THE DISTRIBUTION OF BLOOD FLOW IN THE GILLS OF FISH: APPLICATION OF A NEW TECHNIQUE TO RAINBOW TROUT (SALMO GAIRDNERI)

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SUMMARY

Injecting vitally stained blood cells into the ventral aorta of unrestrained cannulated fish, and rapid freezing in liquid nitrogen, provided a method of investigating blood flow patterns in the gills. Rainbow trout in air-saturated water perfused an average of 58% of the secondary lamellae of the gills. Perfusion of the filamental central compartment was insignificant indicating that the effects of any non-respiratory shunting would be unimportant. If the functional surface area of the gills is variable, it seems likely that this would be accomplished through lamellar recruitment. There was no evidence that pillar cell contraction affected lamellar perfusion. There was preferential perfusion of lamellae near the base of the filaments, and of filaments near the dorsal end of the gill arches.

INTRODUCTION

A large body of indirect evidence, based primarily on changes in the diffusion capacity and vascular resistance of the gills, suggests that fish are capable of regulating the functional capacity of the gills to exchange gases (Keys & Bateman, 1932; Östlund & Fänge, 1962; Steen & Kruysse, 1964; Randall, Holeton & Stevens, 1967; Wood & Randall, 1971; Rankin & Maetz, 1971; Motais & Isaia, 1972; Bergman, Olson & Fromm, 1974; Wood, 1974, 1975). Such regulation might serve to reduce the costs of osmoregulation during periods of rest, when the fish does not require the full gas exchange capacity of the gills (Steen & Kruysse, 1964). While gas exchange depends on several factors, including convection of blood and water, diffusion distance, and permeability, much recent interest has centred on the possibility that fish might be able to alter the proportion of the total gill surface area which is participating in gas exchange.

Two models have been described to explain how such changes in the functional surface area might be achieved. The first proposes a non-respiratory shunt permitting blood to move from the afferent to the efferent filamental artery through a ‘central compartment’ in the body of the filament, or through an anastomosis of the filament arteries at the tip of the filament, instead of through the respiratory pathway across the secondary lamellae (Steen & Kruysse, 1964; Richards & Fromm, 1969). Recent anatomical studies (Gannon, Campbell & Randall, 1973; Morgan & Tovell, 1973; Vogel, Vogel & Kremers, 1973; Fromm, 1974; Laurent & Dunel, 1976; Vogel, Vogel
& Pfautsch, 1976; B. J. Gannon, G. Campbell, and D. J. Randall, in preparation) and a study employing radioactive microbeads 15 µ and 50 µ in diameter (Cameron, 1974) have failed to demonstrate such a shunt pathway.

The initial report of a non-respiratory shunt (Steen & Kruysse, 1964) was based primarily on studies of eels, which possess a considerable capacity for cutaneous gas exchange (Berg & Steen, 1965). Cameron (1974) estimated that in rainbow trout even a small admixture of venous blood from a non-respiratory shunt would considerably reduce the $P_{O_2}$ of the blood in the dorsal aorta. Direct observations of the dorsal aortic $P_{O_2}$ in the resting rainbow trout have shown that this is not the case (Holeton & Randall, 1967b; Randall et al. 1967; Davis, 1971; Holeton, 1971). A non-respiratory shunt would not be of obvious advantage to a fish unless further gas exchange could occur at some other location, such as the skin or an auxiliary breathing organ.

For most fish, the second model, involving the perfusion of varying numbers of secondary lamellae (Steen & Kruysse, 1974; Randall, 1970; Hughes, 1972), would seem to be a better strategy for changing the functional surface area of the gills. Such a system avoids the reduction in dorsal aortic $P_{O_2}$ which is inherent in shunting. Furthermore, it would result in decreased branchial resistance during periods of high oxygen demand, reducing the work load on the heart. During periods of low demand, progressive shutdown of lamellae would reduce the exposure of the blood to unfavourable osmotic conditions or toxic compounds in the water around the fish.

Lamellar recruitment could be regulated by contraction of the pillar cells in the lamellar blood spaces (Hughes & Grimstone, 1965; Newstead, 1967) or by the contraction of muscle cells in the afferent and efferent lamellar arterioles (Richards & Fromm, 1969; Morgan & Tovell, 1973). Apparently contracted pillar cells have been reported (Hughes & Wright, 1970; Morgan & Tovell, 1973; Hughes & Byczkowska-Smyk, 1974) but these have never been correlated with lamellar perfusion.

The only direct evidence favouring lamellar recruitment has been provided by Davis (1972). He observed increased blood flow in the secondary lamellae at the tips of the gill filaments of live intact rainbow trout following injection of adrenaline. The resolution of his technique was not fine enough to obtain observations at the level of the individual secondary lamellae. Other attempts to determine the model which best describes how the functional surface area is regulated in vivo have lacked validity since the techniques have involved either isolated gill arches (Steen & Kruysse, 1964; Richards & Fromm, 1969; Rankin & Maetz, 1971; Bergman et al. 1974) or caused severe disturbances of the fish through anaesthesia, unnatural positions, or surgery (Cameron, 1974; Wood, 1974, 1975).

In order to be able to distinguish between ‘anatomical’ and ‘physiological’ pathways in the gills, a method is required which will permit observations to be made of the distribution of blood flow in live undisturbed fish. This report describes a technique in which blood cells marked with a fluorescent vital dye were injected into the ventral aorta of rainbow trout. Rapid freezing of the gills in liquid nitrogen was used to stop the blood flow before the effects of handling could be expressed. The stained cells provided direct evidence of the distribution of blood flow at the moment of freezing.
MATERIALS AND METHODS

Rainbow trout (Salmo gairdneri) weighing between 200 g and 350 g (Franklin Trout Farm Ltd, Mount Albert, Ontario) were kept indoors in 850 l tanks of aerated, dechlorinated water maintained at 10 °C. The fish had rather large amounts of body fat so they were starved until much of this fat had been metabolized. They were then fed a maintenance diet of commercial trout pellets.

For experimentation, the ventral aorta was cannulated after the method of Holeton & Randall (1967a) using a 23 gauge hypodermic needle attached to 40 cm of PE50 polyethylene tubing. The fish was then placed in an opaque chamber (10 l capacity) in recirculated, aerated water maintained at 5 °C, where it was allowed to recover.

After 24 h, a 0.5 ml sample of blood was withdrawn through the cannula and replaced with heparinized Courtland saline (Wolf, 1963) to maintain blood volume. The blood sample was added to 5 ml of Courtland saline containing 15 i.u./ml heparin. This mixture was then combined with 5 ml of 1.1 x 10⁻³M solution of a fluorescent vital dye, acridine orange (Fisher Scientific), in unheparinized saline. Heparin formed a precipitate with acridine orange, so the above sequence was adopted to permit the heparin to react with the plasma proteins before the dye was added. The blood–dye mixture was left to stand for 10 min and then centrifuged for 1.5 to 2 min at about 7000 g. This was long enough to sediment the blood cells but left most of the remaining precipitate in suspension. The small amount of precipitate which remained with the cells occurred as minute particles (approximately 1–2 μ diameter), much smaller than the blood cells. The supernatant was discarded and the stained blood cells resuspended in 0.5 ml of heparinized Courtland saline. The stained cell suspension was then injected into the fish through the cannula at a rate of 0.8 ml/min.

Preliminary measurements showed that the injection rate, amounting to no more than 15% of the cardiac output (Cameron & Davis, 1970), was sufficiently slow that no changes occurred in the heart rate or dorsal aortic blood pressure. However, the injection rate was sufficiently fast that freezing could be completed before the marked cells could complete a circuit of the circulatory system (Fig. 1). This prevented any confusion which might result if the circulation through some regions of the gills is shut down for a relatively short period of time.

While the stained cells were still being injected, the fish was plunged into a container of either isopentane cooled to −155 °C, or liquid nitrogen, to stop the blood flow through the gills. Temperature measurements obtained with a thermistor bead implanted among the gill filaments revealed that the rate of cooling was so slow that changes in the distribution of blood flow would not be prevented unless the freezing medium could come into direct contact with the gills. Thus, a method of exposing the gills to the coolant was required which did not interfere with the normal breathing movements of the fish before freezing.

Several techniques were tried for opening the opercula at the moment of freezing. The one which proved most reliable, and least disturbing to the fish, involved attaching small loops (about 1.5 cm diameter) of stainless steel suture wire to the opercula while the fish was anaesthetized for cannulation. The loops were passed through two small holes drilled in the operculum, and through two 5 mm diameter plastic buttons placed one on either side of the operculum to prevent the wire from cutting. When the
fish was about to be frozen, the two arms of a specially made steel retractor were slipped into the loops. When the fish was dropped into the cooling medium, the arms of the retractor would spring apart, retracting the opercula and exposing the gills. Placement of the retractor lasted 1–2 s. Measurements obtained with a thermistor implanted among the gill filaments showed that the temperature was reduced from 5 °C to −10 °C less than two seconds after the fish was lifted from the chamber (Fig. 1).

Cold isopentane, which was used for the first six fish, is frequently used to freeze small pieces of tissue because it does not boil on contact with warm tissue, facilitating the transfer of heat. This study required that only a few millimetres depth of tissue be frozen quickly, and it was found that liquid nitrogen alone caused the same rate of cooling of superficial tissues. Since there was no special advantage in using isopentane as well, its use was discontinued in favour of liquid nitrogen for the last four fish.

The frozen gill arches were removed from one side of the fish, separated, and cut into blocks of about 10 filaments each. While still frozen, 8 μ sections of the tissue block were cut along the longitudinal axis of the filaments in the plane of the arch, using a microtome mounted in a cryostat maintained at −15 °C. The sections were thawed and dried on microscope slides and examined without further treatment. Examination was carried out using a Leitz Wetzlar fluorescence microscope equipped with a BG12 3 mm transmission filter (transmission peak at 400 nm) and a K530 barrier filter which transmits all wavelengths greater than 530 nm.
Fig. 2. Sections of trout gill tissue containing stained blood cells, viewed with a fluorescence microscope. (A) A section of part of one filament showing variations in perfusion between adjacent lamellae. Calibration bar 25 μ. (B) A section through part of two filaments showing variations in perfusion between adjacent filaments and perfusion of the central compartment with stained blood cells. Calibration bar 100 μ. Symbols: s, side branch of filament support rod; c, central compartment.
**Blood flow in gills of rainbow trout**

To estimate the degree of perfusion of the gills, the numbers of completely perfused, unperfused, and half-perfused (0.25–0.75 of the blood cells stained) secondary lamellae were obtained for every fifth filament from each hemibranch. Comparison of the perfusion of the gills showed that the proportion of lamellae perfused was essentially the same on both sides of the fish. Thus estimates were made for the arches on one side only. The proportion of secondary lamellae in a hemibranch which were perfused could be obtained from the equation

\[
\text{proportion perfused} = \frac{x + y/2}{z}
\]

where 

- \(x\) = number of completely perfused lamellae,
- \(y\) = number of half-perfused lamellae,
- \(z\) = total of perfused, half-perfused, and unperfused lamellae.

To calculate the proportion of lamellae perfused in a fish, the three perfusion totals were summed for all eight hemibranchs and the totals were applied to the above equation.

**RESULTS**

The nuclei of blood cells stained with acridine orange, when examined under the fluorescence microscope, appeared reddish-orange if a high concentration of dye was used. The colour of the nuclei changed to orange, yellow, yellow-green, or green as the concentration of dye was reduced. Mixing well-stained cells with unstained cells in vitro resulted in all of the cells in the suspension becoming stained. The colour of fluorescence, which was uniform between cells, depended on the original proportions of stained and unstained cells. The dye treatment did not appear to change the size or shape of the blood cells at the concentrations used, when compared with unstained cells in vitro. Also, stained cells appeared to have compressed and bent in the secondary lamellae, in the same manner as unstained cells, suggesting that the dye had little or no effect on the flexibility of the blood cells.

Microscope observations of sections of gill tissue during thawing and drying revealed no movement of blood cells in the secondary lamellae or small blood vessels, and very little movement of cells in the branchial arteries. There was no apparent shrinkage of tissue sections during thawing and drying, as long as all of the section lay flat on the slide. New sections of tissue were almost invisible in the absence of stain. After a few hours, the tissue developed a faint auto-fluorescence, appearing greenish-brown with the filters used.

Observations of the afferent branchial and afferent filamental arteries, using the fluorescence microscope, showed that all of the blood cells fluoresced reddish-orange, orange or yellow. Blood cells in the efferent branchial and efferent filamental arteries were usually yellow or green.

Observations of the gill sections using conventional light microscopy revealed the presence of blood cells in every secondary lamella. Using fluorescence microscopy, it was observed that, while some secondary lamellae contained only stained blood cells fluorescing orange, yellow, or green, others contained few or no stained cells (Fig. 2). Areas of reduced perfusion consisted of (a) single lamellae, (b) groups of lamellae on one or both sides of a filament, (c) whole filaments, (d) groups of filaments (Figs. 2, 3).
Table 1. The proportion of secondary lamellae perfused in the gills of rainbow trout in air-saturated water at 5°C. Values are calculated for each hemibranch (HB) and for each fish.

<table>
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<tr>
<th>Fish no.</th>
<th>Arch 1 HB 1</th>
<th>Arch 2 HB 1</th>
<th>Arch 3 HB 1</th>
<th>Arch 4 HB 1</th>
<th>Whole fish</th>
</tr>
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Fig. 3. Diagram of part of a gill arch showing typical distribution patterns of stained blood cells (darkened regions) in the secondary lamellae of the gill filaments. Patterns (a) and (f) were found less frequently than the others.

The proportion of lamellae perfused for each fish and for each individual hemibranch is shown in Table 1.

Stained cells were observed anywhere within the length of a partially perfused lamella, but were more frequently seen near the base or outer margin (Fig. 2). No particular preference was observed for the marginal channel. While the thickness of
the secondary lamellae occasionally varied, there was no apparent relationship of this variable with the perfusion of the lamellae. The distribution of perfused lamellae was quite variable, but certain basic patterns were observed. Generally, a group of lamellae containing only stained cells at or near the base of the filament was separated by a number of lamellae containing a few stained cells from a group of lamellae near the tip of the filament in which no stained cells were visible (Fig. 3). More lamellae containing stained cells were found in filaments near the dorsal portion of the arch compared to the ventral end.

A central compartment was visible in most of the filaments examined. It appeared to be at least partially divided into subcompartments by cartilaginous side branches of the filament support rods (Fig. 2B). The width of the compartment varied between filaments, even when sectioned at the same location on the filament. Neither the presence of stained cells fluorescing yellow-green or green in the central compartment (Fig. 2B), nor the size of the compartment space, seemed to be related to the level of perfusion of the secondary lamellae in the same filament. In some sections, yellow-green or green fluorescing blood cells were also found in vessels running beside the afferent filamental artery.

DISCUSSION

The techniques described in this paper incorporate two significant improvements over previous methods used to study gill blood flow. The system of rapid freezing permits observations to be made on fish which, until the moment of freezing are alive, intact, unrestrained, and undisturbed. Of course, the most meaningful results will only be obtained if the blood can be stopped before the distribution of blood flow can change in response to the handling. It would be very difficult to determine the precise moment at which blood flow ceases. However, the rapid cooling rate achieved, combined with the very thin tissue involved, make it reasonable to assume that blood flow should stop before the vascular smooth muscle in the vessel walls can significantly alter the flow pattern of blood in the gill filaments (Fig. 1). The second improvement in methodology involves vitally staining the blood cells to obtain information on their movement. Previous studies have used india ink (Steen & Kruysse, 1964; Richards & Fromm, 1969) which behaves more like a plasma marker, or have used rigid microbeads (Cameron, 1974). Neither marker can be expected to accurately mimic the physical properties and movements of the blood cells. Yet this information is important since it is the red blood cells which are the blood component of the greatest respiratory significance. By using a fluorescent vital dye, it was possible to definitely identify vessels which blood cells had entered prior to the moment of freezing. Observations made on the stained cells in vitro and in situ gave no indication that the stained cells behaved any differently than unstained cells.

The in vitro observation that a mixture of stained and unstained cells became uniformly stained explains why all the cells in the afferent branchial artery were stained, even though the injected cells represented only 15% of the cardiac output. Obviously the dye is not firmly bound, and will move out of the cell during mixing in the presence of a suitable concentration gradient. Similarly, if the flow through a region of the gills is slowed, more dye would be lost by stained cells to the vessel walls before freezing. The cells frozen in such a region would appear greener than
those in a region with a high rate of blood flow, where each blood cell would lose less dye.

The number of stained cells found in the central compartment was trivial compared to the number in the secondary lamellae. Also, there was no inverse relationship in the perfusion of the two pathways which might be expected if the exposure of the blood to the respiratory surface was reduced by non-respiratory shunting. Thus the considerable reduction observed in the proportion of the respiratory surface perfused with blood is probably achieved by regulating the recruitment of lamellar blood flow.

Some ability to control blood flow at the level of the filamental arteries is suggested by the observations of complete filaments which remain unperfused. But the distribution of stained cells within filaments indicates that control must also occur at the level of the individual secondary lamellae. The apparent preference for perfusion of lamellae near the base of the filaments and of filaments near the dorsal end of the arch may be the result of attempts by the fish to regulate the local ventilation/perfusion ratio (Wood, 1975).

There was no evidence of any relationship between the thickness of a secondary lamella and the presence of stained blood cells in the lamellar channels. Thus it seems unlikely that lamellar blood flow is mediated by contraction of the pillar cells (Hughes & Grimstone, 1965; Newstead, 1967). This does not preclude variations in flow patterns within single lamellae being affected by pillar cell contraction, but total lamellar flow is probably controlled by muscle tissue in the lamellar arterioles (Richards & Fromm, 1969; Morgan and Tovell, 1973).

The lack of any obstruction of the marginal channel by pillar cells of the secondary lamellae (Hughes & Grimstone, 1965) and its apparently larger diameter compared to the other lamellar channels (Skidmore & Tovell, 1972; Hughes & Byczkowska-Smyk, 1974) have often led to suggestions that it might represent a preferred pathway for lamellar blood flow (Hughes & Grimstone, 1965; Newstead, 1967; Rankin & Maetz, 1971; Hughes & Byczkowska-Smyk, 1974; Laurent & Dunel, 1976). Support for this hypothesis is reduced somewhat by the observations that, in trout gills, the marginal channel is by no means the only unobstructed channel through the lamellae (Morgan & Tovell, 1973), that it is only wider than the other channels at the afferent and efferent ends (B. J. Gannon, G. Campbell and D. J. Randall, in preparation), and that, in casting experiments, it is never exclusively perfused (Laurent & Dunel, 1976). Muir & Brown (1971) observed a similar arrangement of the pillar cells into distinct rows in the secondary lamellae of scombroid fishes. They believed that this arrangement served to organize the blood flow within the lamellae. In the present study, the observed preference for flow through the channels near the base or outer margin, but not the marginal channel exclusively, was probably due to the reduced resistance to flow offered by the channels in these parts of the lamellae, compared to the less regular channels in the middle. Certainly there was no evidence that lamellar blood flow was directed exclusively through the marginal channels by the contraction of the pillar cells.

The present study has demonstrated that the necessary conditions exist for lamellar recruitment (i.e. unperfused lamellae in resting fish). Since 58% of the lamellae were perfused in resting fish, the maximum possible increase in the number of lamellae perfused is about 70%. But fivefold increases in the oxygen uptake have been observed
in moderately exercised fish (Stevens & Randall, 1967). Thus, if variations in functional surface area of the gills do occur, their contribution to changes in the gas exchange capacity of the gills might prove to be rather small.

The direction and role of blood circulation in the central compartment remains uncertain. Gannon et al. (1973), Laurent & Dunel (1976) and Vogel et al. (1976) found small vessels connecting the efferent filamental artery with the central compartment in the gills of rainbow trout. Other vessels connect the central compartment with blood vessels running beside and parallel to the afferent filamental artery (Gannon et al. 1973; Laurent & Dunel, 1976). They proposed that blood flowed from the efferent filamental artery, through the central compartment and back toward the heart through the vessels beside the afferent filamental artery.

Vogel et al. (1973) found a similar arrangement of blood vessels in the gill filaments of Tilapia mossambica. They also found evidence of vessels connecting the afferent filamental artery with the central compartment. However they concluded that these vessels were too narrow and too few in number to represent a significant shunt pathway. Although such vessels have never been observed in rainbow trout, that does not mean that they do not exist. The severity of treatment of a fish required by vascular casting could result in blockage or closing of pathways which might be available in unhandled fish. The technique used by Vogel et al. (1976) was inadequate to screen the afferent lamellar arterioles which, as they suggest, might also have connections with the central compartment.

In the present study the stained cells in the central compartment, as well as those lying in the vessels lying beside the afferent filamental artery, were dull green or slightly brighter yellow-green in colour. They resembled the blood cells in the efferent filamental artery more than the bright orange cells in the afferent filamental artery. While this evidence cannot be considered proof that blood in the central compartment comes from the efferent filamental artery, the amount of dye lost by the cells in the central compartment does indicate that these cells had sustained a longer contact with unstained tissue than would be likely if they had entered the compartment through short vessels directly from the afferent filamental artery.

If the role of blood flow in the central compartment is nutritive, as proposed by Gannon et al. (1973) and Morgan & Tovell (1973), it is difficult to understand the complex structure of the compartment in rainbow trout. A possible explanation, which could account for the striking variability of the dimensions of the compartment, is that changes in hydrostatic pressure, combined with the distensible wall of the compartment (Vogel et al. 1973) could serve to vary the position of the secondary lamellae in the respiratory water flow.

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Blood flow in gills of rainbow trout


