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The blood volumes of the primary and secondary circulatory system in the Atlantic cod *Gadus morhua* L., using plasma bound Evans Blue and compartmental analysis

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Summary

The volume of the primary (PCS) and secondary (SCS) circulatory system in the Atlantic cod *Gadus morhua* was determined using a modified dye dilution technique. Cod (*N*=10) were chronically cannulated in the second afferent branchial artery with PE-50 tubing. Evans Blue dye was bound to harvested fish plasma at a concentration of 1 mg dye ml^{-1} plasma, and injected at a concentration of 1 mg kg^{-1} body mass. Serial sampling from the cannula produced a dye dilution curve, which could be described by a double exponential decay equation. Curve analysis enabled the calculation of the primary circulatory and total distribution volume. The difference between these volumes is assumed to be the volume of the SCS. From the dilution curve, it was also possible to calculate flow rates between and within the systems. The results of these experiments suggest a plasma volume in the PCS of 3.42±0.89 ml 100 g^{-1} body mass, and in the SCS of 1.68±0.35 ml 100 g^{-1} body mass (mean ± S.D.) or approximately 50% that of the PCS. Flow rates to the SCS were calculated as 2.7% of the resting cardiac output. There was an allometric relationship between body mass and blood volumes. Increasing condition factor showed a tendency towards smaller blood volumes of the PCS, expressed as percentage body mass, but this was not evident for the volume of the SCS.

Key words: secondary circulation, volume, flow, leak, Evans Blue, plasma-bound tracer, Atlantic cod, *Gadus morhua*.

Introduction

The circulatory system in fish has been fully described (Mott, 1957; Randall, 1970), and until recently it was thought that fish possess a lymphatic system like that of mammals (Kampmeier, 1969). In 1929, Burne observed the existence of a 'fine-vessel' system, which he did not believe constituted a part of the traditional circulatory system or to be of lymphatic origin, yet he could not demonstrate a connection to the vascular system (Burne, 1929). In 1981, Vogel and Claviez, using corrosion casts and scanning electron microscopy (SEM), rediscovered Burne's fine vessel system and revealed their connection to the primary circulatory system (PCS) through interarterial anastomoses (Vogel and Claviez, 1981). Vogel and Claviez recognised these vessels as a separate system, termed the secondary circulatory system (SCS).

Although the systemic distribution of the SCS is species specific, some common characteristics have been described: the secondary vessels in teleost fish originate directly from the primary vascular system, by way of numerous narrow side branches, interarterial anastomoses, that are approximately 7–15 µm inner diameter (Vogel and Claviez, 1981; Vogel, 1985a; Lahnsteiner et al., 1990; Steffensen and Lomholt, 1992; Dewar et al., 1994). In their study on four teleostean and holostean fishes, Vogel and Claviez (1981) showed that secondary vessels run in parallel with primary arteries from the tail and fins, as well as in the head. However, many studies on the anatomical structure of this system have emphasised the interarterial origins from the caudal aorta and segmental arteries (Steffensen et al., 1986; Lahnsteiner et al., 1990; Chopin and Bennett, 1996; Chopin et al., 1998), and less attention has been devoted to interarterial origins elsewhere. Wherever their origin, these anastomoses coil extensively over 200–300 µm, before re-anastomosing with neighbouring vessels to form progressively larger secondary arteries (Olson, 1996). In the skipjack tuna *Katsuwonis pelamis* and the Atlantic cod *Gadus morhua*, the SCS forms capillary beds (Dewar et al., 1994; Burne, 1929), which are assumed to be typical of water breathing teleosts (Vogel, 1985a), before draining into the primary venous system. However, in *Salaria pavo* (prev. *Blennius*) and *Zosterisessor ophiocephalus*, it fails to do so (Lahnsteiner et al., 1990).

The distribution and volume of the SCS has been widely discussed. To date it has been shown that secondary vessels supply secondary capillary beds in the body surface, the fins, the buccal cavity, the pharynx and the peritoneum, and it may

or may not be present in the intestinal wall (Vogel, 1985a, 1999). In general, body surfaces in contact with the external environment are to some extent perfused by the SCS, yet this is quite species specific (Lahnsteiner et al., 1990; Olson, 1996). The SCS empties *via* secondary veins into the veins of the PCS near the heart, through ductus Cuvieri, or through the caudal heart, a venous pump in the secondary circulation (Vogel, 1985a,b).

Several studies have determined the volume of the circulatory system in teleost fishes using a variety of indicators and methods. The most commonly used tracers include sodium radiochromate-labelled red blood cells (^{51}Cr-RBC) (Wardle, 1971; Gingerich et al., 1987; Duff et al., 1987; Gingerich and Pityer, 1989; Brill et al., 1998), iodonated bovine (^{125}I-BSA) or human serum albumin (^{131}I-HSA) (Conte et al., 1963; Wardle, 1971; Gingerich et al., 1987; Gingerich and Pityer, 1989; Bushnell et al., 1998), and Evans Blue (EB) dye (T-1824) (Conte et al., 1963; Smith, 1966; Twelves, 1972; Nikinmaa et al., 1981; Itazawa et al., 1983; Sleet and Weber, 1983; Nichols, 1987; Tort et al., 1991; Acierno et al., 1995). Studies using fluorescein dye (Ronald et al., 1964) or fluorescein-tagged dextrans (Brill et al., 1998) have also been performed.

In the present study Evans Blue dye (EB) was used to determine the volume of the primary and SCSs. EB has been criticised for not being restricted to the circulatory system, making it unsuitable for blood volume determinations. Fish capillaries were previously thought to be highly permeable (Hargens et al., 1974), in sharp contrast to the properties of the mammalian circulatory system, where water and microsolutes can freely pass through the capillary endothelium through specialised pathways (Michel, 1996), but macromolecular passage is severely restricted (Michel, 1996) and requires vesicular transport (Schnitzer et al., 1988; Ghitescu, 1986). In their study, Bendayan and Rasio (1996) showed that albumin transport across the capillary wall of the eel swim bladder also required vesicular transport. Similar conclusions were reached more recently by Matsuyama and Iida (2001), investigating the swim bladder blood vessels of tilapia, using EB-labelled albumins. Jones and others argue that albumin is retained within the vascular compartment, since albumin sized proteins were absent from the interstitial fluid of muscles (D. R. Jones, P. G. Bushnell, J. F. Steffensen, K. L. Cousins, D. W. Duff, C. D. Taxboel, J. E. Keen and R. W. Brill, manuscript submitted for publication).

These principles of vesicular transport may not hold true for the fenestrated venous capillaries. Electron microscopic observations of venous capillaries in mammals reveal that these fenestrations have diameters of 50–88 nm, implying a high permeability to plasma proteins (Michel and Curry, 1999), but this is not supported experimentally. Levick and Smaje (1987) argue that other structures, such as glycoproteins and the basement membrane, contribute to the resistance of the fenestrae. These authors found that while fenestrated vessels had higher hydraulic and diffusional permeability to small hydrophilic solutes than continuous vessels, there was no change in the reflection coefficient for macromolecules such as albumin.

By binding of EB to the albumin fraction of teleost plasma, we believe that the problem of dye distribution to the extra vascular space has been reduced. Tort et al. (1991) showed that EB primarily bound to the albumin and, to some extent, the globulin fraction of elasmobranch plasma, with a binding capacity of 0.6 mg ml^{-1} plasma. With a mean albumin content in elasmobranch plasma of 5–7 mg ml^{-1} (0.5–0.7 g dl^{-1}), this corresponds to a binding ratio of 1:10. Although albumin is the major transport protein in fish blood (McDonald and Milligan, 1992), its contribution to the composition of elasmobranch plasma protein is low compared to that in teleost fishes. We expected binding capacity of teleost plasma to be at least that of elasmobranchs. The binding capacity for EB in cod plasma was quantified.

Material and methods

General

Atlantic cod *Gadus morhua* Linnaeus, 1758 (N=10) of mass 0.334–1.316 kg were caught by trawl at 17–22 m depth, in the northern part of Øresund in Denmark. Fish were transferred to the laboratory, to 500 liter tanks provided with continuously recirculating (8 l min^{-1}) filtered 10°C seawater (30‰ salinity). Experimental animals had a mean±S.D. body mass of 0.67±0.32 kg, length (L) of 42.4±7.3 cm and condition factor (CF=body mass$\times L^{-3}\times$100) of 0.90±0.16. The cod were acclimated to laboratory conditions for a minimum of 3 weeks prior to experimentation, with a 12 h:12 h light:dark photoperiod. During this time they were fed a diet of chopped herring (*Clupea harengus* L.) *ad libitum* three times weekly. Experimental animals were fasted for 1 week prior to experimentation, to ensure complete gastric evacuation.

Plasma-bound Evans Blue (PBEB)

Two 10 ml samples of blood were harvested from a large cod, and centrifuged at 2630 ***g*** for 5 min (Biofuge A, Heraeus Sepatech GmbH, Germany). Supernatant plasma was harvested and weighed. 1 mg EB mg^{-1} plasma was added, mixed, divided into 1 ml portions in Eppendorf tubes and frozen at –18°C. PBEB portions were thawed immediately before use, at 10°C. The mean dye concentration used was 1.04±0.12 mg kg^{-1} body mass.

To quantify the binding capacity of cod plasma for EB, cod blood was collected from a donor fish and the red blood cell fraction removed by centrifugation at 2630 ***g*** for 5 min. EB was added to 500 µl cod plasma samples at concentrations of 0.25, 0.5, 0.75, 1.0, 2.5, 5, 10, 20, 30 and 40 mg ml^{-1} and to distilled water at 0.25 and 0.75 mg ml^{-1}, and centrifuged through a 30 kDa Nanosep centrifugal membrane (Filtron Technology Corporation, MA, USA) at 14 000 ***g*** for 3 h at 12°C in a Sigma 3K30 centrifuge (Sigma Laborzentrifugen GmbH, Germany). From the distilled water samples, 78–81% EB could be recovered. No dye could be recovered in plasma samples containing [EB] <10 mg ml^{-1}, but at >20–40 mg ml^{-1}, dye recovery increased from 2–15%, indicating an EB binding capacity of approximately 10–20 mg ml^{-1} plasma.

Surgical procedure

Fish were anaesthetised in ethyl *p*-amino benzoate (Benzocaine) ($0.06\,g\,l^{-1}$) and placed on a wet foam rubber mat. Aerated seawater containing $0.03\,g\,l^{-1}$ benzocaine was pumped across the gills to maintain anaesthesia and ensure oxygen delivery.

The second afferent branchial artery on the left side of the fish was cannulated using PE-50 tubing (Portex, England), entering the artery approximately 1–2 cm above the base of the gill arch. The cannula was fastened using silk suture (Kruuse, Denmark) around the gill arch. One suture was placed immediately above the cannula entrance, one 2 cm above the entrance, and one skin suture at the base of the first dorsal fin. The fish were weighed and measured for body mass and length, and transferred to non-transparent plastic tubes (130 mm×540 mm), with a slit along the top, allowing access to the cannula. Plastic grids, while allowing circulation, closed the ends of the tubes. Animals were allowed to recover for 24 h after transfer to experimental chambers.

The cannula was connected to a 3-way stopcock with two 1 ml syringes, one to administer heparinised saline (HS) ($25\,i.u.\,ml^{-1}$ heparin in 1.1% NaCl solution), and another to draw 1 ml blood samples, of which the last 0.18 ml was divided into three heparinised microhaematocrit tubes (Modulohm, Denmark), and sealed. Surplus blood was returned to the fish, followed by an additional 0.2 ml HS to replace lost blood and fill the cannula. The entire blood sampling procedure was completed in less than 45 s. All blood samples were centrifuged (Biofuge A, Heraeus Sepatech GmbH, Germany) at 2630 ***g*** for 5 min.

At the beginning of each experiment, a triplicate blood sample was taken from each fish, to measure initial haematocrit (Hct) and spectral absorption in dye-free plasma. Before returning excess blood from the dye-free sample, the stopcock was removed and PBEB was injected directly into the cannula. The stopcock was replaced and surplus blood from the background reading was used to flush the cannula, which was subsequently filled with HS. Blood samples were taken at 5, 10, 20, 40, 60, 90, 120, 180, 240, 300, 1380 and 1440 min after injection of dye.

Haematocrit values were measured visually using a haematocrit grid (Mikro-Hämatokrit, Heraeus Sepatech GmbH, Germany). The red blood cell fraction was removed, and 40 µl plasma was transferred to a microtestplate (Cliniplate, Labsystems, Finland). The plasma sample was diluted with 40 µl demineralised water, and absorbance was read at 620 nm, on a Multiskan RC standard Elisa reader (Labsystems, Finland) after high-speed mixing for 1 min on the built-in mixer function. The absorbance value of dye-free plasma was subtracted from all subsequent measurements.

Calculations

An absorbance calibration curve was produced from measurements with a variety of PBEB concentrations. A background value from pure fish plasma was obtained as described above, and subtracted from the other values. A linear regression line was inserted, and the relationship between spectral absorption and [PBEB] (mg) was found to be:

$$[\mathrm{PBEB}]\times\mathrm{Volume}^{-1}=0.087\times\mathrm{Absorption}\,, \qquad (1)$$

where volume is in ml.

For simplification, the circulatory system of teleost fishes is viewed as a two-pool open system, where mixing within the PCS is expected to be complete at the time of the first sample (see Discussion). As such, it can be subjected to compartment analysis, as described by Shipley and Clark (1972). Washout curves are presented as double exponential decay equations, where the first part of the equation describes flow from the PCS to the SCS, and the second part of the equation describes the slow leak from the vasculature:

$$[\mathrm{PBEB}]=\mathrm{a}\times\mathrm{e}^{-\mathrm{g}_1 t}+\mathrm{b}\times\mathrm{e}^{-\mathrm{g}_2 t}\,, \qquad (2)$$

where a and b are the intercepts of the washout curve, g_1 and g_2 are the slopes of the two components, and t is time post-injection in min. The decay curve can be produced by ‘curve-peeling’, by calculating the first component of the equation by subtracting a series of values from the late (linear) part of the curve from the first part of the curve, thus producing two components (Fig. 1).

In this case, Equation 2 was fitted to observed values using a computer program (CurveFit 4.0, Jandell Scientific). Plasma volume of the PCS (V_{PPCS}) was calculated by traditional methods (Conte et al., 1963):

$$V_{\mathrm{PPCS}}=VC/(\mathrm{a}+\mathrm{b})\,, \qquad (3a)$$

where V is volume of the injected material and C the concentration in the injected material. The total volume of the circulatory system was calculated as:

$$V_{\mathrm{PTCS}}=VC/\mathrm{b}\,, \qquad (3b)$$

and blood volume V_{B} as:

$$V_{\mathrm{B}}=V_{\mathrm{P}}/(1-\mathrm{Hct})\,. \qquad (3c)$$

The volume of the SCS was calculated as the difference between the plasma volumes of the two systems. In order to produce comparable values for different fish, it was necessary to transform concentration curves to fraction of dose, described by:

$$\frac{q_{\mathrm{at}}}{Q_{\mathrm{a0}}}=\frac{[\mathrm{PBEB}]\times\mathrm{Volume}^{-1}}{CV}\,, \qquad (4)$$

where q_{at} is concentration in plasma sample at time t and Q_{a0} is administered dose. The fraction of dose curve is described by:

$$\frac{q_{\mathrm{at}}}{Q_{\mathrm{a0}}}=\mathrm{H}_1\times\mathrm{e}^{-\mathrm{g}_1 t}+\mathrm{H}_2\times\mathrm{e}^{-\mathrm{g}_2 t}\,, \qquad (5a)$$

which can be normalised by

$$\mathrm{f}(t)=\left(\frac{\mathrm{H}_1}{\mathrm{H}_1+\mathrm{H}_2}\right)\mathrm{e}^{-\mathrm{g}_1 t}+\left(\frac{\mathrm{H}_2}{\mathrm{H}_1+\mathrm{H}_2}\right)\mathrm{e}^{-\mathrm{g}_1 t}\,, \qquad (5b)$$

yielding a new equation

$$f(t) = I_1 \times e^{-g_1 t} + I_2 \times e^{-g_2 t}, \quad (5c)$$

where the coefficients I_1 and I_2 add to unity, because at t=0, the whole dose, 1% or 100% is present.

Having a normalised equation allows the calculation of flux rates between the compartments of the system. Each rate constant is related mathematically to the entire group of parameters in the normalised Equation 5c. These calculations are based on three equations (6–8), as stated by Shipley and Clark (1972):

$$k_{PCS} = H_1 \times g_1 + H_2 \times g_2, \quad (6)$$

$$k_{PCS} + k_{SCS} = g_1 + g_2, \quad (7)$$

and

$$k_{PCS} \times k_{SCS} - k_{SCS \to PCS} \times k_{PCS \to SCS} = g_1 \times g_2, \quad (8)$$

which state that the total turnover in the PCS equals the sum of the two intercepts multiplied by their respective slopes (Equation 6), that the turnover in the entire circulatory system is equal to the sum of the two slopes in the double exponential decay equation (Equation 7), and that the product of the fluxes from one compartment to the other, subtracted from the product of the two turnover constants from the respective compartments, is equal to the product of the two slopes (Equation 8).

The total turnover in the PCS (k_{PCS}), is described by Equation 6. Since it is assumed in the model that the only loss from the system is from the SCS, flux from the PCS to the SCS ($k_{PCS \to SCS}$) is the same as k_{PCS}. Total turnover in the SCS (k_{SCS}) is calculated through Equation 5.

$$k_{SCS} = g_1 + g_2 - k_{PCS}. \quad (9)$$

Return flux from the SCS to the PCS can be calculated from Equation 8:

$$k_{SCS \to PCS} = \frac{(k_{PCS} \times k_{SCS}) - (g_1 \times g_2)}{k_{PCS \to SCS}}. \quad (10)$$

Loss of dye from the SCS to the extra vascular space is the calculated as the difference between total turnover in the SCS and flux from the SCS to the PCS.

$$k_{SCS \to OUT} = k_{SCS} - k_{SCS \to PCS}. \quad (11)$$

To transform flux constants to flow rates, these are multiplied by the volume of the respective systems:

$$F_{PCS \to SCS} = k_{PCS \to SCS} \times V_{PPCS}, \quad (12)$$

$$F_{SCS} = k_{SCS} \times V_{PSCS}, \quad (13)$$

$$F_{SCS \to PCS} = k_{SCS \to PCS} \times V_{PSCS}, \quad (14)$$

$$F_{SCS \to OUT} = F_{SCS} - F_{SCS \to OUT}. \quad (15)$$

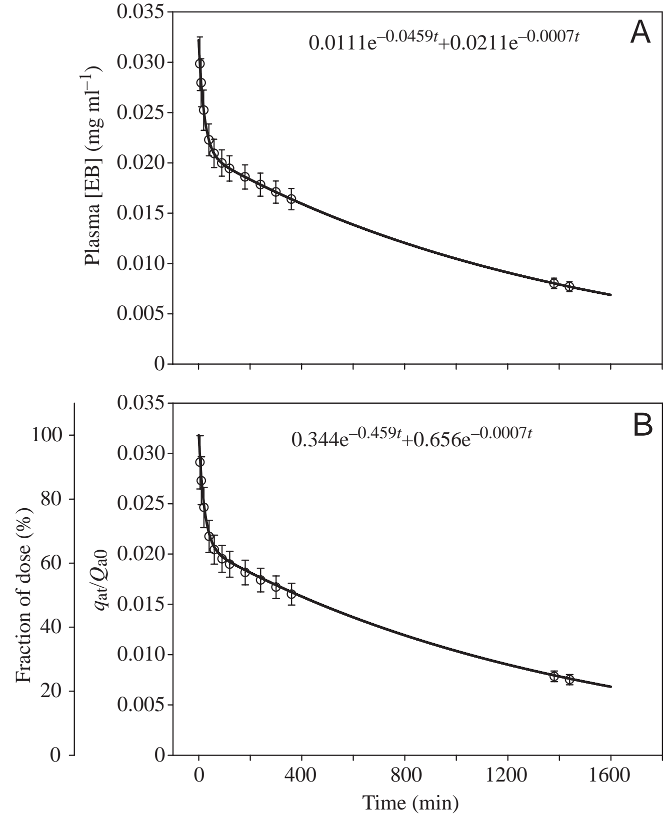

Fig. 1. Decay curves for Evans Blue (EB). (A) Pure dye concentration values. (B) Values normalised to express fraction of dose-injected (q_{at}/Q_{a0}) and normalised to express percentage (see Equations 5a–c). q_{at}, concentration in plasma sample at time t; Q_{a0}, administered dose at time zero.

Results

All washout curves could be described by Equation 2, with an r^2 of 0.98 or better (Fig. 1A).

The average volume of the PCS was found to be 3.42±0.89 ml plasma 100 g^{-1} body mass, and a blood volume of 4.35±1.10 ml 100 g^{-1} body mass, using an average haematocrit value of 21.5±3%. The total plasma volume of the animals was calculated to be 5.10±1.12 ml 100 g^{-1} body mass. The plasma volume of the SCS was 1.68±0.35 ml 100 g^{-1} body mass, or 50% that of the primary circulatory volume, with a haematocrit of zero (Table 1).

Furthermore, there was an allometric relationship between body mass and plasma volume of the PCS (r^2=0.82) and SCS (r^2=0.72) and blood volume of the PCS (r^2=0.83) (Fig. 2). Thus plasma volume of the PCS and SCS, and blood volume of the PCS scale as:

$$V_{PPCS} = 32.03 \times BM^{0.79}, \quad (16)$$

$$V_{PSCS} = 15.13 \times BM^{0.78}, \quad (17)$$

$$V_{BPCS} = 41.41 \times BM^{0.82}, \quad (18)$$

respectively, where BM is body mass. In a similar fashion, there was a correlation between condition factor and volume of the PCS and SCS, when expressed as percentage of body mass, with a decrease in plasma volume of the PCS, with increasing condition. The volume of the SCS did not appear to be affected by alterations in condition factor. All distribution volumes and flow rates are given in Table 1.

Table 1. *Blood and plasma volumes, flow rates, washout times and turnover rates of the primary and secondary circulation in Atlantic cod Gadus morhua*

Parameter	Unit	Value (mean ± S.D.)
Plasma volume of the PCS, V_{PPCS}	ml 100 g^{-1} BM	3.42±0.89
Blood volume of the PCS, V_{BPCS}	ml 100 g^{-1} BM	4.35±1.10
Plasma volume of the SCS, V_{PSCS}	ml 100 g^{-1} BM	1.68±0.35
Total plasma volume V_{P}	ml 100 g^{-1} BM	5.10±1.17
Total blood volume V_{B}	ml 100 g^{-1} BM	6.03±1.30
Plasma volume ratio		
Secondary:primary		0.51±0.14
Secondary:total		0.33±0.06
Blood volume ratio		
Secondary:primary		0.40±0.11
Fractional change		
(k_{out})	min^{-1}	0.0019±0.0003
(k_{out})	h^{-1}	0.1124±0.0194
Plasma flow rate		
Primary→secondary	ml min^{-1} kg^{-1}	0.31±0.19
Secondary→primary	ml min^{-1} kg^{-1}	0.28±0.19
Secondary→out	ml min^{-1} kg^{-1}	0.03±0.01
Time for 50% disappearance from PCS	h	8.22±4.36
Time for 75% disappearance from PCS	h	26.61±9.77
Turnover time for plasma in the SCS	h	1.43±0.94

PCS, primary circulatory system; SCS, secondary circulatory system; BM, body mass.

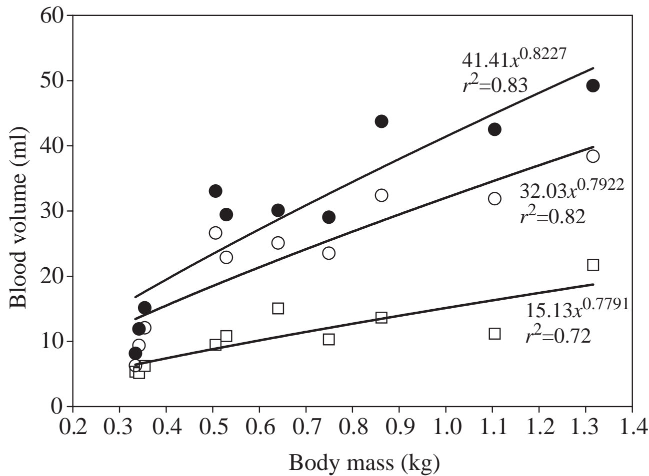

Fig. 2. Relationship between body mass and blood volume (filled circles) and plasma volume (open circles) of the PCS and plasma volume of the secondary (open squares) circulatory system. Best-fit, least-square lines are shown.

Flow rates from the PCS to the SCS were calculated to be 0.32±0.19 ml 100 g^{-1} body mass. With an average resting cardiac output of 12±0.74 ml 100 g^{-1} body mass (Webber et al., 1998) this corresponds to the SCS receiving approximately 2.7% of the cardiac output. Average retention times for plasma in the SCS was thus 78±59 min individual^{-1} (mean ± S.D.).

Discussion

A number of different methods have been used in attempts to estimate the circulatory volumes of teleost fishes. The primary reason for the continuing investigation into methods for blood volume determination is that large discrepancies exist between the results obtained from the various techniques employed. The use of EB as an albumin-bound tracer in fish vascular systems was suspected to be unreliable, as the capillaries were thought to have little or no macromolecular barrier (Hargens et al., 1974). This led to the assumption that blood volume determinations using albumin-bound tracer dilution methods typically overestimate the true volumes (Nichols, 1987; Brill et al., 1998; Bushnell et al., 1998). However, it has recently been shown that fish capillaries do in fact have a highly functional barrier to macromolecules, with a reflection coefficient for proteins that lies within the normal mammalian range (D. R. Jones, P. G. Bushnell, J. F. Steffensen, K. L. Cousins, D. W. Duff, C. D. Taxboel, J. E. Keen and R. W. Brill, manuscript submitted for publication).

Due to the risk of tracer extravasation, there are concerns about the estimated volume of the secondary vascular system, based on the difference between total vascular volume, measured with isotope-labelled albumins, and primary vascular volume, measured with labelled red blood cells (Gingerich and Pityer, 1989; Gingerich et al., 1990; Olson, 1992, 1996; Brill et al., 1998; Bushnell et al., 1998). These concerns are understandable when they are based on a distribution volume several hours after tracer injection. Some of these authors argue against the assumption that

disappearance of tracer from the primary vasculature represents flow to the secondary vascular system. This is based on the fact that if measurements consist of single samples taken several hours after injection of tracer, large fractions of radioactively labelled albumin are recovered in organs that are considered to lack secondary vessels. Tracers that are not wholly restricted to the vascular system are of little or no value in calculating distribution volumes if samples are taken at a single point in time. With reference to Olson's criteria (Olson, 1992) for selecting tracers, none of the substances used to 'measure' blood volume can be said to be truly inert.

The leak rate for PBEB from the vascular system was 0.0007 mg min^{-1}. The fractional change (k_{out}) was calculated (Equation 15) as 0.0019±0.0003 min^{-1}, or 0.1124±0.0194 h^{-1}. This is considerably less than the fractional change for 500 kDa dextran. Jones et al. (D. R. Jones, P. G. Bushnell, J. F. Steffensen, K. L. Cousins, D. W. Duff, C. D. Taxboel, J. E. Keen and R. W. Brill, manuscript submitted for publication) observed k_{out} values of 0.52, 0.18 and 0.21 h^{-1}, for 20, 70–150 and 500 kDa dextrans, respectively, in *G. morhua*, which clearly illustrates that the use of native albumins yields better retention than foreign tracers, even if the latter are several-fold larger. Furthermore, these authors performed clearance studies using BSA, which yielded a k_{out} of 0.18 h^{-1}, almost twice that of PBEB in this study.

Curve analysis

The slope of the first exponent in the decay curve is crucial in determining flow from the PCS to the SCS, as well as the volume of the PCS. However, its importance to the decay curve is short lived. Due to its rapid rate of decrease (0.0459 mg min^{-1}), the value of the first exponent becomes negligible after approximately 20 min. The second exponent of the decay equation illustrates the leak rate (0.0007 mg min^{-1}) from the vascular system, and its intercept dictates the total distribution volume. While the vascular system of fishes is probably permeable to plasma-protein-bound EB over extended time periods, the importance of this in subsequent data analysis is minimal, for two reasons. (1) The point where the value of first exponent approaches zero is the rotation point of the second exponent. This 'fulcrum' is in close proximity to the *y*-axis, i.e. time zero. Hence, it requires large changes in the leak rate to have an effect on the intercept, and larger leak rates would only result in smaller estimates (higher intercept). With the inclusion of samples 24 h post-injection, the range wherein these changes can take place is severely reduced (as opposed to the situation if sampling stopped after 300 min). Furthermore, (2) the intercept of the second exponent is produced by backwards extrapolation. Thus, extravasation that may have occurred since the time of injection is corrected for, and has no impact on the estimated volume.

The values that dictate the intercepts of the decay equation components are established within 20 min of injection of tracer. We expect that the loss of dye in this time period is negligible, since albumin turnover must be minimal and we are only using a fraction of the plasma protein binding capacity. Therefore, observed fluxes are actually indicative of plasma passing from the primary to the secondary vessel system, and not extravasation or other loss of tracer.

It could then be argued that the initial rapid decline of the first exponent merely represents a mixing of tracer within the primary vasculature. When determining initial sampling time, several variables must be taken into consideration; if sampling starts too soon the volume of the primary circulation will be underestimated, with a resulting overestimate in the volume of the secondary circulation. If, on the other hand, sampling is initiated too late, a large fraction of the tracer will have started flowing to the secondary vascular system, affecting both volume of, and flow rate to this system. Traditionally, circulation time equals blood volume divided by cardiac output (Randall, 1970), where mixing time would probably be half a dozen circulation times. However, circulation time through different regions varies greatly; in *Anguilla anguilla* blood appeared in the visceral veins 20 s after leaving the heart (Mott, 1957), while other tissues have much longer circulation times. Observations from blood volume measurements using ^{51}Cr-labelled red blood cells, which we know to be restricted to the primary vasculature, show that tracer distribution is stable after 5 min in plaice *Pleuronectes platessa* (Wardle, 1971) and in rainbow trout *Oncorhynchus mykiss* (Duff et al., 1987; Steffensen et al., unpublished observations; cf. Steffensen and Lomholt, 1992). These observations support that mixing within the primary vasculature is complete at the time of the first blood sample, and that for the determination of the volume of the PCS, it is suitable to initiate sampling at 5 min post-injection.

Results

The only other study presently available on the blood volume of the Atlantic cod is by Ronald et al. (1964). Their results were obtained using fluorescein-tagged native serum protein, and total blood volume was calculated by standard methods (Equation 3b), using an average plasma volume determined from sampling 10 and 20 min post injection. They found an average volume of the PCS of 2.4 ml 100 g^{-1} body mass (101.4 ml individual^{-1}), compared to 4.35±1.10 ml 100 g^{-1} body mass (30.9 ml individual^{-1}) found in the present experiments. However, Ronald et al. (1964) used animals of an average body mass of 4.2 kg *versus* 0.7 kg in the present study. The allometric relationship for body mass and blood volume from Ronald et al.'s study was 30.43×body mass$^{0.8833}$, thus a 1 kg individual has a blood volume of 3.04 ml 100 g^{-1} body mass compared to a blood volume of 4.14 ml 100 g^{-1} body mass in the present study, making the difference less pronounced (Fig. 2).

The volume of the SCS has previously been estimated by Steffensen et al. (Steffensen et al., unpublished observation; cf. Steffensen and Lomholt, 1992) to be 4.8 ml 100 g^{-1} body mass for rainbow trout, calculated as the difference in distribution volumes of two simultaneously injected tracers, ^{51}Cr-RBC and ^{131}I-HSA, and significantly higher than the values obtained for

Table 2. *Plasma and blood volumes of the rainbow trout* Oncorhynchus mykiss *(ml 100 g^{-1} body mass) acclimated to freshwater at various temperatures*

Plasma volume (ml)	Hct	Blood volume (ml)	Acclimation temperature (°C)	Tracer	Method	Reference
–	–	3.35	14	^{51}Cr-RBC	d	Duff et al., 1987
–	–	2.50	14	^{51}Cr-RBC	e	Duff et al., 1987
1.92	0.37	3.08	10	^{131}I-HSA	f	Conte et al., 1963
2.20	0.36	3.48	10	Evans Blue	f	Conte et al., 1963
2.27	0.36	3.52	?	^{51}Cr-RBC	k	Gingerich and Pityer, 1989
2.68	0.29	3.75	7	Evans Blue	g	Nikinmaa et al., 1981
2.83	0.23	3.70	11	Evans Blue	g	Nikinmaa et al., 1981
2.92	0.29	4.09	12	^{51}Cr-RBC	c	Gingerich et al., 1987
3.05	0.25	4.04	14	^{51}Cr-RBC	a	Bushnell et al., 1998
3.15	0.21	3.99	16	Evans Blue	g	Nikinmaa et al., 1981
3.24	0.30	4.63	?	^{51}Cr-RBC and ^{125}I-BSA	b_1	Gingerich et al., 1990
3.25	0.36	5.06	?	^{125}I-BSA	k	Gingerich and Pityer, 1989
3.34	0.19	4.13	16	Evans Blue	j	Nichols, 1987
3.74	0.29	5.27	?	^{51}Cr-RBC and ^{125}I-BSA	b_2	Gingerich et al., 1990
5.60	0.21	6.90	8–12	Evans Blue	h	Smith, 1966
5.80	0.19	7.18	16	Evans Blue	i	Nichols, 1987
11.8	–	–	14	^{125}I-BSA	a	Bushnell et al., 1998

Hct, haematocrit fraction; ^{51}Cr-RBC, sodium chromate-labelled red blood cells; ^{125}I-BSA, iodonated bovine albumin; ^{131}I-HSA, iodonated human serum albumin.

[a]Distribution volumes of tracer 16 h post-injection.

[b]Extrapolation of dilution curve based on samples taken 60, 120, 180 and 240 min post-injection. The two values are from two strains of *O. mykiss*; the Wytheville (b_1) and the Kamloops (b_2) strains.

[c]Extrapolation of linear regression line based on samples taken at 30, 60, 90 and 120 min post-injection.

[d]Dilution volume of tracer at 240 min post-injection.

[e]Extrapolation of linear regression line based on samples taken 30, 60, 150 and 240 min post-injection.

[f]Extrapolation of linear regression line based on samples taken 5, 10, 15 and 28 min post-injection. Note that fish received 42 000 i.u. heparin prior to experimentation, animals remained anaesthetised and their hearts were exposed during experimentation.

[g]Extrapolation of linear regression line based on samples taken at 60, 90 and 120 min post-injection. The sample taken at 40 min was consequently discarded, since it did not fall on the regression line. Blood volume calculations have been altered from $V_B=V_P+(\text{Hct}/100)\times V_P$ to $V_B=V_P/(1-\text{Hct})$, since the former is incorrect.

[h]Extrapolation of linear regression line based on samples taken at 60, 90 and 120 min post-injection.

[i]Based on intercept of single-exponential decay equation.

[j]Based on first intercept of double-exponential decay equation.

[k]Distribution volume of tracer 240 min post-injection.

G. morhua in these experiments. In this study, however, the disappearance rate of labelled human serum albumin was significantly higher. The time for 50% and 95% disappearance from the PCS was 1.8 h and 7.8 h, respectively, compared to 8.2 h for 50% and 26.6 h for 75% disappearance of EB-labelled plasma proteins in this study. To our knowledge, there has been no study on the possible implications of using non-native albumins as tracer carrier, with respect to their fate upon injection.

The only previous estimates of flow to the SCS are those of Steffensen et al. (Steffensen et al., unpublished observation; cf. Steffensen and Lomholt, 1992), determined to be 0.3% of resting cardiac output in *O. mykiss*, using essentially the same method of calculation. This value is several times lower than our calculated value of 2.7%. Vogel (1985a) believes that flow to the SCS is well controlled by a neurotransmitter- or humoral-controlled adjustment of the luminal diameter of the interarterial anastomoses. If in fact teleosts are able to regulate the flow to the SCS, discussions on the physiological functions of this system can be reopened. A number of possibilities have previously been excluded on the basis of low flow rates to this system. However, to what extent teleosts are able to regulate perfusion of the secondary vasculature has yet to be investigated.

Several different fish species have been used as experimental animals in estimates of blood volume, but experiments on the rainbow trout *O. mykiss* are dominant. The numbers of observations on this species are sufficient to enable a comparison of volumes obtained and methods used (Table 2). The variations in results obtained in these studies, seem primarily to arise from methodology, yielding blood volume estimates of 1.9–5.8% body mass. The calculated values in

these experiments are based on samples taken at a given time, varying from minutes to hours after injection, or based on an extrapolation of a series of samples taken at various intervals. It is apparent that ongoing attempts to increase the accuracy of blood volume determinations have focused on tracer material, retarding any direct comparison of results.

Some of the first attempts to estimate blood volume of fish, using a dye as tracer, were by Conte et al. (1963), Ronald et al. (1964) and Smith (1966), using EB or Fluorescein dye. Smith (1966) was aware of the problem of the disappearance of unbound dye, and attempted to bind EB to plasma protein by *in vivo* dialysis. He injected dye into a donor fish and harvested plasma bound EB. This yielded a 27% reduction in blood volume estimates in Coho salmon *O. kisutch* Walbaum, when using reinjected plasma-bound EB rather than traditional EB methods. In spite of this, subsequent publications on blood volume using EB have all used dye in aqueous solutions, often at quite high concentrations. At the same time, the use of radioactively labelled tracers has increased. Human and bovine serum albumins have been iodinated and red blood cells labelled with chromium. Yet the fact remains that this has not produced a more homogenous range of results. Although the primary criticism of EB has been directed at excess dye leaking to the extravascular space, values from EB measurements have no tendency to accumulate at the high end of the range. Rather, values from different tracers seem to be randomly distributed. The key issues when determining blood volumes seem to be time of sampling, concentration of tracer and method of calculation.

With the exception of the use of erythrocytes, there are such large variations in the methodology of blood volume studies that there really is no basis for a comparison of results. With the use of labelled red blood cells, distribution time plays a lesser role, since complete mixing in the PCS is complete within a short period of time. However, since the disappearance of ^{51}Cr-RBC appears to be negligible, and the distribution is restricted to the primary system, the time of sampling plays a smaller role than when using other tracers. Labelled erythrocytes seem to provide an accurate and, within a reasonable time span, stable indicator for the volume of the PCS. Yet in the determination of the total circulatory volume, this marker is rendered useless. For this purpose, a smaller marker that is not excluded from the SCS, yet is not extravasated, is required. Albumins all share the advantage of being high molecular mass compounds, whose passage across the vascular endothelium is restricted. Being the major transport protein in the blood, albumin is capable of reversibly binding with a variety of substances (McDonald and Milligan, 1992), and its passage throughout the vascular tree is ensured by its biological function. In principal, a variety of markers can be used; their common feature is the utilisation of albumin's transportation properties. This said, there is some evidence to suggest that the radioactive labelling of red blood cells and albumins results in a higher loss from circulation. Duff et al. (1987) observed that ^{51}Cr-labelled red blood cells were removed from the vascular system, presumably by the spleen, while Ronald et al. (1964) observed that the use of radio labelled albumins appeared to result in thyroid uptake of the iodine, causing mortality in the experimental animals. It appears that non-native albumins are scavenged or sequestered to a higher degree than native albumins. Whether this is due to iodination (or the process hereof), or because the albumins have a slight difference in protein structure, is unknown. Sørensen et al. (1998) showed that formaldehyde-damaged native albumin in *G. morhua* was endocytosed and degraded by atrial endocardial endothelial cells. Whether this is also the cause of the higher rate of removal of non-native albumins is unknown.

It is likely that the use of radioactively labelled albumin has been more common than the use of dye, because large amounts of dye have previously been required in order to perform spectrophotometric analysis. This meant using dye concentrations that exceeded the binding capacity of the albumin fraction in the blood, resulting in large initial leaks from the vascular compartment. With modern spectrophotometry, this is no longer an issue. Spectral absorbance in samples taken 24 h after injection can be read in an 80 µl sample, compared to the 1.6 ml volume of traditional spectrophotometry cuvettes, which results in a significant reduction of the amount of dye required for serial sampling over an extended time period, and in the required volume of the individual samples. Furthermore, native albumin provides a stable carrier of EB that, compared with all other studies so far, have the lowest loss rate of tracer. We have still not identified a tracer that complies with all the previously listed requirements for 'the optimal tracer', and while albumin does not freely diffuse out of the vascular system, there is still a risk that some of the EB-labelled albumin is trapped in the matrix of the capillary endothelium, which may result in a slight overestimate of distribution volumes. However, in our opinion, the method described herein represents a relatively simple and accurate method to obtain the circulatory volumes of the primary and secondary [total] circulatory system in fish.

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References

Acierno, R., MacDonald, J. A., Agnisola, C. and Tota, B. (1995). Blood volume in the hemoglobinless Antarctic teleost *Chionodraco hamatus* (Lönnberg). *J. Exp. Zool.* **272**, 407-409.

Bendayan, M. and Rasio, E. A. (1996). Transport of insulin and albumin by the microvascular endothelium of the rete mirabile. *J. Cell Sci.* **109**, 1857-1864.

Brill, R. W., Cousins, K. L., Jones, D. R., Bushnell, P. G. and Steffensen, J. F. (1998). Blood volume, plasma volume and circulation time in a high-energy-demand teleost, the yellowfin tuna (*Thunnus albacares*). *J. Exp. Biol.* **201**, 647-654.

Burne, R. H. (1929). A System of 'Fine' Vessels Associated with the Lymphatics in Cod (*Gadus morrhua*). *Phil. Trans. R. Soc. Lond. B* **217**, 335-367.

Bushnell, P. G., Conklin, D. J., Duff, D. W. and Olson, K. R. (1998). Tissue and whole-body extracellular, red blood cell and albumin spaces in the rainbow trout as a function of time: a reappraisal of the volume of the secondary circulation. *J. Exp. Biol.* **201**, 1381-1391.

Chopin, L. K., Amey, A. P. and Bennett, M. B. (1998). A systemic secondary vessel system is present in the teleost fish *Tandanus tandanus* and absent in the elasmobranchs *Carcharhinus melanopterus* and *Rhinobatus typus* and in the dipnoan *Neoceratodus forsteri*. *J. Zool. Lond.* **246**, 105-110.

Chopin, L. K. and Bennett, M. B. (1996). Morphology and tyrosine-hydroxylase immunohistochemistry of the systemic secondary vessel system of the blue catfish, *Arius graeffei*. *J. Morphol.* **229**, 347-356.

Conte, F. P., Wagner, H. H. and Harris, T. O. (1963). Measurement of blood volume in the fish (*Salmo gairdneri gairdneri*). *Am. J. Physiol.* **205**, 533-540.

Dewar, H., Brill, R. W. and Olson, K. R. (1994). Secondary circulation of the vascular heat exchangers in skipjack tuna, *Katsuwonus pelamis*. *J. Exp. Zool.* **570**, 566-570.

Duff, D. W., Fitzgerald, D., Kullman, D., Lipke, D. W. and Olson, K. R. (1987). Blood volume and red cell space in tissues of the rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol.* **87A**, 393-398.

Ghitescu, L., Fixman, A., Simionescu, M. and Simionescu, N. (1986). Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: Receptor-mediated transcytosis. *J. Cell Biol.* **102**, 1304-1311.

Gingerich, W. H. and Pityer, R. A. (1989). Comparison of whole body and tissue blood volumes in rainbow trout (*Salmo gairdneri*) with ^{125}I bovine serum albumin and ^{51}Cr-erythrocyte tracers. *Fish Physiol. Biochem.* **6**, 39-47.

Gingerich, W. H., Pityer, R. A. and Rach, J. J. (1987). Estimates of plasma, packed cell and total blood volume in tissues of the rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **87A**, 251-256.

Gingerich, W. H., Pityer, R. A. and Rach, J. J. (1990). Whole body and tissue blood volumes of two strains of rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. A* **97**, 615-620.

Hargens, A. R., Millard, R. W. and Johansen, K. (1974). High capillary permeability in fishes. *Comp. Biochem. Physiol.* **48A**, 675-680.

Itazawa, Y., Takeda, T., Yamamoto, K. and Azuma, T. (1983). Determination of circulating blood volume in three teleosts, carp, yellowtail and porgy. *Japan. J. Ichtyol.* **30**, 94-105.

Kampmeier, O. F. (1969). Lymphatic system of the bony fishes. In *Evolution and Comparative Morphology of the Lymphatic System*, pp. 232-265. Charles C. Thomas, Springfield.

Lahnsteiner, F., Lametschwandtner, A. and Patzner, R. A. (1990). The secondary blood vessel system of segmental arteries and dorsal aorta in *Blennius pavo* and *Zosterisessor ophiocephalus*. Histology, fine structure and SEM of vascular corrosion casts. *Scan. Microsc.* **4**, 111-124.

Levick, J. R. and Smaje, L. H. (1987). An analysis of the permeability of a fenestra. *Microvasc. Res.* **33**, 233-256.

Matsuyama, T. and Iida, T. (2001). Influence of tilapia mast cell lysate on vascular permeability. *Fish Shellfish Immunol.* **11**, 549-556.

McDonald, D. G. and Milligan, C. L. (1992). Chemical properties of the blood. In *Fish Physiology*, vol. XIIB (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 56-135. San Diego, Academic Press.

Michel, C. C. (1996). Review: Transport of macromolecules through microvascular walls. *Cardiovasc. Res.* **32**, 644-653.

Michel, C. C. and Curry, F. E. (1999). Microvascular permeability. *Physiol. Rev.* **79**, 703-761.

Mott, J. C. (1957). The Cardiovascular System. In *The Physiology of Fishes*, vol. I (ed. M. E. Brown), pp. 81-108. New York, Academic Press.

Nichols, D. J. (1987). Fluid volumes in Rainbow trout, *Salmo gairdneri*: application of compartmental analysis. *Comp. Biochem. Physiol.* **87A**, 703-709.

Nikinmaa, M., Soivio, A. and Railo, E. (1981). Blood volume of *Salmo gairdneri*: Influence of ambient temperature. *Comp. Biochem. Physiol.* **69A**, 767-769.

Olson, K. R. (1992). Blood and extracellular fluid volume regulation: role of the renin-angiotensin system, kallikrein-kinin system, and atrial natriuretic peptides. In *Fish Physiology*, vol. XIIB (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 135-254. San Diego, Academic Press.

Olson, K. R. (1996). Secondary circulation in fish: Anatomical organization and physiological significance. *J. Exp. Zool.* **275**, 172-185.

Randall, D. J. (1970). The circulatory system. In *Fish Physiology*, vol. IV (ed. W. S. Hoar and D. J. Randall), pp. 133-172. New York, Academic Press.

Ronald, K., Macnab, H. C., Stewart, J. E. and Beaton, B. (1964). Blood properties of aquatic vertebrates. 1. Total blood volume of the atlantic cod, *Gadus morhua* L. *Can. J. Zool.* **42**, 1127-1132.

Schnitzer, J. E., Carley, W. W. and Palade, G. E. (1988). Albumin interacts specifically with a 60-kDa microvascular endothelial glycoprotein. *Proc. Natl. Acad. Sci. USA* **85**, 6773-6777.

Shipley, R. A. and Clark, R. E. (1972). *Tracer Methods for* in vivo *Kinetics – Theory and Applications*. New York, Academic Press.

Sleet, R. B. and Weber, L. J. (1983). Blood volume of a marine teleost before and after arterial cannulation. *Comp. Biochem. Physiol.* **76A**, 791-794.

Smith, L. S. (1966). Blood volumes of three salmonids. *J. Fish. Res. Bd. Can.* **23**, 1439-1446.

Steffensen, J. F. and Lomholt, J. P. (1992). The secondary vascular system. In *Fish Physiology*, vol. XIIA (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 185-217. San Diego, Academic Press.

Steffensen, J. F., Lomholt, J. P. and Vogel, W. O. P. (1986). *In vivo* observations on a specialized microvasculature, the primary and secondary vessels in fishes. *Acta Zool.* **67**, 193-200.

Sørensen, K. K., Melkko, J. and Smedsroed, B. (1998). Scavenger-receptor-mediated endocytosis in endocardial endothelial cells of Atlantic cod *Gadus morhua*. *J. Exp. Biol.* **201**, 1707-1718.

Tort, L., González-Arch, F., Torres, P. and Hidalgo, J. (1991). On the blood volume of the Mediterranean dogfish, *Scyliorhinus canicula*. *Fish Physiol. Biochem.* **9**, 173-177.

Twelves, E. L. (1972). Blood volume of two Antarctic fishes. *Brit. Ant. Surv. Bull.* **31**, 85-92.

Vogel, W. O. P. (1985a). The caudal heart of fish: not a lymph heart. *Acta Anat.* **121**, 41-45.

Vogel, W. O. P. (1985b). Systemic vascular anastomoses, primary and secondary vessels in fish, and the phylogeny of lymphatics. *Alfred Benz. Symp. Copenhagen* **21**, 143-159.

Vogel, W. O. P. (1999). Anatomy and relationships of the secondary vascular system of fish. [Abstract] *Comp. Biochem. Physiol.* **124A** (Suppl.), S39.

Vogel, W. O. P. and Claviez, M. (1981). Vascular specialization in fish, but no evidence for lymphatics. *Z. Naturforsch.* **36C**, 490-492.

Wardle, C. S. (1971). New Observations on the lymph system of the plaice *Pleuronectes platessa* and other teleosts. *J. Mar. Biol. Assn UK* **71**, 977-990.

Webber, D. M., Boutilier, R. G. and Kerr, S. R. (1998). Cardiac output as a predictor of metabolic rate in cod *Gadus morhua*. *J. Exp. Biol.* **201**, 2779-2789.