Energy cost of NaCl transport in isolated gills of cutthroat trout

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Morgan, John D., and George K. Iwama. Energy cost of NaCl transport in isolated gills of cutthroat trout. *Am. J. Physiol.* 277 (*Regulatory Integrative Comp. Physiol.* 46): R631–R639, 1999.—Few studies have made direct estimates of the energy required for ion transport in gills of freshwater (FW) and seawater (SW) fish. Oxygen consumption was measured in excised gill tissue of FW-adapted cutthroat trout (*Oncorhynchus clarki clarki*) to estimate the energy cost of NaCl transport in that osmoregulatory organ. Ouabain (0.5 mM) and bafilomycin A_1 (1 μM) were used to inhibit the Na^+-K^+ and H^+ pumps, respectively. Both inhibitors significantly decreased gill tissue oxygen consumption, accounting for 37% of total tissue respiration. On a whole mass basis, the cost of NaCl uptake in the FW trout gill was estimated to be 1.8% of whole animal oxygen uptake. An isolated, saline-perfused gill arch preparation was also used to compare gill energetics in FW- and SW-adapted trout. The oxygen consumption of FW gills was significantly (33%) higher than SW gills. On a whole animal basis, total gill oxygen consumption in FW and SW trout accounted for 3.9 and 2.4% of resting metabolic rate, respectively. The results of both experiments suggest that the energy cost of NaCl transport in FW and SW trout gills represents a relatively small (<4%) portion of the animal's total energy budget.

oxygen consumption; bafilomycin; proton pump; ouabain; sodium pump; *Oncorhynchus clarki clarki*

THE TELEOST FISH GILL plays an important role in maintaining whole animal ionic homeostasis in both freshwater (FW) and seawater (SW) environments (13). Fish gills have been shown to be metabolically very active (23, 33), and biochemical studies suggest that much of gill tissue metabolism is due to the oxidative demands of the chloride cell, using both glucose and lactate as substrates (37, 47). The current model for the mechanism of active NaCl uptake by the gill in FW involves an apical proton pump (V-type H^+-ATPase) and basolateral sodium pump (Na^+-K^+-ATPase) (32). NaCl excretion by SW chloride cells is thought to be driven by an $Na^+/K^+/Cl^-$ carrier system and the Na^+-K^+-ATPase enzyme (24). The energy cost of NaCl transport in FW and SW gills has been estimated on a theoretical basis by Eddy (11) and Kirschner (26, 27), with slightly different results. Eddy (11) used a thermodynamic analysis and calculated the energy cost of ion regulation in rainbow trout (*Oncorhynchus mykiss*) gills to be 1% of resting metabolic rate in FW and 0.5% in SW. Applying a "molecular" approach, Kirschner (26, 27) estimated the energy required for ion regulation in FW trout gills to be ~1.6% of resting metabolic rate and the energy demands for ion regulation to be higher in SW gills (5.7%).

An experimental approach to estimating the energy cost of NaCl transport is to measure oxygen consumption rates in isolated preparations of osmoregulatory organs. Inhibitors of ion translocating enzymes can be used on these tissues to estimate the ion transport-related portion of total tissue respiration. Ouabain is known to specifically inhibit Na^+-K^+-ATPase activity, and this drug has often been used on excised tissues to estimate the sodium pump-dependent portion of oxygen consumption (25, 39, 42, 54). A number of inhibitors have been used to assess V-type H^+-ATPase activity (e.g., Refs. 4, 44), although none have been used to examine the effects on proton pump-dependent oxygen consumption. Bafilomycin A_1 is a macrolide antibiotic that is a very specific and potent inhibitor of V-type H^+-ATPase (4). These inhibitors were used in the present study to assess the oxygen cost of NaCl uptake in excised FW gill tissue of cutthroat trout (*O. clarki clarki*). Calculations were also made on a total mass basis to estimate the percentage of energy required for NaCl uptake in the FW gill compared with the resting metabolic rate of the whole animal.

Isolated, saline-perfused gill arch preparations have been used extensively in studies of branchial hemodynamics, ion exchange, and gas transfer and have been used to make significant contributions to the understanding of these mechanisms in the fish gill (see review, Ref. 46). Perfused gill preparations have been criticized for their inability to duplicate in vivo conditions, due to possible abnormal mucus production, edema, inadequate irrigation, and high vascular resistance to flow (14, 45, 46). In addition, saline has a lower capacitance for oxygen and carbon dioxide than whole blood, making it less than ideal for mimicking in vivo conditions (23). Despite these problems, isolated, saline-perfused gills have an advantage over excised, chopped gill filaments in ion regulation studies because they effectively separate serosal and mucosal media and thus more closely simulate ionic gradients found between blood and water in the in vivo state. Furthermore, perfused gill preparations have been used to measure gill oxygen consumption requirements in two species of marine fish, the Atlantic cod *Gadus morhua* (23) and European flounder *Platichthys flesus* (33). Most studies using isolated, saline-perfused gills have used saline as the bathing medium and only that of Lyndon (33) has involved the measurement of oxygen uptake of perfused gills in a natural external medium (SW). In addition, few studies have compared the

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metabolic demands of intact gills between FW and SW fish.

A second objective of this study, therefore, was to measure gill oxygen consumption in FW- and SW-adapted cutthroat trout under natural conditions (i.e., FW gills immersed in an FW bath, and SW gills in an SW bath), using the isolated, saline-perfused gill preparation. The addition of ouabain to the saline perfusate has been found to inhibit the uptake of Na^+ in the isolated FW trout gill (49) and abolish the transepithelial potential across isolated SW flounder gills (51), consistent with its inhibitory effects on Na^+-K^+-ATPase. Ouabain was therefore used in the present study to assess the oxygen cost of the sodium pump in perfused FW and SW trout gills.

MATERIALS AND METHODS

Fish

Adult cutthroat trout (Chehalis River, BC, Canada) were kept in an 800-liter oval fiberglass tank receiving dechlorinated Vancouver City tap water (Na^+ <1 mM, Cl^- <1 mM, Ca^{2+} 0.03 mM) and were fed a maintenance diet of commercial salmon pellets (EWOS Canada). The water temperature was increased from ambient (6°C) to 10°C over a 1-wk period using an immersion heater, and the fish were acclimated to that temperature for at least 1 wk before testing or salinity acclimation.

Salinity Acclimation and Sampling

After the temperature acclimation, 25 fish were transferred into a second 800-liter tank (density 8 g/l) set up as a saltwater recirculation system (e.g., biofilter, ultraviolet sterilization, aeration, and temperature control). Stock SW was prepared from artificial sea salts (Deep Ocean Synthetic Sea Salt) and added to the tank at a rate of 4–5‰ per day to achieve a final test salinity of 25‰. This salinity was chosen because it approaches the highest salinity (24–26‰) that would be encountered by that stock of sea-run cutthroat trout in the outer Fraser River estuary (8). The fish were acclimated to 10°C SW for at least 2 wk before whole animal respirometry, and 6 wk before gill oxygen consumption measurements. After the 2-wk acclimation period, gill tissue and plasma samples were also taken from FW and SW trout. The fish were anesthetized with tricaine methanesulfonate (TMS, 100 mg/l) buffered with $NaHCO_3$ (100 mg/l), killed by a blow to the head, and blood was collected from the caudal vessels using heparinized syringes. The blood samples were centrifuged (2,000 *g* for 5 min), and the plasma was removed and frozen at −75°C for later cortisol, glucose, and ion analyses. Immediately after blood collection, gill filaments (~20 mg) were removed from the first branchial arch on the left side of the fish, blotted dry, placed in 0.5 ml of ice-cold sucrose buffer (in mM: 150 sucrose, 10 Na_2EDTA, 50 imidazole, pH 7.3) in a 1.5-ml microcentrifuge tube and stored at −75°C for later measurement of Na^+-K^+-ATPase and H^+-ATPase activities.

Respirometry

Whole animal oxygen consumption measurements. Oxygen consumption rates of trout acclimated to FW and SW were measured using a flow-through respirometer that consisted of a Plexiglas cylinder with plastic cones and baffle plates at each end (6). The total volume of the respirometer was 2.8 liter and the chamber was 36 cm long and 8.5 cm in diameter. Inflowing FW or SW was fed by gravity from a head tank to the respirometer using vinyl tubing. Before each trial, individual fish were lightly anesthetized with buffered TMS (50 mg/l), weighed, introduced to the chamber, and allowed to acclimate in flow-through water for 24 h. Flow rates were set to 0.5–1 l/min depending on fish size to achieve a difference in oxygen concentration between inflowing and outflowing water of ~0.5 mg/l. The respirometer was covered with black plastic throughout acclimation and testing to shield the fish from visual disturbances. After the 24-h acclimation period, water oxygen concentrations in the inflowing and outflowing water were measured using a dissolved oxygen meter (Oxyguard Mk III, Point Four Systems, Port Moody, BC, Canada). The trials were conducted at approximately the same time each day (1130–1230) to minimize diurnal variation in metabolism due to entrainment to a feeding schedule or photoperiod (5). Water temperatures during the trials were kept similar to the holding tank (9.8 ± 0.5°C). Oxygen consumption rates (mg $O_2 \cdot kg^{-1} \cdot h^{-1}$), were calculated using the equation

$$[CO_2(I) - CO_2(O)](V_w)/(M)$$

where $CO_2(I)$ is the oxygen concentration in inflowing water (mg O_2/l), $CO_2(O)$ is the oxygen concentration in outflowing water (mg O_2/l), V_w is the water flow rate through the respirometer (l/h), and M is body mass (kg).

Gill tissue oxygen consumption measurements. Oxygen consumption rates were measured in gill tissue excised from FW trout, using the procedure described in Morgan et al. (39). The fish were anesthetized (buffered TMS, 100 mg/l), weighed, and injected in the caudal vessels with heparinized saline (5,000 U/kg) using a 1-ml syringe. After 5 min in the anesthetic bath, the fish were killed and their heads were severed just behind the pectoral fins. The ventral aorta was exposed by a ventral, midline incision into the pericardial cavity and the bulbus arteriosis/ventral artery was cannulated with polyethylene tubing (Clay-Adams PE-90), secured in place with an alligator clip. The head was then immersed in 10°C FW, and the gills were cleared of blood by perfusing for 5 min with filtered (0.2 μm), heparinized (10 U/ml) fish saline (composition in mM: 139 NaCl, 5.1 KCl, 1.1 $CaCl_2$, 0.9 $MgSO_4$, 11.9 $NaHCO_3$, 3.0 NaH_2PO_4, 5.6 glucose, pH 7.5) using a peristaltic pump (Piper P-10T). The first gill arch on the left side of the fish was dissected free, placed in ice-cold saline, and gill filaments were cut into thin slices using a scalpel. Oxygen consumption of gill tissue was measured using a Strathkelvin model 781 oxygen meter and microelectrode (Strathkelvin Instruments, Glasgow, UK). The microelectrode was inserted into a glass respiration chamber, which was thermostatted to 10°C with running FW. Appropriate amounts (12–26 mg) of tissue were placed in 2 ml of air-saturated fish saline and allowed to acclimate for 5 min before testing. The decline in water oxygen tension (to the nearest 0.1 mmHg) was then monitored for 10 min, with values recorded every minute using a computer data acquisition system (Labtech Notebook version 7.11). Gentle stirring was provided by a magnetic stir bar to facilitate gas diffusion, and the tissue sat on a raised stainless steel mesh platform to prevent contact with the stir bar.

After the control trial was completed, fresh fish saline containing 0.5 mM ouabain or bafilomycin A_1 (0.1 and 1 μM treatments) was added to the respiration chamber and the oxygen consumption trial was repeated. All drugs were purchased from Sigma Chemical (St. Louis, MO). Bafilomycin A_1 was dissolved in DMSO, and the actual concentration of the stock solution was determined spectrophotometrically using the molar extinction coefficients provided in Werner et al. (60). The final concentration of DMSO in the bafilomycin trials did not exceed 0.1%, and the control trials for bafilomy-

cin also contained 0.1% DMSO. Separate control trials were run consecutively to ensure that tissue respiration in saline alone remained constant throughout the experimental period. At the end of each trial, the gill tissue was removed from the respiration chamber, blotted dry on tissue paper, and weighed to the nearest milligram. Gill filaments were also cut free from the remainder of the arches, pooled, blotted dry, and weighed to the nearest 0.01 g. Total gill mass was expressed as a percentage of body mass.

Oxygen consumption measurements for each gill tissue slice were completed within 1 h of dissection. Final water oxygen tensions in the respiration chamber were maintained >120 mmHg, and blank trials without tissue were also run to correct for electrode oxygen consumption. Oxygen consumption rates were estimated using linear regression analysis and expressed as micromoles O_2 per gram wet mass per hour.

Isolated, perfused gill preparation. Gill oxygen consumption rates were determined in FW- and SW-adapted trout (224–455 g), using the isolated, perfused gill arch preparation described by Lyndon (33), with the following modifications. The gills were cleared of blood using the procedure described above, and the first gill arch on the left side of the fish was then excised and placed in ice-cold saline for cannulation. The afferent and efferent branchial arteries were cannulated using blunt 23-gauge needles inserted into saline-filled PE 50 tubing (ID 0.58 mm). The cannulation sites were dried with a surgical sponge spear (Weck-Cel), and the cannulas were secured in place using a cyanoacrylate tissue adhesive (Vetbond, 3M). An additional drop of tissue glue was used to seal off the ends of the arch, including the branchial vein. Dissection and cannulation were completed within 5 min, and the arch was kept submerged in saline during the procedure, with the exception of the cannulation site. After cannulation, the gill arch was suspended in a cylindrical glass respiration chamber (volume 160 ml), thermostatted to 10°C and containing either aerated FW or SW (Fig. 1). Stirring was provided by a magnetic stir bar to facilitate gas diffusion. The gill was perfused with a pulsatile flow (pulse frequency 17.9 ± 0.1 per min) of fish saline using a peristaltic pump (Labconco). The efferent pressure head was set to ~15 cm above the level of the chamber. Afferent pressure was monitored using a pressure transducer (Statham P23Db) connected to the perfusion circuit and ranged from 35 to 40 cm H_2O. These afferent and efferent pressures were similar to previous saline-perfused trout gill preparations (46). Perfusion flows, determined from the perfusate effluent, were 346 ± 53 $\mu l \cdot min^{-1} \cdot g\ gill^{-1}$ ($n = 24$).

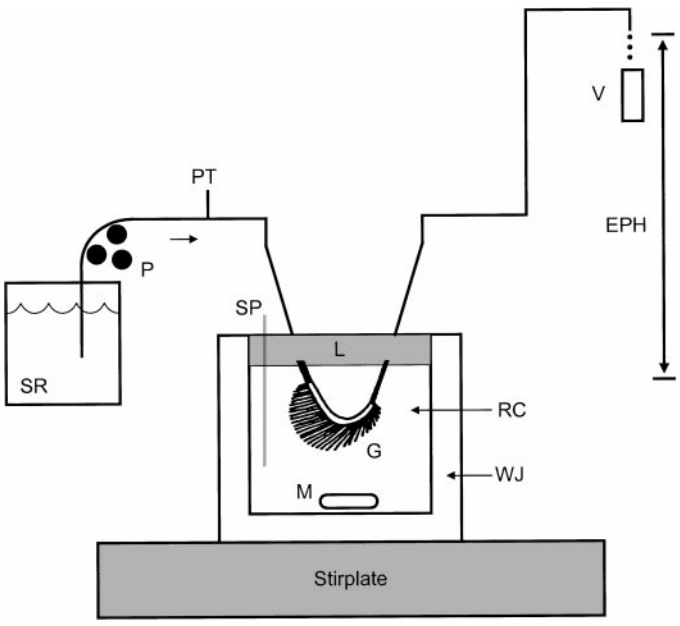

Fig. 1. Schematic diagram of setup used for measuring oxygen consumption in isolated, saline-perfused cutthroat trout gills (adapted from Lyndon, Ref. 33). EPH, efferent pressure head; G, gill; L, lid; M, magnetic stir bar; P, peristaltic pump; PT, pressure transducer; RC, respiration chamber; SP, sampling port; SR, saline reservoir; V, effluent sampling vial; WJ, water jacket. Not to scale.

The respiration chamber was closed by a fitted lid, and the cannulas were inserted through holes in the lid before the gill was cannulated, so that the gill could be sealed in the chamber immediately after the cannulation procedure. The cannulas were held in place with plastic pipette tips, and any gaps in the holes were sealed with plasticine putty. A small capillary tube was also inserted through the top of the lid to extrude air bubbles while closing the lid and to check for leakage during perfusion. Any preparations that were found to leak were discarded. The gills were allowed to equilibrate for 15–20 min before measurements were taken, these being made over the following 30 min. Measurements were made on FW gills in FW and SW gills in SW, and trials were conducted with a saline-only perfusate and a saline perfusate containing 0.5 mM ouabain ($n = 6$ for each group).

Samples for oxygen tension determinations (to the nearest 0.1 mmHg) were taken anaerobically in syringes from the aerated saline perfusate reservoir (afferent PO_2), perfusate effluent (efferent PO_2), and from the sampling port located in the lid of the respiration chamber. PO_2 measurements were made using a polarographic oxygen microelectrode (Microelectrodes, Londonderry, NH) connected to an oxygen meter (OM200, Cameron Instruments, Port Aransas, TX). Before use, the oxygen electrode was calibrated to zero with a sodium bisulphite solution, and at the beginning of each day the electrode was recalibrated to air-saturated saline equilibrated at 10°C. One microelectrode was used for all sample determinations to eliminate the problem of signal drift between multiple sensors. Final PO_2 values in the perfusate or respiration chamber never fell below 125 mmHg. Measured PO_2 values in the saline (8‰ salinity) and FW and SW (25‰) were converted to oxygen content (mg/l) using the conversion tables found in Colt (7).

At the end of each perfusion, the gill arch was removed from the chamber, blotted dry on tissue paper, and weighed to the nearest 0.01 g. The gill filaments were then removed using a scalpel, and the weight of the supporting arch alone (i.e., bone and muscle) was determined. The remaining seven arches were removed from the gill basket and weighed in a similar manner to estimate total gill mass.

Gill oxygen consumption rates ($\mu mol\ O_2 \cdot g\ wet\ mass^{-1} \cdot h^{-1}$) were calculated using the formula given in Lyndon (33), as follows

$$[(P_a - P_e) \cdot F] + [(P_i - P_f) \cdot V/t]/M$$

where P_a and P_e are the afferent and efferent oxygen contents of the perfusion saline (µmol/l), F is the efferent flow rate (l/h), P_i and P_f are the initial and final oxygen contents of the respiration chamber (µmol/l), V is the volume of the respiration chamber (l), t is the time over which the measurement was made (h), and M is the wet mass of the gill, including the arch (g).

ATPase Activity Measurements

Na^+-K^+-ATPase and H^+-ATPase activities ($\mu mol\ ADP \cdot mg\ protein^{-1} \cdot h^{-1}$) in crude gill homogenates were determined at 25°C using the coupled-enzyme assay described by Penefsky

and Bruist (43) with incorporated modifications specified by Lin and Randall (31) and Kültz and Somero (28) for H^+-ATPase and by McCormick (34) for Na^+-K^+-ATPase measurements in a microplate reader. In this kinetic assay, the ouabain-sensitive and *N*-ethylmaleimide-sensitive hydrolysis of ATP is coupled in an equimolar ratio to the oxidation of NADH, which is directly measured in 96-well microplates at 340 nm for 10 min. Protein content in the gill homogenate was determined using the bicinchoninic acid procedure (52).

Plasma Analysis

Plasma cortisol titers were determined using a ^{125}I-labeled cortisol radioimmunoassay kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA). Plasma glucose concentrations were measured using a modification of Trinder's (58) glucose oxidase method (Sigma Procedure 315). Plasma [Na^+] and [K^+] were measured on a flame photometer (Corning model 410), and plasma [Cl^-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer).

Statistical Analysis

Data are presented as means ± SE. Oxygen consumption measurements of FW gill tissue before and after the addition of ouabain or bafilomycin A_1 were compared using paired *t*-tests ($P < 0.05$). Isolated, perfused gill oxygen consumption results were analyzed using a two-way ANOVA, and significant treatment means were identified using Student-Newman-Keuls multiple comparison test ($P < 0.05$). Whole animal oxygen consumption rates, gill enzyme activities, and plasma constituents were compared using unpaired *t*-tests ($P < 0.05$).

RESULTS

FW Trout: Excised Gill Tissue Experiments

Whole animal measurements. Table 1 shows the body and gill mass, whole animal oxygen consumption rate, gill Na^+-K^+-ATPase and H^+-ATPase activities, and plasma concentration of cortisol, glucose, and ions in the FW cutthroat trout used in the excised gill tissue oxygen consumption trials. The resting metabolic rate of FW cutthroat trout measured in this experiment (~100 mg $O_2 \cdot kg^{-1} \cdot h^{-1}$) was similar to values obtained for rainbow trout (15, 48, 50). There were no significant differences between the activities of Na^+-K^+-ATPase and H^+-ATPase in gill tissue, suggesting an equivalent capacity for Na^+ transport in the FW trout gill. Plasma cortisol, glucose levels, and [Na^+], [K^+], [Cl^-] were within the normal range reported for FW salmonids (59).

Table 1. *Body and gill mass, whole animal M_{O_2}, gill Na^+-K^+-ATPase and H^+-ATPase activities, and plasma glucose and ion concentrations in FW cutthroat trout used for excised gill tissue respiration experiments*

Variable	Mean ± SE ($n = 8$)
Body mass, g	103.0 ± 3.6
Gill mass	
g	0.77 ± 0.04
% of body mass	0.75 ± 0.02
Whole animal M_{O_2}	
mg $O_2 \cdot kg^{-1} \cdot h^{-1}$	103.9 ± 8.2
mmol $O_2 \cdot kg^{-1} \cdot h^{-1}$	3.25 ± 0.26
Gill Na^+-K^+-ATPase activity, μmol ADP · mg protein$^{-1} \cdot h^{-1}$	1.27 ± 0.10
Gill H^+-ATPase activity, μmol ADP · mg protein$^{-1} \cdot h^{-1}$	1.44 ± 0.11
Plasma constituents	
Cortisol, ng/ml	57.2 ± 14.5
Glucose, mM	4.4 ± 0.4
Na^+, mM	147.8 ± 1.9
K^+, mM	2.4 ± 0.5
Cl^-, mM	132.4 ± 1.9

Values are means ± SE. FW, freshwater; M_{O_2}, whole animal oxygen consumption rate.

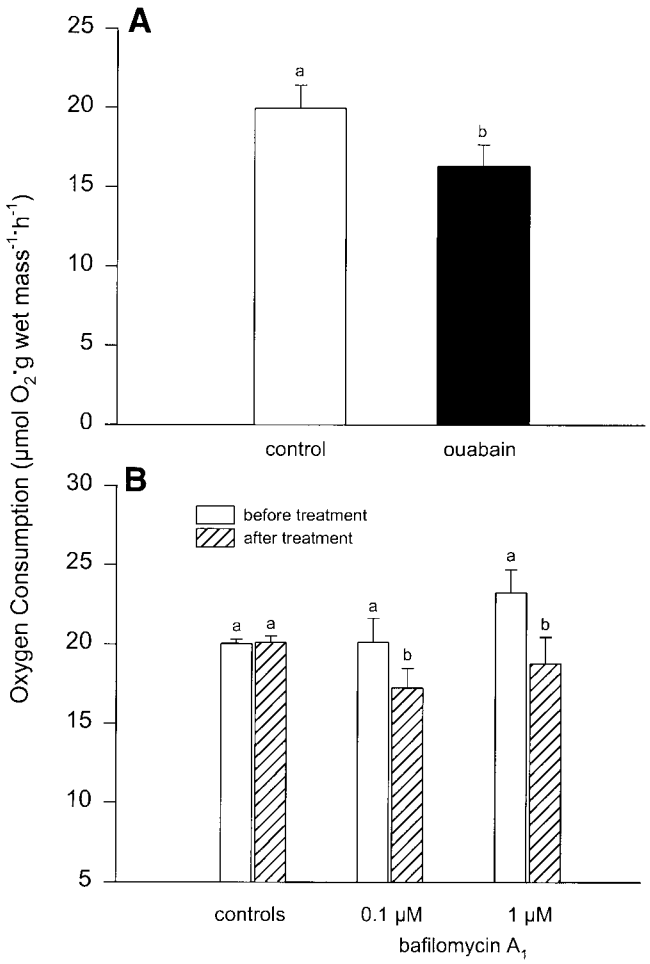

Fig. 2. Oxygen consumption of freshwater (FW) cutthroat trout gill tissue after treatment with 0.5 mM ouabain (*A*), and 0.1 and 1 μM bafilomycin A_1 (*B*). Data are shown as means ± SE ($n = 4$–6). Means with different letters are significantly different ($P < 0.05$, paired *t*-tests).

Excised gill tissue oxygen consumption. The oxygen consumption rates of excised gill tissue from FW cutthroat trout in this study averaged ~20 μmol $O_2 \cdot g$ wet mass$^{-1} \cdot h^{-1}$, which is consistent with values reported for other teleost species (19–26 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) (15, 29, 40, 54). The addition of ouabain resulted in significantly (18%) lower gill tissue oxygen consumption rates (Fig. 2*A*). The addition of bafilomycin A_1 to excised gill tissue also resulted in significantly lower gill tissue

Table 2. *Whole animal M_{O_2}, gill Na^+-K^+-ATPase and H^+-ATPase activities, and plasma cortisol, glucose, and ion concentrations in cutthroat trout after 2 wk in FW and SW*

Variable	FW	SW
Whole animal M_{O_2}, mg $O_2 \cdot kg^{-1} \cdot h^{-1}$	106.1 ± 6.8	116.5 ± 4.7
Gill Na^+-K^+-ATPase activity, μmol ADP · mg protein^{-1} · h^{-1}	1.07 ± 0.09	3.65 ± 0.37*
Gill H^+-ATPase activity, μmol ADP · mg protein^{-1} · h^{-1}	1.18 ± 0.20	0.52 ± 0.04*
Plasma constituents		
Cortisol, ng/ml	44.7 ± 14.3	33.3 ± 6.7
Glucose, mM	4.8 ± 0.6	3.4 ± 0.2*
Na^+, mM	148.2 ± 1.9	151.1 ± 2.7
K^+, mM	2.1 ± 0.2	3.7 ± 0.3*
Cl^-, mM	133.0 ± 1.9	139.9 ± 1.8*

Values are means ± SE ($n = 8$). Fish were used for isolated, perfused gill oxygen consumption experiments. *Significant difference between FW and seawater (SW) values ($P < 0.05$, unpaired *t*-test).

oxygen consumption (14% drop at 0.1 μM and 19% drop at 1 μM; Fig. 2*B*).

FW and SW Trout: Isolated, Perfused Gill Experiments

Whole animal measurements. Table 2 shows the whole animal oxygen consumption rates, gill Na^+-K^+-ATPase and H^+-ATPase activities, and plasma concentrations of cortisol, glucose, and ions in the cutthroat trout acclimated to FW and SW in this experiment. The average oxygen consumption rate in FW and SW fish did not differ statistically ($P > 0.05$). Gill Na^+-K^+-ATPase and H^+-ATPase activities were similar in FW trout, similar to the previous experiment. Gill Na^+-K^+-ATPase activity was significantly (3.4-fold) higher in SW fish compared with FW fish, whereas H^+-ATPase activity was ~56% lower after 2 wk in SW. There were no significant differences in plasma cortisol titers between the FW and SW fish, whereas plasma glucose concentrations were significantly lower in the SW group. Plasma [Cl^-] and [K^+] were significantly higher in SW fish compared with FW fish, whereas plasma [Na^+] did not differ between the two groups.

Isolated, perfused gill oxygen consumption. The oxygen consumption of isolated, perfused FW trout gills bathed in FW (10.1 ± 0.4 μmol O_2 · g wet mass^{-1} · h^{-1}) was significantly higher than SW gills bathed in SW (6.7 ± 0.6 μmol O_2 · g wet mass^{-1} · h^{-1}; Fig. 3). The addition of 0.5 mM ouabain to the saline perfusate resulted in a significant reduction of oxygen consumption in both FW (25%) and SW (37%) gills.

Table 3 shows the gill mass measurements for the FW and SW trout used in this study. The first gill arch on the left side of the fish, used for the isolated, perfused gill preparations, comprised 16% of the mass of all eight gill arches, and the gill filaments accounted for 55% of the first arch mass. The mass of all eight gill arches comprised 1.26% of the total body mass, with filament tissue accounting for 0.77% of total body mass.

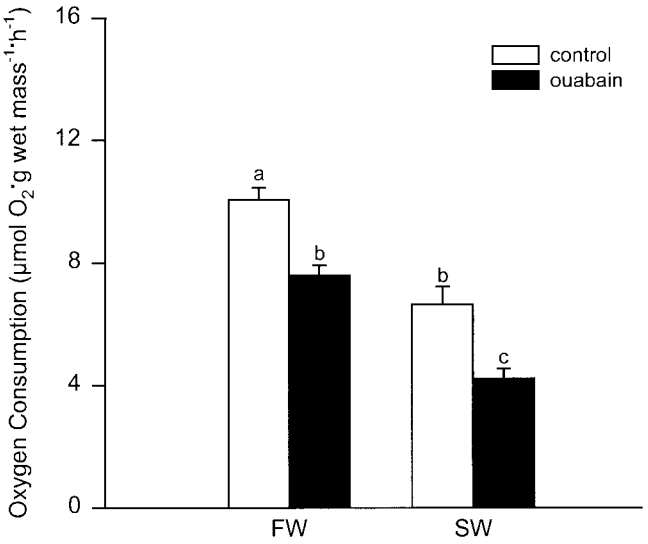

Fig. 3. Oxygen consumption of isolated, saline-perfused cutthroat trout gills in FW and saltwater (SW) and after addition of 0.5 mM ouabain to perfusate. Data are shown as means ± SE ($n = 6$). Means with different letters are significantly different ($P < 0.05$, 2-way ANOVA).

DISCUSSION

FW Trout: Excised Gill Tissue Experiments

There was a significant reduction in the oxygen consumption of excised trout gill tissue after treatment with ouabain. Ouabain-sensitive oxygen consumption represents that portion of tissue respiration that is related to the energy requirements of the sodium (Na^+-K^+) pump (39), and thus it can be estimated that ~18% of the total gill oxygen consumption was used by the Na^+-K^+ pump. Stagg and Shuttleworth (54) found that ouabain caused a decline of ~25% in the oxygen consumption of flounder gill tissue, which suggests that the portion of branchial respiration required for the Na^+-K^+ pump is similar in the two species.

Bafilomycin A_1 is a specific inhibitor of H^+-ATPase activity (9) and caused significantly lower gill tissue oxygen consumption rates (19% drop at 1 μM; Fig. 2*B*).

Table 3. *Body and gill mass of cutthroat trout acclimated for 6 wk in FW and SW and used in isolated, perfused gill preparations*

Variable	Mean ± SE ($n = 12$)
Body mass, g	310.8 ± 28.3
Gill mass, g	
1st arch	
Total, % of gill basket	0.62 ± 0.08
	15.7 ± 1.0
Filaments, % of arch	0.34 ± 0.04
	54.8 ± 1.2
8 arches	
Total, % of body mass	3.95 ± 0.43
	1.26 ± 0.04
Filaments, % of body mass	2.44 ± 0.29
	0.77 ± 0.03

Values are means ± SE. FW and SW values did not differ ($P > 0.05$, *t*-test), therefore the data were pooled.

This is the first reported effect of bafilomycin A_1 on gill tissue oxygen consumption in fish. Fenwick (18) recently reported that 1 μM bafilomycin A_1 reduced in vivo whole body influx of Na^{22} by >90% in young tilapia (*Oreochromis mossambicus*). Berenbrink and Pelster (1) also found that 0.1 μM bafilomycin A_1 significantly reduced the extracellular acidification rate in secondary lamellae preparations of the rainbow trout pseudobranch. These studies provide strong in vivo evidence for the importance of the proton pump in NaCl uptake and H^+ excretion in FW teleost fish. Nominal concentrations of bafilomycin A_1 in the 1–5 μM range have also been shown to inhibit V-type H^+-ATPase activity in other intact epithelia, e.g., insect Malpighian tubules (2), FW crab gills (41), and frog skin (12). The micromolar concentration range of bafilomycin A_1 necessary to inhibit H^+-ATPase activity in intact epithelia is higher than the nanomolar concentration range used in most biochemical studies of H^+-ATPase activity (4, 10). The in vitro V-ATPase assay uses highly purified membrane fractions (3), which greatly increases the accessibility of the enzyme binding sites to the inhibitor. Lin and Randall (31) found that 25 μM bafilomycin was necessary to inhibit H^+-ATPase activity in crude homogenates of trout gill tissue. We have also found that concanamycin A, a macrolide antibiotic structurally and functionally similar to bafilomycin (10), was not effective in significantly inhibiting H^+-ATPase activity in crude gill homogenates at concentrations up to 10 μM (unpublished data). It is possible that the difference in inhibitor sensitivities between these in vitro assay systems and intact epithelia is related to the accessibility of the macrolide antibiotics to the enzyme binding sites.

The Na^+-K^+ and H^+ pumps accounted for similar portions of total gill oxygen consumption rates (18 and 19%, respectively). This result is consistent with the similar Na^+-K^+-ATPase and H^+-ATPase activities measured in FW gill tissue (Table 1) and the equivalent theoretical stoichiometries of Na^+ and H^+ ions transported per ATPs hydrolyzed in the FW trout gill (3 H^+/ATP, 3 Na^+/ATP) (27).

Cost of NaCl Uptake in the FW Cutthroat Trout Gill

The mass-specific oxygen consumption in gill tissue (~20 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) was about sixfold higher than the whole animal oxygen uptake rate (3.25 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$; Table 1), indicating that the gills are more active metabolically than most other body tissues, reflecting their role in ion transport. This has also been found in previous measurements of fish gill oxygen consumption (23, 33) and might be expected because most (>60%) of the mass of the intact fish is composed of the trunk region (i.e., muscle, skin, scales, and bones), which has a low rate of oxygen consumption (22). Itazawa and Oikawa (22) found that the brain and kidney have the highest mass-specific oxygen consumption rates in the carp, and these organs also have higher ion-motive ATPase activities than found in FW fish gills (36). When compared on a whole mass basis for the average-sized trout used in this study (~100 g), gill oxygen consumption accounted for 4.6% of resting metabolic rate (Table 4). This value is quite similar to the estimate of 3.2% obtained by Itazawa and Oikawa (22) using excised gill tissue of carp.

Table 4. *Cost of NaCl uptake in FW cutthroat trout gill*

Whole animal M_{O_2}, μmol/h	325
Total gill O_2 consumption, μmol/h	15
Percentage of whole animal M_{O_2}	4.6
Ouabain-sensitive O_2 consumption, μmol/h	2.7
Bafilomycin-sensitive O_2 consumption, μmol/h	3.3
NaCl transport-related O_2 consumption, μmol/h	6.0
ATP required, μmol/h	36
Percentage of whole animal M_{O_2}	1.8

Whole animal M_{O_2} calculated for 100 g fish at 10°C. Total gill mass based on 0.75% of body mass. Ouabain-sensitive O_2 consumption equivalent to Na^+-K^+ pump O_2 requirement. Bafilomycin-sensitive O_2 consumption (1 μM bafilomycin A_1) equivalent to H^+ pump O_2 requirement. ATP stoichiometry, 6 ATP/O_2.

The fish gill is a heterogeneous organ comprised of several cell types, in addition to chloride cells. Most of the Na^+-K^+-ATPase enzymes in the FW trout gill are located in the basolateral membrane of chloride cells (62), whereas H^+-ATPase is concentrated in the apical region of both chloride cells and pavement cells (30, 57). If one assumes that the majority of these ion pumps in the FW trout gill is involved in active NaCl uptake, then the energy cost of this process can be calculated using the values for both ouabain-sensitive (Na^+-K^+ pump) and bafilomycin-sensitive (H^+-pump) oxygen consumption. This amounts to 6 μmol O_2/h or 1.8% of resting metabolic rate, when expressed on the basis of total fish mass (Table 4). The cost of NaCl uptake estimated for the FW cutthroat trout gill in this study is similar to the theoretical value of 1% determined by Eddy (11) and almost identical to the calculation of Kirschner (27) for the rainbow trout gill (1.6%). Eddy (11) and Kirschner (27) also provided estimates of 0.2 and 0.9% for the cost of NaCl reabsorption in the FW trout kidney for overall osmoregulatory costs of 1.2 and 2.5% of standard metabolism, respectively. The experimental approach used in this study is therefore in good agreement with the theoretical calculations and suggests that the direct energy cost of NaCl uptake in FW trout is a relatively small portion (i.e., <3%) of whole animal oxygen uptake.

FW and SW Trout: Isolated, Perfused Gill Experiments

Whole animal measurements. Plasma ion and cortisol concentrations were similar between the FW and SW cutthroat trout, indicating that the fish were fully acclimated to SW after a 2-wk period. Furthermore, resting metabolic rates in trout acclimated for 2 wk to FW and SW also did not differ in the present study. It appears that, after an initial adjustment period of a few days, whole animal oxygen uptake in SW trout returns close to FW values (50).

Gill Na^+-K^+-ATPase activity was threefold higher in SW fish compared with FW fish, whereas gill H^+-ATPase activity decreased by half after 2 wk in SW. Similar changes in the activity of these enzymes after acclimation to different salinities have been found for

rainbow trout by Lin and Randall (31) and for long-jawed mudsuckers (*Gillichthys mirabilis*) by Kültz and Somero (28). The direction, and possibly magnitude, of the changes in these ion translocating enzymes reflect the relative importance of H^+-ATPase in active NaCl uptake in FW (32) and Na^+-K^+-ATPase in active NaCl extrusion in SW (35). A small amount of gill H^+-ATPase activity is presumably required in SW fish to maintain acid-base balance (61). Although it is not possible to use in vitro ATPase activities to calculate an absolute ATP requirement of the Na^+-K^+ pump and H^+ pump, the results do suggest, at least qualitatively, that the metabolic capacity for ion transport is greater in SW gills than in FW gills (4.2 vs. 2.2 ATPase activity units).

Isolated, perfused gill oxygen consumption. The average oxygen consumption of FW cutthroat trout gills was 10.1 ± 0.4 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$. This value includes both the gill filaments and the bone, skin, and muscle of the arch. The gill tissue oxygen consumption can be calculated from this value, knowing the portion of the arch that is filaments (55% for the first arch) and assuming that the bone has a much lower rate of oxygen consumption (~2.5 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) (22). In accordance with this calculation, the oxygen consumption rate of gill tissue from the FW-adapted trout was 17.4 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$, which is quite similar to the value reported above for FW trout excised gill tissue (20 μmol $O_2 \cdot g$ wet $mass^{-1} \cdot h^{-1}$). This result indicates that the isolated, perfused gill arch preparation used in this study provided reliable estimates of gill oxygen consumption rates.

The oxygen consumption of SW trout gills bathed in SW was significantly (33%) lower than FW gills bathed in FW. Similar decreases in gill tissue oxygen consumption rates were found for cutthroat trout by Holmes and Stott (21) after 7 days in SW and for rainbow trout by Leray et al. (29) after a 10-day SW exposure period. The reasons for this decrease are not clear, because the net increase in ATPase activity measured in SW gills would predict that the gill respiration rates should be higher in SW, if ion pumping costs were a significant portion of total gill respiration. Leray et al. (29) found that after a marked drop in ATP-to-ADP ratios and energy charge during the first 3 days of SW acclimation in the rainbow trout, the adenylate pool returned to initial levels in fully adapted SW fish (*day 10*). This high energy demand in the early stages of SW acclimation is consistent with the changes that occur in gill carbohydrate metabolism (53) and may be related to the increased recruitment of chloride cells and Na^+-K^+-ATPase that occurs after 3–4 days in SW (19). In a well-acclimated SW trout, however, the energy requirements of the gill appear to decrease in SW relative to a FW fish. It is possible that protein synthesis costs, which can approach 80% of total respiration in some tissues (42), are higher in FW gills than in SW gills, however this has yet to be determined.

The addition of ouabain to the saline perfusate significantly reduced oxygen consumption in both FW (25%) and SW (37%) gills, but the ouabain-sensitive portion was similar between the FW and SW gills (2.5 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$). The difference in the percentages is due to the higher total gill oxygen consumption in the FW gills. Thus it appears that the oxygen cost of the Na^+-K^+ pump alone is not sufficient to explain the difference in total gill respiration between FW and SW trout gills. The ouabain-induced decrease in oxygen consumption in the intact FW gill arch was similar to that found in the FW excised gill tissue, suggesting that the cost of the Na^+-K^+ pump in the FW trout gill is ~20–25% of total tissue respiration. Stagg and Shuttleworth (54) also found that ouabain significantly reduced the oxygen consumption of excised gill tissue from both FW- and SW-adapted flounder by ~25%. It should be noted, however, that interpreting ouabain-sensitive respiration rates from isolated, perfused gill arches in terms of ion transport costs can be confounded by two factors. First, 45% of the gill arch mass is composed of supporting tissue (e.g., bone, muscle, skin) and the Na^+-K^+ pumps in these tissues, which are probably less abundant than in the gill filaments, are not directly involved in NaCl extrusion. Second, there is a possibility that ouabain can have a vasosensitive effect on the branchial arteries, although the data are inconclusive. For example, Farmer and Evans (17) found that adding ouabain to the perfusate caused a marked vasoconstriction and increase in afferent pressure in isolated, perfused gills of the pinfish (*Lagodon rhomboides*), whereas Stagg and Shuttleworth (55) found that ouabain had only a slight effect on vascular resistance in perfused gills of the flounder. There were no noticeable effects of ouabain on afferent pressure in this study, although the flow rates were quite variable among preparations (coefficient of variation 44%). The use of an inhibitor specific to the gill epithelia, such as bafilomycin, would overcome some of the potential vasosensitive effects that ouabain may have on arterial pressure and flow. Bafilomycin could not be used to inhibit H^+-ATPase activity in perfused FW gills in this study because of the large amounts needed for an effective concentration (>1 μM) in the external bath (160 ml). The results with the excised gill tissue suggest that the oxygen cost for the proton pump would be similar to the Na^+-K^+ pump in FW, and this would increase the ion transport-related costs in the FW gill. The effect of specific ion pump inhibitors on the oxygen consumption of the isolated, perfused gill arch requires further study.

The oxygen consumption of isolated, perfused gill arches have been compared on a total mass basis to the resting metabolic rates of the intact fish as an indication of the relative energy requirements of the gill. The value of 7% calculated by Johansen and Pettersson (23) for the marine cod gill has often been quoted in this regard (38). In making these estimates, most workers have assumed that the gill arches are of equal size (23, 33). In fact, the first gill arch is larger than the others (16% of the total gill arch mass in cutthroat trout) and this will therefore overestimate the contribution of gill oxygen consumption. The mass of all eight gill arches in the trout comprised ~1.3% of the total body mass. For an averaged-size trout in this study (~310 g; Table 3),

gill oxygen consumption accounted for 3.9% of resting metabolic rate in FW trout and 2.4% of resting metabolic rate in SW trout. The FW value is similar to the estimates obtained using excised gill tissue from FW fish (22; this study). The value for SW trout gills is lower than has been estimated for cod gills bathed in saline (7%) (23) and for flounder gills bathed in SW (31%) (33), although it should be noted that these authors probably overestimated gill mass (see above) and used literature values for resting metabolic rates that were much lower (78 and 31 mg $O_2 \cdot kg^{-1} \cdot h^{-1}$, respectively) than we have measured for SW cutthroat trout (116 mg $O_2 \cdot kg^{-1} \cdot h^{-1}$). A substantial difference in the standard metabolic rate of trout and flounder in SW has also been reported in other studies (26, 48, 56) and this is likely due to a difference in lifestyles between the active trout and more sedentary flounder. Nevertheless, it appears that the gill oxygen requirements in SW-adapted cutthroat trout are lower than in the marine flounder. This is also reflected in the higher mass-specific gill oxygen consumption rates measured in flounder gills bathed in 10°C SW (12.1 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) (33) than was measured in SW trout gills in this study (6.7 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$).

In summary, the respiration rate of FW trout gills bathed in FW was found to be higher than in SW trout gills bathed in SW. The oxygen cost of the Na^+-K^+ pump did not differ between FW and SW gills, suggesting that other metabolic processes (e.g., protein synthesis; H^+ pump) may be contributing to the higher oxygen consumption in FW gills. There was, however, a discrepancy between the ouabain-sensitive oxygen consumption results and the in vitro Na^+-K^+-ATPase activities in the FW and SW gills, which may be related to the use of ouabain on isolated, perfused gill arch preparations. The contribution of total gill arch oxygen consumption was relatively small (<4%) and did not have a significant influence on whole animal oxygen uptake, which did not differ between FW and SW trout.

Perspectives

The results of this study showed that the direct energy cost of ion transport in the fish gill was quite small. This is in agreement with previous theoretical calculations (11, 26, 27); however, a few whole animal experiments conducted in the past suggested that the energy cost of osmoregulation in teleost fish is much higher (e.g., 16, 48). These studies measured oxygen consumption rates in different water salinities and found a 20–30% increase in metabolism in FW and SW compared with that in an isoosmotic environment, at which the osmoregulatory cost was assumed to be zero. The discrepancies between the theoretical and experimental approaches are likely due to the metabolic costs of other salinity-related processes in the whole animal studies that are not directly related to osmoregulation (e.g., hormonal changes). The use of an isolated preparation in the present study allowed the separation of direct energy costs of ion transport from other whole animal metabolic responses to salinity change. Future studies in this area should focus on estimating the direct energy costs of ion transport in other osmoregulatory organs, such as the kidney and intestine.

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