ENERGETIC ASPECTS OF OSMOREGULATION IN FISH

by

JOHN DAVID MORGAN

B.Sc., The University of British Columbia, 1979

M.Sc., The University of British Columbia, 1991

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Animal Science)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Animal Science

The University of British Columbia
Vancouver, Canada

Date September 29, 1997

DE-6 (2/88)

ABSTRACT

The energetic aspects of osmoregulation in several species of fish were examined, using an experimental approach on both a whole-animal and tissue level. The first series of experiments examined the metabolic response of temperate and tropical fish species to acute and gradual salinity change, using whole-animal oxygen consumption rates and gill Na^+,K^+-ATPase activity as indicators of osmoregulatory energetics. Juvenile dolphin fish (*Coryphaena hippurus*) were exposed for 24 h to a reduced water salinity (34 to 20 ppt). They responded by decreasing oxygen consumption and gill Na^+,K^+-ATPase activity, suggesting a decrease in osmoregulatory costs. Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), showed an elevation in plasma growth hormone levels, gill Na^+,K^+-ATPase activity, and a 20% increase in oxygen uptake after 4 d. No increases in these variables were seen in tilapia transferred from FW to isosmotic salinity (ISO). These results indicated that the physiological changes associated with SW entry represent a significant short-term cost, whereas ISO did not impose (or reduce) an energy demand in tilapia during the acclimation process. In a long-term study (6 wk), coho salmon (*Oncorhynchus kisutch*) smolts did not show any differences in metabolic rate between FW, ISO and SW, whereas gill Na^+,K^+-ATPase activity was lowest in ISO, higher in FW and highest in SW. In this case, there was no correlation between whole-animal oxygen consumption rates and the relative activity of ion transport enzymes in the gills. An acute (24 h) transfer of cutthroat trout (*O. clarki clarki*) from FW to SW resulted in a significant elevation of both oxygen uptake and plasma cortisol levels.

To further examine the influence of cortisol on oxygen consumption and osmoregulatory variables, cutthroat trout parr were given cortisol implants that elevated plasma cortisol titres to a

level similar to that found in fish following SW exposure. Cortisol significantly increased oxygen consumption rates and plasma glucose levels of trout in FW, consistent with its glucocorticoid role. This study suggests that some of the increases in oxygen consumption that occurred during the intitial stages of SW exposure may have been related to the metabolic effects of cortisol, rather than the direct costs usually associated with osmoregulation.

To separate the energy costs of NaCl transport from other whole-animal metabolic responses to salinity change, experiments were conducted using isolated preparations of osmoregulatory tissues. Oxygen consumption and Na^+,K^+-ATPase activity were measured in excised rectal gland and gill tissue of the spiny dogfish (*Squalus acanthias*), using ouabain to estimate the portion of tissue respiration required by the Na^+/K^+-pump. Ouabain-sensitive oxygen consumption of the rectal gland accounted for 55% of tissue respiration, compared to 22% for the gill. On a whole-mass basis, the cost of NaCl secretion in the rectal gland was estimated to be 0.5% of whole-animal oxygen uptake. A similar approach was used on excised gill tissue from FW-adapted cutthroat trout, to assess the oxygen cost of NaCl uptake in the FW trout gill. In that study, bafilomycin was also used to inhibit H^+-pump activity in the gill tissue. A similar portion of gill tissue respiration was required by the Na^+/K^+-pump (18%) and H^+-pump (19%), and the cost of NaCl uptake in the FW trout gill was estimated at 1.8% of resting metabolic rate. Finally, an isolated, perfused gill arch preparation was used to compare gill energetics in FW- and SW-adapted cutthroat trout. The total gill oxygen consumption of FW gills was significantly (33%) higher than SW gills, and accounted for 3.9% and 2.4% of resting metabolic rate, respectively. The results of those experiments indicate that the energy demands of ion transport in osmoregulatory organs, such as the rectal gland and gill, represent a relatively small portion of the total energy budget in fish.

TABLE OF CONTENTS

		Page
Abstract		ii
Table of Contents		iv
List of Tables		vi
List of Figures		vii
List of Abbreviations		ix
Acknowledgements		x
General Introduction		1
Section I.	**Whole-animal metabolic responses to salinity change**	12
Chapter 1	Physiological responses to hyposaline exposure, handling and confinement stress in juvenile dolphin fish	13
	Introduction	13
	Materials and Methods	14
	Results	17
	Discussion	21
Chapter 2	Physiological and respiratory responses of the Mozambique tilapia to salinity acclimation	25
	Introduction	25
	Materials and Methods	27
	Results	31
	Discussion	35
Chapter 3	Salinity effects on oxygen consumption, gill Na^+,K^+-ATPase and ion regulation in juvenile coho salmon smolts	43
	Introduction	43
	Materials and Methods	44
	Results	50
	Discussion	55
Chapter 4	Metabolic response of cutthroat trout to acute salinity change	58
	Introduction	58
	Materials and Methods	59
	Results	61
	Discussion	61

Page

Section II. Hormonal effects associated with whole-animal responses to salinity change 66

Chapter 5 Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout parr 67
Introduction 67
Materials and Methods 68
Results 74
Discussion 78

Section III. Detailed components of ion transport-related costs in osmoregulatory tissues 84

Chapter 6 Oxygen consumption and Na^+,K^+-ATPase activity of rectal gland and gill tissue in the spiny dogfish 85
Introduction 85
Materials and Methods 87
Results 92
Discussion 96

Chapter 7 Energy cost of NaCl uptake in freshwater cutthroat trout gill tissue 101
Introduction 101
Materials and Methods 102
Results and Discussion 108

Chapter 8 Oxygen consumption in isolated, perfused gills of freshwater- and seawater-adapted cutthroat trout 116
Introduction 116
Materials and Methods 118
Results 124
Discussion 128

General Discussion 135

References 139

LIST OF TABLES

Page

Table 1 Body mass, liver glycogen content, plasma glucose, protein and ion concentrations, blood hemoglobin concentration, hematocrit values, and erythrocyte counts in juvenile dolphin fish, before and after a 24 h exposure to 34 ppt and 20 ppt salinity. 20

Table 2 Chemical composition of water samples collected from the tilapia salinity treatment tanks. 28

Table 3 Chemical composition of water samples collected from the coho salmon salinity treatment tanks. 46

Table 4 Body mass, and plasma cortisol, glucose and ion levels in cutthroat trout in FW and 24 h after transfer to SW. 62

Table 5 Length, mass, and plasma cortisol and ion concentrations of cutthroat trout parr following a 24 h seawater challenge test. 70

Table 6 Plasma cortisol, glucose and ion concentrations, and gill Na^+,K^+-ATPase activity in non-implanted and cortisol-implanted cutthroat trout parr, before and after a 24 h seawater challenge test. 77

Table 7 Size characteristics and concentrations of constituents in dogfish plasma and seawater at the Bamfield Marine Station. 93

Table 8 Ouabain-sensitive oxygen consumption and Na^+,K^+-ATPase activity in rectal gland and gill tissue of the spiny dogfish. 95

Table 9 Body and gill mass, oxygen consumption rate, gill Na^+,K^+-ATPase and H^+-ATPase activities, and plasma glucose and ion concentrations in cutthroat trout reared in fresh water. 109

Table 10 Cost of NaCl uptake in the freshwater cutthroat trout gill. 114

Table 11 Oxygen consumption rates, gill Na^+,K^+-ATPase and H^+-ATPase activities, and plasma cortisol, glucose and ion concentrations in cutthroat trout acclimated for 2 wk to FW and SW. 125

Table 12 Body and gill mass of cutthroat trout used in the isolated, perfused gill preparations. 127

LIST OF FIGURES

		Page
Figure 1	Salt and water exchange in (A) hagfish, (B) elasmobranchs, (C) marine teleosts, and (D) freshwater teleosts.	2
Figure 2	Model for the movement of NaCl by chloride cells of seawater teleosts.	4
Figure 3	Model of ion transfer across the gill epithelium of a freshwater teleost.	6
Figure 4	Metabolic rates of juvenile dolphin fish after a 24 h exposure to 34 ppt and 20 ppt salinity.	18
Figure 5	Plasma cortisol concentrations in juvenile dolphin fish, before and after a 24 h exposure to 34 ppt and 20 ppt salinity.	19
Figure 6	Gill Na^+,K^+-ATPase activity in juvenile dolphin fish, before and after a 24 h exposure to 34 ppt and 20 ppt salinity.	22
Figure 7	Plasma cortisol and glucose levels in tilapia after transfer from FW to FW, ISO and 75% SW.	32
Figure 8	Plasma growth hormone (GH) and prolactin (PRL_{177} and PRL_{188}) levels in tilapia after transfer from FW to FW, ISO and 75% SW.	33
Figure 9	Plasma [Na^+], [K^+] and [Cl^-] of tilapia after transfer from FW to FW, ISO and 75% SW.	34
Figure 10	Gill Na^+,K^+-ATPase activity of tilapia after transfer from FW to FW, ISO and 75% SW.	36
Figure 11	Oxygen consumption rates of tilapia in FW, ISO and 75% SW, 4 days after transfer from FW.	37
Figure 12	Plasma cortisol and glucose levels in juvenile coho salmon reared in FW, ISO and SW.	51
Figure 13	Plasma [Na^+], [K^+] and [Cl^-] in juvenile coho salmon reared in FW, ISO and SW.	52
Figure 14	Gill Na^+,K^+-ATPase activity of juvenile coho salmon reared in FW, ISO and SW.	53
Figure 15	Oxygen consumption rates of juvenile coho salmon after 6 wk in FW, ISO and SW.	54

Page

Figure 16 Oxygen consumption rates of cutthroat trout in FW and 24 h after SW entry. 63

Figure 17 The effect of cortisol treatment (50 μg/g) on oxygen consumption rate in cutthroat trout. Control fish were not treated, while sham fish received an oil implant only. 75

Figure 18 Plasma cortisol and glucose concentrations in control, sham and cortisol-implanted (50 μg/g) cutthroat trout, sampled from the respirometer and holding tanks. 76

Figure 19 Schematic diagram of the respiration chamber used to determine oxygen consumption rates in rectal gland and gill tissue of the spiny dogfish. 89

Figure 20 A representative example showing the time course of oxygen consumption in rectal gland tissue of the spiny dogfish. 90

Figure 21 Oxygen consumption in rectal gland and gill tissue of the spiny dogfish, before and after the addition of 0.5 mM ouabain. 94

Figure 22 Oxygen consumption of cutthroat trout gill tissue following treatment with A) 0.5 mM ouabain, 1 mM NEM, and B) 0.1 μM and 1 μM bafilomycin A_1. 111

Figure 23 Schematic diagram of the setup used for measuring oxygen consumption in isolated, saline-perfused cutthroat trout gills. 121

Figure 24 Oxygen consumption of isolated, saline-perfused cutthroat trout gills in FW and SW, and following the addition of 0.5 mM ouabain to the perfusate. 126

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
Ca^{2+}	calcium ion
Cl^{-}	chloride ion
DMSO	dimethylsulphoxide
EDTA	ethylenediamine-tetraacetic acid
FW	fresh water
ISO	isosmotic water
K^{+}	potassium ion
Mg^{2+}	magnesium ion
Na^{+}	sodium ion
NADH	nicotinamide adenine dinucleotide
NEM	*N*-ethylmaleimide
ppt	parts per thousand
RIA	radioimmunoassay
SE	standard error
SEI	sucrose-EDTA-imidazole
SO_4^{2-}	sulphate ion
SW	seawater
TMAO	trimethylamine oxide
TMS	tricaine methanesulfonate

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. George Iwama, for his support and guidance throughout the completion of this thesis. I have thoroughly enjoyed working in his lab for the better part of a decade, and have been greatly influenced by his approach to science and his sense of life's priorities. I would also like to thank Kim Cheng, Don McPhail and Dave Randall for serving on my research committee and providing helpful comments during the preparation of this thesis. The comments of the examining committee (Maryanne Hughes, Steve McCormick and Jim Thompson) were also most helpful in producing the final version of the thesis. Financial support for this research was provided by NSERC and CBDN operating grants to Dr. Iwama, and an NSERC post-graduate scholarship and University of B.C. graduate fellowship to myself.

There are many people in the Iwama and Randall labs that have helped me during this thesis, and in particular I would like to thank Paige Ackerman, Shannon Balfry, Robert Forsyth, Erick Groot, Carl Mazur, Ellen Teng, Matt Vijayan, and Jonathan Wilson for their assistance in various aspects of the work.

Finally, I would like to acknowledge the support of my family. I could not have completed this thesis without the patience and moral support of my wife, Louise Robbins. My two daughters, Emily and Sophie, provided the perspective needed for a balanced life as a graduate student, and helped me keep things in order at the "fish place". I would also like to thank my mother-in-law, Betty Robbins, and my father, Bert Morgan, for their encouragement and material support of this venture that has taken me a little further into life than most. This thesis is dedicated to the memory of my mother, Jean Audrey Morgan, for instilling in me the importance of learning and discovery.

GENERAL INTRODUCTION

Strategies for Ionic and Osmotic Regulation in Fish

For the proper function of metabolic processes, all animals must maintain a stable internal composition of water and solutes in the face of an often changing external environment (Moyle and Cech 1988). Many cellular and tissue-based mechanisms have evolved in the animal kingdom to maintain hydromineral balance in a diverse array of environments. As a group, the fishes employ four general strategies for ionic and osmotic regulation (osmoregulation) in an aquatic environment (Fig. 1). The first strategy is that of the marine and stenohaline hagfish (family: Myxinidae). Hagfish plasma is approximately ISO with SW and the fish does not actively regulate blood ion concentrations, although some mechanisms for sodium regulation may be present (Evans 1979). The second strategy for osmoregulation is employed by the marine elasmobranchs (i.e., sharks, skates and rays), holocephalans (i.e., ratfish) and the coelocanth (*Latimeria chalumnae*). These fish maintain the plasma concentration of Na^+ and Cl^- at about one-half that of SW, excreting these ions from a special excretory organ called the rectal gland (Shuttleworth 1988). The osmotic pressure of their blood is kept ISO with SW, however, by using urea and TMAO as organic osmolytes (Yancey and Somero 1980).

The third strategy for osmoregulation is that of marine teleosts. These fish maintain the salt content of their blood at approximately one-third that of SW. They are therefore hyposmotic to the surrounding SW and tend to lose water by osmosis to the environment, primarily across the permeable gill epithelia. Water losses are replaced by continually drinking SW, which is absorbed across the intestine into the blood. This process is driven by a large active uptake of salts which

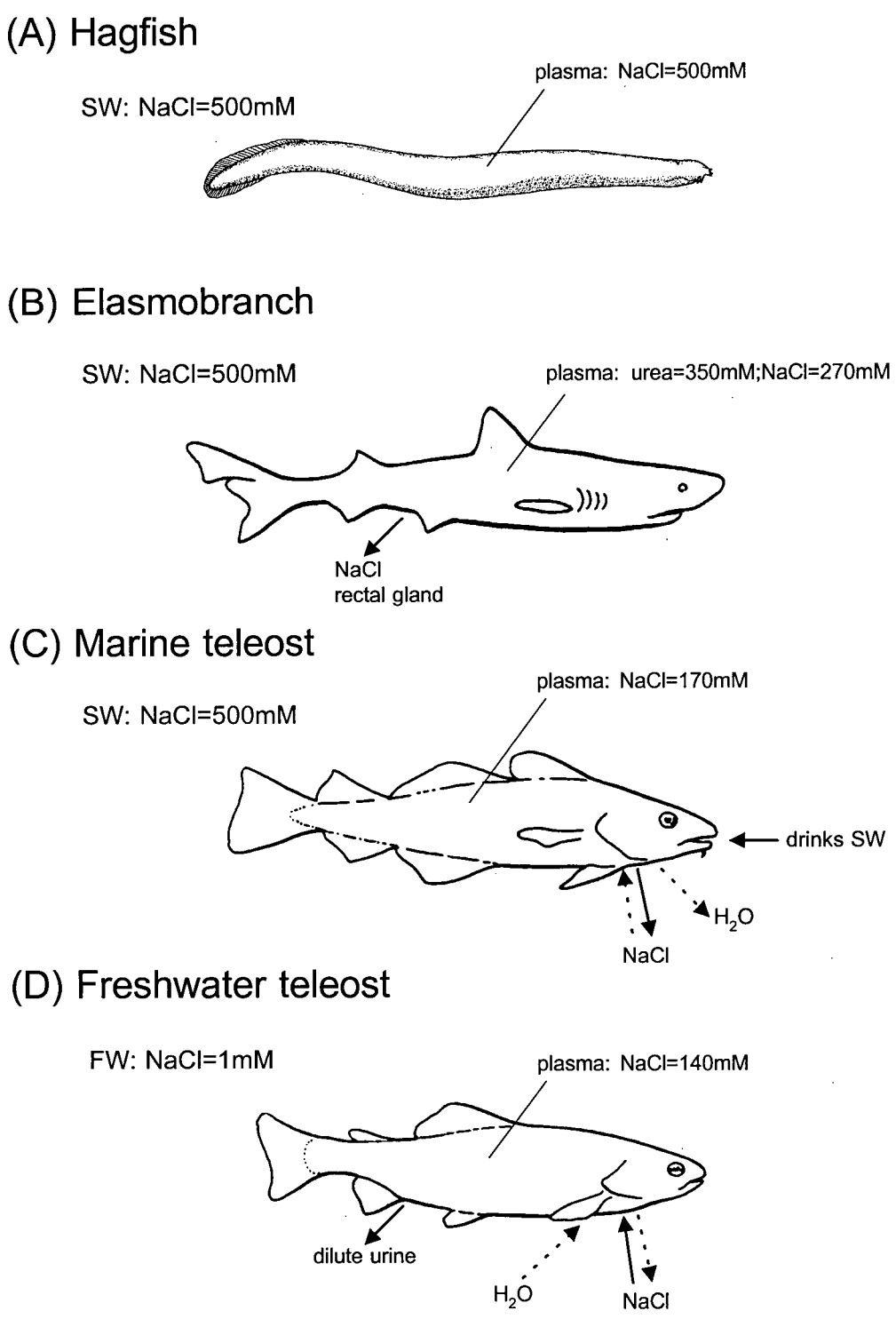

Fig. 1. Salt and water exchange in (A) hagfish, (B) elasmobranchs, (C) marine teleosts, and (D) freshwater teleosts (drawings modified from Eckert *et al.* 1988 and Withers 1992). Solid arrows indicate active processes; broken arrows, passive processes.

must be eliminated from the blood against a concentration gradient back into the surrounding SW. Na^+ and Cl^- also move passively from SW into the blood following electrochemical gradients through the gill epithelial cells, which have a relatively high permeability to monovalent ions (Evans 1993). Excess Na^+ and Cl^- in the blood are excreted out of the fish by active transport in specialized chloride cells, first described by Keys and Willmer (1932), and located on the primary filaments of the gills. The currently accepted model for salt excretion by chloride cells involves a coupled $Na^+/K^+/Cl^-$ carrier system and a Na^+/K^+-pump, which is catalyzed by the enzyme Na^+,K^+-ATPase located on the basolateral membrane of the cell (Fig. 2; Karnaky 1986). This pump actively transports Na^+ into the extracellular space in exchange for K^+. Energy is provided by ATP produced in the numerous mitochondria located within the cell, which are connected to the membrane with an extensive tubular network. This process creates a Na^+ gradient that favours Na^+ entry back into the cell, and drives a linked $Na^+/K^+/Cl^-$ carrier system which increases the intracellular concentration of Cl^-. The build up of Cl^- inside the chloride cell enables Cl^- to follow its electrochemical gradient and move passively across the apical membrane into SW. The movement of Cl^- outside the cell has been localized to a region of the membrane called the apical crypt (Foskett *et al.* 1983). Na^+ is thought to concentrate paracellularly and diffuse passively from blood to SW, exiting via "leaky" junctions between chloride cells and accessory cells (Karnaky 1986). This mechanism of salt excretion is, with minor differences, similar to that found in the rectal gland of elasmobranchs and salt glands of marine birds. The teleost kidney can only produce a urine which is isotonic to blood (Schmidt-Nielson 1975), and, therefore, is not an important organ for monovalent ion excretion. Urine flows are also greatly reduced in marine teleosts to assist in water retention. The kidney does, however, secrete excess divalent ions such as Mg^{2+}, Ca^{2+} and SO_4^{2-}, which are present in much lower amounts in SW than Na^+ and Cl^-.

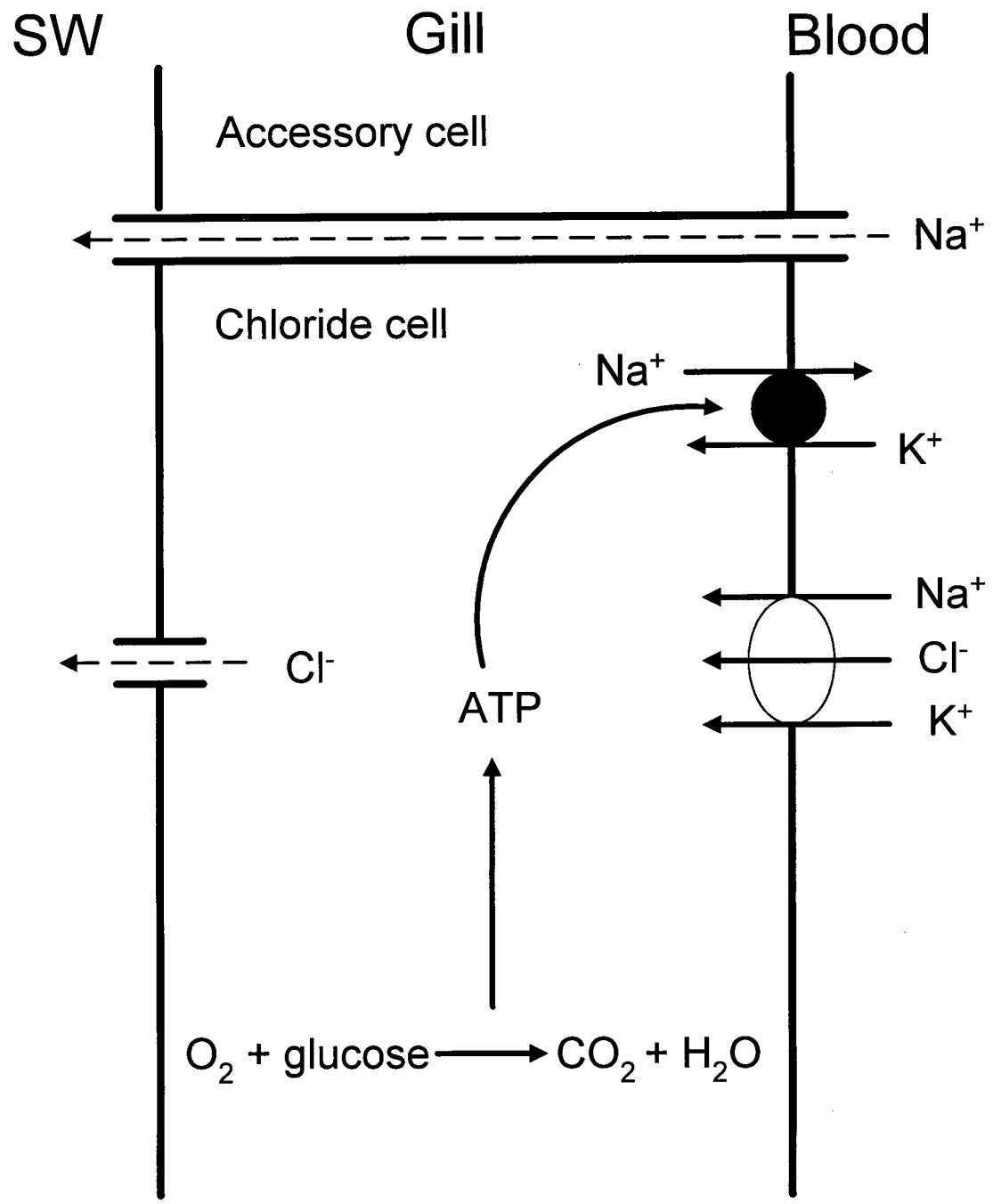

Fig. 2. Model for the movement of NaCl by chloride cells of seawater teleosts. ATP-driven pumps are denoted by solid circles, and neutral co-transporters by an open ellipse. See Karnaky (1986) for more details.

The fourth strategy for osmoregulation has evolved in FW teleosts, the lampreys, and a few FW species of elasmobranchs (eg., *Potamotrygon*; Evans 1979). These fish also maintain the salt concentration of their blood at about one-quarter to one-third that of SW. They are therefore hyperosmotic to the surrounding FW in which they live, and water continually enters their bodies by diffusion through permeable membranes. The excess water is removed by a copious production of a dilute urine in the kidneys. Salts are also lost through the urine and by passive diffusion through the gills. The salt losses are compensated by active ion uptake mechanisms located in the gills (Evans 1993). The current model for the uptake of Na^+ and Cl^- by the gill in FW involves an electrogenic proton pump (vacuolar, or V-type, H^+-ATPase) that extrudes H^+ to the external water and generates a potential across the apical membrane which, in turn, drives Na^+ entry into the gill through a sodium channel. Sodium ions are then transferred to the blood via the sodium pump (Na^+,K^+-ATPase) located on the basolateral membrane of the gill epithelium (Fig. 3; Lin and Randall 1995). H^+-ATPase is located in both chloride cells and pavement cells in the gill epithelia (Lin *et al.* 1994), whereas Na^+,K^+-ATPase is located mostly in the chloride cells (Witters *et al.* 1996). Freshwater chloride cells also contain many mitochondria and an extensive tubular system on the basolateral membrane, however they lack the apical crypt and accessory cells characteristic of SW chloride cells. Fish which live in ion-deficient FW have numerous chloride cells distributed on the secondary gill lamellae as well as on primary filaments (Laurent *et al.* 1985; Laurent and Hebibi 1989). Chloride ions are thought to enter gill epithelial cells via an electroneutral Cl^-/HCO_3^- exchanger in the apical membrane, and a favourable gradient at the basolateral membrane allows passive Cl^- transfer into the body fluids through a chloride channel (Heisler 1993). In cases of respiratory acidosis (e.g., hypercapnia), regulation of plasma Cl^- can be compromised in order to compensate for disturbances in acid-base balance (i.e., increased plasma

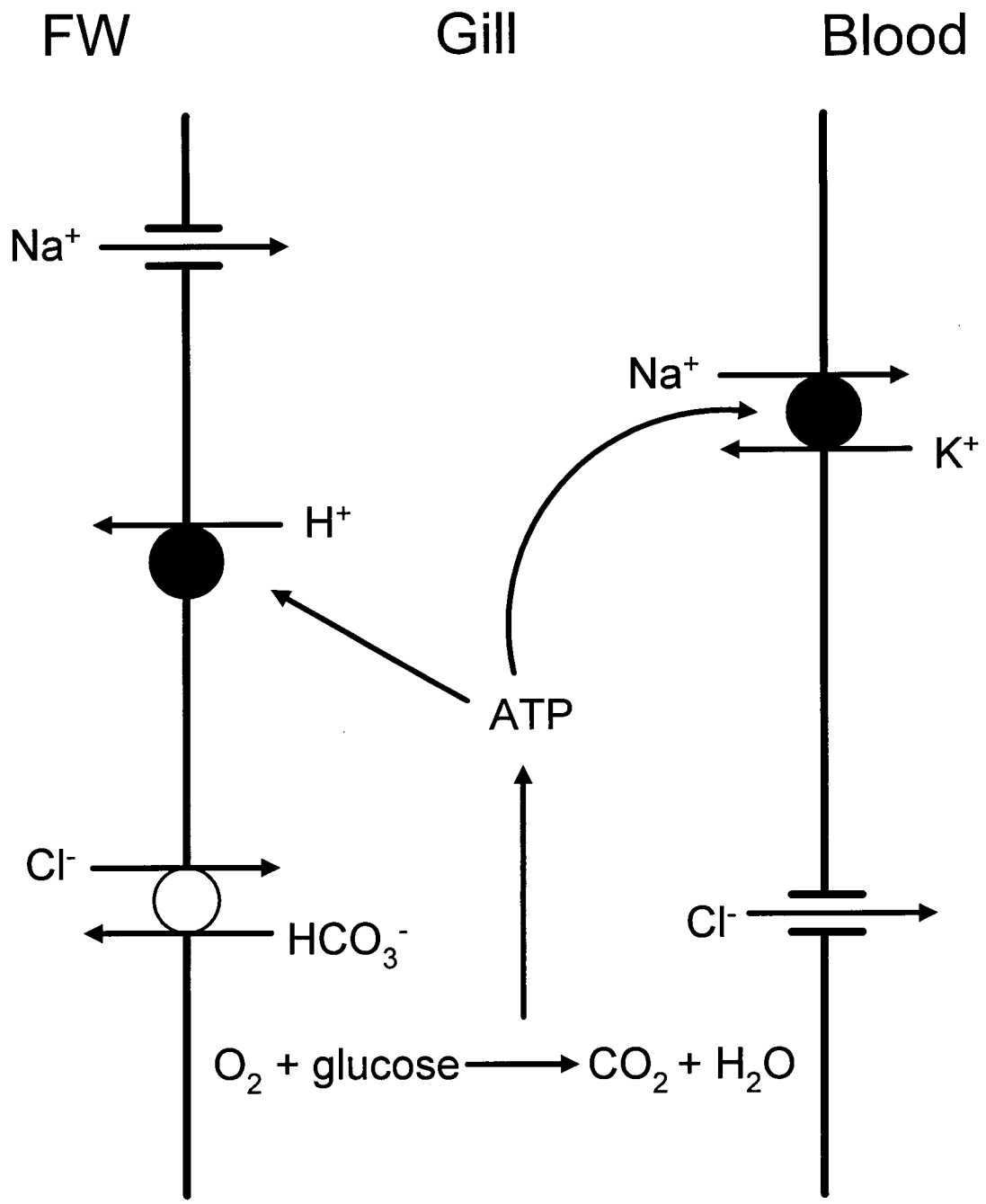

Fig. 3. Model of ion transfer across the gill epithelium of a freshwater teleost fish (modified from Lin and Randall 1995). ATP-driven pumps are denoted by solid circles and neutral exchangers by open circles.

HCO_3^-; Cameron and Iwama 1989). Similar models for active NaCl uptake have been proposed for frog skin (Ehrenfeld and Klein 1997) and freshwater crab gills (Onken and Putzenlechner 1995).

Modulators of Osmoregulation

Although most fishes are stenohaline (i.e., tolerate a narrow range of salinity), there are a number of euryhaline fish species which have the capacity to maintain ionic and osmotic balance in a wide range of salinities. These include estuarine or brackishwater fish such as killifish, tilapia, flounder, stickleback, etc., which may move between waters of varying salinity, as well as diadromous fishes (e.g., salmonids, eels, lampreys) which migrate between FW and SW during specific life history stages (see Evans 1984 for a complete list). Euryhaline fish have provided a good model for studying mechanisms of osmoregulation (see reviews by Eddy 1982; Karnaky 1986; Wood and Marshall 1994), and numerous studies have also demonstrated the importance of the endocrine system in the control and modulation of gill permeability and ion transport. The two most important (or at least most studied) osmoregulation hormones are prolactin and cortisol. Prolactin secretory cells are located in the pituitary gland of fish, and prolactin is essential for preventing diffusive Na^+ loss across permeable membranes in FW fish (see review by Hirano 1986). The steroid hormone cortisol, produced in the interrenal cells of the head kidney, plays an important role in the adaptation of euryhaline fish to SW (Hoar 1988). Cortisol promotes branchial ion excretion in euryhaline teleosts by stimulating chloride cell proliferation, differentiation and secretory activity (Foskett *et al.* 1983; McCormick and Bern 1989; Madsen 1990a). Since plasma cortisol levels increase during periods of migration or transfers from FW to SW (Specker and Schreck 1982; Young *et al.* 1989; Avella *et al.* 1990) it has become known as the 'SW hormone'. Recent studies, however, indicate that cortisol may also play an osmoregulatory role in some FW fish as well

(Laurent and Perry 1990; Bindon *et al.* 1994). Other hormones that have been implicated in osmoregulation, and that may act synergistically, include growth hormone and insulin-like growth factor (Sakamoto *et al.* 1993), thyroxine and tri-iodothyrosine (Dickhoff and Sullivan 1987), catecholamines, glucagon, somatostatin, stanniocalcin, urotensin and, most recently, natriuretic peptides (see review by Wendelaar Bonga 1993).

Energy Cost of Osmoregulation

There have been several attempts in the literature to quantify the energy required for osmoregulation in fish. Despite these efforts, the energy demands of active ion transport processes remains unclear (Evans 1984, 1993). A discrepancy exists between theoretical and experimental approaches to estimating the energy cost of osmoregulation. Theoretical estimates are based on observed active fluxes of Na^+ and Cl^- in the gills and kidneys of FW and SW fish, and the amount of energy that would be required to transport these ions against a measured electrochemical gradient between blood and water. The amount of work done by the transport system can then be calculated, given that the movement of 1 mole of an ion against a potential difference of 1 volt requires 96,487 Joules of energy. Using this method, Eddy (1982) calculated the energy cost of osmoregulation in rainbow trout (*Oncorhynchus mykiss*) to be about 1.2% of the resting metabolic rate in FW and 0.5% in SW. Similar values were determined by Potts *et al.* (1973) for the flounder, *Platichthys flesus*, in SW (1%) and by Eddy (1975) for the goldfish, *Carassius auratus*, in FW (2%). A different approach was used by Kirschner (1993, 1995) to estimate the energy cost of osmoregulation in the flounder and the rainbow trout in SW, and the rainbow trout in FW. In this case, energy was calculated as the amount of ATP required by the Na^+/K^+-pump and H^+-pump in the gills and kidney to transport Na^+ and Cl^-, based on observed

fluxes of these ions, and assuming 1 ATP for every 6 NaCl transferred. The cost of NaCl transport was then expressed as oxygen consumption, given that 6 ATP molecules are produced from 1 ml O_2/kg/h. The energy demands for osmoregulation calculated in this manner were 7.5% of standard metabolism for rainbow trout and 15% for the flounder in SW (Kirschner 1993), and about 2.5% for rainbow trout in FW (Kirschner 1995).

In contrast with the theoretical calculations, several experimental studies have suggested that the energy cost of osmoregulation in teleost fish is much higher. Measurements of whole animal oxygen consumption rates in different water salinities have generally been used to estimate the energy cost of osmoregulation. The major assumption made in these studies is that energy demands for ion transport processes are lowest in an ISO environment (generally 8-12 ppt salinity), where the ionic gradients between blood and water are minimal. Rao (1968) reported a 20% and 27% increase in the metabolic rate of rainbow trout in FW and SW, respectively, over that in ISO salinity. Farmer and Beamish (1969) found that the oxygen uptake of the Nile tilapia (*Oreochromis niloticus*) was 19% higher in FW and 29% higher in SW, compared to the ISO salinity. Febry and Lutz (1987) also reported salinity-related differences in metabolic rate in the Florida red hybrid tilapia (*O. mossambicus* x *O. hornorum*). In that study, oxygen consumption rates were 16% higher in FW and 12% higher in SW, compared to ISO salinity, which is opposite to the trend found in the Nile tilapia by Farmer and Beamish (1969). In one of the highest estimates reported, Pérez-Pinzón and Lutz (1991) found that the basal metabolic rate of juvenile snook (*Centropomus undecimalis*) was 65% and 63% higher in FW and SW, respectively, than at ISO salinity.

One explanation for the discrepancy between these theoretical and experimental estimates is the definition of the processes involved in fish osmoregulation. The theoretical estimates focus

only on the cost of moving ions against electrochemical gradients, and do not consider the energy requirements of supporting metabolic processes in osmoregulatory organs such as the gills, kidneys and intestines (e.g., protein synthesis costs for recruiting ion-motive ATPases, etc.). Likewise, the experimental estimates using whole-animal respiratory responses to different salinities may include metabolic changes which provide energy for the salinity adaptation process, but which are not directly involved in organismal ion transport (e.g., carbohydrate metabolism in the liver; Nakano *et al.* 1997). These indirect effects are undoubtedly important to the energy budget of the whole animal, but would serve to overestimate the actual costs associated with ion transport processes in the osmoregulatory organs. It is evident, therefore, that the energetic aspects of fish osmoregulation can be examined on many different levels, from the whole-animal to cellular level.

Thesis Objectives and Organization

The primary objective of this thesis was to investigate the energetic aspects of osmoregulation in fish, in an attempt to reconcile some of the large differences reported in the literature between theoretical and measured estimates of the energy cost of osmoregulation. I used an experimental approach, in which studies were designed to examine osmoregulatory energetics at both a whole-animal and tissue level. The body of the thesis is divided into three main sections. The first section describes the metabolic responses of several fish species to salinity change, using whole-animal oxygen consumption rates and gill Na^+,K^+-ATPase activity as indicators of energy requirements for osmoregulation. The second section explores the possibility that a significant portion of the increase in oxygen consumption that occurs during seawater transfer in salmonids is due to the glucocorticoid effects of cortisol, rather than just the increased energy required for

ion transport. The third section outlines experiments using isolated preparations of osmoregulatory tissues, in an attempt to separate ion transport-related costs from other whole-animal metabolic responses to salinity change. Further details on the rationale for each experiment are given in the Section Introductions.

SECTION I: Whole-Animal Metabolic Responses to Salinity Change

There have been many studies on the respiratory responses of fish to different salinities, and several have been interpreted in terms of the energy cost of osmoregulation (e.g., see Nordlie 1978). In a previous review of the literature (Morgan and Iwama 1991), we categorized five different patterns of metabolic responses of teleost fish to salinity change, indicating the importance of considering species, life stage, habits and season when interpretating whole-animal metabolic rate measurements in terms of osmoregulatory costs. Furthermore, in whole-animal studies, a distinction must also be made between metabolic rates measured in fish during salinity acclimation, and those made in long-term acclimated fish.

The goal of the experiments described in this section was to measure the metabolic response of several fish species to salinity change. These experiments were conducted as a follow-up to my M.Sc. thesis work (Morgan 1991), in an attempt to correlate whole-animal changes in oxygen uptake rate with indicators of ion transport energetics. Two tropical fish species, the stenohaline marine dolphin fish (Chapter 1) and the euryhaline freshwater tilapia (Chapter 2) were subjected to acute salinity changes and a number of physiological variables were measured, including oxygen consumption rates, gill Na^+,K^+-ATPase activities, and plasma hormone and ion levels. In a chronic study, coho salmon smolts (temperate, anadromous) were reared for 6 wk in FW, ISO and SW and sampled for similar variables (Chapter 3). In Chapter 4, cutthroat trout were acutely transferred from FW to SW and the associated changes in metabolic rate and plasma cortisol levels were measured.

CHAPTER 1: Physiological Responses to Hyposaline Exposure, Handling and Confinement Stress in Juvenile Dolphin Fish[1]

INTRODUCTION

The dolphin fish (mahimahi: *Coryphaena hippurus*) is an epipelagic predator widely distributed in tropical and subtropical marine environments. It is an excellent candidate for commercial aquaculture due to its fast growth in captivity and high demand in the marketplace (Kraul 1993). There have been many recent advances in the larviculture (Kraul 1991) and understanding of the nutritional requirements of this species (Szyper and Ako 1990; Iwai *et al.* 1992); however, very little is known about its physiological responses to environmental stressors such as changes in salinity or handling and confinement. The dolphin fish does not normally encounter variations in salinity in its natural environment, but tolerates salinities as low as 15 ppt in captivity (Kraul 1993), and can even survive in FW for at least 1 h (Szyper and Lutnesky 1991). Increased understanding of the physiological responses of dolphin fish to factors such as stress and salinity change may assist in the husbandry of this species in culture operations. Therefore, the objective of this study was to examine the changes in selected aspects of metabolism, ionic regulation and hematology in juvenile dolphin fish subjected to a reduced water salinity. In addition, the physiological responses to stress associated with handling and confinement were also studied in this species.

---

[1] A version of this chapter has been published in:
J.D. Morgan, S.K. Balfry, M.M. Vijayan and G.K. Iwama. 1996. Can. J. Fish. Aquat. Sci. 53: 1736-1740.

MATERIALS AND METHODS

Fish

Three-month-old dolphin fish (20-40 g) reared in captivity at the Waikiki Aquarium (Honolulu, Hawaii) were used in the study. Fifty fish were kept in an outdoor 2500-L circular tank with running SW (temp = 25°C, salinity = 34 ppt) and constant aeration. The fish were fed a daily diet of chopped squid, but feeding was stopped 24 h prior to the experiment.

Salinity Exposure

Prior to the salinity challenge test, eight fish were sampled from the 2500-L stock tank to establish baseline values for the variables measured (see below). Twelve fish were then transferred into each of two 200-L experimental tanks: control fish were transferred to full-strength SW (34 ppt) as in the stock tank, and treatment fish were transferred directly to diluted SW (20 ppt). After 24 h, the fish were anesthetized with TMS (Syndel Laboratories, Vancouver, British Columbia), the caudal peduncle was severed and blood was collected for determination of hematocrit, hemoglobin and erythrocyte numbers. The remaining blood samples were centrifuged (2000 *g* for 5 min), and the plasma was removed and stored at -75°C for later analyses of cortisol, glucose, protein and ions. Liver samples were quickly dissected out and frozen on dry ice for analysis of glycogen content, and gill tissue was then cut out with fine-point scissors and frozen in 1 mL of SEI buffer (0.3 M sucrose, 0.02 M Na_2EDTA, 0.1 M imidazole, pH 7.1) for measurement of Na^+,K^+-ATPase activity.

Respirometry

Oxygen consumption rates of juvenile dolphin fish in the two salinity treatments were measured 24 h after transfer using a respirometer which consisted primarily of a plexiglass swimtube (Cech 1990). The total volume of the respirometer was 2.75 L and the swimtube was 36 cm long and 8.5 cm in diameter. Water flow was generated using a centrifugal pump (Eheim model 1250) connected to the swimtube with vinyl tubing. A valve assembly allowed the respirometer to operate in a flow-through (acclimation) or closed (measurement) mode. Water oxygen concentrations were measured using a dissolved oxygen meter (Oxyguard® Mk III, Point Four Systems, Port Moody, British Columbia) with the electrode mounted inside the respirometer. The oxygen electrode was calibrated in air to 101% saturation before use, according to the meter's operating manual.

Prior to each respirometry trial, individual fish from an experimental tank were introduced to the swimtube and allowed to acclimate for 20 min in flow-through water. The swimming speed was set to 0.5 body length/s to standardize the level of activity (Barton and Schreck 1987). The respirometer was covered with black plastic throughout the acclimation and measurement periods to shield the fish from outside disturbances. After acclimation, the respirometer was closed and the subsequent decline in water oxygen concentration (to the nearest 0.1 mg/L) was monitored at 2 min intervals for 10 min. After a trial was completed, the fish was removed from the swimtube and its length and mass measured. To adjust for possible bacterial oxygen consumption within the system, blank trials without any fish were run throughout the experiment. Water temperatures in the respirometer were controlled by immersing the apparatus in a flow-through reservoir, and temperatures were kept similar to the experimental tanks (25 ± 0.5°C).

Water oxygen concentrations decreased at a constant rate with approximately 25% of the initial oxygen consumed during each trial. Dissolved oxygen levels were maintained above 5 mg/L to minimize stress-related effects of low oxygen concentration (Lutnesky and Szyper 1990). Oxygen consumption rates were estimated using linear regression analyses. The regression slope of oxygen uptake was multiplied by the respirometer volume and expressed in terms of wet weight over a 1 h period (i.e., mg O_2/kg/h). Oxygen consumption rates of five fish were determined for each salinity treatment.

Analytical procedures

Blood hemoglobin levels were determined using the Ames™ Minilab (Iwama *et al.* 1995), while hematocrit (% red blood cells), erythrocyte numbers and size were determined using methods described by Houston (1990). Plasma cortisol levels were determined using a commercially available [^{125}I] cortisol radioimmunoassay (RIA) kit (GammaCoat, Incstar Corporation, Stillwater, Minnesota), validated for fish plasma by Heath (1993). Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Chemical Co., St. Louis, Missouri), while plasma protein levels were measured by a micro-modification of Lowry's (1951) folin phenol method (Sigma). Plasma [Na^+] and [K^+] were measured using an ion chromatograph (Shimadzu Model HIC-6A, Shimadzu Corporation, Kyoto, Japan). Briefly, plasma samples were deproteinated with acetonitrile, diluted with distilled deionized water and injected into the ion chromatograph with 5 mM nitric acid as the mobile phase. Plasma [Cl^-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer), whereas plasma [Ca^{2+}] and [Mg^{2+}] were measured colorimetrically (Sigma).

Liver glycogen content was determined according to Perry *et al.* (1988). Na^+,K^+-ATPase activity (μmoles of ADP/mg of protein/h) in crude gill homogenates was determined at 25°C in a temperature-controlled plate reader (Thermomax, Molecular Devices Corp., Menlo Park, California) according to McCormick (1993). In this kinetic assay, the ouabain-sensitive hydrolysis of ATP is enzymatically coupled to the oxidation of NADH, which is directly measured in 96-well microplates at 340 nm for 10 min. Protein content in the gill homogenate was determined using the bicinchoninic acid procedure (Smith *et al.* 1985).

Statistical Analyses

Data are presented as means ± 1 SE. When ANOVA indicated significant differences, Student-Newman-Keuls multiple comparison test was used to identify significantly different treatment means ($P<0.05$). Comparison between the two metabolic rate measurements was accomplished using a Student's t-test ($P<0.05$).

RESULTS

Metabolic Effects

The average metabolic rate of juvenile dolphin fish was significantly lower in 20 ppt salinity compared to full-strength SW (Fig. 4). Plasma cortisol levels were significantly higher in 20 ppt salinity after 24 h compared to the stock group (34 ppt salinity at 0 h), but not the control group (34 ppt at 24 h, Fig. 5). There were no differences in liver glycogen content, plasma glucose or plasma protein levels during the experiment (Table 1).

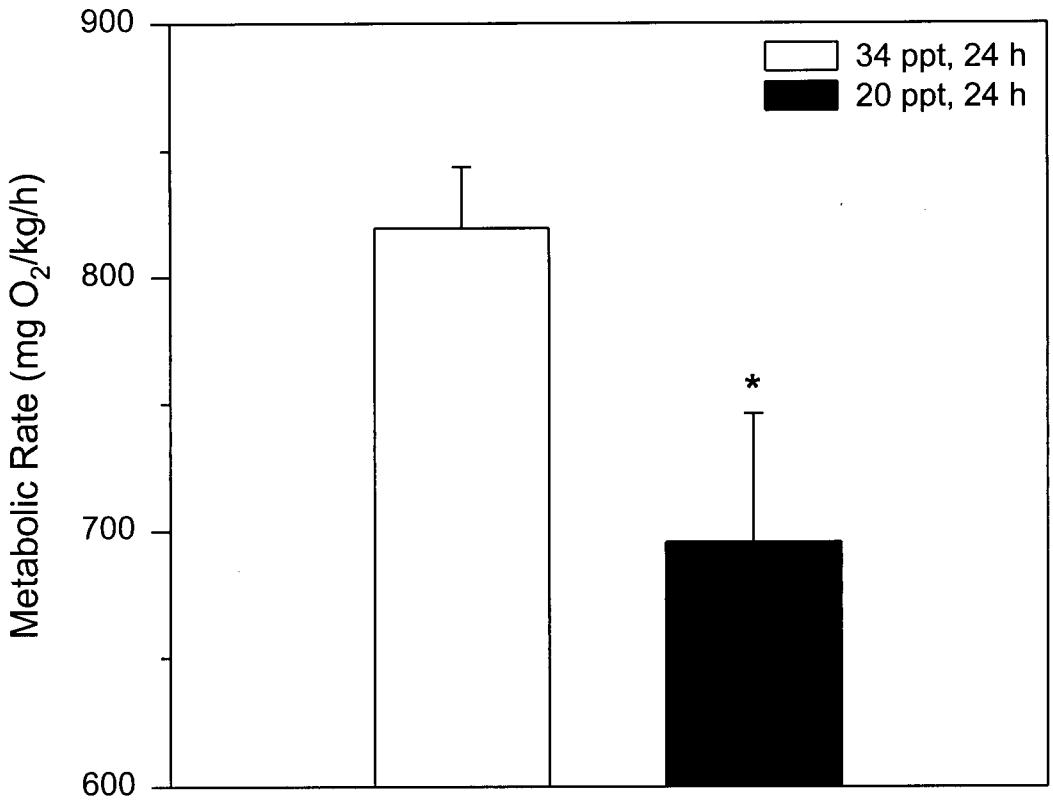

Fig. 4. Metabolic rates of juvenile dolphin fish after a 24 h exposure to 34 ppt and 20 ppt salinity. Data are shown as means ± SE (n=5). Asterisk denotes significant difference (unpaired t-test, $P<0.05$).

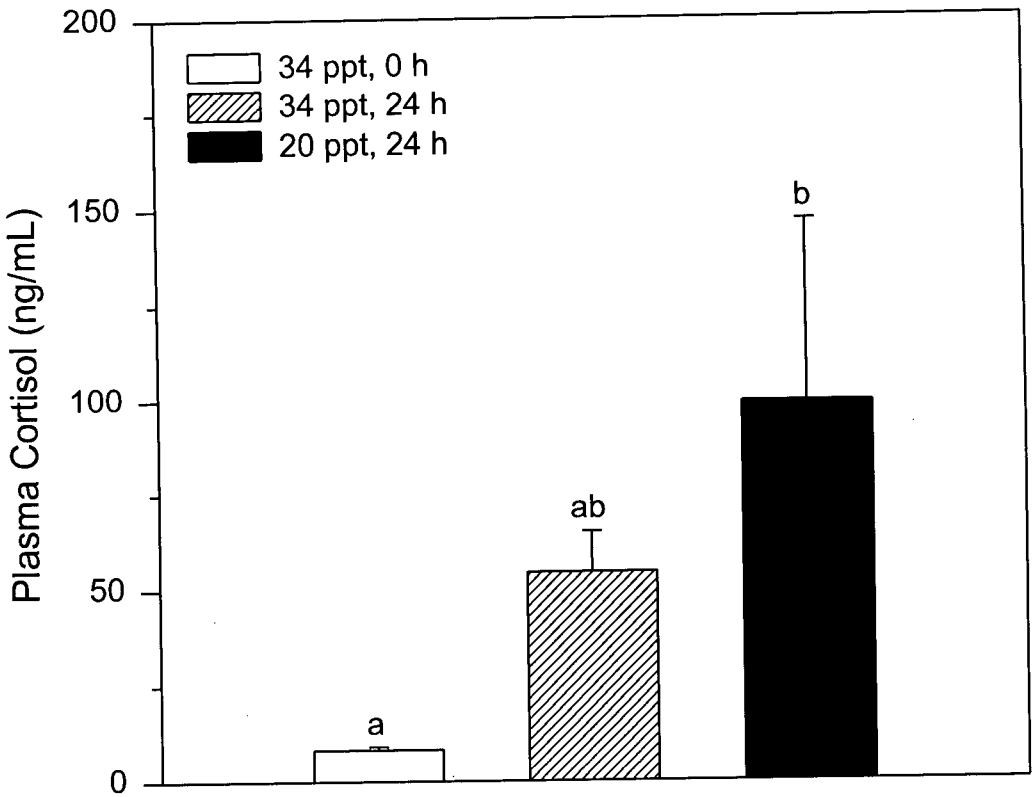

Fig. 5. Plasma cortisol concentrations in juvenile dolphin fish, before and after a 24 h exposure to 34 ppt and 20 ppt salinity. Data are shown as means ± SE (n=6-8). Means with different letters are significantly different (one-way ANOVA, $P<0.05$).

Table 1. Body mass, liver glycogen content, plasma glucose, protein and ion concentrations, blood hemoglobin concentration, hematocrit values, and erythrocyte counts in juvenile dolphin fish, before and after a 24 h exposure to 34 ppt and 20 ppt salinity. Values are the mean (±SE) of 6-8 fish per treatment. Means within a row with different superscript letters are significantly different ($P<0.05$).

	Salinity treatment (ppt), Exposure time (h)		
Variable	34 ppt, 0 h	34 ppt, 24 h	20 ppt, 24 h
Mass (g)	28.1 ± 2.8^{a}	32.0 ± 3.3^{a}	32.9 ± 2.3^{a}
Liver glycogen (µmol/g)	84.3 ± 4.0^{a}	89.5 ± 1.6^{a}	87.9 ± 1.2^{a}
Plasma glucose (mM)	5.1 ± 0.3^{a}	4.9 ± 0.4^{a}	5.3 ± 1.0^{a}
Plasma protein (g/dL)	1.9 ± 0.1^{a}	1.5 ± 0.2^{a}	1.7 ± 0.3^{a}
Plasma ions (mM)			
Na^{+}	174.0 ± 5.1^{a}	175.5 ± 5.4^{a}	173.1 ± 3.6^{a}
Cl^{-}	151.5 ± 6.7^{a}	161.1 ± 2.8^{a}	160.2 ± 1.5^{a}
K^{+}	6.0 ± 0.2^{a}	5.1 ± 0.4^{a}	5.0 ± 0.3^{a}
Ca^{2+}	2.1 ± 0.2^{a}	1.9 ± 0.2^{a}	1.9 ± 0.1^{a}
Mg^{2+}	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}
Hemoglobin (g/dL)	7.0 ± 0.4^{a}	8.3 ± 0.2^{b}	8.2 ± 0.3^{b}
Hematocrit (% RBC)	29.9 ± 1.6^{a}	36.6 ± 1.1^{b}	36.2 ± 0.8^{b}
Erythrocytes (10^{9}/mL)	2.7 ± 0.2^{a}	3.9 ± 0.3^{b}	4.3 ± 0.6^{b}
Erythrocyte size (µm)	5.3×10^{a}	5.5×9.5^{a}	5.3×10.5^{a}

Plasma Ion Concentrations and Gill Na^+,K^+-ATPase

There were no differences in plasma [Na^+], [Cl^-], [K^+], [Ca^{2+}] or [Mg^{2+}] between the salinity treatments (Table 1), but there was a significant decrease in gill Na^+,K^+-ATPase activity in 20 ppt salinity compared to full-strength SW (Fig. 6).

Hematology

There were significant increases in hematocrit and hemoglobin values, and red blood cell numbers in both salinity treatments after 24 h compared to the stock group (34 ppt at 0 h, Table 1). There was no change in erythrocyte size during the experiment.

DISCUSSION

This study is the first to measure metabolic rate in juvenile dolphin fish subjected to a change in environmental salinity. The metabolic rate of juvenile dolphin fish in SW in this study is similar to values obtained by other workers for this species (Lipskaya 1974; Waller 1989; Benetti 1992). As in tunas, dolphin fish have high energy demands to support an active predatory lifestyle, and have metabolic rates several times higher than salmonids, even when corrected for temperature differences (Benetti *et al.* 1995). To achieve these high levels of oxygen consumption, dolphin fish have exceptionally large gill surface areas (1389 cm^2 for a 100 g fish) similar to that of tunas (Hughes 1972, 1984). These large surface areas of permeable gill epithelia may increase the passive uptake of salt into the dolphin fish, resulting in high osmoregulatory costs. This is supported by the significant decline in both the rate of oxygen consumption and gill Na^+,K^+-ATPase activity observed in the lower water salinity (Figs. 4 and 6), where ionic concentration

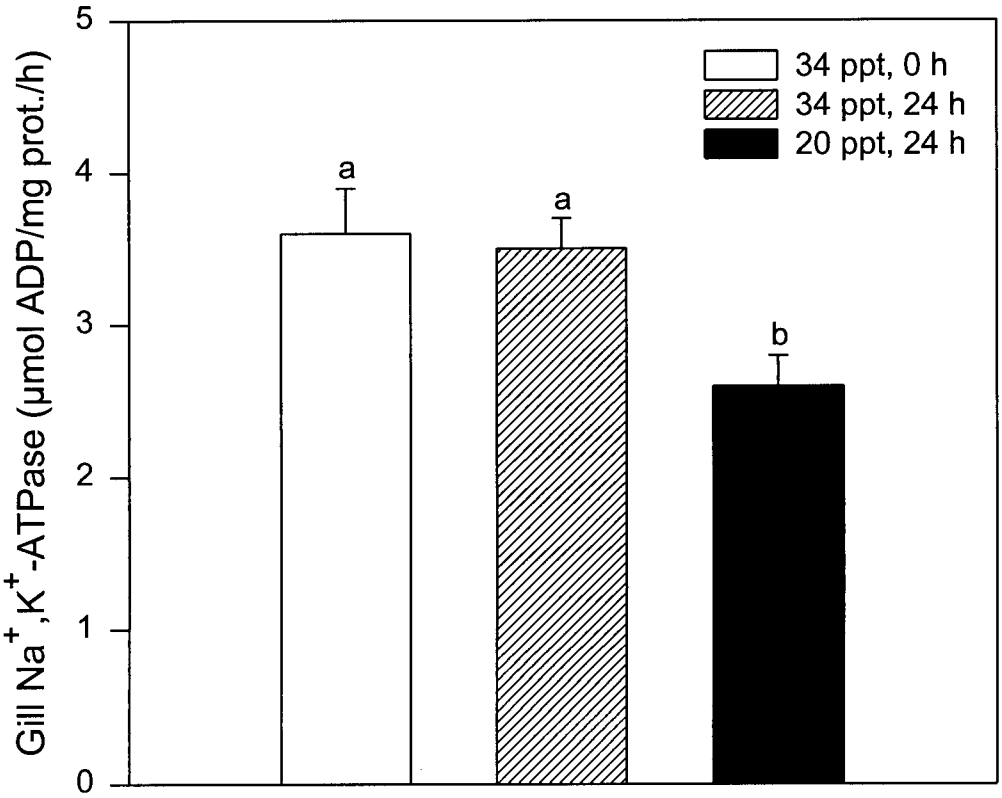

Fig. 6. Gill Na^+,K^+-ATPase activity in juvenile dolphin fish, before and after a 24 h exposure to 34 ppt and 20 ppt salinity. Data are shown as means ± SE (n=6-8). Means with different letters are significantly different (one-way ANOVA, $P<0.05$).

gradients between blood and water would be reduced. Benetti *et al.* (1995) also suggested that the high standard metabolic rate they recorded for adult dolphin fish reflects a high maintenance cost for osmoregulation resulting from large thin gills. Future studies on the cost of osmoregulation in dophin fish should include metabolic rate measurements of isolated gills, as proposed by Brill (1996).

In the present study, juvenile dolphin fish (a relatively stenohaline marine species) responded to a reduced salinity by decreasing their metabolic rate, thus providing evidence for the possibility of a sixth metabolic response pattern to salinity change, in addition to the five patterns listed in Morgan and Iwama (1991). As stated above, this decrease in metabolic rate at a lower salinity may have been associated with a decrease in osmoregulatory costs, indicated by the decrease in gill Na^+,K^+-ATPase activity. Hence, dolphin fish appear to conserve energy at the reduced salinity, unlike many euryhaline marine fish species that exhibit higher metabolic rates in water with lower salinities (Prosser 1973; Morgan and Iwama 1991). The absence of any changes in plasma glucose concentration or liver glycogen content with exposure to water with lower salinity in this study also suggests that carbohydrate reserves were not being mobilized for energy purposes.

There is a general lack of published information on the hematology and blood chemistry of dolphin fish. The plasma cortisol, glucose and protein concentrations of dolphin fish in SW were within the range of values reported for resting fish in the literature (e.g., Hille 1982; Miller *et al.* 1983; Wedemeyer *et al.* 1990; Barton and Iwama 1991). Plasma ion concentrations were also similar to those reported for other marine teleosts (McDonald and Milligan 1992). Fast swimmers, such as tuna and herring, generally tend to have high hematocrit and hemoglobin concentrations (Fänge 1992), although the values for juvenile dolphin fish in this study were

closer to those reported for salmonids (Miller *et al.* 1983; Wedemeyer *et al.* 1990; Culloty *et al.* 1991). Like the tuna, dolphin fish have a relatively high number of small red blood cells (Table 1), compared to less active species (Fänge 1992). A large number of small erythrocytes would be advantageous for increasing oxygen carrying capacity in an active swimmer such as the dolphin fish, as the small red cell size would facilitate gas exhange processes by a favorable surface area/volume ratio.

There were no other salinity-related effects observed in this study; however, transferring and holding dolphin fish in smaller tanks (200 L) resulted in higher plasma cortisol concentrations, indicating an endocrine stress response that may be attributed to handling and (or) confinement (see Barton and Iwama 1991; Gamperl *et al.* 1994). There were increases in hematocrit, hemoglobin and red cell numbers in the experimental tanks compared to the stock tank that are consistent with a splenic release of red bloods cells which occurs during stress (Fänge 1992). There were also decreases in plasma lysozyme activities in the experimental tanks (S.K. Balfry, UBC, personal communication), that may have been indicative of a stress-related impairment of immune function.

In summary, this study provides information on selected physiological variables of juvenile dolphin fish kept in captivity. A short-term (24 h) exposure to a reduced water salinity (20 ppt) resulted in a decreased rate of oxygen consumption and lower gill Na^+,K^+-ATPase activity. This decrease in metabolic rate may have been associated with a reduction in osmoregulatory costs. There were also stress-related effects, caused by handling and (or) confinement in the experimental tanks, on plasma cortisol concentrations and some aspects of hematology.

CHAPTER 2: Physiological and Respiratory Responses of the Mozambique Tilapia to Salinity Acclimation[2]

INTRODUCTION

The Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline cichlid which has been introduced from its native Africa to tropical FW and marine environments around the world (Balarin and Hatton 1979). This species has provided a good model for studying the mechanisms of osmoregulation in teleost fishes (e.g., Foskett *et al.* 1981; Foskett *et al.* 1983), due to its euryhaline nature and hardiness in captivity (Evans 1984).

There have been many studies on the physiological changes that occur during the SW acclimation process in Mozambique tilapia. Following transfer from FW to SW there is a temporary elevation in plasma $[Na^+]$, $[Cl^-]$ and osmolality (Assem and Hanke 1979a; Hwang *et al.* 1989), accompanied by a transient rise in plasma cortisol concentrations (Assem and Hanke 1981), and a more gradual increase in plasma growth hormone (GH) levels (Yada *et al.* 1994). There is an alteration in branchial chloride cell morphology (Hwang 1987) and an increase in gill Na^+,K^+-ATPase activity (Dharmamba *et al.* 1975; Dange 1985; Hwang *et al.* 1989) in SW. Furthermore, there is recent evidence to link the actions of cortisol and GH to these processes in Mozambique tilapia (McCormick 1990; Borski *et al.* 1994). Tilapia possess two forms of prolactin ($tPRL_{177}$ and $tPRL_{188}$) (Specker *et al.* 1985; Yamaguchi *et al.* 1988), which have a sodium retention effect in FW and decrease to low levels in the plasma of fish transferred to SW

[2] A version of this chapter has been published in:
J. D. Morgan, T. Sakamoto, E.G. Grau and G.K. Iwama. 1997. Comp. Biochem. Physiol. 117A: 391-398.

(Ayson *et al.* 1993; Yada *et al.* 1994). The result of these biochemical and hormonal responses is a net efflux of Na^+ and Cl^- to maintain ionic balance in a hyperosmotic environment (Potts *et al.* 1967; Dharmamba *et al.* 1975).

The metabolic response of tilapia during the SW acclimation process is less clear. Oxygen consumption has commonly been used as an indirect indicator of metabolism in fish (Cech 1990), and measurements of oxygen consumption rates in different salinities have been employed in an attempt to determine the energy cost of osmoregulation in tilapia. Farmer and Beamish (1969), using the Nile tilapia, and Febry and Lutz (1987), using the Florida red hybrid tilapia, found that oxygen consumption rates of swimming fish were lowest in ISO (12 ppt salinity) compared to FW and SW. In contrast, Job (1969) reported ISO to have the highest rate of oxygen consumption in Mozambique tilapia, but activity was not controlled in that study and therefore statements regarding osmoregulatory costs cannot be made unequivocally. Furthermore, the fish in the three studies above were acclimated to the test salinities for at least a month before oxygen consumption rates were determined and, therefore, do not reflect metabolic requirements of the animals during the acclimation process.

The purpose of the present study was to examine the physiological and respiratory responses of Mozambique tilapia during the acclimation to ISO and SW. Simultaneous measurements of oxygen consumption rates, plasma constituents and gill Na^+,K^+-ATPase activities were carried out in tilapia after transfer from FW to FW, ISO and SW.

MATERIALS AND METHODS

Fish

Adult male and female tilapia maintained in FW at the Hawaii Institute of Marine Biology (Coconut Island, Hawaii) were used in the study. These fish originated from a population on Oahu that was introduced to Hawaii from Singapore in 1951 (Hida *et al.* 1962; Uchida and King 1962). While in captivity, they were kept in a 5000-L circular fibreglass tank under a natural photoperiod (approx. 14 h light : 10 h dark). The fish were fed a daily diet of Purina trout chow during the experiment, but were not fed for 24 h before sampling.

Salinity Exposure

In April 1994, tilapia (50-80 g) were transferred randomly into three 60-L oval fiberglass tanks at a density of 25 fish per tank. The fish were checked to ensure that no brooding females were used in the experiment. One tank was supplied with FW and two contained ISO (12 ppt salinity; Febry and Lutz 1987). The salinity in one of the ISO tanks was then increased over the next 6 h to 75% SW (25 ppt). An initial attempt to acclimate fish to 100% SW (34 ppt) over 30 h resulted in 92% mortality. Previous studies have shown that Mozambique tilapia cannot tolerate an abrupt transfer from FW to full-strength SW, and require a gradual (1 wk) acclimation period (see review by Stickney 1986). Water temperatures were kept similar in each treatment tank (22 ± 1°C) and aeration was provided to maintain dissolved oxygen levels above 95% saturation. Water samples were collected to measure the ionic composition of each salinity treatment (Table 2). Eight fish were sampled prior to the transfer, and at 1 and 4 d after transfer to the treatment tanks. The fish were anesthetized in 2-phenoxyethanol (1 mL/L), killed by a blow to

Table 2. Chemical composition of water samples collected from the tilapia salinity treatment tanks.

	Medium			
Variable	FW	ISO	75% SW	SW
Salinity (ppt)	0	12	25	34
Na^+ (mM)	2	161	322	482
Cl^- (mM)	<1	182	377	553
K^+ (mM)	BDL[a]	3.0	6.9	9.2
Ca^{2+} (mM)	0.4	3.4	6.7	8.4
Mg^{2+} (mM)	0.7	15.8	27.9	35.2

[a]Below detection limits

the head, and blood was collected from the caudal vessels using heparinized syringes (Houston 1990). The blood samples were centrifuged (2000 *g* for 5 min) and the plasma was removed and stored at -75°C for later analyses of cortisol, GH, the two PRLs, glucose and ions. Immediately following blood collection, gill filaments were removed from the second gill arch on the left side of the fish, placed in 1 mL of ice-cold SEI buffer (0.3 M sucrose, 0.02 M Na_2EDTA, 0.1 M imidazole, pH 7.1), and stored at -75°C for measurement of Na^+,K^+-ATPase activity.

Respirometry

Oxygen consumption rates of tilapia in the salinity treatments were measured at the last sampling period (4 d after transfer) using the same respirometer described in Chapter 1. Prior to each respirometry trial, individual fish from a treatment tank were introduced to the swimtube and allowed to acclimate in flow-through water. The fish were not fed for 24 h prior to testing, in order to ensure a postabsorptive digestive state (Beamish 1978). The respirometer was covered with black plastic throughout acclimation and testing to shield the fish from visual disturbances. In each trial, the swimming speed was set to 0.5 body length/s to standardize the level of activity (Barton and Schreck 1987). After acclimation, the respirometer was closed and the subsequent decline in water oxygen concentration (to the nearest 0.1 mg/L) was monitored at 5 min intervals for 20 min. After a trial was completed, the fish was removed from the swimtube and its length and mass measured. To adjust for possible bacterial oxygen consumption within the system, blank trials without any fish were run throughout the experiment. Water temperatures during the trials were controlled by immersing the respirometer in a flow-through reservoir, and temperatures were kept similar to the treatment tanks (23 ± 1°C).

Water oxygen concentrations decreased at a constant rate with about 30% of the initial oxygen consumed during each trial. Oxygen consumption rates were estimated using linear regression analyses and expressed as milligrams of oxygen per hour per kilogram of fish (i.e., mg O_2/kg/h). Oxygen consumption rates of six fish were determined for each salinity treatment.

Analytical Procedures

Plasma cortisol levels were determined using a commercial RIA kit ([^{125}I] cortisol; GammaCoat, Incstar Corporation, Stillwater, Minnesota). Plasma GH, $tPRL_{177}$ and $tPRL_{188}$ levels were measured using the homologous RIA developed by Ayson *et al.* (1993). Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Chemical Co., St. Louis, Missouri). Plasma [Na^+] and [K^+] were measured on an ion chromatograph (Shimadzu Model HIC-6A, Shimadzu Corporation, Kyoto, Japan). Plasma [Cl^-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer). Na^+,K^+-ATPase activity (μmoles of ADP/mg of protein/h) in crude gill homogenates was determined at 25°C in a temperature-controlled plate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA) according to McCormick (1993).

Statistical Analyses

Data are presented as means ± 1 SE. Two-way ANOVAs were used to test for treatment and time effects, whereas oxygen consumption results were analyzed using a one-way ANOVA. Significant treatment means were identified using Student-Newman-Keuls multiple comparison test ($P<0.05$).

RESULTS

Plasma Cortisol and Glucose Levels

The transfer of fish from the FW stock tank into the 60-L FW and ISO treatment tanks resulted in a significant increase in plasma cortisol titres after 1 d (Fig. 7A). In contrast, plasma cortisol levels in the SW fish were not elevated 1 d after transfer and were significantly lower than the FW and ISO treatments. Cortisol levels in all three treatment groups were not significantly different at the 4 d sampling period. Plasma glucose levels were significantly higher in FW and ISO on both days 1 and 4, compared to SW values which did not change during the experiment (Fig. 7B).

Plasma Growth Hormone and Prolactin Levels

Plasma GH levels increased significantly 4 d after transfer to SW, whereas no significant change was observed in the FW and ISO groups (Fig. 8A). Plasma $tPRL_{177}$ decreased to low levels in SW after 1 and 4 d compared to FW (Fig. 8B). Levels of $tPRL_{177}$ in ISO were intermediate between the FW and SW values. Plasma $tPRL_{188}$ levels were significantly lower in both ISO and SW after 4 d compared to FW (Fig. 8C). The ratio of $tPRL_{188}$:$tPRL_{177}$ was significantly higher in SW (7.0) than in FW (0.9) and ISO (1.0) after 4 d.

Plasma Ion Concentrations and Gill Na^+,K^+-ATPase Activity

Plasma [Na^+] and [Cl^-] in SW were significantly elevated over FW values 1 d after transfer, but returned to FW levels on day 4 (Fig. 9A,C). There was a slight rise in plasma [Na^+] and [Cl^-] after 1 d in ISO. Plasma [K^+] did not differ significantly among salinity treatments at each

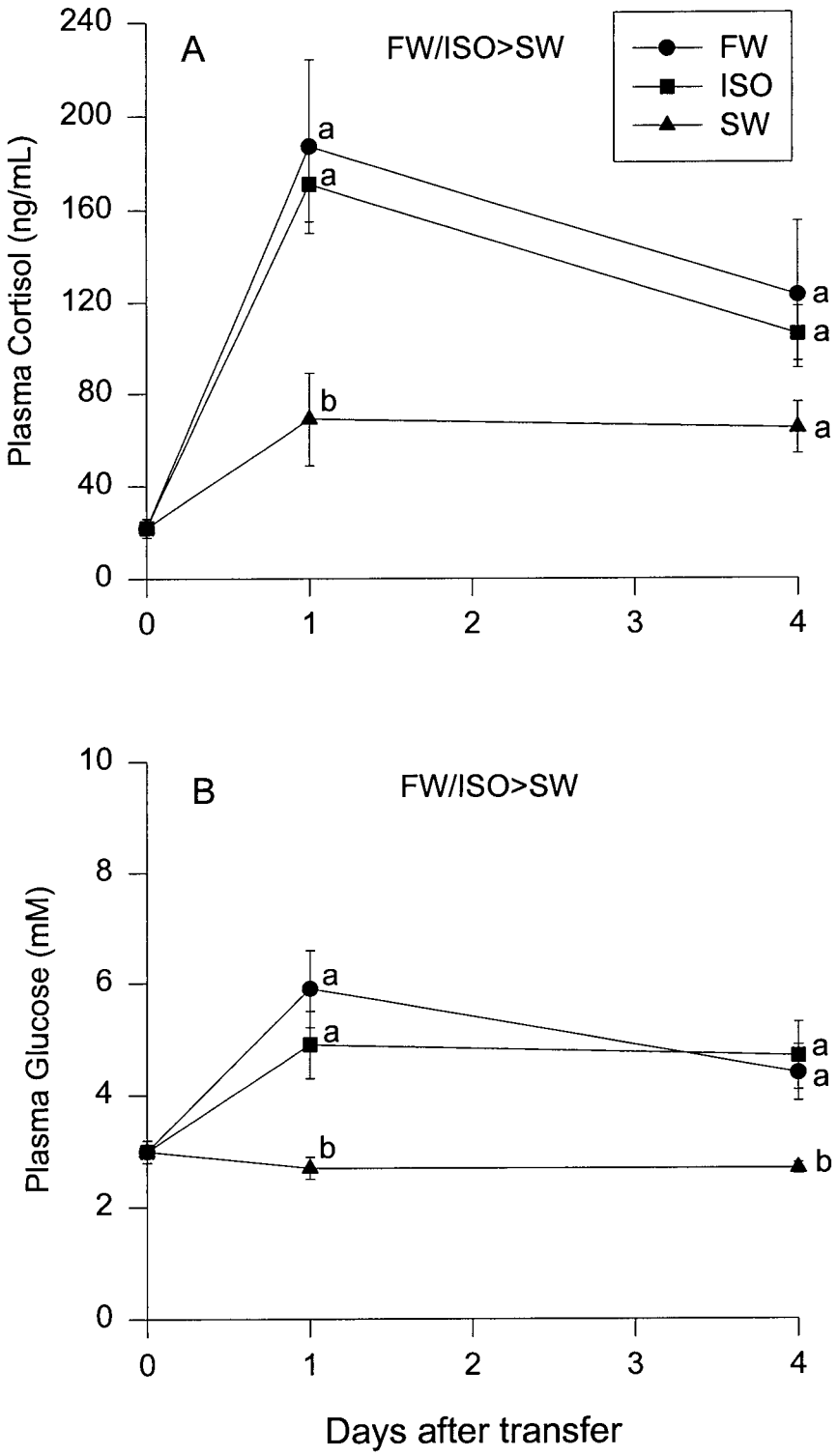

Fig. 7. Plasma cortisol and glucose levels in tilapia after transfer from FW to FW, ISO and 75% SW. Data are presented as means ± SE (n=6-8). Inset shows significant treatment effects; significant interaction at each time period is shown by letters next to symbols, and means with different letters are significantly different ($P<0.05$, two-way ANOVA).

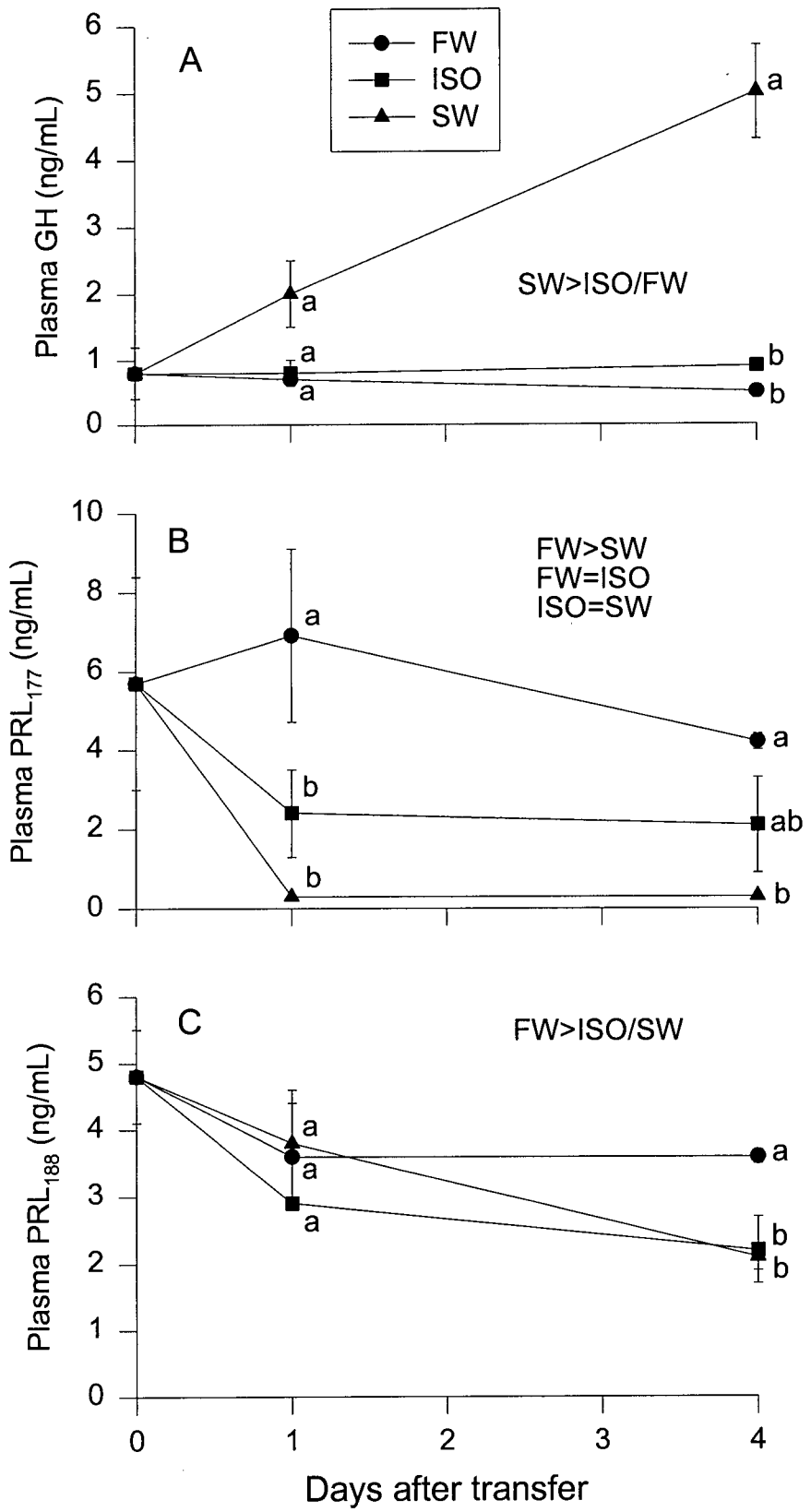

Fig. 8. Plasma growth hormone (GH) and prolactin (PRL_{177} and PRL_{188}) levels in tilapia after transfer from FW to FW, ISO and 75% SW. Data are presented as means ± SE (n=3-8). Inset shows significant treatment effects; significant interaction at each time period is shown by letters next to symbols, and means with different letters are significantly different ($P<0.05$, two-way ANOVA).

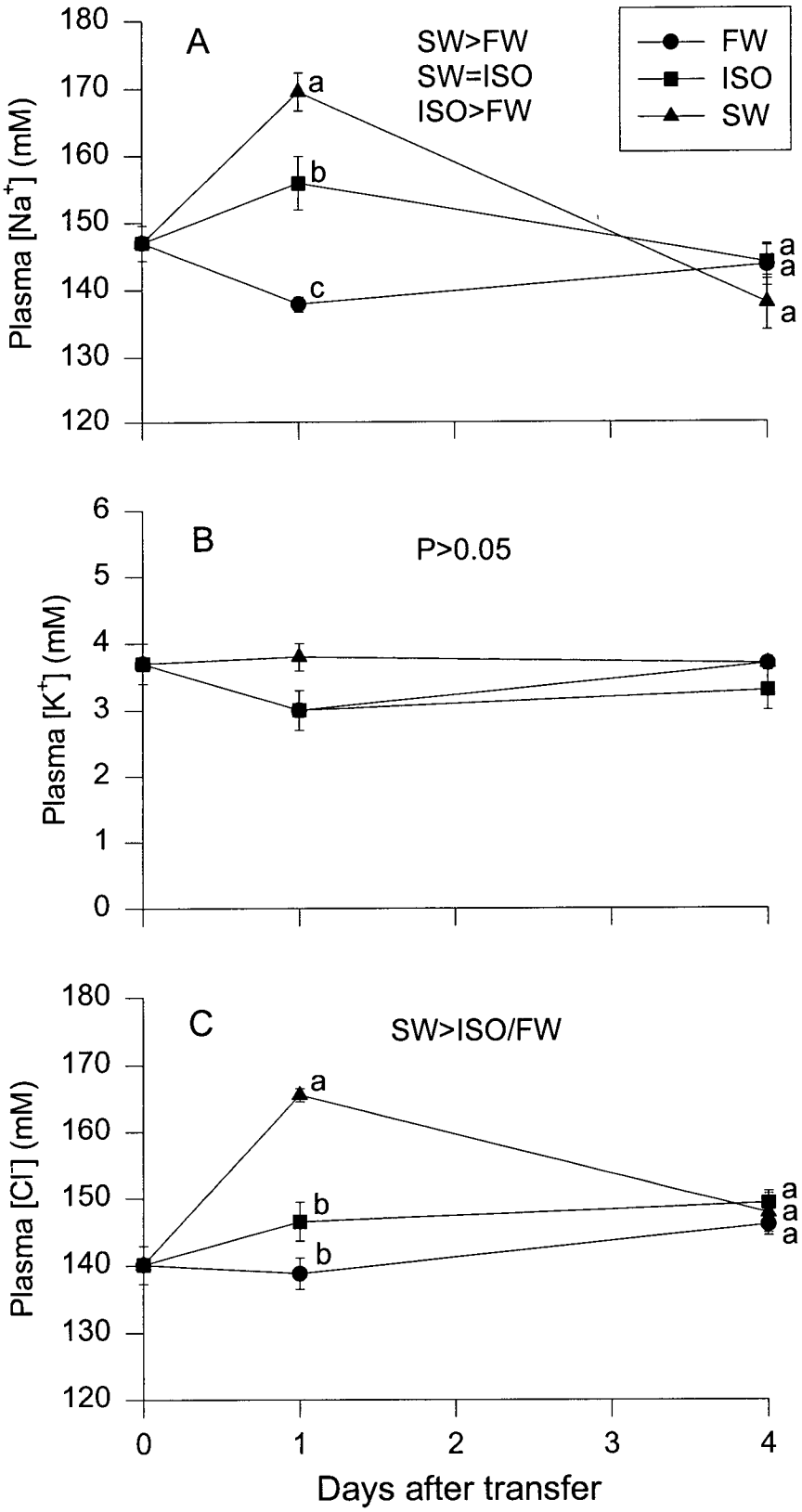

Fig. 9. Plasma [Na^+], [K^+] and [Cl^-] of tilapia after transfer from FW to FW, ISO and 75% SW. Data are presented as means ± SE (n=6-8). Inset shows significant treatment effects; significant interaction at each time period is shown by letters next to symbols, and means with different letters are significantly different ($P<0.05$, two-way ANOVA).

sampling period (Fig. 9B). Gill Na^+,K^+-ATPase activity of tilapia was significantly greater in SW than in both FW and ISO 4 d after transfer (Fig. 10).

Oxygen Consumption Rates

The average oxygen consumption rate of tilapia 4 d after transfer was significantly (20%) higher in SW than in FW or ISO (Fig. 11). There was no difference in the oxygen consumption rate of tilapia between FW and ISO.

DISCUSSION

The oxygen consumption rate of Mozambique tilapia in FW measured in the present study is comparable to values obtained for this, and other, species of tilapia at a similar temperature, body size and activity level (Farmer and Beamish 1969; Job 1969; Abdel Magid and Babiker 1975; Caulton 1977, 1978; Zohar 1992). In this study, the metabolic response of Mozambique tilapia to salinity change was measured during the acclimation process, whereas most previous studies have focused on tilapia which have undergone long-term salinity exposure (4-5 wk), when all acclimatory processes could be considered complete (e.g., Farmer and Beamish 1969; Job 1969; Febry and Lutz 1987). Oxygen consumption rates of Mozambique tilapia in SW (25 ppt) were significantly elevated 4 d after transfer compared to FW and ISO (12 ppt). This increase in oxygen consumption rate was associated with an increase in plasma GH levels and gill Na^+,K^+-ATPase activity in SW fish. The oxygen consumption data suggested that the metabolic cost of acclimating to SW after 4 d was at least 20%. This does not necessarily imply that the direct energetic cost of active ion transport processes in osmoregulatory organs such as the gills, intestine and kidneys was increased by 20%, only that the metabolism of the whole animal was raised by this

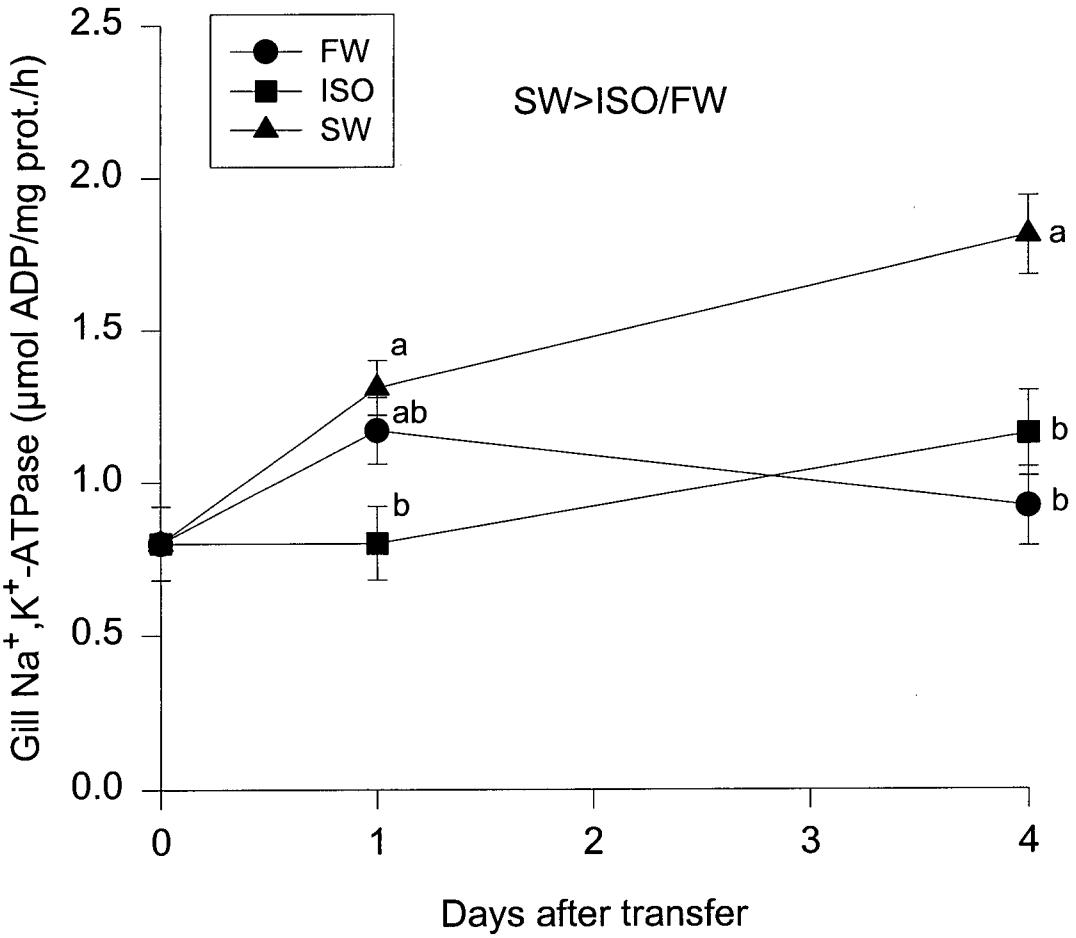

Fig. 10. Gill Na^+,K^+-ATPase activity of tilapia after transfer from FW to FW, ISO and 75% SW. Data are presented as means ± SE (n=6-8). Inset shows significant treatment effects; significant interaction at each time period is shown by letters next to symbols, and means with different letters are significantly different ($P<0.05$, two-way ANOVA).

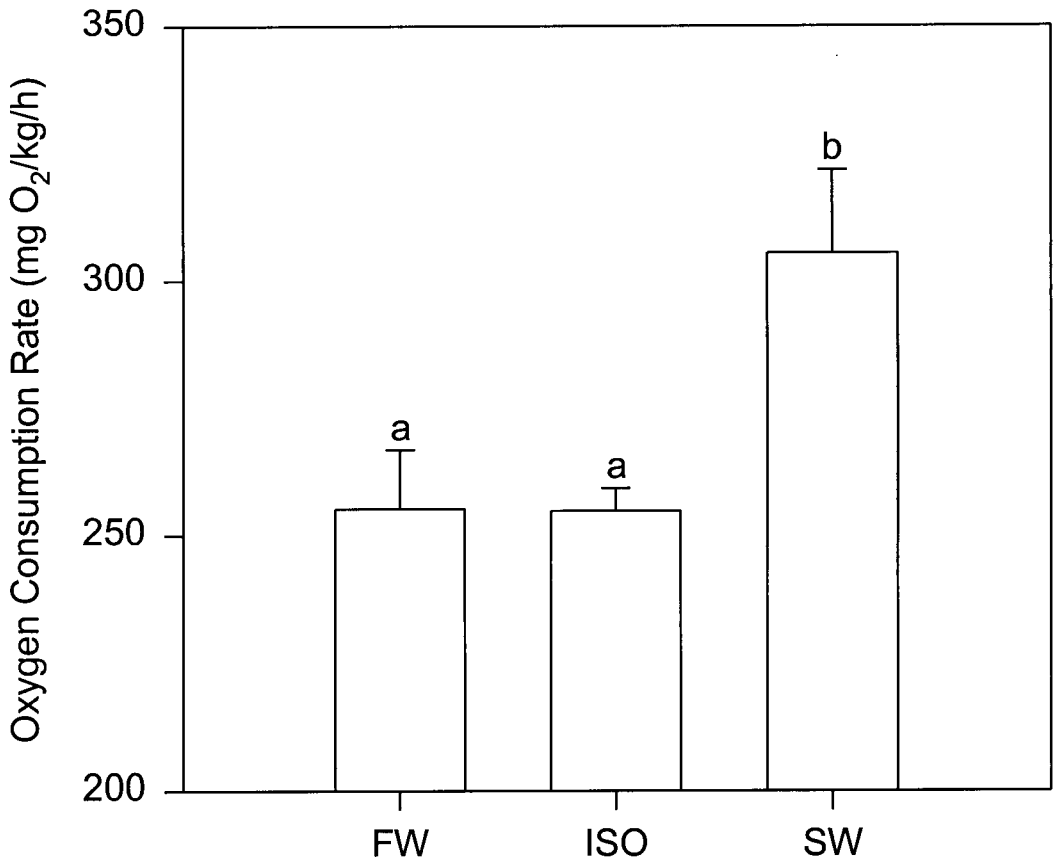

Fig. 11. Oxygen consumption rates of tilapia in FW, ISO and 75% SW, 4 days after transfer from FW. Data are presented as means ± SE (n=6). Means with different letters are significantly different ($P<0.05$, one-way ANOVA).

amount in SW. Increased GH production following SW transfer is likely to stimulate other aspects of metabolism (e.g., increased rates of protein synthesis), in addition to inducing the required osmoregulatory adjustments (e.g., increased gill Na^+,K^+-ATPase activity). Seddiki *et al.* (1995), for example, have recently reported that treatment with trout recombinant GH increased standard oxygen consumption in rainbow trout by 18% in FW and a further 12% after 4 d in SW. Physiological changes initiated by GH, and possibly other hormones, during the SW acclimation process in tilapia likely caused most of the elevated rate of oxygen uptake observed in this study. In a related study, it has been found that Mozambique tilapia fasted for 2 wk could not osmoregulate properly compared to fed fish following a gradual transfer to SW, which also suggests that there is a significant energy requirement for SW acclimation in this species of tilapia (Vijayan *et al.* 1996). Further studies are required to delineate the time course of the metabolic response of Mozambique tilapia to SW, by making repeated oxygen consumption measurements throughout the acclimation period.

No difference in the oxygen consumption rate of Mozambique tilapia was observed between FW and ISO in this acute study, suggesting that acclimation to ISO does not impose (or reduce) an energetic demand on this species of tilapia. Febry and Lutz (1987) reported that, after a 1 month acclimation, the cost of osmoregulation in Florida red hybrid tilapia, based on differences in oxygen consumption rates, was more expensive in FW than in SW, and was cheapest in ISO. Kültz *et al.* (1992) also found that gill Na^+,K^+-ATPase activity was lowest in ISO compared to FW and SW in long-term (5 wk) acclimated Mozambique tilapia. These results are consistent with the theory that the energetic cost of osmoregulation is lowest in an isosmotic environment, where the ionic gradients between blood and water would be minimal. A recent study by Ron *et al.* (1995) found that when Mozambique tilapia were reared from yolksac fry for 20 months in FW or SW, the

oxygen consumption rate in SW tilapia was half of that measured in FW fish. Taken together with the present study, this suggests that the metabolic cost of acclimating to SW may be high in the short-term, but that in a fully-acclimated tilapia the energy requirements of a marine existence may be lower than in FW. It is also now clear that meaningful measurements of oxygen consumption rate in relation to salinity change require a careful consideration of the physiological history of the fish. For example, tilapia which have been reared entirely in SW cannot tolerate direct transfers into FW, whereas FW fish that have been acclimated to SW have no problem making the transition back to FW (E.G. Grau, Hawaii Institute of Marine Biology, personal communication). It is likely then, that the metabolic cost of SW adaptation in Mozambique tilapia depends to a considerable extent on its individual developmental experience with different osmotic challenges.

Plasma GH levels in tilapia increased after transfer from FW to SW, but ISO was not sufficient to trigger an increase in plasma GH. Similar increases in plasma GH levels during SW acclimation have been reported for Mozambique tilapia by Yada *et al.* (1994) and for salmonids by a number of studies (reviewed by Sakamoto *et al.* 1993). Borski *et al.* (1994) found that tilapia reared in SW for 7 months from the yolksac stage had more active GH cells in their pituitaries than did FW fish, even though plasma levels may not be elevated long-term (3-4 wk; Ayson *et al.* 1993). Work with salmonids suggests that the initial rise in plasma GH levels is followed by a concomitant increase in the metabolic clearance rate of GH (Sakamoto *et al.* 1990, 1993). The chronic elevation of GH cell activity in SW tilapia may help to explain the higher growth rates observed in SW tilapia compared to FW fish (Kuwaye *et al.* 1993; Ron *et al.* 1995). Plasma levels of the two PRLs in the present study declined after transfer from FW to SW; this is in agreement with previous observations in Mozambique tilapia transferred from FW to SW (Nicoll *et al.* 1981; Ayson *et al.* 1993; Yada *et al.* 1994). The reduction of plasma PRL levels

after SW transfer is related to its sodium-retaining action (Clarke 1973), which would be counterproductive to maintaining proper ionic balance in SW fish. The concentration of $tPRL_{177}$ was somewhat intermediate in the ISO treatment compared to FW and SW, and a similar progressive response to salinity was seen in total PRL levels by Nicoll *et al.* (1981). The higher plasma $tPRL_{188}$:$tPRL_{177}$ ratio observed in SW in this study suggests that the two prolactins may be differentially regulated during SW acclimation, as proposed earlier by Yoshikawa-Ebesu *et al.* (1995) using *in vitro* pituitary preparations of Mozambique tilapia.

Gill Na^+,K^+-ATPase activity of tilapia in SW increased 2.2-fold over the FW value after 4 d. A similar result was reported by Hwang *et al.* (1989), who found that it took gill Na^+,K^+-ATPase activity of Mozambique tilapia 2 d to increase significantly (1.3-fold) above the FW control after transfer to 20 ppt salinity. The timing of the decrease in plasma [Na^+] and [Cl^-] and the increase in gill Na^+,K^+-ATPase activity in the present study are consistent with the known role of this enzyme in salt secretion to maintain ionic balance in SW fish (Zadunaisky 1984). GH treatment has recently been found to increase gill Na^+,K^+-ATPase activity in Mozambique tilapia (Borski *et al.* 1994; Sakamoto *et al.* 1997; Shepherd *et al.* 1997) and the increase in both plasma GH levels and enzyme activity during SW acclimation in the present study lends further support to a possible osmoregulatory role for GH in Mozambique tilapia. In contrast, GH does not appear to increase the adaptability of a related species, the Nile tilapia, to brackish water (Auperin *et al.* 1995). Transfer to ISO did not have a significant effect on gill Na^+,K^+-ATPase activity after 4 d in this study. Dange (1985) showed that a salinity of at least 17 ppt was required to increase gill Na^+,K^+-ATPase activity of Mozambique tilapia 1 wk after transfer.

Plasma cortisol levels in FW and ISO were significantly elevated 1 d after transfer. This was probably a result of the stress associated with being transferred into the smaller treatment tanks.

Assem and Hanke (1981) found that plasma cortisol levels in Mozambique tilapia transferred to 27 ppt salinity increased significantly above the FW value at 2 h and returned to normal 6 to 72 h after transfer. In contrast, I found that plasma cortisol levels in the SW fish were significantly lower than the FW and ISO treatments 24 h after transfer. The lower plasma cortisol levels in SW fish after 1 d may have been related to an increase in the clearance rate of cortisol, rather than a decrease in cortisol secretion. Nichols and Weisbart (1985), for example, reported that plasma cortisol concentrations in Atlantic salmon (*Salmo salar*) were significantly lower after transfer to SW, and that the metabolic clearance rate of cortisol was significantly higher in SW compared to FW. Balm *et al.* (1995) have recently shown that cortisol production in SW tilapia was several-fold higher than in FW fish, even though plasma cortisol levels between the two groups were similar, again suggesting an increase in clearance rate. Redding *et al.* (1984a) have further demonstrated that gills of SW-adapted coho salmon (*Oncorhynchus kisutch*) take up and retain more cortisol than do gills of FW fish. Therefore, during the initial stages of the SW acclimation process in Mozambique tilapia, it is possible that an increased release of cortisol in the blood is soon followed by an enhanced uptake by osmoregulatory organs. Plasma glucose levels followed the same pattern as cortisol, being elevated in FW and ISO compared to SW. Assem and Hanke (1979b) also found glucose concentrations in Mozambique tilapia to increase in FW for 6 h due to transfer stress, but levels in SW were elevated above FW values for up to 24 h after transfer. As in cortisol, the lower glucose levels in SW in the present study may have reflected an elevated uptake by metabolizing cells, but a glucose turnover study would be necessary to verify this. Plasma protein levels and hematocrit values did not change with increasing salinity (data not shown), thus the lower plasma glucose and cortisol concentrations in SW observed in this study were unlikely to be due to plasma volume changes.

Following transfer from FW to SW, plasma [Na^+] and [Cl^-] peaked after the first day and declined to FW levels (about 145 mM) on day 4. Similar patterns for these ions were observed by Assem and Hanke (1979a) and Hwang *et al.* (1989) after direct transfer of Mozambique tilapia from FW to 27 and 20 ppt salinity, respectively. In the present study, plasma [Na^+] and [Cl^-] were slightly elevated in 12 ppt after 1 d, and although this salinity is generally considered to be isosmotic for tilapia (Farmer and Beamish 1969; Febry and Lutz 1987), [Na^+] and [Cl^-] in ISO were, in fact, higher than in the plasma of FW fish (Table 2). It is the presence of organic osmolytes, such as glucose and albumin, in the plasma that gives it the same osmotic pressure as 12 ppt salinity water. Plasma [K^+] did not change significantly after transfer to SW, which was also observed for Mozambique tilapia by Assem and Hanke (1979a). This is possibly due to the lower gradient for K^+ between the blood and SW compared to Na^+ and Cl^-. Alterations in plasma [K^+] can also be buffered by transfer to and from the large intracellular pool of K^+ and therefore may not pose a problem for regulation compared to Na^+ and Cl^- (McDonald and Milligan 1992).

In conclusion, the SW acclimation process in Mozambique tilapia involves several hormonal and osmoregulatory adjustments in order to re-establish ionic homeostasis. The present study indicates that these physiological changes represent a significant short-term energetic cost, elevating metabolic rate by about 20% after 4 d in SW. In contrast, acclimation to ISO appears not to require substantial physiological adjustment, and after acute exposure did not impose (or reduce) an energetic demand in Mozambique tilapia.

CHAPTER 3: Salinity Effects on Oxygen Consumption, Gill Na^+,K^+-ATPase and Ion Regulation in Juvenile Coho Salmon

INTRODUCTION

Salmonid fry reared in FW respond to an increase in salinity with an elevated rate of oxygen consumption (Nagibina 1983; Morgan and Iwama 1991). Isosmotic salinity does not significantly lower metabolic rate in salmonid fry, despite the notion that it would provide the lowest energy demand for osmoregulation. Although some of the increase in oxygen consumption in SW may be related to a higher cost for osmoregulation (Kirschner 1993, 1995), it is also likely that the metabolic response to salinity in juvenile salmonids is dependent to a large extent on developmental state, and that the lowest metabolic rate will be found in the environment that is natural for a particular species and life history stage (Morgan and Iwama 1991). It seems reasonable to propose, therefore, that smolts, which have undergone a preparatory adaptation for life in the sea (Folmar and Dickhoff 1980; Hoar 1988), might be expected to show a different metabolic response to long-term salinity acclimation than in fry. To test this assumption, oxygen consumption rates were measured in coho salmon smolts acclimated to FW, ISO and SW for 6 wk. Gill Na^+,K^+-ATPase activities are useful indicators of the relative energy requirements for ion transport, and were determined in the present study in an attempt to correlate changes in gill energetics with whole-animal oxygen consumption measurements in the three salinities.

MATERIALS AND METHODS

Fish

Coho salmon gametes were collected from mature adults returning to the Capilano River Hatchery (North Vancouver, B.C.) in November 1992, and were fertilized and incubated at The University of British Columbia (UBC) Aquaculture Unit. The juvenile fish were maintained in 800-L oval tanks continuously supplied with dechlorinated Vancouver City tap water (Na^+ = 1 mM, Cl^- = 1 mM, Ca^{2+} = 0.03 mM, seasonal temperature variation: 4-16°C) and the lighting was controlled to provide a simulated natural photoperiod. The fish were fed a diet of commercial salmon pellets (EWOS Canada). The coho were 10 months of age (mean length = 11.3 cm, mean mass = 19.8 g) when the experiment began in January 1994. The fish appeared silver in colour, and an initial 96 h SW challenge test indicated that they could tolerate the highest salinity tested (30 ppt, data not shown). Coho salmon juveniles typically smolt after spending one year in FW (Sandercock 1995).

Salinity Acclimation

At the start of the experiment, the fish were lightly anaesthetized in TMS (50 mg/L) buffered with $NaHCO_3$, and transferred randomly into three 200-L oval fibreglass tanks, at a density of 50 fish per tank. One tank was supplied with flowing FW, and two tanks were set up as saltwater recirculation systems. Stock seawater was obtained from the Vancouver Aquarium and added to the tanks to achieve test salinities of 10 ppt (ISO) and 28 ppt (SW). The fish in the ISO and SW treatments were gradually acclimated to the test salinities in a stepwise manner at a rate of 4-5 ppt per day. The water was continually pumped through a bio-filter (Eheim model 2213,

Germany) and UV-sterilizer (Aquafine, Valencia, California) to maintain water quality. Water in the ISO and SW tanks was also partially replaced at a rate of 25% per wk. Salinity, temperature, dissolved oxygen, pH and mortalities were recorded daily in each tank. Water temperatures were kept similar in each treatment tank (8 ± 0.5°C) using an immersion heater in the FW tank, and stainless steel cooling coils in the static saltwater tanks. Aeration was provided in each tank to maintain dissolved oxygen levels near saturation. Dissolved oxygen levels ranged from 9.2-11.9 mg/L, due to differences in oxygen solubility with increasing salt content. Water samples were collected to measure the ionic composition of each salinity treatment (Table 3).

Gill Tissue and Plasma Sampling

Ten fish per treatment were sampled after the acclimation period (day 7), and at 21 and 42 d after transfer to the treatment tanks. The fish were anaesthetized in buffered TMS (100 mg/L), killed by a blow to the head, and blood was collected from the caudal vessels into heparinized syringes. The blood samples were centrifuged (2000 *g* for 5 min) and the plasma was removed and stored at -75°C for later analyses of cortisol, glucose and ion concentrations. Immediately following blood collection, gill filaments were removed from the second gill arch on the left side of the fish, placed in 1 mL of ice-cold SEI buffer (0.3 M sucrose, 0.02 M Na_2EDTA, 0.1 M imidazole, pH 7.1), and stored at -75°C for measurement of Na^+,K^+-ATPase activity.

Table 3. Chemical composition of water samples collected from the coho salmon salinity treatment tanks.

	Treatment		
Variable	FW	ISO	SW
Salinity (ppt)	0	10	28
pH	5.8	7.6	7.8
Na^+ (mM)	<1	128	379
Cl^- (mM)	<1	155	488
K^+ (mM)	<1	3.2	9.0

Respirometry

After 6 wk in the salinity treatments (late February), oxygen consumption rates of the coho were measured in a Brett-type swimming respirometer (e.g., Brett 1964, Steffenson *et al.* 1984, Gerhke *et al.* 1990), modified for use with juvenile salmonids. A swimming respirometer design was chosen to control the swimming speed of the fish and reduce variations in oxygen consumption caused by spontaneous activity (Brett and Glass 1973). The total volume of the respirometer was 5.3 L, and the transparent swimming section was 21.0 cm long and 6.5 cm in diameter. Water flow in the respirometer was generated by a stainless steel centrifugal pump (Jabsco), powered by a 0.25 HP electric motor (Reliance DC-1, Reliance Electric Co., Cleveland, Ohio). Motor speed, and thus water velocity, was controlled by a variable speed controller. Velocities in the swimming section could be regulated up to 52 cm/s and were measured by a Signet flow transmitter (Signet Scientific Co., El Monte, California) installed upstream of the swimming section. The respirometer apparatus was oriented in a horizontal plane and supported in a 200-L reservoir, with the exception of the pump and motor which were externally mounted. Water supplied to the reservoir was directed into the respirometer through a valve assembly, which allowed the system to be operated in a flow-through (acclimation) or recirculation (measurement) mode. Water temperature in the reservoir was controlled by an attached temperature control unit (Lauda RM6 circulator, Germany), and temperature was monitored using a thermistor (Cole Parmer, Chicago, Illinois) mounted inside the respirometer.

Water oxygen partial pressure (P_{O_2}) in the respirometer was measured using a Radiometer oxygen electrode (E-5046) mounted in a thermostatted jacket (D-616) and connected to an oxygen meter (manufactured by the Max Planck Institute for Experimental Medicine, Göttingen,

Germany). The oxygen electrode was calibrated to zero with a sodium bisulphite solution and to saturation with FW equilibrated with air. A constant water flow from the respirometer was passed over the electrode, using a Piper peristaltic pump (Dungey Inc., Agincourt, Ontario) and gas impermeable tubing, before being returned into the respirometer. Signals from the thermistor, oxygen and flow meters were transferred to an IBM-compatible microcomputer using a 12-bit analog-to-digital converter (PC-LabCard 812, Advantech Co., Sunnyvale, California). Data acquisition was performed using the program Labtech Notebook version 7.1.1 (Laboratory Technologies Corp., Wilmington, Massachusetts).

Prior to each respirometry trial, individual fish from a salinity treatment were placed into the swimming section through a removable lid, and were allowed to acclimate in flow-through water for 24 h. The fish were not fed for 48 h prior to testing to ensure a postabsorptive digestive state (Beamish 1978). The respirometer was covered throughout acclimation and testing to shield the fish from visual disturbances. After acclimation, the swimming speed was set to 1 body length/s and the water flow to the respirometer was stopped. The subsequent decline in water P_{O_2} was then monitored for 90 min, with values recorded every 2 min. After a trial was completed, the fish was removed from the swimming chamber and weighed. The trials were conducted at approximately the same time each day to minimize diurnal variation in metabolism due to entrainment to a feeding schedule or photoperiod (Brett and Zala 1975). Water temperatures during the trials were kept similar to the holding tanks ($8.5 \pm 0.5^{\circ}C$). Background oxygen consumption was measured by running blanks (i.e., no fish) throughout the experimental period; no corrections were necessary.

Water P_{O_2} decreased at a constant rate and about 20-25% of the initial oxygen was consumed during each trial. Measured P_{O_2} values (mm Hg) were converted to oxygen content (mg/L) using

the conversion tables found in Colt (1984). Oxygen consumption rates were estimated using linear regression analysis and expressed as milligrams of oxygen per hour per kilogram of fish (i.e., mg O_2/kg/h).

Analytical Procedures

Plasma cortisol levels were determined using a commercially available [^{125}I] cortisol RIA kit (GammaCoat, Incstar Corporation, Stillwater, Minnesota). Plasma glucose levels were measured using the Ames™ Minilab (Iwama *et al.* 1995). Plasma [Na^+] and [K^+] were measured on an ion chromatograph (Shimadzu Model HIC-6A, Shimadzu Corporation, Kyoto, Japan). Plasma [Cl^-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer). Na^+,K^+-ATPase activity (μmoles of ADP/mg of protein/h) in crude gill homogenates was determined at 25°C in a temperature-controlled plate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA) according to McCormick (1993).

Statistical Analyses

Data are presented as means ± 1 SE. When ANOVA indicated significant differences, Student-Newman-Keuls multiple comparison test was used to identify significantly different treatment means ($P<0.05$).

RESULTS

Plasma Cortisol and Glucose Levels

Plasma cortisol levels in fish in the three salinities averaged about 40 ng/mL and were generally not different, with the exception of significantly lower values in FW and SW on day 21 (Fig. 12A). Similarly, plasma glucose levels did not vary with salinity (averaging about 4 mM), except for slightly lower values in SW fish on day 21 (Fig. 12B).

Plasma Ion Concentrations and Gill Na^+,K^+-ATPase Activity

Plasma [Na^+] and [Cl^-] in SW were significantly elevated over FW values after the acclimation period (day 7), but returned close to FW levels by day 42 (Fig. 13A,C). Plasma [Na^+] and [Cl^-] in ISO showed only slight disturbances during the acclimation period compared to FW values. Plasma [K^+] showed only slight increases in SW fish on day 21 and did not differ among the salinity treatments on day 42 (Fig. 13B). Gill Na^+,K^+-ATPase activity did not differ among the three salinities after the acclimation period (day 7), but were lowest in ISO, higher in FW and highest in SW on days 21 and 42 (Fig. 14).

Oxygen Consumption Rates

There were no significant differences in the oxygen consumption rates of coho after 6 wk in FW, ISO and SW (Fig. 15).

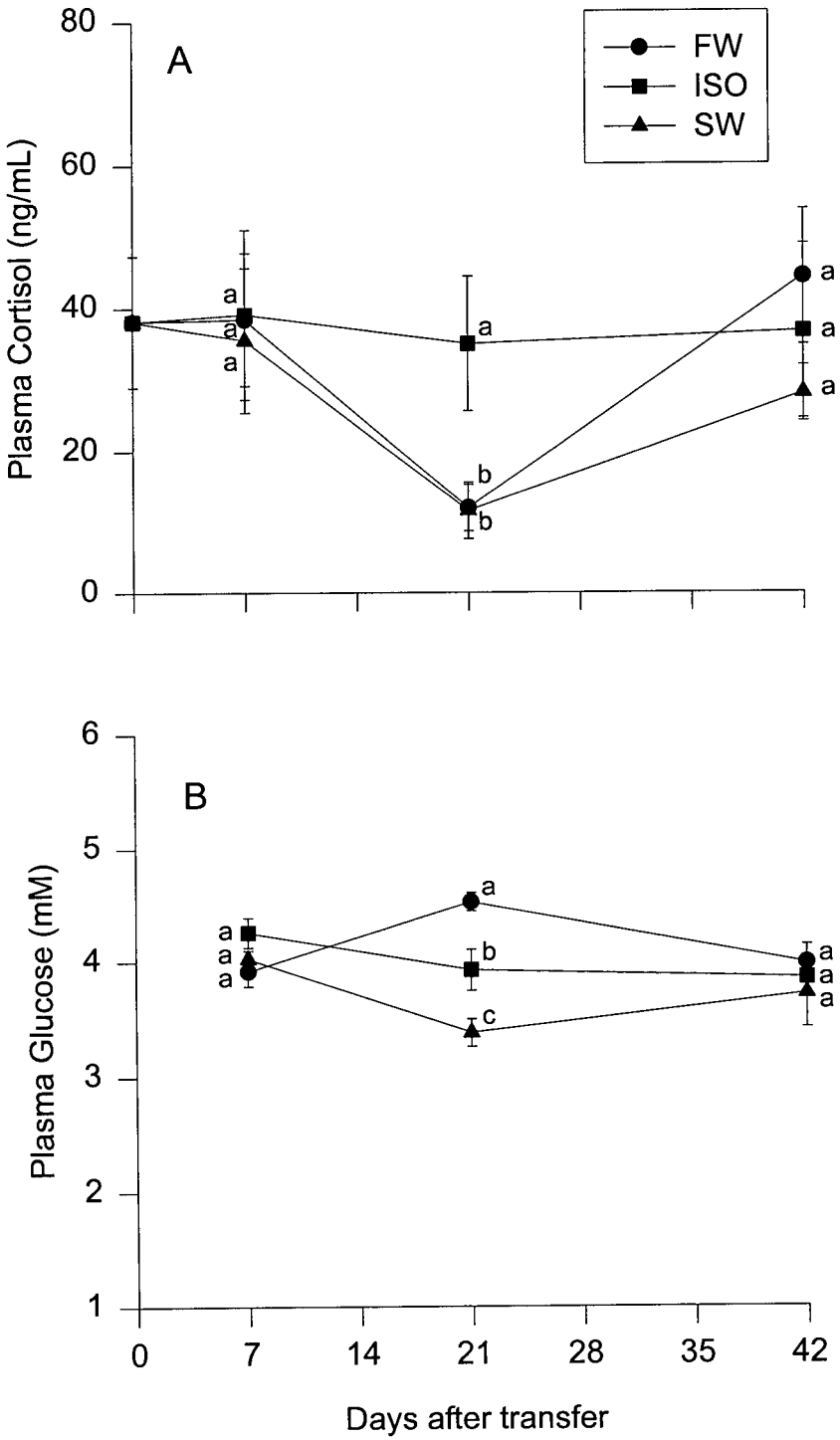

Fig. 12. Plasma cortisol and glucose levels in juvenile coho salmon reared in FW, ISO and SW. Data are presented as means ± SE (n=8-10). Means with different letters at each sampling period are significantly different ($P<0.05$, one-way ANOVA).

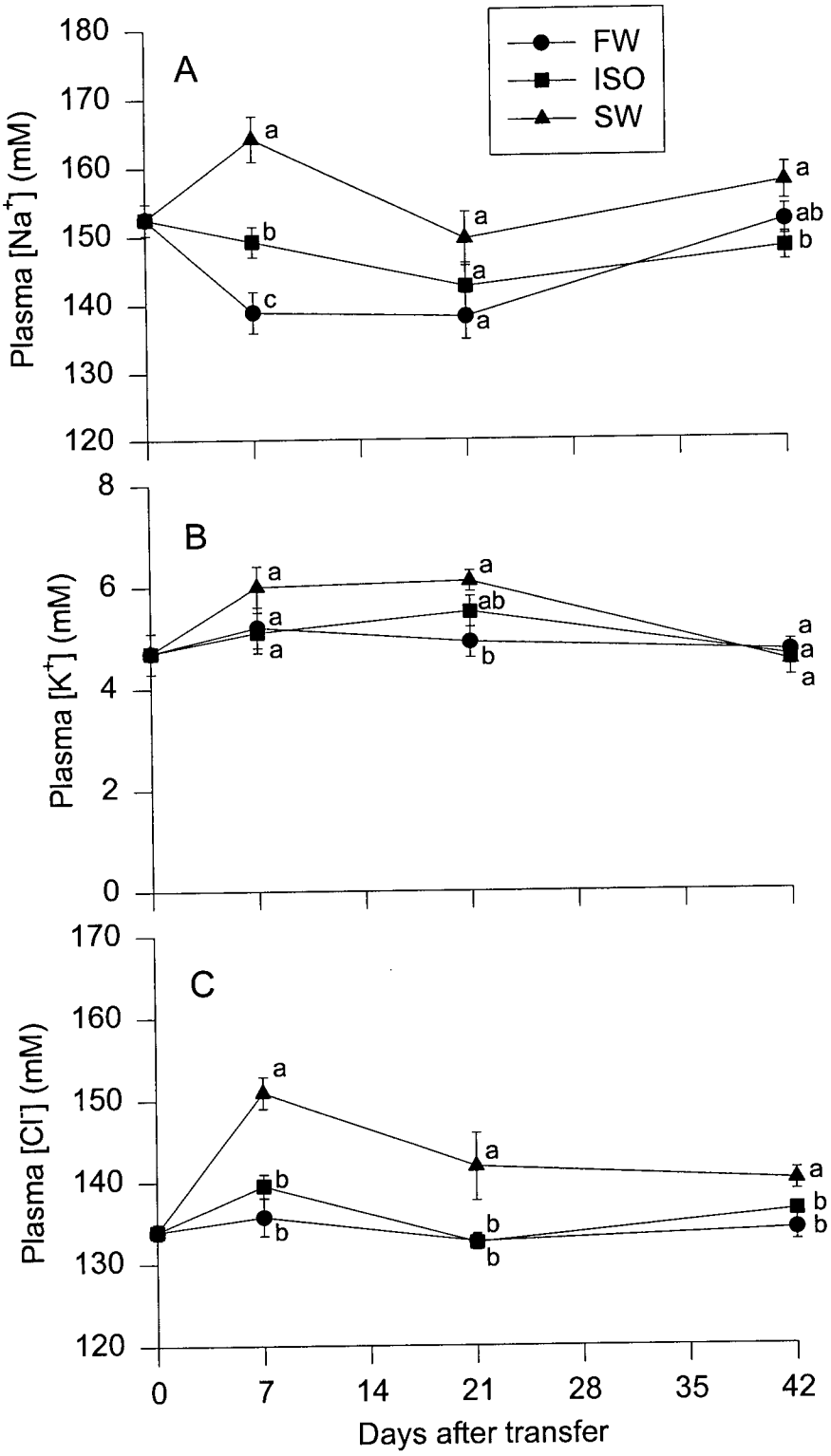

Fig. 13. Plasma [Na^+], [K^+] and [Cl^-] in juvenile coho salmon reared in FW, ISO and SW. Data are presented as means ± SE (n=8-10). Means with different letters at each sampling period are significantly different ($P<0.05$, one-way ANOVA).

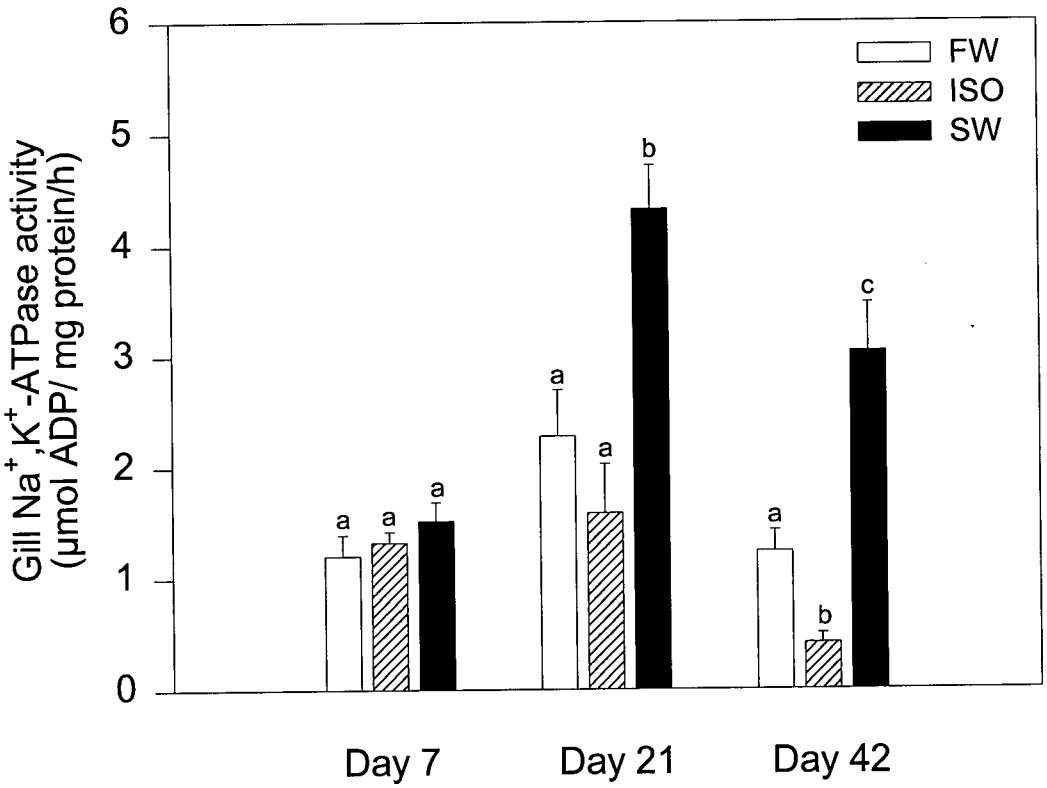

Fig. 14. Gill Na^+,K^+-ATPase activity of juvenile coho salmon reared in FW, ISO and SW. Data are presented as means ± SE (n=8). Means with different letters at each sampling period are significantly different ($P<0.05$, one-way ANOVA).

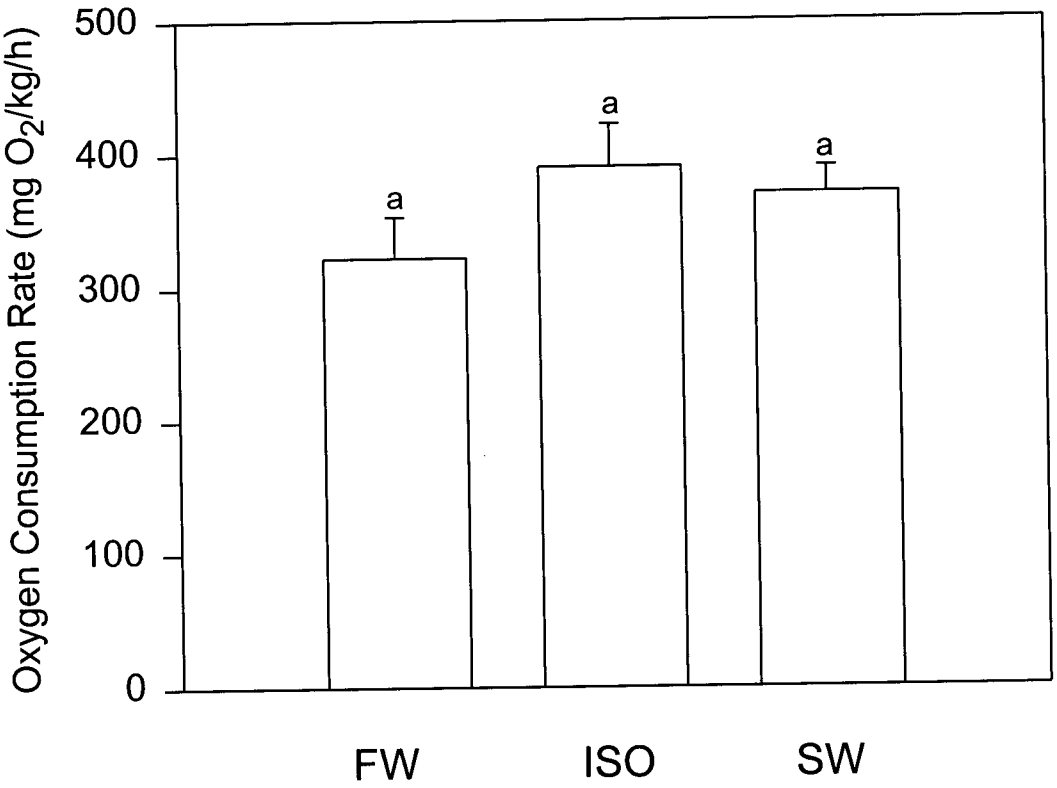

Fig. 15. Oxygen consumption rates of juvenile coho salmon after 6 wk in FW, ISO and SW. Data are presented as means ± SE (n=6). Means with the same letter are not significantly different (P>0.05).

DISCUSSION

Seawater adaptability in juvenile salmonids can be assessed by a number of physiological variables, including plasma ion and hormone levels after SW transfer (Franklin *et al.* 1992a). Following an initial elevation during the salinity acclimation period, plasma ion levels in the juvenile coho salmon returned to near-FW values by 3 wk. Plasma cortisol and glucose levels were also similar between the FW and SW fish. Based on these criteria, juvenile coho salmon in the present study were fully acclimated to the test salinities when oxygen consumption rates were determined (late February).

Oxygen consumption rates in coho salmon smolts acclimated for 6 wk to FW, ISO and SW were not significantly different. Similar results have been reported for yearling chinook (*O. tshawytscha*; Bullivant 1961) and coho salmon smolts (Zinichev *et al.* 1993), where rearing salinity did not affect the rate of oxygen consumption. These results are different from that reported for FW salmonid fry, which respond to an increase in salinity with an elevated rate of oxygen uptake (Nagibina 1983; Morgan and Iwama 1991). These data support the notion that the metabolic response to salinity in juvenile salmonids will depend to a large extent on developmental stage. Coho and chinook salmon fry, for example, are generally found in FW environments and do not yet possess the physiological mechanisms necessary for hypo-osmoregulation in SW. Long-term exposure to SW in these fish may result in a generalized stress response (i.e., chronic elevation of plasma cortisol) which may elevate metabolic rate (see Chapter 5). Salmon smolts, which have undergone the parr-smolt transformation to enhance their hypo-osmoregulatory ability in SW, show only a brief physiological disturbance following SW transfer (e.g., Franklin *et al.* 1992b; Young *et al.* 1995). These fish have undergone a preparatory

adaptation to live in estuaries (close to ISO) and the ocean, and therefore do not show large changes in whole-animal metabolic rates when acclimated to ISO or SW. It is interesting to note that the study by Rao (1968), where higher metabolic rates were observed in FW and SW than in ISO, was conducted on the landlocked form of rainbow trout, a salmonid which does not smolt or make migrations to the sea. This further emphasizes the importance of considering species and life stage when interpretating metabolic rate measurements in terms of osmoregulatory costs, even within the same family (in this case Salmonidae).

In the present study, gill Na^+,K^+-ATPase activity in juvenile coho salmon after 3 and 6 wk was lowest in ISO, higher in FW and highest in SW. Higher gill Na^+,K^+-ATPase activity following SW adaptation is generally characteristic of diadromous teleosts (Kirschner 1980; McCormick 1995). The lowest gill Na^+,K^+-ATPase activity found in ISO is consistent with the idea that less energy would be required to maintain ionic balance in an ISO environment, where the ionic gradients between extracellular fluid and the ambient water would be minimal. A similar result was found by Kültz *et al.* (1992) in long-term acclimated tilapia (*O. mossambicus*), who also suggested that the energy required for osmoregulation would be minimized in ISO conditions. Gill Na^+,K^+-ATPase activity of the euryhaline killifish (*Fundulus heteroclitus*) and pupfish (*Cyprinodon salinus*) was also lower at near ISO conditions (15-16 ppt) compared to either FW or SW (Towle *et al.* 1977; Stuenkel and Hillyard 1980). Nevertheless, there are a few studies in teleost fish where gill Na^+,K^+-ATPase activity was not lowest in ISO. McCormick *et al.* (1989), for example, found that gill Na^+,K^+-ATPase activity of Atlantic salmon smolts acclimated to FW, ISO and SW for 2 months was positively correlated with salinity, being 2.5- and 5-fold higher in ISO and SW than in FW, respectively. There is the possibility, therefore, that changes in gill Na^+,K^+-ATPase activity in response to ISO may be species-dependent.

While the gill Na^+,K^+-ATPase activity results in this study supported the theory that the cost of ion transport is minimized in an ISO environment, there was no correlation with whole-animal oxygen consumption rates, which did not differ between the salinity treatments in swimming fish. This suggests that the energy demands of the Na^+/K^+-pump in the gill may constitute a relatively small component of the overall metabolism in juvenile coho salmon. Taken together with results from previous salinity studies with salmonid fry (e.g., Morgan and Iwama 1991), this study also indicates that the metabolic response of juvenile salmonids to changes in salinity is largely dependent on life history stage (e.g., parr vs smolts), and that whole-animal oxygen consumption rates measured in different salinities may not necessarily reflect osmoregulatory costs.

CHAPTER 4: Metabolic Response of Cutthroat Trout to Acute Salinity Change

INTRODUCTION

The studies described in the first three chapters of this thesis have demonstrated a variety of metabolic responses to salinity change depending on species, life stage, and direction and duration of exposure. The purpose of this study was to provide information on the metabolic response to acute salinity change in the coastal cutthroat trout (*O. clarki clarki*), which was used for most of the remaining experiments in this thesis. The coastal cutthroat trout was chosen as it is an anadromous trout (sometimes called sea-run cutthroat) and makes numerous migrations between FW and SW throughout its life. Typically, sea-run cutthroat juveniles smolt at age two and move seaward in the spring. The fish generally remain inshore while in salt water, and return to their home rivers in the fall or winter to overwinter. Seaward migration occurs again in the spring following spawning (Trotter 1989; Behnke 1992). The sea-run cutthroat trout therefore provides a good model for salinity acclimation studies, as it can be found in either FW or SW depending on the time of year. Despite this, much less is known about the osmoregulation physiology of cutthroat trout compared to rainbow trout and both Pacific and Atlantic salmon. In the present study, cutthroat trout were transferred from FW to SW, and oxygen consumption rates and plasma cortisol and ion levels were measured after a 24 h period.

MATERIALS AND METHODS

Fish

Cutthroat trout, weighing 180-360 g, were kept in an 800-L oval fibreglass tank receiving flowing dechlorinated Vancouver City tap water (Na^+ = 1 mM, Cl^- = 1 mM, Ca^{2+} = 0.03 mM). The water temperature was increased from ambient (6°C) to 10°C over a 2 wk period using immersion heaters, and the fish were acclimated to this temperature for at least 1 wk before testing.

Respirometry

Oxygen consumption rates were measured in fish transferred from FW to FW and from FW to SW (25 ppt), using a flow-through respirometer which consisted of a plexiglass cylinder with plastic cones and baffle plates at each end (Cech 1990). The total volume of the respirometer was 2.75 liters and the chamber was 36 cm long and 8.5 cm in diameter. Inflowing FW or SW was fed by gravity from a head tank to the respirometer using vinyl tubing. Prior to each trial, individual fish from the FW holding tank were lightly anesthetized with TMS (50 mg/L), introduced to the chamber and allowed to acclimate in either flow-through FW or SW for 24 h. Flow rates were set to 0.5-1 L/min depending on fish size to achieve a difference in oxygen concentration between inflowing and outflowing water of about 0.5 mg/L. The respirometer was covered with black plastic throughout acclimation and testing to shield the fish from visual disturbances. After the 24 h acclimation period, water oxygen concentrations in the inflowing and outflowing water were measured using a dissolved oxygen meter which allowed for immediate correction of oxygen values in saline waters (Oxyguard® Mk III, Point Four Systems,

Port Moody, British Columbia). The oxygen electrode was calibrated in air to 101% saturation before use, according to meter's operating manual. The trials were conducted at approximately the same time each day (1030-1130h) to minimize diurnal variation in metabolism due to entrainment to a feeding schedule or photoperiod (Brett and Zala 1975). Water temperatures during the trials were kept similar to the FW holding tank (9.8 ± 0.5°C).

After each trial was completed, the fish was removed from the respirometer, anesthetized (TMS, 100 mg/L), weighed, killed by a blow to the head, and blood was collected from the caudal vessels using heparinized syringes. The blood samples were centrifuged (2000 *g* for 5 min) and the plasma was removed and frozen at -75°C for cortisol, glucose and ion analyses.

Oxygen consumption rates for each fish were calculated using the equation:

$$\dot{M}O_2 = (Co_2(I) - Co_2(O))\ (V_w) / (M)$$

where,

$\dot{M}O_2$ = oxygen consumption rate (mg O_2/kg/h)

$Co_2(I)$ = oxygen concentration in inflowing water (mg O_2/L)

$Co_2(O)$ = oxygen concentration in outflowing water (mg O_2/L)

V_w = water flow rate through the respirometer (L/h)

M = body mass (kg)

Analytical Procedures

Plasma cortisol levels were determined using a [^{125}I] cortisol RIA kit (Coat-a Count, Diagnostic Products Corporation, Los Angeles, California). Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Procedure 315). Plasma [Na^+] and [K^+] were measured on a flame photometer (Corning model 410), and plasma [Cl^-] was

determined by coulometric titration (Haake Buchler Instruments digital chloridometer). Data are presented as means ± 1 SE. Results between FW and SW fish were compared using unpaired t-tests ($P<0.05$).

RESULTS

Plasma Constituents

Plasma cortisol levels in SW fish sampled from the respirometer were about 4.4-fold higher than in the FW fish (Table 4). Plasma glucose levels did not differ significantly between the two groups. Plasma [Na^+] and [Cl^-] were significantly elevated in the SW fish, but there was no change in plasma [K^+] following the 24 h exposure to SW (Table 4).

Oxygen Consumption Rates

The average oxygen consumption rate in the SW-exposed fish was 161.2 ± 5.8 mg O_2/kg/h. This was significantly (~52%) higher than measured in the FW fish (106.1 ± 6.8 mg O_2/kg/h; Fig. 16).

DISCUSSION

The transfer of cutthroat trout from FW to SW in this study resulted in a significant elevation in oxygen consumption rate after 24 h. Similar increases in oxygen consumption rate over a 24 h period have been reported for rainbow trout transferred abruptly from FW to SW (Maxime *et al.* 1991; Seddiki *et al.* 1995). Madsen *et al.* (1996) observed a decrease in oxygen uptake for 48 h in an anadromous whitefish (*Coregonus lavaretus*) transferred from FW to SW, but attributed the

Table 4. Body mass, and plasma cortisol, glucose and ion levels in cutthroat trout in FW and 24 h after transfer to SW.

Variable	FW	SW
Body mass (g)	274.9 ± 22.1	249.2 ± 19.8
Cortisol (ng/mL)	53.7 ± 13.6	236.8 ± 40.4*
Glucose (mM)	5.4 ± 0.9	6.6 ± 0.8
Na^+ (mM)	135.0 ± 2.7	169.8 ± 2.5*
K^+ (mM)	3.1 ± 0.2	3.2 ± 0.2
Cl^- (mM)	126.0 ± 1.5	151.0 ± 3.6*

Values are shown as means ± SE (n=6).

An asterisk denotes a significant difference between FW and SW values ($P<0.05$, unpaired t-test).

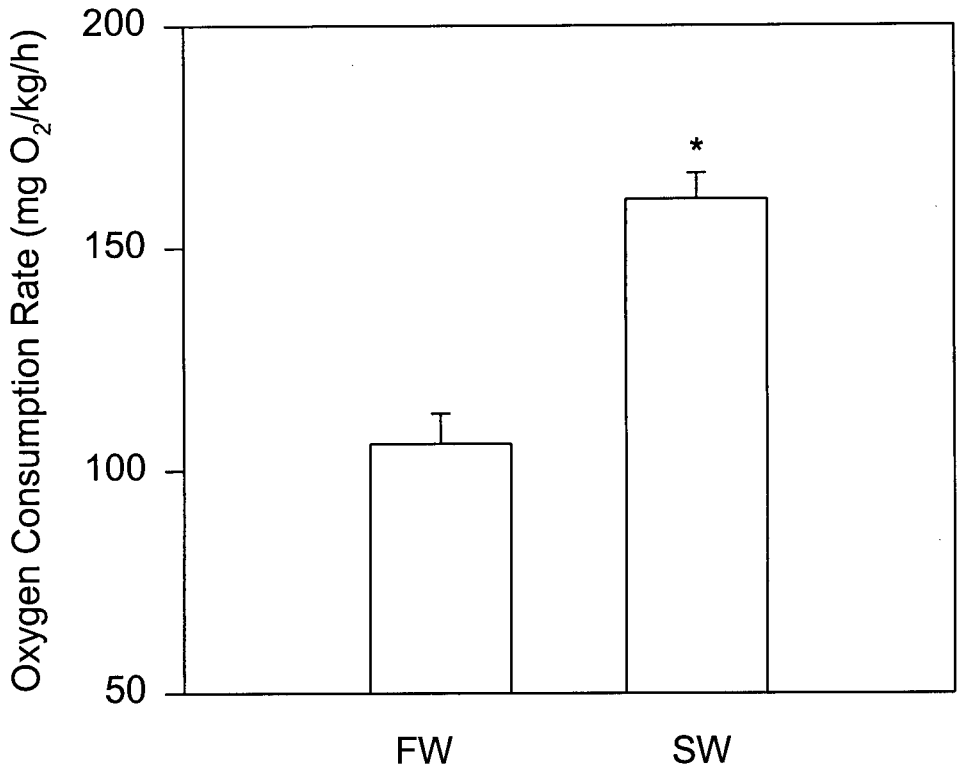

Fig. 16. Oxygen consumption rates of cutthroat trout in FW and 24 h after SW entry. Data are presented as means ± SE (n=6). The asterisk denotes a significant difference using a t-test ($P<0.05$).

decline in metabolic rate to reduced swimming activity in this naturally cruising species. In the present study, visual inspection of the trout in the respirometer at the end of each trial did not indicate any differences in swimming activity between FW and SW fish. Abrupt transfer of rainbow trout from FW to SW has also been accompanied by an increase in gill tissue dehydration (Leray *et al.* 1981), which results in a transient elevation in arterial P_{CO_2} and reduction in P_{O_2} due to the decrease in gas diffusing capacity of the gills (Bath and Eddy 1979; Larsen and Jensen 1983; Maxime *et al.* 1991; Seddiki *et al.* 1995). There is also a decrease in blood pH following SW exposure in rainbow trout, presumably caused by a metabolic acidosis (Leray *et al.* 1981; Seddiki *et al.* 1995). This drop in blood pH causes an increase in gill ventilation (Perry and Wood 1989), primarily from the increase in amplitude of opercular movements (Maxime *et al.* 1991; Seddiki *et al.* 1995). The energy cost of ventilation in fish can be quite substantial (>10% of resting metabolic rate; Milsom 1989), and it is possible that an increased ventilatory response to SW exposure in this study caused some of the elevated oxygen uptake, as was proposed by Maxime *et al.* (1991) and Seddiki *et al.* (1995) for rainbow trout. This is a matter of speculation, however, as ventilation frequency and amplitude were not measured in the present study.

Plasma cortisol levels in SW cutthroat trout in this study were several-fold higher than measured in FW fish. Although some of this increase may be related to the osmoregulatory role of cortisol in SW adaptation in salmonids (e.g., Madsen 1990a,b; Bisbal and Specker 1991), it is likely that much of it was due to a generalized stress response to a novel environment (Franklin *et al.* 1992a; Young *et al.* 1995). It is also possible that the elevated plasma cortisol levels in SW fish resulted in some of the increases in oxygen consumption rate, through its stimulatory effect on intermediary metabolism (e.g., Vijayan *et al.* 1994).

Plasma [Na^+] and [Cl^-] were significantly elevated in the trout following SW exposure, but the values were maintained at a level that could be considered normal for a marine salmonid (e.g., $Na^+ < 170$ mM; $Cl^- < 150$ mM; Holmes and Donaldson 1969). Yeoh *et al.* (1991) found that larger coastal cutthroat survived better following a 24 h SW challenge, and that 50 g was the critical size for the fish to successfully regulate blood ions. The fish in this study exceeded 180 g and showed good hypo-osmoregulatory ability, based on plasma ion levels following the 24 h SW exposure. Adult rainbow trout (>150 g) require a longer period (about 1 wk) to reestablish ionic balance in SW (Leray *et al.* 1981).

In summary, the results of this study show that increases in oxygen consumption during short-term SW exposure in cutthroat trout are associated with elevated plasma cortisol levels. The idea there may there may be a glucocorticoid effect on oxygen consumption during SW exposure, that is not directly related to osmoregulatory costs, was explored in more detail in the next section of the thesis.

SECTION II: Hormonal Effects Associated with Whole-Animal Responses to Salinity Change

The above studies with coho salmon and cutthroat trout suggest that oxygen consumption rates of salmonids transferred to different salinities may not be a direct reflection of the energy required for osmoregulation. The experiment described in this section (Chapter 5) was designed to explore the possibility that some of the increases in oxygen consumption observed in SW fish may be related to the hormonal changes that occur when FW salmonids are transferred to SW. Specifically, the dual role of cortisol as a glucocorticoid and mineralcorticoid was investigated. Cortisol implants were used to examine its effects on oxygen consumption and osmoregulatory variables in sea-run cutthroat trout parr.

CHAPTER 5: Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout parr [3]

INTRODUCTION

Considerable attention has been given in recent years to the physiological aspects of SW adaptation in salmonids. The transfer of juvenile salmonids from FW to SW is accompanied by a number of physiological changes, including increases in plasma cortisol levels, gill Na^+,K^+-ATPase activity and metabolism (see reviews by Folmar and Dickhoff 1980; McCormick and Saunders 1987; and Hoar 1988). For example, SW exposure of cutthroat trout (Chapter 4) resulted in an increase in plasma cortisol levels and oxygen consumption rate. In a recent study, I also found that oxygen consumption rates of rainbow and steelhead trout and fall chinook salmon fry were higher in SW compared to FW (Morgan and Iwama 1991). The data suggested that the energy demands for osmoregulation increased in SW, but I also speculated that some of the increase in oxygen consumption rates in the SW fish may have been related to the secondary effects of increased cortisol production on other metabolic processes (Morgan and Iwama 1991). That assertion was supported indirectly by published evidence that plasma cortisol levels are elevated in the long term (2-3 wk) in juvenile salmonids after entry into SW (Redding *et al.* 1984b; Young *et al.* 1989; Avella *et al.* 1990), and that elevated cortisol levels have been associated with higher rates of oxygen consumption in FW fish (Chan and Woo 1978; Barton and Schreck 1987). In order to better understand the effects that elevated plasma cortisol levels may have on oxygen consumption rates in juvenile salmonids, I examined the effects of

[3] A version of this chapter has been published in:
J.D. Morgan and G.K. Iwama. 1996. Fish Physiol. Biochem. 15: 385-394.

exogenous cortisol addition on oxygen consumption rates of coastal cutthroat trout parr. In the only other study of cortisol effects on oxygen consumption rate in fish (Chan and Woo 1978) Japanese eels (*Anguilla japonica*) were given a single intramuscular injection of cortisol, and oxygen consumption rates were found to increase over the following 24 h. Plasma cortisol levels were not measured and may have been outside of the physiological range due to the mode of administration.

The primary objective of the present study was to achieve elevated cortisol levels similar to those observed in cutthroat trout parr following SW entry, and observe their effects on the rate of oxygen consumption. In addition, changes in plasma levels of glucose and major ions, as well as branchial Na^+,K^+-ATPase activity were measured to examine the effects of plasma cortisol elevation on glucose metabolism and SW adaptability in cutthroat trout parr. Cortisol treatment has been shown to stimulate hypo-osmoregulatory ability in several species of salmonids (e.g., Madsen 1990a,b; Bisbal and Specker 1991), but comparable data are lacking for cutthroat trout.

MATERIALS AND METHODS

Fish

Sea-run cutthroat trout eggs were taken at the Chehalis River hatchery near Harrison, B.C. in March, 1993 and reared at the UBC Aquaculture Unit. The fish were maintained in 800-L tanks continuously supplied with dechlorinated Vancouver City tap water (Na^+ = 1 mM, Cl^- = 1 mM, Ca^{2+} = 0.03 mM, seasonal temperature variation: 4-16°C) and the lighting was controlled to provide a simulated natural photoperiod. The fish were fed a diet of commercial salmon pellets (EWOS Canada). The cutthroat trout were 16-month-old parr (mean length = 15.6 cm, mean

mass = 37.6 g) at the time of the experiment in the fall of 1994. Sea-run cutthroat trout juveniles typically spend 2 years in FW before migrating to the sea (Trotter 1989).

Determination of Dose for Cortisol Implants

To determine plasma cortisol levels after SW entry and establish a dosage for the cortisol implants, the fish were subjected to a 24 h seawater challenge (SWC) test (Blackburn and Clarke 1987). Ten fish were transferred into each of two 200-L tanks, one supplied with 15°C FW and one containing aerated 15°C SW (30 ppt, Instant Ocean®) with continuous filtration. After 24 h, the fish were anesthetized in neutralized TMS (100 mg/L), killed by a blow to the head, and blood was collected from the caudal vessels into heparinized syringes. Blood was centrifuged and the plasma removed and frozen at -75°C for cortisol and ion analyses. From this preliminary experiment (Table 5), it was decided to use a dose for the slow-releasing cortisol implants that would achieve a chronic plasma cortisol concentration of about 150-200 ng/mL (see below).

Cortisol Implantation

The cutthroat trout were injected with slow-releasing cortisol implants, using the method described by Specker *et al.* (1994). The cortisol (hydrocortisone, Sigma Chemical Co., St. Louis, Missouri) was dissolved in a 1:1 mixture (w/w) of coconut oil:vegetable oil heated to liquification (42°C), at a concentration of 10 mg cortisol/mL of oil. The injections were made while the mixture was still fluid (25°C) and the implant solidified in the ambient water temperatures. The injections were made using a 1 mL plastic syringe attached to a 21 gauge 1" needle, and the insertion point into the peritoneal cavity was on the ventral body surface, midway between the pelvic and pectoral fins.

Table 5. Length, mass, and plasma cortisol and ion concentrations of cutthroat trout parr following a 24 h seawater challenge test.

			Plasma			
Treatment	Length (cm)	Body mass (g)	Cortisol (ng/mL)	$[Na^+]$ (mM)	$[Cl^-]$ (mM)	$[K^+]$ (mM)
FW	16.3 (0.5)	45.6 (5.1)	59.2 (12.6)	156.5 (4.5)	130.3 (1.7)	4.0 (0.1)
SW	15.2 (0.2)	33.5 (1.5)	151.2 (12.6)*	184.4 (6.4)*	182.5 (2.7)*	4.0 (0.2)

Values are the mean (± SE) of 10 fish per treatment.

* indicates significant difference between treatments ($P<0.05$).

To achieve plasma cortisol concentrations similar to those seen in the SWC, fish were implanted with a dose of 50 μg cortisol/g body mass. The fish were lightly anesthetized (50 mg/L TMS) prior to injection and weighed to determine injection volume (50 μL per 10 g). The treatment groups were: untreated controls; sham-implants (oil only); and cortisol-implanted fish. The three groups were held in separate 200-L tanks at a stocking density of 40 fish per tank (8 g/L). The fish were fed once daily, and food was withheld 24 h prior to sampling or being placed into the respirometer. Water temperature and dissolved oxygen were also monitored daily in the tanks and ranged from 14.0-15.5°C and 9.3-10.2 mg/L (93-99% saturation), respectively, during the experiment.

Respirometry

Respirometry experiments were performed on fish from the three treatment groups in the following order: controls; cortisol implants; and sham implants. Measurements for the sham and cortisol implant groups began 1 and 3 d after injection, respectively. Oxygen consumption rates were measured in the modified Brett-type swimming respirometer described in Chapter 3.

Prior to each respirometry trial, individual fish from a treatment tank were immobilized with buffered TMS, measured for length, and then placed into the swimming section through a removable lid. The fish were acclimated to the respirometers in flow-through water for 24 h and were not fed for 48 h prior to testing to ensure a postabsorptive digestive state (Beamish 1978). The respirometer was covered throughout acclimation and testing to shield the fish from visual disturbances. After acclimation, the swimming speed was set to 1 body length/s and the water flow to the respirometer was stopped. The subsequent decline in water P_{O_2} was then monitored for 90 min, with values recorded every 5 min. After a trial was completed, the fish was removed

from the swimming chamber within 30 s, killed, weighed, and blood sampled for plasma cortisol and glucose as described above. Concurrent blood samples were also taken from fish maintained in the holding tank, to assess the effect of the respirometry trial on plasma cortisol and glucose levels. The fish were dissected to locate the implants, which formed a soft pellet and were generally found at the posterior end of the body cavity. The trials were conducted at approximately the same time each day to minimize diurnal variation in metabolism due to entrainment to a feeding schedule or photoperiod (Brett and Zala 1975). Water temperatures during the trials were kept similar to the holding tanks (15 ± 1°C). Background oxygen consumption was measured by running blanks (i.e., no fish) throughout the experimental period, and a correction was applied to all measurements with fish.

Water P_{O_2} decreased at a constant rate and about 25% of the initial oxygen was consumed during each trial. Measured P_{O_2} values (mm Hg) were converted to oxygen content (mg/L) using the conversion tables found in Colt (1984). Oxygen consumption rates were estimated using linear regression analysis and expressed as milligrams of oxygen per hour per kilogram of fish (i.e., mg O_2/kg/h).

Seawater Challenge

Ten days after implantation and 2 d after respirometry, control and cortisol-implanted trout were sampled in FW and then subjected to a SWC test, to determine the effect of exogenous cortisol addition on SW adaptability. Ten fish were transferred from the two treatment groups into a common 200-L tank containing aerated SW (30 ppt, 15°C). The two groups were identified by adipose fin-clips. After 24 h, the fish were anesthetized and sampled for plasma cortisol, glucose and ion determinations as described above. Immediately following blood collection, gill tissue

was removed from the first branchial arch on the left side, blotted dry, and stored in 1 mL of SEI buffer (0.3 M sucrose, 0.2 M Na_2EDTA, 0.1 M imidazole, pH 7.1) at -75°C for measurement of Na^+,K^+-ATPase activity. The criterion used for proper SW adaptation was the ability to maintain plasma $[Na^+]$ less than 170 mM, 1 d after transfer to SW (Clarke *et al.* 1981).

Analytical procedures

Plasma cortisol levels were determined using a $[^{125}I]$ cortisol RIA kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California). Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Procedure 315). Plasma $[Na^+]$ and $[K^+]$ were measured on an ion chromatograph (Shimadzu Model HIC-6A, Shimadzu Corporation, Kyoto, Japan). Plasma $[Cl^-]$ was determined by coulometric titration (Haake Buchler Instruments digital chloridometer). Gill Na^+,K^+-ATPase activity (μmoles of ADP/mg of protein/h) measurements were made at 25°C in a temperature-controlled plate reader (Thermomax, Molecular Devices Corp., Menlo Park, California), using the method described by McCormick (1993).

Statistical Analyses

Data are presented as means ± 1 SE. When ANOVA indicated significant differences, Student-Newman-Keuls multiple comparison test was used to identify significantly different treatment means ($P<0.05$). Comparisons between two sample means were accomplished using a Students t-test ($P<0.05$).

RESULTS

Oxygen Consumption Rates

The average oxygen consumption rate of cortisol-implanted fish was 477.6 ± 13.2 mg O_2/kg/h. This was significantly (53%) higher than measured for the control (304.6 ± 20.6 mg O_2/kg/h) and sham (319.8 ± 37.6 mg O_2/kg/h) groups (Fig. 17). Implantation with oil alone did not significantly affect the rate of oxygen consumption compared to the untreated controls.

Plasma Cortisol and Glucose Levels

During the respirometry trials, plasma cortisol levels in the cortisol-implanted fish averaged 257.0 ± 62.9 ng/mL, and were about five times higher than measured for the control and sham groups (46.4 ± 18.4 and 44.0 ± 23.5 ng/mL, respectively). There was no significant difference in plasma cortisol titres between the control and sham groups (Fig. 18A). Plasma cortisol levels in fish sampled from the respirometer were slightly higher than the concurrently sampled tank fish, but the differences were not statistically significant. Following the 24 h exposure to SW, plasma cortisol levels in the control group were significantly elevated, but there was no further increase in the cortisol-implanted group (Table 6).

Plasma glucose levels were significantly higher in the cortisol-implanted fish than in the control and sham groups, both in the respirometer and the holding tanks (Fig. 18B). In the SWC test, plasma glucose increased significantly in the control group, but showed no further increases in cortisol-implanted fish (Table 6).

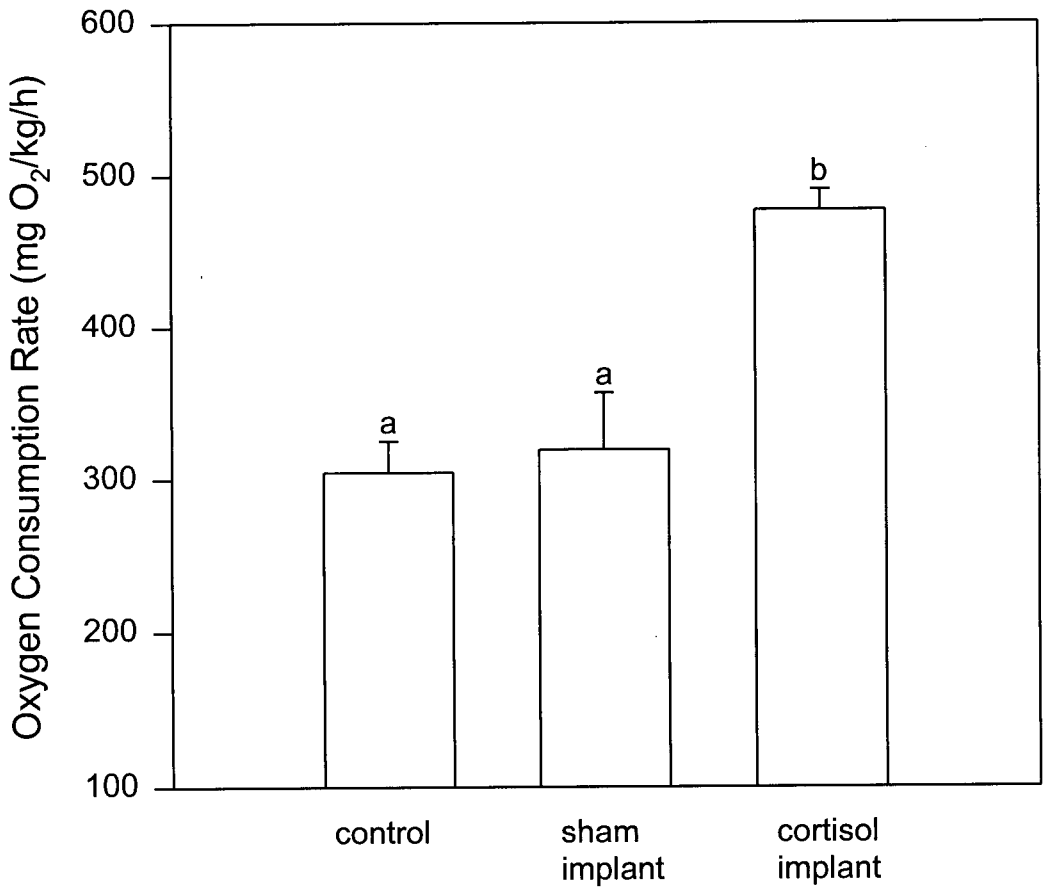

Fig. 17. The effect of cortisol treatment (50 μg/g) on oxygen consumption rate in cutthroat trout. Control fish were not treated, while sham fish received an oil implant only. Data are shown as means ± SE (n=6). Means with different letters are significantly different by Student-Newman-Keuls test ($P<0.05$).

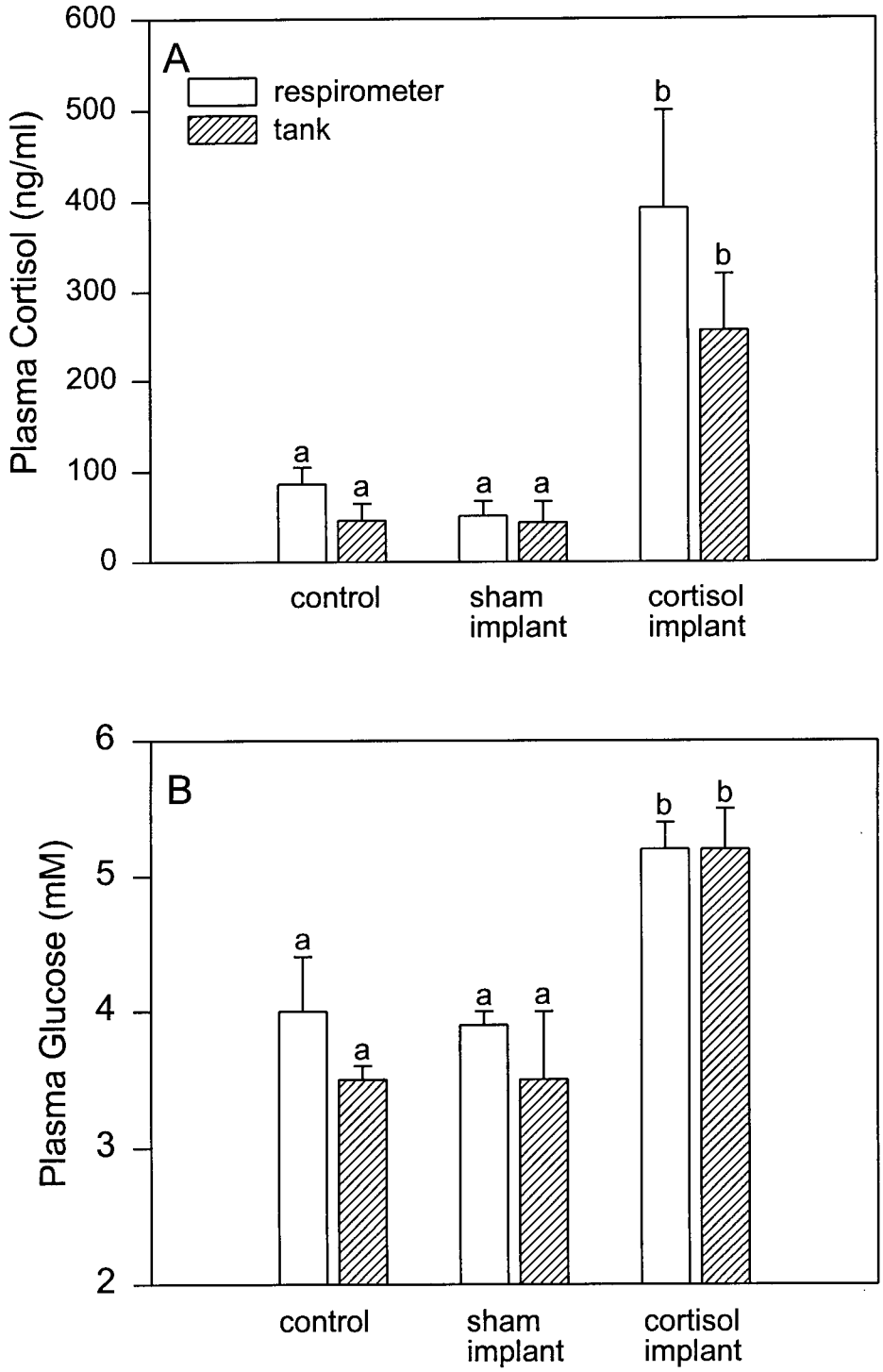

Fig. 18. Plasma cortisol and glucose concentrations in control, sham and cortisol-implanted (50 μg/g) cutthroat trout, sampled from the respirometer and holding tanks. Data are shown as means ± SE (n=6). Means for each group with different letters are significantly different by Student-Newman-Keuls test ($P<0.05$). There were no significant differences between respirometer and tank values within a treatment ($P>0.05$, t-test).

Table 6. Plasma cortisol, glucose and ion concentrations, and gill Na^+,K^+-ATPase activity in non-implanted and cortisol-implanted cutthroat trout parr, before and after a 24 h seawater challenge test.

	Plasma					
Treatment	Cortisol (ng/mL)	Glucose (mM)	$[Na^+]$ (mM)	$[Cl^-]$ (mM)	$[K^+]$ (mM)	Gill Na^+,K^+-ATPase (μmol ADP/mg prot./h)
FW-Control	29.6 (8.6)	3.4 (0.1)	141.8 (4.0)	135.8 (1.1)	3.0 (0.1)	0.75 (0.06)
FW-Cortisol	203.8 (23.7)*	5.1 (0.2)*	134.4 (4.3)	135.0 (2.5)	2.7 (0.1)	0.69 (0.06)
SW-Control	282.3 (49.1)†	5.7 (0.4)†	197.1 (4.0)†	178.8 (4.2)†	3.1 (0.1)	0.90 (0.09)
SW-Cortisol	249.8 (36.3)	5.9 (1.6)	169.5(7.2)*†	174.0 (3.1)†	3.0 (0.2)	1.18 (0.16)†

Values are the mean (± SE) of 10 fish per treatment.

* indicates significant difference between control and cortisol-implanted groups in fresh water or seawater ($P<0.05$).
† indicates significant difference between freshwater and seawater values within a treatment group ($P<0.05$).

Plasma Ion Concentrations

Cortisol treatment did not significantly affect plasma [Na^+], [Cl^-] or [K^+] of cutthroat trout parr in FW (Table 6). After the 24 h SWC, plasma [Na^+] and [Cl^-] were significantly elevated in both groups, but plasma [Na^+] in the cortisol-implanted fish was significantly lower than the control fish, and below the 170 mM threshold value (Clarke *et al.* 1981). There were no changes in plasma [K^+] in control or cortisol-implanted fish following exposure to SW.

Gill Na^+,K^+-ATPase

Cortisol treatment for 10 d did not affect gill Na^+,K^+-ATPase activity of cutthroat trout parr in FW (Table 6). After the 24 h SWC, only the cortisol implant group in SW showed a significant increase in enzyme activity compared to the cortisol implant group in FW.

DISCUSSION

Plasma cortisol levels in the cutthroat trout parr following the two 24 h SWC tests were quite high (means: 151 and 280 ng/mL) and resembled FW stenohaline fish rather than euryhaline fish during acclimation to SW. A number of studies with salmonids have shown that following an initial peak at 1-2 h, plasma cortisol levels return to basal levels within 6-12 h after SW entry (e.g., Strange and Schreck 1980; Nichols and Weisbart 1985; Franklin *et al.* 1992b). These cases generally involve smolt and post-smolt stages which can readily adapt to SW and may involve an increase in the clearance rate of cortisol in SW-adapted fish (Redding *et al.* 1984a; Nichols and Weisbart 1985; Patino *et al.* 1987; Balm *et al.* 1995). In contrast, the plasma cortisol values obtained for cutthroat trout in this study were more comparable with other salmonid parr stages

and non-salmonid species during exposure to saline environments. For example, Franklin *et al.* (1992b) found that 15-month-old sockeye salmon (*O. nerka*) smolts which adapted to SW had a marked (to 231 ng/mL) but brief (6 h) rise in plasma cortisol, while nine-month-old parr that did not fully acclimate had sustained increases in plasma cortisol levels (150-250 ng/mL). In another teleost, carp (*Cyprinus carpio*) showed elevated cortisol concentrations (120-190 ng/mL) for up to 3 d when transferred from FW to 15 ppt salinity (Abo Hegab and Hanke 1984). Goldfish also showed a chronic (9 d) elevation of plasma cortisol (100-250 ng/mL) when exposed to 9 ppt salinity (Singley and Chavin 1975). These cyprinid species are considered to be FW, stenohaline fish which do not normally encounter SW, and the increase in cortisol can be interpretated as being related to a stress response rather than having any osmoregulatory function (Strange and Schreck 1980). In fact, the plasma cortisol values in the SWC tests were very similar to those associated with acute handling stress in juvenile salmonids (see Barton and Iwama 1991). The increase in plasma glucose levels following SW exposure is also consistent with the concept of a stress response, and has been recorded previously for rainbow trout (Abo Hegab and Hanke 1986; Madsen 1990a). It is apparent then, that the cutthroat trout parr used in this study were stressed by the abrupt exposure to SW, and that the measured cortisol levels reflect a generalized stress response in addition to any osmoregulatory role.

The cortisol implant procedure employed in the present study proved effective in producing an elevation in plasma cortisol titres similar to those experienced by the cutthroat trout parr soon after SW entry. This increase in plasma cortisol concentration with the cortisol implant in FW fish resulted in increased plasma glucose levels and oxygen consumption rates. The increase in plasma glucose levels observed in the cortisol-implanted fish is similar to results obtained in other studies (Chan and Woo 1978; Leach and Taylor 1982; Vijayan and Leatherland 1989) and

probably resulted from a stimulatory effect of cortisol on gluconeogenesis (Vijayan *et al.* 1994). In this study, the SWC test did not result in any further increases in plasma cortisol or glucose levels in cortisol-implanted fish. A similar result was obtained by Redding *et al.* (1984b) using coho salmon, and suggests that there may be an upper limit to the glucocorticoid response of juvenile salmonids to SW exposure.

The increases in oxygen consumption rate following cortisol implantation corroborate the findings of Chan and Woo (1978) using Japanese eels, and support the hypothesis that at least some of the increases in oxygen consumption rate in juvenile salmonid parr in SW may be caused by the glucocorticoid effects of cortisol, in addition to energy required to maintain ionic and osmotic homeostasis (e.g., Morgan and Iwama 1991). As mentioned above, SW is a novel environment for life stages which are not pre-adapted for a marine existence, and elicits a generalized stress response. The subsequent increase in cortisol production would stimulate several aspects of intermediary energy metabolism and result in an elevated rate of oxygen uptake. This type of response might be expected for life stages such as salmonid fry and parr (e.g., Morgan and Iwama 1991; Franklin *et al.* 1992b), salmonid smolts which have been held beyond the normal smolting 'window' prior to SW transfer and undergo parr-reversion (e.g., Folmar *et al.* 1982; Avella et al. 1990), and other stenohaline FW species (e.g., Toepfer and Barton 1992). The metabolic response of these fish to SW undoubtedly includes both stress and osmoregulation components, but the relative energetic demands of these processes cannot be discerned from whole-animal oxygen consumption measurements and requires further study. The metabolic cost of acute physical stress in juvenile salmonids has been described by Barton and Schreck (1987), who demonstrated a linear association between oxygen consumption rates and plasma cortisol levels in stressed juvenile steelhead trout kept in FW. In that study, a five fold

increase in plasma cortisol levels was associated with a 70% increase in oxygen consumption rate; a response similar to that observed in the present study.

Given the apparent relationship between cortisol and oxygen consumption rate in fish presented here, it is also reasonable to propose that oxygen consumption rates would be elevated during the parr-smolt transformation in salmonids when there is a large transient increase in plasma cortisol levels (Specker and Schreck 1982; Hoar 1988). Resting oxygen consumption rates of Atlantic salmon smolts have been shown to be higher than that reported for parr, when corrected for differences in body size (Higgins 1985; Maxime *et al.* 1989).

Although cortisol plays an important role during SW acclimation in salmonids, it is unlikely that cortisol acts exclusively in causing an increase in oxygen consumption rate in SW. There are other osmoregulatory hormones which show increases following SW entry and also have metabolic actions, most notably growth hormone (Sakamoto *et al.* 1993) and thyroid hormones (Dickhoff and Sullivan 1987), which are thought to interact with cortisol. Seddiki *et al.* (1995) have recently reported an increase in oxygen consumption in rainbow trout treated with trout recombinant growth hormone, both in FW and SW.

Gill Na^+,K^+-ATPase activity of untreated cutthroat trout parr in FW was similar to levels found for juvenile Atlantic salmon by McCormick (1993) in the fall, when activity of this enzyme is at its lowest level. Cortisol treatment did not significantly affect plasma ion concentrations or gill Na^+,K^+-ATPase activity in FW after 10 d, contrary to the findings of Richman and Zaugg (1987), Madsen (1990a,b), and Bisbal and Specker (1991), but in agreement with Redding *et al.* (1984b), Langdon *et al.* (1984), and Eib and Hossner (1985). These differences among various studies may be due to interspecific differences, or changes in responsiveness of the gill tissue to cortisol treatment related to life history stage and season.

McCormick *et al.* (1991), for example, found that coho salmon parr in the fall were not responsive to cortisol treatment in increasing gill Na^+,K^+-ATPase activity, compared to smolts in the following spring. Recent evidence has also indicated that cortisol can stimulate gill H^+-ATPase activity, which is linked to Na^+ uptake in FW fish (Lin and Randall 1993). If significant, this would result in an increase in plasma $[Na^+]$ following cortisol treatment in FW, rather than a decrease normally associated with increased gill Na^+,K^+-ATPase activity (Madsen 1990a). H^+-ATPase activity was not measured in the present study, but as mentioned above, cortisol did not significantly change plasma ion concentrations in FW.

Plasma $[Na^+]$ and $[Cl^-]$ of untreated fish were significantly elevated in both SWC tests, indicating that the cutthroat parr were not fully acclimated to SW. Similar plasma $[Na^+]$ values following 24 h SWC tests were reported for coastal cutthroat trout by Yeoh *et al.* (1991). Plasma $[K^+]$ was unaffected by entry into SW, which was also observed for yearling coho salmon by Avella *et al.* (1990). The electrochemical gradient for K^+ between the blood and SW is much less than for Na^+ and Cl^- and does not appear to pose a problem for regulation (Evans 1993). Cortisol treatment resulted in lower plasma $[Na^+]$ following SW exposure compared to untreated controls, similar to the findings of Madsen (1990a,b). This is consistent with the known stimulatory effects of cortisol on the mechanisms for branchial ion excretion (McCormick and Bern 1989). The increase in plasma $[Cl^-]$ after SW entry was not ameliorated by cortisol treatment. The reason for this is not clear, as the transport of Na^+ and Cl^- from the chloride cells into the external medium are thought to be linked to maintain electroneutrality (Zadunaisky 1984). Following the 24 h SWC test, gill Na^+,K^+-ATPase activity showed a significant increase only in the cortisol-implanted fish. The relatively short exposure time may have prevented detection of this response,

as increases in gill Na^+,K^+-ATPase associated with SW entry usually occur after a delay of several days (Folmar and Dickhoff 1980).

In summary, the present study demonstrates that oxygen consumption rates in juvenile salmonids may be elevated by cortisol concentrations similar to those found in pre-smolts following SW entry. The increases in cortisol which occur after SW transfer may be related to a stress response in addition to any mineralocorticoid role. The metabolic cost of the novel SW environment to the animal may be substantial, however its impact on the energy demands for osmoregulation are not yet clear. It is apparent that studies which attempt to quantify the energetic cost of osmoregulation based on comparisons of oxygen consumption rates in salmonids acutely exposed to different salinities may be overestimating the true cost of this homeostatic process. Lastly, cortisol treatment did not significantly stimulate gill Na^+,K^+-ATPase activity of cutthroat trout parr in FW, but it did appear to improve plasma Na^+ regulation following an acute (24 h) exposure to SW.

SECTION III: Detailed Components of Ion Transport-Related Costs in Osmoregulatory Tissues

The study in Chapter 5 illustrated that metabolic processes other than those usually associated with osmoregulation (e.g., stress) can affect oxygen consumption rates of salmonids transferred from FW to SW. In an attempt to separate the energy costs of NaCl transport from other whole-animal metabolic responses to salinity change, the experiments in this section were conducted using isolated osmoregulatory tissues. Isolated preparations have the advantage of separating the influence that stressors and other factors may have on whole-animal oxygen consumption measurements, and they also allow the use of inhibitors (which would be toxic to the intact animal) to identify specific ion transport-related costs.

In Chapter 6, oxygen consumption and Na^+,K^+-ATPase activity was reported for excised rectal gland and gill tissue of the spiny dogfish, using ouabain to estimate the portion of tissue respiration required by the Na^+/K^+-pump in those osmoregulatory organs. A similar approach to the dogfish study was used on excised gill tissue from FW-adapted cutthroat trout, to assess the oxygen cost of NaCl uptake in the FW trout gill (Chapter 7). In that experiment, ouabain was used to inhibit the Na^+/K^+-pump, whereas bafilomycin A_1 was used to inhibit H^+-pump activity in the gill tissue. Finally, to compare gill energetics in FW and SW fish, an isolated, perfused gill arch preparation was used to measure oxygen consumption in intact gill arches from FW- and SW-adapted cutthroat trout (Chapter 8).

CHAPTER 6: Oxygen Consumption and Na^+,K^+-ATPase Activity of Rectal Gland and Gill Tissue in the Spiny Dogfish[4]

INTRODUCTION

Marine elasmobranchs (i.e., sharks, skates and rays) maintain the salt (NaCl) concentration of their blood at about one-half that of SW, but are slightly hyperosmotic to SW owing to the presence of two organic solutes, TMAO and urea (Shuttleworth 1988). They are faced, therefore, with a passive influx of both water and ions, which must be eliminated to maintain osmotic and ionic homeostasis. The kidney of elasmobranchs is well developed and the osmotic influx of water is balanced by a glomerular filtration rate that is similar to that of freshwater teleosts (Evans 1993). Since the first description of its function by Burger and Hess (1960), the role of the rectal gland in the secretion of excess salt in sharks has been well documented (see reviews by Shuttleworth 1988; Evans 1993). The rectal gland secretes a plasma isosmotic solution composed almost entirely of NaCl. The current model for the mechanism of NaCl secretion by the rectal gland was described by Silva *et al.* (1977) and involves the active transport of these ions driven by the Na^+,K^+-ATPase enzyme (for more details see Greger *et al.* 1986). The role of the elasmobranch gill in NaCl secretion is less certain. Although the gills are a major site for the passive influx of ions (Bentley *et al.* 1976), there is no direct evidence to support a role for active ion efflux (Shuttleworth 1988). The gill epithelium of elasmobranchs does possess mitochondrion-rich cells similar to the chloride cells of teleosts (Laurent 1982), but the specific activity of branchial Na^+,K^+-ATPase in elasmobranchs is much lower than that of the rectal gland

[4]A version of this chapter has been published in:
J.D. Morgan, J.M. Wilson and G.K. Iwama. 1997. Can. J. Zool. 75: 820-825.

or the gills of marine teleosts (Jampol and Epstein 1970). Nevertheless, a number of studies have shown that removal of the rectal gland does not impair the salt balance of sharks (e.g., Burger 1965; Chan *et al.* 1967; Wilson *et al.* 1996), which suggests that elasmobranchs may indeed secrete NaCl across the gill epithelium, as is the case in marine teleosts.

There has been considerable interest in quantifying the metabolic cost of ion transport processes in the osmoregulatory organs of teleost fish, but little attention has been given to elasmobranchs. The energy cost of NaCl transport by the rectal gland has recently been estimated on a theoretical basis by Kirschner (1993). Energy consumption was calculated from the amount of ATP required by the Na^+/K^+-pump to move Na^+ out of the cell, measured secretion rates of Na^+ from the rectal gland, and the known ratio of ATP to O_2 consumption. Using this method, Kirschner (1993) calculated the cost of NaCl secretion in the rectal gland of the dogfish *Scyliorhynus canicula*, and the skate *Raja erinacea*, to be about 0.1% and 0.4% of standard metabolism, respectively. No estimates were provided for the gills because of the lack of information on ion secretion rates for the elasmobranch gill as mentioned above.

An alternative approach to estimating the energy cost of NaCl transport in osmoregulatory organs is to measure the rate of oxygen uptake related to ion transport processes. Ouabain is known to specifically inhibit Na^+,K^+-ATPase activity, and this drug can therefore be used on isolated preparations to estimate the ouabain-sensitive (or Na^+/K^+ pump-dependent) portion of oxygen consumption (Stagg and Shuttleworth 1982a). I used this experimental approach to assess the relative costs of ion transport in rectal gland and gill tissue from the spiny dogfish, *Squalus acanthias*. The cost of NaCl secretion in these tissues was also calculated on a total mass basis, to provide an estimate of the metabolic contribution of the rectal gland and gills to the energy budget of the whole animal.

MATERIALS AND METHODS

Fish

Spiny dogfish were obtained by angling or trawling in Barkley Sound, British Columbia, Canada, in August 1995. The fish were held at the Bamfield Marine Station in a 3000-L circular fibreglass tank supplied with running SW (temp = 10.5°C, salinity = 31 ppt) and were used within 3 d of capture.

Experimental Protocol

Each dogfish was anesthetized with TMS (200 mg/L), weighed and blood was collected from the caudal vessels using a heparinized syringe. The blood was centrifuged at 2000 *g* for 5 min and the separated plasma was frozen at -75°C for later analyses of glucose, protein, ion, and urea concentrations. The fish were then killed by pithing the brain with a scalpel and the second gill arch on the left side of the fish was removed. The primary lamellae were scraped free from the arch and placed in ice-cold dogfish saline for use after the rectal gland trial was completed. The composition of the dogfish saline (in mM) was as follows: Na, 267; Cl, 276; K, 6; Ca, 5; Mg, 3; bicarbonate, 6; phosphate, 1; sulphate, 3; glucose, 5; urea, 350; TMAO, 70; pH 7.3. The rectal gland was removed and weighed, and thin slices (approx. 1 mm thick) were cut on a chilled aluminum plate using a scalpel. Oxygen consumption of the tissues was measured using a Strathkelvin Model 781 oxygen meter and microelectrode (Strathkelvin Instruments, Glasgow, Scotland). The microelectrode was inserted into a glass respiration chamber that was thermostatted to 10.5°C with running SW. Appropriate amounts (20 to 40 mg) of tissue were placed in 1.8 mL of air-saturated dogfish saline and allowed to acclimate for 5 min prior to

testing. The decline in water oxygen tension (to the nearest 0.1 mm Hg) was then monitored for 10 min, with values recorded every 2 min. After the control trial was completed, fresh dogfish saline containing ouabain (0.5 mM) was added to the respiration chamber and the oxygen consumption trial was repeated. Gentle stirring was provided by a magnetic stir bar to facilitate gas diffusion, and the tissue sat on a raised stainless steel mesh platform to prevent contact with the stir bar (Fig. 19). Preliminary experiments showed that tissue respiration remained constant for at least 1 h (data not shown), and the addition of ouabain caused a rapid decrease in oxygen consumption that was stable throughout the test period (Fig. 20). At the end of each experiment, the tissue was removed, blotted dry on tissue paper, and weighed to the nearest 1 mg. Oxygen consumption measurements were completed within 30 min and 1 h of dissection for rectal gland and gill tissue, respectively. Final water oxygen tensions in the respiration chamber were maintained above 110 mm Hg, and blank trials without tissue were also run to correct for electrode oxygen consumption. Oxygen consumption rates were estimated using linear regression analysis and expressed as μmol O_2/g wet wt/h.

Samples of freshly dissected gill and rectal gland tissue were placed in 1 mL of ice-cold SEI buffer (150 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.3) and stored at -75°C for measurement of Na^+,K^+-ATPase activity.

Analytical Procedures

Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Chemical Co., St. Louis, Missouri), while plasma protein levels were measured using the bicinchoninic acid procedure (Smith *et al.* 1985). Plasma urea concentrations were measured by the diacetyl monoxime method (Sigma), following dilution to the assay range

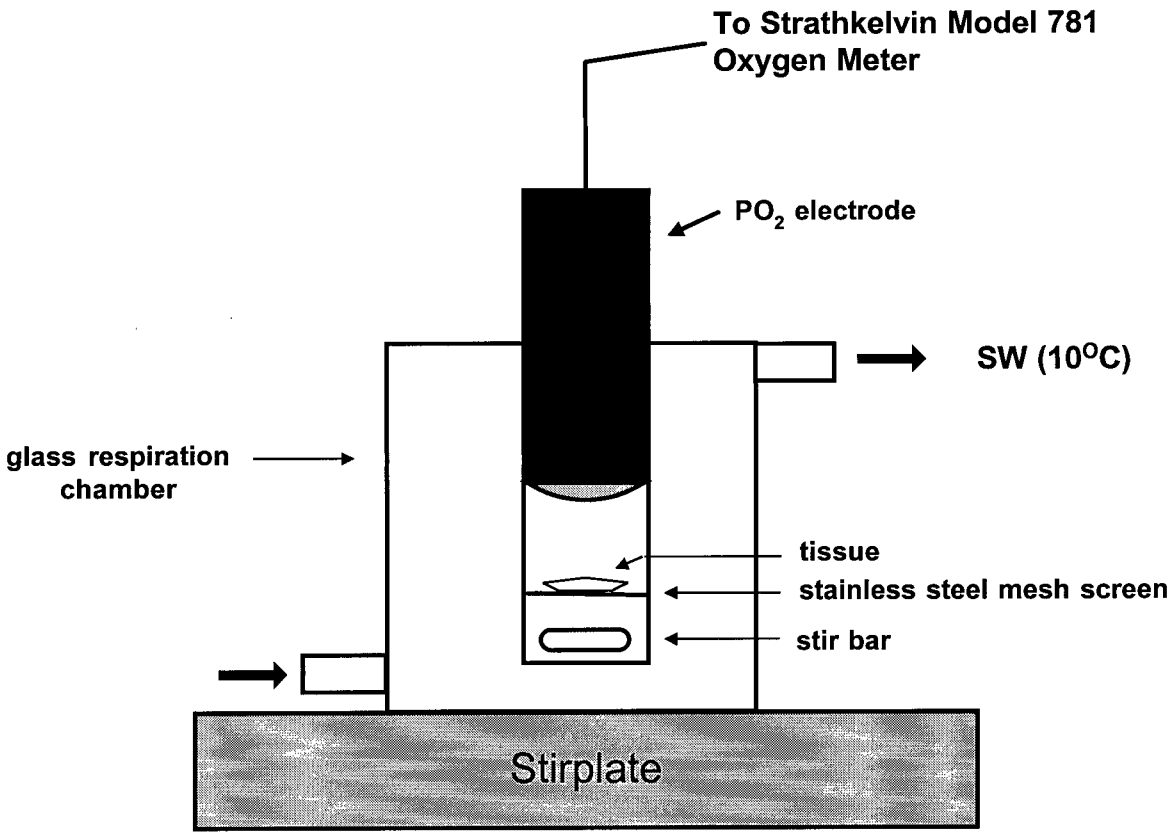

Fig. 19. Schematic diagram of the respiration chamber (~2 mL volume) used to determine oxygen consumption rates in rectal gland and gill tissue of the spiny dogfish. Not to scale.

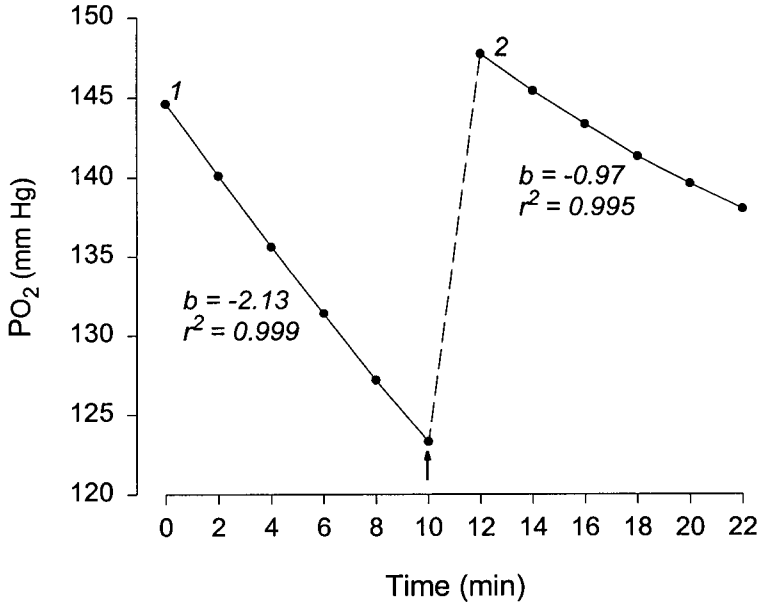

Fig. 20. A representative example showing the time course of oxygen consumption in rectal gland tissue of the spiny dogfish. Line 1 shows the control trace with dogfish saline in the respiration chamber. Line 2 shows the treatment trace after the addition of fresh saline containing 0.5 mM ouabain. The arrow indicates the time when ouabain was added. Results of linear regression analyses are given in the slopes (*b*, mm Hg/min) and r^2 values. See the text for details.

with distilled water. Plasma osmolality was determined using a freezing point depression osmometer (Osmette Precision Osmometer, Precision Systems Inc., Sudbury, Massachusetts). Plasma [Na^+] and [K^+] were measured using an ion chromatograph (Shimadzu Model HIC-6A, Shimadzu Corporation, Kyoto, Japan). Plasma [Cl^-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer), whereas plasma [Ca^{2+}] and [Mg^{2+}] were measured colorimetrically (Sigma). Na^+,K^+-ATPase activity (μmoles of ADP/mg of protein/h) in rectal gland and gill tissue homogenates was determined at 25°C in a temperature-controlled plate reader (Thermomax, Molecular Devices Corp., Menlo Park, California), according to McCormick (1993).

Statistical Analyses

Data are presented as means ± 1 SE. Oxygen consumption results were analyzed using a two-way ANOVA, and significant treatment means were identified using Student-Newman-Keuls multiple comparison test ($P<0.05$). Na^+,K^+-ATPase activities were compared between rectal gland and gill tissues using an unpaired t-test ($P<0.05$).

RESULTS

Size Characteristics and Plasma Constituents

Table 7 shows the size characteristics and plasma concentration of ions, urea, glucose and protein in the dogfish used in this study. The dogfish averaged 3.0 ± 0.3 kg body mass, and the rectal gland and gills comprised 0.05% and 0.68% of total body mass, respectively. The values for the plasma constituents were very similar to the dogfish saline used in the oxygen consumption experiments, and although [Na^+] and [Cl^-] were about 55% of the SW values, the plasma was slightly hyperosmotic to SW owing to high levels of urea.

Oxygen Consumption and Na^+,K^+-ATPase Activity

The oxygen consumption in rectal gland tissue (14.2 ± 1.2 μmol O_2/g wet wt/h) was significantly higher than in gill tissue (9.6 ± 1.4 μmol O_2/g wet wt/h), measured under identical conditions (Fig. 21). The addition of 0.5 mM ouabain resulted in a significant reduction of oxygen consumption in both rectal gland (54.9%) and gill tissue (21.8%). The residual oxygen consumption rates of each tissue did not differ. The ouabain-sensitive portion of oxygen consumption in the rectal gland was 3.7 times that of the gills, calculated on a per gram tissue basis (Table 8). Na^+,K^+-ATPase activity was also much higher in rectal gland tissue, exceeding that measured for gills by a factor of 5.9 (Table 8).

Table 7. Size characteristics and concentrations of constituents in dogfish plasma and seawater at the Bamfield Marine Station. Data for the dogfish are shown as means ± SE (n=6).

Variable	Dogfish	SW (31 ppt)
Size characteristics		
Body weight (kg)	3.0 ± 0.3	
Rectal gland (g)	1.4 ± 0.1	
Gill tissue (g)	20.5 ± 2.1	
Plasma		
Na^+ (mM)	265.9 ± 6.8	436
Cl^- (mM)	257.8 ± 5.2	507
K^+ (mM)	3.5 ± 0.4	7.9
Ca^{2+} (mM)	3.7 ± 0.2	10.6
Mg^{2+} (mM)	2.3 ± 0.4	57.8
Osmolality (mOsm/L)	969.2 ± 9.0	925
Urea (mM)	330.3 ± 8.8	-
Glucose (mM)	4.8 ± 0.6	-
Protein (g/dL)	3.6 ± 0.3	-

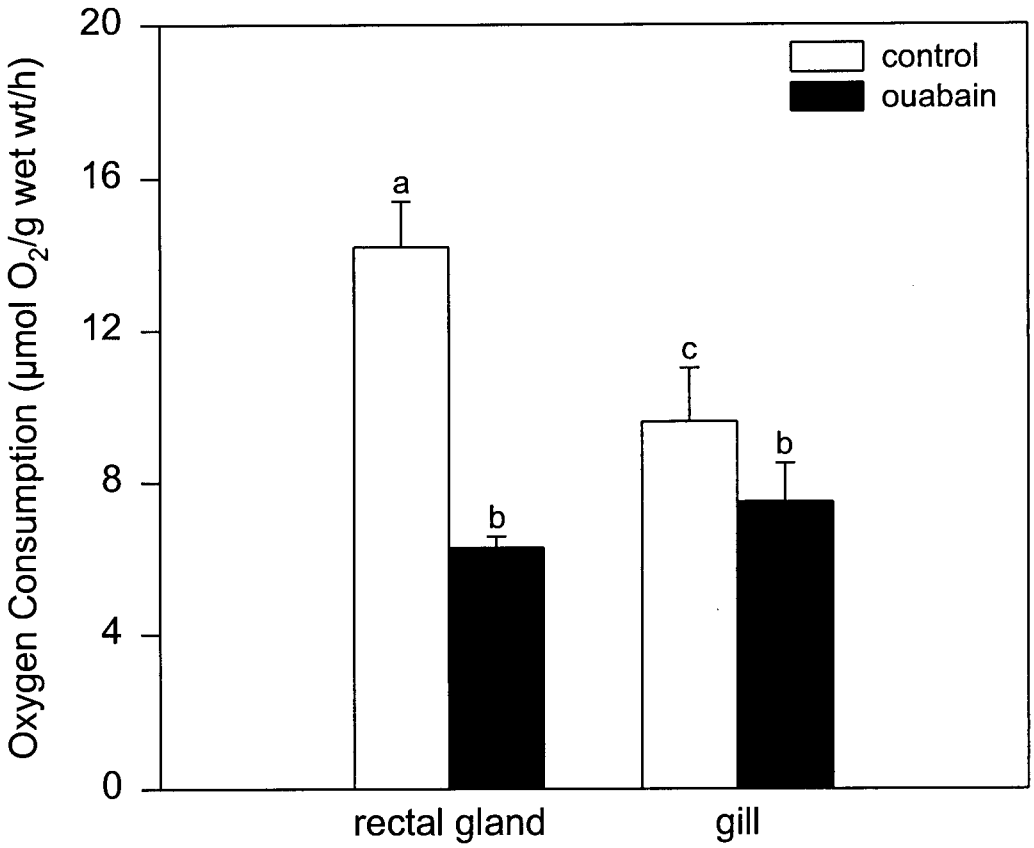

Fig. 21. Oxygen consumption in rectal gland and gill tissue of the spiny dogfish, before and after the addition of 0.5 mM ouabain. Data are shown as means ± SE (n=6). Means with different letters are significantly different ($P<0.05$, two-way ANOVA).

Table 8. Ouabain-sensitive oxygen consumption and Na^+,K^+-ATPase activity in rectal gland and gill tissue of the spiny dogfish.

	Rectal gland tissue	Gill tissue
Ouabain-sensitive oxygen consumption (μmol O_2/g wet wt/h)	7.92 ± 0.94	2.16 ± 0.52*
Na^+,K^+-ATPase activity (μmol ADP/mg prot./h)	3.20 ± 0.31	0.54 ± 0.04*

Data are shown as means ± SE (n=6). An asterisk denotes significant difference between rectal gland and gill tissue values ($P<0.05$, unpaired t-test).

DISCUSSION

There was a significant reduction in the oxygen consumption of rectal gland and gill tissue of dogfish following treatment with ouabain. Ouabain is generally considered to specifically inhibit Na^+,K^+-ATPase activity, and thus ouabain-sensitive oxygen consumption may represent that portion of tissue respiration that is directly related to the energy requirement of the Na^+/K^+-pump (Stagg and Shuttleworth 1982a, Kelly *et al.* 1991). Alternatively, the ouabain effect could be secondary in nature, resulting from changes in intracellular ionic concentrations that could disrupt cellular metabolism. Arguing against this is the fact that the inhibitory effect of ouabain on oxygen consumption was quite rapid (within the 2 min period required to replace saline solutions and equilibrate the system) and was stable throughout the test period. If the addition of ouabain caused a general disruption of cellular metabolism, then a slow and progressive change in oxygen consumption might have been expected (Shuttleworth and Thompson 1980). This did not occur and I therefore suggest that the ouabain-sensitive oxygen consumption measured in this study was primarily a reflection of the energy demand of the Na^+/K^+-pump. On this basis, I estimate that approximately 55% of the total oxygen consumption of the rectal gland was related to the Na^+/K^+-pump, compared with 22% for the gill. This observation was supported by Na^+,K^+-ATPase activity measurements of fresh tissue samples, which were six-fold higher in the rectal gland than in the gill. The higher ouabain-sensitive oxygen consumption value in the rectal gland probably reflects the fact that its tubular cells are specialized for the secretion of Na^+ and Cl^-, while the gill is composed of several cell types in addition to chloride cells (e.g., pavement, pillar, mucous cells, etc.), which have a lower energy demand for ion transport. The residual oxygen consumption rates in the rectal gland and gill did not differ in this study, suggesting that

the energy requirements for other metabolic functions were similar in the two tissues. It is likely that in tissues with high metabolic activity such as the rectal gland and gills, a large portion of this energy for other metabolic functions would be required for protein synthesis. Pannevis and Houlihan (1992), for example, reported that the cost of protein synthesis in isolated hepatocytes of rainbow trout, determined using cycloheximide-sensitive oxygen consumption, constituted 80% of total cellular respiration, compared with an energy requirement of about 3% for the Na^+/K^+-pump, as estimated by ouabain-sensitive oxygen consumption. The energy cost of protein synthesis has not yet been determined in the shark rectal gland or gills (Houlihan *et al.* 1995).

The oxygen consumption rates of rectal gland and gill tissue from dogfish in this study were quite consistent with values reported by other workers. Silva *et al.* (1980), using the isolated perfused rectal gland of spiny dogfish, obtained resting oxygen consumption rates of 18 μmol O_2/g/h at 15°C, which decreased by 50% when the gland was perfused with 0.1 mM ouabain. Shuttleworth and Thompson (1980) measured the oxygen consumption in rectal gland tissue of *S. canicula* at 11°C as 10.4 μmol O_2/g/h, with the proportion that was ouabain-sensitive estimated at 22%. In a recent study using late-term embryos of spiny dogfish, Kormanik and Totten (1993) recorded an oxygen uptake in rectal gland tissue of 9.8 μmol O_2/g/h at 15°C, and found that the ouabain-sensitive portion represented 64% of total tissue respiration. That study also measured the oxygen consumption in dogfish gills, which was 4.9 μmol O_2/g/h and was reduced by 24% following the addition of ouabain, similar to the findings of our study. No other values for oxygen consumption in the dogfish could be found in the literature, but it is interesting that in the gills from SW-adapted flounder (*P. flesus*), ouabain also caused a decline of about

25% in oxygen consumption (Stagg and Shuttleworth 1982a). This suggests that the proportion of branchial respiration that is required for the Na^+/K^+-pump is similar in the two species.

The oxygen consumption in rectal gland and gill tissue measured in this study can be compared with the oxygen uptake in the whole animal, which was measured in spiny dogfish, at 10°C and normal ventilation, as 0.67 mmol O_2/kg/h (Hanson and Johansen 1970). For an average-sized dogfish used in this study (3.0 kg; Table 7), total rectal gland and gill oxygen consumption would be 19.8 and 196.8 μmol O_2/h, respectively. These values account for 1% and 9.8% of standard metabolic rate in spiny dogfish. The higher gill component is due to its greater overall mass in the fish, and is similar to the estimate of 7% obtained by Johansen and Pettersson (1981) using isolated perfused gills of the marine cod *Gadus morhua*.

The rectal gland is a relatively homogeneous organ, composed primarily of secretory cells whose sole function is NaCl transport (Shuttleworth 1988). It can therefore be assumed that most of the ouabain-sensitive oxygen consumption measured in the rectal gland was related to organismal NaCl transport. This amounts to 11.1 μmol O_2/h or 0.5% of standard metabolism, when expressed on the basis of total fish mass. The cost of NaCl tranport estimated for the spiny dogfish rectal gland in this study is quite comparable to the theoretical calculations of Kirschner (1993) for *S. canicula* and *R. erinacea* (0.1% and 0.4% of standard metabolism, respectively). Kirschner (1993) actually considered rectal gland energetics to be a minor component in the overall cost of osmoregulation in ureotelic elasmobranchs. He determined that most of the energy demand for osmoregulation arises from the synthesis and renal reabsorption of urea, accounting for 10.3-14.6% of whole-animal oxygen consumption. In the study using late-term spiny dogfish embryos, Kormanik and Totten (1993) provided the ion transport-related components of oxygen consumption in rectal gland tissue for a 100 g fish, but did not express their data as a function of

whole-animal metabolism. Applying the whole-animal metabolic rate of Hanson and Johansen (1970) to their data gives a value of 0.3% of standard metabolism for the rectal gland, which is quite similar to the value obtained in the present study.

The energy demand of NaCl transport by the elasmobranch gill was not considered in Kirschner's (1993) calculations, as direct evidence for its role in ion regulation is lacking (Shuttleworth 1988; Evans 1993). The elasmobranch gill does possess chloride cells that morphologically resemble those of teleosts (Laurent 1982). Furthermore, in spiny dogfish, chloride cell density shows a significant correlation with Na^+,K^+-ATPase activity (Wilson *et al.* 1996), which is characteristic of NaCl secretion in teleost gills (McCormick 1995). As mentioned above, the gill is a heterogeneous organ comprised of several cell types in addition to chloride cells, and whose primary functions do not involve NaCl secretion. Calculating the cost of NaCl transport on a whole-animal basis therefore requires an estimate of the portion of gill tissue that contains chloride cells. Sargent and Thomson (1974) have reported 5-10% chloride cells in gills from SW-adapted eels (*A. anguilla*), whereas Perry and Walsh (1989) determined the proportion of chloride cells in a gill cell suspension from the marine toadfish (*Opsanus beta*) to be about 13%. Based on area measurements of spiny dogfish gills stained for chloride cells with Champy-Maillet's fixative (Wilson *et al.* 1996), I have estimated the proportion of chloride cells in the dogfish gill to be about 6% (unpublished data, obtained from scanned light micrographs using SigmaScan®, Jandel Scientific Software). Assuming a homogeneous distribution of Na^+,K^+-ATPase in the entire gill, the amount of energy associated with organismal NaCl transport would be about 2.7 μmol O_2/h, accounting for 0.14% of standard metabolic rate in spiny dogfish. This is likely a conservative estimate, as several studies have shown that most of the Na^+,K^+-ATPase in teleost fish gills is located in the chloride cells (e.g., Karnaky *et al.* 1976, Witters *et al.* 1996).

If this is shown to be the case in dogfish, the energy required for NaCl transport in the gill would be about 2.2% of standard metabolism. Further studies are therefore needed to characterize the location of Na^+,K^+-ATPase in the dogfish gill (e.g., using autoradiographic or immuno-cytochemical techniques) and refine the cost estimate provided in this study. Nevertheless, the results of the present study suggest that the energy cost of NaCl secretion in the rectal gland and gill is a relatively small portion of the total energy budget in the spiny dogfish.

CHAPTER 7: Energy Cost of NaCl Uptake in Freshwater Cutthroat Trout Gill Tissue

INTRODUCTION

Freshwater salmonids maintain the salt content of their blood at about 25-30% that of SW. They are therefore hyperosmotic to the surrounding FW environment, and are faced with a passive volume load and NaCl loss, which occurs across permeable membranes such as gill epithelia. The excess water is removed by a copious production of dilute urine in the kidneys, and salt losses are compensated by active ion uptake mechanisms located in the gills (Fig. 3). Theoretical estimates of the energy cost for NaCl uptake in FW trout gills have been calculated to be about 1-2% of resting metabolic rate (Eddy 1982; Kirschner 1995; see General Introduction). In contrast, measurement of whole-animal oxygen consumption rates at differing salinities suggests that there is a 20% increase in standard metabolic rate associated with osmoregulation in FW, compared with that in an ISO environment, at which the osmoregulatory cost is assumed to be zero (e.g., Rao 1968; Farmer and Beamish 1969).

An alternative experimental approach to estimating osmoregulatory costs rather than using whole-animal oxygen uptake is to measure respiration rates in isolated preparations of osmoregulatory organs (eg., gills and kidney). Inhibitors of ion translocating enzymes can be used on these tissues to estimate the ion transport-related portion of total tissue respiration. Ouabain is known to specifically inhibit Na^+,K^+-ATPase activity, and this drug has often been used on excised tissues to estimate the sodium pump-dependent portion of oxygen consumption (eg., Stagg and Shuttleworth 1982a; Kelly *et al.* 1991; Pannevis and Houlihan 1992). A number

of inhibitors have been used to assess V-type H^+-ATPase activity, although none have been used to examine the effects on proton pump-dependent oxygen consumption. Bafilomycin A_1 is a macrolide antibiotic that is a very specific and potent inhibitor of V-type H^+-ATPase (Bowman *et al.* 1988). NEM is a metabolic inhibitor that affects enzymes containing sulphydryl (SH-) groups, and is also a potent inhibitor of V-type H^+-ATPase (Pederson and Carafoli 1987; Forgac 1989). These inhibitors were used in the present study to assess the oxygen cost of NaCl uptake in isolated gill tissue of cutthroat trout. Calculations were also made on a total mass basis to estimate the percentage of energy required for NaCl uptake in the gill compared to the metabolic rate of the whole animal.

MATERIALS AND METHODS

Fish

Cutthroat trout, weighing 100-220 g, were kept in flowing dechlorinated Vancouver City tap water (Na^+ = 1 mM, Cl^- = 1 mM, Ca^{2+} = 0.03 mM). The water temperature was increased from ambient (6°C) to 10°C over a 4 d period using immersion heaters, and the fish were acclimated to this temperature for at least 1 wk before testing.

Respirometry

Whole-animal oxygen consumption measurements. Oxygen consumption rates of eight cutthroat trout were measured using the flow-through respirometer described in Chapter 4. Prior to each trial, individual fish were lightly anesthetized with TMS (50 mg/L), weighed, introduced to the chamber and allowed to acclimate in flow-through water for 24 h. Flow rates were set to 0.5-1

L/min depending on fish size to achieve a difference in oxygen concentration between inflowing and outflowing water of about 0.5 mg/L. The respirometer was covered with black plastic throughout acclimation and testing to shield the fish from visual disturbances. After the 24 h acclimation period, water oxygen concentrations in the inflowing and outflowing water were measured using a dissolved oxygen meter (Oxyguard® Mk III, Point Four Systems, Port Moody, British Columbia). The trials were conducted at approximately the same time each day (1130-1230h) to minimize diurnal variation in metabolism due to entrainment to a feeding schedule or photoperiod (Brett and Zala 1975). Water temperatures during the trials were kept similar to the holding tank (9.8 ± 0.5°C). Oxygen consumption rates for each fish were calculated using the equation given on page 60, and were expressed as mg O_2/kg/h.

Gill tissue oxygen consumption measurements. Each trout was anesthetized (TMS, 100 mg/L) and injected in the caudal artery/vein with heparinized saline (5000 units/kg) using a 1 mL syringe. After 5 min in the anesthetic bath, the fish were killed and the head was severed just behind the pectoral fins. The ventral aorta was exposed and cannulated with polyethylene (PE 90) tubing that was secured in place with an alligator clip. The head was then immersed in 10°C FW and the gills were cleared of blood by perfusing for 5 min with fish saline (composition in mM: NaCl, 139; KCl, 5.1; $CaCl_2$, 1.1; $MgSO_4$, 0.9; $NaHCO_3$, 11.9; NaH_2PO_4, 3.0; glucose, 5.6; pH = 7.5) using a peristaltic pump (Piper P-10T). The first gill arch on the left side of the fish was dissected free, placed in ice-cold saline, and gill filaments were cut into thin slices using a scalpel. Oxygen consumption of gill tissue was measured using a Strathkelvin Model 781 oxygen meter and microelectrode (Strathkelvin Instruments, Glasgow, Scotland). The microelectrode was inserted into a glass respiration chamber which was thermostatted to 10°C with running FW.

Appropriate amounts (12 to 26 mg) of tissue were placed in 2 mL of air-saturated fish saline and allowed to acclimate for 5 min prior to testing. The decline in water oxygen tension (to the nearest 0.1 mm Hg) was then monitored for 10 min, with values recorded every minute using a computer data aquisition system (Labtech Notebook version 7.11). Gentle stirring was provided by a magnetic stir bar to facilitate gas diffusion, and the tissue sat on a raised stainless steel mesh platform to prevent contact with the stir bar (see Fig. 19 in Chapter 6).

After the control trial was completed, fresh fish saline containing 0.5 mM ouabain, 0.5 mM ouabain and 1 mM NEM, or bafilomycin A_1 (0.1 μM and 1 μM dissolved in 0.1% DMSO) was added to the respiration chamber and the oxygen consumption trial was repeated. All drugs were purchased from Sigma Chemical Co. (St. Louis, Missouri), and the stock concentration of bafilomycin A_1 was determined spectrophotometrically using the molar extinction coefficients provided in Werner *et al.* (1984). The control trials for bafilomycin also contained 0.1% DMSO. Separate control trials were run consecutively to ensure that tissue respiration in saline alone remained constant throughout the experimental period. At the end of each trial, the gill tissue was removed, blotted dry on tissue paper and weighed to the nearest milligram.

Oxygen consumption measurements for each gill tissue slice were completed within 1 h of dissection. Final water oxygen tensions in the respiration chamber were maintained above 120 mm Hg, and blank trials without tissue were also run to correct for electrode oxygen consumption. Oxygen consumption rates were estimated using linear regression analysis and expressed as μmol O_2/g wet wt/h.

Gill Tissue and Plasma Sampling

Gill tissue and plasma samples were taken from eight cutthroat trout. The fish were anesthetized (TMS, 100 mg/L), killed by a blow to the head, and blood was collected from the caudal vessels using heparinized syringes. The blood samples were centrifuged (2000 *g* for 5 min) and the plasma was removed and frozen at -75°C for glucose and ion analyses. Immediately following blood collection, gill filaments (~20 mg) were removed from the first branchial arch on the left side of the fish, blotted dry, placed in 0.5 mL of ice-cold SEI buffer (150 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.3) in a 1.5-mL microcentrifuge tube, and stored at -75°C for measurement of Na^+,K^+-ATPase and H^+-ATPase activity.

The total mass of gill tissue was determined for eight additional trout. The fish were anesthetized, killed, weighed, and the gill basket was removed using dissecting scissors. The gill filaments were cut free from the eight arches using a scalpel, pooled, blotted dry, and weighed to the nearest 0.01 g. Gill mass was expressed as a percentage of body mass.

ATPase Activity Measurements

Na^+,K^+-ATPase and H^+-ATPase activities in crude gill homogenates were determined using the coupled-enzyme assay described by Penefsky and Bruist (1984), and incorporated modifications presented by Lin and Randall (1993) and Kültz and Somero (1995) for H^+-ATPase, and by McCormick (1993) for Na^+,K^+-ATPase measurements in a microplate reader. In this kinetic assay, the ouabain-sensitive and NEM-sensitive hydrolysis of ATP is coupled in an equimolar ratio to the oxidation of NADH, utilizing the conversion of phosphoenolpyruvate (PEP) to lactate as catalyzed by pyruvate kinase (PK) and lactate dehydrogenase (LDH).

An assay mixture (solution A) containing final concentrations of 45 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 2 mM PEP, 0.5 mM ATP, 0.16 mM NADH, 5 U/mL PK, 5 U/mL LDH, 1 mM NaN_3, 1 mM EGTA, and 50 mM imidazole (pH 7.5) was prepared just prior to the assay and kept at 4°C. Assay solution B was prepared as above but also contained 0.5 mM ouabain. Assay solution C was prepared by adding 1 mM NEM to solution B. The presence of sodium azide (NaN_3) and EGTA in the assay mixtures ensured the inhibition of F-type (mitochondrial) ATPase and Ca^{2+}-ATPase, respectively. A few minutes before use, the assay mixtures were placed in a 25°C water bath. Prior to thawing the samples, assay mixture A was checked by running an ADP standard curve. The slope of the standard curve was generally between 18 and 20 mOD units/nmol ADP (McCormick 1993).

The gill tissue samples were thawed immediately prior to assay and kept on ice throughout homogenization. Sodium deoxycholate (0.1% in SEI buffer) was added to each microcentrifuge tube and the gill filaments were homogenized in the tubes using a motorized polypropylene pestle (Kontes, Vineland, New Jersey). The tissue was ground for 10-15 s and then centrifuged at 7500*g* for 30 s to remove the insoluble cartilaginous material. With the 96-well microplate sitting on a paper towel-covered ice pack, 10 µL of homegenate was added to each of six wells for every sample. The remaining homogenate was removed for later analysis of protein content, which was determined using the bicinchoninic acid procedure (Smith *et al.* 1985). To begin the ATPase assay, 200 µL of solution A was added to the first two wells per sample, 200 µL of solution B to the next two wells per sample, and 200 µL of solution C to the remaining two wells per sample. The plate was then placed in a temperature-controlled microplate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA) set to 25°C, and the linear rate of NADH oxidation (i.e.,

disappearance) was measured at 340 nm for 10 min. For each assay (16 samples per plate), activity measurements were completed within 0.5 h of tissue homogenization.

ATPase activity in the three assay mixtures was calculated from the slope of the linear portion of the reaction, the standard curve slope, and the protein content of the crude gill homogenates:

$$\text{ATPase activity } (\mu\text{mol ADP/mg protein/h}) = \frac{\text{slope (mOD/10 } \mu\text{L/min)}}{\text{standard curve slope (mOD/nmol ADP)}} \times \frac{1}{\text{protein } (\mu\text{g/10 } \mu\text{L})} \times 60 \text{ min/h}$$

The difference in the ATPase activity between solutions A and B represented the ouabain-sensitive Na^+,K^+-ATPase activity, and the difference in the ATPase activity between solutions B and C represented the NEM-sensitive H^+-ATPase activity.

Plasma Analysis

Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Procedure 315). Plasma $[Na^+]$ and $[K^+]$ were measured on a flame photometer (Corning model 410), and plasma [Cl-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer).

Statistical Analyses

Data are presented as means ± 1 SE. Gill tissue oxygen consumption rates following treatment with ouabain and NEM were analyzed using a one-way ANOVA, and significant treatment means were identified using Student-Newman-Keuls multiple comparison test ($P<0.05$). Oxygen consumption measurements before and after the addition of bafilomycin A_1 were compared using

paired t-tests ($P<0.05$). Comparison of Na^+,K^+-ATPase and H^+-ATPase activities in gill tissue were made using an unpaired t-test ($P<0.05$).

RESULTS AND DISCUSSION

Whole-Animal Measurements

Table 9 shows the body and gill mass, whole-animal oxygen consumption rate, gill Na^+,K^+-ATPase and H^+-ATPase activities, and plasma concentration of glucose and ions in the cutthroat trout used in this study. Gill tissue comprised about 0.75% of the total body mass, which is similar to the values reported for dogfish in Chapter 6 (0.68%) and for Atlantic salmon (0.52%) by McCormick *et al.* (1989). The resting metabolic rate of cutthroat trout in this study (~100 mg O_2/kg/h) was comparable with values obtained for rainbow trout (e.g., Evans 1962; Rao 1968; Maxime *et al.* 1991; Seddiki *et al.* 1995). There were no significant differences between the activities of Na^+,K^+-ATPase and H^+-ATPase in gill tissue, suggesting an equivalent capacity for Na^+ transport in the FW trout gill. This is in agreement with the identical stoichiometries of the proton pump and sodium pump (3 H^+/ATP, 3 Na^+/ATP; Kirshner 1995) that results in the movement of Na^+ into the fish. Plasma glucose levels and $[Na^+]$, $[K^+]$, $[Cl^-]$ were within the normal range reported for FW salmonids (e.g., Wedemeyer *et al.* 1990).

Gill Tissue Oxygen Consumption

The oxygen consumption rates of gill tissue from FW-adapted cutthroat trout in this study (~20 μmol O_2/g wet wt/h) were consistent with values reported for other teleost species (e.g., 19-26 μmol O_2/g/h; Evans 1962; O'Hara 1971; Leray *et al.* 1981; Stagg and Shuttleworth 1982a).

Table 9. Body and gill mass, oxygen consumption rate, gill Na^+,K^+-ATPase and H^+-ATPase activities, and plasma glucose and ion concentrations in cutthroat trout reared in fresh water.

Variable	Mean ± SE (n=8)
Body mass (g)	103.0 ± 3.6
Gill mass (g)	0.77 ± 0.04
(% of body wt.)	0.75 ± 0.02
Whole-animal oxygen consumption	
(mg O_2/kg/h)	103.9 ± 8.2
(mmol O_2/kg/h)	3.25 ± 0.26
Gill Na^+,K^+-ATPase activity	
(μmol ADP/mg protein/h)	1.27 ± 0.10
Gill H^+-ATPase activity	
(μmol ADP/mg protein/h)	1.44 ± 0.11
Plasma constituents	
Glucose (mM)	4.4 ± 0.4
Na^+ (mM)	147.8 ± 1.9
K^+ (mM)	2.4 ± 0.5
Cl^- (mM)	132.4 ± 1.9

The addition of ouabain resulted in a significant reduction in gill tissue oxygen consumption (Fig. 22). Ouabain-sensitive oxygen consumption represents that portion of tissue respiration that is related to the energy requirements of the Na^+/K^+-pump (see discussion in Chapter 6), and thus it can be estimated that about 18% of the total gill oxygen consumption was utilized by the Na^+/K^+-pump. Stagg and Shuttleworth (1982a) found that ouabain caused a decline of about 25% in the oxygen consumption of flounder (*P. flesus*) gill tissue, suggesting that the portion of branchial respiration required for the Na^+/K^+ pump is similar in the two species.

The addition of NEM to the gill tissue resulted in a further drop in oxygen consumption, accounting for 51% of total tissue respiration (Fig. 22). It is unlikely that this was related solely to the energy requirements of the H^+-pump, as NEM is known to inhibit the activity of over 90 cellular enzymes, many of which utilize ATP (Zollner 1993). When used in combination with other inhibitors, NEM can be used in an *in vitro* assay to measure H^+-ATPase activity (e.g., Lin and Randall 1993; Kültz and Somero 1995; this study), but it is not specific enough to be used alone on intact gill tissue. Bafilomycin A_1 is a specific inhibitor of H^+-ATPase activity (Dröse and Altendorf 1997), and resulted in a significant decline in gill tissue oxygen consumption (14% at 0.1 μM and 19% at 1 μM; Fig. 22). This is the first reported effect of bafilomycin A_1 on gill tissue oxygen consumption in fish. Berenbrink and Pelster (1996) found that 0.1 μM bafilomycin A_1 significantly reduced the extracellular acidification rate in secondary lamellae preparations of the rainbow trout pseudobranch. Nominal concentrations of bafilomycin A_1 in the 1-5 μM range have also been shown to inhibit V-type H^+-ATPase activity in other intact epithelia (e.g., insect Malpighian tubules, Bertram *et al.* 1991; FW crab gills, Onken and Putzenlechner 1995; frog skin, Ehrenfeld and Klein 1997). The micromolar concentration range of bafilomycin A_1 necessary to inhibit H^+-ATPase activity in intact epithelia is higher than the nanomolar

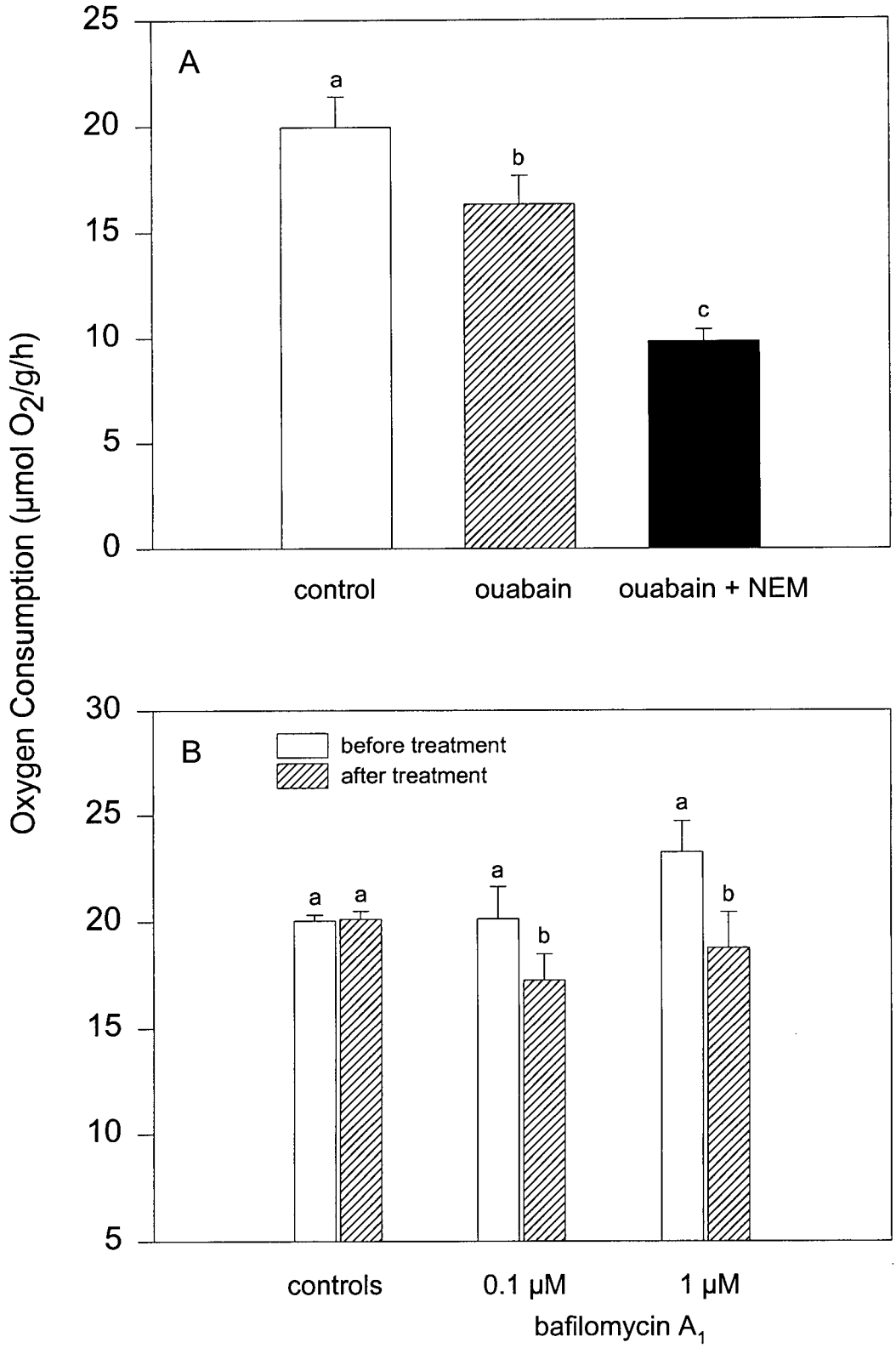

Fig. 22. Oxygen consumption of cutthroat trout gill tissue following treatment with *A)* 0.5 mM ouabain, 1 mM NEM, and *B)* 0.1 µM and 1 µM bafilomycin A_1. Data are shown as means ± SE (n=4-6). Means with different letters are significantly different ($P<0.05$; *A)* one-way ANOVA, *B)* paired t-tests).

concentration range used in most biochemical studies of H^+-ATPase activity (Bowman *et al.* 1988; Dröse *et al.* 1993). The *in vitro* V-ATPase assay utilizes highly purified membrane fractions (Bowman and Bowman 1988), which greatly increases the accessibility of the enzyme binding sites to the inhibitor. Lin and Randall (1993) found that 25 μM bafilomycin was necessary to inhibit H^+-ATPase activity in crude homegenates of trout gill tissue. I have also found that concanamycin A, a macrolide antibiotic structurally and functionally similar to bafilomycin (Dröse *et al.* 1993), was not effective in significantly inhibiting H^+-ATPase activity in crude gill homegenates at concentrations up to 10 μM (data not shown). It is possible that the difference in inhibitor sensitivities between these *in vitro* assay systems and intact epithelia is related to the accessibility of the macrolide antibiotics to the enzyme binding sites.

A similar portion of gill oxygen consumption was required by the Na^+/K^+-pump and the H^+-pump in the present study (18 and 19%, respectively). This result is consistent with the comparable Na^+,K^+-ATPase and H^+-ATPase activities measured in the gill tissue (Table 9), and the equivalent theoretical stoichiometries of Na^+ and H^+ ions transported per ATPs hydrolyzed in the FW trout gill (Kirschner 1995). In a classic study on the relationship between the rate of oxygen consumption and Na^+ transport in isolated frog skin, Zehran (1956) found that about 30% of tissue respiration was related to Na^+ uptake. His results were obtained by measuring oxygen consumption with and without Na^+ present in the apical bathing solution. At that time, the mechanisms for the active transport of NaCl in the frog skin were not known (see Ehrenfeld and Klein 1997), but it is interesting to note that the portion of tissue respiration related to the Na^+/K^+-pump and the H^+-pump in the FW trout gill and frog skin appears to be similar (37 vs 30%).

Cost of NaCl Uptake in the FW Cutthroat Trout Gill

The mass-specific oxygen consumption in gill tissue (~20 μmol O_2/g/h) was about 6-fold higher than the whole-animal oxygen uptake rate (3.25 μmol O_2/g/h; Table 9), indicating that the gills are more metabolically active than most other body tissues, reflecting their role in ion transport. This has also been found in previous measurements of fish gill oxygen consumption (e.g., Johansen and Pettersson 1981; Lyndon 1994), and might be expected because most (>60%) of the mass of the intact fish is composed of the trunk region (i.e., muscle, skin, scales, and bones) which has a low rate of oxygen consumption (Itazawa and Oikawa 1983). Itazawa and Oikawa (1983) found that the brain and kidney have the highest mass-specific oxygen consumption rates in the carp, and these organs also have higher ion-motive ATPase activities than found in FW fish gills (e.g., McCormick *et al.* 1989). When compared on a whole mass basis for the average-sized trout used in this study (~100 g), gill oxygen consumption accounted for 4.6% of resting metabolic rate (Table 10). This value is quite similar to the estimate of 3.2% obtained by Itazawa and Oikawa (1983) using excised gill tissue of carp.

As mentioned in Chapter 6, the fish gill is a heterogeneous organ comprised of several cell types, in addition to chloride cells. Most of the Na^+,K^+-ATPase enzymes in the FW trout gill are located in the chloride cells (Witters *et al.* 1995), whereas H^+-ATPase is located in both the chloride cells and pavement cells (Lin *et al.* 1994; Sullivan *et al.* 1995). If one assumes that the majority of these ion pumps in the FW trout gill are involved in active NaCl uptake, then the energy cost of this process can be calculated using the values for both ouabain-sensitive (Na^+/K^+-pump) and bafilomycin-sensitive (H^+-pump) oxygen consumption. This amounts to 6 μmol O_2/h or 1.8% of resting metabolic rate, when expressed on the basis of total fish mass (Table 10). The cost of NaCl uptake estimated for the FW cutthroat trout gill in this study is similar to the

Table 10. Cost of NaCl uptake in the freshwater cutthroat trout gill.

Whole-animal O_2 consumption[1] (μmol/h)	325
Total gill O_2 consumption[2] (μmol/h)	15
Percent of whole-animal MO_2	**4.6**
Ouabain-sensitive O_2 consumption[3] (μmol/h)	2.7
Bafilomycin-sensitive O_2 consumption[4] (μmol/h)	3.3
NaCl transport-related O_2 consumption (μmol/h)	6.0
ATP required[5] (μmol/h)	36
Percent of whole-animal MO_2	**1.8**

[1]100 g fish at 10°C
[2]total gill mass = 0.75% of body mass
[3]Na^+/K^+-pump O_2 requirement
[4]1 μM bafilomycin A_1; H^+-pump O_2 requirement
[5]6 ATP/O_2

theoretical value of 1% determined by Eddy (1982), and almost identical to the calculation of Kirschner (1995) for the rainbow trout gill (1.6%). Eddy (1982) and Kirschner (1995) also provided estimates of 0.2% and 0.9% for the cost of NaCl reabsorption in the FW trout kidney, for overall osmoregulatory costs of 1.2% and 2.5% of standard metabolism, respectively. The experimental approach used in this study is therefore in good agreement with the theoretical calculations, and suggests that the direct energy cost of NaCl uptake in FW trout is a relatively small portion (i.e., <3%) of whole-animal oxygen uptake.

CHAPTER 8: Oxygen Consumption in Isolated, Perfused Gills of Freshwater- and Seawater-Adapted Cutthroat Trout

INTRODUCTION

The fish gill plays an important role in maintaining ionic homeostasis in both FW and SW environments (Evans 1993). Fish gills have been shown to be metabolically very active (Johansen and Petterson 1981; Lyndon 1994; also see Chapter 7) and biochemical studies suggest that much of gill tissue metabolism is due to the oxidative demands of the chloride cell, utilizing both glucose and lactate as substrates (Mommsen 1984a; Perry and Walsh 1989). Few studies, however, have compared the metabolic demands of intact gills between FW and SW fish. The energy cost of ion transport in FW and SW gills has been estimated on a theoretical basis by Eddy (1982) and Kirschner (1993, 1995), with differing results. Eddy (1982) used a thermodynamic analysis and calculated the energy cost of osmoregulation in rainbow trout gills to be 1% of resting metabolic rate in FW and 0.5% in SW. Applying a "molecular' approach (see General Introduction), Kirschner (1993, 1995) estimated the energy required for osmoregulation in FW trout gills to be about 1.6% of resting metabolic rate, but calculated the energy demands for osmoregulation to be higher in SW (5.7%). A comparison of the gills' energy requirements in FW and SW cannot be made using whole-animal metabolic rate measurements, due to the confounding effects of salinity on other metabolic processes in the fish (Chapter 5), and therefore requires a different experimental approach.

Isolated, saline-perfused gill arch preparations have been used extensively in studies of branchial hemodymamics, ion exchange and gas transfer, and have made significant

contributions to the understanding of these mechanisms in the fish gill (see review by Perry and Farrell 1989). Perfused gill preparations have been criticized for their inability to duplicate *in vivo* conditions, including abnormal mucus production, edema, inadequate irrigation, and high vascular resistance to flow (Evans *et al.* 1982; Perry *et al.* 1984; Perry and Farrell 1989). In addition, saline has a lower capacitance for oxygen and carbon dioxide than whole blood, making it a less than ideal perfusate (Johansen and Pettersson 1981). Despite these problems, isolated, saline-perfused gills have an advantage over excised, chopped gill filaments in ion regulation studies because they effectively separate serosal and mucosal media, and thus more closely simulate ionic gradients found between blood and water in the *in vivo* state. Furthermore, perfused gill preparations have been used successfully to measure gill oxygen consumption requirements in two species of marine fish (*G. morhua*, Johansen and Pettersson 1981; *P. flesus*, Lyndon 1994). Most studies using isolated, saline-perfused gills have used saline as the bathing medium, and only that of Lyndon (1994) has involved the measurement of oxygen uptake of perfused gills in a natural external medium (SW).

The purpose of the present study was to measure gill oxygen consumption in FW- and SW-adapted cutthroat trout under natural conditions (i.e., FW gills immersed in a FW bath, and SW gills in a SW bath). The addition of ouabain to the saline perfusate has been found to inhibit the uptake of Na^+ in the isolated FW trout gill (Richards and Fromm 1970) and abolish the transepithelial potential across isolated SW flounder gills (Shuttleworth *et al.* 1974), consistent with its inhibitory effects on Na^+,K^+-ATPase. Ouabain was therefore used in the present study to assess the oxygen cost of the Na^+/K^+-pump in FW and SW trout gills.

MATERIALS AND METHODS

Fish

Adult cutthroat trout (4 years of age) were maintained in an 800-L oval fibreglass tank receiving flowing dechlorinated Vancouver City tap water, and were fed a maintenance diet of commerical salmon pellets (EWOS Canada). The water temperature was increased from ambient (6°C) to 10°C over a 1 wk period using an immersion heater, and the fish were acclimated to this temperature for 1 wk before salinity acclimation.

Salinity Acclimation and Sampling

After the temperature acclimation, 25 fish were transferred into a second 800-L tank (density = 8 g/L) set up as a saltwater recirculation system (described in Chapter 3). Stock salt water was prepared from artificial sea salts (Deep Ocean Synthetic Sea Salt) and added to the tank at a rate of 4-5 ppt per day, to achieve a final test salinity of 25 ppt. This salinity was chosen as it approaches the highest salinity that would be encountered by this stock of sea-run cutthroat trout in the outer Fraser River estuary (24-26 ppt; Crean *et al.* 1988; Stronach *et al.* 1988). The fish were acclimated to SW for at least 2 wk before whole-animal respirometry and 6 wk before gill oxygen consumption measurements. After the 2 wk acclimation period, gill tissue and plasma were also taken from FW and SW fish for Na^+,K^+- and H^+-ATPase activities and cortisol, glucose and ion determinations, according to the sampling protocol described in Chapter 7.

Whole-Animal Respirometry

Oxygen consumption rates of trout (average mass = 252 g) acclimated to FW and SW were measured using the flow-through respirometer and protocol described in Chapters 4 and 7.

Isolated, Perfused Gill Preparation

Gill oxygen consumption rates were determined in FW- and SW-adapted trout (body mass: 224-455 g), using the isolated, perfused gill arch preparation described by Lyndon (1994), with certain modifications. The fish were anesthetized (TMS, 100 mg/L), weighed, and injected in the caudal vessels with heparinized saline (5000 units/kg). After 5 min in the anesthetic bath, the fish were killed and their heads were severed just behind the pectoral fins. The ventral aorta was exposed by a ventral, midline incision into the pericardial cavity and the bulbus arteriosis/ventral artery was cannulated with polyethylene tubing (Clay-Adams PE 90), secured in place with an alligator clip. The head was then immersed in either 10°C FW or SW, and the gills were cleared of blood by perfusing for 5 min with filtered (0.2 μm), heparinized (10 units/mL) fish saline (composition in mM: NaCl, 139; KCl, 5.1; $CaCl_2$, 1.1; $MgSO_4$, 0.9; $NaHCO_3$, 11.9; NaH_2PO_4, 3.0; glucose, 5.6; pH = 7.5) using a peristaltic pump (Piper P-10T). The branchial basket was removed and the first gill arch on the left side of the fish was then excised and placed in ice-cold saline for cannulation. The afferent and efferent branchial arteries were cannulated using blunt 23 gauge needles inserted into saline-filled PE 50 tubing (i.d. 0.58 mm). The cannulation sites were dried with a surgical sponge spear (Weck-Cel®), and the cannulae were secured in place using a cyanoacrylate tissue adhesive (Vetbond, 3M Corporation). An additional drop of tissue glue was used to seal off the ends of the arch, including the branchial vein and exposed tissues. Dissection and cannulation were completed within 5 min, and the arch was kept submerged in saline during

the procedure, with the exception of the cannulation site. After cannulation, the gill arch was suspended in a cylindrical glass respiration chamber (volume=160 mL), thermostatted to 10°C, and containing either aerated FW or SW (Fig. 23). Stirring was provided by a magnetic stir bar to facilitate gas diffusion. The gill was perfused with a pulsatile flow (pulse frequency = 17.9 ± 0.1 per min) of fish saline using a peristaltic pump (Labconco). The efferent pressure head was set to about 15 cm above the level of the chamber, giving an efferent pressure similar to previous saline-perfused trout gill preparations (Perry and Farrell 1989). Afferent pressure was monitored using a pressure transducer (Statham P23Db) connnected to the perfusion circuit. Perfusion flows, determined from the perfusate effluent, were 346 ± 53 μL/min/g gill (n=24).

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

Samples for oxygen tension determinations (to the nearest 0.1 mm Hg) were taken anaerobically in syringes from the aerated saline perfusate reservoir (afferent P_{O_2}), perfusate effluent (efferent P_{O_2}) and from the sampling port located in the lid of the respiration chamber. P_{O_2} measurements were made using a polarographic oxygen microelectrode (Microelectrodes,

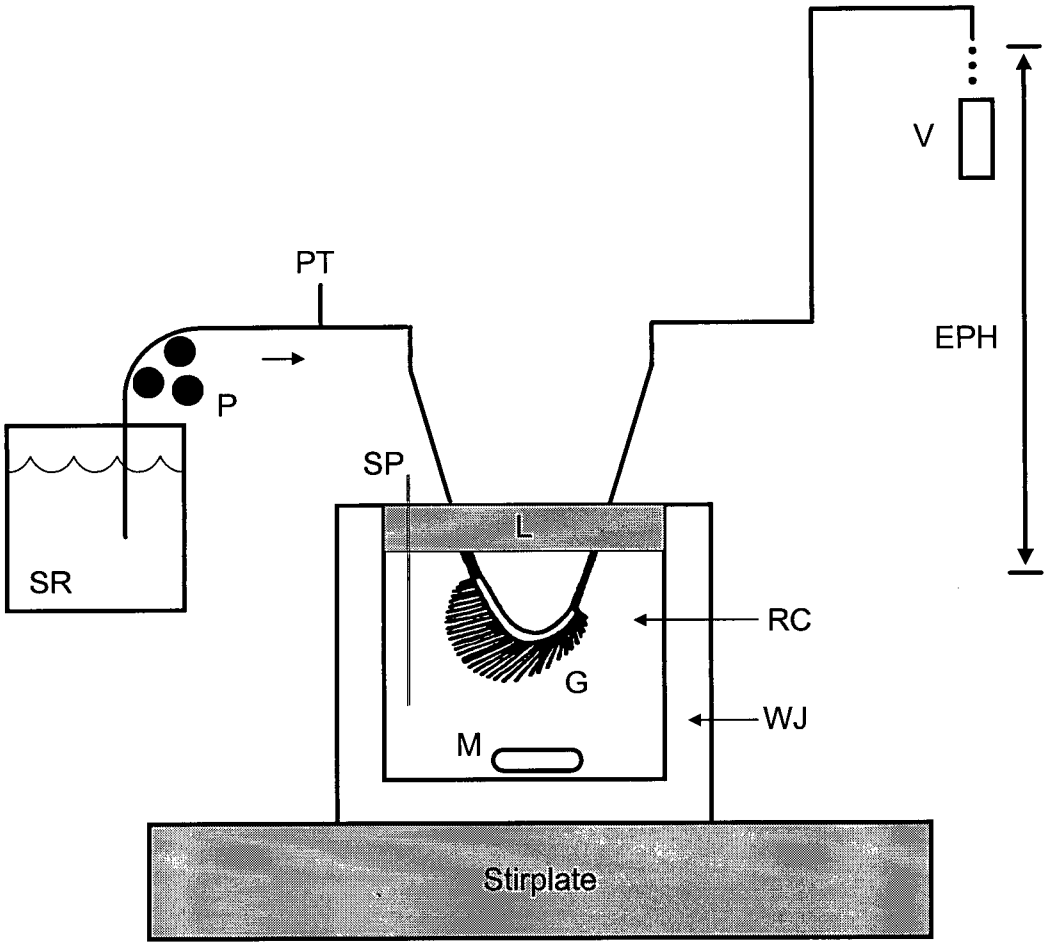

Fig. 23. Schematic diagram of the setup used for measuring oxygen consumption in isolated saline-perfused cutthroat trout gills (adapted from Lyndon 1994). EPH, efferent pressure head; G, gill; L, lid; M, magnetic stir bar; P, peristaltic pump; PT, pressure transducer; RC, respiration chamber; SP, sampling port; SR, saline reservoir; V, effluent sampling vial; WJ, water jacket. Not to scale.

Inc., Londonderry, New Hampshire), connected to an oxygen meter (OM200, Cameron Instrument Co., Port Aransas, Texas). Before use, the oxygen electrode was calibrated to zero with a sodium bisulphite solution, and at the beginning of each day the electrode was recalibrated to air-saturated saline equilibrated at 10°C. One microelectrode was used for all sample determinations, to eliminate the problem of signal drift between multiple sensors. Final P_{O_2} values in the perfusate or respiration chamber never fell below 125 mm Hg. Measured P_{O_2} values in the saline (8 ppt salinity), FW and SW (25 ppt) were converted to oxygen content (mg/L) using the conversion tables found in Colt (1984).

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

Gill oxygen consumption rates (μmol/g wet wt/h) were calculated using the formula given in Lyndon (1994), as follows:

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

where P_a and P_e are the afferent and efferent oxygen contents of the perfusion saline (μmol/L), F is the efferent flow rate (L/h), P_i and P_f are the initial and final oxygen contents of the respiration chamber (μmol/L), V is the volume of the respiration chamber (L), t is the time over which the measurement was made (h), and M is the wet mass of the gill, including the arch (g). It should be noted that the above calculation assumes that oxygen in the perfusate is taken up by tissues facing the internal gill vasculature, while the oxygen in the chamber is consumed by the gill tissues facing the ambient medium. The afferent P_{O_2} in the saline is saturated (~155 torr), which

is unlike the *in vivo* situation (ventral aorta $P_{O_2} \cong 30$ torr; Perry and Farrell 1989), so that the main driving force for passive oxygen diffusion into the gill is created by the oxygen consumption of the outward facing tissues.

Analytical Procedures

Plasma cortisol levels were determined using a [^{125}I] cortisol RIA kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California). Plasma glucose levels were measured using a modification of Trinder's (1969) glucose oxidase method (Sigma Procedure 315). Plasma [Na^+] and [K^+] were measured on a flame photometer (Corning model 410), and plasma [Cl-] was determined by coulometric titration (Haake Buchler Instruments digital chloridometer). Gill Na^+,K^+-ATPase and H^+-ATPase activities were determined according to the procedure described in Chapter 7.

Statistical Analyses

Data are presented as means ± 1 SE. Gill oxygen consumption results were analyzed using a two-way ANOVA, and significant treatment means were identified using Student-Newman-Keuls multiple comparison test ($P<0.05$). Whole-animal oxygen consumption rates, gill enyme activities, and plasma constituents were compared between FW and SW fish using unpaired t-tests ($P<0.05$).

RESULTS

Whole-Animal Measurements

Table 11 shows the whole-animal oxygen consumption rates, gill Na^+,K^+-ATPase and H^+-ATPase activities, and plasma concentrations of cortisol, glucose and ions in the cutthroat trout acclimated to FW and SW in this study. The average oxygen consumption rate in FW fish was slightly (9.8%) higher than measured in SW fish, however the difference was not statistically significant. Gill Na^+,K^+-ATPase activity was significantly (3.4-fold) higher in SW fish compared to FW fish, whereas H^+-ATPase activity decreased about 56% after 2 wk in SW. There were no significant differences in plasma cortisol titres between the FW and SW fish, whereas plasma glucose levels were significantly lower in the SW group. Plasma $[Cl^-]$ and $[K^+]$ were significantly higher in SW fish compared to FW fish, while plasma $[Na^+]$ did not differ between the two groups.

Gill Oxygen Consumption

The oxygen consumption of isolated, perfused FW trout gills bathed in FW (10.1 ± 0.4 μmol O_2/g wet wt) was significantly higher than SW gills bathed in SW (6.7 ± 0.6 μmol O_2/g wet wt/h; Fig. 24). The addition of 0.5 mM ouabain to the saline perfusate resulted in a significant reduction of oxygen consumption in both FW (25%) and SW (37%) gills. The ouabain-sensitive portion of gill oxygen consumption was similar between FW and SW gills (2.5 μmol O_2/g wet wt/h).

Table 12 shows the gill mass measurements for the trout used in the present study. The first gill arch on the left side of the fish, used for the isolated, perfused gill preparations, comprised

Table 11. Oxygen consumption rates, gill Na^+,K^+-ATPase and H^+-ATPase activities, and plasma cortisol, glucose and ion concentrations in cutthroat trout acclimated for 2 wk to fresh water (FW) and seawater (SW).

Variable	FW	SW
Whole-animal MO_2 (mg O_2/kg/h)	106.1 ± 6.8	116.5 ± 4.7
Gill Na^+,K^+-ATPase activity (µmol ADP/mg protein/h)	1.07 ± 0.09	3.65 ± 0.37*
Gill H^+-ATPase activity (µmol ADP/mg protein/h)	1.18 ± 0.20	0.52 ± 0.04*
Plasma Constituents		
Cortisol (ng/mL)	44.7 ± 14.3	33.3 ± 6.7
Glucose (mM)	4.8 ± 0.6	3.4 ± 0.2*
Na^+ (mM)	148.2 ± 1.9	151.1 ± 2.7
K^+ (mM)	2.1 ± 0.2	3.7 ± 0.3*
Cl^- (mM)	133.0 ± 1.9	139.9 ± 1.8*

Values are given as means ± SE (n=8)

An asterisk denotes a significant difference between FW and SW values ($P<0.05$, unpaired t-test)

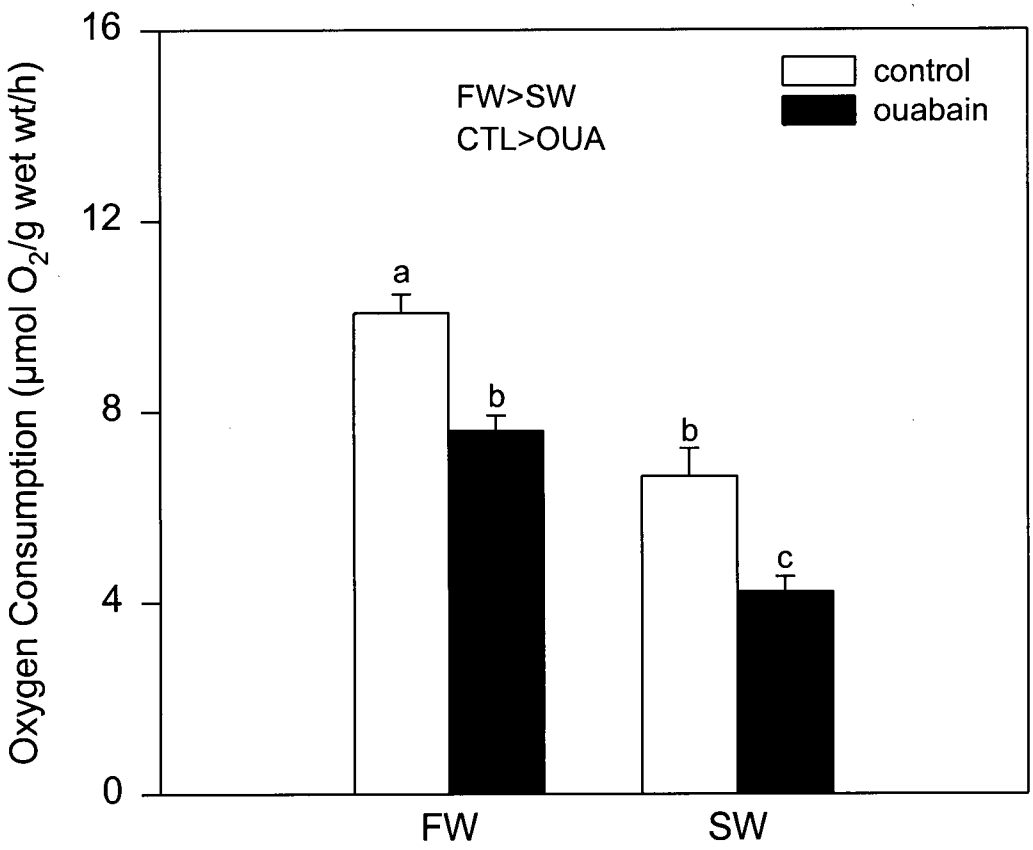

Fig. 24. Oxygen consumption of isolated, saline-perfused cutthroat trout gills in FW and SW, and following the addition of 0.5 mM ouabain to the perfusate. Data are shown as means ± SE (n=6). Inset shows significant treatment effects, and means with different letters are significantly different ($P<0.05$, two-way ANOVA).

Table 12. Body and gill mass of cutthroat trout used in the isolated, perfused gill preparations. FW and SW values did not differ (P>0.05, t-test), therefore the data was pooled.

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

16% of the mass of all eight gill arches, and the gill filaments accounted for 55% of the first arch mass. The mass of all eight gill arches comprised 1.26% of the total body mass, with filament tissue accounting for 0.77% of total body mass.

DISCUSSION

Whole-Animal Measurements

Plasma ion and cortisol levels were similar between the FW and SW cutthroat trout, indicating that the fish were fully acclimated to SW after a 2 wk period. Furthermore, resting metabolic rates in trout acclimated for 2 wk to FW and SW also did not differ in the present study. It appears that, following an initial adjustment period (see Chapter 4), whole-animal oxygen uptake in SW returns close to FW values, as is the case with adult rainbow trout (e.g., Seddiki *et al.* 1995). This is somewhat different than is found with the Mozambique tilapia, where the metabolic cost of SW acclimation is also significant (see Chapter 2), but oxygen consumption rates in long-term acclimated SW fish are half of that measured in FW fish (Ron *et al.* 1995).

Gill Na^+,K^+-ATPase activity was significantly elevated (3-fold) in SW fish, whereas gill H^+-ATPase activity decreased by half after 2 wk in SW. Similar changes in the activity of these enzymes following acclimation to different salinities have been found for rainbow trout by Lin and Randall (1993) and for long-jawed mudsuckers (*Gillichthys mirabilis*) by Kültz and Somero (1995). The direction, and possibly magnitude, of the changes in these ion translocating enzymes reflect the relative importance of H^+-ATPase in active NaCl uptake in FW (Lin and Randall 1995) and Na^+,K^+-ATPase in active NaCl extrusion in SW (McCormick 1995). A small amount of gill H^+-ATPase activity is presumably required in SW fish to maintain acid-base balance

(Wilson *et al.* 1997). Although it is not possible to use *in vitro* ATPase activities to calculate an absolute ATP requirement of the Na^+/K^+-pump and H^+-pump, the results do suggest, at least qualitatively, that the metabolic capacity for ion transport is greater in SW gills than in FW gills (4.2 vs 2.2 ATPase activity units).

General Viability of the Isolated,Perfused Gill Arch Preparation

The isolated, perfused gill arch preparation used in this study was able to approximate *in vivo* conditions, with a few notable exceptions. Pulse frequency (18 per min) was substantially lower than the resting heart rate normally found in rainbow trout (40-70 beats per min; Maxime *et al.* 1991), this being a technical consequence of the peristaltic pump speed required to maintain an appropriate afferent flow rate. This difference in pulse rates did not appear to have an adverse effect on gill performance, as indicated by normal flow rates and pressures. As mentioned previously, the afferent P_{O_2} in the saline was higher in the perfused gill than is found in the *in vivo* situation. This makes it difficult to quantify the relative importance of the perfusate and the bathing medium for oxygen supply to the gill in the intact fish (Lyndon 1994). This does have the advantage, however, of eliminating the gas exchange function of the gill, and therefore the oxygen requirements of the gill tissue alone can be examined (Johansen and Pettersson 1981). In the isolated, perfused trout gill preparation, the oxygen tension in the perfusate leaving the gill was about 12% lower than in the perfusate entering the gill, which is similar to the afferent-efferent P_{O_2} difference found in other perfused gill preparations (e.g., Stagg and Shuttleworth 1984; Lyndon 1994). The remainder of the oxygen is supplied to the tissues by diffusion from the external bath, and was in fact the major source of oxygen for the gill arch in this study. Finally, because only the afferent and efferent branchial arteries were cannulated and the ends of

the arches were sealed off, this effectively occluded the brachial vein. The venous flow was therefore restricted and it is possible that this resulted in an elevation in the compartmental pressure of the central venous sinus, and a redirection of flow into the arterial system. A similar approach was taken by Johansen and Pettersson (1981) in their measurement of oxygen consumption in isolated, perfused cod gills. Cannulation of the branchial vein in trout is a relatively lengthy procedure (Ishimatsu *et al.* 1988), and it was therefore not possible to both cannulate the branchial vein and maintain the viability of the gill for oxygen consumption measurements. The isolated, saline-perfused gill preparation of Lyndon (1994) allowed the venous flow to drain into the external bath, however this was not done in the present study to maintain the ionic gradients between the internal and external media. Despite these deficiencies in the preparation, it was possible to measure whole gill oxygen consumption, as discussed below.

Gill Oxygen Consumption

The average oxygen consumption of FW cutthroat trout gills was 10.1 ± 0.4 μmol O_2/g wet wt/h. This value includes both the gill filaments, and the bone, skin and muscle of the arch. The gill tissue oxygen consumption can be calculated from this value, knowing the portion of the arch that is filaments (55% for the first arch), and assuming that the bony arch has a much lower rate of oxygen consumption (~2.5 μmol O_2/g/h; Itazawa and Oikawa 1983). According to this calculation, the oxygen consumption rate of gill tissue from the FW-adapted trout was 17.4 μmol O_2/g/h, which is quite comparable to the value reported for FW trout gill tissue in Chapter 7 (20 μmol O_2/g/h). This result indicates that the isolated, perfused gill arch preparation used in the present study provided reliable estimates of gill oxygen consumption rates.

The oxygen consumption of SW trout gills bathed in SW was significantly (33%) lower than FW gills bathed in FW. Similar decreases in gill tissue oxygen consumption rates were found for cutthroat trout by Holmes and Stott (1960) after 7 d in SW, and for rainbow trout by Leray *et al.* (1981) following a 10 d SW exposure period. The reasons for this decrease are not clear, as the net increase in ATPase activity measured in SW gills would predict that the gill respiration rates should be higher in SW, if ion pumping costs were a significant portion of total gill respiration. Leray *et al.* (1981) found that after a marked drop in ATP/ADP ratios and energy charge during first 3 d of SW acclimation in the rainbow trout, the adenylate pool returned to initial levels in fully adapted SW fish (day 10). This high energy demand in the early stages of SW acclimation is consistent with the changes that occur in gill carbohydrate metabolism (Soengas *et al.* 1995), and may be related to the increased recruitment of chloride cells and Na^+,K^+-ATPase that occurs after 3-4 d in SW (Folmar and Dickhoff 1980). In a well acclimated SW trout, however, the energy requirements of the gill appear to decrease in SW relative to a FW fish. It is possible that protein synthesis costs, which can approach 80% of total respiration in some tissues (e.g., Pannevis and Houlihan 1992), are higher in FW gills than in SW gills, however this has yet to be determined.

The addition of ouabain to the saline perfusate significantly reduced oxygen consumption in both FW (25%) and SW (37%) gills, but the ouabain-sensitive portion was similar between the FW and SW gills (2.5 μmol O_2/g/h). The difference in the percentages is due to the higher total gill oxygen consumption in the FW gills. Thus it appears that the oxygen cost of the Na^+/K^+-pump alone is not sufficient to explain the difference in total gill respiration between FW and SW trout gills. The ouabain-induced decrease in oxygen consumption in the intact FW gill arch was similar to that found in the FW gill tissue (Chapter 7), suggesting that the cost of the

Na^+/K^+-pump in the FW trout gill is about 20-25% of total tissue respiration. Stagg and Shuttleworth (1982a) also found that ouabain significantly reduced the oxygen consumption of excised gill tissue from both FW- and SW-adapted flounder (*P. flesus*) by about 25%. It should be noted, however, that interpretating ouabain-sensitive respiration rates from isolated, perfused gill arches in terms of ion transport costs can be confounded by two factors. Firstly, 45% of the gill arch mass is composed of supporting tissue (e.g., bone, muscle, skin) and the Na^+/K^+-pumps in these tissues, which are probably less abundant than in the gill filaments, are not directly involved in NaCl extrusion. Secondly, there is a possibility that ouabain can have a vasosensitive effect on the branchial arteries, although the data is inconclusive. For example, Farmer and Evans (1981) found that adding ouabain to the perfusate caused a marked vasoconstriction and increase in afferent pressure in isolated, perfused gills of the pinfish (*Lagodon rhomboides*), whereas Stagg and Shuttleworth (1982b) found that ouabain had only a slight effect on vascular resistance in perfused gills of the flounder (*P. flesus*). There were no noticable effects of ouabain on afferent pressure in this study, although the flow rates were quite variable among preparations (CV=44%). The use of an inhibitor specific to the gill epithelia, such as bafilomycin, would overcome some of the potential vasosensitive effects that ouabain may have on arterial pressure and flow. Bafilomycin could not be used in the present study to inhibit H^+-ATPase activity in perfused FW gills, due to the prohibitive costs involved in purchasing the amounts needed for an effective concentration (>1 μM) in the external bath (160 mL). The results in Chapter 7 suggest that the oxygen cost for the proton pump would be similar to the Na^+/K^+-pump in FW, and this would increase the ion transport-related costs in the FW gill. The effect of specific ion pump inhibitors on the oxygen consumption of the isolated, perfused gill arch requires further study.

The oxygen consumption of isolated, perfused gill arches have been compared on a total mass basis to the resting metabolic rates of the intact fish, as an indication of the relative energy requirements of the gill. The value of 7% calculated by Johansen and Pettersson (1981) for the marine cod gill has often been quoted in this regard (e.g., Mommsen 1984b). In making these estimates, most workers have assumed that the gill arches are of equal size (e.g., Johansen and Petterson 1981; Lyndon 1994). In fact, the first gill arch is larger than the others (16% of the total gill arch mass in cutthroat trout) and this will therefore overestimate the contribution of gill oxygen consumption. The mass of all eight gill arches in the trout comprised about 1.3% of the total body mass. For an averaged-size trout in this study (~310 g), gill oxygen consumption accounted for 3.9% of resting metabolic rate in FW trout and 2.4% of resting metabolic rate in SW trout. The FW value is comparable to the estimates obtained using excised gill tissue from FW fish (e.g., Itazawa and Oikawa 1983; Chapter 7). The value for SW trout gills is lower than has been estimated for cod gills bathed in saline (7%, Johansen and Pettersson 1981) and for flounder gills bathed in SW (31%, Lyndon 1994), although it should be noted that these authors probably overestimated gill mass (see above) and used literature values for resting metabolic rates that were much lower (78 and 31 mg O_2/kg/h, respectively) than I have measured for SW cutthroat trout (116 mg O_2/kg/h). A substantial difference in the standard metabolic rate of trout and flounder in SW has also been reported in other studies (e.g., Rao 1968; Steffenson *et al.* 1982; Kirschner 1993), and this is likely due to a difference in lifestyles between the active trout and more sedentary flounder. Nevertheless, it appears that the gill oxygen requirements in SW-adapted cutthroat trout are lower than in the marine flounder. This is also reflected in the higher mass-specific gill oxygen consumption rates measured in flounder gills bathed in 10°C SW (12.1 μmol O_2/g/h; Lyndon 1994) than was measured in SW trout gills in this study (6.7 μmol O_2/g/h).

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

GENERAL DISCUSSION

The experiments presented in this thesis were conducted with several fish species, involved both acute and chronic exposures to different salinities, and covered several levels of biological organization from the whole-animal to the tissue level. From this diversity of experimental approaches, there are a number of general observations that can be made regarding the study of osmoregulatory energetics in fish.

Acute salinity exposure studies using tilapia (Chapter 2) and trout (Chapter 4) suggest that there is a significant metabolic cost to SW acclimation in some species. Physiological adjustments must be made for the fish to adapt to the new physical and chemical environment, including changes in hormone profiles (e.g., cortisol, growth hormone), intermediary metabolism (e.g., glycolytic and gluconeogenic enzymes), and osmoregulatory organs (e.g., gills). The cortisol-implant study (Chapter 5) illustrated the some of the hormonal changes that occur during SW acclimation in salmonids may result in changes to whole-animal oxygen consumption that are not directly related to the energy demands of the osmoregulatory organs. The acute exposure of dolphin fish to a lower salinity resulted in a decrease in metabolic rate and gill Na^+,K^+-ATPase activity (Chapter 1), suggesting a possible reduction in osmoregulatory costs. It should be noted that a number of other marine fish species respond quite differently (i.e., increase oxygen uptake at lower salinities; Prosser 1973), emphasizing the influence that species and life habits can have on whole-animal responses to salinity change. The chronic salinity acclimation studies with coho salmon (Chapter 3) and trout (Chapter 8), demonstrate that once a euryhaline salmonid has adapted to SW, most blood chemistry variables return to a steady state, and there are no significant differences in the whole-animal oxygen consumption rates between FW, ISO and SW. In such cases, whole-animal

metabolic rate measurements in different salinities cannot be used to quantify the energy required for osmoregulation. There are actually only a few studies that show reduced oxygen uptake rates in ISO relative to FW and SW (e.g., Rao 1968; Farmer and Beamish 1969), while there are many studies that indicate at least five other metabolic response patterns to salinity change in teleost fish (see Morgan and Iwama 1991; Chapter 1). The metabolic cost of acclimating to a new salinity is certainly an important consideration in the study of fish energetics, but it does not provide quantitative information on the direct energy requirements of the osmoregulatory organs in regulating plasma ions.

The experimental approach taken in Chapters 6-8 was to use isolated osmoregulatory tissues, in order to separate the energetic aspects of NaCl transport from other salinity-related metabolic differences in the whole-animal. Ouabain-inhibited oxygen consumption in rectal gland tissue from the spiny dogfish indicated that the Na^+/K^+-pump accounts for a large portion (55%) of tissue respiration, but this amounts to only about 0.5% of standard metabolism when expressed on the basis of total fish mass. A similar study with FW cutthroat trout gill tissue suggested that the oxygen costs of the Na^+/K^+-pump and H^+-pump represent about 37% of tissue respiration, accounting for 1.8% of resting metabolic rate. These values are in good agreement with theoretical calculations (e.g., Eddy 1982; Kirschner 1993, 1995), and indicate that the direct costs for NaCl transport in the dogfish rectal gland and FW trout gill are a relatively small portion of the fish's total energy budget. It can be argued that the oxygen consumption of the entire osmoregulatory organ should be used to represent osmoregulatory costs, as the ion pumps obviously do not operate in isolation. Turnover of chloride cells and Na^+,K^+-ATPase enzymes, for example, would require a high rate of protein synthesis (Houlihan *et al.* 1995). This would increase the values to about 1% for the dogfish rectal gland and 4% for the FW trout gill, and would also suggest that the FW trout

gill consumes more oxygen for osmoregulatory purposes than the SW trout gill (4% vs 2.5%, Chapter 8).

The experiments in Section 3 of the thesis concentrated mainly on the energetics of NaCl transport in the gills (and rectal gland in dogfish). There are, of course, other osmoregulatory organs (i.e., kidney, intestine) and ions (e.g., Mg^{2+}, Ca^{2+}) that involve energy-requiring ATPases. These components were not addressed in the thesis, but deserve some mention here. The kidney plays an important role in reabsorbing Na^+ and Cl^- in FW teleosts, and the intestine actively takes up NaCl in marine fish, using the Na^+,K^+-pump, to drive water absorption (Evans 1993). Theoretical estimates for the cost of NaCl transport have been provided by Kirschner (1993, 1995) for the kidney in FW (0.9% of standard metabolic rate) and the intestine in SW trout (1.9%), and these values would add to the overall cost of osmoregulation in fish. In addition to Na^+ and Cl^- (and possibly K^+), there are passive movements of divalent ions between the blood and water that require active regulation. Magnesium poses a problem in SW where its concentration is relatively high (about 40 mM) compared to plasma (about 1 mM). The kidney actively excretes Mg^{2+}, but its theoretical energy cost is not known (Kirschner 1995). Another variable is Ca^{2+} regulation; Ca^{2+} is actively transported inward in the FW gill and kidney via Ca^{2+}-ATPase (e.g., Flik and Verbost 1993; Bijvelds *et al.* 1995), and is excreted by the kidney in SW fish. These factors will all contribute to the total energy cost of osmoregulation in fish, and have not been examined in detail on an experimental level.

In conclusion, the results of this thesis indicate that the direct energy costs for ion transport in fish are probably closer to theoretical estimates (2-4% of resting metabolic rate), rather than the higher values (20-30%) obtained from whole-animal metabolic rate measurements in different salinities (see Withers 1992). The differences between the two methods are likely related to the

metabolic cost of other salinity-related activities (e.g., endocrine responses) that occur in FW and SW fish.

REFERENCES

Abdel Magid, A.M., and Babiker, M.M. 1975. Oxygen consumption and respiratory behaviour of three Nile fishes. Hydrobiologia 46: 359-367.

Abo Hegab, S., and Hanke, W. 1984. The significance of cortisol for osmoregulation in carp (*Cyprinus carpio*) and tilapia (*Sarotherodon mossambicus*). Gen. Comp. Endocrinol. 54: 409-417.

Abo Hegab, S., and Hanke, W. 1986. Electrolyte changes, cell volume regulation and hormonal influences during acclimation of rainbow trout (*Salmo gairdneri*) to saltwater. Comp. Biochem. Physiol. 83A: 47-52.

Assem, H., and Hanke, W. 1979a. Volume regulation of muscle cells in the euryhaline teleost, *Tilapia mossambica*. Comp. Biochem. Physiol. 64A: 17-23.

Assem, H., and Hanke, W. 1979b. Concentrations of carbohydrates during osmotic adjustment of the euryhaline teleost, *Tilapia mossambica*. Comp. Biochem. Physiol. 64A: 5-16.

Assem, H., and Hanke, W. 1981. Cortisol and osmotic adjustment of the euryhaline teleost, *Sarotherodon mossambicus*. Gen. Comp. Endocrinol. 43: 370-380.

Auperin, B., Leguen, I., Rentier-Delrue, F., Smal, J., and Prunet, P. 1995. Absence of a tiGH effect on adaptability to brackish water in tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol. 97: 145-159.

Avella, M., Young, G., Prunet, P., and Schreck, C.B. 1990. Plasma prolactin and cortisol concentrations during salinity challenges of coho salmon (*Oncorhynchus kisutch*) at smolt and post-smolt stages. Aquaculture 91: 359-372.

Ayson, F.G., Kaneko, T., Tagawa, M., Hasegawa, S., Grau, E.G., Nishioka, R.S., King, D.S., Bern, H.A., and Hirano, T. 1993. Effects of acclimation to hypertonic environment on plasma and pituitary levels of two prolactins and growth hormone in two species of tilapia, *Oreochromis mossambicus* and *Oreochromis niloticus*. Gen. Comp. Endocrinol. 89: 138-148.

Balarin, J.D., and Hatton, J.P. 1979. Tilapia: a guide to their biology and culture in Africa. University of Stirling, UK.

Balm, P.H.M., Haenen, H.E.M.G., and Wendelaar Bonga, S.E. 1995. Regulation of interrenal function in freshwater and seawater adapted tilapia (*Oreochromis mossambicus*). Fish Physiol. Biochem. 14: 37-47.

Barton, B.A., and Iwama, G.K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1: 3-26.

Barton, B.A., and Schreck, C.B. 1987. Metabolic cost of acute physical stress in juvenile steelhead. Trans. Am. Fish. Soc. 116: 257-263.

Bath, R.N., and Eddy, F.B. 1979. Ionic and respiratory regulation in rainbow trout during rapid transfer to seawater. J. Comp. Physiol. 134: 351-357.

Beamish, F.W.H. 1978. Swimming capacity. *In* Fish physiology, vol. 7. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, New York. pp. 101-187.

Behnke, R.J. 1992. Native trout of western North America. American Fisheries Society Monograph 6. American Fisheries Society, Bethesda, Maryland. 275 p.

Benetti, D.D. 1992. Bioenergetics and growth of dolphin, *Coryphaena hippurus*. Ph.D. thesis, University of Miami, Florida.

Benetti, D.D., Brill, R.W., and Kraul, S.A. 1995. The standard metabolic rate of dolphin fish. J. Fish Biol. 46: 987-996.

Bentley, P.J., Maetz, J., and Payan, P. 1976. A study of the unidirectional fluxes of Na and Cl across the gills of the dogfish *Scyliorhinus canicula* (Chondrichthyes). J. Exp. Biol. 64: 629-637.

Berenbrink, M., and Pelster, B. 1996. Acid secretion of mitochondria rich cells from the teleost pseudobranch. Society for Experimental Biology Annual Meeting, University of Lancaster. Abstract no. A1.34.

Bertram, G., Schleithoff, L., Zimmerman, P., and Wessing, A. 1991. Bafilomycin A_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*: Is a vacuolar-type ATPase involved in ion and fluid secretion? J. Insect Physiol. 37: 201-209.

Bijvelds, M.J.C., van der Heijden, A.J.H., Flik, G., Verbost, P.M., Kolar, Z.I., and Wendelaar Bonga, S.E. 1995. Calcium pump activities in the kidneys of *Oreochromis mossambicus*. J. Exp. Biol. 198: 1351-1357.

Bindon, S.D., Fenwick, J.C., and Perry, S.F. 1994. Branchial chloride cell proliferation in the rainbow trout, *Oncorhynchus mykiss*: implications for gas transfer. Can. J. Zool. 72: 1395-1402.

Bisbal, G.A., and Specker, J.L. 1991. Cortisol stimulates hypo-osmoregulatory ability in Atlantic salmon, *Salmo salar*. J. Fish Biol. 39: 421-432.

Blackburn, J., and Clarke, W.C. 1987. Revised procedure for the 24 hour seawater challenge test to measure seawater adaptability of juvenile salmonids. Can. Tech. Rep. Fish. Aquat. Sci. 1515: 35 p.

Borski, R.J., Yoshikawa, J.S.M., Madsen, S.S., Nishioka, R.S., Zabetian, C., Bern, H.A., and Grau, E.G. 1994. Effects of environmental salinity on pituitary growth hormone content and cell activity in the euryhaline tilapia, *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 95: 483-494.

Bowman, E.J., and Bowman, B.J. 1988. Purification of vacuolar membranes, mitochondria, and plasma membranes from *Neurospora crassa* and modes of discriminating among the different H^+-ATPases. Methods Enzymol. 157: 562-573.

Bowman, E.J., Siebers, A., and Altendorf, K. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. 85: 7972-7976.

Brett, J.R. 1964. The respiratory metabolism and swimming performance of young sockeye salmon. J. Fish. Res. Board Can. 21: 1183-1226.

Brett, J.R., and Glass, N.R. 1973. Metabolic rates and critical swimming speeds of sockeye salmon (*Oncorhynchus nerka*) in relation to size and temperature. J. Fish. Res. Board Can. 30: 379-387.

Brett, J.R., and Zala, C.A. 1975. Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. J. Fish. Res. Board Can. 32: 2479-2486.

Brill, R.W. 1996. Selective advantages conferred by the high performance physiology of tunas, billfishes, and dolphin fish. Comp. Biochem. Physiol. 113A: 3-15.

Bullivant, J.S. 1961. The influence of salinity on the rate of oxygen consumption of young quinnat salmon (*Oncorhynchus tshawytscha*). N.Z. J. Sci. 4: 381-391.

Burger, J.W. 1965. Roles of the rectal gland and the kidneys in salt and water excretion in the spiny dogfish. Physiol. Zool. 38: 191-196.

Burger, J.W., and Hess, W.N. 1960. Function of the rectal gland in the spiny dogfish. Science 131: 670-671.

Cameron, J.N., and Iwama, G.K 1989. Compromises between ionic regulation and acid-base regulation in aquatic animals. Can. J. Zool. 67: 3078-3084.

Caulton, M.S. 1977. The effect of temperature on routine metabolism in *Tilapia rendalli* Boulenger. J. Fish Biol. 11: 549-553.

Caulton, M.S. 1978. The effect of temperature and mass on routine metabolism in *Sarotherodon (Tilapia) mossambicus* (Peters). J. Fish Biol. 13: 195-201.

Cech, J.J. 1990. Respirometry *In* Methods for fish biology. *Edited by* C.B. Schreck and P.B. Moyle. American Fisheries Society, Bethesda. pp. 335-363.

Chan, D.K.O., Phillips, J.G., and Chester Jones, I. 1967. Studies on electrolyte changes in the lip-shark, *Hemiscyllium plagiosum* (Bennett), with special reference to hormonal influence on the rectal gland. Comp. Biochem. Physiol. 23: 185-198.

Chan, D.O., and Woo, N.Y.S. 1978. Effect of cortisol on the metabolism of the eel, *Anguilla japonica*. Gen. Comp. Endocrinol. 35: 205-215.

Clarke, W.C. 1973. Sodium-retaining bioassay of prolactin in the intact teleost *Tilapia mossambica* acclimated to sea water. Gen. Comp. Endocrinol. 21: 498-512.

Clarke, W.C., Shelbourn, J.E., and Brett, J.R. 1981. Effect of artificial photoperiod cycles, temperature, and salinity on growth and smolting in underyearling coho (*Oncorhynchus kisutch*), chinook (*O. tshawytscha*), and sockeye (*O. nerka*) salmon. Aquaculture 22: 105-116.

Colt, J. 1984. Computation of dissolved gas concentrations in water as functions of temperature, salinity, and pressure. American Fisheries Society, Special Publication 14. 154 p.

Crean, P.B., Stronach, J.A., and Murty, T.S. 1988. Salt and freshwater exchange on Roberts Bank, British Columbia. Water Poll. Res. J. Can. 23: 160-178.

Culloty, S.C., Cross, T.F., and O'Halloran, J. 1991. Comparison of two methods of hemoglobin measurement in rainbow trout, *Oncorhynchus mykiss*. Aquacult. Fish. Manage. 22: 379-383.

Dange, A.D. 1985. Branchial Na^+-K^+-ATPase activity during osmotic adjustments in two freshwater euryhaline teleosts, tilapia (*Sarotherodon mossambicus*) and orange chromid (*Etroplus maculatus*). Mar. Biol. 87: 101-107.

Dharmamba, M., Bornancin, M., and Maetz, J. 1975. Environmental salinity and sodium and chloride exchanges across the gill of *Tilapia mossambica*. J. Physiol., Paris 70: 627-636.

Dickhoff, W.W., and Sullivan, C.V. 1987. Involvement of the thyroid gland in smoltification, with special reference to metabolic and developmental processes. American Fisheries Society Symposium 1: 197-210.

Dröse, S., and Altendorf, K. 1997. Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. J. Exp. Biol. 200: 1-8.

Dröse, S., Bindseil, K.U., Bowman, E.J., Siebers, A., Zeeck, A., and Altendorf, K. 1993. Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphatases. Biochemistry 32: 3902-3906.

Eckert, R., Randall, D., and Augustine, G. 1988. Animal physiology: mechanisms and adaptations. W.H. Freeman and Company, New York. 683 p.

Eddy, F.B. 1975. The effect of calcium on gill potentials and on sodium and chloride fluxes in the goldfish, *Carassius auratus*. J. Comp. Physiol. 96: 131-142.

Eddy, F.B. 1982. Osmotic and ionic regulation in captive fish with particular reference to salmonids. Comp. Biochem. Physiol. 73B: 125-141.

Ehrenfeld, J., and Klein, U. 1997. The key role of the H^+ V-ATPase in acid-base balance and Na^+ transport processes in frog skin. J. Exp. Biol. 200: 247-256.

Eib, D.W., and Hossner, K.L. 1985. The effects of cortisol and actinomycin D injections on chloride cells and branchial Na^+,K^+-ATPase in rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 59: 449-452.

Evans, D.H. 1979. Ionic and osmotic regulation in fish. *In* Osmotic and ionic regulation in animals. *Edited by* G.M.D. Maloiy. Academic Press, Orlando. pp. 305-390.

Evans, D.H. 1984. The roles of gill permeability and transport mechanisms in euryhalinity. *In* Fish physiology, vol. 10B. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, New York. pp. 239-283.

Evans, D.H. 1993. Osmotic and ionic regulation. *In* The physiology of fishes. *Edited by* D.H. Evans. CRC Press, Boca Raton. pp. 315-341.

Evans, D.H., Claiborne, J.B., Farmer, L., Mallery, C., and Krasny, E.J. 1982. Fish gill ionic transport: methods and models. Biol. Bull. 163: 108-130.

Evans, R.M., Purdie, F.C., and Hickman, C.P. 1962. The effect of temperature and photoperiod on the respiratory metabolism of rainbow trout (*Salmo gairdneri*). Can. J. Zool. 40: 107-118.

Fänge, R. 1992. Fish blood cells *In* Fish ohysiology, vol. 12B. *Edited by* W.S. Hoar, D.J. Randall and A.P. Farrell. Academic Press, San Diego. pp. 1-54.

Farmer, G.J., and Beamish, F.W.H. 1969. Oxygen consumption of *Tilapia nilotica* in relation to swimming speed and salinity. J. Fish. Res. Board Can. 26: 2807-2821.

Farmer, L.L., and Evans, D.H. 1981. Chloride extrusion in the isolated perfused teleost gill. J. Comp. Physiol. 141: 471-476.

Febry, R., and Lutz, P. 1987. Energy partitioning in fish: the activity-related cost of osmoregulation in a euryhaline cichlid. J. Exp. Biol. 128: 63-85.

Flik, G., and Verbost, P.M. 1993. Calcium transport in fish gills and intestine. J. Exp. Biol. 184: 17-29.

Folmar, L.C., and Dickhoff, W.W. 1980. The parr-smolt transformation (smoltification) and seawater adaptation in salmonids. A review of selected literature. Aquaculture 21: 1-37.

Folmar, L.C., Dickhoff, W.W., Mahnken, C.V.W., and Waknitz, F.W. 1982. Stunting and parr-reversion during smoltification of coho salmon (*Oncorhynchus kisutch*). Aquaculture 28: 91-104.

Forgac, M. 1989. Structure and function of vacuolar class of ATP-driven proton pumps. Physiol. Rev. 69: 765-796.

Foskett, J.K., Bern, H.A., Machen, T.E., and Conner, M. 1983. Chloride cells and the hormonal control of teleost fish osmoregulation. J. Exp. Biol. 106: 255-281.

Foskett, J.K., Lodgson, C.D., Turner, T., Machen, T.E., and Bern, H.A. 1981. Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. J. Exp. Biol. 93: 209-224.

Franklin, C.E., Davison, W., and Forster, M.E. 1992a. Seawater adaptability of New Zealand's sockeye (*Oncorhynchus nerka*) and chinook salmon (*O. tshawytscha*): physiological correlates of smoltification and seawater survival. Aquaculture 102: 127-142.

Franklin, C.E., Forster, M.E., and Davison, W. 1992b. Plasma cortisol and osmoregulatory changes in sockeye salmon transferred to sea water: Comparison between successful and unsuccessful adaptation. J. Fish Biol. 41: 113-122.

Gamperl, A.K., Vijayan, M.M., and Boutilier, R.G. 1994. Experimental control of stress hormone levels in fishes: techniques and applications. Rev. Fish Biol. Fish. 4: 215-255.

Gehrke, P.C., Fidler, L.E., Mense, D.C., and Randall, D.J. 1990. A respirometer with controlled water quality and computerized data acquisition for experiments with swimming fish. Fish Physiol. Biochem. 8: 61-67.

Greger, R., Schlatter, E., and Gogelein, H. 1986. Sodium chloride secretion in rectal gland of dogfish, *Squalus acanthias*. News Physiol. Sci. 1: 134-136.

Hanson, D., and Johansen, K. 1970. Relationship of gill ventilation and perfusion in Pacific dogfish, *Squalus suckleyi*. J. Fish. Res. Board. Can. 27: 551-564.

Heath, D.D. 1993. Genetic, environmental, and physiological factors involved in the precocious sexual maturation of chinook salmon (*Oncorhynchus tshawytscha*). Ph.D. thesis, University of British Columbia, Vancouver, B.C. 166 p.

Heisler, N. 1993. Acid-base regulation. *In* The physiology of fishes. *Edited by* D.H. Evans. CRC Press, Boca Raton. pp. 343-378.

Hida, T.S., Harada, J.R., and King, J.E. 1962. Rearing tilapia for tuna bait. Fishery Bulletin 62: 1-20.

Higgins, P.J. 1985. Metabolic differences between Atlantic salmon (*Salmo salar*) parr and smolts. Aquaculture 45: 33-53.

Hille, S. 1982. A literature review of the blood chemistry of rainbow trout, *Salmo gairdneri* G. J. Fish Biol. 20: 535-569.

Hirano, T. 1986. The spectrum of prolactin actions in teleosts. *In* Comparative endocrinology: developments and directives. *Edited by* C.L. Ralph. Alan Liss, Inc., New York. pp. 53-74.

Hoar, W.S. 1988. The physiology of smolting salmonids. *In* Fish physiology, vol. 11B. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, San Diego. pp. 275-343.

Holmes, W.N. and Donaldson, E.M. 1969. The body compartments and the distribution of electrolytes. *In* Fish Physiology, vol. 1. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, New York. pp. 1-89.

Holmes, W.N., and Stott, G.H. 1960. Studies on the respiration rates of excretory tissues in the cutthroat trout (*Salmo clarki clarki*). II. Effect of transfer to sea water. Physiol. Zool. 33: 15-20.

Houlihan, D.F., Carter, C.G., and McCarthy, I.D. 1995. Protein synthesis in fish. *In* Biochemistry and molecular biology of fishes, vol. 4. *Edited by* P.W. Hochachka and T.P. Mommsen. Elsevier Science, Amsterdam. pp. 191-220.

Houston, A.H. 1990. Blood and circulation. *In* Methods for fish biology. *Edited by* C.B. Schreck and P.B. Moyle. American Fisheries Society, Bethesda. pp. 273-334.

Hughes, G.M. 1972. Morphometrics of fish gills. Respir. Physiol. 14: 1-25.

Hughes, G.M. 1984. General anatomy of the gills. *In* Fish physiology, vol. 10A. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, Orlando. pp. 1-72.

Hwang, P.P. 1987. Tolerance and ultrastructural responses of branchial chloride cells to salinity changes in the euryhaline teleost *Oreochromis mossambicus*. Mar. Biol. 94: 643-649.

Hwang, P.P., Sun, C.M., and Wu, S.M. 1989. Changes of plasma osmolality, chloride concentration and gill Na^+,K^+-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. Mar. Biol. 100: 295-299.

Ishimatsu, A., Iwama, G.K., and Heisler, N. 1988. *In vivo* analysis of partitioning of cardiac output between systemic and central venous sinus circuits in rainbow trout: a new approach using chronic cannulation of the branchial vein. J. Exp. Biol. 137: 75-88.

Itazawa, Y., and Oikawa, S. 1983. Metabolic rates in excised tissues of carp. Experientia 39: 160-161.

Iwai, T.I., Ako, H., and Yasukochi, L.E. 1992. Use of beef liver in the diet of juvenile mahimahi, *Coryphaena hippurus* L. World Aquacult. 23(3): 49-51.

Iwama, G.K., Morgan, J.D., and Barton, B.A. 1995. Simple field methods for monitoring stress and general condition of fish. Aquacult. Res. 26: 273-282.

Jampol, L.M., and Epstein, F.H. 1970. Sodium-potassium-activated adenosine triphosphatase and osmotic regulation by fishes. Am. J. Physiol. 218: 607-611.

Job, S.V. 1969. The respiratory metabolism of *Tilapia mossambica* (Teleostei). I. The effect of size, temperature and salinity. Mar. Biol. 2: 121-126.

Johansen, K., and Pettersson, K. 1981. Gill O_2 consumption in a teleost fish, *Gadus morhua*. Respir. Physiol. 44: 277-284.

Karnaky, K.J. 1986. Structure and function of the chloride cell of *Fundulus heteroclitus* and other teleosts. Amer. Zool. 26: 209-224.

Karnaky, K.J., Kinter, L.B., Kinter, W.B., and Stirling, C.E. 1976. Teleost chloride cell. II. Autoradiographic localization of gill Na,K-ATPase in killifish *Fundulus heteroclitus* adapted to low and high salinity environments. J. Cell Biol. 70: 157-177.

Kelly, J.M., Summers, M., Park, H.S., Milligan, L.P., and McBride, B.W. 1991. Cellular energy metabolism and regulation. J. Dairy Sci. 74: 678-694.

Keys, A.B., and Willmer, E.N. 1932. Chloride-secreting cells in the gills of fishes with special reference to the common eel. J. Physiol. 76: 368-377.

Kirschner, L.B. 1980. Comparison of vertebrate salt-excreting organs. Am. J. Physiol. 238: R219-R223.

Kirschner, L.B. 1993. The energetics of osmotic regulation in ureotelic and hypoosmotic fishes. J. Exp. Zool. 267: 19-26.

Kirschner, L.B. 1995. Energetics of osmoregulation in freshwater vertebrates. J. Exp. Zool. 271: 243-252.

Kormanik, G.A., and Totten, R.K. 1993. Ion transport-related oxygen consumption by isolated gills and rectal gland of embryos of the spiny dogfish, *Squalus acanthias*. Bull. Mt. Desert Isl. Biol. Lab. 32: 107-109.

Kraul, S. 1991. Hatchery methods for the mahimahi, *Coryphaena hippurus*, at Waikiki Aquarium. *In* CRC handbook of mariculture, vol. 2. *Edited by* J.P. McVey. CRC Press, Boca Rotan, Florida. pp. 241-250.

Kraul, S. 1993. Larviculture of the mahimahi, *Coryphaena hippurus*, in Hawaii, USA. J. World Aquacult. Soc. 24: 410-421.

Kültz, D., and Somero, G. 1995. Osmotic and thermal effects on *in situ* ATPase activity in permeabilized gill epithelial cells of the fish *Gillichthys mirabilis*. J. Exp. Biol. 198: 1883-1894.

Kültz, D., Bastrop, R., Jürss, K., and Siebers, D. 1992. Mitochondria-rich (MR) cells and the activities of Na^+/K^+-ATPase and carbonic anhydrase in the gill and opercular epithelium of *O. mossambicus* adapted to various salinities. Comp. Biochem. Physiol. 102B: 293-301.

Kuwaye, T.T., Okimoto, D.K., Shimoda, S.K., Howerton, R.D., Lin, H.R., Pang, P.K.T., and Grau, E.G. 1993. Effect of 17α-methyltestosterone on the growth of the euryhaline tilapia, *Oreochromis mossambicus*, in fresh water and in sea water. Aquaculture 113: 137-152.

Langdon, J.S., Thorpe, J.E., and Roberts, R.J. 1984. Effects of cortisol and ACTH on gill Na^+, K^+-ATPase, SDH and chloride cells in juvenile Atlantic salmon, *Salmo salar*. Comp. Biochem. Physiol. 77A: 9-12.

Larsen, B.K., and Jensen, F.B. 1983. Arterial P_{O_2}, acid-base status, and red cell nucleoside triphosphates in rainbow trout transferred from fresh water to 20‰ sea water. J. Fish Biol. 42: 611-614.

Laurent, P. 1982. Structure of vertebrate gills. *In* Gills. *Edited by* D.F. Houlihan, J.C. Rankin and T.J. Shuttleworth. Cambridge University Press, Cambridge. pp. 25-43.

Laurent, P., and Hebibi, N. 1989. Gill morphometry and fish osmoregulation. Can. J. Zool. 67: 3055-3068.

Laurent, P., and Perry, S.F. 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell Tissue Res. 259: 429-442.

Laurent, P., Hobe, H., and Dunel-Erb, S. 1985. The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. Cell Tissue Res. 240: 675-692.

Leach, G.J., and Taylor, M.H. 1982. The effects of cortisol treatment on carbohydrate and protein metabolism in *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 48: 76-83.

Leray, C., Colin, D.A., and Florentz, A. 1981. Time course of osmotic adaptation and gill energetics of rainbow trout (*Salmo gairdneri*) following abrupt changes in external salinity. J. Comp. Physiol. 144: 175-181.

Lin, H., and Randall, D.J. 1993. H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. J. Exp. Biol. 180: 163-174.

Lin, H., and Randall, D.J. 1995. Proton pumps in fish gills. *In* Cellular and molecular approaches to fish ionic regulation. Fish physiology, vol. 14. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, San Diego. pp. 229-255.

Lin, H., Pfeiffer, D.C., Vogl, A.W., Pan, J., and Randall, D.J. 1994. Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. J. Exp. Biol. 195: 169-183.

Lipskaya, N.Y. 1974. Metabolic rates in the young of some tropical fish species. J. Ichthyol. 14: 934-943.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lutnesky, M.M.F., and Szyper, J.P. 1990. Respiratory and behavioral responses of juvenile dolphin fish to dissolved oxygen concentration. Prog. Fish-Cult. 52: 178-185.

Lyndon, A.R. 1994. A method for measuring oxygen consumption in isolated perfused gills. J. Fish Biol. 44: 707-715.

Madsen, S.S. 1990a. Cortisol treatment improves the development of hypo-osmoregulatory mechanisms in the euryhaline rainbow trout, *Salmo gairdneri*. Fish Physiol. Biochem. 8: 45-52.

Madsen, S.S. 1990b. The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). Gen. Comp. Endocrinol. 79: 1-11.

Madsen, S.S., Larsen, B.K., and Jensen, F.B. 1996. Effects of freshwater to seawater transfer on osmoregulation, acid-base balance and respiration in river migrating whitefish (*Coregonus lavaretus*). J. Comp. Physiol. 166: 101-109.

Maxime, V., Boeuf, G., Pennec, J.P., and Peyraud, C. 1989. Comparative study of the energetic metabolism of Atlantic salmon (*Salmo salar*) parr and smolts. Aquaculture 82: 163-171.

Maxime, V., Pennec, J.P., and Peyraud, C. 1991. Effects of direct transfer from freshwater to seawater on respiratory and circulatory variables and acid-base status in rainbow trout. J. Comp. Physiol. 161: 557-568.

McCormick, S.D. 1990. Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. Am. J. Physiol. 259: R857-R863.

McCormick, S.D. 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50: 656-658.

McCormick, S.D. 1995. Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. *In* Cellular and molecular approaches to fish ionic regulation. Fish physiology, vol. 14. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, San Diego. pp. 285-315.

McCormick, S.D., and Bern, H.A. 1989. *In vitro* stimulation of Na^+,K^+-ATPase activity and ouabain binding by cortisol in coho salmon gill. Am. J. Physiol. 256: R707-R715.

McCormick, S.D., and Saunders, R.L. 1987. Preparatory physiological adaptations for marine life of salmonids: osmoregulation, growth and metabolism. American Fisheries Society Symposium 1: 211-219.

McCormick, S.D., Dickhoff, W.W., Duston, J., Nishioka, R.S., and Bern, H.A. 1991. Developmental differences in the responsiveness of gill Na^+,K^+-ATPase to cortisol in salmonids. Gen. Comp. Endocrinol. 84: 308-317.

McCormick, S.D., Moyes, C.D., and Ballantyne, J.S. 1989. Influence of salinity on the energetics of gill and kidney of Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem. 6: 243-254.

McDonald, D.G., and Milligan, C.L. 1992. Chemical properties of the blood. *In* Fish physiology, vol. 12B. *Edited by* W.S. Hoar, D.J. Randall and A.P. Farrell. Academic Press, San Diego, California. pp. 55-133.

Miller, W.R., Hendricks, A.C., and Cairns, J. 1983. Normal ranges for diagnostically important hematological and blood chemistry characteristics of rainbow trout, *Salmo gairdneri*. Can. J. Fish. Aquat. Sci. 40: 420-425.

Milsom, W.K. 1989. Mechanisms of ventilation in lower vertebrates: adaptations to respiratory and non-respiratory constraints. Can. J. Zool. 67: 2943-2955.

Mommsen, T.P. 1984a. Biochemical characterization of the rainbow trout gill. J. Comp. Physiol. 154: 191-198.

Mommsen, T.P. 1984b. Metabolism of the fish gill. *In* Fish physiology, vol. 10B. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, Orlando. pp. 203-238.

Morgan, J.D. 1991. The effects of salinity on survival, growth, aerobic metabolism and ion regulation in early life history stages of Pacific salmonids. M.Sc. thesis, University of British Columbia, Vancouver, B.C. 90 p.

Morgan, J.D., and Iwama, G.K. 1991. Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish. Aquat. Sci. 48: 2083-2094.

Moyle, P.B., and Cech, J.J. 1988. Fishes: an introduction to icthyology. 2nd edition. Prentice-Hall, Englewood Cliffs, New Jersey.

Nagibina, G.V. 1983. Influence of salinity on the metabolic rate in coho salmon (*Oncorhynchus kisutch*) in early periods of ontogenesis. *In* Physiological basis of reproduction of marine and anadromous fish. *Edited by* I.A. Shekhanova, L.B. Klyashtorin, and E.A. Kamenskaya. VNIRO, Moscow, USSR. pp. 57-61.

Nakano, K., Tagawa, M., Takemura, A. and Hirano, T. 1997. Effects of ambient salinities on carbohydrate metabolism in two species of tilapia, *Oreochromis mossambicus* and *O. niloticus*. Fisheries Sci. 63: 338-343.

Nichols, D.J., and Weisbart, M. 1985. Cortisol dynamics during seawater adaptation of Atlantic salmon, *Salmo salar*. Am. J. Physiol. 248: R651-R659.

Nicoll, C.S., Walker Wilson, S.W., Nishioka, R., and Bern, H. 1981. Blood and pituitary prolactin levels in tilapia (*Sarotherodon mossambicus*, Teleostei) from different salinities as measured by a homologous radioimmunoassay. Gen. Comp. Endocrinol. 44: 365-373.

Nordlie, F.G. 1978. The influence of environmental salinity on respiratory oxygen demands in the euryhaline teleost, *Ambassis interrupta*. Comp. Biochem. Physiol. 59A: 271-274.

O'Hara, J. 1971. Relationship of the metabolic rate of excised gill tissue to body size in two species of sunfish. Can. J. Zool. 49: 373-375.

Onken, H., and Putzenlechner, M. 1995. A V-ATPase drives active, electrogenic and Na^+ independent Cl^- absorption across the gills of *Eriocheir sinensis*. J. Exp. Biol. 198: 767-774.

Pannevis, M.P., and Houlihan, D.F. 1992. The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. 162: 393-400.

Patino, R., Redding, J.M., and Schreck, C.B. 1987. Interrenal secretion of corticosteroids and plasma cortisol and cortisone concentrations after acute stress and during seawater acclimation in juvenile coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 68: 431-439.

Pederson, P.L, and Carafoli, E. 1987. Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. Trends Biochem. Sci. 12: 146-150.

Penefsky, H.S., and Bruist, M.F. 1984. Adenosinetriphosphatases. *In* Methods of enzymatic analysis, vol. 4. *Edited by* H.U. Bergmeyer. Verlag Chemie, Weinham. pp. 324-328.

Pérez-Pinzón, M.A., and Lutz, P.L. 1991. Activity related cost of osmoregulation in the juvenile snook (*Centropomus undecimalis*). Bull. Mar. Sci. 48: 58-66.

Perry, S.F., and Farrell, A.P. 1989. Perfused preparations in comparative respiratory physiology. *In* Techniques in comparative respiratory physiology: an experimental approach. *Edited by* C.R. Bridges and P.J. Butler. Cambridge University Press, Cambridge. pp. 223-257.

Perry, S.F., and Walsh, P.J. 1989. Metabolism of isolated fish gill cells: contribution of epithelial chloride cells. J. Exp. Biol. 144: 507-520.

Perry, S.F., and Wood, C.M. 1989. Control and coordination of gas transfer in fishes. Can. J. Zool. 67: 2961-2970.

Perry, S.F., Davie, P.S., Daxboeck, C., Ellis, A.G., and Smith, D.G. 1984. Perfusion methods for the study of gill physiology. *In* Fish physiology, vol. 10B. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, Orlando. pp. 325-388.

Perry, S.F., Walsh, P.J., Mommsen, T.P., and Moon, T.W. 1988. Metabolic consequences of hypercapnia in the rainbow trout, *Salmo gairdneri*: ß-adrenergic effects. Gen. Comp. Endocrinol. 69: 439-447.

Potts, W.T.W., Fletcher, C.R., and Eddy, F.B. 1973. An analysis of the sodium and chloride fluxes in the flounder, *Platichthys flesus*. J. Comp. Physiol. 87: 21-28.

Potts, W.T.W., Foster, M.A., Rudy, P.P., and Howells, G.P. 1967. Sodium and water balance in the cichlid teleost, *Tilapia mossambica*. J. Exp. Biol. 47: 461-470.

Prosser, C.L. 1973. Comparative animal physiology. W.B. Saunders Co., Philadelphia. 966 p.

Rao, G. 1968. Oxygen consumption of rainbow trout in relation to activity and salinity. Can. J. Zool. 46: 781-786.

Redding, J.M., Patino, R., and Schreck, C.B. 1984a. Clearance of corticosteroids in yearling coho salmon, *Oncorhynchus kisutch*, in fresh water and seawater and after stress. Gen. Comp. Endocrinol. 54: 433-443.

Redding, J.M., Schreck, C.B., Birks, E.K., and Ewing, R.D. 1984b. Cortisol and its effects on plasma thyroid hormone and plasma electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 56: 146-155.

Richards, B.D., and Fromm, P.O. 1970. Sodium uptake by isolated-perfused gills of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 33: 303-310.

Richman, N.H., and Zaugg, W.S. 1987. Effects of cortisol and growth hormone on osmoregulation in pre- and desmoltified coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 65: 189-198.

Ron, B., Shimoda, S.K., Iwama, G.K., and Grau, E.G. 1995. Relationships among ration, salinity, 17α-methyltestosterone and growth in the euryhaline tilapia, *Oreochromis mossambicus*. Aquaculture 135: 185-193.

Sakamoto, T., Iwata, M., and Hirano, T. 1991. Kinetic studies of growth hormone and prolactin during adaptation of coho salmon, *Oncorhynchus kisutch*, to different salinities. Gen. Comp. Endocrinol. 82: 184-191.

Sakamoto, T., McCormick, S.D., and Hirano, T. 1993. Osmoregulatory actions of growth hormone and its mode of action in salmonids: A review. Fish Physiol. Biochem. 11: 1-6.

Sakamoto, T., Ogasawara, T., and Hirano, T. 1990. Growth hormone kinetics during adaptation to a hyperosmotic environment in rainbow trout. J. Comp. Physiol. 160: 1-6.

Sakamoto, T., Shepherd, B.S., Madsen, S.S., Nishioka, R.S., Siharath, K., Richman, N.H., Bern, H.A., and Grau, E.G. 1997. Osmoregulatory actions of growth hormone and prolactin in an advanced teleost. Gen. Comp. Endocrinol. 106: 95-101.

Sandercock, F.K. 1991. Life history of coho salmon. *In* Pacific salmon life histories. *Edited by* C. Groot and L. Margolis. UBC press, Vancouver, B.C. pp. 395-445.

Sargent, J.R., and Thomson, A.J. 1974. The nature and properties of the inducible sodium-plus-potassium ion-dependent adenosine triphosphatase in the gills of eels (*Anguilla anguilla*) adapted to fresh water and sea water. Biochem. J. 144: 69-75.

Schmidt-Nielson, K. 1975. Animal physiology: adaptation, and environment. Cambridge Univ. Press, London. 560 p.

Seddiki, H., Maxime, V., Boeuf, G., and Peyraud, C. 1995. Effects of growth hormone on plasma ionic regulation, respiration and extracellular acid-base status in trout (*Oncorhynchus mykiss*) transferred to seawater. Fish Physiol. Biochem. 14: 279-288.

Shepherd, B.S., Sakamoto, T., Nishioka, R.S., Richman, N.H., Mori, J., Madsen, S.S., Chen, T.T., Hirano, T., Bern, H.A., and Grau, E.G. 1997. Somatotropic actions of the homologous growth hormone and prolactins in the euryhaline teleost, the tilapia, *Oreochromis mossambicus*. Proc. Natl. Acad. Sci. 94: 2068-2072.

Shuttleworth, T.J. 1988. Salt and water balance - extrarenal mechanisms. *In* Physiology of elasmobranch fishes. *Edited by* T.J. Shuttleworth. Springer-Verlag, Berlin. pp. 171-199.

Shuttleworth, T.J., and Thompson, J.L. 1980. Oxygen consumption in the rectal gland of the dogfish *Scyliorhinus canicula* and the effects of cyclic AMP. J. Comp. Physiol. 136: 39-43.

Shuttleworth, T.J., Potts, W.T.W., and Harris, J.N. 1974. Bioelectric potentials in the gills of the flounder *Platichthys flesus*. J. Comp. Physiol. 94: 321-329.

Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J.N., and Epstein, F.H. 1977. Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. Am. J. Physiol. 233: F298-F306.

Silva, P., Stoff, J.S., Solomon, R.J., Rosa, R., Stevens, A., and Epstein, J. 1980. Oxygen cost of chloride transport in perfused rectal gland of *Squalus acanthias*. J. Membr. Biol. 53: 215-221.

Singley, J.A., and Chavin, W. 1975. The adreno-cortical-hypophyseal response to saline stress in the goldfish, *Carassius auratus*. Comp. Biochem. Physiol. 51A: 749-756.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85.

Soengas, J.L., Barciela, P., Aldegunde, M., and Andres, D. 1995. Gill carbohydrate metabolism of rainbow trout is modified during gradual adaptation to sea water. J. Fish Biol. 46: 845-856.

Specker, J.L., and Schreck, C.B. 1982. Changes in plasma corticosteroids during smoltification of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 46: 53-58.

Specker, J.L., King, D.S., Nishioka, R.S., Shirahata, K., Yamaguchi, K., and Bern, H.A. 1985. Isolation and partial characterization of a pair of prolactins released in vitro by the pituitary of a cichlid fish, *Oreochromis mossambicus*. Proc. Natl. Acad. Sci. 82: 7490-7494.

Specker, J.L., Portesi, D.M., Cornell, S.C., and Veillette, P.A. 1994. Methodology for implanting cortisol in Atlantic salmon and effects of chronically elevated cortisol on osmoregulatory physiology. Aquaculture 121: 181-193.

Stagg, R.M., and Shuttleworth, T.J. 1982a. Na^+,K^+-ATPase, ouabain binding and ouabain-sensitive oxygen consumption in gills from *Platichthys flesus* adapted to seawater and freshwater. J. Comp. Physiol. 147: 93-99.

Stagg, R.M., and Shuttleworth, T.J. 1982b. The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (*Platichthys flesus*). J. Comp. Physiol. 149: 83-90.

Stagg, R.M., and Shuttleworth, T.J. 1984. Hemodynamics and potentials in isolated flounder gills: effects of catecholamines. Am. J. Physiol. 246: R211-R220.

Steffensen, J.F., Johansen, K., and Bushnell, P.G. 1984. An automated swimming respirometer. Comp. Biochem. Physiol. 79A: 437-440.

Steffensen, J.F., Lumholt, J.P., and Johansen, K. 1982. Gill ventilation and oxygen extraction during graded hypoxia in 2 ecologically distinct species of flatfish, the flounder (*Platichthys flesus*) and the plaice (*Pleuronectes platessa*). Env. Biol. Fishes 7: 157-164.

Stickney, R.R. 1986. Tilapia tolerance of saline waters: a review. Prog. Fish-Cult. 48: 161-167.

Strange, R.J., and Schreck, C.B. 1980. Seawater and confinement alters survival and cortisol concentration in juvenile chinook salmon. Copeia 1980: 351-353.

Stronach, J.A., Crean, P.B., and Murty, T.S. 1988. Mathematical modelling of the Fraser River plume. Water Poll. Res. J. Can. 23: 179-212.

Stuenkel, E.L., and Hillyard, S.D. 1980. Effects of temperature and salinity on gill Na^+,K^+-ATPase activity in the pupfish, *Cyprinodon salinus*. Comp. Biochem. Physiol. 67A: 179-182.

Sullivan, G.V., Fryer, J.N., and Perry, S.F. 1995. Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. J. Exp. Biol. 198: 2619-2629.

Szyper, J.P., and Ako, H. 1990. Feed formulas producing improved palatability and growth with juvenile mahimahi, *Coryphaena hippurus*. World Aquacult. 21(3): 104-105.

Szyper, J.P., and Lutnesky, M.M.F. 1991. Ventilation rate and behavioral responses of juvenile mahimahi to temperature and salinity. Prog. Fish-Cult. 53: 166-172.

Toepfer, C., and Barton, M. 1992. Influence of salinity on the rates of oxygen consumption in two species of freshwater fishes, *Phoxinus erythrogaster* (family Cyprinidae), and *Fundulus catenatus* (family Fundulidae). Hydrobiologia 242: 149-154.

Towle, D.W., Gilman, M.E., and Hempel, J.D. 1977. Rapid modulation of gill $Na^+ + K^+$-dependent ATPase activity during acclimation of the killifish *Fundulus heteroclitus* to salinity change. J. Exp. Zool. 202: 179-186.

Trinder, P. 1969. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. Biochem. 6: 24.

Trotter, P.C. 1989. Coastal cutthroat trout: a life history compendium. Trans. Am. Fish. Soc. 118: 463-473.

Uchida, R.N., and King, J.E. 1962. Tank culture of tilapia. Fishery Bulletin 62: 21-52.

Vijayan, M.M., and Leatherland, J.F. 1989. Cortisol-induced changes in plasma glucose, protein, and thyroid hormone levels, and liver glycogen content of coho salmon (*Oncorhynchus kisutch*). Can. J. Zool. 67: 2746-2750.

Vijayan, M.M., Morgan, J.D., Sakamoto, T., Grau, E.G., and Iwama, G.K. 1996. Food-deprivation affects seawater acclimation in tilapia: hormonal and metabolic changes. J. Exp. Biol. 199: 2467-2475.

Vijayan, M.M., Reddy, P.K., Leatherland, J.F., and Moon, T.W. 1994. The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. Gen. Comp. Endocrinol. 96: 75-84.

Waller, U. 1989. Respiration and low oxygen tolerance of two fish species from the Arabian Sea, *Cubiceps whiteleggi* and *Coryphaena hippurus*. J. Appl. Ichthyol. 5: 141-150.

Wedemeyer, G.A., Barton, B.A., and McLeay, D.J. 1990. Stress and acclimation. *In* Methods for fish biology. *Edited by* C.B. Schreck and P.B. Moyle. American Fisheries Society, Bethesda. pp. 451-489.

Wendelaar Bonga, S.E. 1993. Endocrinology. *In* The Physiology of fishes. *Edited by* D.H. Evans. CRC Press, Boca Raton. pp. 469-502.

Werner, G., Hagenmaier, H., Drautz, H., Baumgartner, A., and Zähner, H. 1984. Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. J. Antibiot. 37: 100-117.

Wilson, J.M., Randall, D.J., and Vogl, A.W. 1996. Branchial mitochondria-rich cells in the elasmobranch *Squalus acanthias*: a life without a rectal gland. Society for Experimental Biology Annual Meeting, University of Lancaster. Abstract no. A9.47.

Wilson, J.M., Randall, D.J., Vogl, A.W., and Iwama, G.K. 1997. Immunolocalization of proton-ATPase in the gills of the elasmobranch, *Squalus acanthias*. J. Exp. Zool. 278: 77-85.

Withers, P.C. 1992. Comparative animal physiology. Saunders College Publishing, Orlando. 949 p.

Witters, H., Berckmans, P., and Vangenechten, C. 1996. Immunolocalization of Na^+,K^+-ATPase in the gill epithelium of rainbow trout, *Oncorhynchus mykiss*. Cell Tissue Res. 283: 461-468.

Wood, C.M., and Marshall, W.S. 1994. Ion balance, acid-base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus* - a euryhaline estuarine teleost. Estuaries 17: 34-52.

Yada, T., Hirano, T., and Grau, E.G. 1994. Changes in plasma levels of the two prolactins and growth hormone during adaptation to different salinities in the euryhaline tilapia, *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 93: 214-223.

Yamaguchi, K., Specker, J.L., King, D.S., Yokoo, Y., Nishioka, R., Hirano, T., and Bern, H.A. 1988. Complete amino acid sequences of a pair of fish (Tilapia) prolactins, $tPRL_{177}$ and $tPRL_{188}$. J. Biol. Chem. 263: 9113-9121.

Yancey, P.H., and Somero, G.N. 1980. Methylamine osmoregulatory solutes of elasmobranch fishes counteract urea inhibition of enzymes. J. Exp. Zool. 212: 205-213.

Yeoh, C.G., Kerstetter, T.H., and E.J. Loudenslager. 1991. Twenty-four-hour seawater challenge test for coastal cutthroat trout. Prog. Fish-Cult. 53: 173-176.

Yoshikawa-Ebesu, J.S.M., Borski, R.J., Richman, N.H., and Grau, E.G. 1995. Effects of acclimation salinity and *in vitro* medium osmotic pressure on the incorporation of ^{3}H-Leucine into the two prolactins of the tilapia, *O. mossambicus*. J. Exp. Zool. 271: 331-339.

Young, G., Bjornsson, B.T., Prunet, P., Lin, R.J., and Bern, H.A. 1989. Smoltification and seawater adaptation in coho salmon (*Oncorhynchus kisutch*): plasma prolactin, thyroid hormones, and cortisol. Gen. Comp. Endocrinol. 74: 335-345.

Young. G., McCormick, S.D., Thrandur, B.T., and Bern, H. 1995. Circulating growth hormone, cortisol and thyroxine levels after 24 h seawater challenge of yearling coho salmon at different developmental stages. Aquaculture 136: 371-384.

Zadunaisky, J.A. 1984. The chloride cell: The active transport of chloride and the paracellular pathways. *In* Fish physiology, vol. 10B. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, Orlando. pp. 129-176.

Zehran, K. 1956. Oxygen consumption and active sodium transport in the isolated and short-circuited frog skin. Acta Physiol. Scand. 36: 300-318.

Zinichev, V.V., Varnavskii, V.S., and Saravanski, O.N. 1993. Effect of salinity on oxygen consumption by young Pacific salmons. Voprosy Ikhtiologii 33: 225-229.

Zohar, G., Bejerano, I., and Wishovsky, A. 1992. The effect of fish weight, water temperature, and dissolved oxygen concentration on the oxygen consumption of tilapia. Fish. Fishbreed. Isr. 25: 166-172.

Zollner, H. 1993. Handbook of enzyme inhibitors. VCH Publishers, Weinheim.