EFFECTS OF EXERCISE, STRESS AND TRAINING ON ION REGULATION

THE CONSEQUENCES OF SHORT TERM EXERCISE, VARIOUS LEVELS OF STRESS AND TRAINING ON ION REGULATION IN DIFFERENT SPECIES OF FISH

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ABSTRACT

Initially, this study examined the mechanisms by which Na^+ and Cl^- are regulated in freshwater rainbow trout during exercise and stress. Aerobic exercise (~2 body lengths sec^{-1}) caused a brief increase in diffusive Na^+ efflux ($J_{out}^{Na^+}$) and a brief decline in plasma Na^+ and Cl^-. This disturbance was rapidly compensated by a 3 fold increase in $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ (over the first 10-12 h exercise), and by a reduction in $J_{out}^{Na^+}$ to 40% of routine by 7 h of exercise. The compensation produced a significant increase in whole body Na^+ while whole body Cl^- remained unchanged. In contrast, confinement stress (for 4 or 8 h) caused an 8 fold increase in $J_{out}^{Na^+}$and $J_{out}^{Cl^-}$ 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 $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ (of similar magnitude and timing to that of exercise) as well as reductions in $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ to nearly zero. We conclude that J_{in} increased because of an activation of inactive transport sites in the gills while J_{out} was reduced by a reduction in branchial ionic permeability, both responses mediated hormonally. Although the hormonal control mechanisms are as yet poorly defined, we argue that growth hormone and prolactin are responsible for the J_{in} and J_{out} regulation, respectively, and rule out either cortisol or epinephrine as having any role, at least with respect to the rapid NaCl regulation evident during exercise.

The ability of rainbow trout to rapidly regulate ion balance was then investigated to determine whether it is unique to rainbow trout, exists in stream-dwelling animals or whether it is wide spread in fish regardless of preferred habitat. Common shiners, considered to be an active species and smallmouth bass, considered to be less active, were the two species of comparison. Common shiners demonstrated rapid increases in $J_{in}^{Na^+}$ during exercise and confinement, a lack of change in whole body Na^+ and Cl^- during exercise and a large Na^+ and Cl^- loss during stress. In contrast, smallmouth bass experienced minimal increases in $J_{in}^{Na^+}$ during exercise and no change during stress with ion loss occurring during both exercise and stress. It was concluded that the relative ability to regulate ion balance in response to stress and exercise may reflect the frequency with which the animal experiences that challenge in its natural habitat. Consequently, common shiners probably possess a similar uptake mechanism to that of rainbow trout while the mechanisms in smallmouth bass may exist, but in attenuated form.

The final analysis investigated whether or not exercise training affected the magnitude of the disturbance to Na^+ balance produced by both acute and chronic stress. This was important in that it could be applied to the improvement of fish stocking techniques. Trained fish demonstrated the ability to reduce ion loss produced by stress despite significantly high levels of cortisol, glucose and oxygen consumption. Similar results were produced by both acute and chronic stress and it was established that the rainbow trout's ability to regulate ions during stress, without altering the release of cortisol and catecholamines was improved by training.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS....x

LIST OF TABLES AND FIGURES....xi

CHAPTER 1: MECHANISMS OF Na^+ AND Cl^- REGULATION IN FRESHWATER ADAPTED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)....1

INTRODUCTION....1

MATERIALS AND METHODS....5
- Experimental animals....5
- Exercise apparatus....5
- Stress protocol....6
- Experimental Series....6
  1. Plasma and whole body Na^+ and Cl^- during continous aerobic exercise....6
  2. Na^+ and Cl^- uptake during rest and exercise....6
  3. Na^+ and Cl^- uptake kinetics....7
  4. Na^+ efflux at rest and during exercise....7
  5. Na^+ and Cl^- balance during and following confinement stress....8
  6. Plasma cortisol during exercise and stress....9
  7. Effects of growth hormone and prolactin....9
- Analytical methods....10
- Calculations....11
- Data analysis....11

RESULTS....12
- Effects of aerobic exercise on plasma and whole body NaCl....12
- Na^+ and Cl^- uptake at rest and during exercise....12
  - Figure 1.1....14
  - Figure 1.2....16
  - Figure 1.3....18
  - Figure 1.4....20
- Na^+ efflux prior to and during exercise....21
- NaCl balance during and following stress....21
- Cortisol levels during stress and exercise....22
  - Figure 1.5....24
  - Figure 1.6....26
- Effects of mammalian hormones on Na+ influx....27

Figure 1.7 29

DISCUSSION 30
Regulation of ionic uptake 30
Regulation of ion permeability 34

CHAPTER 2: ION REGULATION DURING AEROBIC EXERCISE AND CONFINEMENT STRESS IN FRESHWATER ADAPTED SMALLMOUTH BASS AND COMMON SHINERS 38

INTRODUCTION 38

MATERIALS AND METHODS 40
Experimental animals 40
Experimental series- common shiners 40
1. Na^+ uptake during rest and exercise. 40
2. Na^+ and Cl^- balance during confinement stress and rest. 41
Experimental series- smallmouth bass 41
1. JinNa+ during rest 41
2. Plasma Na^+ and Cl^- and $J_{in}Na^+$ during exercise. 42
3. Plasma Na^+ and Cl^- and $J_{in}Na^+$ during confinement stress 42
Analytical methods 43
Calculations 43
Data analysis 44

RESULTS 45
Whole body, plasma Na^+ and Cl^- responses to aerobic exercise 45
J_{in} at rest and during exercise 45
Figure 2.1 47
Figure 2.2 49
Figure 2.3 51
Electrolyte balance during stress 52
$J_{in}Na^+$ during confinement stress 52
Figure 2.4 54
Figure 2.5 56
Figure 2.6 58
Table 2.1 59
Cortisol levels in smallmouth bass during stress and exercise 60
Figure 2.7 62
Figure 2.8 64

DISCUSSION 65

CHAPTER 3: EFFECTS OF EXERCISE TRAINING ON RAINBOW TROUT 68

INTRODUCTION 68

MATERIAL AND METHODS 70
Experimental animals 70
Experimental series 70
1. Effects of training on growth and condition factor 70
2. Effects of training on critical swimming speed 70
3. Responses to acute stress 71
4. Responses to chronic stress 72
Calculations- $J_{out}Na^+$ and $M0_2$ 72

RESULTS 73
Effects of continuous exercise on growth and Na+ levels 73
Effects on critical swimming speed 73
Training effects on stress responses 73
1. Acute stress- exhaustive exercise 73
Figure 3.1 75
Table 3.1 76
2. Acute stress- isoproterenol injection 77
Chronic stress- confinement 77
Figure 3.2 79
Figure 3.3 81
Figure 3.4 83

DISCUSSION 84

REFERENCES 86

LIST OF ABBREVIATIONS

$BL \cdot sec^{-1}$:	body lengths per second
$cm \cdot s^{-1}$:	centimeter per second
Cl^-:	chloride ion
c.f.:	condition factor
cpm:	counts per minute
U_{crit}:	critical swimming velocity
°C:	degree Celcius
t:	duration in hours
g:	gram
h:	hour
HCl:	hydrochloric acid
l:	length (fork)
L:	liter
$L \cdot min^{-1}$:	liters per minute
mL:	milliliter
min:	minute
µL:	microliter
µCi:	microcuries
µeq:	microequivalents
neq:	nanoequivalent
ng:	nanogram
N:	number/ Normal
PO_2:	partial pressure of oxygen
^{36}Cl :	radioactive chloride
^{22}Na:	radioactive sodium
^{24}Na:	radioactive sodium
s:	second
SEM:	standard error measurement
Na^+:	sodium ion
Na_2CO_3:	sodium carbonate
NaCl:	sodium chloride
SA:	specific activity
T:	time
$J_{in}^{Na^+}$:	uptake of sodium
$J_{in}^{Cl^-}$:	uptake of chloride
W:	weight

LIST OF TABLES AND FIGURES

Table 2.1: $J_{in}^{Na^+}$ during rest and confinement stress in common shiners.....59

Table 3.1: U_{crit} in rainbow trout exercised for 16 days.....76

Figure 1.1: Plasma Na^+ and Cl^- and whole body Na^+ and Cl^- during exercise in rainbow trout.....14

Figure 1.2: $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ prior to and during exercise in rainbow trout.....16

Figure 1.3: Diffusive Na^+ loss prior to and during exercise in rainbow trout....18

Figure 1.4: $J_{in}^{Na^+}$, $J_{in}^{Cl^-}$, whole body Na^+ and whole body Cl^- in rainbow trout during confinement stress.....20

Figure 1.5: Recovery of $J_{in}^{Na^+}$, $J_{in}^{Cl^-}$, whole body Na^+ and whole body Cl^- from confinement stress in rainbow trout.....24

Figure 1.6: Cortisol levels in rainbow trout during exercise and confinement stress.....26

Figure 1.7: $J_{in}^{Na^+}$ in rainbow trout following injection of saline, ovine growth hormone and ovine prolactin.....29

Figure 2.1: Whole body Na^+ and Cl^- during exercise in common shiners.....47

Figure 2.2: Plasma Na^+ and Cl^- during exercise in smallmouth bass.....49

Figure 2.3: $J_{in}^{Na^+}$ during exercise in common shiners, smallmouth bass and rainbow trout.....51

Figure 2.4: $J_{in}^{Cl^-}$ during exercise in common shiners and rainbow trout.....54

Figure 2.5: Whole body Na^+ and Cl^- during confinement stress in common shiners.....56

Figure 2.6: Plasma Na^+ and Cl^- during confinement stress in smallmouth bass.....58

Figure 2.7: $J_{in}^{Na^+}$ during confinement stress in common shiners, smallmouth bass and rainbow trout.....62

Figure 2.8: Plasma cortisol in smallmouth bass during exercise and confinement stress.....64

Figure 3.1: Weight, condition factor, temperature, whole body Na^+ and plasma Na^+ in rainbow trout during 16 days of exercise.....75

Figure 3.2: Diffusive Na^+ loss ($J_{out}^{Na^+}$), oxygen consumption and ion/gas ratio during recovery from acute stress in trained and untrained rainbow trout.....79

Figure 3.3: Diffusive Na^+ loss ($J_{out}^{Na^+}$), oxygen consumption and ion/gas ratio during recovery from isoproterenol injection in trained and untrained rainbow trout.....81

Figure 3.4: Plasma Na^+, cortisol and glucose levels in trained and untrained rainbow trout prior to and following 4 h of confinement stress.....83

CHAPTER 1

MECHANISMS OF Na^+ AND Cl^- REGULATION IN FRESHWATER ADAPTED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

INTRODUCTION

To meet their oxygen demand from 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 on a daily basis through branchial diffusion (Gonzalez and McDonald, 1992). These losses are normally compensated by the active, independent branchial absorption of Na^+ and Cl^- from the medium with the result that blood NaCl homeostasis is maintained within quite narrow limits (McDonald and Milligan, 1992).

Nevertheless, a variety of circumstances will act to challenge that homeostasis. One of the most frequently studied is exposure to dilute media. Here, maintaining fish in low external NaCl (and, in many instances, low Ca^{++} as well) causes a depletion of Na^+ and Cl^- to which the most widely reported response is an increase in branchial Na^+ and Cl^- transport activity (see Perry and Laurent, 1989 for recent review). This increased activity has been characterized, in rainbow trout, as requiring at least 9 days to fully develop (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 day treatment of rainbow trout in normal fresh water with daily cortisol injections provoked chloride cell proliferation and corresponding increases in $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$.

A reduction in 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 $J_{out}^{Na^+}$and $J_{out}^{Cl^-}$ declined to about 1/3 of routine levels within the first 24h of exposure to ion-poor water. Similarly, Perry and Laurent (1989) showed a reduction in $J_{out}^{Cl^-}$ in rainbow trout occurring before there was any significant increase in $J_{in}^{Cl^-}$. Earlier studies on ammocoete larvae, Lampetra planeri, and goldfish, Carassius auratus (Morris and Bull, 1970; Cuthbert and Maetz, 1972, respectively) also showed reductions in J_{out} in response to low ion exposure.

Much less is known about how freshwater fish respond to other challenges to ion balance. Two such challenges are exercise and stress, both more likely encountered than ion-poor water, and having 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 functional surface area of the gills (Randall et al., 1972), while stressful treatments of any kind will lead to an elevation in circulating epinephrine (Mazeaud and Mazeaud, 1981). Recent studies have emphasized the profound effect of epinephrine on diffusional losses of NaCl across the gills; effects produced by changes in gill hemodynamics and also through direct increases in gill ionic permeability (Gonzalez and McDonald, 1992).

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. 1h forced exercise by manual chasing, Wood and Randall, 1973a ; 5-6 min exhaustive exercise, Gonzalez and McDonald, 1992) or strenuous (6 h exercise at 85% of maximum sustainable swimming speed; Gonzalez and McDonald, 1992) and the impact on ion balance significant. For voluntary routine exercise, at speeds salmonids can maintain for long periods, the impacts, if any, on ion balance are unknown. Such impacts are, in contrast, well established with respect to stress. For stressful treatments persisting for 4h or more, substantial depressions in plasma Na^+ and Cl^-, in excess of 10 mEq L^{-1}, have been widely reported for salmonid species (reviewed in McDonald and Robinson, 1993). However, in neither stress or exercise is the time course or nature of the ionoregulatory response known.

Therefore, the objective of this study was to more thoroughly characterize 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 ~2 $BL \cdot sec^{-1}$ 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 whether the time course of the ionoregulatory response is as slow as that reported for exposure to ion-poor fresh water, the relative contributions of uptake increase and permeability reduction to the correction, and the specific feedback controls responsible for the ionoregulatory response. With regard to the latter we have launched a preliminary investigation into hormonal control mechanisms by measuring cortisol concentrations during both exercise and

stress and by examining responses of Na^+ transport to growth hormone and prolactin injection.

MATERIALS AND METHODS

Experimental animals

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

Exercise apparatus

Three different sizes of annular swim chambers were employed to exercise fish. Each chamber consisted of an outer and inner circular tank with the fish exercising in the volume contained between the tanks. The nominal operating volumes of the swim chambers were 19 L, 60 L and 450 L, with average velocities of 23, 34 and 17 $cm \cdot sec^{-1}$, respectively. Dimensions were as follows; 19L model, 56 cm outside diameter X 27 cm inside diameter X 17 cm water depth; 60 L model, 56 X 27 X 24 cm; 450 L model, 92 X 42 X 68 cm. Centrifugal pumps (Little Giant model 5-MSP, 0.5 hp) located either at the bottom of the inner cylinder (60 L and 450 L models, 2 and 3 pumps respectively) or outside the outer cylinder (19L model, 1 pump) were used to generated flow. The flow was directed through tubing and nozzles to create 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 into 3 and 4 sections respectively with plastic mesh partitions in order to separate groups of fish. Aeration was provided by air stones and temperature control by either

continuous supply of water (1.5 - 2.0 $L \cdot min^{-1}$ in the 450 L model), 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 previously employed (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 all fish were in close physical contact 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 vigorous enough to ensure that bubbles penetrated the bags.

Experimental Series

1. Plasma and whole body Na^+ and Cl^- during continous aerobic exercise.

Rainbow trout (8.1 ± 0.3 g, N = 86) were exercised (~1.8 body lengths$\cdot sec^{-1}$) for up to 96 h in the 450 L swim chamber. At periodic intervals (0, 3, 6, 12, 24, 48 and 96 h) fish ($N \geq 10$ per sampling period) were removed and killed by a quick blow to the head. Weight and fork length were recorded and a blood sample (≥ 0.25 mL) was collected by caudal severance, centrifuged, and the plasma drawn off and stored frozen for later analysis of Na^+ and Cl. The carcass was then dried to a constant weight (48 h at 70°C) for analysis of body Na^+ and Cl^- and % water.

2. 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 ~2.0 BL sec^{-1}, was measured in separate trials by the absorption from the water over 2 h periods of ^{22}Na (as NaCl, 2 $\mu Ci \cdot L^{-1}$ obtained from Amersham, Inc) and ^{36}Cl (as NaCl, 2 $\mu Ci \cdot L^{-1}$ obtained from 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 J_{in}^{Na+} and J_{in}^{Cl-} measurement) were allowed to acclimate to the swim chamber with the current off for 4 h before isotope addition. For the exercise trials, the addition of groups of fish to the 3 compartments of the swim chamber (current on) was staggered so that by the time of isotope addition they had been exercising for either 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 and ^{36}Cl & ^{35}Cl. Water samples (10 mL) were also collected for analysis of ^{22}Na & ^{23}Na and ^{36}Cl & ^{35}Cl at the beginning (after mixing) and end of the 2 h isotope period.

3. Na^+ and Cl^- uptake kinetics.

The effect of external Na^+ and Cl^- on J_{in}^{Na+} and J_{in}^{Cl-} was determined in separate trials. J_{in}^{Na+} was measured in artificial hard water (1 mM $CaCl_2$ adjusted to pH 7.5) to which ^{22}Na (0.5 $\mu Ci\ mM^{-1}$) was added as NaCl to give $[Na^+]$ of 0.1, 0.25, 0.6, 1.2 or 2.5 mM. J_{in}^{Cl-} was measured in 1mM $CaCO_3$ (adjusted to pH 7.5) to which ^{36}Cl (3 $\mu Ci \cdot mM^{-1}$) was added as NaCl to give $[Cl^-]$ of 0.1, 0.25, 0.6, 1.2 or 2.5 mM. Fish (11.7 ± 0.6 g) were held in black plastic containers (N = 4 per container) containing 1.5 L of the artificial media. The chambers were fitted with air lines to provide aeration and mixing of 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.

4. 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 ~1.8 body lengths·sec^{-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 chambers. Sixteen hours before the start of efflux measurement all fish were injected intraperitoneally with 50 μL of ^{24}Na solution (54.0 mM $^{24}Na_2CO_3$, produced in the McMaster Nuclear Reactor, 10 μCi 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 6 times with 15-20 injected fish per trial.

5. 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 either 1, 3 or 6 h. At 6 h, ^{22}Na (as NaCl, 3 $\mu Ci \cdot L^{-1}$ obtained from Amersham, Inc) and ^{36}Cl (as HCl, 3 $\mu Ci \cdot L^{-1}$ obtained from ICN) were added to the water and allowed to circulate for the next 2 h. At 8 h, all fish were removed, 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, 6 fish were immediately sampled for analysis of whole body NaCl and the remainder were transfered to 'recovery' chambers (6 black plastic chambers with 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 recovery from the confinement. Two hours

before the fish were removed ^{22}Na and ^{36}Cl were added to the chambers as above.

6. Plasma cortisol 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 either 0, 1, 3 or 4 h of exercise at 2 $BL \cdot sec^{-1}$, fish were removed one at a time and killed with a blow to the head. Blood samples ($\geq$ 0.05 mL) were obtained by caudal severance, centrifuged and the plasma frozen at -20°C for later analysis of cortisol. The number of fish per compartment was kept to 3 to minimize the effects of cohort sampling on cortisol levels; the experiment was repeated until N = 18 for T0 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.

7. Effects of growth hormone and prolactin.

The acute effects of growth hormone and prolactin on $J_{in}^{Na^+}$ was assessed by monitoring ^{22}Na uptake for a 2 h period starting 1 h after hormone injection. Fish (10.5 $\pm$ 0.6 g, N = 28) were injected intraperitoneally with either saline (5 $\mu l \cdot g^{-1}$), 1 $\mu g \cdot g^{-1}$ of ovine growth hormone (obtained from NIH, Bethesda, MD) or 2 $\mu g \cdot g^{-1}$ of ovine prolactin (obtained from Sigma Chemical Co., St. Louis, MO). The hormones were dissolved in saline, both prolactin and growth hormone were used to check for possible cross reactivity of these mammalian hormones (Hirano, 1986). Following the injections, fish were transferred to black plastic chambers containing 1.5 L of aerated water at 14° C.

2 μCi of ^{22}Na was added to the water at +1 h. Fish were sacrificed at +3 h and then counted immediately for ^{22}Na activity.

Analytical methods

Plasma Na^+ was measured on 10 μL aliquots by atomic absorption spectrophotometry (AAS, Varian 1275) following 1:1000 dilution with de-ionized water. Plasma Cl^- was determined directly on 10 μL aliquots by coulometric titration using a Radiometer CMT10 Chloride Titrator. For whole body ion analysis, each fish was digested in 1 vol. of 1N HNO_3 at 80°C for 48 h. The digests were centrifuged, filtered and the supernatants read 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 aliquots using a radio-immunoassay kit (Quanticoat™ Cortisol, Kallestad Laboratories, Inc.) validated for use on trout plasma (Hontela et al., 1992) that involves the competitive binding of plasma cortisol and ^{125}-I labeled cortisol to cortisol-specific antibody coated tubes.

^{24}Na and ^{22}Na were read directly (tissues, plasma and water) on a Canberra-Packard AutoGamma 5000 deep well gamma counter with a 3" NaI crystal. ^{24}Na samples were corrected for decay ($t_{1/2}$ = 15 h). ^{36}Cl activity was measured on a 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 de-ionized water. Aliquots (0.35 mL) were then digested for 12 h @ 50°C in 1mL 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 $nEq \cdot g^{-1} \cdot h^{-1}$) were calculated according to the following equation:

$$J_{in} = \frac{\Sigma Q}{SA \bullet W \bullet t} \qquad (1)$$

where 'ΣQ' is the whole body radioactivity (in cpm), 'SA' is specific activity of the water in $cpm \cdot nEq^{-1}$, 'W' is body weight in grams and 't' is duration in hours.

The effects of external NaCl on J_{in} was analyzed by non linear regression (SAS, 1982) using the Michaelis-Menton kinetics equation:

$$J_{in}^{X} = \frac{J_{max} \bullet [X]_e}{K_m + [X]_e} \qquad (2)$$

where X_e is the ion concentration (Na^+ or Cl^-) in $\mu Eq \cdot L^{-1}$ in the external medium, K_m is the inverse of affinity (in $\mu Eq \cdot L^{-1}$) and J_{max} (in $nEq \cdot g^{-1} \cdot h^{-1}$) is the maximum rate of transport.

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

$$J_{out} = \frac{\Delta Q}{SA \bullet W \bullet t} \qquad (3)$$

where ΔQ is the increase in radioactivity in the water over time t (in cpm), 'SA' is the average specific activity of the ^{24}Na in the fish (in $cpm \cdot nEq^{-1}$), 'W' is the body weight in grams and 't' is the time in hours.

Data analysis.

Means ± one standard error of the mean (SEM) are reported throughout. Comparisons amongst time series data were analyzed by analysis of variance ($P < 0.05$). If significant, Dunnett's 't' test (Dunnett, 1955) was used to compare treatment effects to control ($P < 0.05$).

RESULTS

Effects of aerobic exercise on plasma and whole body NaCl

When rainbow trout juveniles were exercised at ~1.8 body lengths per second, a significant 8% depression in plasma Na^+ and Cl^- developed by 3 h of exercise (also at 6 h for Na^+ only, Fig. 1.1A). Plasma Na^+ and Cl^- levels then subsequently increased so that by 12 h of exercise they were not significantly different from controls (Fig. 1.1A).

The changes in whole body Na^+ and Cl^- during the 96 h exercise period (Fig. 1.1B) followed a somewhat different pattern. There was no detectable decrease in either whole body Na^+ or Cl^- in the initial hours of exercise. Instead, whole body Na^+ increased steadily until 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. Also, there were no significant changes in either wet weight or dry weight relative to controls at any point during the exercise period. Wet and dry weights averaged 8.1 ± 0.3 g and 3.0 ± 0.1 g respectively, yielding an average percent body water of 82.9 ± 0.7%.

In a subsequent 12 h exercise experiment conducted for measurements of ion uptake (Fig. 1.4), the absence of any depression in whole body ions in the initial hours of exercise was confirmed (Fig. 1.4 B,D). A significant increase in whole body Na^+ (but not Cl^-) by 12 h of exercise was also found (data not shown).

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

Prior to exercise, $J_{in}^{Na^+}$ averaged 390 ± 31 $nEq \cdot g^{-1} h^{-1}$ (N = 10) at external Na^+ of 571 $neq \cdot mL^{-1}$ and $J_{in}^{Cl^-}$ averaged 304 ± 21 $nEq \cdot g^{-1} h^{-1}$ (N = 7) at $[Cl^-]$ of 760 $neq \cdot mL^{-1}$ (Fig. 1.2 A,B, control). These values are closely comparable to the maximum uptake (J_{max}) during rest based on in vivo uptake

Figure. 1.1. The effect of exercise at ~1.8 BL sec^{-1} on (A) plasma Na^+ and Cl^- and (B) whole body Na^+ and Cl^- of rainbow trout. Values are means ± one SEM, N = ≥10 per time point, asterisks indicate means significantly different ($P < 0.05$) from controls (i.e. T0) by Dunnett's 't' test.

Figure 1.1

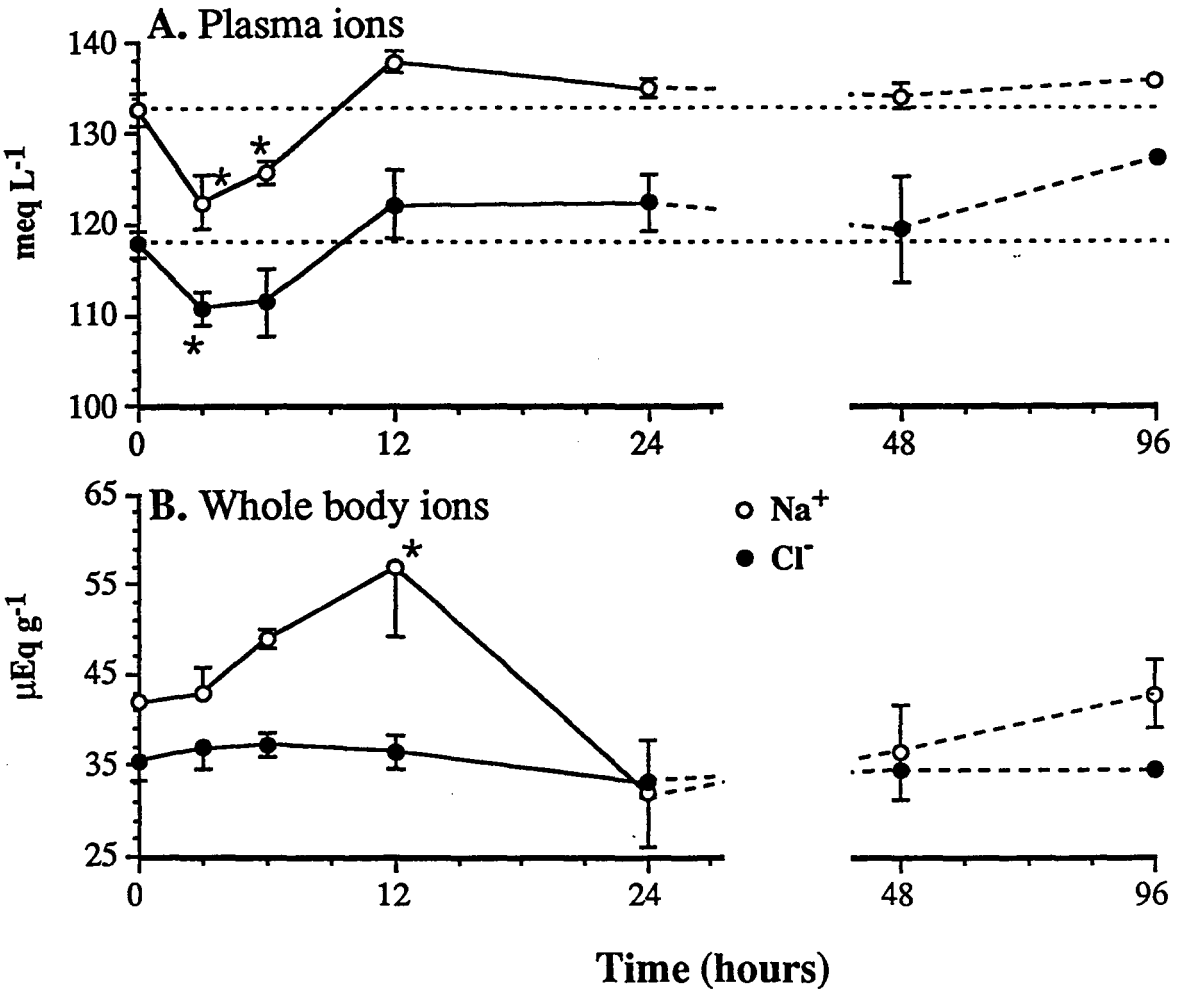

A. Plasma ions
meq L-1
140
130
120
110
100
0
12
24
48
96
B. Whole body ions
µEq g-1
65
55
45
35
25
Na+
Cl-
Time (hours)

Figure 1.2. (A) Na^+ uptake ($J_{in}^{Na^+}$) in relation to external [Na^+] (open circles) and prior to (control) and during exercise at ~2 BL sec^{-1} (solid circles). (B) Cl^- uptake ($J_{in}^{Cl^-}$) in relation to [Cl^-] (open circles) and prior to (control) and during exercise. Values are means ± one SEM, N = 8-10 per time point for exercise, N = 4 per time point for uptake kinetics. Asterisks indicate means significantly different ($P < 0.05$) from controls by Dunnett's 't' test. Curves fitted to kinetic data by non-linear regression using the Michaelis-Menten equation. $J_{max}^{Na^+} = 560 \pm 55$ nEq g^{-1} h^{-1}, $K_m = 138 \pm 56$ $\mu Eq \cdot l^{-1}$; $J_{max}^{Cl^-} = 524 \pm 81$ nEq $\cdot g^{-1}$ h^{-1}, $K_m = 152 \pm 46$ $\mu Eq \cdot l^{-1}$.

Figure 1.2

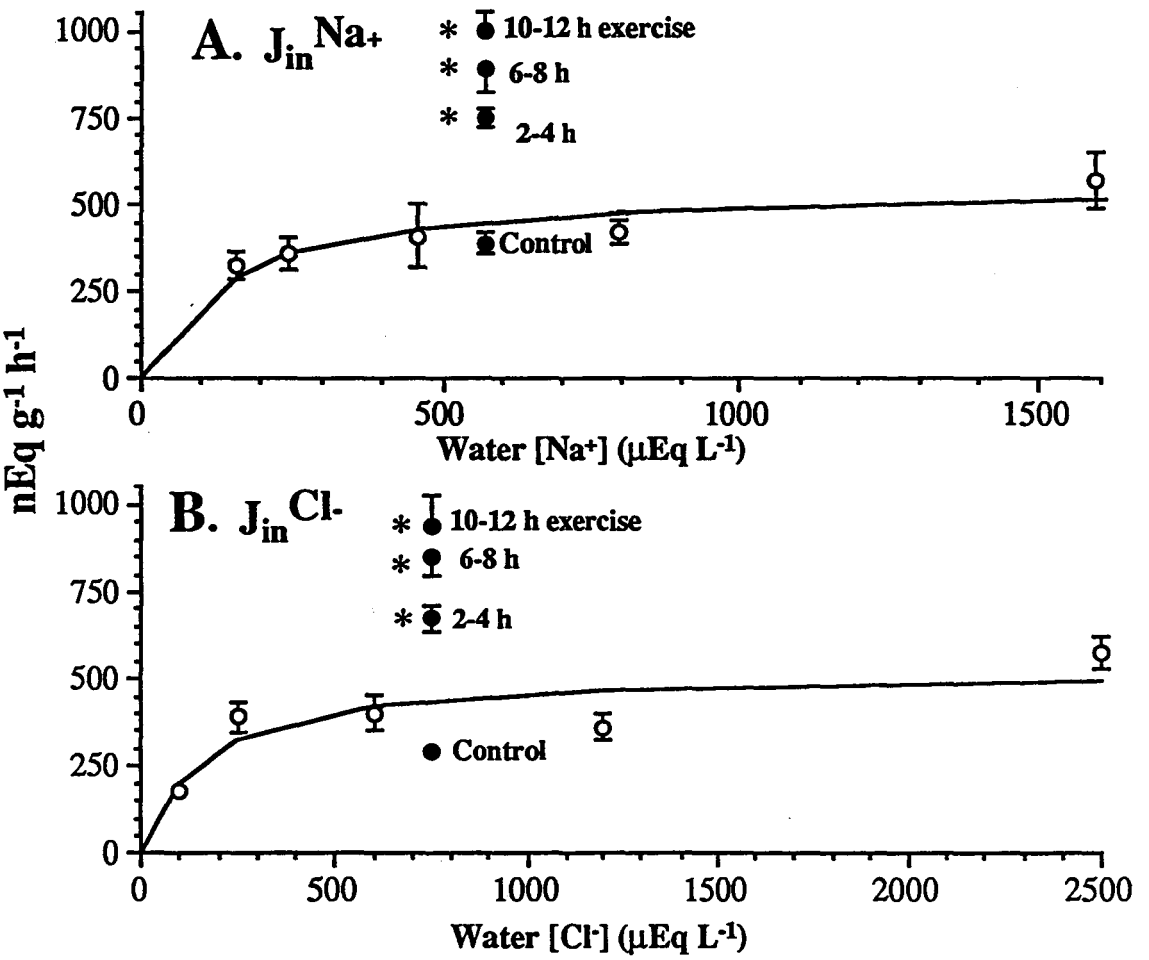

A. $J_{in}Na_+$
* 10-12 h exercise
* 6-8 h
* 2-4 h
Control
1000
750
500
250
0
0
500
1000
1500
Water [Na^+] (µEq L^{-1})
nEq g^{-1} h^{-1}
B. $J_{in}Cl^-$
* 10-12 h exercise
* 6-8 h
* 2-4 h
Control
1000
750
500
250
0
0
500
1000
1500
2000
2500
Water [Cl^-] (µEq L^{-1})

Figure 1.3 Diffusive Na^+ loss ($J_{out}^{Na^+}$) prior to (control) and during exercise @ ~1.8 $BL \cdot sec^{-1}$ in rainbow trout. Each trial comprised 15 - 20 fish; 6 trials, total N = 104. Values are means ± one SEM (N = 6). Asterisks indicate means significantly different ($P < 0.05$) from controls (i.e. T0) by Dunnett's 't' test.

Figure 1.3

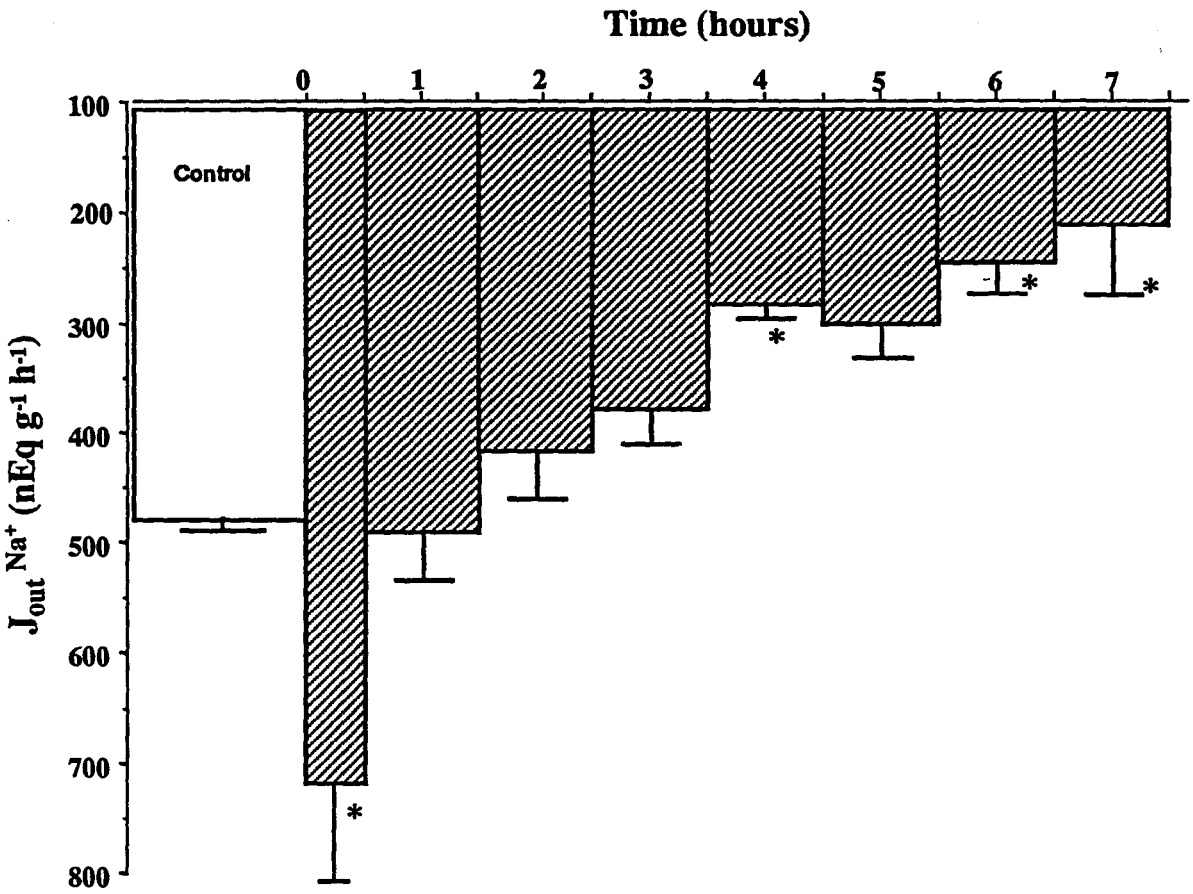
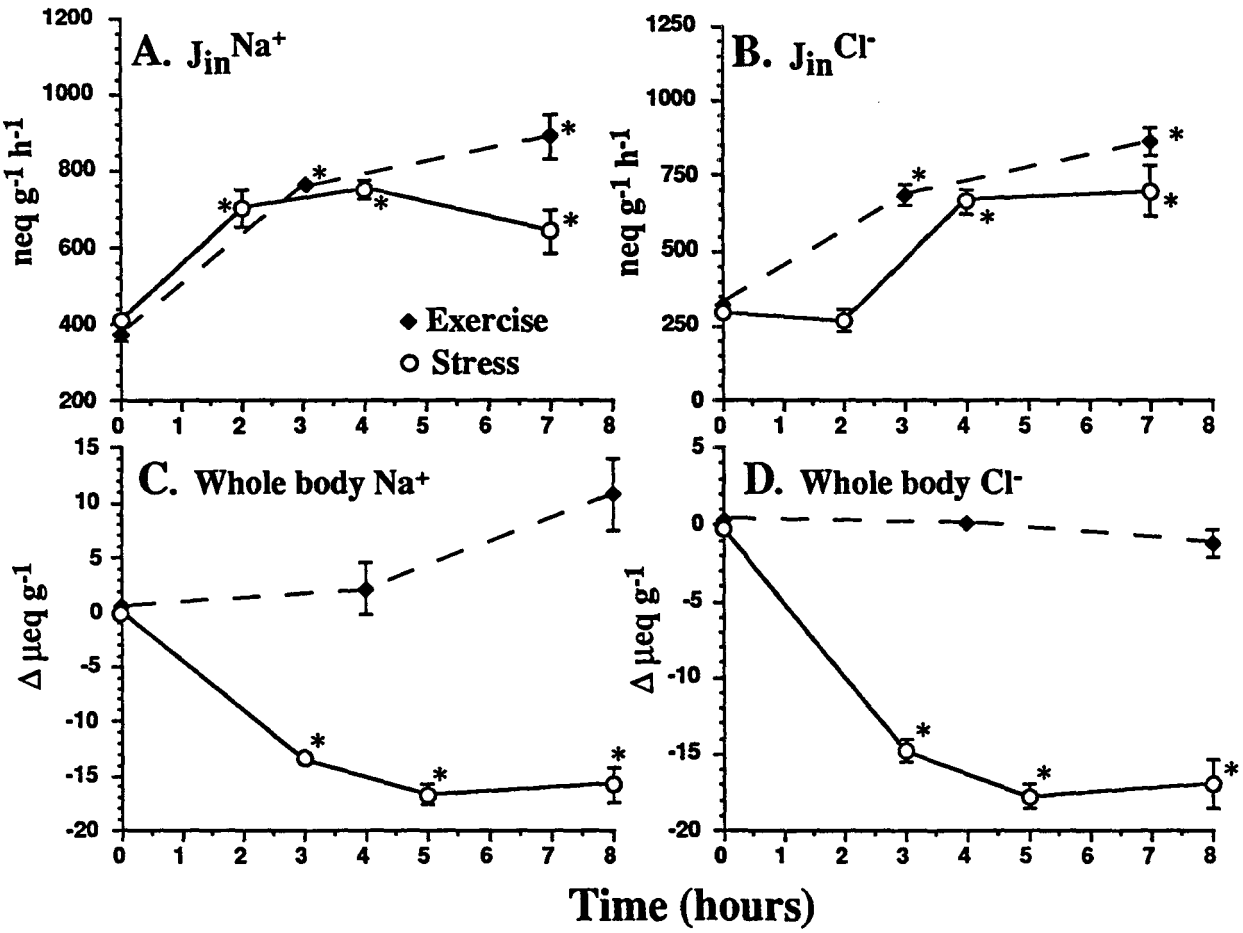

Time (hours)
0
1
2
3
4
5
6
7
100
200
300
400
500
600
700
800
Control
J_{out} Na^+ (nEq g^{-1} h^{-1})
*
*
*
*

Figure 1.4. The effects of 8 h confinement stress (open symbols, 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 ± one SEM, N = 9-11 per time point, 41 in total. J_{in} measured over 2 h periods; values are displayed at mid-point. Data for exercise (solid symbols, dashed lines) from Fig. 1.2 included for comparison. Asterisks indicate means significantly different ($P < 0.05$) from T0 by Dunnett's 't' test.

Figure 1.4


A. $J_{in}Na^+$
neq g^{-1} h^{-1}
1200
1000
800
600
400
200
◆ Exercise
○ Stress
B. $J_{in}Cl^-$
neq g^{-1} h^{-1}
1250
1000
750
500
250
0
C. Whole body Na^+
Δ µeq g^{-1}
15
10
5
0
-5
-10
-15
-20
D. Whole body Cl^-
Δ µeq g^{-1}
5
0
-5
-10
-15
-20
0 1 2 3 4 5 6 7 8
Time (hours)

kinetic measurements (J_{max} 560 ± 55 and 524 ± 81 nEq·g^{-1}·h^{-1}, respectively, Fig. 1.2). K_m values for $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ were found to be 138 ± 56 and 152 ± 46 μEq·l^{-1}, respectively.

Exercise stimulated rapid increases in both $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ (Fig. 1.2 A,B). Over 2 - 4h exercise, $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ had increased by 1.9 and 2.3 fold respectively, above control levels 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 routine conditions, Na^+ efflux ($J_{out}^{Na^+}$) averaged 471 ± 14.4·nEq g^{-1}·h^{-1} (N = 6 trials, 104 fish, Fig. 1.3). 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 h to 1 h, $J_{out}^{Na^+}$ had already declined to levels found during rest, and over the successive 7 h continued to decline; by 6 h $J_{out}^{Na^+}$ was only 47 % of routine 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. 1.4 C,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 μEq·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. 1.4 A,B) averaged about 4000 nEq·g·h^{-1} for each of Na^+ and Cl^-, i.e. efflux rates that are at least 8 fold higher than routine (Fig. 1.3). These net losses were accompanied by significant increases in $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ (Fig. 1.4 A,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. 1.4 A,B).

In a subsequent experiment examining recovery from 4 h of confinement stress (Fig. 1.5), whole body Na^+ and Cl^- levels were still depressed at 16 h post-stress, by 24% and 37%, respectively (Fig. 1.5B). 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. 1.5). $J_{in}^{Na^+}$ 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}'s 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. 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 remained relatively low, averaging 18 ± 3 ng·mL^{-1} (N = 7), with no tendency for increasing cortisol with sample number (Fig. 1.6, T0 for confinement). However, cortisol levels in the controls from the exercise chamber (no current, fish removed one at a time from groups of 3) did increase; from 28 ± 20.7 (N = 6) for sample #1, to 61.7 ± 12.2 (N = 6) for sample #2, to 94.0 ± 13.5 ng·mL^{-1} for sample #3. As a result of this sampling effect the average T0 value for exercise was about 3 fold higher than the average T0 value for confinement (Fig. 1.6). Exercising fish (also sampled in groups of 3)

Figure 1.5. Recovery of (A) $J_{in}^{Na^+}$ (open circles) and $J_{in}^{Cl^-}$ (solid circles), and (B) whole body Na^+ (open circles) and Cl^- (closed circles) from 4 h of confinement stress. Values are means ± one SEM.; N = 5 - 7 for each time point. Asterisks indicate means significantly different ($P < 0.05$) from T0 by Dunnett's 't' test. J_{in} measured over 2 h periods; values are displayed at mid-point.

Figure 1.5

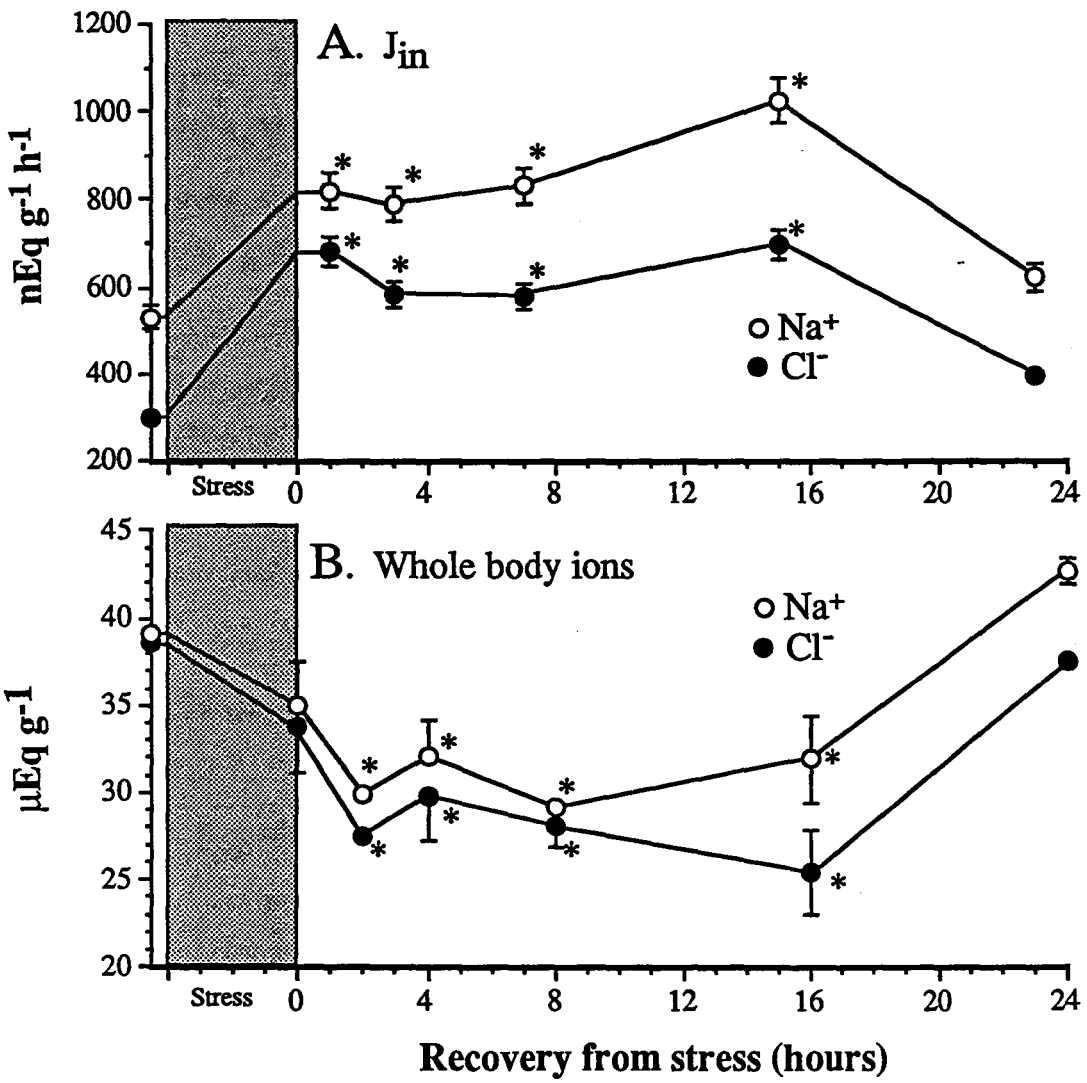
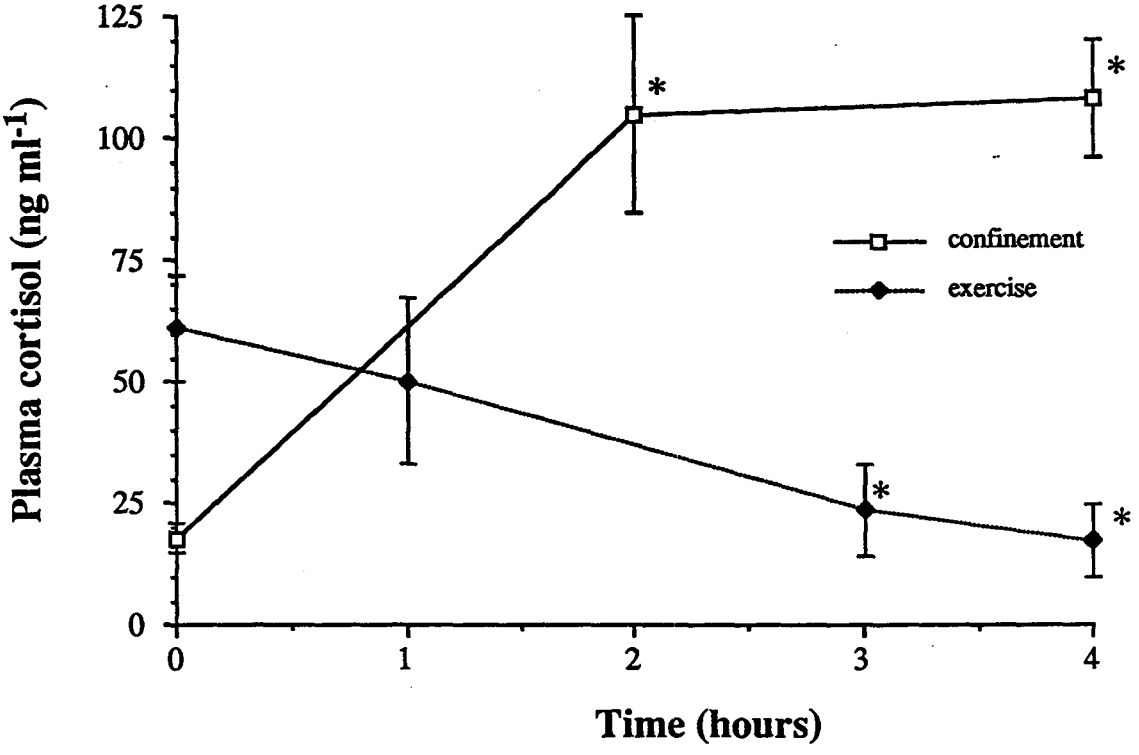

A. J_{in}
nEq g^{-1} h^{-1}
1200
1000
800
600
400
200
Stress
0
4
8
12
16
20
24
o Na+
● Cl-
B. Whole body ions
µEq g^{-1}
45
40
35
30
25
20
Recovery from stress (hours)

Figure 1.6. Effects of exercise at ~2 $BL \cdot sec^{-1}$ and confinement stress on plasma cortisol in rainbow trout. Values are means ± one SEM, N = 18 for T0 (exercise), N = 6 for 1, 3 & 4 h exercise; N = 8 for T0 (stress), N = 7 for 2 & 4 h stress. Asterisks indicate means significantly different ($P < 0.05$) from controls (i.e. T0) by Dunnett's 't' test.

Figure 1.6


Plasma cortisol (ng ml-1)
125
100
75
50
25
0
0
1
2
3
4
Time (hours)
confinement
exercise
*
*
*
*

also showed increasing cortisol with sample number. However, the sampling effect was smaller, and the average cortisol levels tended to decline with exercise (Fig. 1.6), 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 $ng \cdot mL^{-1}$ (N = 7), a 6 fold increase relative to pre-confinement stress controls. With confined fish, there was no sampling effect on cortisol levels; i.e. no increase in cortisol with sample number.

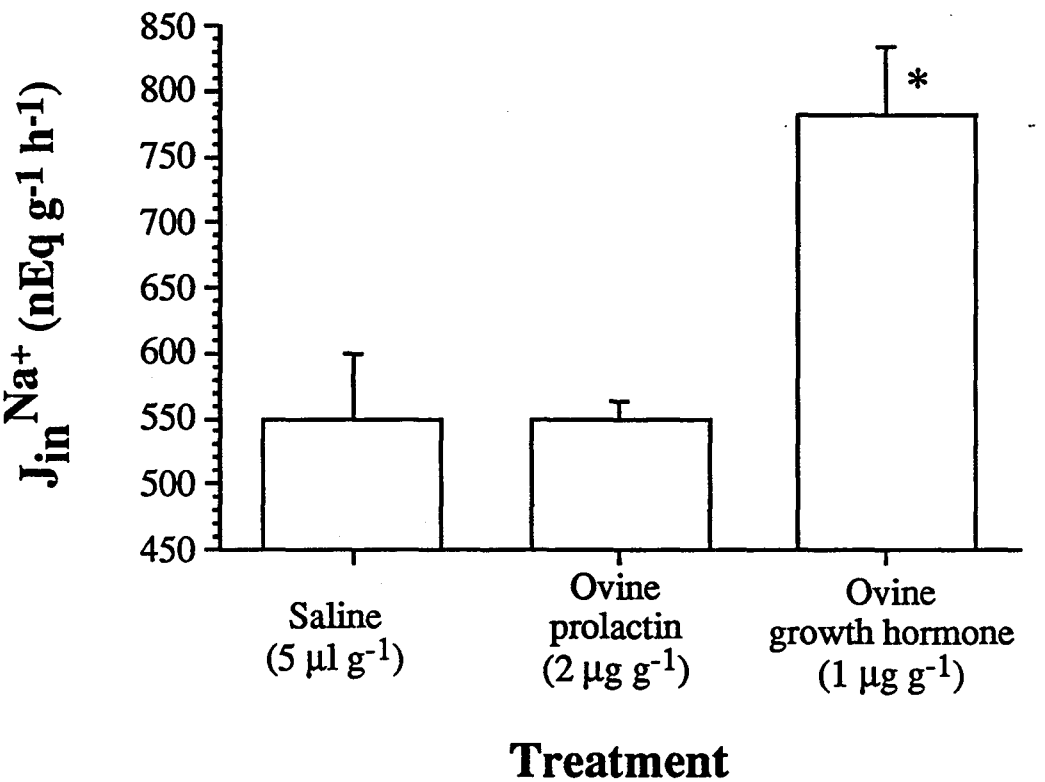
Effects of mammalian hormones on Na^+ influx

In this experiment (Fig. 1.7), $J_{in}^{Na^+}$ measurement was not initiated until 1 h post-injection to allow for the stimulating effect of injection stress to subside (c.f. Gonzalez and McDonald, 1992). Nonetheless, $J_{in}^{Na^+}$for saline injection was significantly higher than $J_{in}^{Na^+}$ in uninjected controls (549 ± 52 vs 390 ± 31 $nEq\ g^{-1} \cdot h^{-1}$, Fig. 2).

Ovine prolactin had no significant effect on $J_{in}^{Na^+}$ relative to saline-injected controls but ovine growth hormone has a pronounced stimulatory effect. For the period of 1 - 3 h post-injection, $J_{in}^{Na^+}$ in GH-injected fish was 1.4 times greater than controls (Fig. 1.7). This is a similar level of stimulation to that found with 2 - 4 h of exercise (Fig. 1.2).

Figure 1.7. The effects of saline, ovine growth hormone and ovine prolactin injection on $J_{in}^{Na^+}$ measured for 2 h starting at 1h post-injection. Values are means ± one SEM, N = 7-11 per treatment. Asterisk indicates mean significantly different ($P < 0.05$) from T0 by Dunnett's 't' test.

Figure 1.7


850
800
750
700
650
600
550
500
450
$J_{in}^{Na^+}$ (nEq g^{-1} h^{-1})
*
Saline
(5 µl g^{-1})
Ovine
prolactin
(2 µg g^{-1})
Ovine
growth hormone
(1 µg g^{-1})
Treatment

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 differed in the magnitude of the disturbance and the rapidity of the correction. In exercise, a small depression in plasma Na^+ and Cl^- (Fig. 1.1A) was rapidly followed by a >3 fold increase in J_{in}, almost 3 fold higher than the routine J_{max} (Fig. 1.2 A,B), and a similar reduction in J_{out} (Fig. 1.3). Consequently, the NaCl imbalance was corrected in the early stages of exercise and, in fact, there was an overshoot in whole body Na^+ (Fig. 1.1B). In contrast, $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ increased with confinement stress to levels at least 8 fold higher than controls, remained elevated for at least 5 h (compared to only 0.5 h at the start of exercise) and produced much larger net Na^+ and Cl^- losses (Fig. 1.4 C,D). Adjustments to J_{in} followed a similar time course and were of the same magnitude to that of exercise (Fig. 1.4 A,B), but the reduction in J_{out} occurred much later with NaCl balance taking 24 h to be corrected (Fig. 1.5 A,B).

These observations can now be used to develop a general model for ion regulation in freshwater fish, a model which 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 challenges to ion balance. Furthermore, simultaneous increases in $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ can take place much more rapidly than 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 by the gills of freshwater fish were those reported following experimentally induced acid-base disturbances, where acidoses or alkaloses were corrected by the appropriate manipulation of Na^+/H^+ and Cl^-/HCO_3^- exchanges (see McDonald et al., 1989; Goss et al., 1992 for reviews). Kinetic analysis by Wood and Goss (1990) revealed that a major part of the response to acid-base imbalance was alterations in 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 suggests the activation of inactive carriers rather than the synthesis of new carriers. The notion of there being inactive transport sites in the gills is unprecedented for life in freshwater, but is consistent with the rapid changes in ion transport that occur in anadromous fish upon transfer to seawater (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 review by 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 that 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. With exercise the initial drop in plasma Na^+ and Cl^- (Fig. 1.1A) was small relative to the initial decrease with stress (Fig. 1.4) and yet the J_{in} response was very similar for both (Fig. 1.4). Furthermore, with exercise, the initial decrease of plasma Na^+ and Cl^- was not accompanied by a decrease in whole body ions (Fig. 1.1), indicating that an actual 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 fact that there was a disturbance in plasma but not whole body Na^+ and Cl^- is worthy of comment. It is most likely the result of a combination of a net influx of water at the gills (c.f. Wood and Randall, 1973b), a redistribution of water between extracellular and intracellular compartments, and an increase in diffusive ion flux at the gills. None of these effects was, however, particularly prominent. For example, there was no detectable increase in total body water in the initial stages of exercise, and the increased J_{out} was minor and quite temporary (Fig. 1.3).

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), as far as we are aware, only cortisol and

epinephrine have previously been shown to specifically 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 et al., 1989; Madsen, 1990a; Perry et al., 1992). However, it is probable that cortisol was not important to the adjustments to ion uptake in the present study for two reasons. Cortisol concentrations were elevated in stress but not during exercise and yet similar increases in ion uptake occured in both circumstances. Secondly, Laurent and Perry (1990) showed that acute (3h) 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 a similar reasoning, epinephrine can also be discounted because, while 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 (e.g. Ristori and Laurent, 1985; Butler et al., 1986). Furthermore, although acute infusion of epinephrine into rainbow trout will stimulate J_{in}^{Na+} and J_{in}^{Cl-} within a few minutes of the start of infusion (McDonald and Rogano, 1986), longer term infusions ($\geq$ 6h) actually inhibit J_{in}^{Na+} and J_{in}^{Cl-} (Vermette and Perry, 1987). In addition, epinephrine infusion also greatly stimulates branchial diffusion of Na^+ and Cl^- (McDonald and Rogano, 1986; Gonzalez and McDonald, 1992) so any stimulatory effect on ion transport may, in fact, be indirect through the stimulation of ion losses.

Of the remaining hormones, current evidence points to growth hormone as having the greatest potential for regulating NaCl uptake in freshwater. Although its main osmoregulatory function is thought to be in the preparation for seawater migration and is 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 efficiency to cortisol in modulating ionoregulatory activities of the gills (see Borgatti et al., 1992 for review). Madsen, (1990b), for example, found that 7 days of ovine GH injection (2 μg/g every other 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 $J_{in}^{Na^+}$ (Fig. 1.7), much more rapidly than Madsen's study would suggest. Parallel to this observation, Barrett and McKeown (1988), found that aerobic exercise (1.5 BL sec^{-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, such an explanation 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 (c.f. Maetz, 1974) previous studies on rainbow trout have suggested that regulation of ionic permeability of the gills is at least equally important. McDonald and Rogano (1986) showed that $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ were reduced to 25% of control levels by 24 h of exposure to ion-poor freshwater, whereas J_{in} in this medium 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. 1.3) was at least as rapid as the stimulation of J_{in} (Fig. 1.2). 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. 1.5A) were never more than about 2 fold higher than routine rates. These rates would only be adequate to explain the recovery of ion balance (Fig. 1.5B) 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 since the location and nature of the branchial NaCl diffusion path(s) are not established. However, Gonzalez and McDonald (1992) presented indirect evidence suggesting that the paracellular channels were the main diffusion path and that permeability control was 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} are such to 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 fishes (see Hirano, 1986; Prunet et al., 1990 for reviews), and the Na^+ retaining effect of PRL in fresh water, which is thought largely to occur through inhibition of Na^+ loss since it has no stimulating effect on Na^+ uptake (Dharmamba and Maetz, 1972), is well established for a number of non-salmonid euryhaline fish species (see Hasegawa et al., 1986 for review). For freshwater salmonids, PRL's role is much less certain since the removal of

PRL by hypophysectomy (Komourdjian and Idler, 1977; Björnsson and Hansson, 1983) and the injection of homologous PRL (Hasegawa et al, 1986) has slight, if any, effects on Na^+ balance. Some of the uncertainty as to PRL's actions in salmonids may be attributed to the lack of rapid responses of osmoregulatory surfaces to PRL treatment (Bern et al., 1981) and perhaps the masking effects of other hormones. For example, stresses associated with handling and intra-peritoneal injection of PRL would evoke substantial increases in cortisol and epinephrine. The latter would cause rapid and marked increases in Na^+ and Cl^- efflux (Gonzalez and McDonald, 1992) masking any effect of PRL. Consequently, any action of PRL in reducing J_{out} may be difficult to detect with an experimental approach employing a single injection of PRL. It was for this reason that we did not attempt to measure the effects of PRL injection on Na^+ efflux in the present study.

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

Thus, while we have established the likelihood of hormonal control in the ionoregulatory responses reported here, there is still considerable uncertainty

as to 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 apparently strikingly different; growth hormone prominent in exercise, cortisol and epinephrine prominent in stress, and uncertainty over PRL in either circumstance. 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, which has 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.

CHAPTER 2

ION REGULATION DURING AEROBIC EXERCISE AND CONFINEMENT STRESS IN FRESHWATER ADAPTED SMALLMOUTH BASS AND COMMON SHINERS

INTRODUCTION

As shown in Chapter 1, rainbow trout experience only brief ionic disturbances when exercising and are able to rapidly regulate and restore ion balance. This is clearly a benefit to an animal that prefers fast moving head water streams and moderately flowing shallow rivers (Scott and Crossman, 1979). Indeed, rainbow trout are considered to be good swimmers and when tested do not exhaust at swimming speeds less than 5.5 $BL \cdot sec^{-1}$ (Graham and Wood, 1981). Thus, the question that arises is whether this ability to rapidly regulate ion balance is unique to rainbow trout, is found only in stream-dwelling animals, or is widespread in fish irrespective of preferred habitat.

The two species chosen for comparison are common shiners, Notropis cornutus, and smallmouth bass, Micropterus dolomieu. Shiners are cyprinids that dwell in fast flowing streams (Lee et al, 1980) and clear water lakes (Scott and Crossman, 1973). Shiners can be considered active swimmers similar to rainbow trout, although their actual swimming ability has not been documented.

Unlike common shiners and rainbow trout, smallmouth bass are less active sit-and-wait predators that commonly live in lakes but can also be found in slow moving streams (Scott and Crossman, 1973). Smallmouth bass are only capable of sustaining swimming speeds of low velocities depending on the environmental temperature. Between 5°C and 20°C, critical swimming speeds for smallmouth bass range from 2 $BL \cdot sec^{-1}$ to 3.7 $BL \cdot sec^{-1}$ (Kolok, 1992).

In addition to habitat preference, there are other differences amongst the three species that might have a bearing on their ionoregulatory abilities. The gill epithelium in smallmouth bass is twice as thick as that in common shiners and rainbow trout (McDonald et al, 1991) and also, common shiners are less acid tolerant than rainbow trout as indicated by changes in the gill epithelium, whole body ion loss and shorter survival times in response to low pH exposure (Freda and McDonald, 1988).

Therefore, the objective of the study was to determine whether the ionoregulatory changes described in chapter 1 are unique to rainbow trout. Similarities and differences in ion regulation between active and less active species are assessed. Finally, possible mechanisms of control and reasons for any variation in rates and magnitude of change are discussed.

MATERIALS AND METHODS

Experimental animals

Smallmouth bass were obtained from Aquaresearch Limited, Quebec and common shiners from Steel City Bait in Hamilton, Ontario. Both species were maintained for at least two weeks prior to experimentation in 500 L cylindrical tanks continuously supplied with dechlorinated tap water at 20 ± 2°C. Smallmouth bass were fed Rangen Inc. salmon pellet feed from Iofoten Aqua supplies in British Columbia, while shiners were fed trout chow. Both received food ad libitum in the holding facilities until 2 days before the beginning of experiments.

Experimental series

A. common shiners

1. Na^+ uptake during rest and exercise.

Common shiners, (7.9 ± 0.5 g, N = 32) were exercised in the 19 L swim chamber at a velocity of 23 $cm \cdot s^{-1}$ (~2 body lengths$\cdot sec^{-1}$) for up to 8 h at 16 ± 1°C. The experimental regime employed was similar to that described in Chapter 1 for rainbow trout. Fish (N = 8) were added at T = 0 h, +3 h and +5 h to the 3 compartments with the current started at T = 0. At +6 h, ^{22}Na (as NaCl, 2$\mu Ci \cdot L^{-1}$ obtained from Amersham, Inc) and ^{36}Cl (as HCl, 2 $\mu Ci \cdot L^{-1}$,obtained from ICN) were added to the water, allowed to circulate, and radiotracer uptake followed for the next 2 h. At +8 h, all fish were removed, sacrificed with a blow to the head, and analyzed for whole body levels of ^{22}Na and ^{36}Cl. Na^+ and Cl^- were determined from whole body digests following radioactivity analysis. Water samples (10 mL) were also collected for analysis of ^{22}Na, ^{36}Cl and Na^+ and Cl^- at the beginning and end of the 2 h period. Na^+ and Cl^- uptake ($J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$) during rest were determined on fish held in 17 L of still water. Fish (N

= 8) were transferred to the chamber at 0 h, isotope was added at +4 h and animals were removed and sacrificed at +6 h.

2. Na^+ and Cl^- balance during confinement stress and rest.

Three separate, but similar experiments were conducted to analyze J_{in}^{Na+} during confinement stress and rest (experiments 1-3) and whole body Na^+ and Cl^- (expt.'s 2 and 3). The confinement procedure was similar to that previously employed with rainbow trout in Chapter 1. Fish (N = 24 in expt. 1, N = 8 in expt. 2, and N = 5 in the third) were transferred to nylon mesh bags (1 cm mesh) suspended in the 19 L tank (4-6 fish per bag) maintained at 17.5°C with water flow at a rate of 1 $L \cdot min^{-1}$. The tanks were aerated by airstones, positioned underneath each of the bags, and supplied with compressed air at a rate vigorous enough to ensure that bubbles penetrated the bags. The fish were confined for 3, 5 or 8 h in the first expt. or 5 h in the second and third expt.'s. ^{24}Na (as Na_2CO_3, produced in the McMaster Nuclear Reactor, 2 $\mu Ci \cdot L^{-1}$) was added to the water and allowed to circulate during the last hour in expt. 1 and during the last two hours in expt 2 and 3. J_{in}^{Na+} in resting fish was measured during 3-4 h in expt. 1 (N = 8) and 3-5 h in expt.'s 2 (N = 7) and 3 (N = 4). All fish were then removed, sacrificed by a blow to the head, rinsed, weighed and analyzed for whole body ^{24}Na activity and later digested for whole body ion analysis. Water samples (10 mL) were also collected for analysis of ^{24}Na at the beginning (after mixing) and end of the uptake period.

Experimental series

B. smallmouth bass

1. J_{in}^{Na+} during rest.

J_{in}^{Na+} was determined on individual fish (253.3 ± 15.9 g, N = 12 fish in total) held in 3 L black chambers containing 1.5 L of aerated water. Isotope (2.5 $\mu Ci \cdot L^{-1}$ of ^{22}Na) was added to fish and J_{in}^{Na+} measured for 2 h. Water samples

(7 mL) were taken at +10 min and each 0.5 h thereafter throughout the time period. After 2 h, fish were sacrificed and a 100 µL blood sample was obtained, centrifuged and the plasma analyzed for $^{22}Na^+$ activity ($cpm \cdot mL^{-1}$).

2. Plasma Na^+ and Cl^- and $J_{in}^{Na^+}$ during exercise.

In this experiment, smallmouth bass (237.1 ± 6.5 g, N = 30) were exercised in the 60 L swim chamber with a velocity of 34 $cm \cdot s^{-1}$ (~2 $BL \cdot sec^{-1}$) for up to 12 h. Fish (N = 6) were added to one compartment and the current was started (T = 0). Additional groups of fish were then added to the other three compartments at +4 h, +7 h and at +9 h. At +10 h, ^{22}Na (as NaCl, 2µCi·L^{-1} obtained from Amersham, Inc) was added to the water and allowed to circulate. Radiotracer uptake then followed for the next 2 h. At 12 h, all fish were removed, anaesthetized in MS 222 (0.3 $g \cdot L^{-1}$) for approximately 4 min, rinsed and sampled for blood by caudal puncture. Following blood sampling, fish were sacrificed by a blow to the head. Blood samples were immediately centrifuged and plasma analyzed for ^{22}Na activity, Na^+ ,Cl^- and cortisol. Water samples (10 mL) were also collected for analysis of ^{22}Na at the beginning (after mixing) and end of the 2 h period. $J_{in}^{Na^+}$ during 3-5 h was also determined on fish (N = 6) held in the chamber without the flow generating pumps in operation.

3. Plasma Na^+ and Cl^- and $J_{in}^{Na^+}$ during confinement stress.

In this series of experiments, plasma Na^+ and Cl^- and $J_{in}^{Na^+}$ were examined during confinement stress in smallmouth bass (234.9 ± 7 g, N = 18). The confinement procedure was similar to that previously employed in chapter 1. Fish were transferred to nylon mesh bags (1 cm mesh) and suspended in 80 L tanks (3 bags per tank, 5-6 fish per bag). Fish were confined for 3, 5 or 8 h and during the final two hours of confinement, ^{22}Na (as NaCl, 2.5 µCi·L-1 obtained from Amersham, Inc) was added to the water and allowed to circulate, and radiotracer uptake followed for the next 2 hours. All fish were then

removed, anaesthetized in MS 222 (0.3 $g \cdot L^{-1}$) for approximately 4 min, rinsed and sampled for blood by caudal puncture. Blood samples were centrifuged and plasma was collected for measurements of plasma Na^+, Cl^- and cortisol. Water samples (10 mL) were also collected for analysis of ^{22}Na at the beginning (after mixing) and end of the 2 h period.

Analytical methods

Methods for the following procedures are described under analytical methods in Chapter 1: plasma Na^+ and Cl^-, whole body Na^+ and Cl^- analysis, ^{22}Na, ^{24}Na and ^{36}Cl activity analysis in tissue, plasma and water samples, ion analysis in water samples and plasma cortisol.

Calculations

$J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ in $neq \cdot g^{-1} \cdot h^{-1}$ for common shiners were calculated according to the following formula:

$$J_{in} = \frac{\Sigma Q}{SA \bullet W \bullet t} \qquad (1)$$

where 'ΣQ' is the whole body radioactivity in $cpm \cdot kg^{-1}$, 'SA' is the specific activity of the water in $cpm \cdot neq^{-1}$, 'W' is the body weight in grams and 't' is the duration in hours.

$J_{in}^{Na^+}$ for smallmouth bass at rest was calculated from equation 1 and during exercise and confinement stress according to the following formula:

$$J_{in}^{Na^+} = \frac{[^{22}Na]_p \bullet Na^+_{sp}}{SA \bullet t} \qquad (2)$$

where '$[^{22}Na]_p$' is the $^{22}Na^+$ in plasma in $cpm \cdot mL^{-1}$ and 'Na^+_{sp}' is the Na^+ space in $mL \cdot kg^{-1}$.

Na^+ space was calculated from the two hour ^{22}Na uptake measurements made on resting smallmouth bass according to the following formula:

$$Na^{+}_{sp} = \frac{\Sigma Q}{[^{22}Na]_p \bullet t} \qquad (3)$$

where ΣQ is the total ^{22}Na uptake over 2 h in cpm and $[^{22}Na]_p$ is the plasma ^{22}Na activity in cpm mL^{-1} at the end of the 2 h period. Na^+ space averaged 260 $mL \cdot kg^{-1}$.

Data analysis.

Means ± one standard error of the mean (SEM) are reported throughout. Comparisons amongst time series data were analyzed by analysis of variance ($P < 0.05$). If significant, Dunnett's 't' test (Dunnett, 1955) was used to compare treatment effects to control ($P < 0.05$).

RESULTS

Whole body, plasma Na^+ and Cl^- responses to aerobic exercise

When common shiners were continuously exercised for 8 h at 2 $BL \cdot s^{-1}$, there were no detectable changes in whole body Na^+ and Cl^- (Fig. 2.1). The average Na^+ during the 8 h regime was 41.5 ± 0.9 $\mu eq \cdot g^{-1}$ while Cl^- averaged 23.0 ± 0.6 $\mu eq \cdot g^{-1}$.

With the onset of exercise, there were significant alterations in plasma Na^+ and Cl^- in smallmouth bass (Fig. 2.2 A,B). There was an initial depression in plasma Na^+ and Cl^- after 5 h of continuous exercise (4% and 13%, respectively). Plasma Na^+ levels continued to remain 5% lower than controls at 8 h, but had returned to routine levels after 12 h of exercise. In contrast, plasma Cl^- showed a downward trend throughout and was 17.5% lower than routine levels after 12 h of exercise.

J_{in} at rest and during exercise

There were significant differences in routine $J_{in}^{Na^+}$ amongst the three species (Fig. 2.3, T = 0). Shiners had significantly lower routine uptake levels in comparison to rainbow trout (250.5 ± 24.1 $neq \cdot g \cdot h^{-1}$ versus 390.3 ± 31 $neq \cdot g \cdot h^{-1}$, Table 2.1), even though average weights were similar (9.6 ± 0.7 g and 8.5 ± 2.6 g, respectively). In smallmouth bass, which were ~26 fold larger than common shiners and rainbow trout, (237 ± 6.5 g versus ~9.0 g), $J_{in}^{Na^+}$ was similar to common shiners (172.7 ± 27.2 $neq \cdot g \cdot h^{-1}$ versus 250.5 ± 24.1 $neq \cdot g \cdot h^{-1}$, respectively) and significantly lower than rainbow trout (172.7 ± 27.2 $neq \cdot g \cdot h^{-1}$ versus ~390.3 ± 31 $neq \cdot g \cdot h^{-1}$, respectively).

Exercise stimulated a significant increase in $J_{in}^{Na^+}$ in common shiners. Levels increased 2.3 fold by 1 h of exercise and remained at this level for the

Figure 2.1. The effect of exercise at 2 $BL \cdot sec^{-1}$ on whole body Na^+ and Cl^- of common shiners (7.9 ± 0.5 g). Values are means ± one SEM, N = 8 fish per time point (32 fish in total).

Figure 2.1

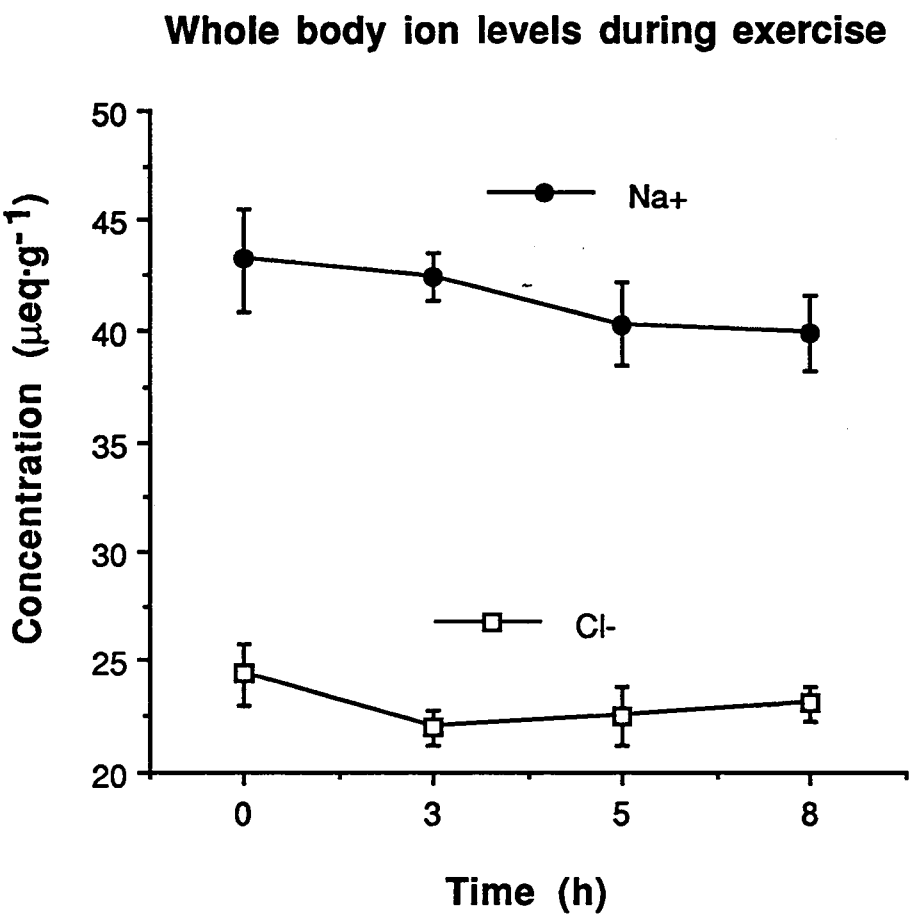
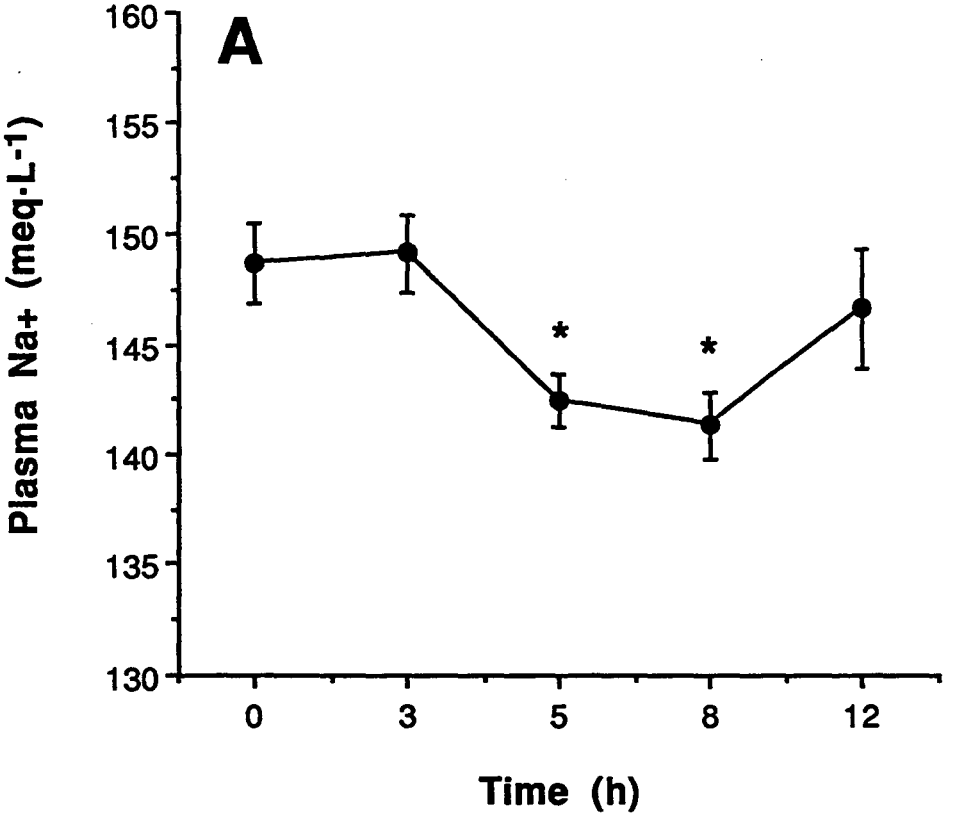

Whole body ion levels during exercise
Concentration (μeq·g-1)
50
45
40
35
30
25
20
Na+
Cl-
0
3
5
8
Time (h)

Figure 2.2. The effect of exercise on plasma Na^+ (A) and Cl^- (B) of smallmouth bass (237.1 ± 6.5 g). Values are means ± one S.E.M, N = 6 per time point (42 fish in total), asterisks indicate means significantly different ($P<0.05$) from controls (i.e. T = 0) by Dunnett's 't' test.

Figure 2.2


A
160
155
150
145
140
135
130
Plasma Na+ (meq·L-1)
*
*
0
3
5
8
12
Time (h)

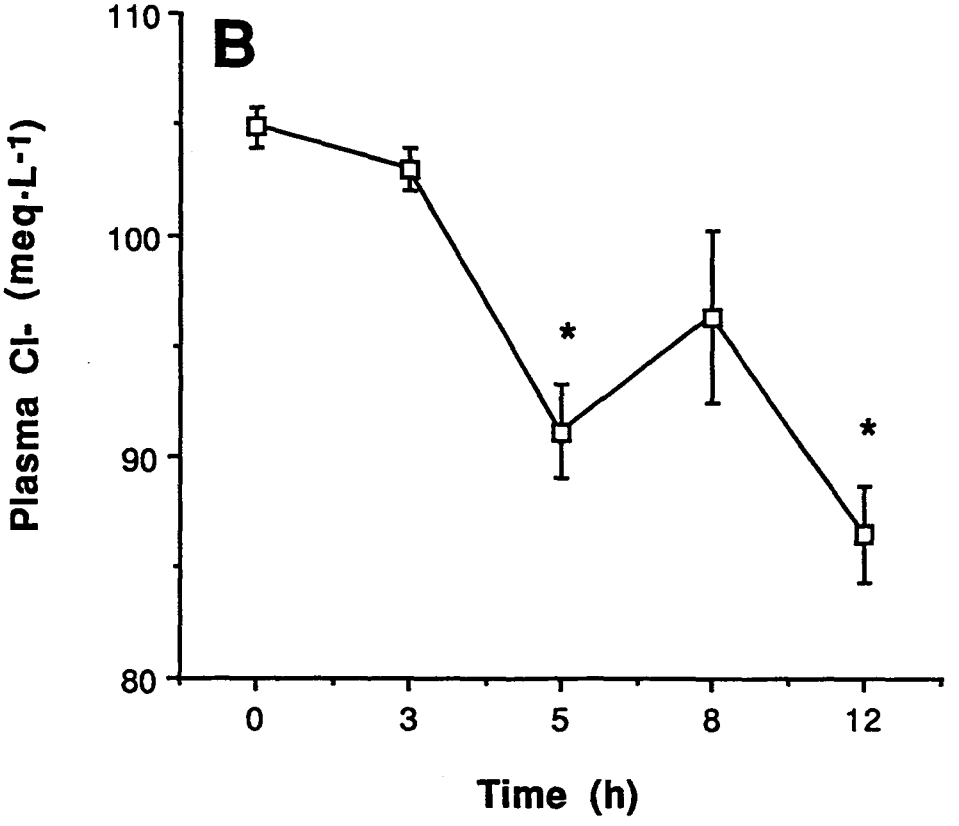
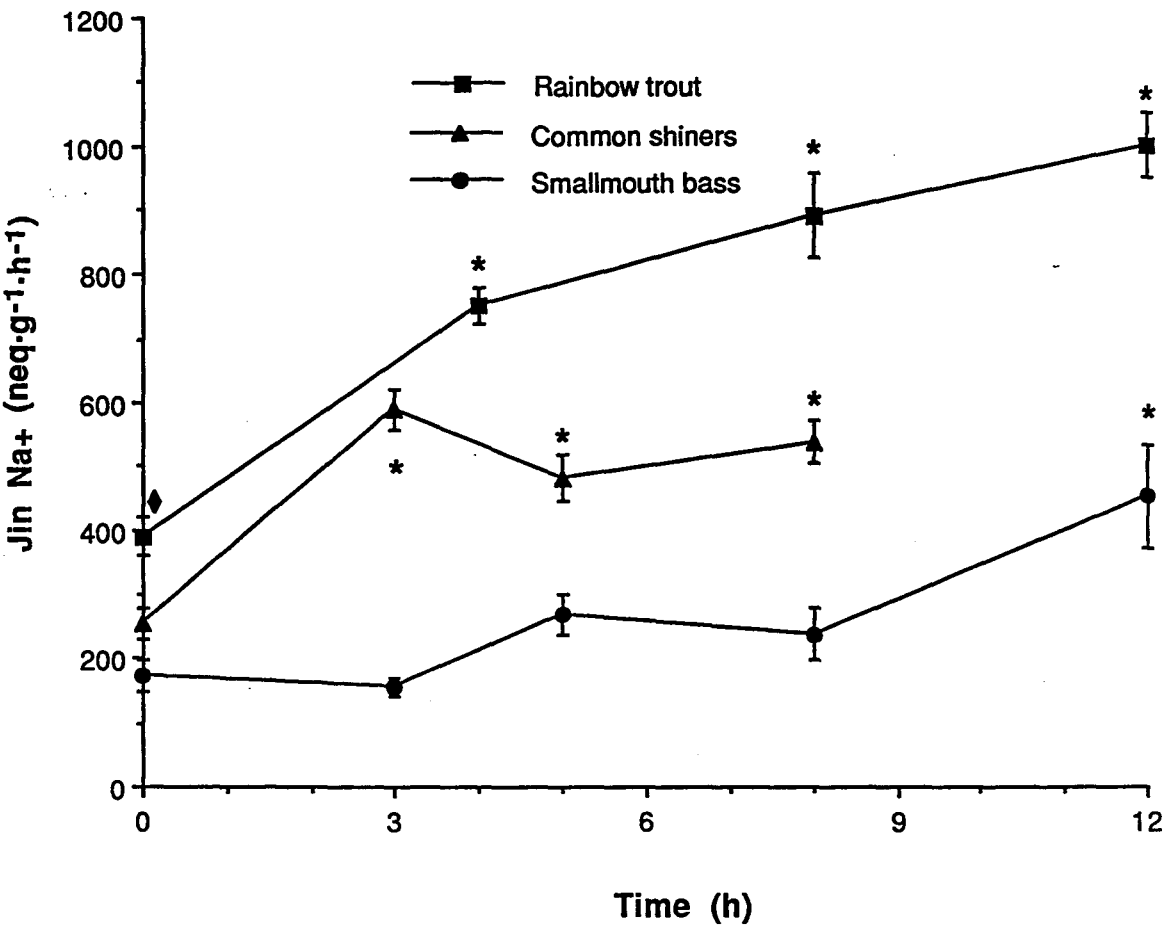

B
110
100
90
80
Plasma Cl- (meq·L-1)
*
*
0
3
5
8
12
Time (h)

Figure 2.3. Changes in Na^+ influx ($J_{in}Na^+$) during exercise in rainbow trout, common shiners and smallmouth bass. Influx was measured for 2 h immediately prior to the point indicated (4-6 h = 6 h). Values are means ± one S.E.M., N= 8-10 per time point for rainbow trout (38 in total); 8 per time point for shiners (32 in total) and 6 per time point for smallmouth bass (30 in total), asterisks indicate a significant difference ($P<0.05$) from routine levels at T = 0. A solid diamond indicates a significant difference ($P<0.05$) in resting influx in rainbow trout compared to common shiners and smallmouth bass

Figure 2.3


Rainbow trout
Common shiners
Smallmouth bass
Jin Na+ (neq·g-1·h-1)
Time (h)
0
200
400
600
800
1000
1200
0
3
6
9
12

duration of the 8 h exercise period (Fig. 2.3). Smallmouth bass, on the other hand, showed no significant elevation in $J_{in}^{Na^+}$ until 10-12 h of exercise. At this point however, $J_{in}^{Na^+}$ was still only about 45 % of $J_{in}^{Na^+}$ in rainbow trout at this time.

Routine $J_{in}^{Cl^-}$ in common shiners averaged 110.3 ± 11.5 neq·g·h^{-1} (N = 8). These values were only about 1/3 of the routine $J_{in}^{Cl^-}$ in rainbow trout (303 ± 20.6 neq·g·h^{-1}). Furthermore, exercise did not stimulate any increase in $J_{in}^{Cl^-}$ during an 8 h period. $J_{in}^{Cl^-}$ at 6 - 8 h of exercise in common shiners was 18% of $J_{in}^{Cl^-}$ in rainbow trout during the same time period (Fig. 2.4). $J_{in}^{Cl^-}$ was not measured in smallmouth bass.

Electrolyte balance during stress

In shiners, 5 h of confinement caused significant losses in whole body Na^+ and Cl^- (whole body ion levels were measured in expts 2 &3 only). In the two experiments, the amounts of ion loss were not significantly different from one another and the results were therefore pooled (Fig. 2.5). On average, losses of Na^+ and Cl^- amounted to 7.7 and 8.7 μeq·g^{-1} (Fig. 2.5), or approximately one-half of the Na^+ and Cl^- losses by 5 h in confined rainbow trout (Fig. 1.4).

Confinement stress in smallmouth bass was found to produce significant decreases in plasma Na^+ (whole body Na^+ and Cl^- levels were not measured). The 25.8 mEq·L^{-1} loss of Na^+ at 8 h amounted to a 17% reduction in plasma Na^+ (Fig. 2.6A). In contrast, plasma Cl^- remained constant throughout the 8 h stress period (Fig. 2.6B).

$J_{in}^{Na^+}$ during confinement stress

$J_{in}^{Na^+}$ in common shiners varied from one experiment to another, not only during stress but also during rest (Table 2.1). Since no stimulation of

Figure 2.4. Cl^- uptake (Jin Cl^-) in common shiners in relation to Cl^- uptake in rainbow trout during exercise at 2 BL-sec^{-1}. Values are means ± one S.E.M., N = 8 per time point for rainbow trout (24 in total) and 8 per time point for shiners (32 in total), asterisks indicate a significant difference ($P<0.05$) from routine levels at 0 h.

Figure 2.4

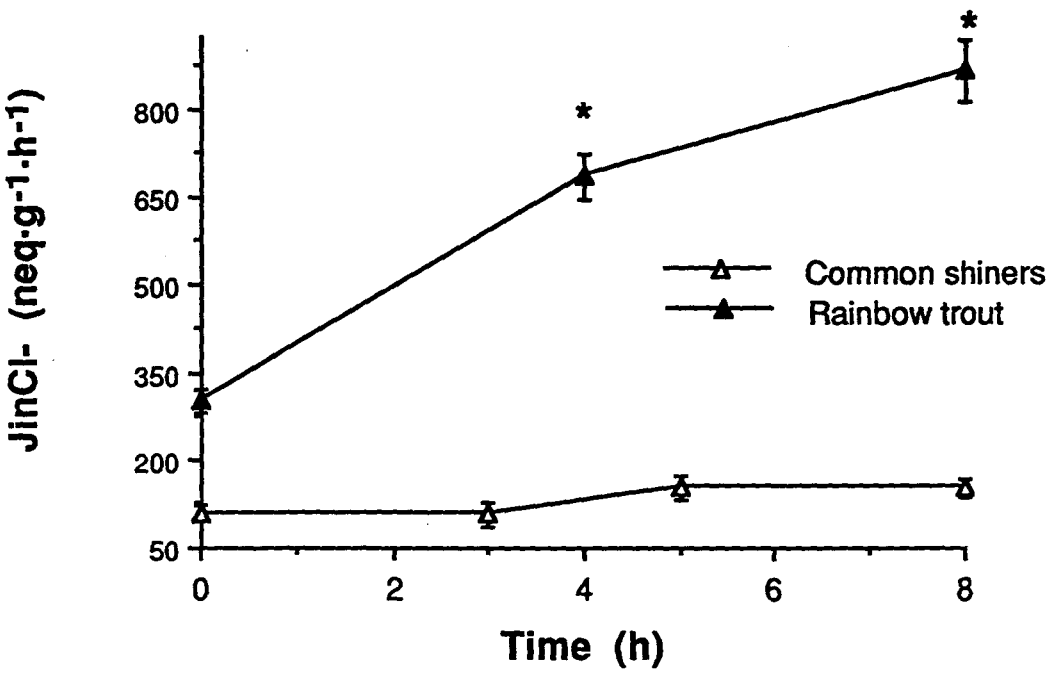

JinCl- (neq·g-1·h-1)
800
650
500
350
200
50
0
2
4
6
8
Time (h)
*
*
Common shiners
Rainbow trout

Figure 2.5. The effect of 3 - 5 h of confinement stress on whole body Na^+ in Cl^- of common shiners (expt.'s 2 and 3). Values are means ± one S.E.M., N= 11 - 13 per time point (24 in total), asterisks indicate a significant difference ($P<0.05$) from controls at T = 0.

Figure 2.5

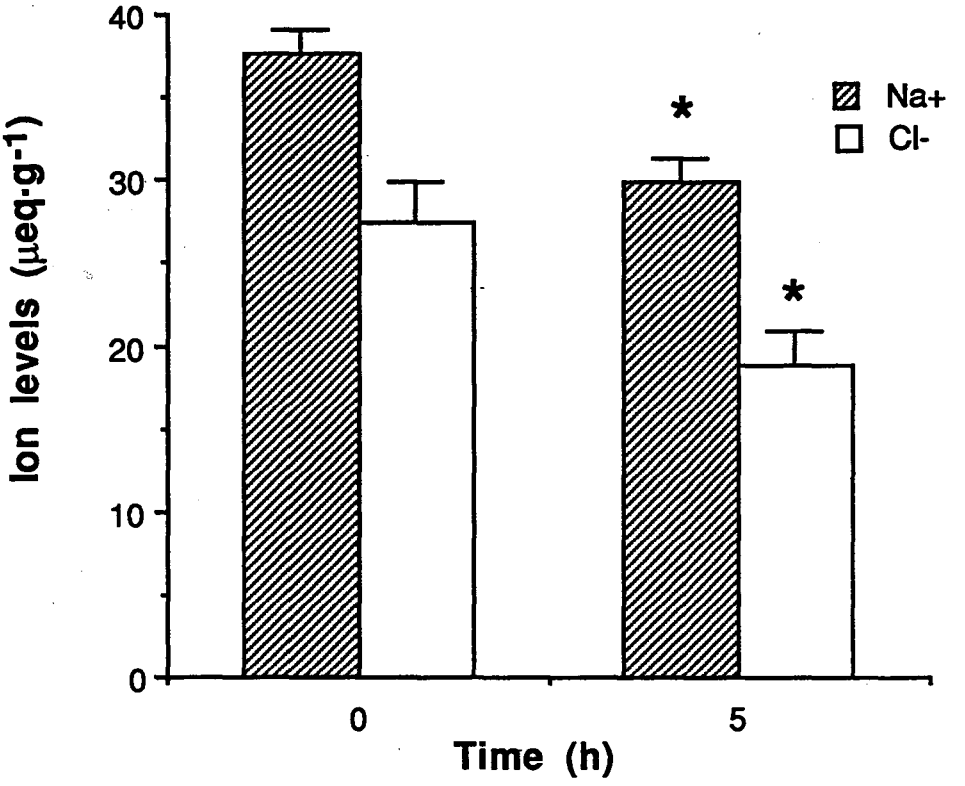


Figure 2.6. The effect of confinement stress on (A) plasma Na^+ and (B) plasma Cl^- of smallmouth bass. Values are means ± one S.E.M., N= 6-8 per time point (26 in total), asterisks indicate a significant difference ($P<0.05$) from controls at T = 0.

Figure 2.6

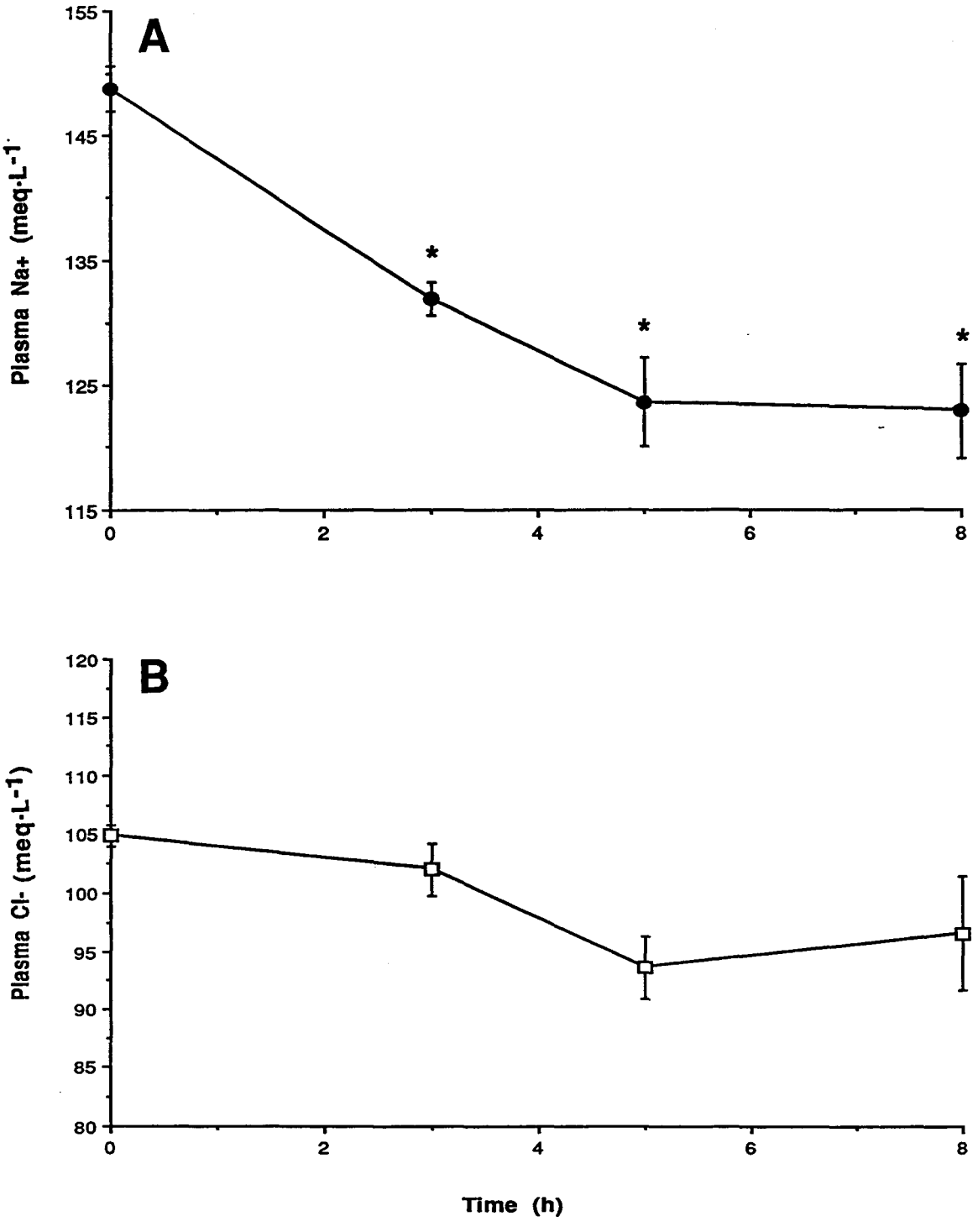

A
B
Plasma Na+ (meq·L-1)
Plasma Cl- (meq·L-1)
155
145
135
125
115
120
115
110
105
100
95
90
85
80
0
2
4
6
8
*
*
*
Time (h)

Table 2.1 Experiment number, fish weight and Na^+ influx ($J_{in}^{Na^+}$) for common shiners at 17.5°C during 1 h of rest and 3-4 h of confinement in expt. 1 and during 2 h of rest and during 3-5 h of confinement stress in expt.'s 2 and 3. Values are means ± one SEM, (N)

Experiment	Wt (g)	Jin Na+-rest ($neq \cdot g^{-1} \cdot h^{-1}$)	JinNa+-stress ($neq \cdot g^{-1} \cdot h^{-1}$)
1	10.2 ± 1.2	255.7 ± 24.6 (8)	221.1 ± 19.8 (8)
2	6.59 ± 0.6	306.9 ± 52.8 (7)	635.3 ± 61.5 (8)
3	16.21 ± 1.2	141.3 ± 29.2 (4)	812.0 ± 10.3 (5)
Average	**9.6 ± 0.7**	**250.5 ± 24.1**	**519.6 ± 65.8**

J_{in}^{Na+} occurred during the 8 h confinement period in expt. 1, the experiment was repeated two more times. Confinement stress for 3-5 h in expt. 2 produced a 2 fold increase in J_{in}^{Na+} and a 6 fold increase in J_{in}^{Na+} by this time in expt. 3. The J_{in}^{Na+} values from expt.'s 2 and 3, when averaged give a similar increase in uptake to that found in rainbow trout (Fig. 2.7). J_{in}^{Na+} was unaltered by 8 h of confinement in smallmouth bass (Fig. 2.7).

Cortisol levels in smallmouth bass during stress and exercise

Both exercise and confinement stress significantly elevated cortisol levels in smallmouth bass (Fig 2.8). After 8 h of confinement stress, cortisol levels in smallmouth bass had increased 5.7 fold above routine levels which is a comparable increase to that seen in rainbow trout after 4 h of confinement (Fig. 1.6). During exercise, cortisol levels also steadily increased to values that were 6.5 fold larger than routine levels by 12 h of exercise (Fig. 2.8). In contrast, cortisol levels declined during exercise in rainbow trout (Fig. 1.6).

Figure 2.7. Changes in Na^+ influx ($J_{in}Na^+$) during confinement stress in rainbow trout, common shiners (expt.'s 2 and 3) and smallmouth bass. Influx was measured for 2 h immediately prior to the point indicated (4-6 h = 6 h). Values are means ± one S.E.M., N = 8-10 per time point for rainbow trout (38 in total); 11 - 13 per time point for shiners (24 in total) and 6-8 per time point for smallmouth bass (26 in total), asterisks and cross indicate a significant difference ($P<0.05$) from routine levels at 0 h.

Figure 2.7

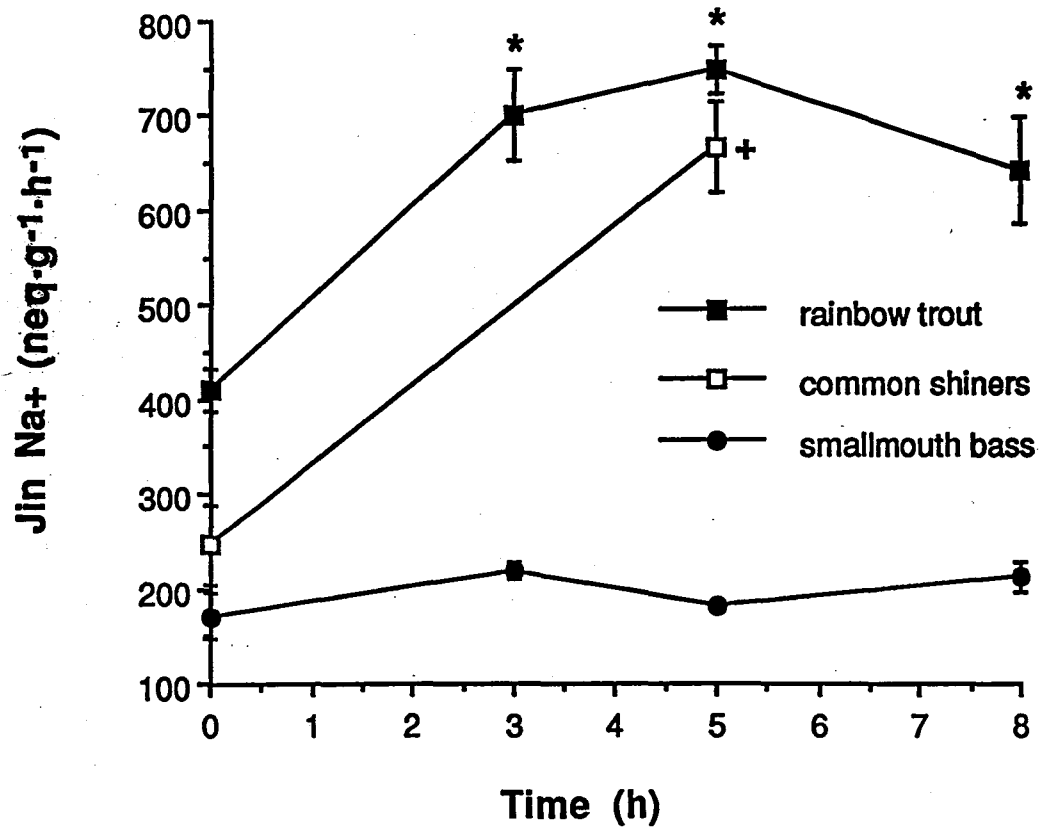
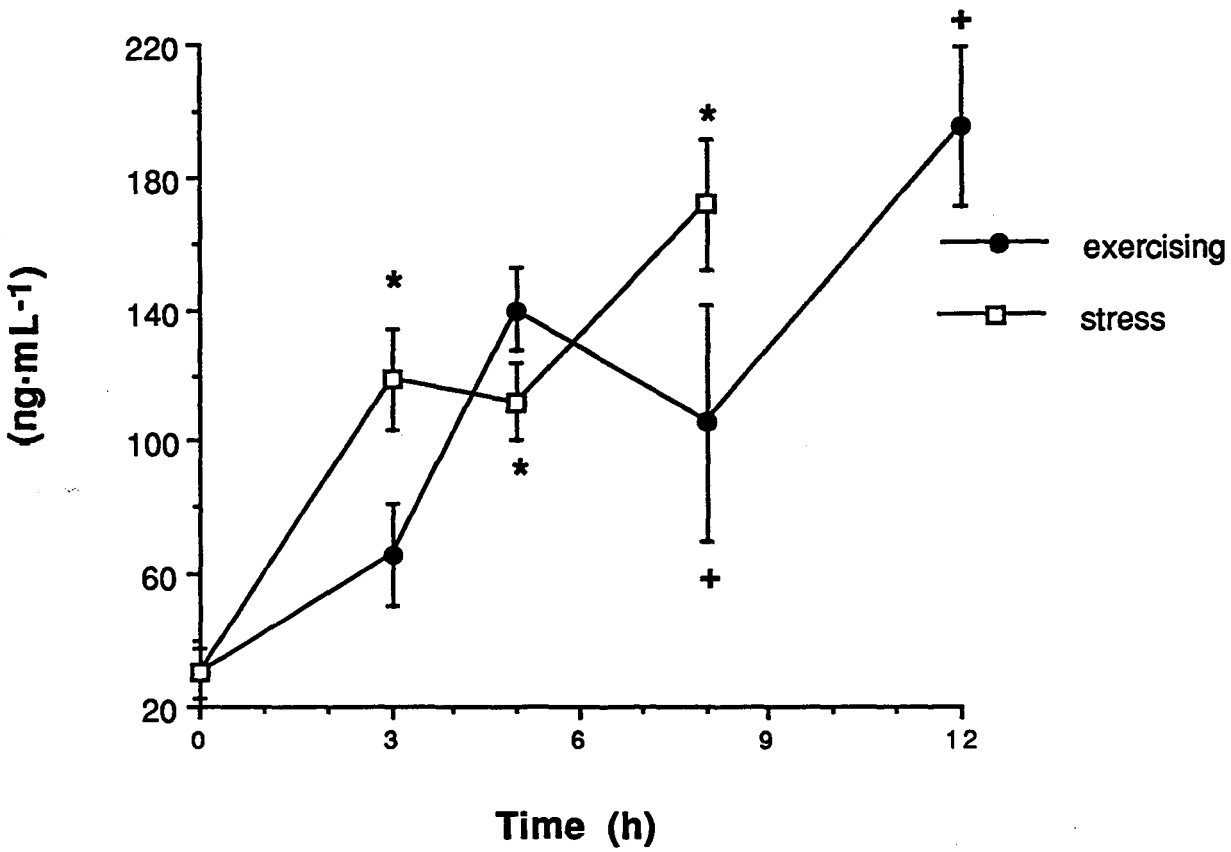

800
700
600
500
400
300
200
100
Jin Na+ (neq-g-1-h-1)
0
1
2
3
4
5
6
7
8
Time (h)
*
*
*
+
rainbow trout
common shiners
smallmouth bass

Figure 2.8. The effect of exercise at 2 BL·sec^{-1} and confinement stress on plasma cortisol levels of smallmouth bass. Values are means ± one S.E.M., N = 6 per time point during exercise (30 in total) and N = 6-8 per time point during stress (26 in total); asterisks indicate a significant difference ($P<0.05$) from routine levels at 0 h.

Figure 2.8



DISCUSSION

During exercise and confinement stress, common shiners exhibit ionoregulatory responses similar to rainbow trout while smallmouth bass show very different responses. In common shiners,there was a rapid increase in $J_{in}^{Na^+}$ during confinement stress (Fig. 2.7) and exercise (Fig. 2.3), a lack of change in whole body Na^+ and Cl^- during exercise (Fig. 2.1), and large Na^+ and Cl^- losses during stress (Fig. 2.5). In contrast, smallmouth bass experience Na^+ and Cl^- loss during exercise (Fig 2.2), minimal or no increase in $J_{in}^{Na^+}$ during either exercise (Fig. 2.3) or stress (Fig. 2.7) and, unlike rainbow trout, increases in cortisol during exercise (Fig. 2.8). The fact that shiners, but not smallmouth bass, show similar responses to rainbow trout, supports the hypothesis that rapid ion regulation during exercise and confinement stress is a common feature of stream-dwelling animals. This, in turn, suggests that exercise is probably a routine feature of the preferred habitat of common shiners and rainbow trout. In contrast, the absence of rapid increases in $J_{in}^{Na^+}$, plasma Na^+ ion loss and high cortisol levels during exercise in smallmouth bass are three lines of evidence suggesting that exercise is stressful and probably not a routine behavior of this species. Nonetheless, the small and delayed increase in $J_{in}^{Na^+}$ during exercise in smallmouth bass (Fig. 2.3), indicates that an uptake mechanism similar to common shiners and rainbow trout may exist but in an attenuated form.

During stress, common shiners and rainbow trout showed increases in $J_{in}^{Na^+}$ in response to whole body ion losses indicating the ability to compensate for losses incurred. In contrast, ion loss in smallmouth bass failed to stimulate increases in $J_{in}^{Na^+}$. Again, the results suggest that stress may be more routinely

increases in $J_{in}^{Na^+}$. Again, the results suggest that stress may be more routinely encountered in the environment of common shiners and rainbow trout than in the environment of smallmouth bass.

One of the notable differences between common shiners and smallmouth bass is the marked variability of $J_{in}^{Na^+}$ in the former, both at rest and in the magnitude of response to stress. The origin of the variability is unknown since all animals in all three expts were apparently healthy at the time of experimentation, were of similar body size and were held at similar temperatures. Furthermore each group of experimental animals had been held in laboratory conditions for at least 3 weeks. Although the cause of the variability is unknown, it at least does suggest that ion balance may be less tightly regulated in common shiners compared to rainbow trout.

It is worth noting that shiners (expts 2 and 3) also demonstrated a greater response to stress, with more effective ion regulation compared to rainbow trout while their response to exercise was less effective. These findings suggest that common shiners may experience stress more often, and exercise less often than rainbow trout. In conclusion, the relative ability to regulate ion balance in response to a particular challenge, whether stress or exercise, may reflect the likelihood with which the animal experiences that challenge in its natural habitat.

To further clarify these species differences, future studies should include measurements of efflux rates during exercise in smallmouth bass and common shiners. This would increase the understanding of what mechanisms are involved in ion regulation and what is initially triggering that particular mechanism. Cortisol levels during exercise in common shiners would be useful in determining the extent of similarity to rainbow trout. Although growth hormone and prolactin were not used in the present study in determining mechanistic

involvement, it would be worth investigating the role of these or other hormones which may be responsible for uptake, similar to their involvement in rainbow trout. Finally, other species, in particular less active species, from similar habitats should be compared to determine the variability if any, that exists from one species to another.

CHAPTER 3

EFFECTS OF EXERCISE TRAINING ON RAINBOW TROUT

INTRODUCTION

Domestication of salmonids has resulted in stocks of fish which have been selected for fast growth (Duthie, 1989) and good food conversion efficiencies (Green, 1964) but which have placid behaviors and poor swimming abilities (Davison, 1989). Past training studies, although limited and often contradictory (Davison, 1989) generally indicate that training of hatchery reared fish enhances swim performance (Farlinger and Beamish, 1978), growth rates (Burrows, 1969), ability to resist disease (Burrows, 1969) and survival rates in the wild (Woodward and Smith, 1985). The latter two benefits may reflect alterations to the stress response. In fish, as in other vertebrates, stress begins with the perception of a harmful stimulus which initiates the release of cortisol and adrenaline (Wedemeyer et al, 1990). These primary responses, in turn, produce a myriad of secondary physiological responses (Mazeaud et al, 1977). Epinephrine leads to the mobilization of energy stores, in particular, glucose, and promotes increases in blood flow and pressure which in turn produce ion losses across the gills of fish. Continued elevation of cortisol can lead to supression of the immune system, reduced growth rate and appetite loss which can all lead to eventual death through increased susceptability to opportunistic infections (Pickering and Pottinger, 1989).

Woodward and Smith (1985) provide evidence to suggest that training leads to an attenuation of particular secondary stress responses in fish. However, conclusions were based only on changes to plasma catecholamines and glucose levels following stress and not on ion regulation. Consequently,

the objective of the present study was to investigate whether or not exercise training affects the magnitude of the disturbance to Na^+ balance evoked by both acute and chronic stress. Two types of acute stress were administered, the first was brief exhaustive exercise and the second was isoproterenol injection. Exhaustive exercise elevates MO_2 and stimulates an increase in Na^+ loss across the gills through increases in functional surface area (FSA) and through direct increases in Na^+ permeability, the latter mediated by the adrenergic component of stress (Gonzalez and McDonald, 1992). Isoproterenol is a pure beta-adrenergic agonist and was used to specifically reproduce the adrenergic effects of acute stress. During chronic stress, fish were confined for a 4 h period in nets similar to the confinement protocol described in Chapter 1. Measurements of diffusive Na^+ loss (J_{out} Na^+) and plasma Na^+ were used to test the hypothesis that training reduces the disturbance to Na^+ balance, while oxygen consumption (MO_2), cortisol and glucose measurements were used to assess the level of stress. Simultaneous measurements of MO_2 and J_{out} Na^+ allow for the determination of Na^+ loss to oxygen uptake ratio (IGR) which Gonzalez and McDonald (1992) argue is a conservative and approximate measure of branchial Na^+ permeability.

MATERIAL AND METHODS

Experimental animals

Rainbow trout were obtained and maintained as described in Chapter 1. Both continuously swimming and resting fish were fed approximately 2% of their body weight on a daily basis in holding facilities until 2 days before the beginning of experimental procedures.

Experimental series

1. Effects of training on growth and condition factor

Rainbow trout (9.2 ± 0.6 g, N = 72) were exercised continuously for 16 days at 2 BL·s^{-1} or left to rest in 500 L tanks. After 1, 2, 5, 8, 12, and 16 days of exercise or rest, 6 fish were removed by netting from each of the tanks and sacrificed by a flick to the head. Whole bodies were weighed, fork length measured and digested for ion analysis. Tank temperatures in the exercise and control tanks were taken on a daily basis throughout the experimental series. The condition factor (c.f.) was calculated using the following equation:

$$c.f. = \frac{W}{l^3} \cdot 100 \qquad (1)$$

where 'W' is the final weight in grams and 'l' is the fork length in cm.

2. Effects of training on critical swimming speed

Fish (11.6 ± 1.1 g, N = 7) were either exercised for 16 days at ~1.7 BL·s^{-1} or left to rest (12.3 ± 1.2 g, N = 6) for 16 days at 14.5 ± 0.5°C before being placed in a Brett type respirometer described in Graham and Wood (1981). Speeds were increased in 6 cm increments beginning at 24 cm·s^{-1} (2.1 BL·s^{-1}) for 1 h. After this period of acclimation, the water velocity was increased every 30 min. The duration and the velocity at which each fish was overcome with exhaustion, indicated by resting on the rear grid, was recorded along with the

length and weight. Fatigued fish were removed from the swim chamber with the use of a siphon which did not interfere with the flow or swimming of the other fish. Maximum sustainable or critical swimming speed (U_{crit}) was then calculated as follows (after Brett, 1964):

$$U_{crit} = V_{ls} + (\frac{t_f}{60} \times V_{inc}) \qquad (2)$$

where 'Vls' is the velocity of the last completed time period in cm/sec, 't_f' is the time spent swimming at the final swimming speed in minutes, and 'V_{inc}' is the velocity increment.

3. Responses to acute stress

Oxygen consumption (M_{O2} in $nmol \cdot g^{-1} \cdot min^{-1}$) and diffusive Na^+ loss (J_{out}^{Na+}) in $nmol \cdot g^{-1} \cdot min^{-1}$) were investigated in trained (6 weeks at 2 $BL \cdot s^{-1}$ throughout) and untrained fish (6 weeks in tank with no current) following one of two stress treatments. Treatment 1 involved vigorous chasing of each individual fish in a cylindrical chamber containing 12 L of tap water at 13°C. Treatment 2 fish received a 50 µL intraperitoneal injection containing 0.6 mg of isoproterenol in 0.6% NaCl. After treatment, the fish were rinsed in distilled 'ion free' water before being placed in individual 150 mL cylindrical respirometers supplied with 'Na+ free' hard water (<0.03 mM Na^+, 1 mM Ca^{++}, pH 7.5) at a rate of 90 $mL \cdot min^{-1}$. Water for the respirometers was supplied from a 150 L recirculating system which was fed by gravity. Simultaneous measurements of MO_2 and J_{out}^{Na+} were made +10 min after placing fish in respirometers and again at +30 min, +60 min and +120 min. Ten mL water samples were collected from each individual respirometers for analysis of O_2 levels on a Radiometer E5046 P_{O2} electrode connnected to a Radiometer PHM71 meter and for determination of J_{out} Na^+ read on an A. A. At the conclusion of the

series, fish were sacrificed and weights were recorded. Temperature was maintained at 13°C throughout the experimental procedures.

4. Responses to chronic stress

Fish (16.8 ± 0.6 g) were trained for 22 days in 500 L tanks at 15 ± 2 °C. Following the exercise regime, groups (N = 6) were confined in nets for 4 h and placed with proper aeration in 50 L tanks similar to that described in Chapter 1. Control fish (16.0 ± 1.3 g) that had been resting for 22 days in 500 L tanks at a similar temperature was also confined. After 4 h, fish were sacrificed and a blood sample was obtained, centrifuged and the plasma analyzed for cortisol, glucose, Na^+.

Calculations- $J_{out}^{Na^+}$ and $M0_2$

$J_{out}^{Na^+}$ was calculated from the changes in Na^+ concentration in water with time and was expressed as $neq \cdot g^{-1} \cdot h^{-1}$.

$$J_{out} = \frac{([Na+]_f - [Na+]_i) \bullet F}{W} \qquad (3)$$

where 'F' is the flow rate in $mLs \cdot min^{-1}$ and 'W' is the weight in grams.

Oxygen consumption (MO_2) was calculated using the following equation:

$$M0_2 = \frac{(F \bullet (\Delta P_{O_2} \cdot 2.09))}{W} \qquad (4)$$

where 'F' is the flow rate in $mLs \cdot min^{-1}$,'$\Delta P0_2$' is the O_2 partial pressure in mmHg, '2.09' is the βO_2 (oxygen solubility coefficient) at 13 °C in $\mu Mol \cdot L^{-1} \cdot mmHg^{-1}$ and 'W' is the weight in grams.

RESULTS

Effects of continuous exercise on growth and Na^+ levels

During a 16 day period, body weight increased significantly in rainbow trout exercised at 2 $BL \cdot s^{-1}$ (Fig. 3.1A), but not in still water control fish even though both groups were fed exactly the same amount of food each day. Condition factor also significantly increased in swimming fish (Fig. 3.1B). Over the time course of the experiment, temperature in both the still water and exercise tanks dropped by 9 °C, but the exercise tank was consistently 2 °C higher because of heat imparted to the water by the circulating pumps (Fig. 3.1C). Despite the changes in temperature, whole body and plasma Na^+ levels remained constant in the still water controls (Fig. 3.1D), while plasma Na^+ was significantly elevated in the exercising fish at 24 h (Fig. 3.1E).

Effects on critical swimming speed

Continuous swimming for 16 days significantly increased the maximum critical swimming speed (U_{crit}) by 10% compared to still water controls (Table 3.1). Maximum swimming speed was not assessed prior to this time so it is unknown as to whether training would have had an effect earlier in the training regime.

Training effects on stress responses

1. Acute stress- exhaustive exercise

Under routine conditions, $J_{out}^{Na^+}$ in juvenile rainbow trout is ~9.0 ± 1$neq \cdot g^{-1} \cdot min^{-1}$ (Gonzalez and McDonald, 1993). Ten min following 5 min of exhaustive exercise, $J_{out}^{Na^+}$ in untrained fish was 5 fold greater than routine while $J_{out}^{Na^+}$ in trained fish was only 2.5 fold higher (Fig. 3.2A). Even though the effect of exhaustive exercise was substantial, the disturbance subsided rapidly (Fig. 3.2A) and the measured values were not different from reported

Figure 3.1 The changes in (A) body weight, (B) condition factor, (C) temperature, (D) whole body Na^+ and (E) plasma Na^+ of rainbow trout during 16 days of continuous exercise or rest (N = 12 per time point, 72 in total) at 2 $BL \cdot s^{-1}$ on . Values are mean ± S.E.M. Asterisks indicate means significantly different ($P < 0.05$) from controls (ie T0) by Dunnett's 't' test.

Figure 3.1

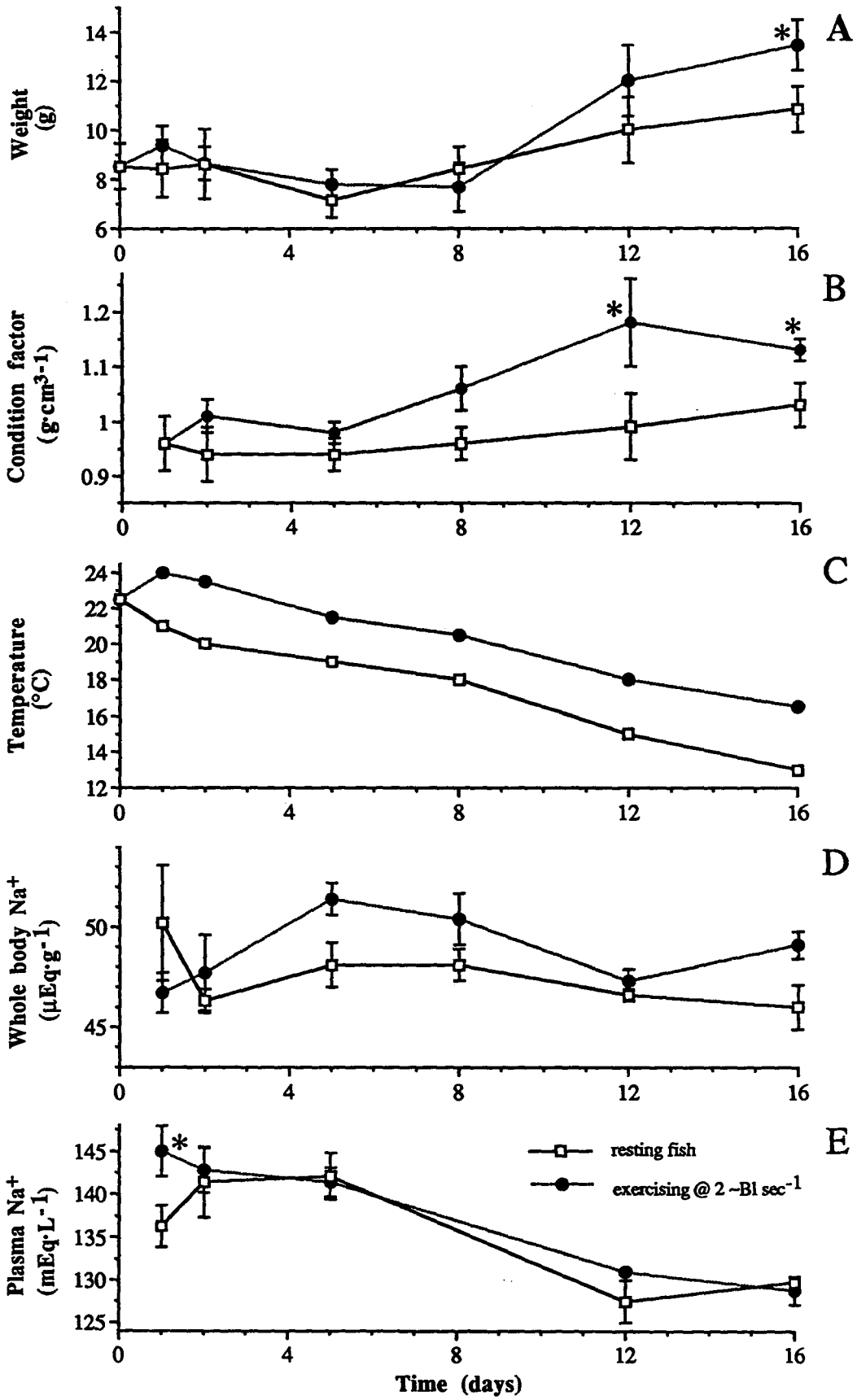

A
Weight (g)
14
12
10
8
6
*
0
4
8
12
16
B
Condition factor (g·cm³·1)
1.2
1.1
1
0.9
*
*
0
4
8
12
16
C
Temperature (°C)
24
22
20
18
16
14
12
0
4
8
12
16
D
Whole body Na+ (µEq·g·1)
50
45
0
4
8
12
16
E
Plasma Na+ (mEq·L·1)
145
140
135
130
125
*
resting fish
exercising @ 2 ~Bl sec-1
0
4
8
12
16
Time (days)

Table 3.1 Body mass, length, number used and critical swimming speed (U_{crit}) in rested rainbow trout and rainbow trout that had been previously exercised for 16 days at 14.5 ± 0.5°C.

Treatment	body mass (g)	length (cm)	N (number)	U ($BL \cdot s^{-1}$)
Rested	12.9 ± 1.2	10.3 ± 0.3	6	4.39 ± 0.11
Exercised	11.6 ± 1.1	9.7 ± 0.3	7	4.84 ± 0.11*

Values are means ± one SEM

Asterisk indicates mean significantly different ($P<0.05$) from control (rested) values by Dunnett's 't' test.

routine levels after 30 min. The magnitude of J_{out} Na^+ produced by 5 min of stress was less than half of the J_{out} Na^+ produced by confinement stress in Chapter 1 (Fig. 1.4A).

Both trained and untrained fish experienced significant increases in MO_2 following 5 min of acute exercise stress. Unlike $J_{out}^{Na^+}$, MO_2 remained high throughout the 60 min recovery period in both trained and untrained fish (Fig. 3.2B) and levels were not significantly different from each other.

Consequently, the IGR at +10 min was 2 fold higher than routine levels in untrained fish while the IGR in trained fish, at the same time point, was virtually identical to routine levels (Fig. 3.2C). The IGR in both treatments decreased at +30 min and +60 min below routine levels.

2. Acute stress- isoproterenol injection

Ten min following injection with isoproterenol J_{out} Na^+ increased 4 fold in untrained fish while $J_{out}^{Na^+}$ in trained fish was only 1.3 fold higher than routine levels (Fig. 3.3A). Similar to 5 min of exhaustive exercise, isoproterenol injection produced similar MO_2 rates in untrained and trained fish (Fig. 3.3B) although IGR values were 6 fold higher in untrained fish (Fig. 3.3C). Even though the initial effect of isoproterenol on IGR in untrained fish was substantial, the disturbance subsided rapidly and calculated values were not significantly different after 30 min. The drop in IGR of untrained fish is attributable to significant reductions in $J_{out}^{Na^+}$ (Fig. 3.3A) since there was no change in MO_2 over the 2 h sampling period (Fig. 3.3B).

3. Chronic stress- confinement

Plasma cortisol and glucose in trained and untrained fish were virtually identical after 4 h of confinement stress (Fig. 3.4B,C). However, untrained fish experienced a greater loss in plasma Na^+ than trained fish (10% versus 4.2%, respectively, Fig. 3.4A).

Figure 3.2 (A) Na+ efflux (J_{out}^{Na+}), (B) oxygen consumption (MO_2), and (C) Ion/gas ratio (IGR) in trained (28.1 ± 5.5g, N = 4) and untrained (19.9 ± 1g, N = 4) rainbow trout during 60 min recovery period from 5 min of exhaustive exercise at 13°C. Estimates of routine levels from Gonzalez and McDonald, (1992) are given for comparison (dashed line). Values are mean ± S.E.M. Asterisks indicate means significantly different ($P < 0.05$) from corresponding mean in trained fish by Dunnett's 't' test.

Figure 3.2

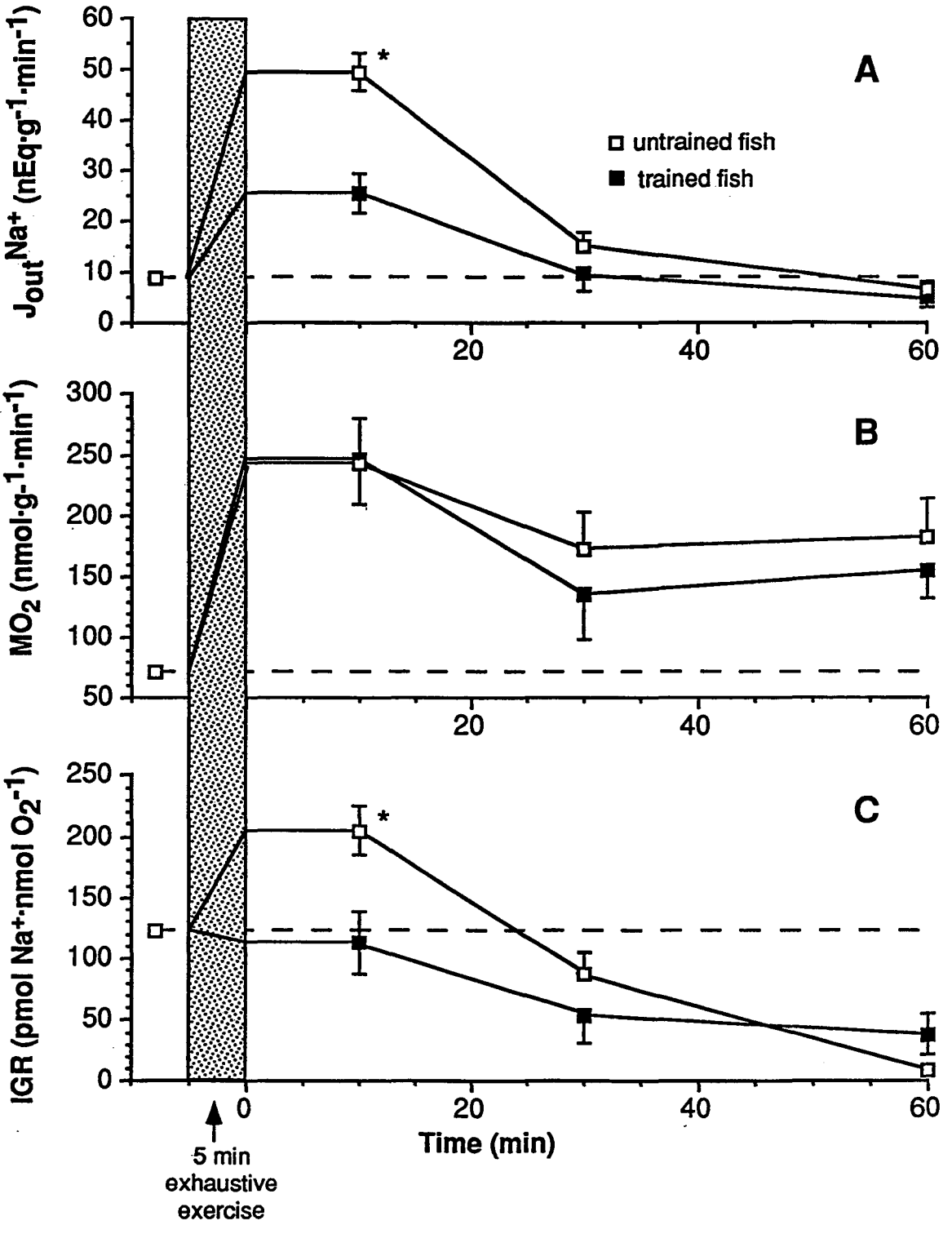

A
B
C
untrained fish
trained fish
*
*
$J_{out}Na^+$ (nEq·g^{-1}·min^{-1})
MO_2 (nmol·g^{-1}·min^{-1})
IGR (pmol Na^+·nmol O_2^{-1})
60
50
40
30
20
10
0
300
250
200
150
100
50
250
200
150
100
50
0
20
40
60
0
5 min
exhaustive
exercise
Time (min)

Figure 3.3 (A) Na+ efflux (J_{out}^{Na+}), (B) oxygen consumption (MO_2), and (C) Ion/gas ratio (IGR) in trained (16.0 ±1.3 g, N = 4) and untrained (15.8 ±1.6 g, n = 4) rainbow trout during 60 min recovery from isoproterenol injection (0.6 mg). Routine values for J_{out}^{Na+}, MO_2 and IGR from Gonzalez and McDonald (1992) are given for comparison (dashed line). Values are mean ± S.E.M. Asterisks indicate means significantly different ($P < 0.05$) from corresponding value in trained fish by Dunnett's 't' test.

Figure 3.3

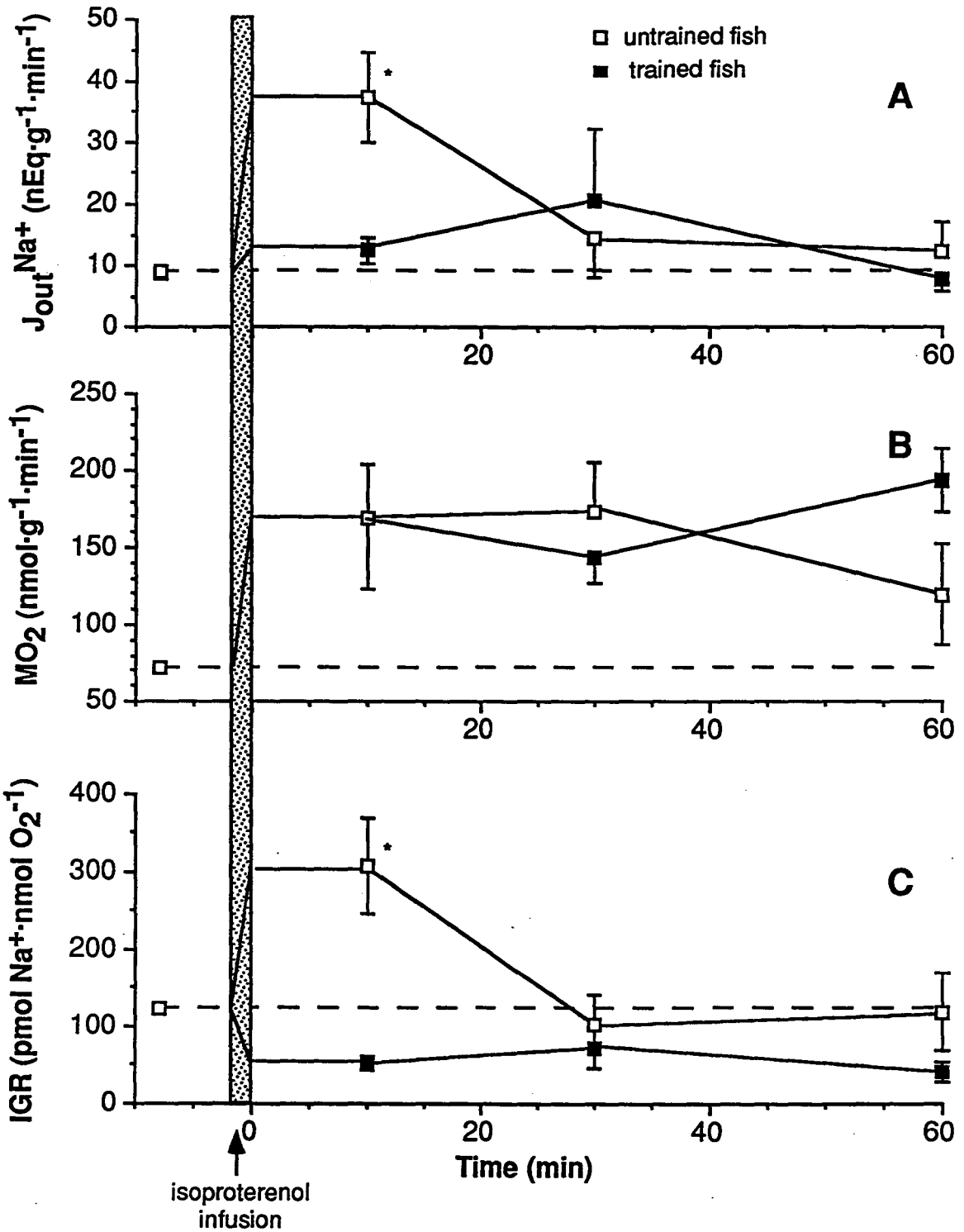
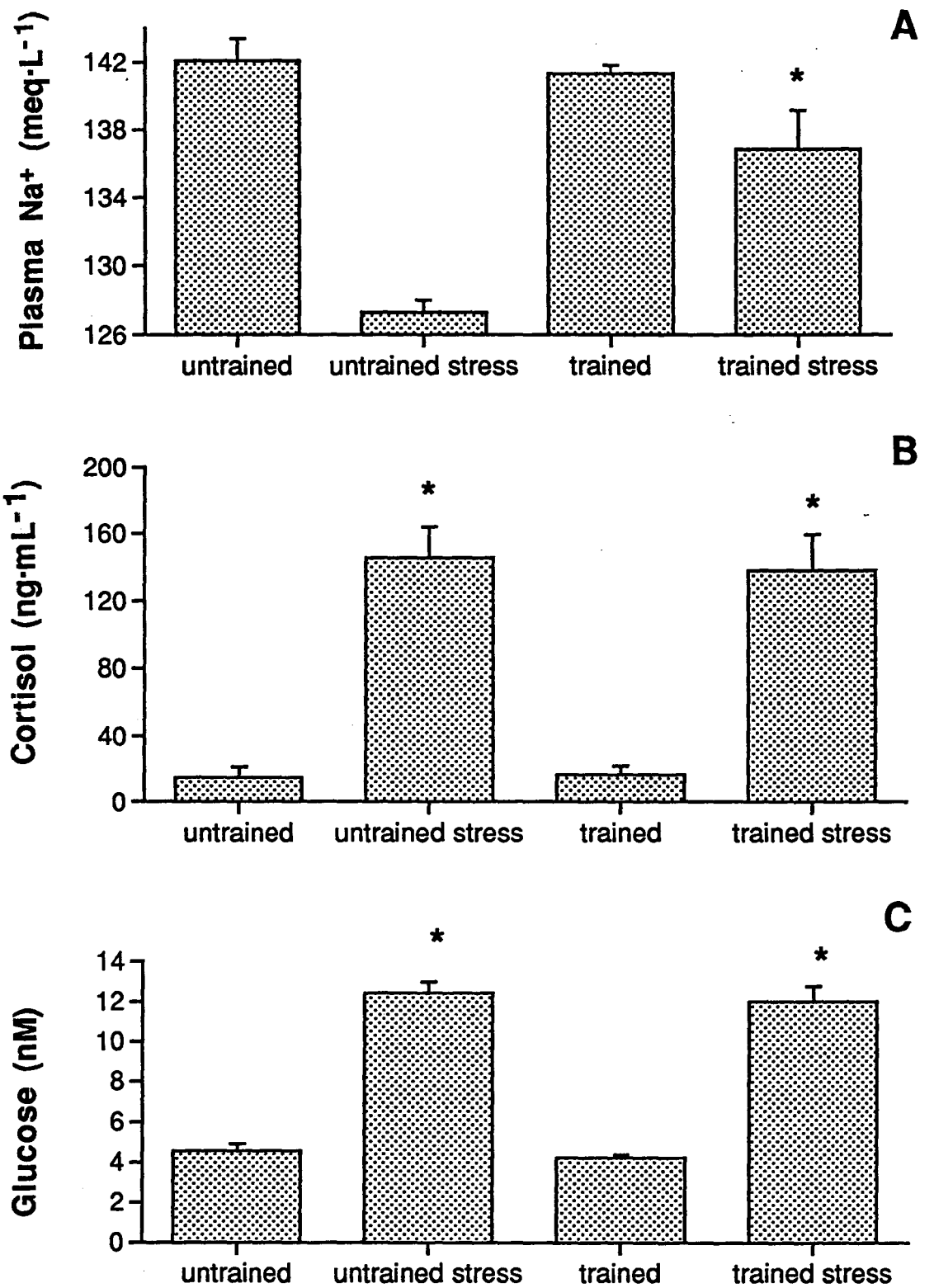

untrained fish
trained fish
A
B
C
$J_{out}^{Na^+}$ (nEq·g^{-1}·min^{-1})
MO_2 (nmol·g^{-1}·min^{-1})
IGR (pmol Na^+·nmol O_2^{-1})
Time (min)
isoproterenol infusion
*

Figure 3.4 (A) Plasma Na+, (B) cortisol and (C) glucose for trained (16.8 ±0.6 g, N = 12) and untrained (16.0 ±1.3 g, N = 6) rainbow trout following 4 h of chronic confinement stress at 15 ± 2°C. Values are mean ± S.E.M. Asterisks indicate means significantly different ($P < 0.05$) from (A) untrained stress mean, (B) and (C) controls at T = 0 stress.

Figure 3.4


A
Plasma Na+ (meq·L-1)
142
138
134
130
126
*
untrained
untrained stress
trained
trained stress
B
Cortisol (ng·mL-1)
200
160
120
80
40
0
*
*
untrained
untrained stress
trained
trained stress
C
Glucose (nM)
14
12
10
8
6
4
2
0
*
*
untrained
untrained stress
trained
trained stress

DISCUSSION

While the objective of the present study was to establish the effects of training on ionoregulatory responses in rainbow trout, we have also confirmed that the exercise regime was, in fact, sufficient in producing training effects. Faster growth (Fig. 3.1A) and increased U_{crit} (Table 3.1), previously reported training effects (Davison, 1989), occurred after a shorter time period than what has been investigated (Woodward and Smith, 1985; Farrell, Johansen and Suarez, 1991). Similarly high cortisol levels in both trained and untrained fish suggested that both groups experienced similar stress levels while similarly high levels of glucose, indicated similar mobilization of energy stores, and also perhaps similar increases in blood catecholamines. However, trained fish demonstrated a striking ability to diminish ion disturbances produced by both acute and chronic stress.

Following both acute stress treatments, trained fish, in spite of similarly elevated MO_2, demonstrated less of an increase in J_{out}^{Na+} relative to untrained fish. Since there was no increase in IGR in trained fish, the increase in J_{out} is most likely attributable to an increase in gill functional surface area and not to an increase in Na^+ permeability. The absence of any increase in IGR could therefore mean that there was less of an increase in circulating catecholamines in trained versus untrained fish or that there was a reduction in catecholamine sensitivity of the gills. However, the results of the isoproterenol injection experiment, which was designed to give similar adrenergic stimulation in both groups, suggests that the latter possibility is more likely.

The effects of exhaustive exercise and isoproterenol injection on J_{out}^{Na+} and IGR did not persist beyond 10 min in either trained or untrained fish (Fig. 3.2,3.3) so the extended effects of the training cannot be determined from these

acute stress tests. However, the much smaller Na^+ losses in trained fish during confinement stress suggest that a reduced responsiveness to catecholamines continued for at least 4h.

Thus, we have established that training improves the animal's ability to regulate ions during both acute and chronic periods of stress, without modifying the primary response to stress, the release of cortisol and catecholamines. Smaller ion disturbances in response to stress may improve the ability of hatchery-reared stocks of fish to cope with situations such as predation, annual fluctuations in the environment and competition once released into the wild (Woodward and Strange, 1987). Further investigation, however, is still needed to determine how long after terminating continuous swimming, the training effects are sustained. Finally, it would be worthwhile assessing the success of trained hatchery reared fish in comparison to untrained fish once they are stocked in lake systems.

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