The Journal of Experimental Biology **198**, 295–304 (1995)
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MECHANISMS OF Na^+ AND Cl^- REGULATION IN FRESHWATER-ADAPTED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) DURING EXERCISE AND STRESS

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Accepted 8 September 1994

Summary

This study examined the mechanisms by which Na^+ and Cl^- are regulated in freshwater rainbow trout during exercise and stress. Aerobic exercise (at approximately 2 body lengths s^{-1}) caused a brief increase in diffusive Na^+ efflux and a brief decline in plasma Na^+ and Cl^- concentrations. This disturbance was rapidly compensated by a threefold increase in Na^+ and Cl^- influx (over the first 10–12 h of exercise) and by a reduction in Na^+ efflux to 40 % of the control value by 7 h of exercise. The compensation produced a significant increase in whole-body [Na^+], whereas whole-body [Cl^-] remained unchanged. In contrast, confinement stress (for 4 or 8 h) caused an eightfold increase in Na^+ and Cl^- efflux which was sustained for at least the first 5 h of stress and resulted in large decreases in whole-body [Na^+] and [Cl^-]. Compensation of the losses was not complete until 24 h post-stress and was achieved by increases in Na^+ and Cl^- influx (of similar magnitude and timing to those observed during exercise) as well as reductions in Na^+ and Cl^- efflux to nearly zero. We conclude that ion influx increased because of an activation of inactive transport sites in the gills, whereas efflux was reduced by a reduction in branchial ionic permeability; both responses are mediated hormonally. Although the hormonal control mechanisms are as yet poorly defined, we argue that growth hormone and prolactin are responsible for the regulation of influx and efflux, respectively, and rule out either cortisol or epinephrine as having any role, at least with respect to the rapid [NaCl] regulation evident during exercise.

Key words: ion transport, ion permeability, gills, rainbow trout, hormonal control, *Oncorhynchus mykiss*.

Introduction

To extract sufficient oxygen from the surrounding water, fish require a large and permeable gill surface. In fresh water, this permeability, together with a steep outward gradient for NaCl diffusion, makes the gills a major route for NaCl loss. The freshwater-adapted rainbow trout, for example, typically loses 20–25 % of its Na^+ and Cl^- content per day through branchial diffusion (Gonzalez and McDonald, 1992). These losses are normally compensated by active, independent branchial absorption of Na^+ and Cl^- from the medium, with the result that blood NaCl homeostasis is maintained within narrow limits (McDonald and Milligan, 1992). Nevertheless, a variety of circumstances challenge this homeostasis. One of the most frequently studied is exposure to dilute media. Maintaining fish in media containing low [NaCl] (and in many instances, low [Ca^{2+}] as well) causes depletion of plasma Na^+ and Cl^-, to which the most widely reported response is an increase in branchial transport of Na^+ and Cl^- (for a review, see Perry and Laurent, 1989). In rainbow trout, this increased activity requires at least 9 days to develop fully (McDonald and Rogano, 1986), is due mainly to an increase in transport capacity (J_{max}) of the Na^+ and Cl^- carriers (McDonald and Rogano, 1986; Avella *et al.* 1987; Perry and Laurent, 1989) and is thought to result from cortisol-mediated hyperplasia and hypertrophy of branchial epithelial chloride cells (Avella *et al.* 1987; Perry and Laurent, 1989). Indeed, Perry and Laurent (1989) showed that 10 days of treatment of rainbow trout in normal fresh water with daily cortisol injections provoked chloride cell proliferation and corresponding increases in Na^+ and Cl^- influx (J_{in}^{Na} and J_{in}^{Cl}, respectively).

A reduction in diffusive ion efflux (J_{out}) has also been reported in some studies as part of the adaptation to ion-poor media and may, in fact, be the first defence employed. McDonald and Rogano (1986) showed that diffusive Na^+ and Cl^- efflux (J_{out}^{Na}, J_{out}^{Cl}) declined to about 33 % of control levels within the first 24 h of exposure to ion-poor water. Similarly, Perry and Laurent (1989) showed that in rainbow trout J_{out}^{Cl} declined before there was any significant increase in J_{in}^{Cl}. Earlier studies on ammocoete larvae, *Lampetra planeri* (Morris and Bull, 1970), and goldfish, *Carassius auratus* (Cuthbert and Maetz, 1972), also showed reductions in J_{out} in response to exposure to low ion concentrations.

Much less is known about how freshwater fish respond to

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other challenges to ion balance. Two such challenges are exercise and stress, both more likely to be encountered than is ion-poor water and with potentially greater impact. With exercise, increased rates of diffusional NaCl loss are expected to accompany the increase in O_2 consumption because of an increase in the functional surface area of the gills (Randall *et al.* 1972), whereas stress of any kind will lead to an elevation in circulating epinephrine levels (Mazeaud and Mazeaud, 1981). Previous studies (McDonald and Rogano, 1986; Gonzalez and McDonald, 1992) have emphasized the profound effect of epinephrine on diffusional losses of NaCl across the gills: effects produced by changes in gill haemodynamics and by direct increases in gill ionic permeability.

Previous studies on rainbow trout have confirmed increased rates of Na^+ efflux either during, or immediately following, exercise. However, the exercise regimes in these studies were either stressful (e.g. 1 h of forced exercise by manual chasing, Wood and Randall, 1973*a*; 5–6 min of exhaustive exercise, Gonzalez and McDonald, 1992) or strenuous (6 h of exercise at 85 % of maximum sustainable swimming speed; Gonzalez and McDonald, 1992), and their impact on ion balance was significant. For voluntary routine exercise, at speeds that salmonids can maintain for long periods, the impacts, if any, on ion balance are unknown. In contrast, the effects of stress are well established. For stressful treatments persisting for 4 h or more, substantial depressions in plasma $[Na^+]$ and $[Cl^-]$, in excess of 10 mequiv l^{-1}, have been widely reported for salmonid species (for a review, see McDonald and Robinson, 1993). However, in neither stress nor exercise is the time course or nature of the ionoregulatory response known.

Therefore, the objective of this study was to characterize more thoroughly Na^+ and Cl^- regulation in freshwater fish by using continuous aerobic exercise and stress to challenge ion balance. Stress was imposed using net-confinement, a procedure previously shown to be highly reproducible (e.g. Woodward and Strange, 1987; McDonald and Robinson, 1993). For continous exercise, a speed of approximately 2 *BL* s^{-1} where *BL* is body length, was chosen as being within the range of migratory swimming speeds for salmonids (Trump and Leggett, 1980) and therefore unlikely to be stressful. In this study, we were specifically interested in (a) whether the time course of the ionoregulatory response is as slow as that reported for exposure to ion-poor fresh water, (b) the relative contributions of uptake increase and permeability reduction to the correction, and (c) the specific feedback controls responsible for the ionoregulatory response. We have begun an investigation into hormonal control mechanisms by measuring cortisol concentrations during both exercise and stress and by examining changes in Na^+ transport following injection of growth hormone.

Materials and methods

Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), (6–25 g) were obtained from Rainbow Springs Fish Hatchery in Thamesford, Ontario, Canada. They were maintained for at least 2 weeks prior to experimentation in 500 l cylindrical tanks continuously supplied with dechlorinated, Lake Ontario municipal tap water (1 mmol l^{-1} Ca^{2+}, 0.6 mmol l^{-1} Na^+, 0.3 mmol l^{-1} Mg^{2+}, 0.8 mmol l^{-1} Cl^-) at 15±2 °C. They were fed trout chow *ad libitum* in the holding facilities until 2 days before the experiments.

Exercise apparatus

Three different sizes of annular swim chambers were employed to exercise fish. Each chamber consisted of a small inner circular tank placed concentrically within a larger circular tank. The fish exercised in the volume contained between the walls of the tanks. The nominal operating volumes of the swim chambers were 19, 60 and 450 l, with average water velocities of 23, 34 and 17 cm s^{-1}, respectively. Dimensions were as follows; 19 l model, 56 cm outside diameter × 27 cm inside diameter × 17 cm water depth; 60 l model, 56 cm × 27 cm × 24 cm; 450 l model, 92 cm × 42 cm × 68 cm. Centrifugal pumps (Little Giant model 5-MSP, 370 W) located either on the bottom of the inner tank (60 l and 450 l models, two and three pumps, respectively) or outside the outer wall (19 l model, one pump) were used to generate water flow. The water was directed through tubing and nozzles to create a uniform flow throughout the swimming chamber. Velocity was measured by an electromagnetic current meter (Marsh-McBirney model 201D). The flow chambers in the 19 l and 60 l models were divided with plastic mesh partitions into three and four sections, respectively, in order to separate groups of fish. Aeration was provided by air-stones, and temperature control by a continuous supply of water (1.5–2.0 l min^{-1} in the 450 l model), by stainless-steel cooling coils (60 l model) or by immersion in a cooling bath (19 l model).

Stress protocol

Stress was imposed with a net-confinement procedure similar to that employed previously (McDonald and Robinson, 1993). Fish were transferred to nylon mesh bags (1 cm mesh) suspended in 19 l tanks (3–6 bags per tank, 5–7 fish per bag) maintained at 15 °C. At the beginning of confinement, the bags were tightened so that the fish were in close physical contact with each other and could not move about freely. The tanks were aerated by air-stones positioned underneath the bags and supplied with compressed air at a rate sufficient to ensure that bubbles penetrated the bags.

Experimental series

Plasma and whole-body $[Na^+]$ and $[Cl^-]$ during continuous aerobic exercise

Rainbow trout (8.1±0.3 g, N=86) were exercised at approximately 1.8 *BL* s^{-1} for up to 96 h in the 450 l swim chamber. At 0, 3, 6, 12, 24, 48 and 96 h, fish ($N \geqslant 10$ per sampling period) were removed and killed by a quick blow to

the head. Body mass and fork length were recorded and a blood sample (at least 0.25 ml) was collected by caudal severance, then centrifuged and the plasma drawn off and stored frozen for later analysis of Na^+ and Cl^-. The carcass was then dried to a constant mass (48 h at 70 °C) for analysis of body Na^+, Cl^- and water content.

Na^+ and Cl^- uptake during rest and exercise

The uptake of Na^+ and Cl^- (J_{in}^{Na} and J_{in}^{Cl}) at rest and during exercise at approximately 2.0 *BL* s^{-1} was measured in separate trials by absorption from the water over 2 h periods of ^{22}Na (as NaCl, 74 kBq l^{-1}; Amersham) and ^{36}Cl (as NaCl, 74 kBq l^{-1}; ICN). Fish (8.5±2.6 g) were added to the swim chamber in groups (8–10 per group, *N*=70). The control groups (one each for measurement of J_{in}^{Na} and J_{in}^{Cl}) were allowed to acclimate to the swim chamber, with the current off, for 4 h before addition of isotope. For the exercise trials, the addition of groups of fish to the three compartments of the swim chamber (current on) was staggered, so that by the time of isotope addition they had been exercising for 2, 6 or 10 h. At the end of the 2 h isotope uptake period, all fish were removed, killed with a blow to the head, rinsed in isotope-free tap water and analyzed for whole-body levels of ^{22}Na, ^{23}Na, ^{36}Cl and ^{35}Cl. Water samples (10 ml) were also collected for analysis of ^{22}Na, ^{23}Na, ^{36}Cl and ^{35}Cl at the beginning (after mixing) and end of the 2 h isotope period.

Na^+ and Cl^- uptake kinetics

The effects of external [Na^+] and [Cl^-] on J_{in}^{Na} and J_{in}^{Cl} were determined in separate trials. J_{in}^{Na} was measured in artificial hard water (1 mmol l^{-1} $CaCl_2$ adjusted to pH 7.5) to which ^{22}Na (18 kBq $mmol^{-1}$) was added as NaCl to give Na^+ concentrations of 0.1, 0.25, 0.6, 1.2 or 2.5 mmol l^{-1}. J_{in}^{Cl} was measured in 1 mmol l^{-1} $CaCO_3$ (adjusted to pH 7.5) to which ^{36}Cl (111 kBq $mmol^{-1}$) was added as NaCl to give Cl^- concentrations of 0.1, 0.25, 0.6, 1.2 or 2.5 mmol l^{-1}. Fish (11.7±0.6 g) were held in black plastic containers (*N*=4 per container) containing 1.5 l of the artificial medium. The chambers were fitted with air lines to aerate and mix chamber contents, and fish were allowed to acclimate for 2 h before the addition of isotopes. After isotope addition, fish were left for 2 h and then removed, rinsed in isotope-free tap water, killed with a blow to the head and analyzed for whole-body levels of ^{22}Na or ^{36}Cl.

Na^+ efflux at rest and during exercise

Na^+ efflux (J_{out}^{Na}) was measured prior to (for 4 h) and during (for 0–7 h) exercise (at approximately 1.8 *BL* s^{-1}) by measuring the loss of ^{24}Na to the water from groups of fish at rest or swimming (22.3±1.5 g, *N*=104) in either the 19 l or 60 l swim chamber. 16 h before the start of efflux measurement, all fish were injected intraperitoneally with 50 μl of ^{24}Na solution (54.0 mmol l^{-1} $^{24}Na_2CO_3$, produced in the McMaster Nuclear Reactor: 370 kBq per fish) and transferred to the swim chambers for recovery (with replacement flow on, current off). During efflux measurement, water samples for analysis of ^{24}Na were collected at 0.5 h intervals, first for a 4 h rest period (replacement flow off, current off) and then for a 7 h exercise period (current on). At the end of the exercise, fish were removed, rinsed in isotope-free water, killed with a blow to the head and analyzed for ^{23}Na and ^{24}Na. This experiment was repeated six times with 15–20 injected fish per trial.

Na^+ and Cl^- balance during and following confinement stress

Whole-body [Na^+] and [Cl^-] and J_{in}^{Na} and J_{in}^{Cl} were examined during and following net confinement in two series of experiments. In the first series, the start of confinement for different groups was staggered so that, by the time of isotope addition, fish had been confined for 1, 3 or 6 h. At 6 h, ^{22}Na (as NaCl, 19 kBq l^{-1}; Amersham) and ^{36}Cl (as HCl, 111 kBq l^{-1}; ICN) were added to the water and allowed to circulate for the next 2 h. At 8 h, all fish were removed and killed with a blow to the head. Whole bodies were analyzed for ^{22}Na and ^{36}Cl activity and later digested for whole-body ion analysis. Water samples (10 ml) were also collected for analysis of ^{22}Na and ^{36}Cl at the beginning (after mixing) and end of the 2 h period. In the second series, fish (*N*=42) were confined for 4 h. At the end of the confinement, six fish were immediately sampled for analysis of whole-body [NaCl] and the remainder were transferred to 'recovery' chambers (six black plastic chambers containing 1.3 l of aerated water, *N*=6 fish per chamber) for measurements of uptake. Fish were sampled after 2, 4, 8, 16 or 24 h of recovery from the confinement. 2 h before the fish were removed, ^{22}Na and ^{36}Cl were added to the chambers as above.

Plasma cortisol levels during exercise and stress

For cortisol measurements prior to and during exercise, fish were placed in each of the three compartments of the 19 l exercise chamber and allowed to acclimate for at least 14 h. After 0, 1, 3 or 4 h of exercise at 2 *BL* s^{-1}, fish were removed one at a time and killed with a blow to the head. Blood samples (at least 0.05 ml) were obtained by caudal severance and centrifuged and the plasma frozen at −20 °C for later analysis of cortisol. The number of fish per compartment was kept to three to minimize the effects of cohort sampling on cortisol levels; the experiment was repeated until *N*=18 for t_0 and *N*=6 for each of the exercise periods. For measurements of cortisol levels during confinement stress, fish (*N*=7 at each of 2 h and 4 h of stress) were removed one at a time from the confinement nets and sampled as above. Cortisol levels prior to stress (i.e. controls for the confinement stress trial) were measured on fish (*N*=8) removed one at a time from a 500 l holding tank.

Effects of growth hormone

The acute effects of growth hormone on J_{in}^{Na} were assessed by monitoring ^{22}Na uptake for a 2 h period, starting 1 h after hormone injection. Fish (10.5±0.6 g) were injected intraperitoneally with either saline (5 μl g^{-1}, *N*=10) or 1 μg g^{-1} ovine growth hormone (NIH, Bethesda, MD) dissolved in saline (*N*=10). Following injection, fish were

transferred to black plastic chambers containing 1.5 l of aerated water at 14 °C. 74 kBq of ^{22}Na was added to the water at 1 h. Fish were killed at 3 h and then counted immediately for ^{22}Na activity.

Analytical methods

Plasma [Na^+] was measured on 10 μl samples by atomic absorption spectrophotometry (AAS, Varian 1275) following 1:1000 dilution with deionized water. Plasma [Cl^-] was determined directly on 10 μl samples by coulometric titration using a Radiometer CMT10 chloride titrator. For whole-body ion analysis, each fish was digested in 1 volume of 1 mol l^{-1} HNO_3 at 80 °C for 48 h. The digests were centrifuged and filtered and the supernatants were analyzed for Na^+ and Cl^- in the same way as the plasma samples. Water samples were analyzed for Na^+ by AAS after appropriate dilution and for Cl^- by the mercuric thiocyanate method (Zall *et al.* 1956).

Cortisol was measured on 20 μl plasma samples using a radioimmunoassay kit (Quanticoat Cortisol, Kallestad Laboratories) validated for use on trout plasma (Hontela *et al.* 1992), which involves the competitive binding of plasma cortisol and ^{125}I-labeled cortisol to tubes coated with cortisol-specific antibody.

^{24}Na and ^{22}Na were read directly (tissues, plasma and water) on a Canberra-Packard AutoGamma 5000 deep-well gamma counter with a 7.6 cm NaI crystal. ^{24}Na samples were corrected for decay ($t_{1/2}$=15 h). ^{36}Cl activity was measured on an LKB Rackbeta 1217 scintillation counter. Water samples (5 ml) were diluted 1:2 in Hionic fluor (Packard Co.). Tissues were first homogenized in 4 volumes of deionized water. Samples (0.35 ml) were then digested for 12 h at 50 °C in 1 ml of tissue solubilizer (Soluene-350, Packard Co.), cooled and diluted in 10 ml of fluor. Quench correction was found to be unnecessary.

Calculations

Na^+ and Cl^- influx (J_{in}^{Na} and J_{in}^{Cl} in nequiv g^{-1} h^{-1}) were calculated according to the following equation:

$$J_{in} = \frac{\Sigma Q}{SA \times M \times t}, \quad (1)$$

where ΣQ is the whole-body radioactivity (in cts min^{-1}), *SA* is specific activity of the water in cts min^{-1} nequiv^{-1}, *M* is body mass in grams and *t* is duration in hours.

The effects of external [NaCl] on J_{in} were analyzed by non-linear regression (SAS, 1982) using the Michaelis–Menten kinetics equation:

$$J_{in}^{X} = \frac{J_{max} \times [X]_e}{K_m + [X]_e}, \quad (2)$$

where $[X]_e$ is the ion concentration (Na^+ or Cl^-) in μequiv l^{-1} in the external medium, K_m is the inverse of affinity (in μequiv l^{-1}) and J_{max} is the maximum rate of transport (in nequiv g^{-1} h^{-1}).

Na^+ efflux (J_{out}^{Na}) was calculated according to the following equation:

$$J_{out} = \frac{\Delta Q}{SA \times M \times t}, \quad (3)$$

where ΔQ is the increase in radioactivity in the water over time *t* (in cts min^{-1}) and *SA* is the specific activity of the ^{24}Na in the fish (in cts min^{-1} nequiv^{-1}). Since each efflux measurement was on a group of fish (*N*=15–20), the specific acitivity used in equation 3 was an average of individual measurements on each fish in the group. Backflux correction was unnecessary as the water *SA* was always less than 5 % of the fish *SA*.

Data analysis

Means ± one standard error of the mean (S.E.M.) are reported throughout. Comparisons amongst time series data were made by analysis of variance (P<0.05). If significant, Dunnett's *t*-test (Dunnett, 1955) was then used to compare treatment effects with control (P<0.05).

Results

Effects of aerobic exercise on plasma and whole-body [NaCl]

When rainbow trout juveniles were exercised at approximately 1.8 *BL* s^{-1}, a significant 8 % depression in plasma [Na^+] and [Cl^-] developed within 3 h of exercise. This was maintained until 6 h for Na^+ only (Fig. 1A). Plasma Na^+ and Cl^- levels then increased so that by 12 h of exercise they were not significantly different from controls (Fig. 1A).

The changes in whole-body [Na^+] and [Cl^-] during the 96 h exercise period (Fig. 1B) followed a somewhat different pattern. There was no detectable decrease in either whole-body [Na^+] or whole-body [Cl^-] in the initial hours of exercise. Instead, whole-body [Na^+] increased steadily until it became significantly elevated at 12 h of exercise. Thereafter, [Na^+] declined to control levels. In contrast, whole-body Cl^- levels remained stable throughout the 96 h period. There were no significant changes in either wet mass or dry mass relative to controls at any point during the exercise period. Wet and dry masses averaged 8.1±0.3 g and 3.0±0.1 g, respectively, yielding an average body water content of 82.9±0.7 %.

The absence of any depression in whole-body ion contents in the initial hours of exercise was confirmed in a subsequent 12 h exercise experiment conducted for measurements of ion uptake (see Fig. 4). There was a significant increase in whole-body [Na^+] (but not [Cl^-]) by 12 h of exercise (data not shown).

Na^+ and Cl^- uptake at rest and during exercise

Prior to exercise, J_{in}^{Na} averaged 390±31 nequiv g^{-1} h^{-1} (*N*=10) at external [Na^+] of 571 nequiv ml^{-1} and J_{in}^{Cl} averaged 304±21 nequiv g^{-1} h^{-1} (*N*=7) at [Cl^-] of 760 nequiv ml^{-1} (Fig. 2A,B; control). These values are closely comparable to the maximum uptake (J_{max}) during rest based on *in vivo* uptake kinetic measurements (J_{max}=560±55 and

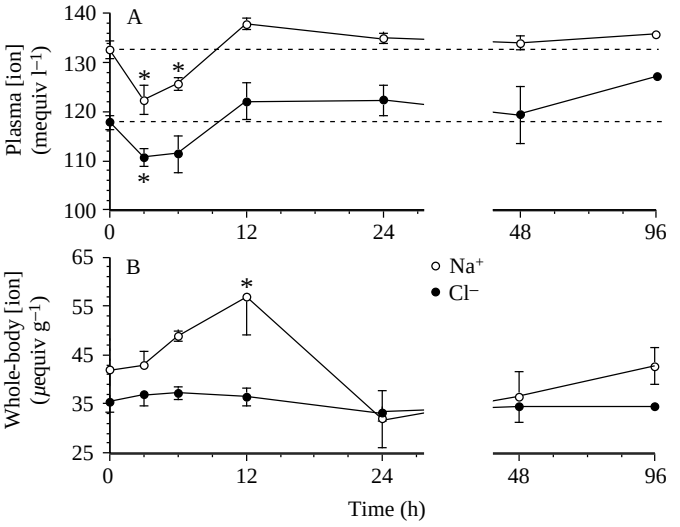

Fig. 1. The effect of exercise at approximately 1.8 *BL* s^{-1}, where *BL* is body length, on (A) plasma [Na^+] and [Cl^-] and (B) whole-body [Na^+] and [Cl^-] of rainbow trout. Values are means ± S.E.M., $N \geqslant 10$ per time point; asterisks indicate means significantly different ($P<0.05$) from controls (i.e. t_0) by Dunnett's *t*-test. Dotted lines in A indicate control levels of plasma ions.

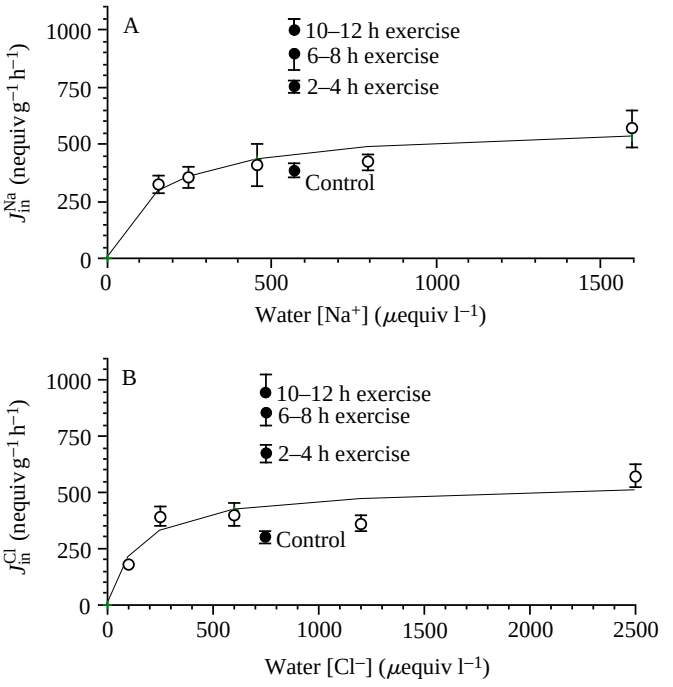

Fig. 2. (A) Na^+ uptake (J_{in}^{Na}) in relation to external [Na^+] (open circles) and prior to (control) and during exercise at approximately 2 *BL* s^{-1} (filled circles) in water containing 571 μequiv l^{-1} Na^+. (B) Cl^- uptake (J_{in}^{Cl}) in relation to [Cl^-] (open circles) and prior to and during exercise (filled circles) in water containing 760 μequiv l^{-1} Cl^-. Values are means ± S.E.M., N=8–10 per time point for exercise, N=4 per time point for uptake kinetics. Curves were fitted to kinetic data by non-linear regression using the Michaelis–Menten equation. J_{max}^{Na}=560±55 nequiv g^{-1} h^{-1}, K_m=138±56 μequiv l^{-1}; J_{max}^{Cl}=524±81 nequiv g^{-1} h^{-1}, K_m=152±46 μequiv l^{-1}.

524±81 nequiv g^{-1} h^{-1}, respectively, Fig. 2). K_m values for J_{in}^{Na} and J_{in}^{Cl} were 138±56 and 152±46 μequiv l^{-1}, respectively.

Exercise stimulated rapid increases in both J_{in}^{Na} and J_{in}^{Cl} (Fig. 2A,B). Over 2–4 h of exercise, J_{in}^{Na} and J_{in}^{Cl} increased by 1.9- and 2.3-fold respectively and by 10–12 h had further increased to 2.6- and 3.2-fold above control levels. These J_{in}^{Na} and J_{in}^{Cl} values were, by 12 h of exercise, 2.5- and 3.4-fold higher, respectively, than J_{max} in resting fish.

Na^+ efflux prior to and during exercise

Under control conditions, Na^+ efflux (J_{out}^{Na}) averaged 471±14.4 nequiv g^{-1} h^{-1} (N=six trials, 104 fish, Fig. 3). With the onset of exercise, there was an abrupt increase in J_{out}^{Na} to values that were 1.5-fold higher than resting values, but this increase lasted for only about 0.5 h. Over the exercise period of 0.5–1 h, J_{out}^{Na} had already declined to levels found during rest and continued to decline over the successive 7 h; by 6 h, $J_{out}^{Na}$was only 47 % of control levels.

NaCl balance during and following stress

Confinement stress for 8 h provoked, in contrast to exercise, substantial net losses of Na^+ and Cl^- (Fig. 4C,D). These losses continued for the first 5 h of confinement, resulting in decreases in whole-body Na^+ and Cl^- levels of 16.6 and 17.8 μequiv g^{-1}, respectively (39 and 44 %, respectively). The rate of efflux during this time (estimated from the changes in whole-body ion levels and corresponding measurements of J_{in}, Fig. 4A,B) averaged about 4000 nequiv g^{-1} h^{-1} for both Na^+ and Cl^-, i.e. efflux rates that are at least eightfold higher than control rates (Fig. 3). These net losses were accompanied by significant increases in J_{in}^{Na} and J_{in}^{Cl} (Fig. 4A,B), reaching a peak by about 5 h, at levels 1.7- and 2.3-fold higher, respectively, than pre-stress levels, i.e. increases very similar in magnitude and timing to those seen with exercise (Fig. 4A,B).

In a subsequent experiment examining recovery from 4 h of confinement stress (Fig. 5), whole-body Na^+ and Cl^- levels were still depressed at 16 h post-stress, by 24 % and 37 %, respectively (Fig. 5B). However, over the next 8 h there was a substantial increase in whole-body [Na^+] and [Cl^-] so that, by 24 h post-stress, recovery was complete (Fig. 5). J_{in}^{Na} and J_{in}^{Cl} remained significantly elevated until at least 16 h post-stress but had returned to pre-stress levels at 22–24 h post-stress. Although J_{out} values were not measured in this experiment, they must have been very low for Na^+ and Cl^- levels to recover in the final 8 h. In fact, assuming that J_{in}^{Na} and J_{in}^{Cl} remained at the 16 h rate for the final 8 h, the recovery of whole-body [NaCl] could only have occurred if J_{out}^{Na} and J_{out}^{Cl} were virtually zero.

Cortisol levels during stress and exercise

Cortisol was measured on fish removed one at a time for blood collection. But, as Laidley and Leatherland (1988) have pointed out, serial removal of fish from a tank (especially one of limited volume) can lead to a rapid elevation of plasma cortisol levels in the remaining fish. This was not a problem

with the controls sampled prior to confinement stress (fish were removed serially over 20 min from a 500 l tank) as cortisol levels remained relatively low, averaging 18±3 ng ml^{-1} (N=7), with no tendency for increasing cortisol level with sample number (Fig. 6, t_0 for confinement). However, cortisol levels in the controls from the exercise chamber (no current, fish removed one at a time from groups of three) did increase; from 28±20.7 ng ml^{-1} (N=6) for sample no. 1, to 61.7±12.2 ng ml^{-1} (N=6) for sample no. 2, and then to 94.0±13.5 ng ml^{-1} for sample no. 3. As a result of this sampling effect, the average t_0 value for exercise was about three times higher than the average t_0 value for confinement (Fig. 6). Exercising fish (also sampled in groups of three) also showed increasing cortisol levels with sample number. However, the sampling effect was smaller and the average cortisol levels tended to decline with exercise (Fig. 6), becoming significantly lower than controls at 3 h and 4 h of exercise. In contrast, confinement stress caused a substantial elevation in cortisol. By 4 h of stress, cortisol had increased to 108±12 ng ml^{-1} (N=7), a sixfold increase relative to pre-confinement stress controls. There was no sampling effect on cortisol levels of confined fish; i.e. no increase in cortisol with sample number.

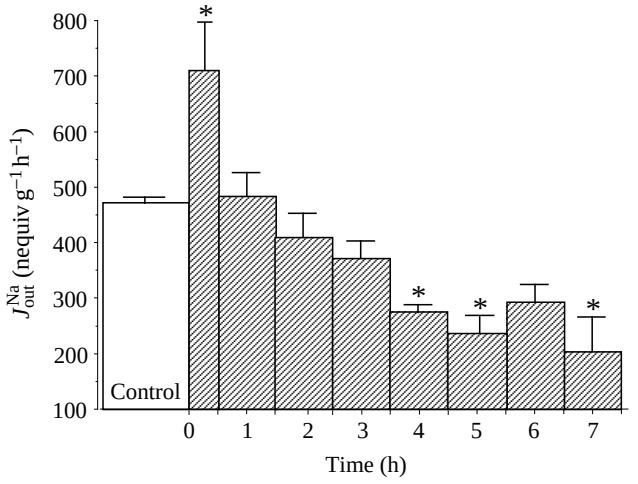

Fig. 3. Diffusive Na^+ loss (J_{out}^{Na}) prior to (control) and during exercise at approximately 1.8 BL s^{-1} in rainbow trout. Each trial consisted of 15–20 fish; six trials, total N=104. Values are means + S.E.M. (N=6). Asterisks indicate means significantly different (P<0.05) from controls (i.e. t_0) by Dunnett's t test.

Effects of growth hormone on Na^+ influx

In this experiment, J_{in}^{Na} measurement was not initiated until 1 h post-injection to allow for the stimulating effect of injection stress to subside (see Gonzalez and McDonald, 1992). Nonetheless, J_{in}^{Na} for fish given a saline injection was significantly higher than J_{in}^{Na} in uninjected controls (549±52 *versus* 390±31 nequiv g^{-1} h^{-1}, Fig. 2).

Ovine growth hormone (GH) had a significant stimulatory effect. For the period of 1–3 h post-injection, J_{in}^{Na} in GH-injected fish was 783±51 nequiv g^{-1} h^{-1}, 1.4 times greater than saline-injected controls. This is a similar level of stimulation to that found with 2–4 h of exercise (Fig. 2).

Discussion

This study has demonstrated substantial ionoregulatory responses by freshwater-adapted rainbow trout both to continuous aerobic exercise and to confinement stress. The responses to exercise and stress were similar to one another, in that there were adjustments to both uptake and loss, but they differed in the magnitude of the disturbance and the rapidity

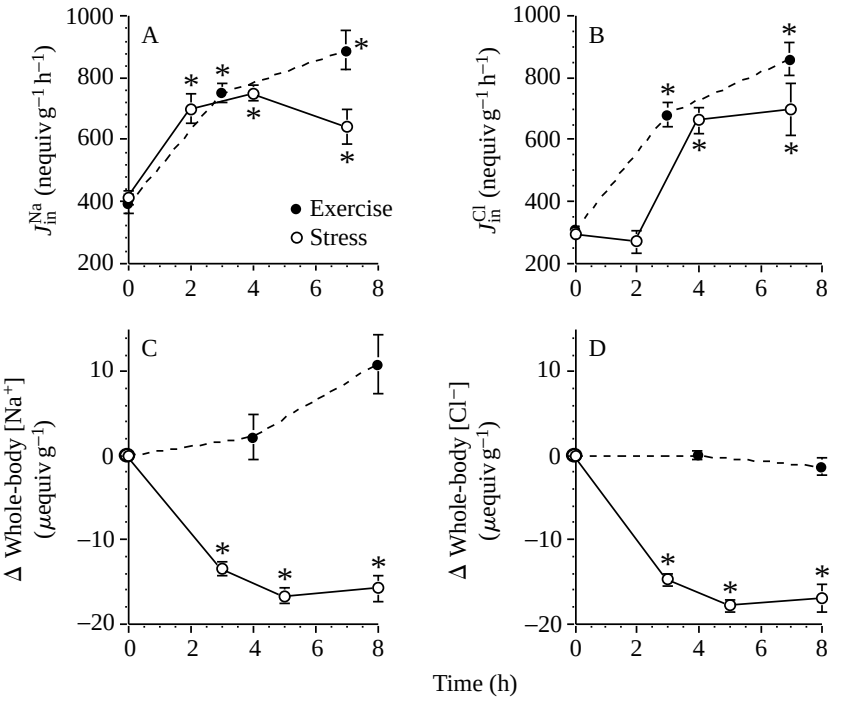

Fig. 4. The effects of 8 h of confinement stress (open circles, solid lines) on (A) J_{in}^{Na}, (B) J_{in}^{Cl}, (C) whole-body [Na^+] and (D) whole-body [Cl^-] in rainbow trout. Values are means ± S.E.M., N=9–11 per time point, 41 in total. J_{in} was measured over 2 h periods; values are displayed at the mid-point. Data for exercise (filled circles, dashed lines) from Fig. 2 are included for comparison. Asterisks indicate means significantly different (P<0.05) from t_0 by Dunnett's t-test.

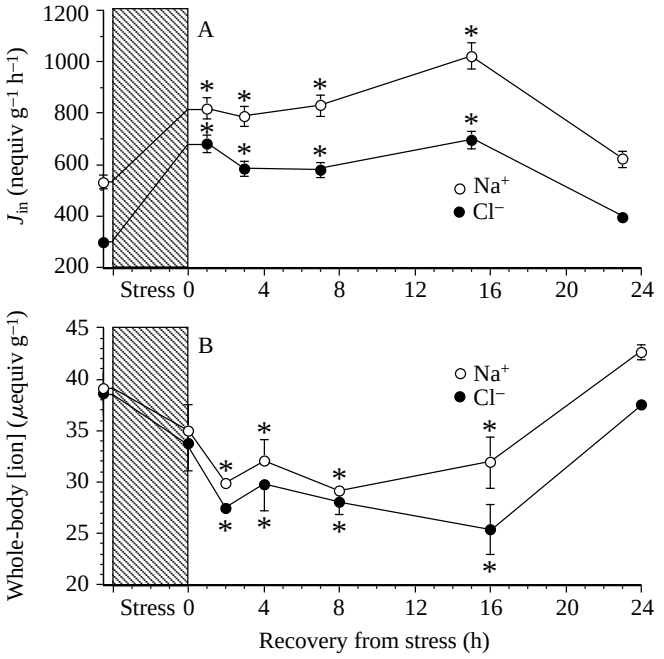

Fig. 5. Recovery of (A) J_{in}^{Na} (open circles) and J_{in}^{Cl} (filled circles) and (B) whole-body [Na^+] (open circles) and [Cl^-] (filled circles) from 4 h of confinement stress. Values are means ± S.E.M., N=5–7 for each time point. Asterisks indicate means significantly different (P<0.05) from t_0 by Dunnett's t-test. J_{in} was measured over 2 h periods; values are displayed at the mid-point.

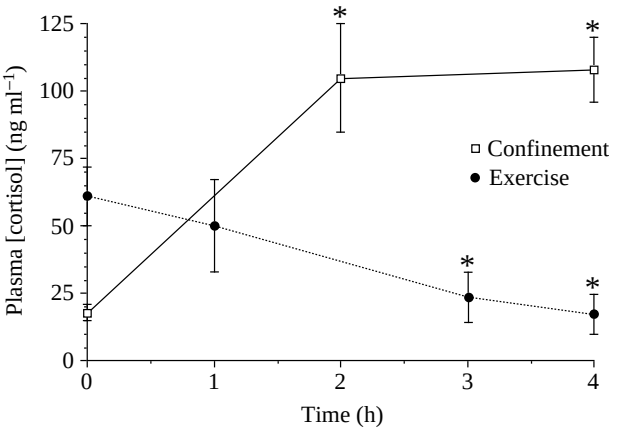

Fig. 6. Effects of exercise at approximately 2 BL s^{-1} and confinement stress on plasma cortisol levels in rainbow trout. Values are means ± S.E.M., N=18 for t_0 (exercise), N=6 for 1, 3 and 4 h exercise; N=8 for t_0 (stress), N=7 for 2 and 4 h stress. Asterisks indicate means significantly different (P<0.05) from controls (i.e. t_0) by Dunnett's t-test.

of the correction. During exercise, a small depression in plasma [Na^+] and [Cl^-] (Fig. 1A) was rapidly followed by a more than threefold increase in J_{in}, almost three times higher than the routine J_{max} (Fig. 2A,B), and a similar reduction in J_{out} (Fig. 3). Consequently, the NaCl imbalance was corrected in the early stages of exercise and, in fact, there was an overshoot in whole-body [Na^+] (Fig. 1B). In contrast, J_{out}^{Na} and J_{out}^{Cl} increased with confinement stress to levels at least eight times higher than controls, remained elevated for at least 5 h (compared with only 0.5 h at the start of exercise) and produced much larger net Na^+ and Cl^- losses (Fig. 4C,D). Adjustments to J_{in} followed a similar time course and were of the same magnitude as responses to exercise (Fig. 4A,B), but the reduction in J_{out} occurred much later, with NaCl balance taking 24 h to be corrected (Fig. 5A,B).

These observations can now be used to develop a general model for ion regulation in freshwater fish; a model that builds upon previous observations but offers some important new insights.

Regulation of ionic uptake

Our results support the conclusion that gill ion transport activity in freshwater fish can be adjusted to compensate for challenges to ion balance. Furthermore, simultaneous increases in J_{in}^{Na} and J_{in}^{Cl} can take place much more rapidly than was previously thought, in a few hours rather than the 1–2 weeks reported for chronic cortisol infusion or exposure to ion-poor water (see Introduction for references). Indeed, prior to the present study, the only similarly rapid ion transport adjustments reported for the gills of freshwater fish were those following experimentally induced acid–base disturbances, where acidoses or alkaloses were corrected by the appropriate manipulation of Na^+/H^+ and Cl^-/HCO_3^- exchanges (for reviews, see McDonald *et al.* 1989; Goss *et al.* 1992). Kinetic analysis by Wood and Goss (1990) revealed that a major part of the response to acid–base imbalance was an alteration in the ion-transport capacity and that J_{max} of both Na^+ and Cl^- transport could be either increased or decreased with respect to control levels for the purpose of acid–base correction. However, J_{max} can change rapidly with acid–base disturbances because the supply of acid or base counter-ions is normally limiting to transport (Wood and Goss, 1990). Such an explanation cannot account for the rapid adjustments in transport activity in the present study, since both J_{in}^{Na} and J_{in}^{Cl} increased simultaneously and simultaneous increases in the supply of the relevant acid–base counter-ions cannot occur. In fact, the most plausible explanation for the increased J_{in} in the present study is an increase in the number of transport carriers for both Na^+ and Cl^- in the gills. Furthermore, the rapidity of the increases argues for the activation of inactive carriers rather than the synthesis of new carriers. We are unaware of any evidence for inactive transport sites in the freshwater-adapted gill, but this idea is consistent with the rapid changes in ion transport that occur in anadromous fish upon transfer to sea water (De Renzis and Bornancin, 1984).

The key questions, then, are: what is the specific signal that is responsible for activation of inactive transport sites and how is that signal transmitted to the ion transport cells in the gills?

The generally accepted view (see Maetz, 1974) is that internal Na^+ and Cl^- concentrations provide feedback control to the Na^+ and Cl^- uptake mechanisms. Decreases or increases in internal [Na^+] and [Cl^-] result in corresponding increases or decreases in J_{max} of the respective transport systems.

Implicit in this view is the idea that the response should be proportional to the disturbance. Our results, while generally supporting this view, indicate that the response need not, in fact, be strictly proportional. During exercise, the initial drops in plasma [Na^+] and [Cl^-] (Fig. 1A) were small relative to the initial decreases during stress (Fig. 4) and yet the J_{in} response was very similar for both (Fig. 4). Furthermore, during exercise, the initial decreases of plasma [Na^+] and [Cl^-] were not accompanied by a decrease in whole-body ion levels (Fig. 1), indicating that an ion depletion *per se* is not required to initiate the response; i.e. the proximate signal to the ion transport regulating mechanism is, most importantly, a change in plasma ion concentrations. The observation that there was an initial decrease in plasma, but not whole-body, [Na^+] and [Cl^-] is worthy of comment. Although the origin of this phenomenon is not entirely clear, it could occur if there was an increased net influx of water at the gills in excess of urine production, for that would lead to an expansion of extracellular fluid volume (ECFV) and to dilution of extracellular ions. Aerobic exercise does, in fact, stimulate branchial water uptake (see Wood and Randall, 1973*b*), but we were unable to detect any change in total body water during exercise. Nonetheless, such measurements may be inadequate to resolve the relatively small changes in ECFV required to produce the observed Na^+ and Cl^- dilution. In future studies, direct measurements of ECFV, through indicator dilution, would help to establish whether there is a significant redistribution of fluid volumes during aerobic exercise and, in turn, to explain why plasma and whole-body ion levels did not change in parallel (Fig. 1).

The time course of the J_{in} response (hours instead of minutes) suggests that the control mechanism is probably hormonal rather than neural. Although a number of hormones are implicated as having some role in osmoregulation in either fresh water or sea water (e.g. arginine vasotocin, atriopeptin, cortisol, thyroid hormones, growth hormone, prolactin, epinephrine, urotensin; McDonald and Milligan, 1992; Takei, 1993), as far as we are aware, only cortisol and epinephrine have previously been shown specifically to stimulate J_{in} in freshwater fish.

It is now well established that cortisol has a prominent role to play in osmoregulation in freshwater fish through its effects on chloride cell density, Na^+/K^+-ATPase levels and Na^+ and Cl^- uptake rates (e.g. McCormick and Bern, 1989; Madsen, 1990*a*; Perry *et al.* 1992). For two reasons, however, it is probable that cortisol was not a major factor affecting adjustments to ion uptake in the present study. First, cortisol concentrations were elevated during stress but not during exercise and yet similar increases in ion uptake occurred in both circumstances. Second, Laurent and Perry (1990) showed that acute (3 h) intra-arterial infusion of cortisol into the rainbow trout had no effect on either J_{in}^{Na} or J_{in}^{Cl}. Cortisol is now known to be rather slow to act. Typically, daily injections for at least 4 days are required to produce significant effects (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.* 1992).

By similar reasoning, epinephrine can also be discounted because, although epinephrine levels are substantially elevated by confinement stress (e.g. Mazeaud and Mazeaud, 1981), a number of studies have shown that aerobic exercise, comparable to that employed here, does not elevate epinephrine levels (e.g. Ristori and Laurent, 1985; Butler *et al.* 1986). Furthermore, although acute infusion of epinephrine into rainbow trout stimulates J_{in}^{Na} and J_{in}^{Cl} within a few minutes of the start of infusion (McDonald and Rogano, 1986), longer-term infusions (for more than 6 h) inhibit J_{in}^{Na} and J_{in}^{Cl} (Vermette and Perry, 1987). In addition, epinephrine infusion greatly stimulates branchial diffusion of Na^+ and Cl^- (McDonald and Rogano, 1986; Gonzalez and McDonald, 1992), so any stimulatory effect on ion transport may be indirect through the stimulation of ion losses.

Of the remaining hormones, current evidence points to growth hormone (GH) as having the greatest potential for regulating NaCl uptake in fresh water. Although its main osmoregulatory function is thought to be in the preparation for seawater migration and it is only one of a number of hormones elevated during smoltification in salmonids (McDonald and Milligan, 1992), GH injections into freshwater fish have a similar time course and efficacy to cortisol in modulating ionoregulatory activities of the gills (for a review, see Borgatti *et al.* 1992). Madsen (1990*b*), for example, found that 7 days of ovine GH injection ($2\,\mu g\,g^{-1}$ every second day) produced significant increases in both chloride cell number and Na^+/K^+-ATPase activity in freshwater rainbow trout. We can now report that ovine GH directly stimulates J_{in}^{Na} much more rapidly than Madsen's study would suggest. Parallel to this observation, Barrett and McKeown (1988) found that aerobic exercise ($1.5\,BL\,s^{-1}$) prompted rapid increases in plasma GH in rainbow trout and coho salmon, significant by 6 h of swimming and reaching a peak at 24 h at levels 800 % higher than controls. These two observations taken together strongly implicate GH in the rapid adjustments to ion uptake, at least during exercise. Unfortunately, this cannot explain the stimulation of J_{in} during stress, for at least two studies have demonstrated continued suppression of GH below control levels during stress (Pickering *et al.* 1991; Farbridge and Leatherland, 1992).

Regulation of ion permeability

Although most studies have regarded adjustments to J_{in} as the principal means of regulating ion balance in fresh water (for a review, see Maetz, 1974), some studies on rainbow trout suggest that regulation of ionic permeability of the gills is at least equally important. McDonald and Rogano (1986), for example, showed that J_{out}^{Na} and J_{out}^{Cl} were reduced to 25 % of control levels by 24 h of exposure to ion-poor fresh water, whereas J_{in} continued to slowly increase for 9 days. Similarly, Gonzalez and McDonald (1992) reported a reduction in J_{out}^{Na} to 10 % of control levels by 6 h of recovery from a brief bout of exhaustive (i.e. stressful) exercise. Indeed, the reduction in J_{out}^{Na} during aerobic exercise in the present study (Fig. 3) was at least as rapid as the stimulation of J_{in} (Fig. 2). Furthermore, reduction of J_{out} had a more prominent role to play in the correction of the ionic deficit produced by stress than did the

stimulation of J_{in}. During the recovery period, J_{in}^{Na} and J_{in}^{Cl} (Fig. 5A) were never more than about twofold higher than control rates. These rates would only be adequate to explain the recovery of ion balance (Fig. 5B) if J_{out} had been reduced to virtually zero.

The control mechanism responsible for the reduction in J_{out} cannot be stated with any certainty, for the location and nature of the branchial NaCl diffusion path(s) are unknown. However, Gonzalez and McDonald (1992) presented indirect evidence suggesting that paracellular channels are the main diffusion path and that permeability control is achieved largely at the level of the tight junctions in a manner similar to that reported for leaky epithelia (Madara, 1988). Again, the time course of the adjustments to J_{out} suggest hormonal rather than neural control.

Here, the most likely candidate is prolactin because of its widely reported role in the regulation of ionic and water permeability in freshwater fish. Plasma prolactin (PRL) levels increase with freshwater adaptation in euryhaline salmonid and non-salmonid fish (for reviews, see Hirano, 1986; Prunet *et al.* 1990), and the Na^+-retaining effect of PRL in fresh water, which is thought largely to occur through inhibition of Na^+ loss, since PRL has no stimulating effect on Na^+ uptake (Dharmamba and Maetz, 1972), is well established for a number of non-salmonid euryhaline fish species (for a review, see Hasegawa *et al.* 1986). For freshwater salmonids, PRL's role is much less certain, for the removal of PRL by hypophysectomy (Komourdjian and Idler, 1977; Björnsson and Hansson, 1983) or the injection of homologous PRL (Hasegawa *et al.* 1986) has slight, if any, effects on Na^+ balance.

Nonetheless, the most compelling recent evidence for PRL's action in NaCl regulation in freshwater salmonids is the finding (Avella *et al.* 1991) of a gradual rise in plasma PRL in coho salmon smolts during 9 days of confinement stress that paralleled an initial decline and subsequent recovery in plasma [Na^+]. This is a controversial finding, however, as a later study on freshwater rainbow trout (Pottinger *et al.* 1992) found the opposite effect; significantly lower PRL levels in stressed fish compared with controls. Plasma ion levels were not reported in the latter study, so it is not known whether ion balance was disturbed. Therefore, in addition to uncertainty concerning the involvement of PRL in permeability regulation in salmonids, there is uncertainty concerning the response of plasma PRL to stress. Furthermore, as far as we are aware, it is not known whether PRL levels change during exercise.

Although we have implicated hormonal control in the ionoregulatory responses reported here, there is still considerable uncertainty about the hormone or hormones involved and their specific effects. Much of this uncertainty stems from the fact that the osmoregulatory responses to stress and exercise were similar to one another but their respective hormonal profiles were strikingly different (growth hormone being prominent in exercise, cortisol and epinephrine being prominent in stress) and uncertainty over PRL in both circumstances. This study emphasizes that hormonal control of NaCl balance in freshwater fish is still a very fruitful area for further research. Future studies should include a more complete description of hormonal responses to stress and exercise, an examination of other putative osmoregulatory hormones, such as the newly discovered teleost pituitary hormone somatolactin, levels of which have been shown to increase during stress in rainbow trout (Rand-Weaver *et al.* 1993), and the administration of homologous rather than mammalian hormones, preferably by some longer-term method than injection, to study responses of gill ion transport and permeability.

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to D.G.M. The authors gratefully acknowledge the assistance of M. Goldstein, D. Legati, K. Self, J. Robinson and V. Thomas and thank Dr H. Saunderson, Wilfred Laurier University, for the loan of the electromagnetic flow meter.

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