaneous epithelia are only one or two cells thick in larval fish, and diffusion distances to underlying tissues are generally short (less than 10 μm) (Lasker, 1962; Jones *et al.* 1966; Roberts *et al.* 1973; Liem, 1981; O'Connell, 1984; Morrison, 1993). No previous study has measured both surface areas and diffusion distances in larval fish.

An increase in gill surface area has been suggested to be a good indicator of transformation from cutaneous respiration to branchial respiration (Blaxter, 1988; Rombough, 1988*a*; Rombough and Moroz, 1990). However, studies that correlate gill development and physiological function are lacking. There is a correlation between the increase in surface area of gills of developing chinook salmon (*Oncorhynchus tshawytscha*) and the onset of branchial respiration (Rombough and Moroz, 1990; Rombough and Ure, 1991), although Rombough and Ure (1991) found that only approximately half of the increase in branchial oxygen uptake could be explained by the increase in surface area; the rest was attributed to an increase in 'efficiency', i.e. the oxygen uptake per unit area. Much of this increased efficiency might be explained by changes in diffusion distance. Morgan (1974) demonstrated that the blood–water distance of rainbow trout gill lamellae (*O. mykiss*) decreased through early development.

The major objective of this study and the companion paper (Wells and Pinder, 1996) is to correlate the changes in the ADF of the body surface skin, yolk sac and gills with the transformation from cutaneous to branchial O_2 uptake to determine whether morphological changes are adequate to explain changes in oxygen uptake partitioning. We also compare our data with values in the literature to evaluate the hypothesis that the shift to branchial respiration is necessitated by increasing body size.

Materials and methods

Spawning and fish rearing

Fertilized ova were obtained from Atlantic salmon (*Salmo salar* L.) spawned artificially at the Cold Brook Fish Station (Cold Brook, Nova Scotia, Canada). In November 1990 and 1991, 5000 eggs were manually stripped from two or more female Atlantic salmon and fertilized with the milt of two or more males. Fertilized eggs were allowed to absorb water for 1 h and then approximately 2500 eggs were transported to Dalhousie on ice. The remaining eggs were laid down on trays for incubation to the 'eyed' stage at the hatchery and were then transported to the laboratory several months after spawning.

Fish were reared at ambient temperatures (4–22 °C). When fish had absorbed approximately 80 % of their yolk sac, they were fed a commercial salmon feed (Martin's Mills starter) and brine shrimp larvae *ad libitum*. Feeding fish were maintained at 10–22 °C on a 12 h:12 h light:dark cycle.

Morphometry

Animals and tissues were preserved in phosphate-buffered 10 % formalin, and tissues were embedded for histology in Paraplast and paraffin. These treatments cause significant tissue shrinkage, treated in detail in Hughes *et al.* (1986). Dimensions were not corrected for shrinkage. Because all samples were treated similarly, shrinkage should be uniform and thus should not affect the slopes of the allometric relationships or the relative importances of the respiratory surfaces. We did not use methacrylate embedding medium because the time required to process the large numbers of samples would be prohibitive.

Cutaneous surface areas

Cutaneous surface areas were estimated from 64 fish, sampled at evenly spaced intervals through development

Table 1. *Numbers of fish sampled for estimation of respiratory surface area (RSA), diffusion distance (*D*) and scanning electron microscopy (SEM)*

ATU*	Fish wet mass (g)	Skin RSA	Gill RSA	*D*	SEM
≈5	0.032–0.060	6	6	6	4
≈83	0.060–0.087	6	4	–	4
≈145	0.100–0.130	6	4	6	2
≈217	0.135–0.174	6	4	–	4
≈315	0.164–0.212	6	4	6	2
≈439	0.190–0.230	6	4	–	2
≈578	0.230–0.380	6	4	6	2
≈723	0.280–0.490	6	4	–	2
≈882	0.350–0.510	6	–	–	2
≈1472	0.860–1.520	6	3	6	2
≈2370	6.430–11.20	4	1	6	2
Total		64	38	34	28

Fish used for estimating gill RSA and *D* were all from within the RSA (skin) sample group.

*ATU, accumulated thermal units (Rombough, 1988*b*). One thermal unit is accumulated by a fish while in water held at 1 °C for 24 h. Ten thermal units (10 ATUs) is the equivalent of one 24 h period in water held at 10 °C.

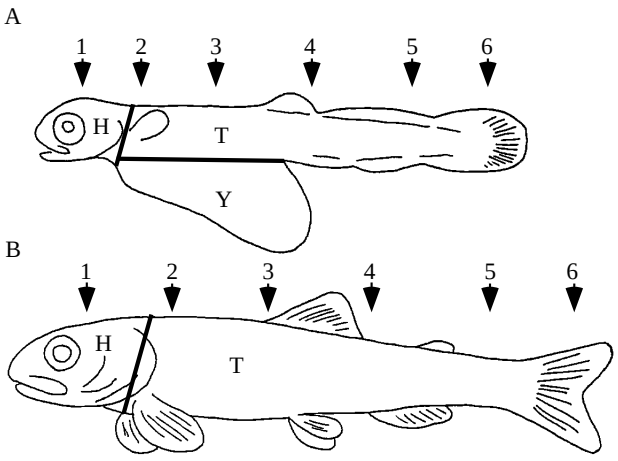

Fig. 1. Divisions of the body used to estimate cutaneous surface areas. All fins and finfolds were included in the measurement of fin area. Four regions were measured in alevins (A): head (H), trunk (T), yolk sac (Y) and fins. Older fish (B), which had absorbed their yolk, had three regions: head (H), trunk (T) and fins. The regions of the fish cross-sectioned for histological study are also shown (numbered) in this figure. Six regions in total, including sections though the the yolk sac (2 and 3), were used to study skin thickness. Red muscle was examined using sections from regions 2–5 (inclusive) and cutaneous capillaries were examined in regions 1–6 (inclusive).

(Table 1). Cutaneous area was partitioned into head, trunk, yolk sac and fins (Fig. 1). The skin from half the body was removed for measurement of area; the other half of the body, with skin intact, was returned to buffered 10 % formalin for histology. The fins and skin of the body, head and yolk sac were each cut into small pieces and their projected images were magnified (10×) onto a digitizing tablet. Areas for each region were calculated using the Videoplan image processing system (Kontron).

Branchial surface areas

Surface areas of gills were estimated from a subset of 38 fish between hatch and juvenile (0.032 g to 11.2 g wet mass) from the group in which cutaneous areas were measured (Table 1). Areas of gill filaments and lamellae at regular intervals along the gill arches and total numbers of filaments and lamellae were measured to calculate total gill area (technique modified from Hughes, 1984*c*).

The gill arches from the right side were used to estimate surface area, while the arches from the left side were saved in buffered 10 % formalin for histology. Individual arches were placed in a Petri dish with phosphate buffer and flattened with a coverslip. The number of filaments was counted on each arch. The length of approximately every fifth filament and the length of filament supporting 10 lamellae were measured on both hemibranchs.

Gill area was calculated for each arch as in Hughes (1984*c*). Areas of filaments for each hemibranch were estimated from approximately every fifth filament (minimum of seven filaments) along the gill arch. Individual filaments were placed on a microscope slide in buffer and gently squashed with a coverslip to flatten both the filament and the lamellae. Filament images were drawn at 100× under a *camera lucida* and digitized. Thirty-six lamellae were sampled from the proximal, medial and distal sections of filaments along the second gill arch and drawn at 400×. Filament lengths for each arch were averaged.

Histology

Sampling and sample preparation

Fish used for estimating cutaneous surface areas (Table 1) were examined histologically to measure the skin thickness of various body regions, the development of the red muscle layer, the red muscle capillary density and the gill filament and lamellar water–blood distances. All fish were infiltrated with Paraplast plus wax (Fisher Scientific) using a Fisher histomatic tissue processor with procedures from Humason (1979).

The body of each fish was dissected before final embedding to make it possible to section six different body regions at once (Fig. 1), oriented so that the skin surface was perpendicular to the plane of sectioning. Sections were cut 7 μm thick and were stained with Haematoxylin and counter-stained with Pollak Trichrome (Humason, 1979). Measurements were made on digitized images.

Water–blood distances in gill filaments and lamellae

Blood–water distances for gill filaments and lamellae were quantified with techniques modified from Hughes *et al.* (1986). The first and second gill arches of each fish were dissected into three evenly spaced regions and embedded in Paraplast and wax. Tissues were oriented so that sections were cut perpendicular to the surfaces to be measured. Sections were cut 5 μm thick and stained with Haematoxylin and Eosin.

Lamellar blood–water distances were measured from the blood channel to the lamellar surface (Hughes *et al.* 1986) in lamellae from the base, middle and tip of the filaments. Five evenly spaced measurements were made on each lamellar blood channel; a total of 12 lamellae per gill region were measured. Similarly, filament blood–water distances were measured at the base, middle and tip of the filament. Again, five measurements were made on each filament and a total of 12 filaments per gill region were measured. Measurements were made on digitized images and harmonic mean diffusion distance was calculated. The harmonic mean is a better measure of diffusion barrier thickness than the arithmetic mean (Hughes *et al.* 1986; Perry, 1990).

Skin thickness

Ten measurements of the shortest distance from the stratum compactum to the epidermal surface were made from each of six sections of the head, body, yolk sac and caudal fin (Fig. 1). Both the arithmetic and harmonic means for each region and for the total body were calculated. Sections were examined for cutaneous blood vessels.

Red muscle

A distinct unicellular layer of red muscle was visible on the

outside of the bulk of the body musculature, as has been reported in other larval teleosts and salmonids (El -Fiky and Wieser, 1988; Proctor *et al.* 1980). The contribution of this red muscle layer to the total body musculature (red and white combined) was estimated from digitized images from four regions of the body (regions 2–5 in Fig. 1). The red muscle layer was also examined for capillaries.

Scanning electron microscopy

The development of gill arches, filaments and lamellae was examined qualitatively using scanning electron microscopy (SEM); see Table 1 for details of sampling times. Gills were prepared for critical-point drying using standard preparation techniques (Humason, 1979) and were dried using Peldri (Ted Pella Inc.). Care was taken to avoid removing the gill arches from the solution to prevent damage resulting from surface tension. Individual gill arches were post-fixed overnight with 0.5 % osmium tetroxide in cacodylate buffer (pH 7.2). Dried specimens were mounted on nickel stubs, with the anterior hemibranch facing up, and sputter-coated with gold (Samsputter 2a Sputter Coater). Samples (N=28 fish, 112 arches) were observed on a Bausch and Lomb ARL Nanolab 2000 scanning electron microscope at 15 kV.

Statistical analyses

Initial data inspection revealed that many relationships appeared to be better fitted by two linear regression lines than by one. The BASIC program of Yaeger and Ultsch (1989) was therefore used to determine whether data were better fitted by one- or two-phase regressions. This program provided line intersection points, midpoint and transition intervals for two-phase regressions. Log-transformed data for each phase were also tested using Pearson and Spearman correlations (FASTAT statistical package) to identify data points with high leverage and to test for significance of relationships. Data are presented as mean ± standard error (S.E.M.) except where otherwise noted.

Results

Morphometry of respiratory surfaces

Distribution between skin and gills

Cutaneous surfaces made up over 95 % of the total surface area available for respiration in recently hatched fish (5 accumulated thermal units, ATUs), weighing 0.032–0.060 g (Fig. 2; regression equations given in Table 2). The gills were poorly developed at hatch, with 200–350 filaments per fish and few or no lamellae. Gill area increased much more rapidly than body mass (proportional to $m^{1.97}$) until the end of yolk absorption and completion of metamorphosis to the juvenile body shape (440 ATUs, 0.19–0.23 g). After yolk absorption, gill surface area increased almost in direct proportion to body mass (proportional to $m^{0.97}$), while cutaneous area increased in proportion to $m^{0.54}$, so that gill surface area made up an increasing proportion of total area as the fish grew. The regression line for gill surface area intersects the line for cutaneous surface area between 5 and 6 g wet body mass.

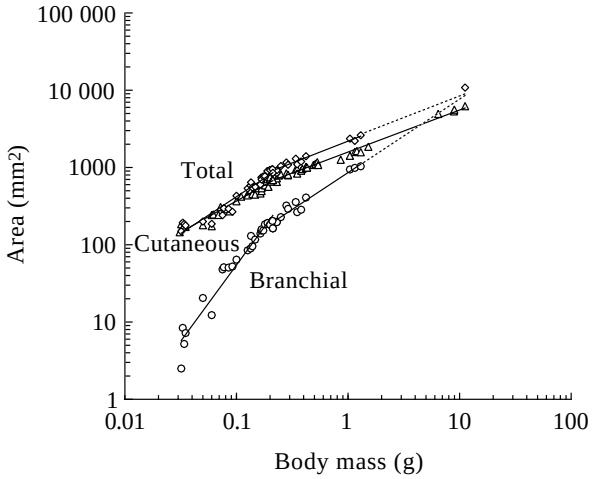

Fig. 2. Allometry of respiratory surface areas: total (◇), cutaneous (△) and branchial surface (○). Data from the largest fish (11 g) for total and branchial surface areas had very high leverage and thus was not included in the calculated regressions. Regression equations are presented in Table 2.

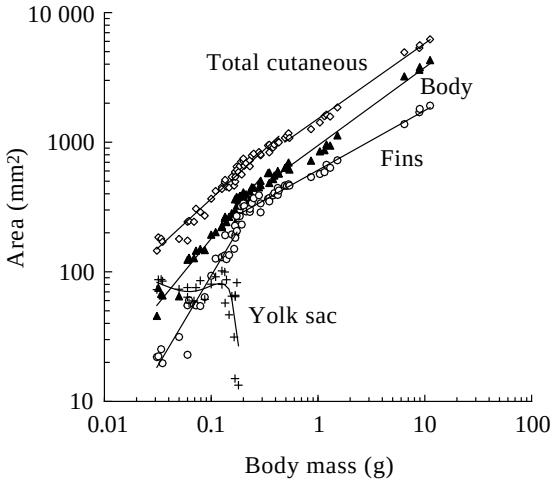

Fig. 3. Allometry of cutaneous surface areas: total (◇), body (i.e. trunk + head; △), fins (○) and yolk sac (+). Regression equations are presented in Table 2.

Cutaneous surfaces

The proportions of cutaneous surface area contributed by yolk sac, trunk, head and fin surface areas changed during early development, reflecting changes in body shape. The proportion of total surface area contributed by the yolk sac decreased from 45±2 % (N=6) in just-hatched fish to 20±1 % (N=6) in the next older sample (83 ATUs, 0.060–0.087 g) and had disappeared completely by 440 ATUs (0.19–0.23 g) (Fig. 3). Just post-hatch, the contributions of trunk and head skin to total cutaneous area were 30±1 % and 10±0.4 % respectively (head and trunk areas have been summed as 'body' in Fig. 3). By the end of yolk absorption, the trunk contributed 38±0.6 % and the head 18±0.6 % to body surface area (N=4). For the remainder of development, the relative contribution of the head to total

Table 2. *Allometric regression equations of body and gill surface areas, diffusion distances and anatomical diffusion factor (ADF)*

Body surface area	Body mass, *m* (g)	*a*	*b*	r^2	*N*	S.E.M. of *b*	*P*
Total area (branchial and cutaneous surfaces)	0.032–0.212	3758	0.95	0.93	25	0.054	***
	0.190–1.15	2167	0.56	0.98	15	0.034	***
Cutaneous surfaces							
Total area	0.032–0.212	2371	0.81	0.95	35	0.033	***
	0.190–11.2	1584	0.54	0.99	35	0.011	***
Head + trunk area	0.032–0.212	2023	1.05	0.94	35	0.046	***
	0.190–11.2	954	0.59	0.98	35	0.014	***
Trunk area	0.032–0.212	1009	0.91	0.94	35	0.040	***
	0.190–11.2	679	0.62	0.99	35	0.013	***
Head area	0.032–0.212	946	1.20	0.94	35	0.052	***
	0.190–11.2	272	0.50	0.93	35	0.024	***
Fin area	0.032–0.212	2831	1.47	0.95	35	0.059	***
	0.190–11.2	610	0.46	0.98	35	0.013	***
Branchial surfaces							
Total area	0.032–0.212	5116	1.97	0.95	25	0.096	***
	0.190–1.15	881	0.97	0.95	15	0.062	***
Lamellar area	0.060–0.212	3265	2.08	0.92	21	0.138	***
	0.190–1.15	612	1.09	0.97	15	0.081	***
Filament area	0.032–0.212	990	1.52	0.93	25	0.088	***
	0.190–1.15	273	0.78	0.94	15	0.054	***
Number of filaments per fish	0.032–0.212	1734	0.57	0.94	22	0.029	***
	0.190–11.2	879	0.15	0.92	16	0.011	***
Number of lamellae per fish	0.060–0.200	11859	1.28	0.95	16	0.077	***
	0.200–11.2	37322	0.51	0.99	16	0.016	***
Diffusion distances (body skin and gills) (μm)							
Skin	0.032–11.2	44	0.25	0.93	29	0.013	***
Lamellae	0.060–11.2	2.79	–0.097	0.60	31	0.015	***
Filaments	0.032–11.2	11.85	–0.06	0.48	31	0.011	***
Anatomical diffusion factor, ADF (skin and gills) ($cm^2\,\mu m^{-1}\,g^{-1}$)							
Total	0.032–1.15	2.55	–0.04	0.14	19	0.024	
Skin	0.032–1.15	0.364	–0.61	0.96	21	0.033	***
Gills	0.032–0.164	51.7	1.82	0.94	10	0.168	***
	0.190–11.2	2.11	–0.02	0.02	11	0.049	
Lamellae	0.060–11.2	1.81	0.30	0.39	17	0.076	***
Filaments	0.032–11.2	0.27	0.161	0.30	21	0.057	*

Asterisks denote probability level of regression: *P<0.05; **P<0.01; ***P<0.001. Regression formula: $y=am^b$, where y is area (mm^2), distance (μm) or ADF ($cm^2\,g^{-1}\,\mu m^{-1}$), a is a constant, m is body mass (g) and b is the mass exponent.

skin area remained constant at 15–18 %. The relative contribution of the trunk, however, increased to 50±1 % of the total skin area in 6.43–11.2 g fish (N=4). The contribution of the fins increased from 14±1 % of total cutaneous area just post-hatch to 45±1 % at the end of yolk absorption, then decreased to 31±1 % in 6.43–11.2 g fish. The break point for the best-fitting lines for both fin and trunk surface area are at the end of yolk absorption (roughly 0.19 g body mass), when both the fins and the body have assumed juvenile form.

Branchial surfaces

The total surface area of the gills increased dramatically during development (Fig. 4). Gill area in newly hatched fish was 9.4±2.6 mm^2 (5 % of total area), with only the largest two of the six fish sampled (weighing 0.050 and 0.060 g) having lamellae that could be measured. Gill area increased quickly, however, so that by the time the fish had resorbed their yolks the area had increased over 20-fold to 189±6 mm^2 concomitant with a fourfold increase increase in body mass. After the end

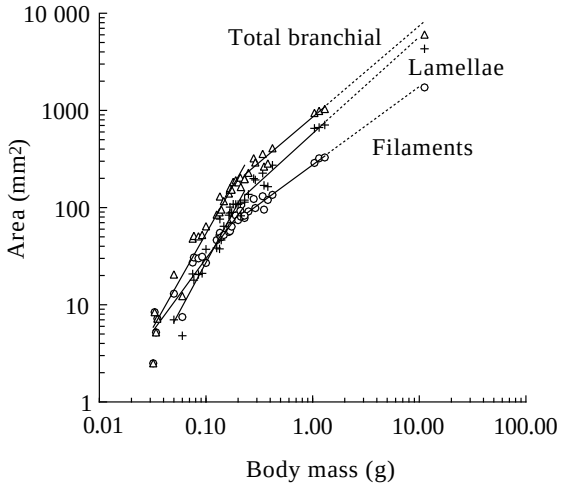

Fig. 4. Allometry of branchial surface areas: total (△), filaments (○) and lamellae (+). Data from the largest fish (11 g) had very high leverage and thus were not included in the calculated regressions. Regression equations are presented in Table 2.

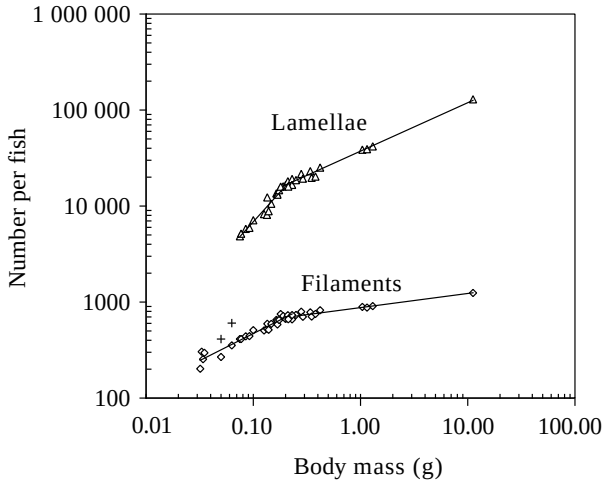

Fig. 5. Allometry of numbers of filaments and lamellae. Regression equations are presented in Table 2.

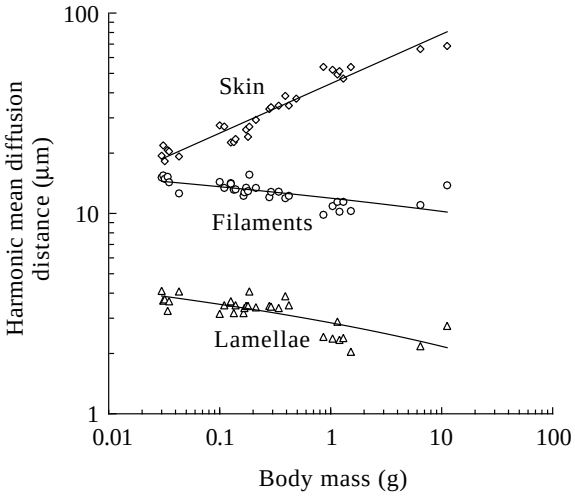

Fig. 6. Allometry of harmonic mean diffusion distances: skin (◇), filaments (○) and lamellae (△). Regression equations are presented in Table 2.

of yolk absorption, gill area increased in proportion to $m^{0.97}$ (Table 2).

Gill area is the sum of the area of the lamellae and filaments. Initially, there were either no lamellae or lamellar area was small (34 and 39 % of the total gill area in fish weighing 0.05 and 0.06 g, respectively; the other four fish had no lamellae). By the next sample (83 ATUs), the lamellae contributed 40±2 % (N=4) and by the end of yolk resorption they contributed 56±1 % of the total gill area. In the largest fish (11.2 g), 71 % of gill area was on lamellae.

The increase in the total surface area of the gills during development was due to increases in numbers of both lamellae and filaments (Fig. 5) and to increases in the mean bilateral surface area of individual lamellae and filaments (mass exponents in Table 2). In fish that had recently hatched, lamellae had not yet differentiated or were small (0.0017 mm^2 bilateral area). At the end of yolk absorption, all gills had lamellae and individual lamellar area had increased by fourfold (from 0.007 to 0.008 mm^2). Areas of individual lamellae (results not shown) increased with a somewhat lower mass exponent (0.57) than would be expected if the fish had remained geometrically similar as they grew (expected mass exponent 0.67). The areas of individual filaments also increased steadily through development, with a mass exponent (0.703) that was not significantly different from 0.67.

The increase in the number of lamellae through development depended on the increase in the length and number of filaments, while the spacing of lamellae on the filament remained constant through development at 20.8±1.8 lamellae mm^{-1} (S.D.). The average length of filaments increased in proportion to $m^{0.63}$ in yolk sac larvae, from 0.16 mm in 0.032 g fish just post-hatch to over 0.7 mm in fish weighing 0.35 g. The rate of increase of filament length decreased in larger fish to be proportional to $m^{0.33}$ (consistent with geometric similarity) in 1–11 g fish. The number of filaments increased biphasically (Fig. 5). From the time of hatch to the end of yolk absorption, the number of filaments nearly tripled from 250 to 650 in 0.032–0.212 g fish; the number of filaments increased in proportion to $m^{0.57}$. After the end of yolk absorption, the rate of appearance of new filaments decreased to be proportional to $m^{0.15}$. The number of lamellae thus also increased biphasically, in proportion to $m^{1.28}$ before the end of yolk absorption (excluding fish just post-hatch, which generally did not have any lamellae) and in proportion to $m^{0.51}$ after the end of yolk absorption.

Diffusion distances

The harmonic mean diffusion distance across the skin increased from 20 μm in newly hatched fish to 70 μm in 11.2 g fish (Fig. 6). Skin thickness varied over the body, with the fins

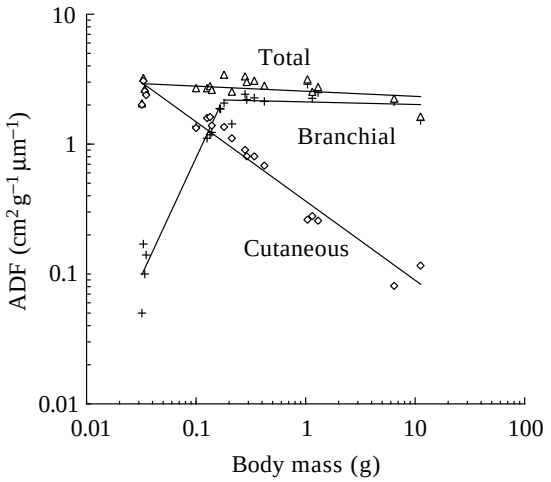

Fig. 7. Allometry of anatomical diffusion factor (ADF): total (△), cutaneous (◇) and branchial (+). Regression equations are presented in Table 2.

and the yolk sac having the shortest diffusion distances. In 10 fish sampled just post-hatch, the diffusion distance across the yolk sac was 13.9±0.8 µm.

In contrast to cutaneous diffusion distance, the harmonic mean diffusion distances across both the filaments and lamellae decreased from 14 µm and 3.7 µm, respectively, in newly hatched fish to 11 µm and 2.4 µm, respectively, in 11.2 g fish (Fig. 6).

Anatomical diffusion factor

Total ADF was 3.0±0.2 $cm^2\,g^{-1}\,\mu m^{-1}$ just after hatching and remained constant through early development (Fig. 7), although the two largest fish sampled (6 and 11 g) had somewhat lower ADF values (2.24 and 1.63 $cm^2\,g^{-1}\,\mu m^{-1}$, respectively).

Cutaneous ADF decreased during development (Fig. 7). In recently hatched fish, cutaneous ADF was 2.8±0.2 $cm^2\,g^{-1}\,\mu m^{-1}$, 92 % of total ADF. Because skin area increased less than body mass while skin thickness increased, cutaneous ADF decreased rapidly with increasing body mass (proportional to $m^{-0.61}$).

Branchial ADF in recently hatched fish was 0.22±0.08 $cm^2\,g^{-1}\,\mu m^{-1}$, only 8 % of total ADF. Branchial ADF increased greatly as lamellae developed up to the end of yolk absorption, scaling as $m^{1.82}$, however, so that by the next sample (217 ATUs) the ADF of the gills was roughly equal to that of the skin (1.3 $cm^2\,g^{-1}\,\mu m^{-1}$). After the end of yolk absorption, branchial ADF remained constant.

Red muscle and cutaneous capillaries

In histological sections, the contribution that the red muscle layer made to the overall muscle area did not change between 83 ATUs (0.082 g fish) and 720 ATUs (0.33 g fish), representing 1.8±0.35 % of total muscle mass in 0.082 g fish and 1.5±0.36 % of total muscle mass in 0.33 g fish. The shape and distribution of the muscle layer did, however, change through development. At 83 ATUs, the red muscle layer extended away from the lateral line area as a unicellular layer surrounding the inner white muscle. Later samples (440 ATUs) showed several cell layers that appeared to be positioned more towards the lateral line. In the older fish sampled (720 ATUs), the band of muscle was 8–10 layers thick and remained constricted towards the lateral line area.

There were almost no superficial capillaries during early development. In fish at 83 ATUs, only four blood vessels could be found under the red muscle layer, and none was found in the dermis. With the exception of the yolk sac, external *in vivo* microscopic examination did not reveal any capillary networks in the skin. Vessels from the yolk sac were not quantified.

Scanning electron microscopy

All four gill arches were present just post-hatch. Only the lower limb of each gill arch had developed, with the filaments closely packed together on the arch (Fig. 8A). The arches had 30–80 filaments on both the anterior and posterior hemibranchs, but no lamellae. New filaments appeared to be developing at the ends of the arches where the filaments were progressively shorter (Fig. 8A–C). On some specimens, bumps were observed on the filaments; these were probably developing lamellae (Fig. 8A). By the end of yolk resorption (Fig. 8B), almost all the filaments on both hemibranchs possessed lamellae, and gill rakers could be seen on all gill arches. The upper limb had developed on all arches, and the filaments were more widely spaced. Lamellae at the proximal end (base) of the filaments were larger than those at the distal ends. By the time fish weighed 1 g (1500 ATUs), the gills had the typical arched limbs found in adult salmonids, with the lower limb being slightly longer than the upper limb (Fig. 8C). The filaments on the upper limb were longer and more numerous, and they had more lamellae than in previous stages. The gill rakers continued to increase in size and number. The lamellae of each filament became larger and appeared to be capable of interlocking with the lamellae of the adjacent filament on the hemibranch. Some lamellae at the distal ends of the filaments appeared to be in the process of differentiating (Fig. 8D). Openings of mucous cells were observed between the epithelial cells along with what appeared to be the apical ends of chloride cells, both on the branchial epithelium and on the general body surface epithelium (Fig. 8E), especially just post-hatch.

Discussion

Anatomical diffusion factor

Total anatomical diffusion factor (ADF), a measure of the morphological potential for gas exchange normalized to body mass, remained constant during early development at approximately 3 $cm^2\,g^{-1}\,\mu m^{-1}$. Branchial ADF in adult fish is lower, approximately 1 $cm^2\,g^{-1}\,\mu m^{-1}$ (Perry, 1990). Thus, newly hatched salmon have a more than adequate morphological gas exchange capacity even before the gills develop. The distribution of ADF between skin and gills

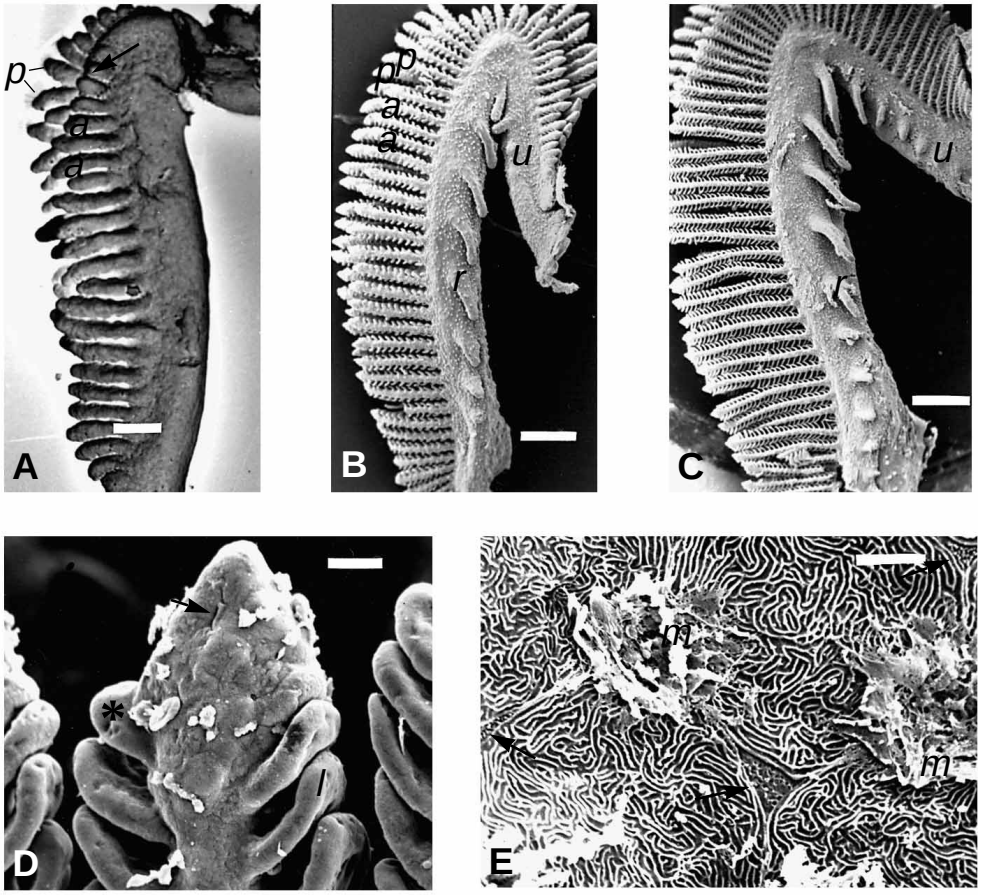

Fig. 8. (A,B,C) The second gill arches of Atlantic salmon (*Salmo salar*) as seen with the scanning electron microscope. (A) Recently hatched fish, 5 ATUs, body mass 0.032 g. No lamellae were visible and only the lower limb of the arch was present. The filaments were closely packed together and new filaments appeared to be differentiating on the upper portion of the anterior hemibranch (arrow). *a*, filaments of the anterior hemibranch; *p*, filaments of the posterior hemibranch. Scale bar, 100 μm. (B) Fish at the end of yolk resorption (439 ATUs, body mass 0.212 g). Lamellae were present, the hemibranchs had more filaments and the upper limb of the arch (*u*) was present. Filaments appeared to differentiate at the ends of the arches. Rakers (*r*) had developed on the anterior and posterior sides of the arch. Scale bar, 200 μm. (C) Juvenile fish (1472 ATUs, body mass 1.15 g). The arch upper limb (*u*) was larger and had more filaments than in B. Scale bar, 400 μm. (D) The distal end of a filament from the second gill arch of the same juvenile. Lamellae (*l*) are typical flattened plate-like structures. The lamella towards the tip of the filament may be in the process of differentiating (*). The arrow indicates the pit of a possible chloride cell. Scale bar, 15 μm. (E) The exterior epidermal surface of a recently hatched fish. There appear to be apical ends of chloride cells (arrows) visible between the epithelial cell borders. Mucous cell secretions (*m*) can be seen at the opening of the mucous cells. Scale bar, 5 μm.

changed dramatically during early development, however, from 92 % cutaneous at hatching to 32 % cutaneous at the end of yolk resorption and less than 5 % in 6–11 g fish, while the branchial contribution concomitantly increased. There are no other comparable studies of ADF or diffusion capacity in the early developmental stages of fish, although it seems likely that similar patterns would appear, at least in salmonids. Branchial diffusion distances decrease through post-hatch development in rainbow trout (*Oncorhynchus mykiss*) (Morgan, 1974), and the cutaneous and branchial surface areas of chinook salmon (*O. tshawytscha*) increase similarly to those of Atlantic salmon (Rombough and Moroz, 1990).

Changes in the distribution of ADF among body surfaces are qualitatively similar to the patterns of change of surface areas during development (Rombough and Moroz, 1990), but are quantitatively different because of the very different thicknesses of the skin, filaments and lamellae. For example, although filaments represent almost 50 % of the total branchial surface area at the end of yolk resorption (0.2 g), they account for less than 10 % of branchial ADF. As soon as lamellae appear, they constitute the major gas exchange surface of the gills, representing more than 80 % of branchial ADF. Similarly, although branchial surface area does not equal cutaneous surface area until the fish are larger than approximately 5 g, branchial ADF accounts for approximately 95 % of the total ADF in 5 g fish (Fig. 7); branchial ADF surpasses cutaneous ADF before the end of yolk resorption (0.2 g). Thus, the changes in distribution of ADF suggest an earlier shift of the relative importances of branchial and cutaneous gas exchange than do surface area changes alone, in better agreement with oxygen uptake partitioning measurements than using surface areas alone (Rombough and Ure, 1991; Wells and Pinder, 1996).

Cutaneous ADF

At hatch, larval salmon epidermis is only 2–3 cells thick and has almost no dermis; harmonic mean diffusion distance across the skin is approximately 20 μm. The skin of larval Atlantic salmon is thinner than that of adult salmonids (Wilkins and Janscar, 1979); however, in comparison with other larval fish, it is quite thick (Rombough, 1988*a*). Epidermal thickness of pelagic larvae such as plaice (*Pleuronectes platessa*, Roberts *et al.* 1973) and herring (*Clupea harengus*, Jones *et al.* 1966) is 5.0 μm and 2.3 μm respectively. Because these larvae are

also smaller than salmon larvae, their cutaneous ADF must be considerably higher than that of salmon larvae.

Although ADF is a better indicator of gas exchange potential than surface area, cutaneous ADF in larval fish is not entirely comparable with the ADF for gills or with the ADF of other larger skin-breathing animals because gas exchange is not between the exterior and an internal vascular bed. Cutaneous morphometric diffusion capacity, or ADF, is usually calculated from capillary density and blood–water diffusion distances (Burggren and Pinder, 1991; Perry, 1990), with the subcutaneous capillary bed acting as the sink for oxygen. Oxygen from cutaneous uptake by larvae, however, is probably delivered by direct diffusion to underlying tissue; thus, although the skin is an important gas exchange surface, it is not a 'respiratory organ' in the usual sense of supplying oxygen *via* the circulation to other tissues of the body.

The red layer of muscle has been suggested to be a 'respiratory organ' in larval cyprinids before the gills develop (El-Fiky *et al.* 1987; El-Fiky and Wieser, 1988). Red muscle of Atlantic salmon is first seen as a single layer of cells immediately under the skin of most of the body and superficial to the bulk of somatic muscle. As the larval salmon develop into juveniles, the red muscle layer thickens and gathers under the lateral line in a pattern similar to that in cyprinids (El-Fiky *et al.* 1987), brown trout *Salmo trutta* (Proctor *et al.* 1980) and rainbow trout *Oncorhynchus mykiss* (Nag and Nursall, 1972). To act as a 'respiratory organ', the red muscle would have to be perfused to permit gas transport to other areas of the body. El-Fiky *et al.* (1987) do not mention vascularization in the red muscle layer of cyprinids, but in Atlantic salmon there were no capillaries in the red muscle layer of recently hatched fish and few capillaries in older fish, all of which were at the inner boundary of the red muscle layer. Thus, the superficial distribution of red muscle in larval salmonids may be important for oxygen delivery to the red muscle itself, but not for O_2 delivery to other body tissues.

The fins of larval fish represent a significant fraction of the total cutaneous surface area, up to approximately 40 % of total cutaneous area, and have also been proposed to act as respiratory surfaces. For example, prenatal seaperch (*Rhacohilus vacca* and *Embioteca lateralis*) have highly vascularized fins with short diffusion distances (less than 5 μm) that may allow for effective oxygen transfer from the ovary to the embryos contained within (Webb and Brett, 1972). The vascularized fins of larval *Monopterus cuchia* (Singh *et al.* 1989) and *Tilapia mossambica* (Lanzing, 1975) are also thought to act as respiratory structures. The fins of Atlantic salmon, however, are poorly vascularized and do not overlie metabolically active tissue and thus they may take up oxygen only to satisfy the oxygen consumption of the fin itself.

In contrast to other cutaneous surfaces, the yolk sac is well-vascularized and, because salmonids have circulating red cells, it has the potential to act as a respiratory organ providing oxygen to other body tissues through circulatory transport. There is little metabolizing tissue directly under the skin of the yolk sac, although there is a layer of endoderm that may be metabolically active in resorbing the yolk (Kamler, 1992). The oxygen uptake through the yolk sac is high relative to the mass of tissue (Wells and Pinder, 1996), suggesting that oxygen is transported away from the yolk sac in the circulation.

Branchial ADF

Branchial ADF increased after hatch until the gills resembled those of adults by the end of yolk absorption (0.2 g). Thereafter, branchial ADF remained constant, or at least there was no significant correlation between ADF and body mass. Perry (1990) calculated ADF for a 25 g rainbow trout to be approximately $1\,cm^2\,g^{-1}\,\mu m^{-1}$, however, and the two largest fish we measured, weighing 6 and 11 g, had ADFs of 2.2 and $1.5\,cm^2\,g^{-1}\,\mu m^{-1}$, respectively. Gill allometry in other salmonids also suggests that branchial ADF decreases with growth: lamellar diffusion distances in other salmonids may remain roughly constant (Hughes, 1984*a*) or decrease slightly (Morgan, 1974) and gill area scales to less than unity with mass (Hughes, 1970, 1972, 1984*a*,*b*). Calculated ADF of tench (*Tinca tinca*) decreases by about 50 % (from 1.06 to $0.46\,cm^2\,g^{-1}\,\mu m^{-1}$) as fish increase in mass from 25 to 376 g (data from Hughes, 1972). Thus, a decrease in ADF with growth from juvenile to adult in fish may be a general feature.

The increase of branchial ADF during early development results from both the rapid increase in gill surface area (lamellae and filaments) and the reduction of lamellar diffusion distances. The increase in total gill surface area, and particularly the area contributed by the lamellae, is the largest contributor to the increase in branchial ADF. Allometric growth of the gill surface area of Atlantic salmon is broadly similar to that of other larval fishes (Table 3). As gills begin to develop, mass exponents for total gill area are high (mass exponent 1.97 from 0.032 to 0.212 g body mass) then decrease to close to unity after metamorphosis (0.97, 0.19–1.15 g). High mass exponents early in development result from a combination of organogenesis and growth, while exponents after metamorphosis are related only to growth and are close to those for oxygen uptake (Oikawa and Itazawa, 1985; Rombough and Ure, 1991; Wells and Pinder, 1996).

The high mass exponent for gill surface area resulted from multiplicative effects (thus additive mass exponents) of increasing filament length and number, differentiation of lamellae and increasing size of lamellae. Before metamorphosis, filament length and number increased with much higher mass exponents than would be expected from geometric similarity (b=0.63 and 0.57, respectively, compared with a value of 0.33 expected for increases of linear dimensions in geometric similarity), indicating that both the arches and filaments lengthened much faster than the other body components were growing. Thus, the total length of filaments increased with an exponent of 1.20 (0.63+0.57), much higher than the value of 0.67 expected from geometric similarity. This was associated with the changes in the shape of the gill arch apparent in Fig. 8A–C, from a straight arch to a sharply curved arch with upper and lower limbs. Because the spacing of lamellae remained constant at $20.8\pm1.8\,lamellae\,mm^{-1}$ (very

Table 3. *Gill surface area allometry in relation to total body length, body shape and mass at metamorphosis in various species of fish*

Fish	*b*	Body length (mm)	Body mass (g)	Reference
Fusiform fish				
Atlantic salmon (*Salmo salar*)				
Premetamorphosis	1.97	<27	<≈0.2	Present study
Juvenile	0.97	>27	>≈0.2	
Chinook salmon (*Oncorhynchus tshawytscha*)				
Premetamorphosis	1.485	<30	<≈0.39	P. J. Rombough (personal communication)*
Juvenile	0.887	>30	0.4–1.9*	
Rainbow trout (*Oncorhynchus mykiss*)				
Premetamorphosis	3.443	<22	<≈0.1	Hughes and Al-Kadhomiy (1985)‡
Juvenile	0.932	>22	>≈0.1	
Carangiform fish				
Herring (*Clupeus harengus*)				
Premetamorphosis	3.36	<40	<≈0.110	DeSilva (1974)
Juvenile	0.78	>40	>≈0.110	
Deep-bodied fish				
Carp (*Carpio carpio*)				
Premetamorphosis	7.066	<9	<≈0.003	Oikawa and Itazawa (1985)
Premetamorphosis	1.222	<18	<≈0.2	
Juvenile	0.794	>18	>≈0.2	
Flatfish				
Plaice (*Pleuronectes platessa*)				
Premetamorphosis	1.59	<12†	<≈0.035	DeSilva (1974)
Juvenile	0.85	>12	>≈0.035	
Flounder (*Platyichthys flesus*)				
Premetamorphosis	2.213	<≈10†	<≈0.05	Al-Kadhomiy (1985)
Juvenile	0.824	>≈10	>≈0.05	

*Range of mass before smoltification.
†Within the size range identified for metamorphosis by Ahlstrom *et al.* (1984).
‡These authors use data from Morgan (1974) and Hughes (1970).
Regression formula: $y=am^b$, where y is the area of the surface in mm^2, a is a constant, m is tissue mass in g and b is the mass exponent.

similar to the value for adult rainbow trout, 18 lamellae mm^{-1}; Hughes, 1972), the total number of lamellae increased with a similar mass exponent (1.28) to that for total filament length. The area of individual lamellae increased at a slightly lower rate than that expected from geometric similarity (0.57 compared with the expected value of 0.67). Total lamellar area thus increased with a very high mass exponent (2.08) during early development. This pattern of gill development is similar to that of *Colisa* (=*Trichogaster*) *fasciatus*, which also has high mass exponents for filament length (1.16) and number (0.71), and thus total length of filaments (1.83), but little change in lamellar spacing, all of which result in a relative increase in gill surface area with a mass exponent of 1.41 (Prasad, 1988).

This pattern differs from the allometry of gills in carp (Oikawa and Itazawa, 1985), in which the high exponent for early larval gill development (7.1) was largely the result of high exponents for the area of individual lamellae (2.27) and the number of lamellae per unit length of filament (3.88), whereas total filament length increased with a modest exponent of 0.74. Some of the explanation of the difference lies in the treatment of the data: we used a single line segment for all of larval development, covering a range of body mass of an order of magnitude, while Oikawa and Itazawa (1985) used two, the first of which covered only a doubling of body mass. Although the mass exponent of gill area in our data also appeared to be higher in the first few points (see Fig. 4), there were not enough data to justify a separate regression. Nonetheless, the difference in scaling of filament number and total filament length through larval development is not due to procedural differences and demonstrates that similar structures can result from somewhat different developmental processes.

Critical size

It has been suggested that there is a critical fish size at which

gills must supplement skin to maintain sufficient O_2 uptake (DeSilva and Tytler, 1973; DeSilva, 1974; Blaxter, 1988). For example, black sea bream (*Acanthopagrus schlegeli*) have been suggested to reach a critical point where surface-to-volume ratio would be low, would limit fish activity and would possibly lead to increased mortality (Iwai and Hughes, 1977).

Gill organogenesis (as opposed to growth) is reflected in high mass exponents for branchial surface area as lamellae develop on the filaments; thus, the timing of gill organogenesis can be inferred from the changes in mass exponents with growth. Table 3 summarizes the patterns of gill allometry of several fish species. In all species, the highest mass exponents, reflecting the appearance of lamellae and presumably the start of gill function, were observed early in development and decreased at about the time of metamorphosis (although the transition points from previous studies should be interpreted cautiously because the regressions were often arbitrarily separated into two or three linear segments). Mass exponents after metamorphosis are similar to those reported for O_2 uptake in adult fishes (0.80; Winberg, 1956; Kamler, 1976). In contrast, the body mass at which lamellae develop is quite variable. Of the larval fish studied to date, mass at metamorphosis varied from 0.035 to 0.40 g and length ranged from 10 to 40 mm (Table 3). This seems too broad a range of mass and length to indicate a critical size. For gills to operate as respiratory organs supplying oxygen to other tissues, the blood must also contain red cells. It would be interesting to know whether red cells always appear in the blood before the end of gill organogenesis. If not, then the gills may not be necessary for gas exchange when they first appear to be morphologically functional.

There is little evidence that respiration in larval fish becomes limited by respiratory surface area. Salmonids, which have the largest larvae among bony fish, nonetheless have a higher ADF at hatch than as adults. Over 90 % of ADF is cutaneous at hatch, and even at the end of yolk absorption cutaneous ADF is about the same as branchial ADF in adult fish, approximately $1\,cm^2\,g^{-1}\,\mu m^{-1}$ (Perry, 1990). Smaller fish larvae, with a thinner skin and a higher surface area to volume ratio, should have an even higher ADF. Some fish, e.g. plaice and flounder, metamorphose at a body mass similar to that of newly hatched salmon yet have much more fully developed gills (DeSilva, 1974; Al-Kadhomiy, 1985). With continued growth, of course, limitation of respiratory surface area would eventually occur unless gills developed. The actual timing of lamellar development, however, seems to be well before the point at which the ADF of the skin becomes limiting to respiration.

In conclusion, this study has shown (1) that the anatomical diffusion factor is high at hatch, approximately $2.6\,cm^2\,g^{-1}\,\mu m^{-1}$; (2) that the distribution of ADF changes from 92 % cutaneous (50 % yolk sac and 42 % body and fins) and 8 % branchial at hatch to 68 % branchial and 32 % cutaneous by the end of yolk absorption; and (3) that the mass exponents of branchial areas and some components of cutaneous areas decrease at metamorphosis. The timing of gill development appears to be more closely linked to developmental stage than to body mass.

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