IN VITRO STUDIES OF TELEOST IONOREGULATION IN FRESHWATER KILLIFISH, TILAPIA AND TROUT

IN VITRO STUDIES OF FRESHWATER TELEOST IONOREGULATION IN THE COMMON KILLIFISH (*Fundulus heteroclitus*), NILE TILAPIA (*Oreochromis niloticus*) AND RAINBOW TROUT (*Oncorhynchus mykiss*)

By

Darryl W. Burgess, B.Sc.

A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

McMaster University

MASTER OF SCIENCE (1997) McMaster University
(Biology) Hamilton, Ontario

TITLE: *IN VITRO* STUDIES OF FRESHWATER TELEOST IONOREGULATION IN THE COMMON KILLIFISH (*Fundulus heteroclitus*), NILE TILAPIA (*Oreochromis niloticus*) AND RAINBOW TROUT (*Oncorhynchus mykiss*)

AUTHOR: Darryl W. Burgess, B.Sc. (McMaster University)

SUPERVISOR: Professor C.M. Wood

Number of Pagers: xv, 152

Abstract

Largely through the use of *in vitro* preparations there is now a generally accepted theory for ion transport for the seawater (SW) gill. However, to date there is no generally accepted freshwater (FW) model for the mechanisms of NaCl transport in the teleost gill. By using an Ussing chamber approach with the opercular epithelia of *Fundulus heteroclitus* and *Oreochromis niloticus*, and the urinary bladder of *Oncorhynchus mykiss*, all acclimated to FW, we hoped to establish one as a possible model for the study of FW ion regulation.

FW *Fundulus* opercular epithelia displayed a serosal negative transepithelial potential (V_t) of -43.9 mV, transepithelial conductance (G_t) of 1.94 $mS \cdot cm^{-2}$, and active transport of Cl^- from the mucosal FW against a strong electrochemical gradient. Na^+ movement was dominated by passive diffusion. The opercular epithelia of *Fundulus* adapted to 10% SW exhibited properties similar to SW *Fundulus* by actively extruding Cl^- while Na^+ moved passively into the mucosal 10% SW. With FW bathing the mucosal surface, FW *Oreochromis* opercular epithelia displayed a serosal positive V_t of +8.0 mV, G_t of 1.78 $mS \cdot cm^{-2}$, and active reabsorption of Na^+, Cl^- and Ca^{2+} against large electrical and/or chemical gradients. The FW *Oreochromis* opercular epithelia is the only FW *in vitro* preparation to date that exhibits active absorption (albeit at small absolute rates) of both Na^+ and Cl^-.

FW *O. mykiss* urinary bladders mounted *in vitro* under symmetrical saline conditions exhibited a transepithelial conductance (G_t) of ~9.15 $mS \cdot cm^{-2}$ and

electroneutral active absorption of Na^+ and Cl^- from the mucosal urine side. The transport of Na^+ and Cl^- was a partially coupled process whereby removal of Na^+ from the mucosal saline decreased Cl^- absorption by a 56% and removal of Cl^- inhibited Na^+ absorption by 69%. However, active net absorption of both ions persisted when the counter-ion was replaced with a non-permeant ion. Under more realistic conditions with artificial urine bathing the mucosal surface, V_t increased to a serosal positive ~+7.6 mV and G_t decreased to ~1.47 $mS \cdot cm^{-2}$. Unidirectional influx rates of both Na^+ and Cl^- were much lower, but active absorption of both ions still occurred. Replacement of Na^+ in the mucosal artificial urine caused no change in unidirectional influx of Cl^- and *vice versa*. The mucosal addition of DIDS, amiloride or bumetanide (10^{-4} M) all had no affect on absorption rates of Na^+ and/or Cl^-, under either artificial urine or symmetrical saline conditions. When the mucosal surface was bathed in artificial urine, removal of mucosal Cl^- significantly reduced the maximum transport rate (J_{max}) of Na^+ (6.1 $\rightarrow$ 2.1 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) but had no effect on affinity for Na^+ (K_m~27 mM). Similarly, removal of mucosal Na^+ significantly reduced the J_{max} for Cl^- uptake (11.4 $\rightarrow$ 2.4 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) but had no effect on Cl^- K_m (~37 mM). The anterior portion of the urinary bladder transported Na^+ and Cl^- at a faster rate than the posterior portion under symmetrical saline conditions, but there was no difference in measured Na^+/K^+-ATPase activities between the two portions.

In conclusion, opercular epithelia of *Fundulus* and *Oreochromis* demonstrated transport properties believed to be typical of FW teleosts; either preparation may eventually prove to be a good working model for studying FW transport mechanisms.

The urinary bladder of *Oncorhynchus* mounted *in vitro* did not behave characteristically as the teleost gill is thought to function, but this model may be useful in characterizing various other mechanisms of ionic transport.

Acknowledgements

Well, where do I begin? I'm actually finished! First and foremost I have to thank Chris Wood for his overall guidance throughout this project. Chris was a great mentor and made me realize that to succeed, you need to have a passion and you must ultimately enjoy your work. I learned a great deal during my stay at Mac, such as work ethic (you learn a lot of that), patience, belief in yourself and yes, even a bit of theory. To anyone in the process of earning your M.Sc. now, I recommend relaxing and enjoying your time with the friends you make - because believe it or not, you will eventually finish.

I've got so many people to thank I hope I mention most. Bill Marshall was a huge asset to this project, there is no doubt I would still be in 211 today if it weren't for his explanatory e-mails. Mike O'Donnell found time to analyze ionic activities when my time was running thin and he also came up with excellent suggestions for the work. Without a doubt, the absolute best people in Wood's lab are the Post-docs, who if it weren't for Ian Morgan's efforts in the initial stages of the project, I would never have been able to set up Bill Marshall's lab at Mac. Jim McGeer also deserves mention because he was always helpful and available to me when I had questions. Derek Alsop (Frenchie), besides having to work on his golf swing in a big way, is a great guy and we had a lot of fun. And yes, I have to add my 211 lab-mate Mary Fletcher (DQ) to the list who inspired me never to lay out in the sun. I would also like to thank Jacqui Dockray for keeping everything in perspective for me during the summer of 96' when Ussing was at his worst.

As for the remainder of the bladder crew, Danielle McDonald, I wish the best of luck to, and I know her supersonic voice will keep her out of trouble. However, Maciej Miarczynski is another story... I just hope that the first person you train on the Ussing chambers is informed that there are indeed some spare chambers! Sorry Bob, it had to be done. I would also like to thank Hans Ussing for inventing a totally neat system, some day I'll have to call Hans up and ask him some remaining questions. I'm sure his fellow Dane, Martin Grossel, will be just as wise some day. Martin, I will make it to DK some day and you'll have to show me the Ussing temple.

I would also like to thank my parents, Bill and Josephine Burgess, for their continual support throughout my academic endeavors. Even though they didn't understand the science, they understood the hard work that went into it, that's all I needed. I would especially like to thank my mother for always listening to me and then steering me in the right direction. Finally, I would like to thank Deb Lorenzin for believing in me and more often than not, helping me to see the brighter side of things, you definitely made this experience a lot easier to handle.

Thesis Format

This thesis is organized into three Chapters and one Appendix. Chapter one is composed of background information and a general overview of the rationale for this research and the conclusions of the work. The second and third chapters describe the experimental work and have been written as manuscripts for submission to scientific journals. The appendix includes general methodology for Ussing chamber techniques and equations used in this thesis. Literature cited follows chapters two and three respectively and general references from chapter 1 follow appendix.

Chapter 1: General Introduction

Chapter 2:

Title: NaCl transport of the opercular epithelia of freshwater acclimated *Fundulus heteroclitus* and *Oreochromis niloticus*

Authors: D.W. Burgess, W.S. Marshall and C.M. Wood

Comments: *In vitro* experiments and analyses performed by D.W.B with advice from W.S.M and supervision of C.M.W. This paper has been submitted to Can. J. Zool.

Chapter 3:

Title: Na^+ and Cl^- transport by the urinary bladder of the freshwater rainbow trout (*Oncorhynchus mykiss*)

Authors: D.W. Burgess, M. Miarczynski and C.M. Wood

Comments: *In vitro* experiments and analyses performed by D.W.B with

the exception of Na^+/K^+-ATPase measurements and kinetic flux experiments which were performed by M.M under the guidance of D.W.B. Overall advice and supervision was provided by C.M.W. This paper will be submitted to J. Exp. Zool.

Appendix 1: General Methodology

Table of Contents

List of Figures

List of Tables

Chapter 1

General Introduction - The Need for a Flat Epithelial Model for the Freshwater Gill.

Background

The freshwater (FW) fish gains water by osmosis while Na^+ and Cl^- are lost by passive diffusion. Excess water is regulated by the kidney and lost salt is replaced by transport mechanisms in the gills and other epithelia, and also by the ingestion of food (Smith *et al.*, 1989). In contrast, the seawater (SW) fish survives in a hypertonic environment and must actively extrude Na^+ and Cl^- at the gills and other epithelia. The seawater fish must also actively drink seawater to replenish water which is continually lost by osmosis across the body surface. In FW teleosts, the primary role of the kidney is to excrete excess water while reabsorbing most of the filtered solutes. In contrast, the renal tubules of SW teleosts maintain a low permeability, and the glomerular filtration rate is less than half that of FW teleosts (Nishimura *et al.*, 1983). The urinary bladder of SW-adapted (Renfro, 1977; Demarest, 1984) and FW-adapted teleost fish (Fossat and Lahlou, 1979a; Marshall, 1986) has also been shown to play an important osmoregulatory role. In FW teleosts, the urinary bladder has a low osmotic permeability, reabsorbing monovalent ions (Na^+ and Cl^-) with a minimum of accompanying water, thereby excreting a very dilute urine (Curtis and Wood, 1991). The urinary bladder of SW teleosts also reabsorbs NaCl but has a higher osmotic permeability and the excreted urine is concentrated with respect to non-transported ions (eg. Mg^{2+} and Ca^{2+}) (Marshall, 1995).

The understanding of ion transport in the gills of seawater teleosts has been successful largely due to the use of *in vitro* studies. Opercular epithelia from the killifish *Fundulus heteroclitus* (Degnan *et al.* 1977; Degnan and Zadunaisky 1979, 1980; Karnaky *et al.* 1977; Karnaky 1980, 1986; Ernst *et al.* 1980; Zadunaisky 1984), Mozambique tilapia *Oreochromis mossambicus* (Foskett *et al.* 1981, 1983; Foskett and Scheffey 1982; Scheffey *et al.* 1983) and the jaw skin epithelia of the goby *Gillichthys mirabilis* (Marshall and Nishioka, 1980; Marshall, 1981) have all been instrumental in this regard. These *in vitro* studies have used flat epithelial preparations mounted in Ussing-style chambers (which facilitate rigorous radioisotopic fluxes and electrophysiology measurements), to characterize the Na^+ and Cl^- transport mechanisms.

The euryhaline *Fundulus* has long been used as an experimental animal for the study of SW ionoregulation *in vivo* (Potts and Evans, 1967; Motais *et al.*, 1966; Maetz *et al.*, 1967a) along with other *in vivo* preparations such as the European flounder *Platichthys flesus* (Motais *et al.*, 1966) and the European eel *Anguilla anguilla* (Maetz *et al.*, 1967b). However, it is largely because of the *in vitro* studies (mentioned above) that there is a generally accepted model for SW teleost ionoregulation.

Seawater opercular epithelia

Opercular epithelia from the *Fundulus*, *Oreochromis* and jaw skin of *Gillichthys* are easily dissected from the animal to yield a planar sheet that can be mounted in an Ussing-style chamber. Under symmetrical saline and open-circuit conditions, the transepithelial potential (V_t) of the *Fundulus* opercular epithelium is serosal positive by about +15 mV, and transepithelial resistance (R_t) is approximately 170 $\Omega \cdot cm^{-2}$ (Degnan *et*

al. 1977). Once the epithelium is clamped to a V_t of zero, there remains a large serosal to mucosal net flux of Cl^- (~5 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) which is not different from the measured short-circuit current (Isc) (138 $\mu A \cdot cm^{-2}$), thus suggesting current is equal to Cl^- secretion ($1 \mu eq \cdot cm^{-2} \cdot h^{-1} = 27 \mu A \cdot cm^{-2}$). There was no net movement of Na^+ under these conditions and application of the Ussing flux ratio criterion to the observed ion fluxes of Cl^- and Na^+ indicated that there was active Cl^- secretion and passive diffusion of Na^+. The V_t of the SW epithelium appears to be a combination of a Na^+ diffusion potential (Potts, 1984) and the active, electrogenic extrusion of Cl^- (Marshall, 1981).

O. mossambicus opercular epithelia have a serosal-positive V_t of about +21 mV and a R_t of approximately 259 $\Omega \cdot cm^{-2}$ under symmetrical saline conditions (Foskett *et al.* 1981). The Isc of this preparation is approximately 65 $\mu A \cdot cm^{-2}$ and is equal to the measured net Cl^- extrusion (~2.1 $\mu mol \cdot cm^{-2} \cdot h^{-1}$). Under similar symmetrical experimental conditions, the opercular epithelia of the *G. mirabilis* display a serosa-positive V_t of about +14 mV and a R_t of approximately 540 $\Omega \cdot cm^{-2}$ (Marshall, 1981). The Isc (24 $\mu A \cdot cm^{-2}$) is also approximately equal to the net flux of Cl^-. The epithelium exhibits active Cl^- extrusion against electrical and concentration gradients and the observed Cl^- flux ratio is in disagreement with the predicted ratio, again suggesting active transport of Cl^-. However, as in the other epithelia previously mentioned, Na^+ moves passively across the membrane based on agreement of the observed and predicted flux ratios.

Each of the epithelia (above) demonstrated a dependency of measurable Isc on the presence of Na^+, such that removal of Na^+ from the basal side (goby, Marshall, 1977; tilapia, Foskett *et al.*, 1983) or apical side (killifish Degnan *et al.*, 1977) resulted in a

reduction of Isc to zero. Na^+/K^+-ATPase is involved in Na^+ and Cl^- secretion by opercular membranes since the addition of ouabain to the basolateral side also results in an inhibition of Isc, due to a decreased Cl^- efflux and increased influx (Foskett *et al.*, 1983).

Seawater mitochondrial-rich cells

The Isc of the *Fundulus* epithelia has been significantly correlated with the number of mitochondria-rich cells (MR cells, also often called "chloride cells") detected by DASPEI fluorescence, thus indicating that MR cells are responsible for Cl^- transport. Further evidence has been obtained through the use of the vibrating probe technique which localized all current and nearly all conductance to the apical crypt of MR cells in the tilapia opercular epithelium (Foskett and Scheffey, 1982; Scheffey *et al.*, 1983; Foskett and Machen, 1985).

The occurrence of mitochondrial-rich (MR) cells appears to be an essential component for an epithelial "model" preparation which successfully mimicks gill function in the intact animal. The seawater opercular epithelium contains a high density of these cells, and the number of cells per mm^2 varies from preparation to preparation and from species to species (Marshall and Nishioka, 1980). The MR cells of the opercular epithelium are identical in ultrastructure to those of the gills, and other cells types (mucous, pavement, and accessory) are also very similar (Karnaky and Kinter, 1977). The opercular epithelia of *Fundulus and Oreochromis* both contain MR cells and it has been shown that these cells are the sites of Cl^- secretion in seawater adapted teleosts (hence the former name, chloride cells) (Foskett and Scheffey, 1982).

Pavement cells form a continuous layer over the surface of the opercular bone interrupted only by mucous cells and apical crypts from MR cells. The MR cell extends from the basal lamina (serosa) to the surface of the epithelia (mucosa) (Zadunaisky, 1984). MR cells contain numerous mitochondria and an extensive branching tubular system that is continuous with the basal and plasma membranes (Degnan *et al.* 1977).

Current seawater model for NaCl transport

The SW transport model proposed by Silva *et al.* (1977), based initially on experiments with the eel *in vivo*, and later validated and elaborated through the use of opercular epithelial "models" *in vitro*, is now widely accepted (Wood and Marshall, 1994) (Fig.1.1). Extrusion of Na^+ and Cl^- from the blood to the external seawater is energized by the transport enzyme Na^+/K^+-ATPase. This enzyme, located on the basolateral membrane, creates a large Na^+ gradient across the basolateral membrane and is the primary source of energy for active Cl^- secretion. The Na^+/K^+-ATPase maintains a low intracellular concentration of Na^+ and contributes to the negative potential inside the cell, thus creating an electrochemical gradient for the entry of Na^+ along with Cl^- and K^+ by way of a Na^+, $K^+/2Cl^-$ cotransporter. Cl^- then leaves the cell passively down an electrical gradient through an apical Cl^- channel (transcellular), and Na^+ moves through a paracellular pathway also following its electrochemical gradient. The large serosal positive V_t (+35-40 mV) found in SW teleost epithelia is established by secondary Cl^- transport and a cation-selective paracellular shunt pathway (Silva *et al.*, 1977; Karnaky, 1986; Wood and Marshall, 1994; McCormick, 1995).

The current model for seawater ion regulation does not however include an acid-base component. There has been circumstantial evidence for the presence of Na^+/acid and Cl^-/base exchange in the gills of several seawater species (Evans *et al.* 1982), but as yet no direct evidence (Degnan *et al.* 1977; Karnaky *et al.* 1977; Zadunaisky 1984). In the freshwater gill, intracellular carbonic anhydrase is believed to provide H^+ and HCO_3^- which are then exchanged on a 1-for-1 basis with Na^+ and Cl^- respectively. However the function of carbonic anhydrase, which has been detected in the chloride cells of SW killifish opercular epithelia (Lacy, 1983), remains uncertain in the SW gill.

Freshwater opercular epithelia

The SW model of ion regulation (Fig. 1) is now firmly established through the contributions of numerous *in vitro* studies (see above). However, for FW teleost ion regulation, there are many theories and as yet no generally accepted model. The current FW transport models are based on experiments *in vivo* and with perfused gill preparations, with an absence of any contribution from *in vitro* model preparations.

The *Fundulus* opercular epithelium, which has been used so successfully in constructing the SW ion transport model, has been largely ignored in FW since an initial study by Degnan *et al.* (1977). Degnan and colleagues adapted *Fundulus* to freshwater (FW) (Na^+ = 5 mM; Cl^- = 0.5 mM; K^+ = 0.5 mM) and mounted the opercular epithelia *in vitro* under symmetrical saline, short-circuited conditions; the membrane exhibited net Cl^- efflux equal to Isc, similar to SW epithelia, however at a reduced efflux rate. These results indicated that MR cells of the FW opercular epithelium retained their SW characteristic of extruding Cl^-.

Only recently have there been further studies directed at establishing an *in vitro* model for FW ion transport. *Fundulus* acclimated to FW (Na^+ = 1mM, Cl^- = 1mM, Ca^{2+} = 0.1 mM), with the opercular epithelium mounted *in vitro* under symmetrical saline, short-circuited conditions (Isc = ~9-12 $\mu A \cdot cm^{-2}$, V_t = ~0, R_t = 562-758 $\Omega \cdot cm^{-2}$) display net transport of Na^+ and Cl^- in an uptake direction (Marshall *et al.*, 1997). With the mucosal side of the epithelium bathed with FW (a more realistic environmental condition), the V_t is serosa negative (-61 mV) and R_t is approximately 590 $\Omega \cdot cm^{-2}$. Under these conditions the opercular epithelia actively take up Cl^- from the FW while Na^+ exhibits passive diffusion (Wood and Marshall, 1994; Marshall *et al.*, 1997). It should be mentioned however, that under these more realistic conditions, the epithelium loses both Na^+ and Cl^- in a net direction to the mucosal bath. The apparent leak component to Cl^- efflux was confirmed by showing a significant linear correlation to conductance indicating that the majority of Cl^- efflux is conductive and passive. The negative Na^+ balance is thought to reflect passive diffusion to the mucosal FW through a cation selective shunt pathway thereby producing a serosal negative V_t. Pharmacological agents such as SITS (blocker of anion exchange and anion channels) and amiloride (Na^+/H^+ exchange and Na^+ channel inhibitor) have no effect on Cl^- and Na^+ unidirectional influx respectively, following mucosal application (10^{-4} M) to the FW epithelia. Nevertheless, the negative results with SITS and amiloride may arise from lack of accessibility of the drugs to the apical side because of the mucous in the apical crypts; they should not convince one that the mechanisms are necessarily absent (Marshall, personal communication). The mucosal addition of thiocyanate anion (SCN^-, a

competitive anion transport inhibitor) decreases unidirectional influx of Cl^- suggesting active transport or ion exchange mechanisms may be present.

Opercular epithelia of *O. mossambicus* acclimated to FW with saline bathing both sides of the membrane had a serosal positive V_t of +1.4 mV, R_t of ~3700 $\Omega \cdot cm^{-2}$ and an Isc of 1.2 $\mu A \cdot cm^{-2}$ (Foskett *et al.*, 1981). These epithelia did not actively transport Cl^- as unidirectional radio-labelled Cl^- fluxes under these conditions were small (~0.2 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) and the net flux was not different from zero.

Kültz and Onken (1993) monitored electrophysiology and MR cell density of opercular epithelia from *O. mossambicus* acclimated to various salinities. FW acclimated membranes demonstrated the lowest values of MR cell density, V_t and I_{sc} and highest R_t. In the salinity range between FW and SW, all electrophysiological parameters increased in parallel. These results suggest that the mechanism for Cl^- secretion increased on the cellular and epithelial level as salinity increased.

The cleithrum skin of FW-adapted *Oncorhynchus mykiss* had a serosal positive V_t of +3.8 mV and a high R_t of ~10400 $\Omega \cdot cm^{-2}$ when the mucosal side was bathed with FW (Marshall *et al.*, 1992). This preparation actively transported only Ca^{2+} (positive balance) while both Na^+ and Cl^- ions were passively distributed.

Branchial epithelia of *Oncorhynchus mykiss* from dispersed gill epithelial cells cultured on permeable supports and exposed to apical FW actively transport Cl^- against an electrochemical grandient in the mucosal to serosal direction (Wood *et al.*, 1997). Na^+ movement is dominated by passive diffusion. Of particular importance is that based on morphological criteria, the cultured epithelia consist entirely of pavement-type cells (Wood and Pärt, 1997).

These *in vitro* studies have all tried to establish their preparation as a model for FW ion transport. However, none of the *in vitro* preparations mentioned above has been able to accurately mimick ion regulation processes of the gills of intact fish under realistic environmental conditions (FW bathing mucosal surface). For example, active Cl^- uptake has been shown in opercular epithelia (*F. heteroclitus* and *O. mossambicus*) and cultured epithelia (*O. mykiss*), but Cl^- tends to be in overall net negative balance. Furthermore, every *in vitro* preparation studied has reported only the passive diffusion of Na^+. In contrast, the FW teleost is believed to actively and independently take up both Na^+ and Cl^- on a net basis through mechanisms in the gills and/or other epithelia (see below), something *in vitro* studies have only partially reproduced to date.

Freshwater mitochondrial-rich cells

MR cells in *Fundulus* and *Oreochromis* opercular epithelia are smaller in diameter and fewer in number when the fish are acclimated to FW, in comparison to MR cells which are hypertrophied and differentiated when the same species is acclimated to SW (Foskett *et al.*, 1981; Kültz and Onken, 1993; Wood and Marshall, 1994). The density of MR cells has been positively correlated to the influx of Ca^{2+} in three FW epithelia, the *Fundulus* (Marshall *et al.*, 1995) and *Oreochromis* (McCormick *et al.*, 1992) opercular epithelia and the cleithrum skin of *Oncorhynchus* (Marshall *et al.*, 1992). However, no *in vitro* study has yet demonstrated evidence for a relationship between MR cells and the FW uptake of Na^+ or Cl^-.

FW MR cells, similar to SW MR cells, contain a large number of mitochondria and an extremely complex system of Na^+/K^+-ATPase populated tubules which represent

infolding of the basolateral membrane. In freshwater teleosts, the apical plasma membrane of the MR cells express a shallow crypt, whereas teleosts acclimated to SW express a deep, narrow apical crypt (Pisam and Rambourg, 1991). FW MR cells also have well developed tight junctions between adjacent MR cells (Karnaky, 1986).

The gill epithelia of teleosts *Lebistes reticulatus* (Pisam *et al.*, 1987), *Salmo salar* (Pisam *et al.*, 1988), *Gobio gobio* (Cyprinidae), *Cobitis taenia* (Cobitidae), *Scophthalmus maximus* (Scophthalmidae) (Pisam *et al.*, 1990) and *Oreochromis niloticus* (Cichlidae) (Pisam *et al.*, 1993) adapted to FW contain two types of MR cells (α an β cells). The α MR cells are pale elongated cells located in close contact with lamellar vessels at the base of the lamallae, and β cells, are darker, ovoid cells located in the interlamellar region of the filament epithelium. However, only one type (α cells) is present after acclimation to SW, which is then joined by another cell type (accessory MR cells) (Jürss and Bastrop, 1995). It has not yet been determined if α and/or β MR cells are present in FW opercular epithelia.

Current ideas about NaCl transport in FW fish

Our current ideas about FW teleost ion regulation (Fig. 1.2) have been developed through whole animal and perfused gill work on the stenohaline goldfish (*Carassius auratus*), eel (*Anguilla anguilla and rostrata*), channel catfish (*Ictalurus punctatus*), European carp (*Cyprinus carpio*) and the euryhaline salmonids (*Oncorhynchus*, *Salmo* and *Salvelinus* sp.).

Na^+ and Cl^- are transported apparently independently, from the dilute FW across the gill against an electrochemical gradient. These independent processes are thought to

occur by electroneutral exchangers on the apical membranes of MR cells and/or pavement cells (undetermined). Na^+ is exchanged for H^+ and/or NH_4^+, and Cl^- for HCO_3^-, processes that may be driven indirectly by a basolateral Na^+/K^+-ATPase. The energy required to drive Na^+ absorption is thought to be provided by the basolateral Na^+/K^+-ATPase. This pump creates low intracellular [Na^+] which creates a suitable gradient for the entry of Na^+. Cl^- uptake is stimulated by addition of HCO_3^- on the serosal side and inhibited by addition on the mucosal side and also addition of SITS (Perry *et al.*, 1981), suggesting Cl^-/HCO_3^- exchange. There is inhibition of Cl^- uptake by addition of thiocyanate while Na^+ uptake is unaffected, indicating independent transport (Kerstetter *et al.*, 1970). Amiloride addition inhibits Na^+ influx and produces internal acidosis and reduction in NH_4^+ efflux, indicating $Na^+/(H^+$ or $NH_4^+)$ exchange (Perry *et al.*, 1981). The enzyme carbonic anhydrase, found in all types of gill epithelial cells (Perry and Laurent, 1994), hydrates respiratory CO_2 and forms H^+ and HCO_3^-. The H^+ formed may also be added to intracellular NH_3 to form NH_4^+. According to the acid/base exchange mechanisms described above, the gill is the principal organ of acid/base regulation in freshwater fish (McDonald *et al.*, 1989; Wood, 1991; Goss *et al.*, 1992).

An alternate proposed mechanism for Na^+ uptake is by diffusion through a Na^+ selective channel on the apical membrane of gill cells, driven by an electrical gradient created by an H^+-ATPase which actively extrudes H^+ ions across the apical membrane of the same or neighbouring cells (Lin and Randall, 1991). This mechanism, as with Na^+/H^+ and/or Na^+/NH_4^+ exchange, operates on a 1-for-1 exchange basis and is linked to an acidic component; however existence of this mechanism in the teleost gill is unproven. Cl^- absorption is far less understood. HCO_3^- efflux may be driven by a pH gradient

developed by Na^+/acidic equivalent exchange and extruded by Cl^-/HCO_3^- exchange (however such exchangers would not be independent). Cl^-/HCO_3^--dependent ATPase has been detected in freshwater gills, however its location and linkage to Cl^- transport remains uncertain (Bornancin *et al.*, 1977). Diffusive loss of Na^+ and Cl^- is through the paracellular channels which may be regulated by extracellular and environmental pH. The mechanisms by which Cl^- and acidic/basic equivalents move across the basolateral membrane is also unknown and largely speculative.

Ca^{2+} transport in freshwater opercular epithelia

A few *in vitro* studies have demonstrated active Ca^{2+} transport in isolated epithelia (McCormick *et al.*, 1992; Marshall *et al.*, 1992; Marshall *et al.*, 1995). However, unlike the FW model for NaCl transport, the mechanism(s) of Ca^{2+} transport is better understood and therefore more complete. The Ca^{2+} transport model (Fig. 1.3) has been generated mostly from whole animal studies on freshwater teleosts (most recently see Perry and Wood, 1985; Bently, 1992; Verbost *et al.*, 1987, 1989, 1993; Flik *et al.*, 1985, 1986, 1993; Flik and Perry, 1989; Hogstrand *et al.*, 1994).

FW adapted *Fundulus* opercular epithelia mounted *in vitro*, with serosal saline and mucosal FW, exhibit net active absorption of Ca^{2+} (Marshall *et al.*, 1995). The transport of Ca^{2+} is significantly correlated with the density of MR cells on the opercular epithelium, and appears to involve passive entry across the apical membrane of these cells through Ca^{2+}-selective channels. Marshall *et al.* (1995) reasoned that Ca^{2+} is then complexed intracellularly by calmodulin and actively transported to the plasma through a high-affinity Ca^{2+}-ATPase. In tilapia branchial epithelia, a Na^+-dependent Ca^{2+}

transporter is present (Verbost *et al.*, 1993) and in intestinal epithelia a $Ca^{2+}/3Na^{+}$ exchanger is driven by the Na^{+}/K^{+}-ATPase (Flik *et al.*, 1993). Both transporters occur on the basolateral membrane.

The opercular epithelia of *O. niloticus*, as with *Fundulus* epithelia, also actively transport Ca^{2+} against ionic and electrical gradients in parallel to the density of mitochondrial-rich cells (McCormick *et al.*, 1992). Tilapia acclimated to low environmental Ca^{2+} display an increased capacity to transport Ca^{2+}. The cleithrum skin of FW *O. mykiss* also actively transports Ca^{2+} from the mucosal FW against an electrochemical gradient (Marshall *et al.*, 1992). Again, the transport is linearly correlated to the density of MR cells.

The urinary bladder: a potential in vitro model

As previously mentioned, the urinary bladder of teleost fish plays an important role in ionoregulation and can be considered an accessory osmoregulatory organ to the kidney (Marshall, 1995). In FW teleosts, the urinary bladder reabsorbs Na^{+} and Cl^{-} from the ureteral urine stored in the bladder and excretes a dilute urine thus conserving ions while surviving in a hyposmotic environment (Curtis and Wood, 1991; 1992). The urinary bladder has a similar function to the teleost gill in that it also reabsorbs Na^{+} and Cl^{-} from a dilute media (ie dilute urine *versus* freshwater). Because the urinary bladder is easily removed from the fish and provides a flat epithelial surface, it can be mounted in an Ussing-style chamber. Potentially, the urinary bladder studied *in vitro* could serve as a possible "surrogate" model for the gill.

Ultrastructure of the urinary bladder

The urinary bladder, like the mesonephric duct, is believed to be derived from mesoderm, and both structures are therefore thought to contribute to ion transport (Kamalaveni, 1961). The urinary bladder of the goby *Gillichthys mirabilis* contains two distinct cell types (tall columnar and low cuboidal cells) (Nagahama *et al.*, 1975). Columnar cells are most abundant in the area closest to the ureters. These cells are rich in mitochondria and rough and smooth endoplasmic reticulum. The remaining portion of the *Gillichthys* urinary bladder is composed mostly of cuboidal cells with fewer MR cells. These two major cell types are also found in the urinary bladder epithelia of *Tinca tinca* and *Ictalurus sp.* (Gerzeli *et al.*, 1973). Columnar cells in *Gillichthys* epithelia are ultrastructurally similar to epithelial columnar cells in bladders of *Pseudopleuronectes americanus*, *Hemitripterus americanus*, *Platichthys stellatus* and *Oreochromis mossambicus* (Loretz and Bern, 1980).

Further observations by Curtis (1990) describes a thin layer of smooth muscle surrounding the entire urinary bladder of *O. mykiss*. The inside (mucosal) surface contains microvilli, which greatly enhance surface area for ion transport. The urinary bladder is also a well vascularized, single layer epithelium, which minimizes the distance transported ions have to traverse before entering the plasma.

Freshwater urinary bladder studies *in vivo*

Until recently all *in vivo* ion transport studies of urine flow and composition were collected using internal bladder catheters (e.g. Holmes and Stainer, 1966; Hunn, 1969; Hunn and Willford, 1970; Hofmann and Butler, 1979; Elger and Hentschel, 1983;

Wheatley *et al.*, 1984; Oikari and Rankin, 1985; Elger *et al.*, 1986; Erickson and Gingerich, 1986; Wood, 1988). This method of urine collection collects ureteral urine, thus bypassing any regulatory role of the bladder (Marshall, 1988). However, Curtis and Wood (1991, 1992) developed a new external catheterization technique that collected naturally discharged urine in *O. mykiss*. This new method of urine collection determined that trout urinate in intermittent bursts at 20-30 minute intervals, and during urine storage time in the bladder, significant ion reabsorption occurs. Thus the urinary bladder contributes significantly to the osmoregulatory function of the entire renal system.

Freshwater urinary bladder studies *in vitro*:

Current freshwater urinary bladder models for NaCl transport

The majority of studies involving urinary bladder ion regulation have been performed *in vitro*. Most of these *in vitro* studies have used Ussing-type chambers (Marshall, 1986, 1988) or bladder sac preparations (Hirano *et al.*, 1973). Studies interested in urinary bladder ion transport have been numerous and included many species (e.g. *Oncorhynchus mykiss, Salvelinus fontinalis, Salmo irideus, Pseudopleuronectes americanus, Gillichthys mirabilis*). However, of particular interest is that two closely related species *Salvelinus fontinalis* and *Salmo irideus* have demonstrated two very different mechanisms for the absorption of Na^+ and Cl^-.

Salmo irideus is generally considered to be the European strain of *Oncorhynchus mykiss*. The bladder of the FW acclimated *Salmo irideus* has been studied extensively in perfused bladder sac preparations and in Ussing-style chambers (Fossat and Lahlou, 1977, 1979a, 1979b, 1982; Lahlou and Fossat, 1971, 1984). Lahlou and Fossat (1971)

determined there was a net transport of Na^+, Cl^- and H_2O in a mucosal to serosal direction using a perfused sac technique. Active transport by the epithelium was suggested because mucosal saline Na^+ and Cl^- concentrations were 10-fold lower than the serosal saline concentrations at the end of the experiment.

In the absence of an osmotic gradient (symmetrical saline), water transport was dependent on active absorption of Na^+ and Cl^- in the urinary bladder of *Salmo irideus* and the removal of Na^+ from the mucosal saline abolished the water transport (Fossat and Lahlou, 1977). The following study by Fossat and Lahlou (1979a) indicated there was a coupling of Na^+ and Cl^- transport whereby the lumenal removal of one ion inhibited the transport of its co-ion. This epithelia appeared to employ an electroneutral transport of Na^+ and Cl^- as found in other preparations such as the rabbit ileum (Nellans *et al.*, 1973, 1974) and the rabbit gall-bladder (Frizzel *et al.*, 1975). The mucosal to serosal fluxes of Na^+ and Cl^- yielded similar linear and saturating (K_m = 8 mM, Michaelis-Menten kinetics) components as a function of the mucosal concentration of the relevant ion. Upon pH changes in the mucosal or serosal bathing media, ionic transport was not affected, suggesting that a mechanism of Cl^-/HCO_3^- or Na^+/H^+ exchange is unlikely (Fossat and Lahlou, 1979a).

Work was also performed to determine whether the Na^+/K^+-ATPase enzyme is involved in the absorption of Na^+ in the urinary bladder of *Salmo irideus* (Fossat *et al.*, 1974). Through the use of bladder sac preparations, separate experiments involving the serosal addition of ouabain (Na^+/K^+-ATPase blocker) and removal of K^+ from the serosal bathing solution were performed. Both treatments caused a similar decrease in Na^+ influx. The enzymatic activity of Na^+/K^+-ATPase also decreased when *Salmo irideus*

were acclimated to higher salinities. These results indicated a relationship between Na^+/K^+-ATPase and Na^+ absorption in the urinary bladder.

A characteristic of some transporting epithelia (ie *Fundulus* opercular epithelium, *Goby* jaw skin preparation) is a cation selective paracellular shunt which usually produces a transepithelial potential. However, the cation 2,4,6-Triaminopyrimidine (TAP) commonly used to block Na^+ permeability was ineffective in the urinary bladder of *Salmo irideus* (Fossat and Lahlou, 1979b). This result also suggested a portion of Cl^- absorption must be through paracellular channels due to the previous finding of Na^+ and Cl^- coupled transport and the overall higher permeability of Cl^- (Fossat and Lahlou, 1979a). A model representing the findings of Fossat and Lahlou (1979a) is presented in Figure 1.4 A.

The mechanism of Na^+ and Cl^- transport in the urinary bladder of the closely related brook trout (*Salvelinus fontinalis*) appears to be fundamentally different from that of *Salmo irideus*. Active transport and kinetics of Cl^- were not affected by the absence of Na^+ and K^+, indicating independent transport and suggesting the presence of Cl^-/HCO_3^- exchange (Marshall, 1986) (Fig. 1.4 B). Na^+ absorption was active and continued in the absence of Cl^- in the mucosal bath. Also, under these conditions net acid was secreted into the mucosal bath suggestive of Na^+/H^+ exchange. Amiloride (Na^+/H^+ exchange blocker) partially inhibited Na^+ uptake, but bumetanide, a classic Na^+/Cl^- co-transport blocker, had no effect. These independent mechanisms of transport appears very similar to models normally presented for the freshwater teleost gill (Wood, 1991; Perry, 1997).

Further voltage clamping experiments on the *S. fontinalis* urinary bladder indicated that Na^+ diffusion occurs primarily through transcellular pathways (Marshall,

1988). The epithelium behaves characteristically as a simple resistive barrier to Cl^-, suggesting that a portion of Cl^- efflux may be paracellular. Non-conductive anion exchange is believed to be located apically which accounts for the remainder of Cl^- efflux. Marshall and Bryson (1991) confirmed the existence of Na^+/H^+ exchange by simultaneously adding amiloride and altering the mucosal pH. This treatment inhibited intracellular pH regulation by an apically located Na^+/H^+ exchange mechanism. Addition of NH_4^+ to the serosal bath (saline) enhanced the mucosa to serosa flux of Na^+. This flux was also blocked by mucosal addition of amiloride; Na^+/NH_4^+ exchange was therefore indicated.

Chapter 2

NaCl transport of the opercular epithelia of freshwater acclimated *Fundulus heteroclitus* and *Oreochromis niloticus*

Chapter 2 describes the results of studies on Na^+ and Cl^- transport in the opercular epithelia of two different FW-adapted species (*F. heteroclitus* and *O. niloticus*). The goal of this chapter was to establish one or both of these *in vitro* preparations as a model to further our understanding of FW ionoregulation in the teleost gill. The first objective of this study was to confirm the findings of Marshall *et al.* (1997) who reported active Cl^- uptake but passive diffusion of Na^+ across the *Fundulus* opercular epithelium. The 10% SW-adapted *Fundulus*, like the FW-adapted *Fundulus*, survives in a hyposmotic environment and must maintain its internal composition by absorbing salts from the environment. Therefore, the second objective of the study was to determine if adapting *Fundulus* to 10% seawater, a more natural situation for this estuarine species, would

promote active NaCl uptake, possibly in a net positive direction. The FW-adapted *Oreochromis* is an endemic FW species, unlike the euryhaline *Fundulus* and it was reasoned that the opercular epithelium of this species may display active Na^+ and/or Cl^- uptake. An additional objective was to test whether avoiding exposure of the *Oreochromis* opercular epithelium to high salt concentrations (saline) during the dissection procedure would facilitate NaCl transport. This final objective was tested because Karnaky (1991) suggested that exposure of transporting membranes to high salt content may change the ultrastructure and physiology of the transporting cells.

The *Fundulus* opercular epithelium exhibited active transport of Cl^- from the mucosal FW against a strong electrochemical gradient. Na^+ movement was dominated by passive diffusion. This *in vitro* study of FW *Fundulus* opercular epithelia recorded larger influx rates of Na^+ and Cl^- than previous studies on the same species (attributed to higher FW Ca^{2+}). The opercular epithelia of *Fundulus* adapted to 10% SW exhibited properties similar to SW *Fundulus*, and not to FW *Fundulus* by actively extruding Cl^- while Na^+ moved passively into the mucosal 10% SW. With FW bathing the mucosal surface, FW *Oreochromis* opercular epithelia displayed active absorption of Na^+, Cl^- and Ca^{2+} against large electrical and/or chemical gradients. Ca^{2+} movement was in a net uptake positive balance direction. Using FW osmotically compensated with mannitol during the dissection procedure instead of saline, did not increase or decrease influx of Na^+ or Cl^-.

Therefore, Chapter 2 confirmed the movement of Na^+ and Cl^- in the *Fundulus* opercular epithelium from past studies and introduced the FW *Oreochromis* opercular epithelium as a new *in vitro* model for the study of branchial ion regulation. The *O.*

niloticus preparation is the only FW *in vitro* preparation to our knowledge that exhibits active absorption of both Na^+ and Cl^-.

Chapter 3

Na^+ and Cl^- transport by the urinary bladder of the freshwater rainbow trout (*Oncorhynchus mykiss*)

Chapter 3 describes the results of studies on the mechanisms by which Na^+ and Cl^- are reabsorbed in the urinary bladder of *O. mykiss* mounted *in vitro*. This chapter examined NaCl transport under numerous experimental conditions to determine whether the transport of these ions was coupled or independent. Ion replacement experiments were performed whereby Na^+ or Cl^- was removed from the mucosal bathing solution and its counter-ion movement was monitored. These ion replacement experiments were conducted under symmetrical saline conditions and with artificial urine bathing the mucosal side. Another objective of this study was to test whether the addition of pharmacological agents, known to block specific transporters or ion channels, would affect ion transport. Electrophysiological and kinetic transport properties of the epithelium were also determined.

FW *O. mykiss* urinary bladders mounted *in vitro* under symmetrical saline conditions displayed electroneutral active absorption of Na^+ and Cl^- from the mucosal urine side. Removal of Na^+ from the mucosal saline decreased Cl^- absorption by 56% and removal of Cl^- inhibited Na^+ absorption by 69%. However, active net reabsorption of neither Na^+ nor Cl^- was abolished when its counter-ion was replaced with a permeable ion. Under more realistic conditions with artificial urine bathing the mucosal surface,

unidirectional influx rates of both Na^+ and Cl^- decreased, and net serosal to mucosal fluxes occurred. Nevertheless active absorption of both ions still occurred according to the Ussing flux ratio criterion. Removal of Na^+ from the mucosal artificial urine yielded no change in unidirectional influx of Cl^- and removal of Cl^- also resulted in no change in unidirectional influx rates of Na^+ from control values. The mucosal addition of DIDS, amiloride or bumetanide (10^{-4} M), when the bladder was bathed with either artificial urine or symmetrical Cortland's saline on the mucosal side, did not affect absorption rates of Na^+ and/or Cl^-. When the mucosal surface of bladder epithelia were bathed in artificial urine and "Na^+ kinetics" were compared in control and mucosal Cl^- free solutions, J_{max} substantially decreased but the affinity of the transport system for Na^+ (K_m) was not affected. Similar analyses of "Cl^- kinetics" revealed a significant decrease in J_{max} values in the low-Na^+ mucosal bathing solution compared to the control artificial urine solution, while K_m was again unaffected. A further observation was that the anterior portion of the urinary bladder transported Na^+ and Cl^- at a faster rate than posterior portions, particularly under symmetrical saline conditions. However, there was no difference in measured Na^+/K^+-ATPase activities between anterior and posterior portions of bladder epithelia.

These results suggest that Na^+ and Cl^- reabsorption in the urinary bladder of *O. mykiss* is a partially coupled process, where both dependent and independent uptake processes occur. The findings from this study in particular reinforce previous data from Fossat and Lahlou (1979a) who studied the European strain of rainbow trout (*Salmo irideus*).

The information in this thesis will hopefully contribute towards a better understanding of freshwater teleost ionoregulation. The three different *in vitro*

preparations presented here each should be further studied and their transport processes expanded upon. By way of future *in vitro* experiments one or all of these preparations could be used as a potential model for the study of Na^+, Cl^- and/or Ca^{2+} transport in the teleost gill.

Figure 1.1. The current model for NaCl transport in the gill epithelia of seawater adapted teleosts. This model was proposed from whole animal studies and later confirmed through *in vitro* use of opercular epithelia from *Fundulus heteroclitus*, *Oreochromis mossambicus* and *Gillichthys mirabilis*. The basolateral Na^+/K^+-ATPase indirectly drives Cl^- extrusion through the operation of a Na^+, K^+, $2Cl^-$ co-transporter. Na^+ diffusion is passive along a net electrochemical gradient from blood to external seawater, through paracellular channels between choride and/or adjacent accessory cells. Cl^- exits along an electrochemical gradient through channels in the apical crypt. Carrier-mediated processes are indicated by solid arrows, diffusive processes by dashed lines.

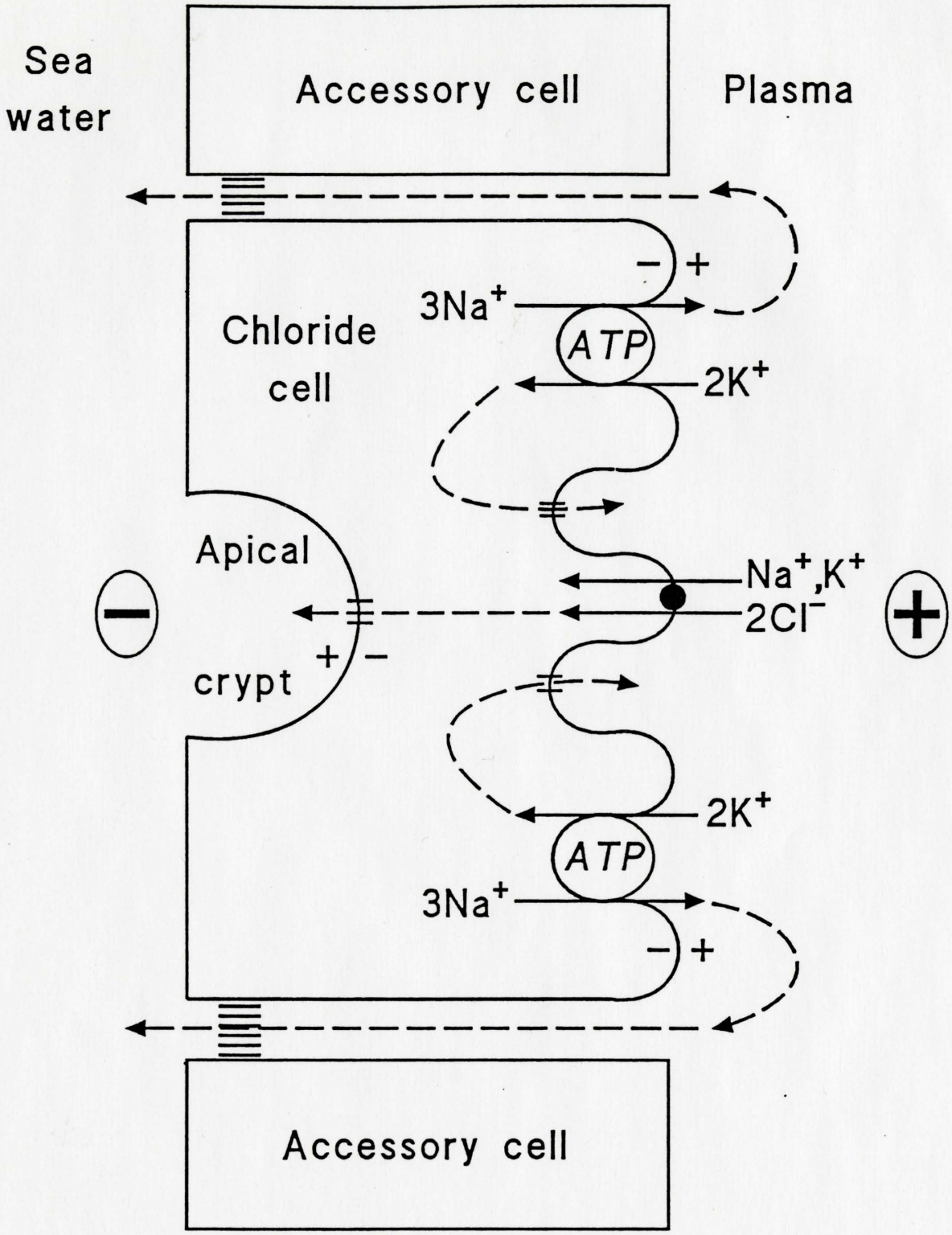

Sea water
Accessory cell
Plasma
Chloride cell
3Na+
ATP
2K+
Apical crypt
Na+,K+
2Cl−
2K+
ATP
3Na+
Accessory cell

Figure 1.2. Current ideas for NaCl transport in the gill epithelia of freshwater adapted teleosts. This model was developed mainly from whole animal studies and perfused gill preparations. Na^+ is exchanged for H^+ and/or NH_4^+ and Cl^- for HCO_3^-, exchange processes that may be driven indirectly by a basolateral Na^+/K^+-ATPase. An alternate proposed mechanism for Na^+ uptake is by diffusion through a Na^+ selective channel on the apical membrane of gill cells, driven by the electrical gradient created by active extrusion of H^+ ions across the apical membrane of the same or neighbouring cells by H^+-ATPase. Carbonic anhydrase (CAH) hydrates respiratory CO_2 and forms H^+ and HCO_3^-. Cell types involved in ion regulation have not been identified and thus are not labelled. Carrier-mediated processes are indicated by solid arrows, diffusive processes by dashed lines.

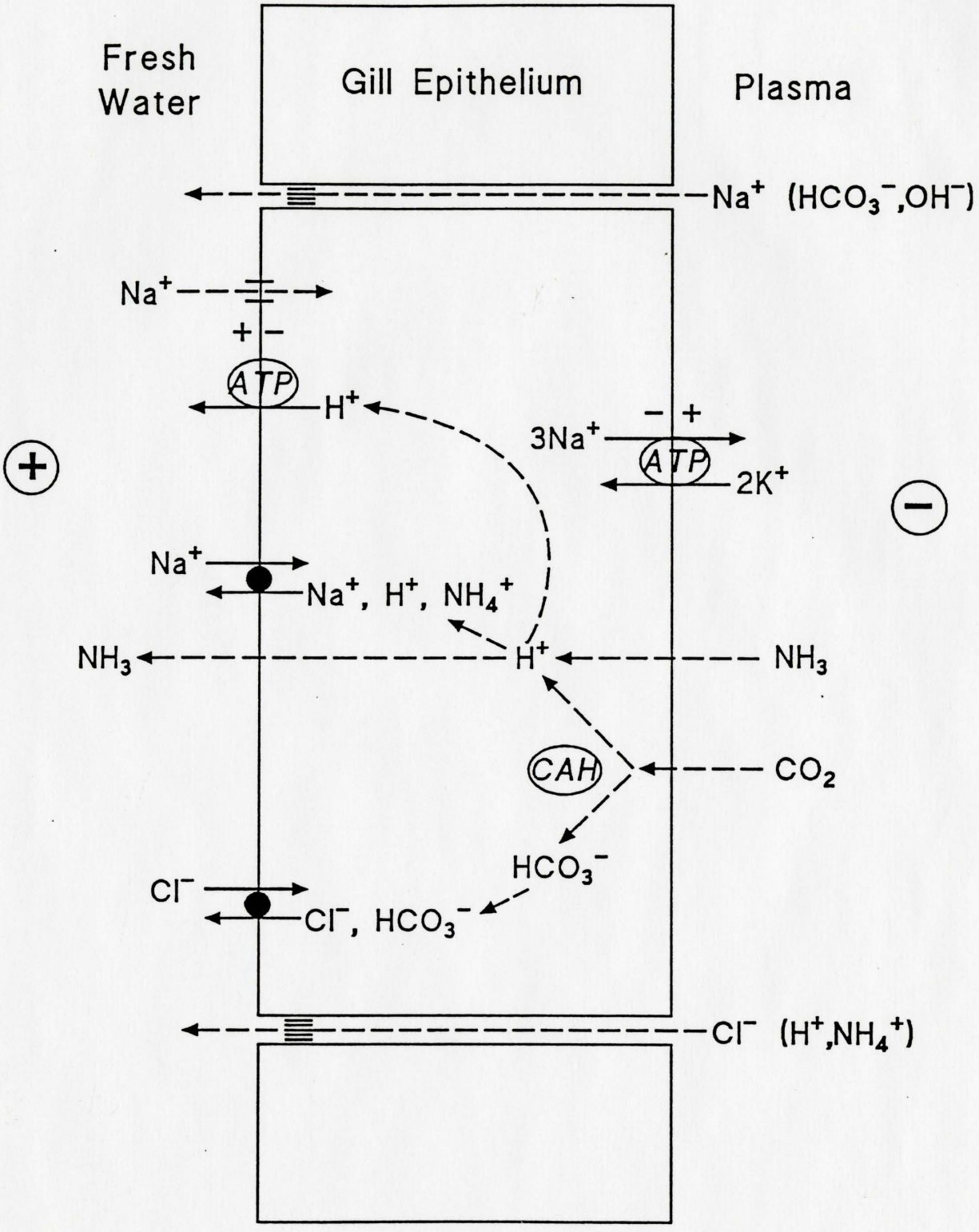

Fresh Water
Gill Epithelium
Plasma
Na+ (HCO3−,OH−)
Na+
+ −
ATP
H+
3Na+
− +
ATP
2K+
+
−
Na+
Na+, H+, NH4+
NH3
H+
NH3
CAH
CO2
HCO3−
Cl−
Cl−, HCO3−
Cl− (H+,NH4+)

Figure 1.3. The current model for Ca^{2+} transport in the gill epithelia of freshwater adapted teleosts. This model was developed from whole animal studies and *in vitro* preparations. Ca^{2+} uptake is believed to occur via a calmodulin-dependent, high affinity Ca^{2+}-ATPase or a $Ca^{2+}/3Na^{+}$ exchanger driven by the enzyme Na^{+}/K^{+}-ATPase. Cell types involved in ion transport have not been identified and thus are not labelled, however strong evidence suggests that they are MR cells. Carrier-mediated processes are indicated by solid arrows, diffusive processes by dashed lines.

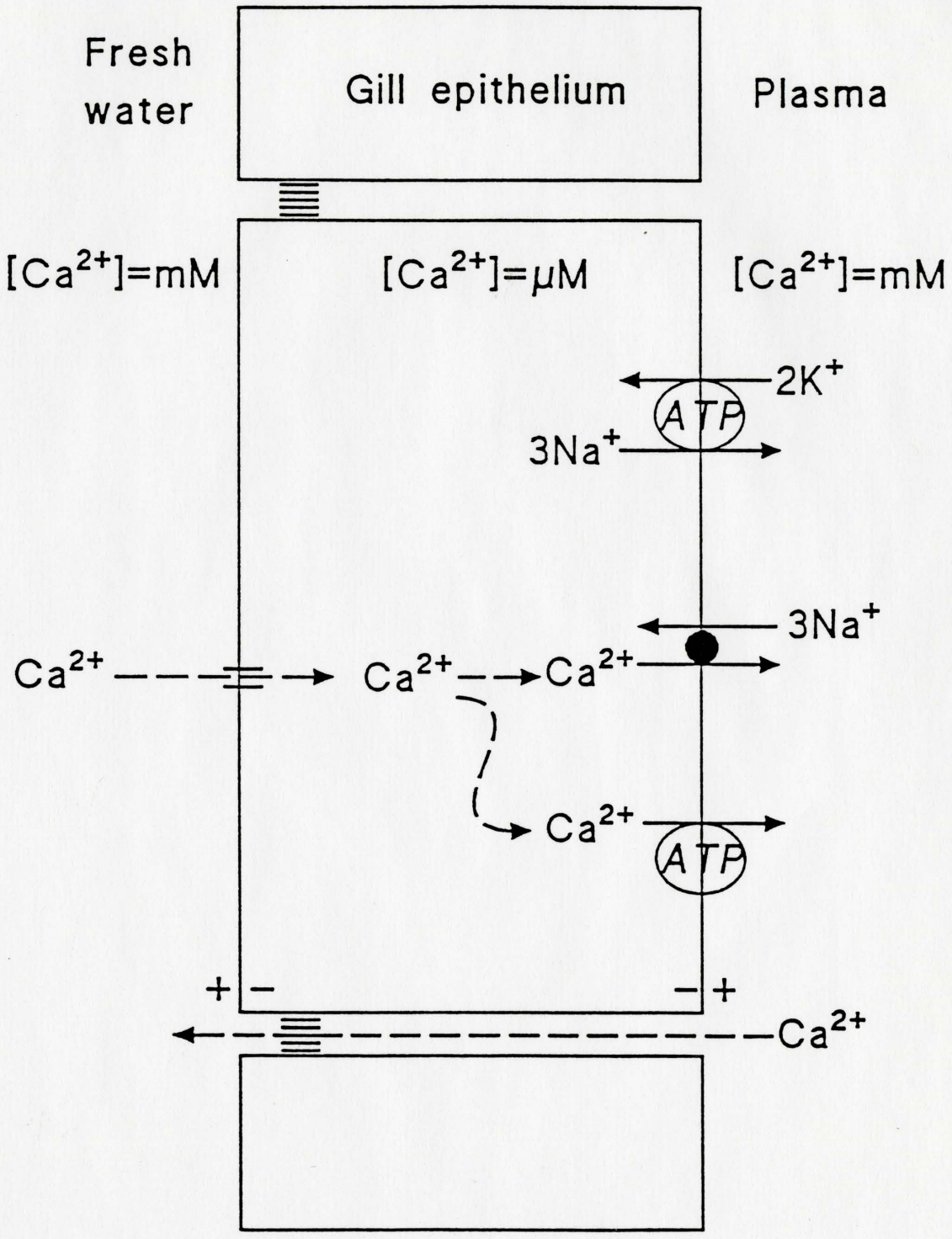
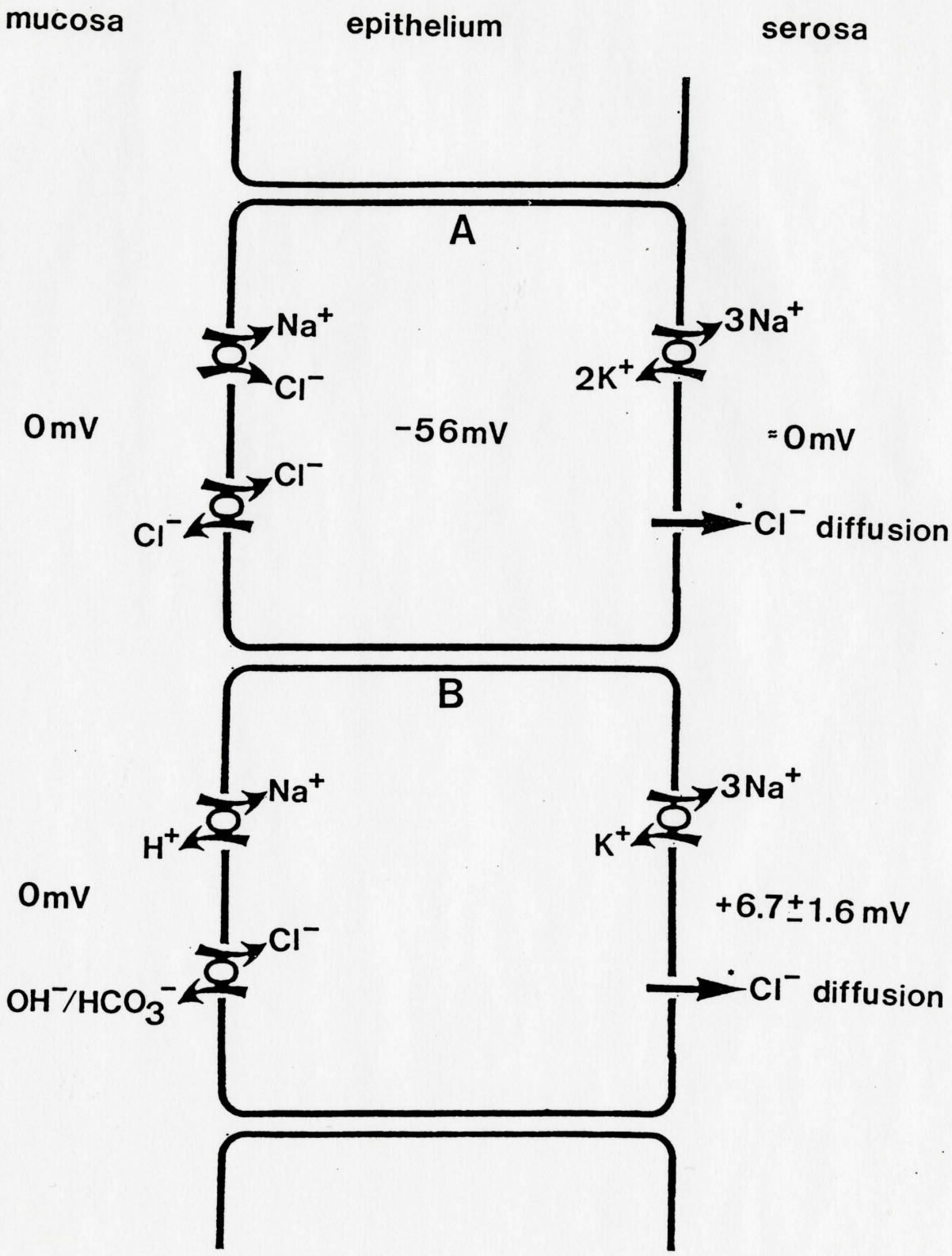

Fresh water
Gill epithelium
Plasma
[Ca2+]=mM
[Ca2+]=μM
[Ca2+]=mM
2K+
ATP
3Na+
3Na+
Ca2+
Ca2+
Ca2+
Ca2+
ATP
+ −
− +
Ca2+

Figure 1.4. A diagrammatic representation of Na^+ and Cl^- transport in the freshwater salmonid trout urinary bladder. A. Transport model based on *in vitro* studies using European rainbow trout *Salmo irideus* (Fossat and Lahlou, 1979a). A coupled transport of Na^+ and Cl^- is shown and driven indirectly by the basolateral enzyme Na^+/K^+-ATPase. B. Transport model based on *in vitro* work on brook trout *Salvelinus fontinalis* (Marshall, 1986, 1988). Independent transport of Na^+ and Cl^- on the apical side of the membrane in exchange for H^+ and OH^-/HCO_3^- respectively. Na^+/K^+-ATPase enzyme is believed to supply the energy required for Na^+ reabsorption. Asterisk in each figure indicates the assumptions of the original authors as to the processes marked.


mucosa
epithelium
serosa
A
Na+
Cl−
3Na+
2K+
0mV
−56mV
≈0mV
Cl−
Cl−
Cl− diffusion
B
Na+
H+
3Na+
K+
0mV
+6.7±1.6 mV
Cl−
OH−/HCO3−
Cl− diffusion

Chapter 2

Abstract

Opercular epithelia from the freshwater (FW) acclimated *Fundulus heteroclitus* and the FW *Oreochromis niloticus* were mounted *in vitro* with FW bathing the mucosal surface. The density of mitochondrial-rich cells was approximately 40-fold greater in the *Fundulus* preparation. The *Fundulus* preparation had larger absolute flux rates of Na^+ and Cl^- than the *Oreochromis* preparation. The *Fundulus* opercular epithelium had an inside negative transepithelial potential (V_t) of -43.9 mV and exhibited non-diffusive transport of Cl^- from the mucosal FW while Na^+ was passively distributed across the membrane according to the flux ratio criterion. Opercular epithelia from *Fundulus* acclimated to 10% seawater (SW) extruded Cl^-, when bathed either with 10% SW on the mucosal side or symmetrical Cortland's saline. Na^+ moved passively across the membrane under both experimental conditions. *Oreochromis* opercular epithelia had a V_t of +8.0 mV and displayed non-diffusive uptake of Cl^- and Na^+ against large concentration gradients from the mucosal FW media, based on disagreement with the Ussing flux ratio equation. Non-diffusive net absorption of Ca^{2+} from the FW bath by the *Oreochromis* membrane was also shown. In experiments with FW osmotically compensated with mannitol as the dissection medium instead of saline, Na^+ and Cl^- influx increased but so did Na^+ and Cl^- efflux. When the membrane was bathed with symmetrical Cortland's saline, the conductance increased approximately 15-fold to 14.6 $mS \cdot cm^{-2}$ and the short-circuit current was less than 1 $\mu A \cdot cm^{-2}$ with no net movement of either Cl^- or Na^+. The FW *Fundulus* and *Oreochromis* opercular epithelia may be good

preparations for studying *in vitro* the mechanisms of Cl^-, Na^+ and Ca^{2+} transport in FW teleosts.

Introduction

There is now a generally accepted model for the mechanisms of ion transport in the gills of seawater teleosts (see Evans 1993; Marshall 1995; McCormick 1995 for recent reviews). Historically, the genesis of this model was largely due to the availability of flat epithelial preparations ("gill surrogates") which allowed mechanistic study of transport details *in vitro*. These *in vitro* studies used opercular epithelia from the killifish *Fundulus heteroclitus* (*e.g.* Degnan et al. 1977; Degnan and Zadunaisky 1979; 1980; Karnaky et al. 1977; Karnaky 1980, 1986; Ernst et al. 1980; Zadunaisky 1984) and the Mozambique tilapia *Sarotherodon* (= *Oreochromis*) *mossambicus* (*e.g.* Foskett et al. 1981, 1983; Foskett and Scheffey 1982; Scheffey et al. 1983), and the jaw skin epithelium of the goby *Gillichthys mirabilis* (*e.g.* Marshall and Nishioka 1980; Marshall 1981).

In contrast, our understanding of the mechanisms of ion transport in the gills of freshwater teleosts remains controversial and incomplete (see Evans 1993; Lin and Randall 1995; Perry 1997 for recent reviews). While it is clear that freshwater fish can take up Na^+ and Cl^- actively and independently from the dilute external environment, there are competing theories as to the mechanisms and sites of Na^+ uptake, a complete lack of knowledge on the energetic basis of Cl^- uptake, and considerable uncertainty as to the mechanisms by which Na^+ and Cl^- fluxes are linked to acidic and basic equivalent fluxes respectively (*e.g.* Péqueux et al. 1988; Wood 1991; Goss et al. 1992; Potts 1994). To a large extent, this deficit of understanding can be linked to the absence of suitable flat epithelial "gill surrogates" for *in vitro* study of the freshwater gill.

To date, the search for such freshwater model preparations has been disappointing. Foskett et al. (1981) found no apparent active Cl^- uptake across the opercular epithelium of freshwater-adapted *Oreochromis mossambicus*, and Marshall et al. (1992) found no apparent active Na^+ or Cl^- uptake across the cleithral epithelium of freshwater-adapted rainbow trout *Oncorhynchus mykiss*, despite the fact that both preparations exhibited populations of mitochondrial-rich (MR) cells. Recently, Marshall et al. (1997) studied the opercular epithelium of freshwater adapted *Fundulus heteroclitus*, which has a much higher density of MR cells than the other two preparations. When the mucosal surface was bathed with symmetrical saline, active uptake of both Na^+ and Cl^- occurred. However when the mucosal surface was bathed with freshwater, only the active uptake of Cl^- could be documented. Interestingly, there is active Ca^{2+} uptake from fresh water in skin preparations from trout (Marshall et al., 1992), killifish (Marshall et al. 1995) and Nile tilapia *Oreochromis niloticus* (McCormick et al. 1992).

The present study extends the search for a suitable freshwater model. The first objective was to confirm the conclusions of Marshall et al. (1997) using a different batch of *Fundulus heteroclitus* and different freshwater quality (higher Ca^{2+} concentration). The second was to evaluate whether acclimation and testing in a more moderate salinity (10% seawater) would promote active NaCl uptake in the preparation, because the killifish is more commonly an estuarine rather than a freshwater inhabitant in its normal environment. A third objective was to test whether Na^+ and/or Cl^- uptake could be detected across the opercular epithelium of freshwater-adapted *Oreochromis niloticus*, inasmuch as the Nile tilapia is native to freshwater. An additional goal was to confirm

the occurrence of active Ca^{2+} uptake across this preparation (McCormick et al. 1992). A final goal was to test whether avoiding exposure to high external NaCl concentrations (*i.e.* isotonic saline) during preparation would promote NaCl transport in the tilapia epithelium, because Karnaky (1991) has suggested that the morphology and physiology of the epithelium, particularly the role of the tight junctions, may change rapidly in response to external salinity.

Materials and Methods

Animals

Adult killifish (*Fundulus heteroclitus*) were obtained from the Antigonish estuary (Antigonish, NS), transferred directly to holding tanks that were ten percent seawater (3.0-3.2 ppt.), and then air-freighted to Hamilton, ON. In Hamilton, the fish were maintained in ten percent synthetic seawater (Marinemix, Baltimore, MD) in a 500 l aerated, charcoal-filtered system, but transferred to freshwater at least two weeks prior to use. During freshwater acclimation, the killifish were held in Hamilton tapwater with an average composition (in mmol l^{-1}) of Na^+ 0.6; Cl^- 0.7; Ca^{2+} 1.05; pH 7.5-8.0. The 60 l acclimation tanks contained charcoal-filter systems and were well aerated. Light was maintained at seasonal photoperiod and water temperature was constant with room temperature (20 ± 1°C). Killifish were fed with commercial food (Wardley, Secaucus, NJ) at a rate of 1% of their body mass per day.

Nile tilapia (*Oreochromis niloticus*) (10-15 g) were obtained from Northern Tilapia, Lindsay, ON, and were kept in a 500-l tank supplied at a rate of 50 ml min^{-1} with a flow through of dechlorinated Hamilton tap water, charcoal filtration and aeration. The

freshwater was maintained at 30 ± 1°C. Tilapia were fed commercial trout pellets (Zeigler, Hazelton, PA) at a rate of 1% of their body mass per day.

Bathing solutions

A modified Cortland saline solution used for dissection and for the basolateral bathing solution was composed of (in mmol l^{-1}) NaCl, 129.9; KCl, 2.55; $CaCl_2.H_2O$, 1.56; $MgSO_4.7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4.H_2O$, 2.97; glucose, 5.55; NH_4Cl, 0.30 (all salts from Sigma Chemical Co.). To substitute for plasma protein the saline was supplemented with 20 mg.ml^{-1} bovine serum albumin. The saline was equilibrated with a 0.3% CO_2, balance O_2, gas mixture and had a measured pH of 7.8-7.9.

In open-circuit experiments using asymmetrical bathing solutions, the solution for the mucosal surface of the membrane was taken from either the freshwater entering the holding tank or directly from the 10% SW in the holding tank. The FW or 10% SW was then equilibrated with 100% O_2 before being used in the Ussing chamber. Prior to the start of the experiment and at the end of each 60 minute flux period (see below), the mucosal solution was rinsed extensively to maintain the original ion composition of the FW (Na^+ = 0.7, Cl^- = 0.6 and Ca^{2+} = 1.05 mmol l^{-1}) or 10% SW (Na^+ = 47.0, Cl^- = 54.8 and Ca^{2+} = 1.02 mmol l^{-1}).

Isolated opercular epithelia preparation

The fish were killed by pithing and both opercular membranes were immediately dissected from their underlying opercular bone, teasing off the epithelia in a ventral-anterior to dorsal-posterior direction. Prior to dissection, mucus was carefully removed

from the epithelial surface using fine forceps. Each opercular epithelium yielded an approximate area of 1-2 cm^2. Throughout the dissection, the preparation was kept moist by the addition of saline.

The Ussing membrane apertures (0.125 cm^2) were prepared with stopcock grease (Dow Corning, Midland, MI) and a thin vinyl mesh was placed over each aperture to support the tissue. The opercular epithelium was carefully pinned down to the aperture mucosal side up and the second half of the aperture then sandwiched the epithelium. After mounting, the mucosal surfaces were thoroughly rinsed with fresh water to remove the saline and any mucus that had accumulated during the dissection. The aperture sandwich was then placed between the two hemi-chambers and tightened into place. The hemi-chambers were filled with the appropriate solutions: fresh water or saline or 10% seawater for the mucosal side, and saline for the serosal side. Each hemi-chamber was filled at an equal rate to ensure there was no 'bagging' of the epithelium. The membranes were then given a 30 min period to adjust to the *in vitro* conditions.

Mannitol dissection procedure

In one set of experiments concerning the *Oreochromis* opercular epithelia, 250 mM mannitol (Sigma Chemical, Co.) was added to FW to serve as the dissection medium in place of saline so as to maintain the correct FW NaCl concentration yet at the same time provide a normal extracellular fluid osmotic pressure which would avoid osmotic stress to the basolateral surface.

Electrophysiology

Polyethylene 4% agar/saline bridges were used to measure transepithelial potential (V_t, mucosal side grounded) and membrane conductance (G_t). Each bridge was connected to the current/voltage clamp (WP Instruments, New Haven, CT, DVC-1000) by Hg/HgCl calomel half-cells. Membrane conductance was corrected for solution resistances. In asymmetrical solutions, corrections for liquid junction potentials were accomplished by measurements against a free flowing 3 M KCl half-cell. Ussing chambers were water jacketed to the killifish and tilapia acclimation temperatures at 20 ± 1°C and 30 ± 1°C respectively. In certain flux experiments with opercular epithelia from *Fundulus* adapted to 10% SW, or *Oreochromis* adapted to FW, the preparation was short-circuited (with saline bathing the mucosal surface) to negate any effect of V_t on ion transport. G_t was determined by clamping the membrane to a set voltage every 10 min. and V_t was recorded at the beginning and end of each 60 min short-circuited flux period.

Experimental protocol

One chamber was set up for each opercular epithelium for pair wise measurements of influx and efflux. Each fish therefore yielded a mucosal to serosal unidirectional influx (J^{ms}) measurement and a serosal to mucosal unidirectional efflux (J^{sm}) measurement. After the initial 30 min adjustment period, the mucosal hemi-chamber volume was gently rinsed again with FW (20 times the chamber volume) and isotope was added to the appropriate side.

Unidirectional fluxes were determined by measuring the specific activity in the labelled side and the appearance of isotope on the unlabelled side. Dual flux

experiments were performed with ^{36}Cl ($Na^{36}Cl$ from I.C.N. Radiochemicals, Irvine, California) and ^{22}Na ($^{22}NaCl$ from NEN-Dupont, Boston, MA) which were added to either the mucosal side (final specific activity of 300 000 CPM μmol^{-1}) for unidirectional influx or serosal side (8 000 CPM μmol^{-1}) to monitor unidirectional efflux. In Ca^{2+} flux experiments, the final specific activity of ^{45}Ca ($^{45}CaCl_2$ from NEN-Dupont, Boston, MA) was 10-100-fold higher. Upon first addition of the radioisotope(s), a 45 min equilibration period was employed. Each experiment consisted of three 60 min periods; within each period samples were taken from the unlabelled bath every 20 min for radioactivity analyses. Flux values for each 20 min period were averaged to produce a final flux rate for each 60 min experimental period. At the start and end of each period, samples were taken from the labeled side to determine the specific activity and ionic concentrations. Between each one hour period the chambers were thoroughly flushed, and new solutions with isotope were added and equilibrated for at least 45 min.

The observed flux ratio (J^{ms}/J^{sm}) was compared to the predicted flux ratio using the Ussing flux ratio equation (Ussing 1949). Disagreement between the observed and predicted value indicated the presence of non-diffusive transport. The predicted flux ratio equation was as follows:

$$J_i^{ms}/J_i^{sm} = (a_i^m/a_i^s)e^{(z_iFV_t/RT)}.$$

The activities of ion *i* are a_i^s and a_i^m on the serosal and mucosal sides respectively; valency of the ion is z_i, V_t is the transepithelial potential and F, R and T have their usual thermodynamic meanings. The ionic activities of Na^+ (109.5 mmol l^{-1}) and Ca^{2+} (0.79 mmol l^{-1}) in Cortland's saline and Na^+ (35.6 mmol l^{-1}) in 10% SW were taken from measurements with microelectrodes filled with the appropriate ionophore (Steiner et al.

1979), while Cl^- was predicted to have the same relative activity (on a % basis) as Na^+ from theory for solutions of these ionic strengths (Lee, 1981). The presence of 20 mg ml^{-1} of bovine serum albumin lowered the activity of Ca^{2+} in the saline, but Na^+ and Cl^- activity were unaffected. The FW ionic activities of Na^+, Cl^- and Ca^{2+} were taken as equal to their measured concentrations.

Fluorescence microscopy

Opercular epithelia from the Ussing chamber were bathed in 10 μmol l^{-1} DASPEI [2-(4-dimethylaminostyrl)-N-ethylpyridium iodide); I.C.N Biomedicals, Costa Mesa, CA] (mitochondrial fluorophore) to determine the density of MR cells. DASPEI was initially dissolved in distilled water to a concentration of 0.2 mg ml^{-1}. The membranes were then incubated in a diluted DASPEI stock solution (oxygenated saline) for 30 min. before being viewed as a wet mount on an epifluorescence microscope (Zeiss, Germany) with an excitation wavelength of 450-490 nm. Cell counts were made at four randomly chosen fields at a magnification of 160 (1.7 mm^2 field of view) and the values were then averaged to yield a density of MR cells per mm^2.

Analytical techniques

Saline Cl^- concentrations were measured by coulometric titration (model CMT10, Radiometer, Copenhagen) and freshwater Cl^- concentrations were determined by colorimetric assay (Zall et al. 1956). Na^+ and Ca^{2+} concentrations in both media were analyzed by atomic absorption spectrophotometry (model AA-1275, Varian, Springvale, Australia). Samples of 40μl from the labelled side and 250μl from the unlabelled side

were added to 4.0 ml of Readysafe fluor (Beckman, Fullerton, CA). Radioactivities of ^{36}Cl and ^{45}Ca were determined by counting on a Rackbeta 1217 liquid scintillation counter (LKB, Wallac, Turku, Finland), and ^{22}Na radioactivity was counted on Minaxi Autogamma 5000 counter (Packard Instrument Co., Downers Grove, IL). ^{22}Na emits both gamma and beta radiation, therefore scintillation counts collected from a ^{22}Na and ^{36}Cl dual flux experiment were from both ^{22}Na and ^{36}Cl. ^{36}Cl counts were determined by a count subtraction procedure. This was accomplished by measuring the CPM of a known concentration of ^{22}Na in both the scintillation and gamma counters and then determining the relative efficiency of the two counters for detecting ^{22}Na. The CPM from the gamma counter was then multiplied by this ratio and subtracted from the CPM of the scintillation counter to yield the beta emission of ^{36}Cl only.

Data are presented as means ± 1 standard error unless indicated otherwise. Comparisons within and between treatments were analyzed by paired or un-paired *t*-tests (two-tailed), as appropriate, at $P<0.05$.

Results

Opercular epithelia from FW acclimated *Fundulus*, bathed on the mucosal side with FW, had a serosa-negative transepithelial potential at open-circuit of -43.9 ± 2.4 mV and a transepithelial conductance of 1.94 ± 0.34 $mS \cdot cm^{-2}$ (Table 1). The influx (Jms) values for Cl^- generally decreased over time, however the difference was not significant from period 1 to period 3 ($P>0.05$). Efflux (Jsm) was about 10 fold greater than influx (Jms); thus there was also a net loss of Cl^- over each of the three one hour flux periods. Figure 1A shows a clear difference between the observed and predicted flux ratios

($P<0.05$) averaged over the three periods for Cl^-, thereby suggesting active chloride absorption by the tissue according to the Ussing flux ratio criterion.

The unidirectional influx and efflux values for Na^+ demonstrated that the epithelium was also losing sodium over each of the three one hour periods. The observed and predicted flux ratios for Na^+ were not significantly different, indicating that this ion moves passively across the epithelium (Figure 1A).

Experiments performed on 10% SW acclimated *Fundulus* opercular epithelia (with 10% SW bathing the mucosal surface and the preparation at open circuit), indicated that the epithelia had a serosa positive V_t of $+3.3 \pm 0.5$ mV in contrast to the very negative V_t seen with FW membranes (Table 1). The G_t of 2.32 ± 0.43 mS·cm^{-2} however, was not different from FW membranes. The preparation displayed significant net extrusion of Cl^- into the mucosal 10% SW, with a similar, but non-significant trend for net extrusion of Na^+. Flux ratio analysis indicated passive diffusion of Na^+ and active extrusion of Cl^- (Fig. 1B). This conclusion was confirmed by placing the epithelia from 10% SW acclimated *Fundulus* under short-circuit conditions with saline bathing both sides of the membrane. V_t increased to $+9.52 \pm 1.9$ mV (prior to short-circuit), G_t was significantly greater at 5.97 ± 0.89 mS·cm^{-2}, and short-circuit current was 34.08 ± 2.47 μA·cm^{-2} (not shown). Under these conditions, the epithelia exhibited higher unidirectional fluxes for both Na^+ and Cl^-, a clear net extrusion of Cl^- into the mucosal saline, but no significant net flux of Na^+ (see hour 3 in bottom part of Table 1). The observed flux ratio for Cl^- was clearly different from the predicted ratio of unity, confirming the active secretion of Cl^-, whereas the observed flux ratio for Na^+ was not

significantly different from unity, confirming passive distribution of this cation (not shown).

The FW-adapted *Oreochromis* opercular membranes, bathed on the mucosal surface with FW had a serosa-positive V_t of +8.0 ± 0.57 mV and G_t of 1.78 ± 0.19 $mS \cdot cm^{-2}$ (Table 2). Both Cl^- and Na^+ unidirectional fluxes indicated that there was a net loss of ions into the mucosal FW bath during the flux experiment. Over time Cl^- influx was more or less stable, while Na^+ influx showed a steady decrease. The observed flux ratios for both Na^+ and Cl^- were significantly greater ($P<0.05$) than the flux ratios predicted on the basis of passive diffusion by the Ussing flux ratio equation (Figure 2A), thus indicating these ions were actively transported in the uptake direction.

Net uptake of Ca^{2+} occurred in each of the three experimental periods. Ca^{2+} influx was against a concentration and an electrical gradient and was almost twice the magnitude of Ca^{2+} efflux (Table 2). The much greater observed flux ratio of Ca^{2+} (Figure 2A) than predicted by the Ussing equation indicated active transport of this ion.

Under symmetrical saline conditions, the ionic conductance of the *Oreochromis* opercular membrane increased approximately 8-fold to 14.6 $mS \cdot cm^{-2}$, while V_t was reduced to approximately zero (0.04 ± 0.04 mV), so, the short-circuit current was less than 1 $\mu A \cdot cm^{-2}$ (Table 2). There was no net movement of either Na^+ or Cl^- that was significant in any direction and the flux ratios for Cl^- and Na^+ were not significantly different from the predicted flux ratios, suggesting that these ions were passively distributed across the epithelium under these conditions.

To evaluate whether exposure to high NaCl levels on the mucosal surface during dissection was a problem, FW was osmotically compensated with mannitol and then used

in place of saline in the *Oreochromis* dissection procedure. Unidirectional influx and efflux of Na^+ and Cl^- were not significantly affected. The observed Na^+ and Cl^- flux ratios were significantly different from their respective predicted ratios (Fig. 2B), but the magnitude of the observed ratios were almost identical to those seen during the normal saline dissection procedure (Fig. 2A).

A comparison of absolute unidirectional flux rates between FW-adapted *Fundulus* and *Oreochromis* preparations when FW was present on the mucosal surface in both (compare Tables 1 and 2) demonstrated that in each period, uptake values (Jms) for both Na^+ (9-fold difference) and Cl^- (4-fold difference) were significantly higher in *Fundulus*. Jsm values for Na^+ were also significantly higher (2.5 fold difference) in *Fundulus*, whereas there was only a slight, non-significant difference in Jsm values for Cl^-.

The FW adapted *Fundulus* opercular epithelium contained 1868 ± 87 MR cells mm^{-2} (n=6). The FW *Oreochromis* epithelium contained a MR cell density of approximately forty-fold less at 45.8 ± 3.0 mm^{-2} (n=6).

Discussion

Fundulus opercular epithelium

The FW acclimated *Fundulus* opercular epithelium actively transported Cl^- from the mucosal FW bath to the serosal saline according to the Ussing flux ratio equation (Fig. 1A), against a large concentration gradient (1:194) and a large electrical gradient (-44 mV). The movement of Na^+ across the FW *Fundulus* opercular epithelium was passive (Fig. 1A). These results confirm those of Marshall et al. (1997) who studied *Fundulus* opercular epithelium acclimated to and bathed mucosally in a FW with a

somewhat higher NaCl and a 10-fold lower Ca^{2+} level. Furthermore, unidirectional Na^+ and Cl^- flux rates and values of G_t were very similar in the two studies. Interestingly, V_t was less negative (-44 mV *vs* -64 mV) than in the study of Marshall et al. (1997), probably reflecting the well known effect of $[Ca^{2+}]$ in modulating the diffusion potential across epithelia of freshwater teleosts (Potts, 1984).

To determine if active transport of Na^+ and Cl^- in the uptake direction could be facilitated, *Fundulus* were acclimated to 10% SW. When the 10% SW adapted opercular epithelium was bathed with 10% SW on the mucosal surface in the Ussing chamber, it exhibited transport properties very similar to those of a SW adapted preparation, and not a FW preparation. The V_t was small but positive (+3.3 mV) and the epithelia exhibited active extrusion of Cl^- and passive movement of Na^+ into the mucosal bath (Fig. 1B). Under short-circuit (Isc) symmetrical saline conditions, Na^+ movement was passive and net extrusion of Cl^- was 4.8 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ compared to 6.07 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ of a SW operculum (Degnan et al. 1977). The Isc of this preparation (34 $\mu A \cdot cm^{-2}$) was intermediate between values previously found for FW adapted (~9-12 $\mu A \cdot cm^{-2}$; Marshall et al. 1997) and SW adapted *Fundulus* (136 $\mu A \cdot cm^{-2}$; Degnan et al. 1977). The small Isc found for the *Fundulus* 10% SW-adapted preparation indicated that the SW adaptive Cl^- secretion was not operating to its full extent. It seems curious that the opercular epithelium would actively excrete salt when the fish is living in 10% SW, because the tonicity of this media is only about one third blood levels. This situation would lead to salt depletion unless compensated in some way. It is possible that the *in vitro* preparation does not properly mimic the *in vivo* condition due to the lack of inhibitory hormones or neurotransmitters. Nevertheless, the result is in accord with structural

studies. *Fundulus* adapted to 10% SW are reported to have MR cells that are identical in ultrastructure to a 100 and 200% SW adapted *Fundulus* (Karnaky et al. 1976). The appearance of FW like MR cells has been found only upon acclimation to very low salinities (1/16 SW) (Philpott and Copeland, 1963). In *O. mossambicus*, Kültz and Onken (1993) found in the salinity range between FW and SW, MR cell density and all electrical parameters (Isc, V_t and G_t) increased in parallel.

Oreochromis opercular epithelium

The *Oreochromis* opercular epithelium had an inside positive V_t of +8.0 mV compared to the -43 mV found for the *Fundulus* preparation (see also Marshall et al. 1997). Both of these species survive in FW, yet each have their own distinct V_t. Marshall et al. (1997) reasoned the large negative inside V_t of *Fundulus heteroclitus* represents the membrane's cation selective diffusion potential. The V_t of intact freshwater fish can be slightly positive or slightly negative (Potts, 1984) and in this study the FW *Oreochromis* membrane's V_t (8 mV) was very comparable to the 10 mV reported for the skin of the same species by McCormick et al. (1992). The closely related *O. mossambicus* in FW had a whole-animal V_t of -1 to +10 mV (Young et al. 1988). In this study the positive V_t of the *Oreochromis* membrane could indicate a diffusion potential that favours anion movement out to the mucosal FW bath (Table 2).

For *Oreochromis*, both Na^+ and Cl^- observed flux ratios were significantly greater than predicted by the Ussing flux ratio equation (Fig. 2A) and therefore Na^+ along with Cl^- transport was active and in the uptake direction. To our knowledge, FW *Oreochromis* opercular epithelium is the only isolated epithelial preparation from a

teleost fish which exhibits *in vitro*, the non-diffusive transport of both Na^+ and Cl^-, as well as Ca^{2+} from a dilute mucosal bathing medium (FW). Other *in vitro* studies have used a variety of isolated preparations in an attempt to elucidate the FW transport of Na^+ and Cl^-. The cleithrum skin of the rainbow trout actively transported only Ca^{2+} while both Na^+ and Cl^- ions were passively distributed (Marshall et al. 1992). The FW killifish opercular epithelium displayed a passive movement of Na^+ and active transport of Cl^- when bathed with FW on the mucosal side (Marshall et al. 1997), an observation confirmed by the present study. Isolated skin from the 5% SW-adapted *Gillichthys mirabilis* demonstrated active transport of Cl^- from serosa to mucosa (Marshall, 1977), rather similar to the 10% SW acclimated *Fundulus* preparations of the present study. Cultured branchial epithelia from rainbow trout exposed to apical freshwater were found to actively transport Cl^- against an electrochemical gradient but Na^+ was again passively distributed (Wood et al. 1997).

Fluxes in symmetrical saline

The FW *Fundulus* opercular epithelium in symmetrical saline has a net uptake of both Na^+ and Cl^- at relatively rapid rates (1-2 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) indicating the presence of active uptake mechanisms for both major ions (Marshall et al. 1997). However, once the FW *Oreochromis* epithelium was bathed in symmetrical saline the V_t was reduced to near zero, the Isc was very small (~1$\mu A \cdot cm^{-2}$), and both Na^+ and Cl^- were passively distributed across the epithelium (Table 2). The isolated opercular membrane of a closely related species, the FW adapted *Oreochromis mossambicus*, had a small Isc (~1 $\mu A \cdot cm^{-2}$) and also did not actively transport Cl^- when bathed in symmetrical saline (Foskett et al. 1981).

Thus, whatever mechanism was operating when the preparation was bathed with FW on the mucosal surface is not detectable when mucosal saline increases shunt conductance and induces large unidirectional fluxes.

There were obvious differences in the electrical and transport parameters of the FW-adapted *Fundulus* and FW *Oreochromis* preparations. The *Fundulus* is a euryhaline, normally estuarine species that can readily adapt to a variety of salinities (Zadunaisky, 1984). Perhaps this species is not characteristic of a "true" FW teleost. The application of SITS, a drug which blocks anion exchange and anion channels had no effect on Cl^- influx in the FW adapted *Fundulus* preparation (Marshall et al. 1997). Amiloride, a well known Na^+/H^+ exchange and Na^+ channel inhibitor, added to the mucosal FW, had no effect on Na^+ unidirectional influx. According to these drug experiments, the transport properties of this FW *in vitro* preparation does not behave as a standard FW teleost is thought to function (Wood 1991; Potts 1994; Lin and Randall 1995; Perry 1997).

Ca^{2+} transport

The transport of Ca^{2+} in the isolated skin of the FW *Oreochromis* against an electrochemical gradient and in a net uptake direction (Table 2) was also shown to be a non-diffusive process (Fig. 2A). The uptake of Ca^{2+} by this isolated FW skin confirms the viability of the preparation. The mean net flux of Ca^{2+} (3.1 nmol$\cdot$cm$^{-2}\cdot$h^{-1}) was markedly higher than the previously reported Ca^{2+} mean net flux of 0.31 nmol$\cdot$cm$^{-2}\cdot$h^{-1} for the same species (McCormick et al. 1992). Possible discrepancies between the two studies exist in the ionic compositions of the serosal and mucosal bathing solutions, thus rendering the Ca^{2+} concentration gradient opposing transport almost seven-times smaller

for the present study. Active transport of Ca^{2+} from the mucosal to serosal surface against electrochemical gradients has also been shown in the cleithrum skin of the rainbow trout (Marshall et al. 1992) and the opercular epithelium of the FW-adapted *Fundulus* (Marshall et al. 1995). However, the opercular epithelium of the *Fundulus* displayed a very high rate of net transport of Ca^{2+} (29 $nmol \cdot cm^{-2} \cdot h^{-1}$) relative to 3.1 $nmol \cdot cm^{-2} \cdot h^{-1}$ by the *Oreochromis* opercular epithelium of the present study. Of interest is the greater density of MR cells (~2000 mm^{-2}) in the *Fundulus* preparation and the greater rate of Ca^{2+} transport when compared to the smaller number of MR cells (~50 mm^{-2}) and transport rate of Ca^{2+} in the *O. niloticus* preparation. MR cells have also been correlated to the rate of Ca^{2+} influx in isolated skin preparations of the *O. niloticus* (McCormick et al. 1992) and rainbow trout (Marshall et al. 1992).

Comparison of unidirectional Na^+ and Cl^- flux between the FW *Fundulus* and *Oreochromis* epithelia indicated that the former had much larger Na^+ and Cl^- influx rates (by 10 and 4 fold respectively) (Tables 1 and 2). The *Fundulus* epithelium contained approximately 40-times more MR cells than the *Oreochromis* epithelium. Therefore, these results may suggest a quantitative relationship between MR cells and influx of Na^+ and Cl^-. MR cells have long been thought to be directly associated with the active uptake of Na^+ and Cl^- (Avella et al. 1987; Laurent and Perry 1990). Conversely, it has also been suggested that these cells are not involved in such ion transport (Girard and Payan 1980).

Based on the proposal of Karnaky (1991), we hypothesized that during the dissection procedure, exposure to high levels of NaCl in the saline that came into contact with the apical side of the membrane might affect the ultrastructure of the membrane, and therefore *in vitro* it would not behave characteristically as a FW membrane. Therefore,

in one series freshwater was osmotically compensated with mannitol and then used instead of saline to bathe the *Oreochromis* preparation during the dissection and setup procedure. This modification did not significantly change Na^+ and Cl^- influx and efflux (Table 2), however observed ratios were not different from control observed ratios (Fig. 2 A/B). Future experiments may be more effective if it is possible to develop a dissection procedure in which FW would moisten only the mucosal surface and saline the serosal surface.

Acknowledgements

The authors thank Dr. M.J. O'Donnell for Ca^{2+} and Na^+ ion activity measurements and for his advice in this work. This work was supported by an NSERC Collaborative Research Grant (D.G. McDonald, P.I.) and by research grants from the Natural Sciences and Engineering Research Council of Canada to C.M.W and W.S.M.

References

Avella, M., Masoni, A., Bornancin, M., and Mayer-Gostan, N. 1987. Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. J. Exp. Zool. **241**: 159-169.

Degnan, K.J., Karnaky, K.J., and Zadunaisky, J.A. 1977. Active chloride transport in the in vitro opercular skin of a teleost (*Fundulus heteroclitus*), a gill-like epithelium rich in chloride cells. J. Physiol. Lond. **271**: 155-191.

Degnan, K.J., and Zadunaisky, J.A. 1979. Open-circuit Na^+ and Cl^- fluxes across isolated opercular epithelium from the teleost *Fundulus heteroclitus*. J. Physiol. Lond. **294**: 483-495.

Degnan, K.J., and Zadunaisky, J.A. 1980. Ionic contributions to the potential and current across the opercular epithelium. Am. J. Physiol. **238**: R231-R239.

Ernst, S.A., Dodson, W.C., and Karnaky, K.J. 1980. Structural diversity of occluding junctions in the low resistance chloride-secreting opercular epithelium of seawater-adapted killifish (*Fundulus heteroclitus*). J. Cell Biol. **87**: 488-497.

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

Foskett, J.K., Logsdon, 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.

Foskett, J.K., and Scheffey, C. 1982. The chloride cell: definitive identification as the salt-secretory cell in teleosts. Science. **215**: 164-166.

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.

Girard, J.P., and Payan, P. 1980. Ion exchanges through respiratory and chloride cells in freshwater- and seawater-adapted teleosteans. Am. J. Physiol. **238**: R260-R268.

Goss, G.G., Perry, S.F., Wood, C.M., and Laurent, P.L. 1992. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. J. Exp. Biol. **106**: 143-159.

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

Karnaky, K.J., Degnan, K.J., and Zadunaisky, J.A. 1977. Chloride transport across isolated opercular epithelium of killifish: A membrane rich in chloride cells. Science.

195: 203-205.

Karnaky, K.J. 1980. Ion-secreting epithelia: chloride cells in the head region of *Fundulus heteroclitus*. Am. J. Physiol. **238**: R185-198.

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

Karnaky, K.J. 1991. Teleost osmoregulation: changes in the tight junction in response to the salinity of the environment. *In* The Tight Junction. *Edited by* M. Cereijido. CRC Press, Boca Raton. pp. 175-185.

Kültz, D., and Onken, H. 1993. Long-term acclimation of the teleost *Oreochromis mossambicus* to various salinities: two different strategies in mastering hypertonic stress. Mar. Biol. **117**: 527-533.

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

Lee, C.O. 1981. Ionic activities in cardiac muscle cells and application of ion-selective microelectrodes. Am. J. Physiol. **241**: H459-H478.

Lin, H., and Randall, D. 1995. Proton pumps in fish gills. *In* Cellular and Molecular Approaches to Fish Ionic Regulation. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, London. pp. 229-255.

Marshall, W.S. 1977. Transepithelial potential and short-circuit current across the isolated skin of *Gillichthys mirabilis* (Teleostei: Gobiidae) acclimated to 5% and 100% seawater. J. Comp. Physiol. **114**: 157-165.

Marshall, W.S., and Nishioka, R.S. 1980. Relation of mitochondria-rich chloride cells to active chloride transport in the skin of a marine teleost. J. Exp. Zool. **214**: 147-156.

Marshall, W.S. 1981. Sodium dependency of active chloride transport across isolated fish skin (*Gillichthys mirabilis*). J. Physiol. Lond. **319**: 165-178.

Marshall, W.S., Bryson, S.E., and Wood, C.M. 1992. Calcium transport by isolated skin of rainbow trout. J. Exp. Biol. **166**: 297-316.

Marshall, W.S. 1995. Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. *In* Cellular and Molecular Approaches to Fish Ionic Regulation. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, London. pp. 1-23.

Marshall, W.S., Bryson, S.E., Burghardt, J.S., and Verbost, P.M. 1995. Ca^{2+} transport by

opercular epithelium of the freshwater adapted euryhaline teleost, *Fundulus heteroclitus*. J. Comp. Physiol. B. **165**: 268-277.

Marshall, W.S., Bryson, S.E., Darling, P., Whitten, C., Patrick, M., Wilkie, M., Wood, C.M., and Buckland-Nicks, J. 1997. NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. J. Exp. Zool. **277**: 23-37.

McCormick, S.D., Hasegawa, S., Hirano, T. 1992. Calcium uptake in the skin of a freshwater teleost. Proc. Nat. Acad. Sci. U.S.A. **89**: 3635-3638.

McCormick, S.D. 1995. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. *In* Cellular and Molecular Approaches to Fish Ionic Regulation. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, London. pp. 285-315.

Péqueux, A., Gilles, R., and Marshall, W.S. 1988. NaCl transport in gills and related structures. *In* Advances in Comparative and Environmental Physiology. Vol. I. *Edited by* R. Greger. Springer-Verlag, Heidelberg. pp. 1-73.

Perry, S.F. 1997. The chloride cell: structure and function in the gills of freshwater fishes. Annu. Rev. Physiol. **59**: 325-347.

Philpott, C.W., and Copeland, D.E. 1963. Fine structure of chloride cells from three

species of *Fundulus*. J. Cell Biol. **18**: 389-404.

Potts, W.T.W. 1984. Trans-epithelial potentials in fish gills. *In* Fish Physiology. Vol. 10B. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, Orlando, Florida. pp. 105-128.

Potts, W.T.W. 1994. Kinetics of sodium uptake in freshwater animals: a comparison of ion-exchange and proton pump hypotheses. Am. J. Physiol. **266**: R315-R320.

Scheffey, C., Foskett, J.K., and Machen, T.E. 1983. Localization of ionic pathways in the teleost opercular membrane by extracellular recording with a vibrating probe. J. Membrane. Biol. **75**: 193-203.

Steiner, R.A., Oehme, M., Ammann, D., and Simon, W. 1979. Neutral carrier ion selective microelectrodes for intracellular studies. Analyt. Chem. **51**: 351-353.

Ussing, H.H. 1949. The distinction by means of tracers between active transport and diffusion. Acta. Physiol. Scand. **19**: 43-56.

Wood, C.M. 1991. Branchial ion and acid-base transfer in freshwater teleost fish: Environmental hyperoxia as a probe. Physiol. Zool. **64**: 68-102.

Wood, C.M., Gilmour, K.M., and Pärt, P. 1997. Passive and active transport properties

of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A, In press.

Young, P.S., McCormick, S.D., Demarest, J.R., Lin, R.J., Nishioka, R.S., and Bern, H.A. 1988. Effects of salinity, hypophysectomy, and prolactin on whole-animal transepithelial potential in the tilapia *Oreochromis mossambicus*. Gen. Comp. Endocrinol. **71**: 389-397.

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

Zall, D.M., Fisher, M.D., and Garner, Q.M. 1956. Photometric determination of chlorides in water. Analyt. Chem. **28**: 1665-1678.

Table 1. Unidirectional efflux (J^{sm}) and influx (J^{ms}) of Cl^- and Na^+ in opercular epithelia of fresh water and 10% seawater adapted *Fundulus heteroclitus* (n = 6) bathed on mucosal side with fresh water and 10% seawater respectively. Open circuit conditions, except as noted.

Bathing Solutions Serosa/Mucosa	V_t (mV)[a]	G_t ($mS \cdot cm^{-2}$)[b]	Hour 1 J^{sm}	Hour 1 J^{ms}	Hour 2 J^{sm}	Hour 2 J^{ms}	Hour 3 J^{sm}	Hour 3 J^{ms}
			($nmol \cdot cm^{-2} \cdot h^{-1}$)					
FW adapted *Fundulus*								
Saline/FW								
Cl^-	-43.9 ± 2.4	1.94 ± 0.34	1110 ± 256	125 ± 42.5	962 ± 265	116 ± 28.9	1082 ± 255	74 ± 21.8
Na^+			1217 ± 215	163 ± 47	1387 ± 286	121 ± 31	1504 ± 365	126 ± 31
10% SW adapted *Fundulus*								
Saline/10% SW								
Cl^-	+3.3 ± 0.5	2.32 ± 0.43	1624 ± 427	664 * ± 153	1998 ± 407	879 * ± 214	7861 *[T] ± 949	2880 *[T] ± 391
Na^+			1868 ± 405	1310 * ± 297	1404 ± 96	944 * ± 265	2810 *[T] ± 242	2784 *[T] ± 277

[a] Transepithelial potential (mucosal ground) corrected for junction potentials; n = 12.

[b] Tissue conductance corrected for solution resistance; n = 12.

[T] Preparation under saline/saline short-circuit conditions, $V_t = 0$ mV.

* Un-paired *t*-test, two-tailed. Comparison of mean flux values (J^{ms} and J^{sm}) between the two preparations; *, $P<0.05$.

Table 2. Unidirectional efflux (J^{sm}) and influx (J^{ms}) of Cl^-, Na^+ and Ca^{2+} in opercular epithelia of fresh water *Oreochromis niloticus* (n = 6) bathed on mucosal side with fresh water. Open circuit conditions, except as noted.

Bathing Solutions Serosa/Mucosa	V_t (mV)[a]	G_t (mS·cm^{-2})[b]	Hour 1 J^{sm}	Hour 1 J^{ms}	Hour 2 J^{sm}	Hour 2 J^{ms}	Hour 3 J^{sm}	Hour 3 J^{ms}
			(nmol·cm^{-2}·h^{-1})					
FW *Oreochromis* Saline/FW								
Cl^-	+8.0 ± 0.6	1.78 ± 0.19	752 ± 122	30.4 ± 6.0	626 ± 158	23.5 ± 2.6	727 ± 147	27 ±4.0
Na^+			520 ± 54	22.3 ± 2.6	552 ± 72	13.1 ± 4.0	587 ± 91	7.6 ± 3.0
Ca^{2+}			4.4 ± 0.6	7.1 ± 0.4	4.7 ± 0.6	7.6 ± 0.8	4.5 ± 0.8	8.4 ± 1.0
250mM Mannitol Dissection Media Saline/FW								
Cl^-	+7.9 ± 0.3	0.95 ± 0.33	1294 ± 313	48 ± 7	1230 ± 325	47 ± 12	1296 ± 197	39 ± 16
Na^+			798 ± 208	21 ± 7	960 ± 203	21 ± 4	887 ± 115	16 ± 4
Saline/Saline, short circuit conditions								
Cl^-	0 ± 0	14.48 ± 1.72	12047* ± 3162	11267* ± 1494	13801* ± 4849	8971* ± 1798	7281* ± 2137	8082* ± 2051
Na^+			9044* ± 1820	7413* ± 999	10607* ± 3316	6410* ± 1171	4720* ± 1675	5948* ± 1430

[a] Transepithelial potential (mucosal ground) corrected for junction potentials; n = 24.

[b] Tissue conductance corrected for solution resistance; n = 24.

* Un-paired *t*-test, two-tailed. Comparison of mean flux values relative to the control saline/FW preparation; *, $P<0.05$.

Figure 2.1. (A). Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in isolated opercular epithelium of FW acclimated *Fundulus heteroclitus* with FW in the mucosal bath (n = 6). Open bars represent the observed flux ratios for Cl^- and Na^+, hatched bars represent the predicted flux ratios for Cl^- and Na^+ based on the Ussing flux ratio equation. (B). Observed and predicted ratios for Cl^- and Na^+ for the 10% SW acclimated *Fundulus* opercular epithelium with 10% SW in the mucosal bath (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired *t*-test, two-tailed) implying evidence for non-diffusive transport.

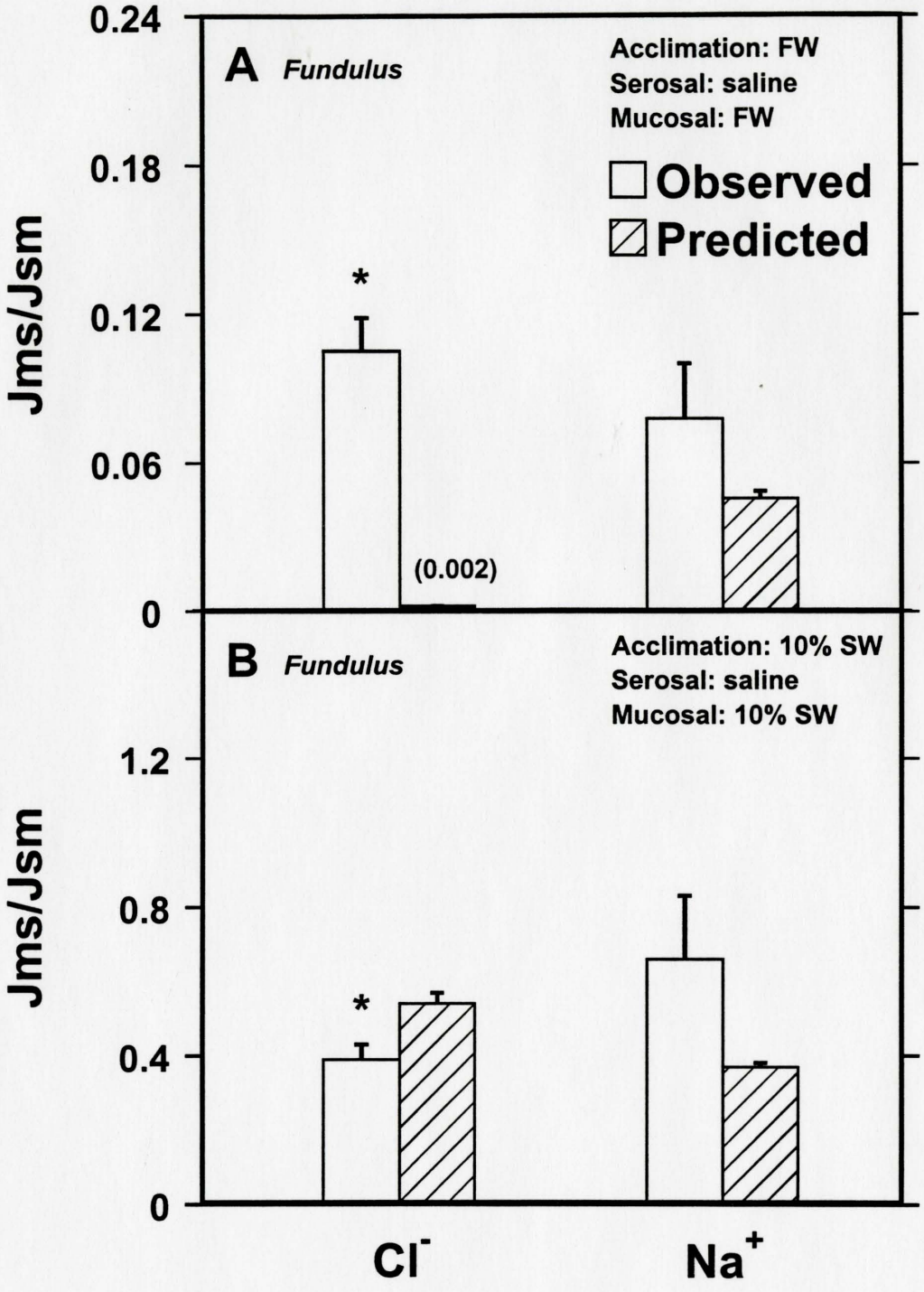
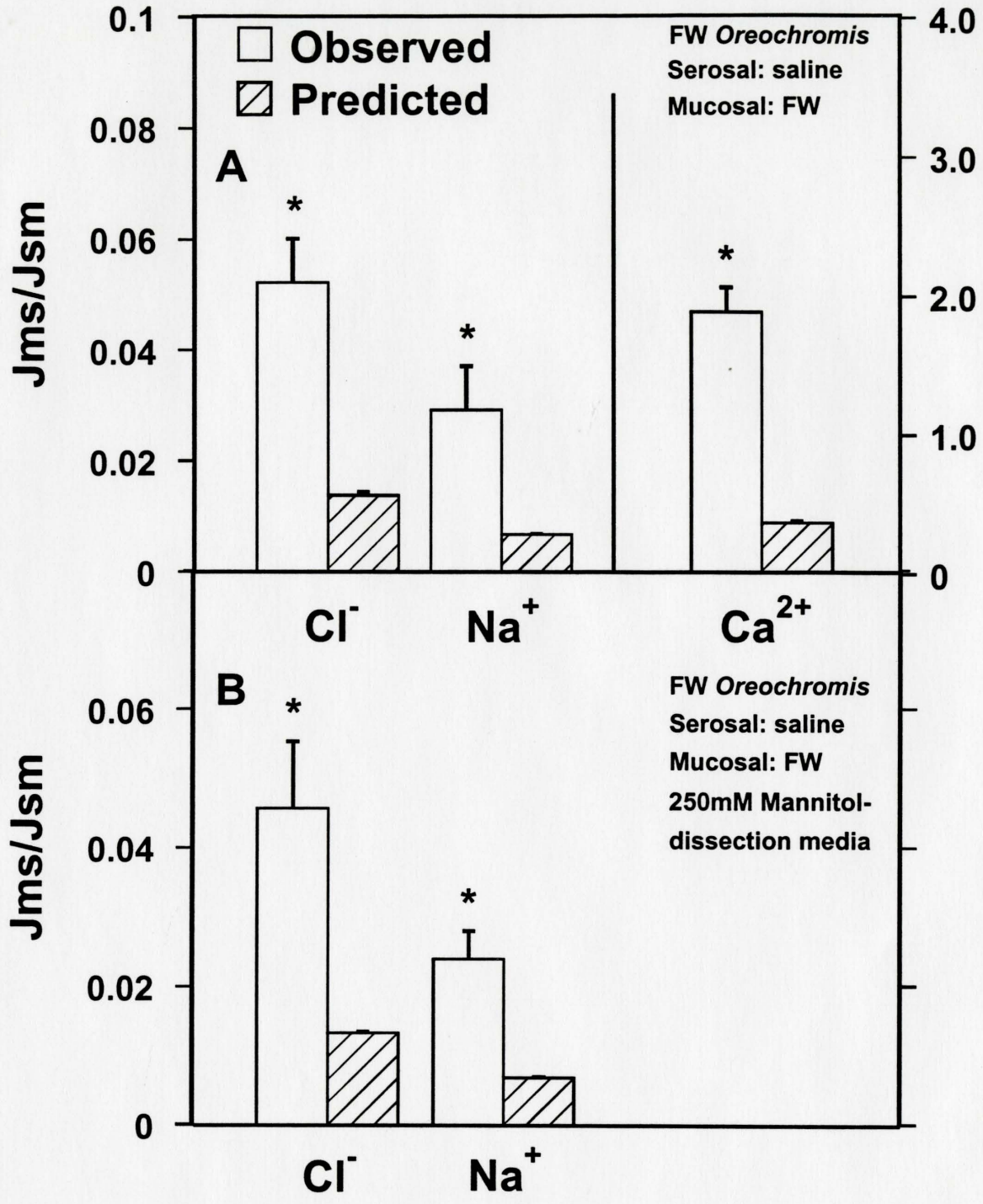

A Fundulus
Acclimation: FW
Serosal: saline
Mucosal: FW
Observed
Predicted
Jms/Jsm
0.24
0.18
0.12
0.06
0
*
(0.002)
B Fundulus
Acclimation: 10% SW
Serosal: saline
Mucosal: 10% SW
Jms/Jsm
1.2
0.8
0.4
0
*
Cl-
Na+

Figure 2.2. (A). Observed and predicted flux ratios for the unidirectional movements of Cl^-, Na^+ and Ca^{2+} in isolated opercular epithelium of FW *Oreochromis niloticus* (n = 6). Open bars represent the observed flux ratios for Cl^-, Na^+ and Ca^{2+}, hatched bars represent the predicted flux ratios for Cl^-, Na^+ and Ca^{2+} based on the Ussing flux ratio equation. (B). Observed and predicted Cl^- and Na^+ flux ratios for the isolated opercular epithelium of *Oreochromis* using FW osmotically compensated with 250mM mannitol, instead of saline during the dissection (n = 6). Other methods the same as in (A). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired *t*-test, two-tailed) implying evidence for non-diffusive transport.


Observed
Predicted
A
FW Oreochromis
Serosal: saline
Mucosal: FW
Jms/Jsm
0.1
0.08
0.06
0.04
0.02
0
4.0
3.0
2.0
1.0
0
*
Cl-
Na+
Ca2+
B
FW Oreochromis
Serosal: saline
Mucosal: FW
250mM Mannitol-
dissection media
Jms/Jsm
0.06
0.04
0.02
0
Cl-
Na+

Chapter 3

Abstract

Freshwater (FW) rainbow trout (*Oncorhynchus mykiss*) urinary bladders mounted *in vitro* under symmetrical saline conditions displayed electroneutral active absorption of Na^+ and Cl^- from the mucosal side; the short-circuit current was less than $1 \mu A \cdot cm^{-2}$. The removal of Na^+ from the mucosal saline decreased Cl^- absorption by 56% and removal of Cl^- decreased Na^+ absorption by 69%. However, active net absorption of both Na^+ and Cl^- were not abolished when the counter-ion was replaced with an impermeant ion (choline or gluconate respectively). Under more realistic conditions with artificial urine ($[Na^+] = 2.12$ mM, $[Cl^-] = 3.51$ mM) bathing the mucosal surface, transepithelial potential (V_t) increased to a serosal positive ~+7.6 mV. Unidirectional influx rates of both Na^+ and Cl^- were much lower but active absorption of both ions still occurred according to the Ussing flux ratio criterion. Replacement of Na^+ with choline, or Cl^- with gluconate in the mucosal artificial urine yielded no change in unidirectional influx of the counter-ion. The mucosal addition of DIDS, amiloride or bumetanide (10^{-4} M) had no effect on either Na^+ or Cl^- transport, under either symmetrical saline or artificial urine/saline conditions. With the mucosal surface bathed in artificial urine, kinetics analyses indicated a decrease in maximum Na^+ transport rate (J_{max}) of 66% with no change in affinity (K_m) in the low Cl^- mucosal solution relative to the control solution. Similarly, there was a 79% decrease in J_{max} values for Cl^-, again with no change in K_m, in the low-Na^+ mucosal bathing solution compared to the control solution. The anterior portion of the urinary bladder transported Na^+ and Cl^- at a faster rate than posterior portions under symmetrical saline conditions, anterior portions also exhibited larger transepithelial conductance (G_t) and

smaller V_t than posterior portions, but there was no difference in Na^+/K^+-ATPase activities. Na^+ and Cl^- reabsorption is a partially coupled process in the urinary bladder of *O. mykiss*, where both dependent and independent transport mechanisms occur.

Introduction

The transport functions of the urinary bladder in freshwater teleosts have been studied extensively *in vitro*, particularly in the European rainbow trout (*Salmo irideus*, generally considered a strain of *Oncorhynchus mykiss*) (Lahlou and Fossat, '71, '84; Fossat *et al.*, '74; Fossat and Lahlou, '77, '79a, b, '82), brook trout (*Salvelinus fontinalis*) (Marshall, '86, '88; Marshall and Bryson, '91), and North American rainbow trout (*O. mykiss*) (Hirano *et al.*, '73; Demarest and Machen, '82; Harvey and Lahlou, '86). The urinary bladder of a teleost fish is an enlargement of the paired mesonephric ducts which leave the kidney and unite to form the urinary bladder (Hickman and Trump, '69). It is a single-layered epithelium containing mitochondria-rich cells, microvilli and cilia surrounded by a contractile muscular wall (Lahlou and Fossat, '84). *In vivo* studies have shown that ureteral urine is modified during residence in the bladder, yielding a urine with lower Na^+ and Cl^- concentrations (Curtis and Wood, '91). Therefore, the urinary bladder functions as an accessory osmoregulatory organ to the kidney in the FW teleost, facilitating hyperosmotic regulation.

The mechanism of Na^+ and Cl^- transport in the urinary bladder of *Salmo irideus* has been characterized as electroneutral co-transport (Fossat and Lahlou, '79a). Thus the removal of either ion from the mucosal side induced the disappearance of the net flux of the co-ion. This coupled NaCl transport is associated with an undetectable transepithelial potential difference (V_t) and short-circuit current (I_{sc}). The urinary bladder is termed a 'leaky' epithelium due to its low transepithelial resistance R_t (~200 $\Omega \cdot cm^{-2}$) and the paracellular pathway did not exhibit cation selective properties, in contrast to other low resistance epithelia (Fossat and Lahlou, '79b).

The brook trout urinary bladder also displays active mucosa to serosa absorption of Na^+ and Cl^- but the transport mechanism appears to be very different (Marshall, '86, '88). The uptake of NaCl was electroneutral, and Na^+ or Cl^- transport continued when Cl^- or Na^+-free solutions, respectively, were placed on the mucosal surface. There was also evidence of independent Cl^--HCO_3^- and Na^+-H^+ exchange mechanisms, and there was no response of Na^+ or Cl^- movement to the mucosal addition of the cotransporter antagonist bumetanide (Na^+-K^+-$2Cl^-$ transport blocker). This "independent model" model appears very similar to that normally presented for the freshwater teleost gill (Wood, '91; Perry, '97).

Of interest is that two related salmonid species, albeit in different genera, demonstrated two very different methods of urinary bladder Na^+ and Cl^- transport. Marshall ('88) has speculated that discrepancies between work done on *Salvelinus fontinalis* and previous work done on the urinary bladder of *Salmo irideus* reflected different techniques, holding conditions or genetic variations among the fish.

By comparison among other transporting epithelia, there appears to be a variety of methods for NaCl uptake. The urinary bladders of the seawater (SW) adapted winter flounder (Renfro, '77) and the SW starry flounder (Demarest and Machen, '84) appear to have both coupled and independent uptake of Na^+ and Cl^-. Other epithelial preparations such as the rabbit ileum (Nellans *et al.*, '73, '74) and rabbit gall-bladder (Frizzell *et al.*, '75) have shown a tight coupling of Na^+ and Cl^- transport.

The aim of the present study was to establish the mechanism(s) of Na^+ and Cl^- transport in the urinary bladder of the North American strain of freshwater rainbow trout (*Oncorhynchus mykiss*). In particular, this *in vitro* study used an Ussing chamber

approach to examine Na^+ and Cl^- transport under a variety of conditions to determine whether the transport of these ions was coupled or independent. First, the urinary bladder was bathed in symmetrical saline, either with both ions present, or with one removed and then flux rates were measured using radiolabelled Na^+ and/or Cl^-. Secondly, artificial urine was used to more closely mimic the conditions found *in vivo* and similar experiments were performed as above. Thirdly, with saline or artificial urine bathing the mucosal surface, drugs known to affect Na^+ and Cl^- transport in other dependent or independent transporting epithelia were applied. Finally, electrophysiological and kinetic transport properties of the epithelium were determined. Our results suggest that Na^+ and Cl^- transport in the urinary bladder of *O. mykiss* is a partially coupled process, where both dependent and independent uptake processes occur.

Materials and Methods

Animals

Adult rainbow trout (*Oncorhynchus mykiss*; 300-750 g) were obtained from Humber Springs Hatchery (Orangeville, ON). The fish were maintained in a 500-l tank, supplied with dechlorinated, aerated Hamilton tap water at a flow rate of 900 $ml \cdot min^{-1}$ and with an average composition (in mmol l^{-1}) of Na^+, 0.6; Cl^-, 0.7; Ca^{2+}, 1.05; pH 7.5-8.0. Light was maintained at seasonal photoperiod fluctuations and water temperature varied according to ambient temperature during September to May (5-14°C). The fish were fed commercial trout pellets (Zeigler, Hazelton, PA) at a rate of 1% of their body mass per day.

Bathing solutions

All salts were obtained from Sigma Chemical Co., St. Louis, MO. The Ringer's solution used for dissection and for the basolateral bathing solution was a modified Cortland saline and composed of (in mmol l^{-1}) NaCl, 129.9; KCl, 2.55; $CaCl_2 \cdot H2O$, 1.56; $MgSO_4 \cdot 7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4 \cdot H2O$, 2.97; glucose, 5.55; NH_4Cl, 0.30. The saline was equilibrated with a 0.3% CO_2, balance O_2, gas mixture and had a measured pH of 7.8-7.9. Phosphate-free and potassium-free saline used for the Na^+/K^+-ATPase assay was composed of (in mmol l^{-1}) NaCl, 129.1; $MgSO_4$, 1.9; $CaCl_2$, 1.4; $NaHCO_3$ 11.9.

In open-circuit experiments using asymmetrical bathing solutions, the artificial urine solution for the mucosal surface of the membrane was formulated from the results of "spot-sampling" of urine from the bladder of undisturbed, non-cannulated rainbow trout, as reported by Curtis and Wood (1991). This artificial urine was composed of (in mmol l^{-1}) KCl, 0.81; $CaCl_2 \cdot H2O$, 1.35; $MgSO_4 \cdot 7H_2O$, 0.79; $NaHCO_3$, 1.66; $NaH_2PO_4 \cdot H2O$, 0.46; urea, 0.55; $Ca(NO_3)_2 \cdot 4H_2O$, 0.045; $(NH_4)_2SO_4$, 0.235. The Ringers and artificial urine solutions were each modified for ion replacement experiments. In the Cl^--free Ringers, sodium, potassium and calcium gluconate ($NaC_6H_{11}O_7$, $KC_6H_{11}O_7$, $\frac{1}{2}CaC_6H_{11}O_7$ respectively) and $(NH_4)_2SO_4$ were substituted for NaCl, KCl, $CaCl_2 \cdot 2H_2O$ and NH_4Cl respectively. The Na^+-free Ringers had choline chloride ($C_5H_{14}NOCl$), choline bicarbonate ($C_5H_{14}NOHCO_3$), and KH_2PO_4 substituted for NaCl, $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ respectively. In Cl^--free artificial urine KCl and $CaCl_2 \cdot 2H_2O$ were replaced with $KC_6H_{11}O_7$ and $\frac{1}{2}CaC_6H_{11}O_7$ respectively. In Na^+-free artificial urine,

$NaHCO_3$ and $NaH_2PO_4 \cdot H2O$ were replaced with $C_5H_{14}NOHCO_3$ and KH_2PO_4 respectively.

For the kinetic experiments, in the initial mucosal "urine", Cl^- was adjusted to equal 2.16 mM and Na^+ was set initially to 2.12 mM, so that Cl^- and Na^+ concentrations were roughly equal. The artificial urine's Na^+ and Cl^- concentrations were then manipulated to the approximate concentrations of 5, 10, 50, and 150 mM by addition of NaCl, $NaC_6H_{11}O_7$ or $C_5H_{14}NOCl$ as appropriate.

The artificial urine solution was equilibrated with a 0.3% CO_2, balance O_2, gas mixture and had a measured pH of 7.2-7.5. Prior to the start of the experiment and at the end of each 60 minute flux period (see below) except in the kinetic experiment, the mucosal solution was rinsed extensively to maintain the original ion composition of the artificial urine ($Na^+ = 2.12$, $Cl^- = 3.51$ and $Ca^{2+} = 1.4$ mmol l^{-1}).

Isolated urinary bladder preparation

The fish were anaesthetized using 0.1 g/l MS-222 and a flanged PE-tubing was inserted into the bladder and tied to the urinary papilla. The bladder was filled with Ringer's to aid in differentiation of its structure from the peritoneum during dissection. Both ureters just anterior to the urinary bladder were ligated with surgical thread and the urinary bladder was separated from surrounding tissues. Following careful removal of remaining fat and connective tissue, the bladder was cut into equal anterior and posterior portions and then both portions were opened by a longitudinal cut. Each portion of the bladder yielded an approximate area of 1-2 cm^2. Throughout the dissection, the preparation was kept moist by the addition of saline.

The Ussing membrane apertures (0.125 cm^2) were prepared with stopcock grease (Dow Corning, Midland, MI) and a thin vinyl mesh was placed over each aperture to support the tissue. Each portion of urinary bladder was stretched gently (to remove macroscopic folds) and carefully pinned down to the aperture mucosal side up and the second half of the aperture then sandwiched the epithelium. After mounting, the mucosal surfaces were thoroughly rinsed with the appropriate solution to remove the saline and any mucus that had accumulated during the dissection. The aperture sandwich was then placed between the two hemi-chambers and tightened into place. The hemi-chambers were filled with the appropriate solutions: Ringers or artificial urine or ion replacement solution for the mucosal side, and saline for the serosal side. Each hemi-chamber was filled at an equal rate to ensure there was no 'bagging' of the epithelium. The membranes were then given a 30 min. period to adjust to the *in vitro* conditions. Appropriate gasses were passed across the surface of each hemi-chamber's media and the mucosal and serosal solutions were mixed by magnetic stirrers.

Electrophysiology

Polyethylene 4% agar/Ringer bridges were used to measure transepithelial potential (V_t, mucosal side grounded) and membrane conductance (G_t). Each bridge was connected to the voltage/current clamp (DVC-1000, WP Instruments, New Haven, CT) by Hg/HgCl calomel half-cells. Membrane conductance was corrected for solution resistances. In asymmetrical solutions, corrections for liquid junction potentials were accomplished by measurements against a free flowing 3 M KCl half-cell. Ussing chambers were water jacketed to 15°C. In flux experiments with saline bathing the

mucosal surface, the preparation was short-circuited to negate any effect of V_t on ion transport. G_t was determined by clamping the membrane to a set voltage every 10 min. and V_t was recorded at the beginning and end of each 60 min. short-circuited flux period.

Experimental protocol

One chamber was set up for each portion of the urinary bladder for independent measurements of either influx or efflux. Therefore either the anterior or posterior portion yielded a mucosal to serosal unidirectional influx (J^{ms}) measurement while the other portion yielded the serosal to mucosal unidirectional efflux (J^{sm}) measurement. These were alternated to obtain equal numbers of both approaches within each treatment group. For data analyses, posterior and anterior portions of the membranes were matched according to G_t. After the initial 30 min. adjustment period, the mucosal hemi-chamber volume was gently rinsed again with the appropriate solution (20 times the chamber volume) and isotope was added to the appropriate side.

Dual flux experiments were performed with ^{36}Cl ($Na^{36}Cl$ from I.C.N. Radiochemicals, Irvine, California) and ^{22}Na ($^{22}NaCl$ from NEN-Dupont, Boston, MA) which were added to either the mucosal side (final specific activity of 300 000 CPM μmol^{-1}, artificial urine) for unidirectional influx or serosal side (8 000 CPM μmol^{-1}, Ringer) to monitor unidirectional efflux. Upon first addition of the radioisotope(s), a 45 min equilibration period was employed. Each experiment consisted of three 60 min. periods; within each period samples were taken from the unlabelled bath every 20 min. for radioactivity analyses. Flux values for each 20 min. period were averaged to produce a final flux rate for each 60 min. experimental period. At the start and end of each

period, samples were taken from the labeled side to determine the specific activity and ionic concentrations and the solutions were replaced at the end of each period. Unidirectional fluxes were determined by measuring the specific activity on the labelled side and the appearance of isotope on the unlabelled side.

A total of five periods were performed for each kinetic flux experiment. Each period represented a different mucosal artificial urine concentration of Na^+ and/or Cl^- at approximately 2, 5, 10, 50 or 150 mM. Appropriate isotope was added and followed by a 30 min. equilibration period prior to the beginning of each 40 min. flux period. At the end of each flux period, both the serosal and mucosal sides were emptied from each hemi-chamber, and new solutions were added to each side, using an increasing mucosal concentration of Na^+ and/or Cl^- throughout the kinetic flux experiment.

For each drug experiment, an initial 60 min. control period was employed (DMSO alone added at the same concentration as in the drug period). The chambers were then emptied, new isotope along with the drug/DMSO solution was added, and allowed to equilibrate for at least 45 min.. The drug experimental flux period was 60 min. in duration and was followed by a second 60 min. control period (DMSO alone again present). Prior to the final control period, the chambers were emptied, the membrane was removed, and the hemi-chambers were flushed extensively with distilled H_2O, the membrane was then replaced, and new solutions with isotope were added and equilibrated for 45 min..

The observed flux ratio (J^{ms}/J^{sm}) was compared to the predicted flux ratio using the Ussing flux ratio equation (Ussing, '49). Disagreement between the observed and

predicted value indicated the presence of non-diffusive transport. The predicted flux ratio equation was as follows:

$$J_i^{ms}/J_i^{sm} = (a_i^m/a_i^s)e^{(z_iFV_t/RT)}.$$

The activities of ion *i* are a_i^s and a_i^m on the serosal and mucosal sides respectively; valency of the ion is z_i, V_t is the transepithelial potential and *F*, *R* and *T* have their usual thermodynamic meanings. The ionic activity of Na^+ (109.5 mmol l^{-1}) in Cortland's saline was taken from measurements with microelectrodes filled with the appropriate ionophore (Steiner et al. 1979), while Cl^- was predicted to have the same relative activity (on a % basis) as Na^+ from theory for a solution of this ionic strength (Lee, 1981). The activities of Na^+ and Cl^- were unaffected in the presence of 20 mg ml^{-1} of bovine serum albumin. The activities of Na^+ and Cl^- in artificial urine were taken as equal to their measured concentrations.

Na^+/K^+-ATPase measurements

Na^+/K^+-ATPase activity was determined by the assay of Holliday ('85). The urinary bladder was removed from the trout (as above) using PO_4^--free and K^+-free saline. After the bladder was cut into equal posterior and anterior portions and medial cuts were performed on each portion, surface area was measured. Membranes were cut into small pieces and stored in homogenizing medium at -70°C overnight. The homogenizing medium contained sucrose 0.25M and EDTA 6 mM. The individual membranes were homogenized by a tissue grinder using a teflon pestle. Total homogenate protein was assayed by adding 40 µl of sample to 2 ml of Bradford reagent (Sigma, St. Louis, MO). Following vortexing and 20 min at room temperature, the samples were read on an

Ultaspec Plus spectrophotometer (model 4054, LKB Biochrom, Cambridge, England) at 595nm. Separate, equal amounts of homogenate were mixed with K^+ assay media (NaCl 167 mM; KCl 50 mM; imidazole 33.3 mM; pH to 7.2 with 1M HCl) and with K^+ free assay media (NaCl 217 mM; imidazole 33.3 mM; ouabain 1.67 mM; pH to 7.2 with 1M HCl). Each sample was then vortexed and incubated in a 30°C waterbath for 10 min. The reaction was then initiated by addition of 'start' solution (Na_2ATP 25mM; $MgCl_2 \cdot 7H_2O$ 50 mM; pH to 7.2 by crystalline imidazole). Samples were then vortexed and returned to the water bath for 30 min., and then the assay was terminated by the addition of Bonting's stop colour reagent (560 mM H_2SO_4; 8.1 mM ammonium molybdate; 176 mM $FeSO_4$). All samples and standards were vortexed and allowed to stand for 15 min. before being read at 700 nm on the Ultraspec.

Pharmaceuticals

DIDS (4,4 diiso thiocyanato-stilbene-2,2 disulfonic acid) was dissolved in dimethylsulphoxide (DMSO, 20 $\mu l \cdot mg^{-1}$) and added to the mucosal side at a final concentration of 10^{-4} M. Amiloride (10^{-4} M) and bumetanide (10^{-4} M) were dissolved using the same protocol as DIDS (all drugs from Sigma Chemical Co., St. Louis, MO). The resulting DMSO concentration in each drug experiment was adjusted to maintain a concentration of no greater than 0.1 %. The same DMSO concentration was added to the mucosal side in both control periods prior to and following the addition of the drug.

Analytical techniques

Saline Cl^- concentrations were measured by coulometric titration (model CMT10, Radiometer, Copenhagen) and artificial urine Cl^- concentrations were determined by colorimetric assay (Zall *et al.*, '56). Na^+ concentrations in both media were analyzed by atomic absorption spectrophotometry (model AA-1275, Varian, Springvale, Australia). Ammonia was determined by a micro-modification of the colorimetric assay of Verdouw *et al.* ('78). Net titratable acid flux was determined by removing 3 mls from each mucosal hemi-chamber at the beginning and end of each flux period, equilibrating with air for 30 min., then titrating down through a pH of 4, using a burette filled with 0.02 N HCl. Net titratable acid flux was calculated by subtracting the final titration value ($\mu mol \cdot cm^{-2} \cdot h^{-1}$) from the initial titration value and taking into account aperture area and flux duration. A negative flux value represented movement of acid from serosa to mucosa.

Samples of 40µl from the labelled side and 250µl from the unlabelled side of the Ussing chamber were added to 4.0 ml of Readysafe fluor (Beckman, Fullerton, CA). Radioactivities of ^{36}Cl were determined by counting on a Rackbeta 1217 liquid scintillation counter (LKB, Wallac, Turku, Finland), and ^{22}Na radioactivity was counted on Minaxi Autogamma 5000 counter (Packard Instrument Co., Downers Grove, IL). ^{22}Na emits both gamma and beta radiation, therefore scintillation counts collected from a ^{22}Na and ^{36}Cl dual flux experiment were from both ^{22}Na and ^{36}Cl. ^{36}Cl counts were determined by a count subtraction procedure. This was accomplished by measuring the CPM of a known concentration of ^{22}Na in both the scintillation and gamma counters and then determining the relative efficiency of the two counters for detecting ^{22}Na. The CPM

from the gamma counter was then multiplied by this ratio and subtracted from the CPM of the scintillation counter to yield the beta emission of ^{36}Cl only.

Data are presented as means ± 1 standard error unless indicated otherwise. Comparisons within and between treatments were analyzed by paired or un-paired *t*-tests (two-tailed), as appropriate, at $P<0.05$. Regressions were performed by the method of least squares, and correlation evaluated by Pearson's linear correlation coefficient. Kinetic values of J_{max} and K_m were determined by Eadie-Hofstee plots, and kinetic plots were generated according to the Michaelis-Menten relationship:

$$J_o = J_{max}[ion]/ K_m + [ion].$$

Initial flux rate is designated as J_o, and J_{max} represents the maximum flux rate of an enzymatic reaction when the binding site is saturated with the ion. K_m is the substrate concentration at which J_o is half-maximal (affinity) and [ion] is the ion concentration at the beginning of the flux.

Results

Bladder bathed with mucosal saline

The isolated urinary bladder bathed in symmetrical saline and open-circuited, had a serosa-positive V_t of 0.10 ± 0.04 mV and a G_t of 9.15 ± 1.50 $mS \cdot cm^{-2}$; the short-circuit current (Isc) of the preparation was <1 $\mu A \cdot cm^{-2}$. Under these symmetrical saline conditions the urinary bladder transported Na^+ and Cl^- in a net uptake direction (Fig. 1). Both unidirectional flux values and net flux values (all periods) for Na^+ were less than those for Cl^- (paired t-test, $P<0.05$). Comparison of both Na^+ and Cl^- observed flux ratios

with their respective predicted flux ratios indicated that both these ions were transported non-diffusively (paired t-test, $P<0.05$) (Fig. 1).

Upon removal of Na^+ from the saline in the mucosal bath and replacement with choline, the V_t increased to 0.7 ± 0.2 mV ($P<0.05$) and Gt decreased to 2.26 ± 0.33 $mS \cdot cm^{-2}$ ($P<0.05$). For both control and experimental treatments, the net transport of Cl^- was in the uptake direction for all three periods, and net movement of Cl^- was not different between the two treatments (unpaired t-test, $P>0.05$) (Fig. 2). However unidirectional influx ($P<0.05$) of Cl^- decreased in the Na^+ free mucosal saline treatment by about 56%; the accompanying decrease in efflux was not significant. The observed flux ratios were different from their predicted ratios (paired t-test, $P<0.05$), indicating that active transport of Cl^- still occurred in the absence of Na^+. The observed Cl^- flux ratios measured under Na^+ free conditions in the mucosal bath were not statistically different from those in the control treatments (unpaired t-test, $P>0.05$).

Replacement of Cl^- with gluconate in the mucosal saline increased V_t to 11.6 ± 0.2 mV and G_t decreased to 3.26 ± 0.4 $mS \cdot cm^{-2}$ ($P<0.05$) from their respective control values (see above). Net flux rates of Na^+ were still in the positive uptake direction but were smaller (unpaired t-test, $P<0.05$; 2 of 3 periods) in the Cl^- free treatment group compared to control values (Fig. 3). The influx of Na^+ was also significantly lower (unpaired t-test, $P<0.05$) than control value by about 69% in all three periods of the Cl^- free treatment; the accompanying decrease in efflux was not significant (Fig. 3.) The observed flux ratios for Na^+ in the Cl^- free solution remained different from the predicted ratios (paired t-test, $P<0.05$) as in the control observed ratios, suggesting active transport of Na^+ was still occurring. For each of the one hour flux periods, the Na^+ flux ratios in the Cl^- free

solution were not significantly different from those in the control treatment (unpaired t-test, $P>0.05$).

The addition of either DIDS (10^{-4} M) or amiloride (10^{-4} M) or bumetanide (10^{-4} M) to the mucosal saline had no effect on Cl^- or Na^+ observed flux ratios or unidirectional flux of either ion (Table 1). There were no effects on V_t or G_t with any of the drug treatments.

Bladder bathed with mucosal artificial urine

When the isolated urinary bladder was bathed on the mucosal side with artificial urine, the V_t increased to 7.6 ± 0.4 mV and the G_t decreased to 1.47 ± 0.1 mS·cm^{-2}, both significant relative to the saline/saline condition (unpaired t-test, $P<0.05$). Net fluxes of Cl^- and Na^+ were in the negative serosal to mucosal direction. Unidirectional influx rates of Cl^- and Na^+ were 0.72 ± 0.12 and 0.37 ± 0.06 μmol·cm^{-2}·h^{-1} respectively and net flux rates were -1.29 ± 0.21 and -0.80 ± 0.13 μmol·cm^{-2}·h^{-1} for Cl^- and Na^+ respectively. This may be compared to the much larger unidirectional influx rates of Cl^- and Na^+ in symmetrical saline (10.6 ± 1.0 and 8.0 ± 0.9 μmol·cm^{-2}·h^{-1} respectively) and net flux rates (6.1 ± 0.6 and 5.0 ± 0.6 μmol·cm^{-2}·h^{-1} respectively).

With artificial urine on the mucosal surface, the net Cl^- flux rates were similar to net Na^+ flux rates except in hour 1 (paired t-test, $P<0.05$) (Fig. 4). Cl^- unidirectional influx was larger than Na^+ influx (paired t-test, $P<0.05$) in two of the three hours and Cl^- unidirectional efflux was greater than Na^+ efflux in all periods ($P<0.05$). Of interest was the noticeable increase in the observed Cl^- flux ratio over time; the observed Na^+ flux ratio did not display any trend over time. The Na^+ and Cl^- observed flux ratios were

significantly different (paired t-test, $P<0.05$) from each of their respective predicted flux ratios suggesting non-diffusive uptake of both ions.

With Na^+ free artificial urine bathing the mucosal surface, V_t (9.7 ± 0.4 mV) and G_t (1.1 ± 0.1 mS·cm^{-2}) were not different from control (unpaired t-test, $P>0.05$). Unidirectional influx and efflux of Cl^- were also the same in both treatments (unpaired t-test, $P>0.05$). The movement of Cl^- was still in the net extrusion direction, and there was no difference between the net flux rates in the two treatments (Fig. 5). In all three periods, control observed Cl^- flux ratios in the Na^+ free treatment were not significantly different (unpaired t-test, $P>0.05$) from the observed ratios in the control treatment. Cl^- observed flux ratios were different from their respective predicted values for each bathing solution (paired t-test, $P<0.05$) indicating non-diffusive transport was maintained. Again observed Cl^- flux ratios increased markedly over time.

When gluconate was substituted for Cl^- in the mucosal artificial urine solution, V_t decreased to 3.3 ± 0.4 mV and G_t increased to 3.0 ± 0.2 mS·cm^{-2} respectively (unpaired t-test, $P<0.05$). The unidirectional and net flux rates of Na^+ were similar for both treatments (unpaired t-test, $P>0.05$) (Fig. 6). The observed ratios for the control and mucosal Cl^- free solution were very similar (unpaired t-test, $P>0.05$), indicating there was no change in the non-diffusive transport of Na^+.

Table 2 compares "cold" Cl^- and Na^+ net fluxes (measured chemically) and "hot" net fluxes (as calculated from the difference between unidirectional influx and efflux rates measured with ^{22}Na or ^{36}Cl) when the urinary bladder was bathed with control, or Cl^--free or Na^+-free artificial urine. In each of the comparisons between Na^+ and Cl^-, "hot" and "cold" net flux measurements, the "hot" net fluxes were substantially smaller than the

measured "cold" fluxes, but because of considerable variability, only a few of the differences were significant. Under the three different mucosal bathing conditions, the "hot" net fluxes generally decreased (not significantly) over time. For each of the three experimental conditions, the "cold" fluxes of Na^+ and Cl^- did not appear to display any trend over time. There was a significant difference (unpaired t-test, $P<0.05$) between "hot" and "cold" net fluxes of Cl^- only in the hour 3 when the bladder was bathed in Na^+ free artificial urine and under the control conditions. "Cold" net fluxes of Na^+ were significantly different (2 of 3 periods) from "hot" net fluxes under Cl^--free artificial urine conditions and in hour 3 of the control condition (unpaired t-test, $P<0.05$).

The addition of either DIDS (10^{-4} M) or amiloride (10^{-4} M) or bumetanide (10^{-4} M) to the mucosal artificial urine had no effect on Cl^- or Na^+ observed flux ratios or unidirectional flux of either ion (Table 3). There were no effects on V_t or G_t with any of the drug treatments.

The effects on Na^+ influx rate (ie "Na^+ kinetics") of increasing the Na^+ concentration in the artificial urine (control) and low Cl^- artificial urine solutions bathing the mucosal surface are shown in Fig. 7. Analysis of the kinetic curves by Eadie-Hofstee plots indicated J_{max} and K_m under control conditions were 6.1 ± 2.3 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and 34 ± 20 mM respectively. The solution low in Cl^- decreased (un-paired t-test, $P<0.05$) the J_{max} (2.1 ± 0.56 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) of the Na^+ influx but did not change K_m (27 ± 12 mM) ($P>0.05$). Comparison of Na^+ influx rates between the two mucosal solutions demonstrated significant differences in the 40-50 and 110-150 mM range (unpaired t-test, $P<0.05$). Unidirectional influx of Na^+ decreased 80% and net uptake was abolished (not

shown) in the 110-150 mM range when the solution low in Cl^- bathed the mucosal surface.

Figure 8 represents the effect of increasing the Cl^- concentration (ie "Cl^- kinetics") in the artificial urine and low Na^+ mucosal solutions on Cl^- influx rate. The control and low Na^+ curves had different (unpaired t-test, $P<0.05$) J_{max} values of 11.4 ± 5.1 and 2.4 ± 0.16 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and similar ($P>0.05$) K_m values of 37 ± 26 and 16 ± 2 mM respectively. Control Cl^- influx rates were significantly greater than those in the low Na^+ treatment at mucosal Cl^- concentration ranges of 5-10, 40-50 and 110-150 mM (unpaired t-test, $P<0.05$). In the range of 110-150 mM of Cl^- in the mucosal low Na^+ artificial urine solution, unidirectional influx of Cl^- decreased 85% from control values and the net uptake of Cl^- was abolished (not shown). There were no significant differences between the K_m and J_{max} values of the Na^+ and Cl^- control curves.

The mean V_t decreased for the control "kinetic" fluxes as the Na^+ and Cl^- concentrations in the mucosal bath increased (Table 4). The V_t for the low Na^+ mucosal solution in the 40-50 and 110-150 mM ranges were significantly different from their respective control values. The low Cl^- mucosal artificial urine displayed an increase in V_t when the Na^+ concentration was increased. The V_t of the low Cl^- treatment in the 0-5, 40-50 and 110-150 ranges were different from their respective control values. The mean G_t increased for each different mucosal treatment (Table 4), most noticeably in the control treatments (12-fold increase), and values in the range 110-150 mM were different from that in the 0-5 mM range (unpaired t-test, $P<0.05$). By increasing the Na^+ and Cl^- concentrations in the low Cl^- and Na^+ solutions respectively, the G_t increased approximately 2-fold each in the 110-150 mM range (unpaired t-test, $P<0.05$).

When the bladder was bathed in symmetrical saline, there was a significant correlation between the efflux of Na^+ and Cl^- and G_t ($P<0.001$ and 0.0001 respectively; Fig. 9). Under the same bathing conditions there was also a significant relationship between Na^+ and Cl^- unidirectional influx and G_t ($P<0.0001$ and 0.0001 respectively). Upon exposure to the more dilute artificial urine as the mucosal bathing media, the overall rate of Na^+ and Cl^- efflux decreased, but there was still a linear correlation with G_t ($P<0.05$ and 0.01 respectively, Fig. 10). However, under these conditions there was no relationship between G_t and the influx of Na^+ or Cl^-.

There was no significant difference at open-circuit in V_t or G_t between anterior and posterior portions of the bladder when bathed on the mucosal surface with artificial urine (Fig. 11). However, when the bladder was bathed in symmetrical saline, the anterior portion had a smaller V_t ($P<0.05$) and larger G_t ($P<0.05$) than the posterior portion.

With artificial urine bathing the mucosal surface of the bladder, Na^+ and Cl^- unidirectional and net fluxes were similar in the anterior and posterior portions of the bladder (Fig. 12). When the bladder was bathed under symmetrical saline, both anterior unidirectional influx and net fluxes for Na^+ ($P>0.05$) and Cl^- ($P<0.05$) were greater than the posterior fluxes.

The Na^+/K^+-ATPase activities in the anterior and posterior portions of the bladder were not statistically different. The posterior portions had a mean activity of 0.15 ± 0.03 $\mu M \cdot cm^{-2} \cdot h^{-1}$ or 1.21 ± 0.3 $\mu M \cdot \mu g$ protein$\cdot h^{-1}$ and the anterior portion mean activity was 0.09 ± 0.03 $\mu M \cdot cm^{-2} \cdot h^{-1}$ or 0.68 ± 0.2 $\mu M \cdot \mu g$ protein$\cdot h^{-1}$.

With artificial urine bathing the mucosal surface of the urinary bladder, net NH_4^+ flux was 0.56 ± 0.87 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ in the uptake direction from urine to saline. Net titratable acid flux was 0.91 ± 0.40 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ also in the urine to saline direction. When Cl^- or Na^+ free artificial urine bathed the mucosal side, net NH_4^+ flux was -0.60 ± 0.2 and -0.22 ± 0.4 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ respectively (serosa to mucosa) and net titratable acid flux was 0.22 ± 0.3 and -1.96 ± 0.9 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (mucosa to serosa and serosa to mucosa) respectively. None of the above net acid or net NH_4^+ fluxes were significantly different from zero ($P>0.05$).

Discussion

Urinary bladder Na^+ and Cl^- transport

The negligible transepithelial potential (~0.1 mV) and apparent active absorption of Na^+ and Cl^- (Fig. 1) suggests an electrically neutral transport mechanism for these ions in the rainbow trout urinary bladder. Electro-neutral transport is a well documented characteristic of many fish transporting epithelia such as the flounder intestine (Field et al., '78), sculpin intestine (House and Green, '65), marine eel intestine (Skadhauge, 1974), SW-adapted (Renfro, '77) and FW-adapted flounder urinary bladder (Demarest, 1984), and the urinary bladder of the European strain of rainbow trout (Lahlou and Fossat, '71).

Replacement of either Na^+ or Cl^- in the mucosal saline significantly decreased influx of the counter-ion by 56 and 69% respectively (Figs. 2 and 3). The absorption of Na^+ and Cl^- appeared saturable at high mucosal concentrations of substrate. However upon removal of the counter-ion from the mucosal bath, absorption rates of both Na^+ and

Cl^- were greatly inhibited (Figs. 7 and 8). These results suggest that a Na^+-Cl^--dependent carrier mediated uptake mechanism is present in the FW *O. mykiss* urinary bladder. Previous studies using the urinary bladder of the closely related *S. irideus* by Fossat and Lahlou ('77, '79a, b, '82) report a tight coupling of Na^+ and Cl^- transport from mucosa to serosa, whereby the removal of one ion completely abolished the net transport of the counter-ion. Noteworthy however, is that in the present study, net active absorption of both ions, albeit reduced, persisted upon the removal of the counter-ion (Figs. 2, 3). Therefore, the lumenal absorption of Na^+ and Cl^- in the FW trout urinary bladder may only be a partially coupled process. These observations are in agreement with studies performed on urinary bladders of FW (Demarest, '84) and SW-adapted flounder (Renfro, '77), both of which display a partial coupling (60 and 75% respectively) of Na^+ and Cl^- transport. In the present study, the mucosal bath in each of the ion replacement experiments contained a small concentration of the "removed" ion (eg. 3 mM) due to either a "rinse-off" effect of the epithelium or passive unidirectional efflux that appeared to increase throughout the experimental period (data not shown). Therefore, the 56 and 69% decrease in influx of Cl^- and Na^+ respectively (as mentioned above) would most likely be greater if their respective counter-ions were completely absent from the mucosal bath, assuming that a coupled transport mechanism is actually present. Further evidence for a coupled transport system in the urinary bladder of *O. mykiss* was observed when net transport of Na^+ or Cl^- was abolished upon the removal of the counter-ion at higher levels (150 mM) of Na^+ or Cl^- in the mucosal artificial urine.

Under more realistic conditions representative of those occurring in the animal *in vivo*, with artificial urine bathing the mucosal surface, the active absorption of Na^+ and

Cl^- continued (Fig. 4). However, unlike results reported under symmetrical saline conditions, there was no effect on the rate of transport of Na^+ or Cl^- upon the removal of the counter-ion (Figs. 5 and 6). It is possible (as mentioned above) that a "rinse-off" effect also occurred in these experiments, such that the removal of Na^+ or Cl^- in the mucosal solutions was not complete. These data suggest that at lower mucosal concentrations of Na^+ and Cl^-, coupled transport did not occur. These results are more consistent with data reported by Marshall ('86) which indicated active independent transport of Na^+ and Cl^- in the urinary bladder of brook trout (*Salvelinus fontinalis*). However unlike results from the present study, in Marshall's ('86) study, the removal of either Na^+ or Cl^- at higher levels of Na^+ and Cl^- did not affect the absorption rate of the respective counter-ion. Furthermore, analyses of net acid and NH_4^+ fluxes in the present study argue against acid-base linkage of an independent transport mechanism.

Under these "more realistic" conditions, bladder epithelia were also in negative balance (net loss of Na^+ and Cl^-) (Fig. 4, Table 2) thus indicating the bladder was most likely not behaving as efficiently as *in vivo* (Curtis and Wood, '91, '92). Therefore, optimal net absorption of Na^+ and Cl^- *in vivo*, may occur only at higher accumulated levels of Na^+ and Cl^- within the bladder (Fig. 1, 7 and 8). The bladder may be "poised" kinetically to transport at the concentrations of Na^+ and Cl^- present in ureteral urine (see Curtis and Wood, '91, '92 for typical concentrations) and not for the concentrations in the final "polished" urine which we supplied.

Electrophysiology

The mucosal Na^+-free saline treatment changed V_t slightly (~0.7 mV, mucosal ground) from the control value (~0.1 mV) in contrast to the large observed change in V_t (~11.6 mV) when bladder epithelia were bathed with Cl^--free saline. It is apparent then, that under symmetrical saline conditions, the epithelia's electroneutral V_t is a diffusion potential generated mostly by a passive mucosa to serosa movement of Cl^-. With artificial urine bathing the mucosal surface, a marked change in V_t to more negative values (~7.6 mV) was observed, which most probably represented greater permeability of the membrane for Cl^- (as seen in symmetrical saline fluxes, Fig. 1) or an anion-selective paracellular leak pathway. With low mucosal Na^+ artificial urine bathing the membrane, observed V_t was not statistically different from control, however low Cl^- in the artificial urine changed V_t to ~2 mV (approximately 75% decrease). Thus, as in the saline/saline preparations, asymmetrical V_t appeared to be dependent on the mucosal concentration of Cl^- (Table 4). Under asymmetrical solutions G_t ranged between 1 and 4 $mS \cdot cm^{-2}$ for all mucosal solutions; when mucosal NaCl concentration increased in the mucosal bath, G_t also increased significantly to approximately 18.3 $mS \cdot cm^{-2}$, however an increase in Na^+ or Cl^- levels in the mucosal solutions low in Cl^- and Na^+ respectively did not yield a large increase in G_t (Table 4). These results are in accord with the findings of Marshall ('86) but presently cannot be explained.

Under symmetrical saline conditions, a relationship between G_t and efflux of Na^+ and Cl^- was demonstrated suggesting that effluxes of these ions occurred by passive diffusion (Fig. 9). However, it was also found that Na^+ and Cl^- influx was correlated to G_t, but as previously mentioned, active net transport of Na^+ and Cl^- was exhibited under

these conditions and therefore a portion of the observed influx component was passive. With artificial urine bathing the mucosal surface, G_t was significantly correlated to the efflux of Na^+ and Cl^- but not influx. These results suggest that the influx rates of these ions observed at these mucosal concentrations were non-conductive, and by active transport alone.

Effects of pharmaceuticals

Mucosal addition of DIDS (Cl^-/HCO_3^- exchange blocker), amiloride (Na^+/H^+ exchange and Na^+ channel inhibitor) or bumetanide (Na^+-Cl^- and Na^+-K^+-Cl^- co-transport blocker) all had no effect on the transport (absorption/extrusion) of Na^+ and/or Cl^- (Tables 1 and 3) in either symmetrical saline or mucosal artificial urine conditions. Active absorption of Na^+ and Cl^- continued in the presence of each drug, which reinforces the suggestion of a dual mechanism type of transport. Thus, for example, if two transport systems were operating in parallel (Na^+ and Cl^- independent and dependent mechanisms), addition of DIDS would impair anion exchange but co-transport mechanisms would be unaffected. In fact in saline/saline conditions, DIDS decreased Cl^- absorption and bumetanide decreased Cl^- and Na^+ absorption (but not significantly). However in each case active transport persisted.

Anterior *vs* posterior portions of urinary bladder

Unidirectional influx, efflux and net flux of Na^+ and Cl^- were similar in the anterior and posterior portions of the bladder under artificial urine conditions (Fig. 12). In contrast, when Na^+ and Cl^- levels were increased in the mucosal bath (saline/saline) Cl^-

($P<0.05$) absorption rates (and also Na^+ absorption rates, but not significantly) were greater in the anterior portion than in the posterior portion of the urinary bladder. The ureters, from the mesonephric duct of the kidney, lead into the anterior portion of the urinary bladder, delivering the more concentrated urine which has exited from the kidney. The anterior portion of the urinary bladder may absorb Na^+ and Cl^- at a greater rate because of its proximity to the ureters. This result is also supported by the observation that G_t was significantly greater in the anterior *vs* the posterior portion of the bladder (Fig. 11). Experiments performed on the goby (*G. mirabilis*) urinary bladder (Loretz and Bern, '80, '83) reported that the columnar cell region is responsible for the active reabsorption of Na^+ and Cl^-. The columnar cells are most abundant in the area closest to the ureters and are rich in mitochondria and rough and smooth endoplasmic reticulum. However, Na^+/K^+-ATPase measurements failed to distinguish greater Na^+/K^+-ATPase activity between the two portions of the bladder.

Further conclusions

The present study observed electroneutral co-transport of NaCl in the urinary bladder of *O. mykiss* under symmetrical saline conditions, thus reinforcing previous studies performed on the urinary bladder of the closely related European strain of rainbow trout (*Salmo irideus*) (Fossat and Lahlou '79a, '82). However, there was not an observed 100% coupling of NaCl transport as reported in *S. irideus* (Fossat and Lahlou '79a). Experiments performed on *S. irideus* did not use an artificial urine, and NaCl transport at these more realistic levels of mucosal NaCl in the present study, were not found to be a coupled process. Therefore a partially coupled transport process of Na^+ and Cl^- appears

to be present. Electrical parameters of *S. irideus* (Fossat and Lahlou '79a, '82) and *O. mykiss* (present study) are also very similar under symmetrical saline conditions (negligible V_t, I_{sc} <1μA·cm^{-2} and Gt = 3-4 mS·cm^{-2}). In contrast, the urinary bladder of the brook trout (*Salvelinus fontinalis*), exhibited independent transport of Na^+ and Cl^- at both higher and lower levels of mucosal NaCl (Marshall '86). The transport of NaCl was reported to be electroneutral, however V_t (6.7 mV) and G_t (0.23 mS·cm^{-2}) were rather different from those of the present study. It is interesting that the brook trout appears to transport Na^+ and Cl^- in a very different manner than the rainbow trout (*O. mykiss*).

Acknowledgements

The authors thank Dr. M.J. O'Donnell for Na^+ ion activity measurements and for his advice in this work. This work was supported by an NSERC Collaborative Research Grant (D.G. McDonald, P.I.) and by research grants from the Natural Sciences and Engineering Research Council of Canada to C.M.W.

Literature Cited

Curtis, B.J., and C.M. Wood (1991) The function of the urinary bladder *in vivo* in the freshwater rainbow trout. J. Exp. Biol., *155:*567-583.

Demarest, J.R., and T.E. Machen (1982) Passive transcellular and paracellular ion movements across urinary bladders of the trout, *Salmo gairdneri* (Abstract). Federation Proc., *41:*1260.

Demarest, J.R., and T.E. Machen (1984) Passive and active ion transport by the urinary bladder of a euryhaline flounder. Am. J. Physiol., *246:*F402-F408.

Field, M., K.J. Karnaky, P.L. Smith, J.E. Bolton, and W.B. Kinter (1978) Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americanus*. I. Functional and structural properties of cellular and paracellular pathways for Na^+ and Cl^-. J. Membr. Biol., *41:*265-293.

Fossat, B., B. Lahlou, and M. Bornancin (1974) Involvement of a Na^+-K^+-ATPase in sodium transport by fish urinary bladder. Experientia, *30:*376-377.

Fossat, B., and B. Lahlou (1977) Osmotic and solute permeabilities of isolated urinary bladder of the trout. Am. J. Physiol., *233:*F525-F531.

Fossat, B., and B. Lahlou (1979a) The mechanism of coupled transport of sodium and chloride in isolated urinary bladder of the trout. J. Physiol., Lond., *294:*211-222.

Fossat, B., and B. Lahlou (1979b) Failure of 2,4,6-triaminopyrimidine (TAP) to block sodium pathways in a 'leaky' epithelium. Pflugers Arch., *379:*287-290.

Fossat, B., and B. Lahlou (1982) Ion flux changes introduced by voltage clamping or by amphotericin B in the isolated urinary bladder of the trout. J. Physiol., Lond., *325:* 111-123.

Frizzell, R.A., M.C. Dugas, and S.G. Shultz (1975) Sodium chloride transport by rabbit gall-bladder. Direct evidence for a coupled NaCl influx process. J. Gen. Physiol., *65:* 769-795.

Harvey, B.J., and B. Lahlou (1986) Ion-selective microelectrode studies of the electrochemical potentials in trout urinary bladder. J. Physiol., Lond., *370:*467-488.

Hickman, C.P., and B.F. Trump (1969) The kidney. In: Fish Physiology, Vol. 1. W.S. Hoar and D.J. Randall, eds. Academic Press, New York, pp. 91-239.

Hirano, T., D.W. Johnson, H.A. Bern, and S. Utida (1973) Studies on water movements in the isolated urinary bladder of selected freshwater, marine and euryhaline teleosts. Comp. Biochem. Physiol., *45A:*529-540.

Holliday, C.W. (1985) Salinity-induced changes in gill Na^+,K^+-ATPase activity in the mud fiddler crab, *Uca pugnax*. J. Exp. Zool., *233:*199-208.

House, C.R., and K. Green (1965) Ion and water transport in isolated intestine of the marine teleost, *Cottus scorpius*. J. Exp. Biol., *42:*177-189.

Lahlou, B., and B. Fossat (1971) Mécanisme du transport de l'eau et du sel à travers la vessie urinaire d'un poisson téléostéen en eau douce, la truite arc-en-ciel. C.R. hebd. Séanc Acad. Sci., Paris., *273:*2108-2110.

Lahlou, B., and B. Fossat (1984) Transport properties of fish urinary bladders in relation to osmoregulation. In: Osmoregulation in Estuarine and Marine Animals. A. Pequex, R. Gilles and L. Bolis, eds. Springer Verlag, Berlin, pp.93-106.

Loretz, C.A., and H.A. Bern (1980) Ion transport by the urinary bladder of the gobiid teleost, *Gillichthys mirabilis*. Am. J. Physiol., *239:*R415-R423.

Loretz, C.A., and H.A. Bern (1983) Control of ion transport by *Gillichthys mirabilis* urinary bladder. Am. J. Physiol., *245:*R45-R52.

Marshall, W.S. (1986) Independent Na^+ and Cl^- active transport by the urinary bladder epithelium of the brook trout. Am. J. Physiol., *250:*R227-R234.

Marshall, W.S. (1988) Passive solute and fluid transport in brook trout (*Salvelinus fontinalis*) urinary bladder epithelium. Can. J. Zool., *66:*912-918.

Marshall, W.S., and S.E. Bryson (1991) Intracellular pH regulation in trout urinary bladder epithelium: Na^+-H^+(NH_4^+) exchange. Am. J. Physiol., *261:*R652-R658.

Nellans, H.N., R.A Frizzell, and S.G. Schultz (1973) Coupled sodium chloride influx across the brush border of rabbit ileum. Am. J. Physiol., *225:*467-475.

Nellans, H.N., R.A. Frizzell, and S.G. Schultz (1974) Brush border processes and transepithelial Na^+ and Cl^- transport by rabbit ileum. Am. J. Physiol., *226:*1131-1141.

Perry, S.F. (1997) The chloride cell: structure and function in the gills of freshwater fishes. Annu. Rev. Physiol., *59:*325-347.

Renfro, J.L. (1977) Interdependence of active Na^+ and Cl^- transport of the isolated urinary bladder of the teleost, *Pseudopleuronectes americanus*. J. Exp. Zool., *199:*383-390.

Skadhauge, E. (1974) Coupling of transmural flows of NaCl and water in the intestine of the eel (*Anguilla anguilla*). J. Exp. Biol., *60:*535-546.

Verdouw, H., C.J.A. Van Echteld, and E.M.J. Dekkers (1978) Ammonia determination based on indophenol formation with sodium salicylate. Water Res., *12:*399-402.

Wood, C.M. (1991) Branchial ion and acid-base transfer in freshwater teleost fish: Environmental hyperoxia as a probe. Physiol. Zool., *64:*68-102.

Zall, D.M., M.D. Fisher, and Q.M. Garner (1956) Photometric determination of chlorides in water. Analyt. Chem., *28:*1665-1678.

Table 1. Effect of the 10^{-4}M mucosal addition of DIDS, amiloride or bumetanide on unidirectional efflux (Jsm), influx (Jms) and flux ratios of Cl^- and Na^+ and on V_t and G_t in urinary bladder of *Oncorhynchus mykiss* (n = 4) bathed on mucosal side with Cortland saline under open circuit conditions.

Experimental Period	Vt (mV)[a]	Gt ($mS \cdot cm^{-2}$)[b]	Jsm ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	Jms ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	Jsm/Jms Observed	Jsm/Jms Predicted
Cl^-						
Control (Hour 1)	0.1 ± 0.1	8.39 ± 2.1	3.5 ± 1.0	12.3 ± 4.6	5.8 ± 2.1	1.00 ± 0.003
DIDS	0.1 ± 0.2	9.20 ± 1.7	3.5 ± 0.6	8.7 ± 2.3	2.7 ± 0.7	1.00 ± 0.008
Control (Hour 3)	-0.1 ± 0.2	8.23 ± 1.9	3.4 ± 0.7	12.9 ± 3.5	5.0 ± 1.6	1.00 ± 0.006
Na^+						
Control (Hour 1)			0.9 ± 0.3	2.9 ± 1.0	11.1 ± 3.0	1.00 ± 0.003
DIDS			1.3 ± 0.4	5.8 ± 0.9	10.1 ± 2.5	1.00 ± 0.008
Control (Hour 3)			1.2 ± 0.5	3.5 ± 0.3	13.5 ± 2.3	1.00 ± 0.006
Cl^-						
Control (Hour 1)	0.2 ± 0.1	3.49 ± 0.9	1.8 ± 0.6	5.0 ± 1.6	3.1 ± 0.5	1.01 ± 0.005
Amiloride	0.2 ± 0.1	3.36 ± 1.1	2.4 ± 0.8	5.6 ± 0.8	3.1 ± 0.6	1.01 ± 0.004
Control (Hour 3)	0.1 ± 0.1	3.56 ± 1.2	1.8 ± 0.6	5.8 ± 0.8	4.2 ± 0.9	1.00 ± 0.005
Na^+						
Control (Hour 1)			0.9 ± 0.3	3.3 ± 1.5	4.2 ± 1.8	0.99 ± 0.005
Amiloride			1.0 ± 0.3	3.9 ± 0.8	4.3 ± 0.6	0.99 ± 0.004
Control (Hour 3)			1.0 ± 0.4	4.2 ± 0.4	6.0 ± 1.6	1.00 ± 0.005
Cl^-						
Control (Hour 1)	0.2 ± 0.1	6.8 ± 1.5	2.7 ± 0.4	5.6 ± 0.6	2.2 ± 0.1	1.01 ± 0.003
Bumetanide	0.3 ± 0.1	4.6 ± 1.0	2.4 ± 0.6	5.2 ± 0.6	2.7 ± 0.6	1.02 ± 0.003
Control (Hour 3)	0.1 ± 0.1	4.3 ± 0.6	2.1 ± 0.4	4.3 ± 0.5	2.2 ± 0.3	1.00 ± 0.005
Na^+						
Control (Hour 1)			1.8 ± 0.8	4.1 ± 0.8	4.6 ± 1.6	0.99 ± 0.003
Bumetanide			1.3 ± 0.6	3.4 ± 0.6	4.7 ± 1.4	0.98 ± 0.003
Control (Hour 3)			1.1 ± 0.2	3.6 ± 0.5	3.9 ± 0.9	1.00 ± 0.005

[b] Tissue conductance corrected for solution resistance; n = 8.

* Paired *t*-test, two-tailed, observed *vs.* predicted values of Jsm/Jms. Comparison of all observed and predicted ratios were significantly different, $P<0.05$.

There were no significant effects of any of the drug treatments on the parameters measured.

Table 2. Comparison of Cl^- and Na^+ "cold" (measured chemically) net fluxes (J_{net}) and "hot" net fluxes as calculated from unidirectional flux measurements with $^{22}Na^+$ and $^{36}Cl^-$ (J_{net}) in the *Oncorhynchus mykiss* urinary bladder (n = 6) bathed on mucosal side with control, or Cl^--free or Na^+-free artificial urine under open circuit conditions.

Bathing Solutions Serosa/Mucosa	Hour 1 Jnet	Hour 2 Jnet	Hour 3 Jnet
	($\mu mol \cdot cm^{-2} \cdot h^{-1}$)		
Saline/Artificial Urine			
$^{36}Cl^-$	-1.7	-1.4	-0.9
	± 0.3	± 0.3	± 0.4
Cl^-	-5.7	-3.3	-9.6*
	± 3.6	± 1.4	± 3.4
$^{22}Na^+$	-0.9	-0.9	-0.6
	± 0.3	± 0.4	± 0.2
Na^+	-9.8	-3.1	-6.7*
	± 3.7	± 2.0	± 3.2
Saline/Na^+ Free Artificial Urine			
$^{36}Cl^-$	-1.5	-1.2	-0.5
	± 0.5	± 0.6	± 0.2
Cl^-	-4.3	-4.6	-7.1*
	± 1.4	± 1.3	± 1.6
Saline/Cl^- Free Artificial Urine			
$^{22}Na^+$	-1.7	-1.4	-1.8
	± 0.4	± 0.3	± 0.6
Na^+	-5.7*	-5.5*	-2.7
	± 1.4	± 1.0	± 1.1

* Un-paired *t*-test, two-tailed. Comparison between "hot" and "cold" net fluxes of Na^+ and Cl^- in each treatment group; *, $P<0.05$.

Table 3. Effect of the 10^{-4}M mucosal addition of DIDS, amiloride or bumetanide on unidirectional efflux (Jsm), influx (Jms) and flux ratios of Cl^- and Na^+ and on V_t and G_t in urinary bladder of *Oncorhynchus mykiss* (n = 4) bathed on mucosal side with artificial urine under open circuit conditions.

Experimental Period	Vt (mV)[a]	Gt ($mS \cdot cm^{-2}$)[b]	Jsm ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	Jms ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	Jsm/Jms Observed	Jsm/Jms Predicted
Cl^-						
Control (Hour 1)	6.5 ± 0.4	1.45 ± 0.2	2.3 ± 0.3	0.3 ± 0.1	0.14 ± 0.02	0.03 ± 0.0004
DIDS	5.2 ± 0.6	1.31 ± 0.2	2.3 ± 0.3	0.3 ± 0.1	0.16 ± 0.06	0.03 ± 0.0004
Control (Hour 3)	5.2 ± 0.6	1.34 ± 0.3	2.7 ± 0.4	0.3 ± 0.1	0.11 ± 0.03	0.03 ± 0.0004
Na^+						
Control (Hour 1)			1.3 ± 0.2	0.1 ± 0.01	0.12 ± 0.03	0.01 ± 0.0001
DIDS			1.3 ± 0.3	0.2 ± 0.03	0.13 ± 0.03	0.01 ± 0.0002
Control (Hour 3)			2.0 ± 0.2	0.1 ± 0.02	0.06 ± 0.01	0.01 ± 0.0001
Cl^-						
Control (Hour 1)	4.4 ± 1.0	2.38 ± 0.8	0.7 ± 0.3	0.3 ± 0.1	0.53 ± 0.11	0.03 ± 0.002
Amiloride	4.7 ± 1.1	2.10 ± 0.8	2.2 ± 0.6	0.5 ± 0.2	0.33 ± 0.16	0.03 ± 0.001
Control (Hour 3)	5.1 ± 0.8	2.70 ± 0.9	1.0 ± 0.1	0.5 ± 0.1	0.56 ± 0.17	0.03 ± 0.001
Na^+						
Control (Hour 1)			0.6 ± 0.2	0.1 ± 0.03	0.23 ± 0.09	0.01 ± 0.0001
Amiloride			1.4 ± 0.5	0.3 ± 0.05	0.34 ± 0.08	0.01 ± 0.0002
Control (Hour 3)			0.6 ± 0.1	0.2 ± 0.02	0.45 ± 0.13	0.01 ± 0.0001
Cl^-						
Control (Hour 1)	4.9 ± 1.0	1.41 ± 0.3	1.0 ± 0.2	0.4 ± 0.1	0.40 ± 0.06	0.03 ± 0.002
Bumetanide	4.0 ± 1.3	1.51 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.60 ± 0.17	0.03 ± 0.002
Control (Hour 3)	3.8 ± 1.2	1.36 ± 0.4	1.0 ± 0.2	0.4 ± 0.1	0.43 ± 0.09	0.03 ± 0.002
Na^+						
Control (Hour 1)			0.4 ± 0.1	0.1 ± 0.02	0.46 ± 0.17	0.01 ± 0.001
Bumetanide			0.3 ± 0.1	0.2 ± 0.03	0.53 ± 0.24	0.01 ± 0.001
Control (Hour 3)			0.6 ± 0.2	0.2 ± 0.03	0.44 ± 0.13	0.01 ± 0.001

[a] Transepithelial potential (mucosal ground) corrected for junction potentials; n = 8.

[b] Tissue conductance corrected for solution resistance; n = 8.

Paired *t*-test, two-tailed, observed *vs.* predicted values of Jsm/Jms. Comparison of all observed and predicted ratios were significantly different, $P<0.05$.

There were no significant effects of any of the drug treatments on the parameters measured.

Table 4. Mean transepithelial potential (V_t) and transepithelial conductance (G_t) for different Na^+ and/or Cl^- concentration ranges for control, low Na^+ and low Cl^- artificial urine mucosal solutions bathing the isolated urinary bladder of the *Oncorhynchus mykiss* (n = 6).

$[Na^+]$ and/or $[Cl^-]$ range (mM)	Mean transepithelial potential (Vt, mV)		
	control (Na^+/Cl^-)	low Na^+	low Cl^-
0-5	7.53 ± 1.6	10.51 ± 0.7	1.90 ± 0.6†
5-10	6.71 ± 0.7	7.69 ± 0.7*	4.76 ± 1.2
10-20	4.85 ± 0.7	-	5.51 ± 0.8*
40-50	2.10 ± 0.4*	9.66 ± 0.6†	5.19 ± 1.0*†
110-150	0.57 ± 0.03*	17.64 ± 0.3*†	10.31 ± 1.6*†
$[Na^+]$ and/or $[Cl^-]$ range (mM)	Mean conductance (G_t, $mS \cdot cm^{-2}$)		
	control (Na^+/Cl^-)	low Na^+	low Cl^-
0-5	3.31 ± 1.5	1.58 ± 0.7	1.82 ± 0.2
5-10	4.95 ± 1.3	1.89 ± 0.8	1.94 ± 0.6
10-20	3.33 ± 1.0	-	2.46 ± 0.8
40-50	7.91 ± 3.3	2.56 ± 1.6	2.48 ± 0.7
110-150	18.3 ± 7.9*	3.63 ± 2.1	3.76 ± 1.0

[a] Transepithelial potential (mucosal ground) corrected for junction potentials; n = 6.

[b] Tissue conductance corrected for solution resistance; n = 6.

* Un-paired *t*-test, two-tailed. Comparison of control Na^+/Cl^- V_t and G_t values to 0-5 range; *, $P<0.05$.

† Un-paired *t*-test, two-tailed. Comparison of low Na^+ and low Cl^- V_t and G_t values to their respective control values; †, $P<0.05$.

Figure 3.1. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *Oncorhynchus mykiss* under symmetrical saline conditions. Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+ respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+ respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant differences from unidirectional influx (above bar), efflux (below bar) or net flux (beside bar) ($P<0.05$, paired t-test, two-tailed) between Cl^- and Na^+.

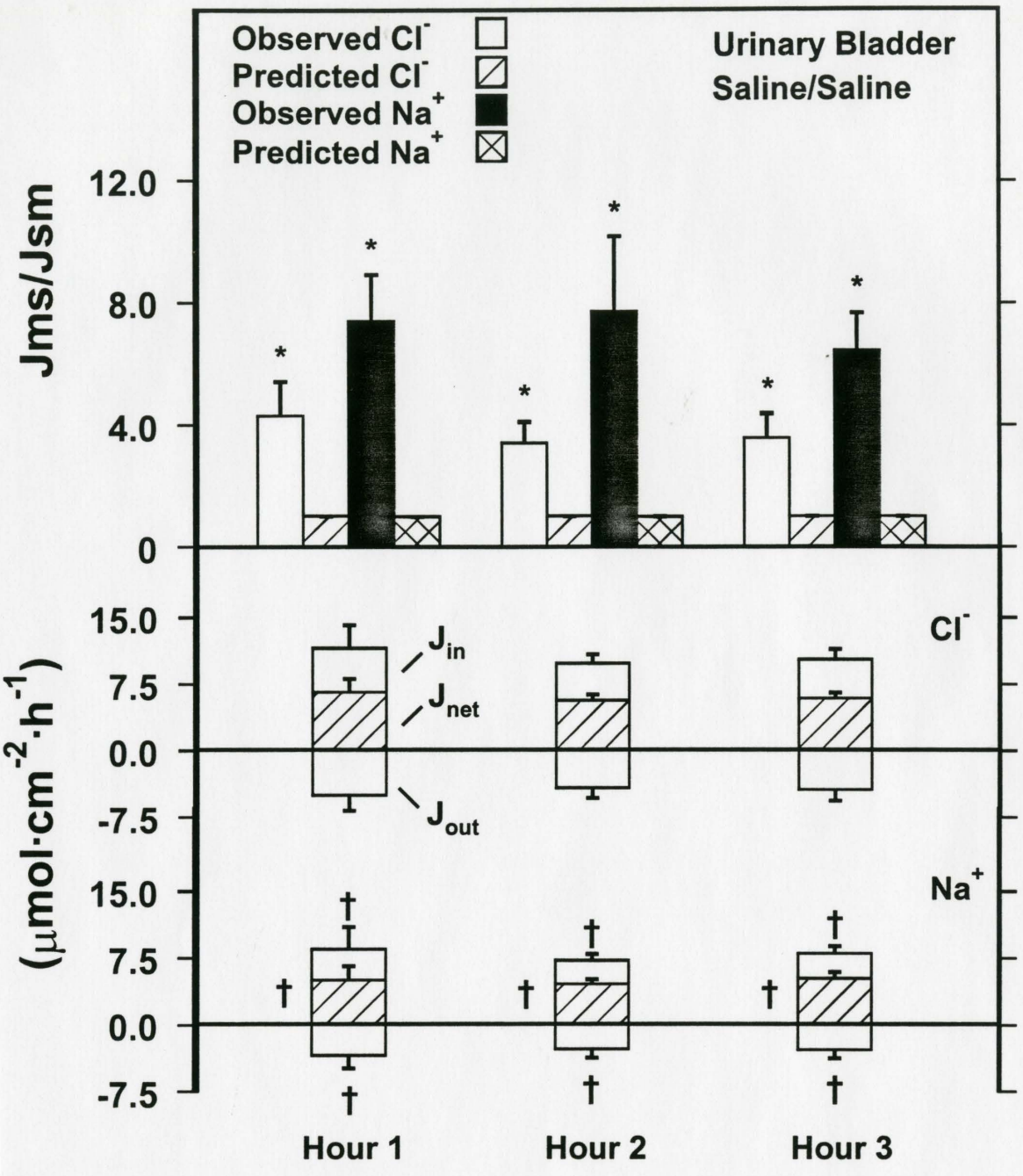

Observed Cl-
Predicted Cl-
Observed Na+
Predicted Na+
Urinary Bladder
Saline/Saline
Jms/Jsm
(μmol·cm-2·h-1)
12.0
8.0
4.0
0
15.0
7.5
0.0
-7.5
Jin
Jnet
Jout
Cl-
Na+
Hour 1
Hour 2
Hour 3

Figure 3.2. Observed and predicted flux ratios for the unidirectional movements of Cl^- with control or Na^+-free saline bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Cl^- in control and in Na^+-free mucosal saline respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- in control and in Na^+-free mucosal saline respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant differences from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- in control and in Na^+-free mucosal saline. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar) or net flux (beside bar) ($P<0.05$, un-paired t-test, two-tailed) of Cl^- between the two treatment groups.

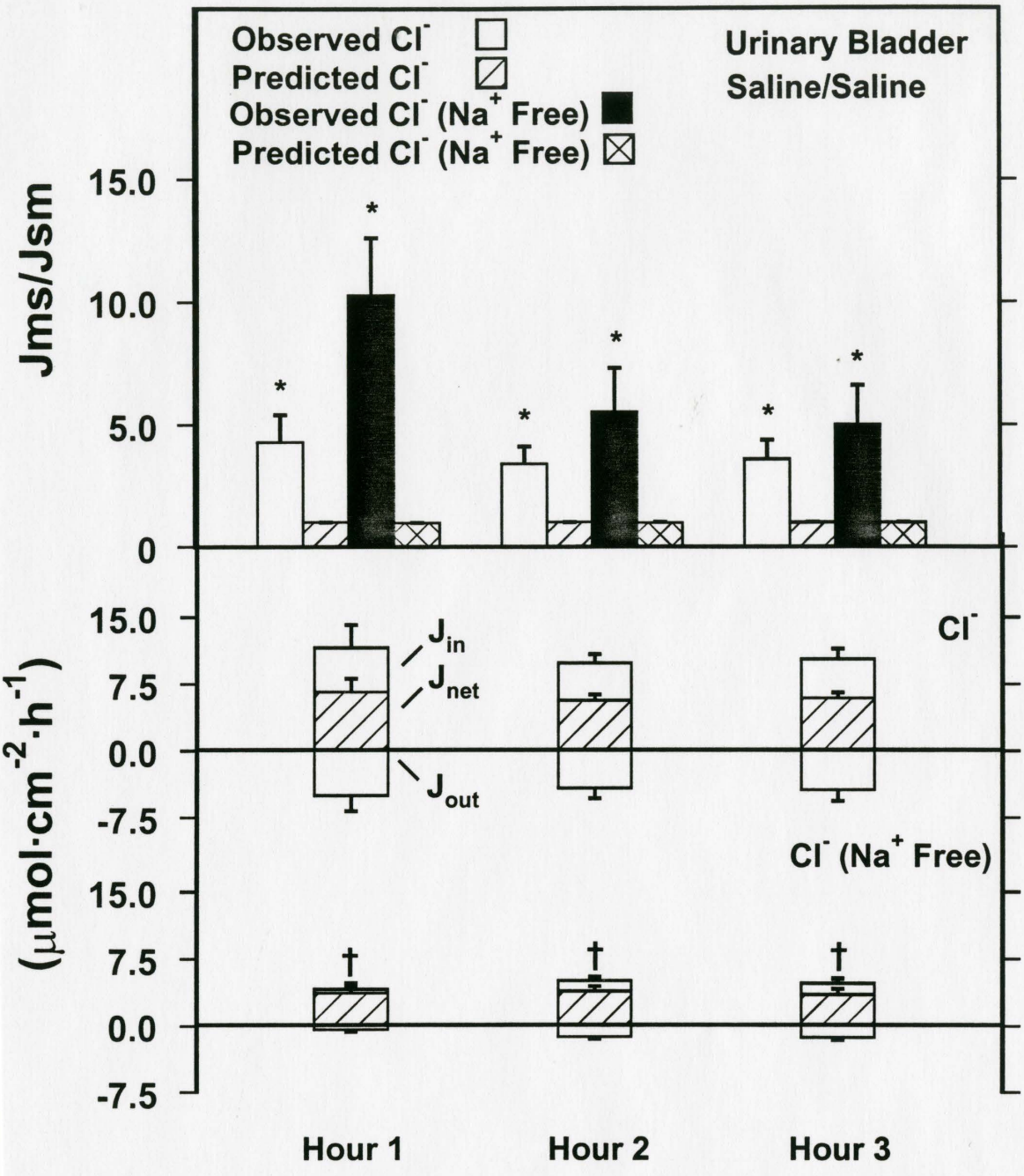

Observed Cl-
Predicted Cl-
Observed Cl- (Na+ Free)
Predicted Cl- (Na+ Free)
Urinary Bladder
Saline/Saline
Jms/Jsm
15.0
10.0
5.0
0
*
15.0
7.5
0.0
-7.5
Jin
Jnet
Jout
Cl-
Cl- (Na+ Free)
(μmol·cm-2·h-1)
†
Hour 1
Hour 2
Hour 3

Figure 3.3. Observed and predicted flux ratios for the unidirectional movements of Na^+ with control or Cl^--free saline bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Na^+ in control and in Cl^--free mucosal saline respectively, hatched and crossed bars represent the predicted flux ratios for Na^+ in control and in Cl^--free mucosal saline respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Na^+ in control and in Cl^--free mucosal saline. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar) or net flux (beside bar) ($P<0.05$, un-paired t-test, two-tailed) of Na^+ between the two treatment groups.

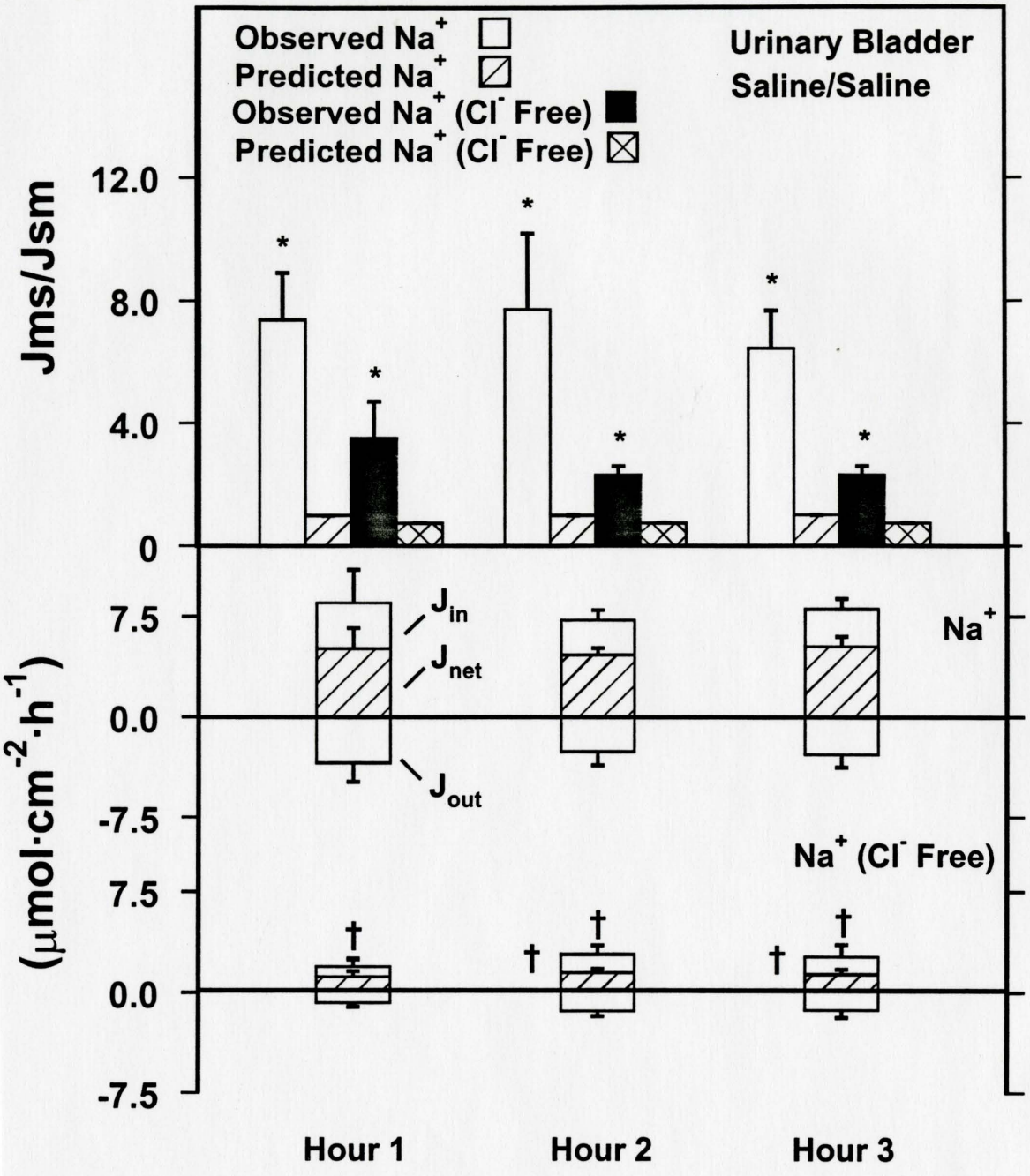

Observed Na+
Predicted Na+
Observed Na+ (Cl- Free)
Predicted Na+ (Cl- Free)
Urinary Bladder
Saline/Saline
Jms/Jsm
(μmol·cm-2·h-1)
12.0
8.0
4.0
0
7.5
0.0
-7.5
7.5
0.0
-7.5
Jin
Jnet
Jout
Na+
Na+ (Cl- Free)
*
†
Hour 1
Hour 2
Hour 3

Figure 3.4. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *Oncorhynchus mykiss* bathed with artificial urine on the mucosal surface. Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+ respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+ respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar) or net flux (beside bar) ($P<0.05$, paired t-test, two-tailed) between Cl^- and Na^+.

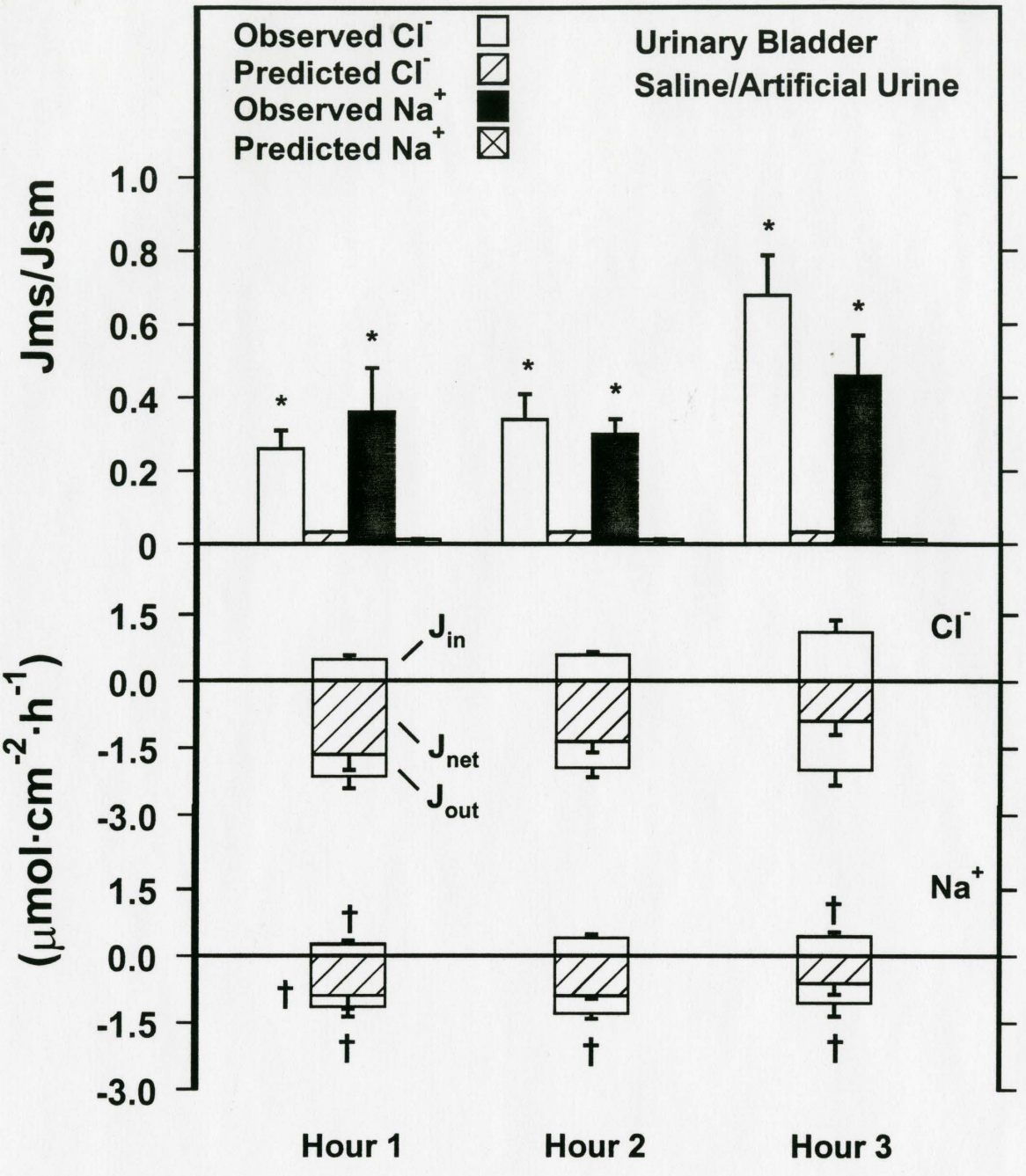

Observed Cl-
Predicted Cl-
Observed Na+
Predicted Na+
Urinary Bladder
Saline/Artificial Urine
Jms/Jsm
(μmol·cm-2·h-1)
1.0
0.8
0.6
0.4
0.2
0
1.5
0.0
-1.5
-3.0
Jin
Jnet
Jout
Cl-
Na+
*
†
Hour 1
Hour 2
Hour 3

Figure 3.5. Observed and predicted flux ratios for the unidirectional movements of Cl^- with control or Na^+-free artificial urine bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Cl^- in control and in Na^+-free mucosal artificial urine respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- in control and in Na^+-free mucosal artificial urine respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- in control and in Na^+-free mucosal artificial urine. There were no significant differences ($P>0.05$) between the two treatment groups.

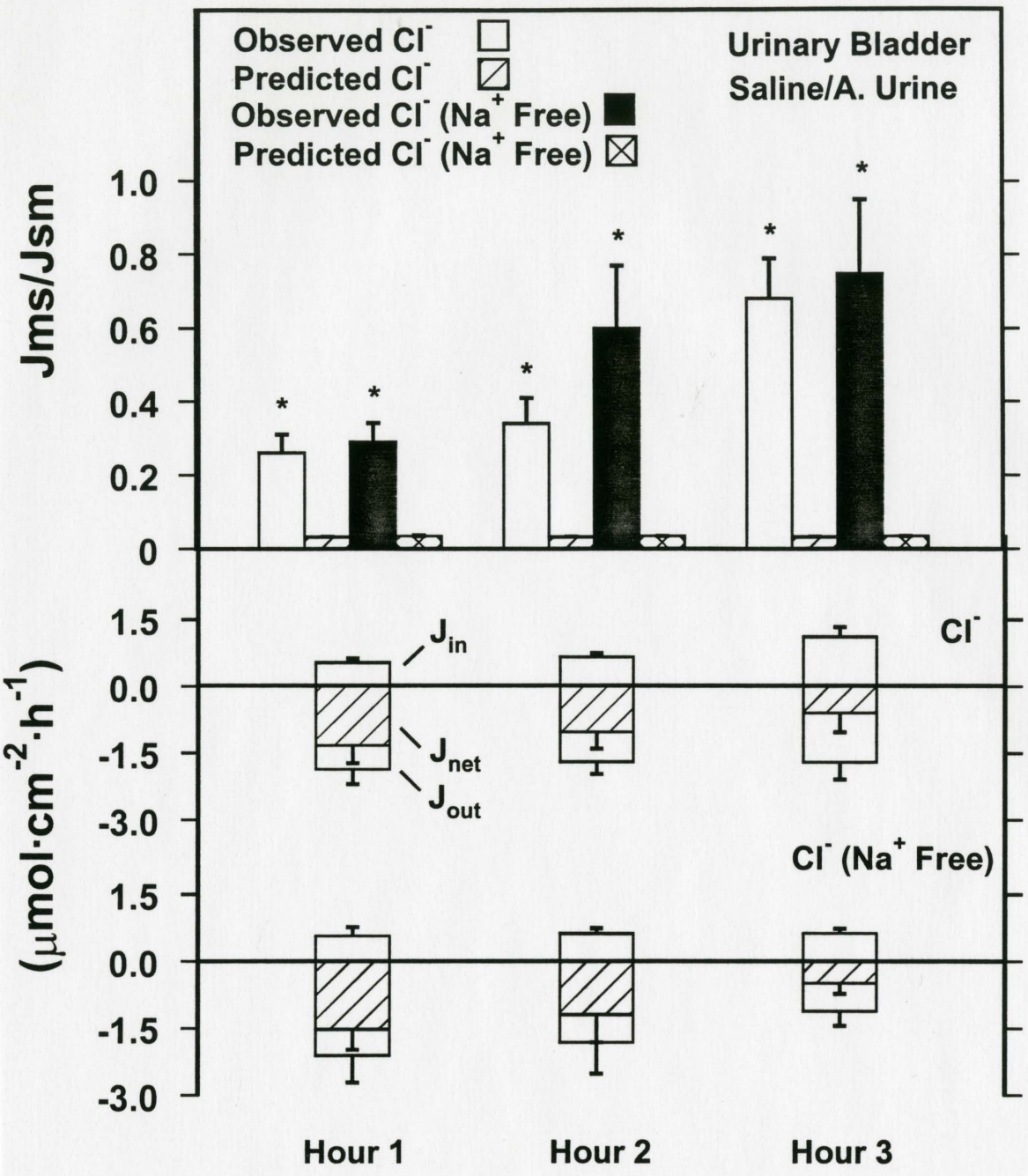

Observed Cl-
Predicted Cl-
Observed Cl- (Na+ Free)
Predicted Cl- (Na+ Free)
Urinary Bladder
Saline/A. Urine
Jms/Jsm
(μmol·cm-2·h-1)
1.0
0.8
0.6
0.4
0.2
0
1.5
0.0
-1.5
-3.0
Jin
Jnet
Jout
Cl-
Cl- (Na+ Free)
Hour 1
Hour 2
Hour 3

Figure 3.6. Observed and predicted flux ratios for the unidirectional movements of Na^+ with control or Cl^--free artificial urine bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Na^+ in control and in Cl^--free mucosal artificial urine respectively, hatched and crossed bars represent the predicted flux ratios for Na^+ in control and in Cl^--free mucosal artificial urine respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed) implying evidence for non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Na^+ in control and in Cl^--free mucosal artificial urine. There were no significant differences ($P>0.05$) between the two treatment groups.

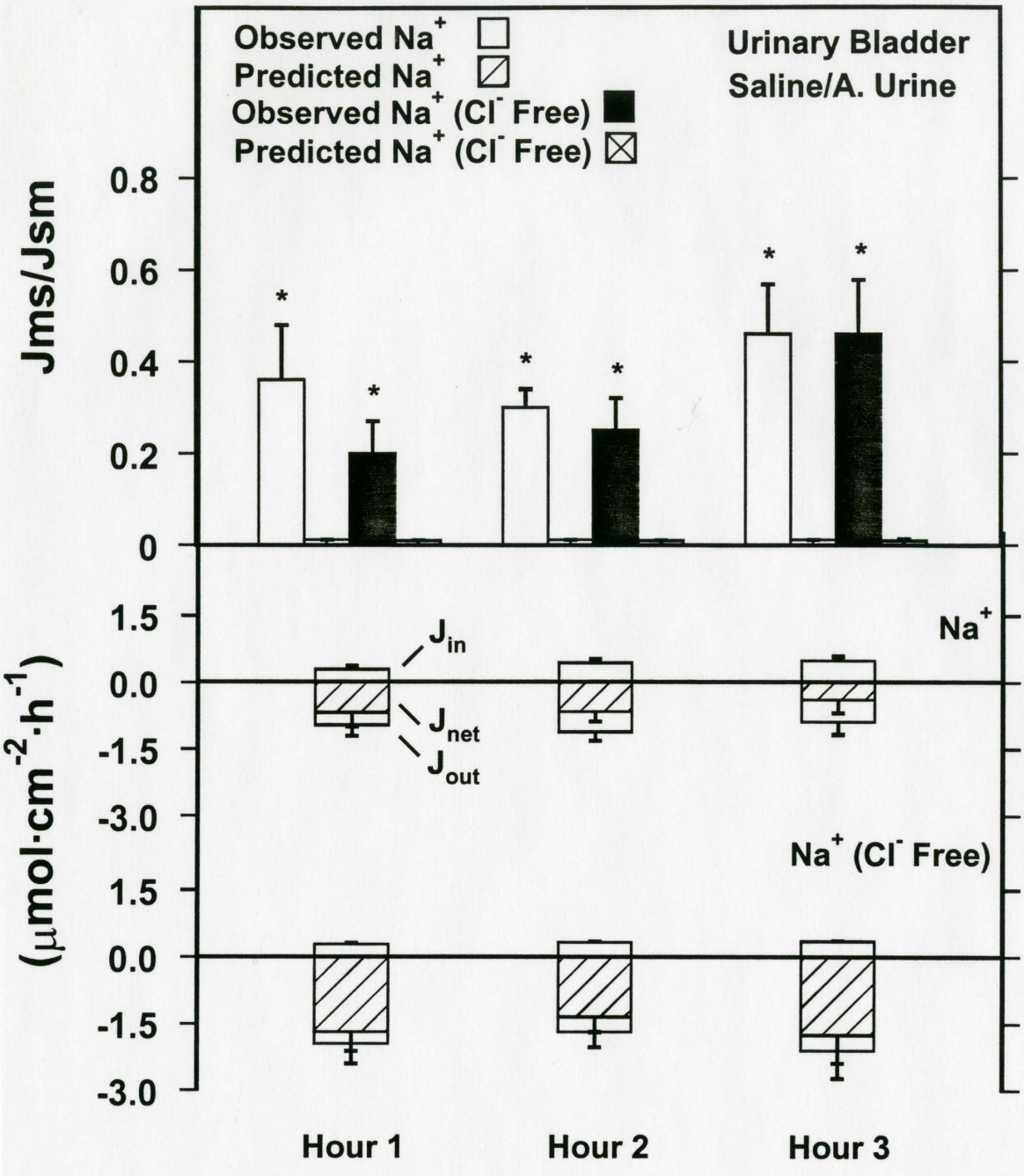

Observed Na+
Predicted Na+
Observed Na+ (Cl- Free)
Predicted Na+ (Cl- Free)
Urinary Bladder
Saline/A. Urine
Jms/Jsm
(μmol·cm-2·h-1)
0.8
0.6
0.4
0.2
0
1.5
0.0
-1.5
-3.0
1.5
0.0
-1.5
-3.0
*
Jin
Jnet
Jout
Na+
Na+ (Cl- Free)
Hour 1
Hour 2
Hour 3

Figure 3.7. Relationship of mucosal to serosal Na^+ flux (Na^+ influx) to the concentration (mM) of Na^+ in control solutions (closed squares) and low Cl^- solutions (closed triangles). Serosal medium was Cortland's saline and mucosal medium was artificial urine with varying concentrations of Na^+ and/or Cl^-. Vertical and horizontal lines represent ± the standard error of mean; n = 4-8 for each concentration range of (0-5, 5-10, 10-20, 40-50 and 110-150 mM). Curves were best fit for Michaelis-Menten kinetics. Asterisk indicates significant difference between concentration range of each treatment ($P<0.05$, un-paired t-test, two-tailed).

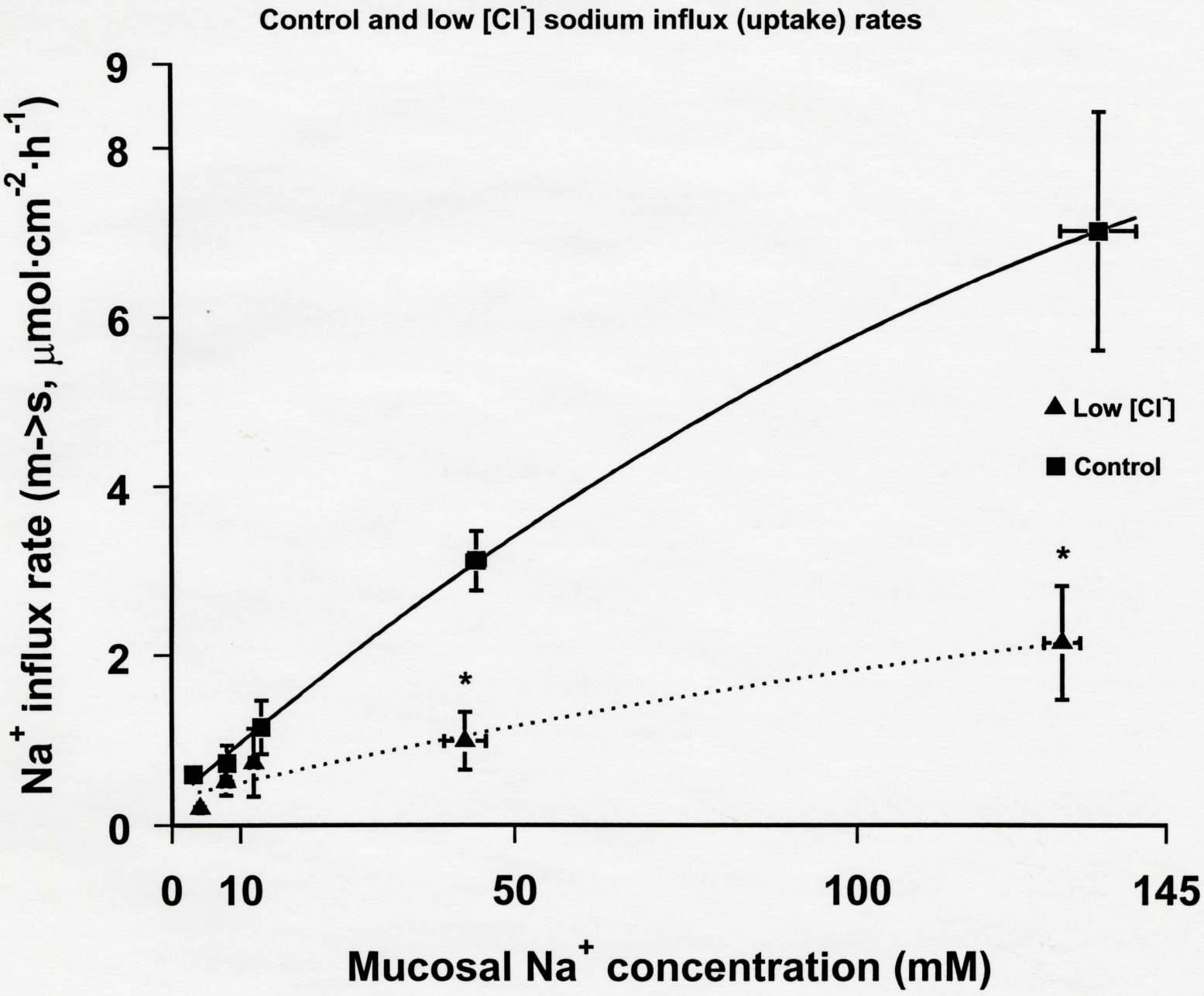

Control and low [Cl-] sodium influx (uptake) rates
Na+ influx rate (m->s, μmol·cm-2·h-1)
9
8
6
4
2
0
0
10
50
100
145
Mucosal Na+ concentration (mM)
Low [Cl-]
Control
*
*

Figure 3.8. Relationship of mucosal to serosal Cl^- flux to the concentration (mM) of Cl^- in control solutions (closed squares) and low Na^+ solutions (closed triangles). Serosal medium was Cortland's saline and mucosal medium was artificial urine with varying concentrations of Na^+ and/or Cl^-. Vertical and horizontal lines represent ± the standard error of mean; n = 4-8 for each concentration range of (0-5, 5-10, 40-50 and 110-150 mM, excluding 10-20 mM range for low Na^+ artificial urine where no observations were recorded). Curves were best fit for Michaelis-Menten kinetics. Asterisk indicates significant difference between concentration range of each treatment ($P<0.05$, un-paired t-test, two-tailed).

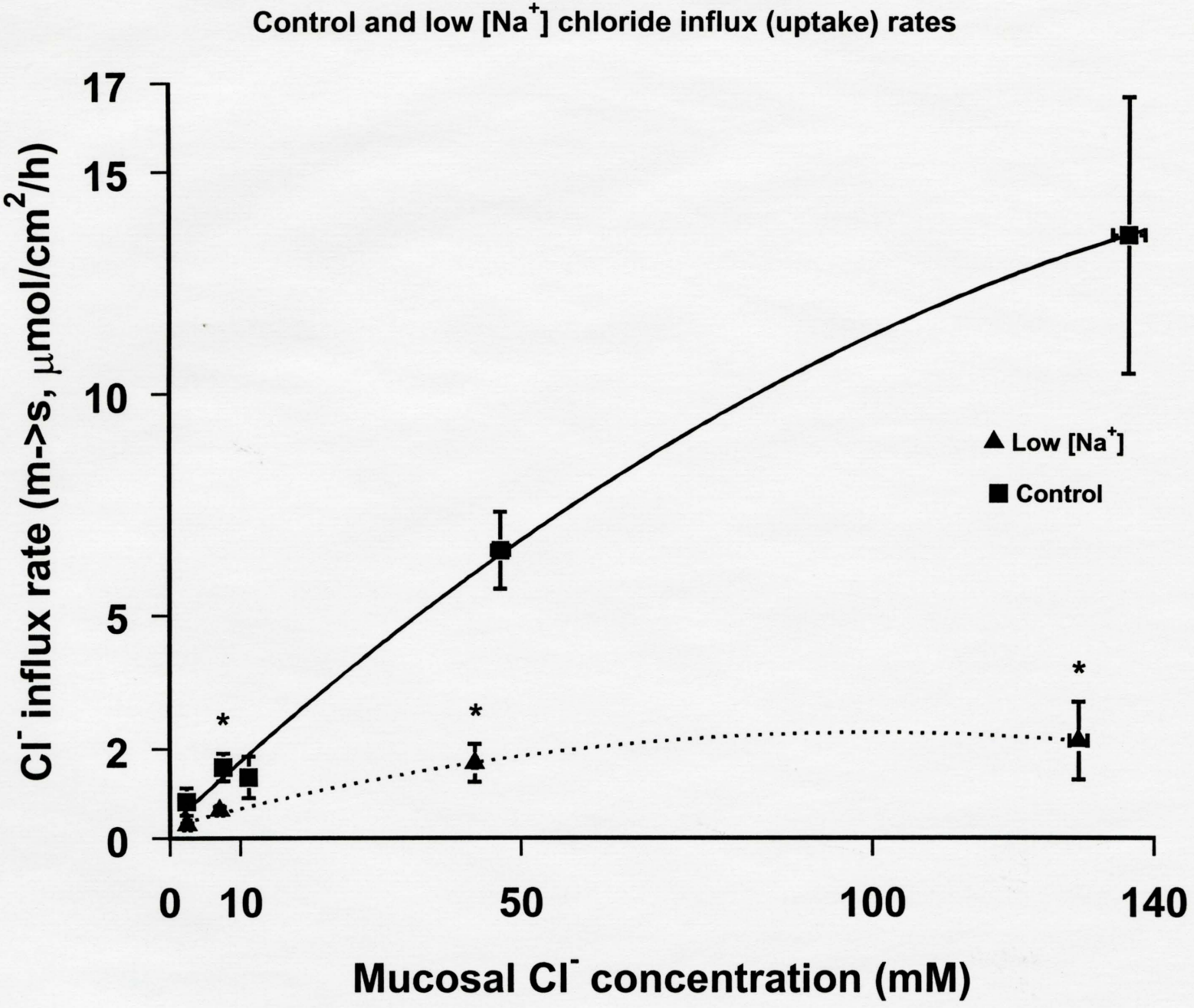

Control and low [Na+] chloride influx (uptake) rates
Cl- influx rate (m->s, μmol/cm2/h)
17
15
10
5
2
0
0
10
50
100
140
Mucosal Cl- concentration (mM)
Low [Na+]
Control
*
*
*

Figure 3.9. (A). Regression of serosal to mucosal Na^+ (open triangles, solid line) and Cl^- flux (open circles, dashed line) (influx) and (B) mucosal to serosal Na^+ and Cl^- flux (efflux) in relation to total ionic conductance (G_t, $mS \cdot cm^{-2}$) under symmetrical saline conditions in urinary bladder epithelia of FW *Oncorhynchus mykiss*.

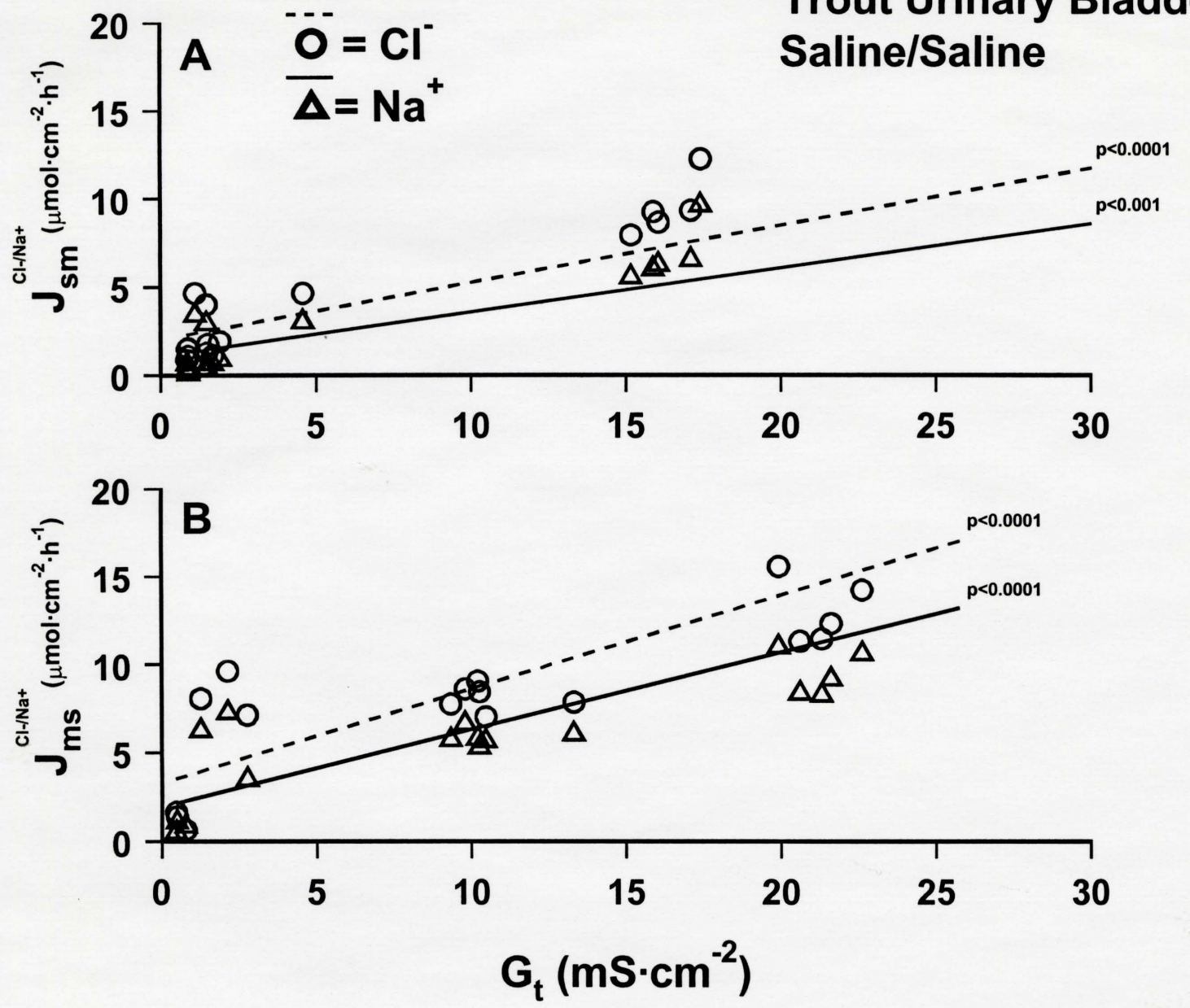

Trout Urinary Bladder
Saline/Saline
A
B
O = Cl-
Δ = Na+
p<0.0001
p<0.001
p<0.0001
p<0.0001
Jsm Cl-/Na+ (μmol·cm-2·h-1)
Jms Cl-/Na+ (μmol·cm-2·h-1)
Gt (mS·cm-2)
0
5
10
15
20
25
30

Figure 3.10. (A). Regression of serosal to mucosal Na^+ (open triangles, solid line) and Cl^- flux (open circles, dashed line) and (B) mucosal to serosal Na^+ and Cl^- flux in relation to total ionic conductance (G_t, $mS \cdot cm^{-2}$) with artificial urine bathing the mucosal surface in urinary bladder epithelia of FW *Oncorhynchus mykiss*.

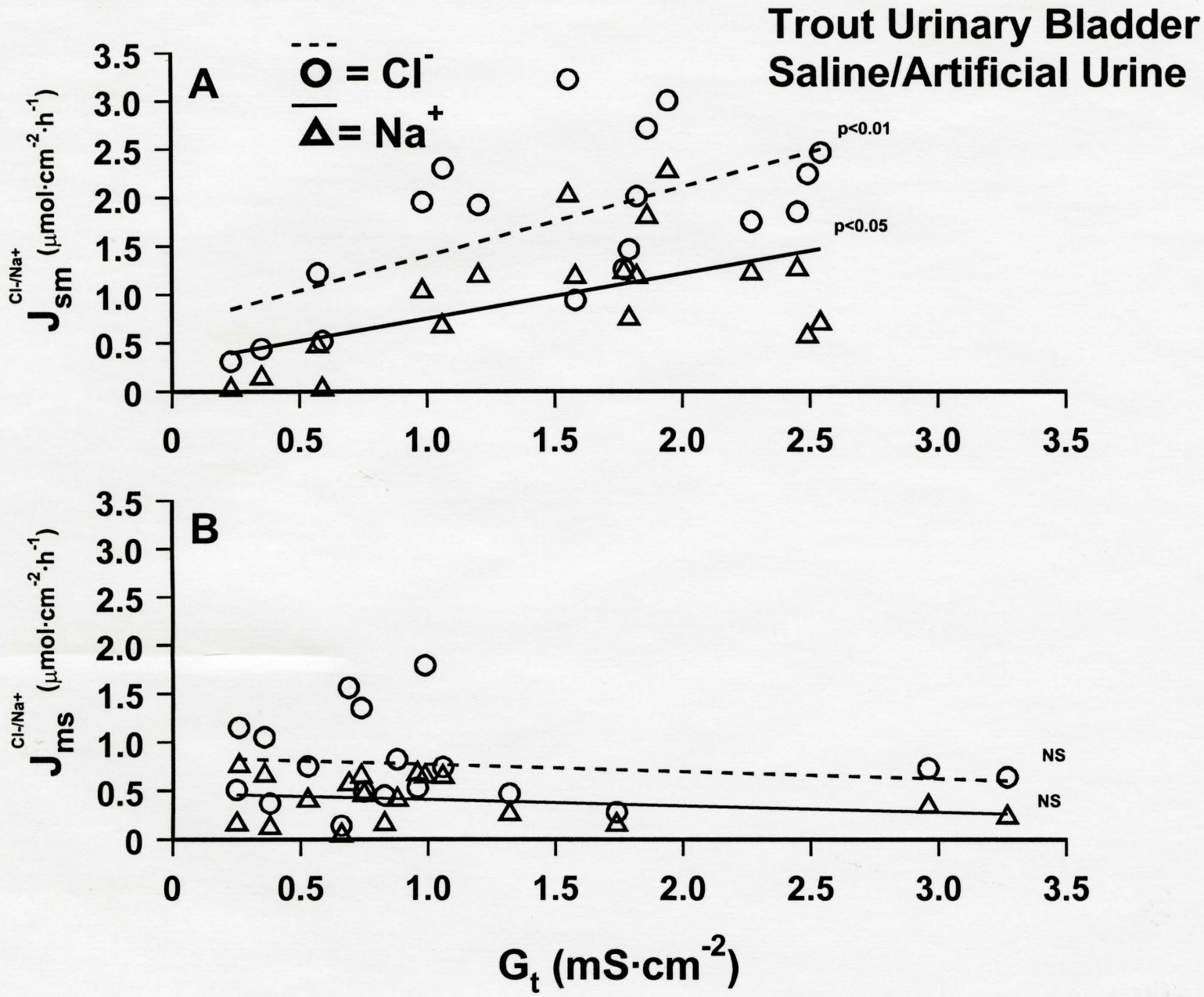

Trout Urinary Bladder
Saline/Artificial Urine
A
B
O = Cl-
Δ = Na+
p<0.01
p<0.05
NS
NS
Cl-/Na+ Jsm (μmol·cm-2·h-1)
Cl-/Na+ Jms (μmol·cm-2·h-1)
Gt (mS·cm-2)
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Figure 3.11. (A). Comparison of transepithelial voltage (V_t, mV) and total tissue conductance (G_t, $mS \cdot cm^{-2}$) in anterior and posterior portions of urinary bladder epithelia from FW *Oncorhynchus mykiss* with artificial urine bathing the mucosal surface. Open bars represent anterior portions, hatched bars represent posterior portions of urinary bladder epithelia. (B). Comparison of transepithelial voltage (V_t, mV) and total tissue conductance (G_t, $mS \cdot cm^{-2}$) in anterior and posterior portions of urinary bladder epithelia from FW *Oncorhynchus mykiss* under symmetrical saline conditions Other details same as in (A). Means ± 1SEM (n = 36). Asterisk indicates significant difference between V_t and G_t values of anterior and posterior portions of bladder epithelia ($P<0.05$, un-paired t-test, two-tailed).

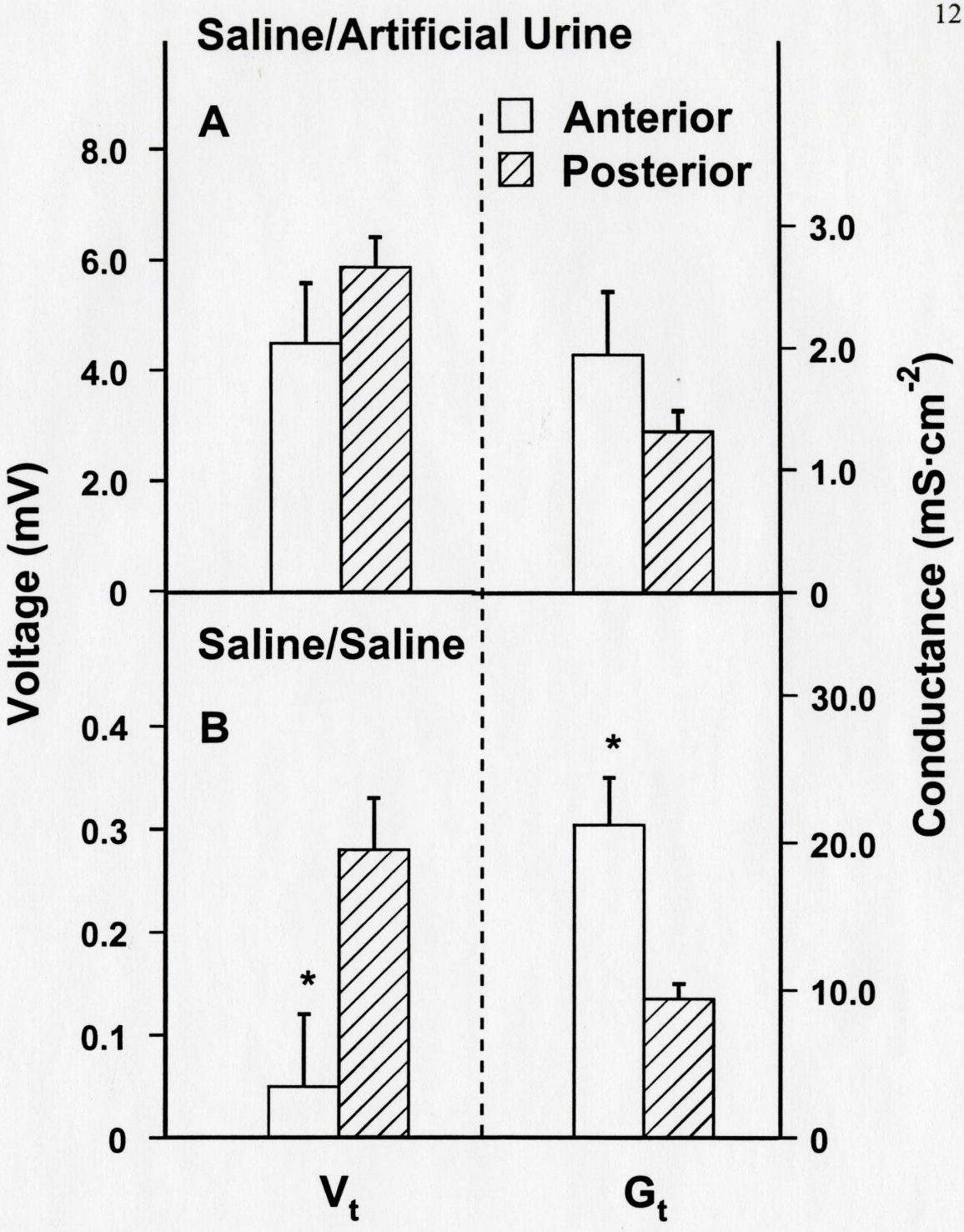
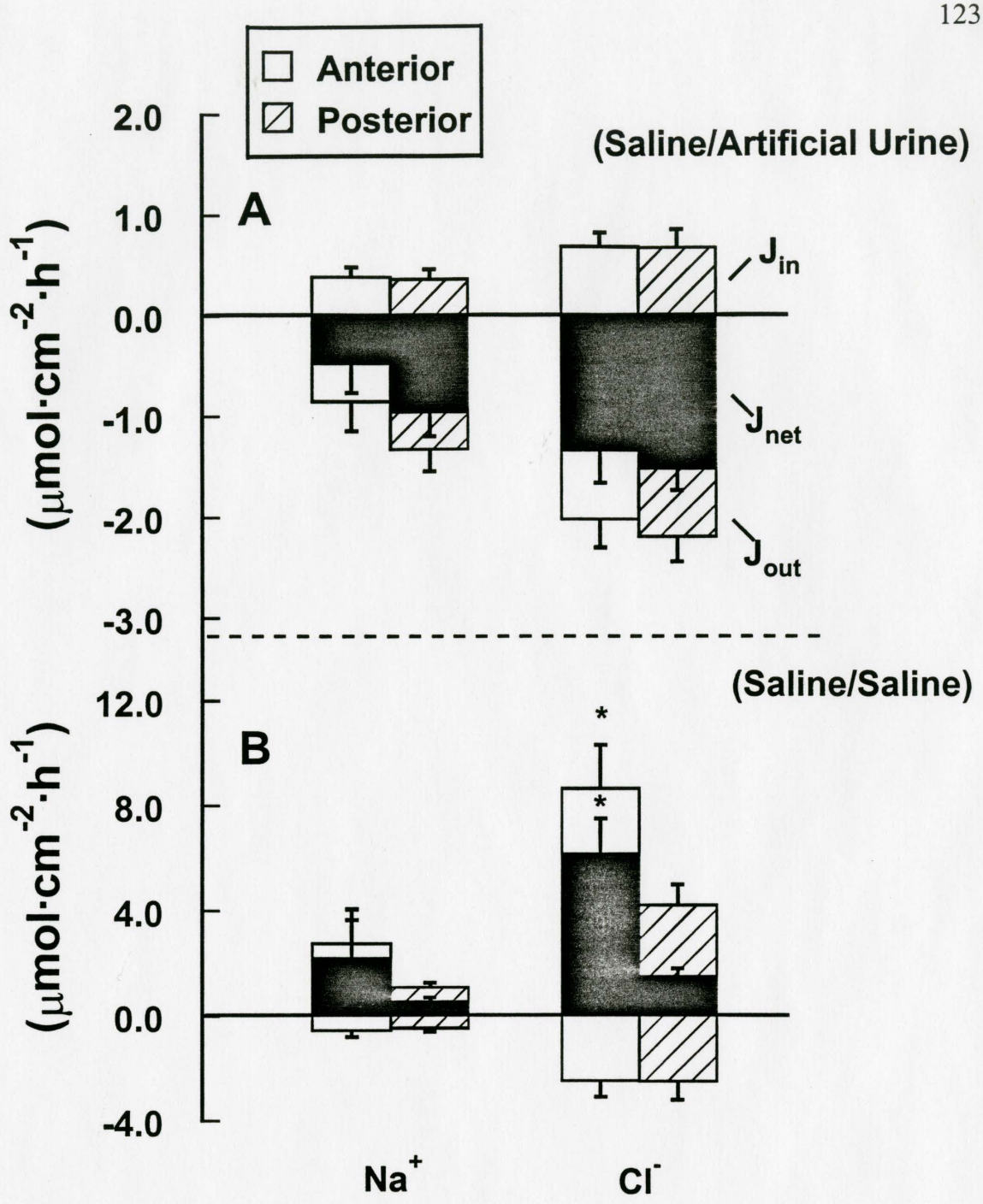

Saline/Artificial Urine
A
Anterior
Posterior
8.0
6.0
4.0
2.0
0
3.0
2.0
1.0
0
Saline/Saline
B
0.4
0.3
0.2
0.1
0
30.0
20.0
10.0
0
*
*
Voltage (mV)
Conductance (mS·cm-2)
Vt
Gt

Figure 3.12. (A). Comparison of unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of Na^+ and Cl^- between anterior and posterior portions of urinary bladder epithelia from FW *Oncorhynchus mykiss* with artificial urine bathing the mucosal surface. Open bars represent anterior portions, hatched bars represent posterior portions of urinary bladder epithelia. (B). Comparison of unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of Na^+ and Cl^- between anterior and posterior portions of urinary bladder epithelia from FW *Oncorhynchus mykiss* under symmetrical saline conditions. Other details same as in (A). Means ± 1SEM (n = 6-15). Asterisk indicates significant difference between unidirectional influx, efflux or net flux between anterior and posterior portions of bladder epithelia ($P<0.05$, paired t-test, two-tailed).


Anterior
Posterior
(Saline/Artificial Urine)
A
2.0
1.0
0.0
-1.0
-2.0
-3.0
(μmol·cm-2·h-1)
Jin
Jnet
Jout
(Saline/Saline)
B
12.0
8.0
4.0
0.0
-4.0
(μmol·cm-2·h-1)
*
*
Na+
Cl-

Appendix: General Methodology

Ussing Chambers

The Ussing-style chambers used for this study were made in Antigonish, Nova Scotia. Figure A.1 is an over-head diagrammatic representation of two hemi-chambers chambers resting on their platform. The three different preparations used in this thesis (opercular epithelia of both the *Fundulus* and *Oreochromis* and urinary bladder of *Oncorhynchus*) were dissected carefully from the animal and pinned down on a lucite adaptor that had an aperture area of 0.125 cm^2. Subsequently, one matching adaptor (no pin-holes) would 'sandwich' the epithelia, and the completed sandwich was tightened into place between the two hemi-chambers.

The lucite adaptors were made using a lathe and a dremel tool was used to make holes for the pins, agar bridges and apertures. Following the making of the adaptors from lucite they were originally rough in appearance and to eliminate macroscopic scratches, a cotton tip (mixed with diatemaceous earth and water) was carefully dremelled over the surface of the lucite adaptors. The adaptors were then fitted with ~3mm stainless steel insect pins (000) (Fine Science Tools, Vancouver, B.C.) using epoxy resin and hardener.

Agar bridges

Agar bridges were constructed from Clay-Adams poly-ethylene cannula: PE 90 for voltage measuring bridges and PE 260 for current passing bridges. The PE tubing was cut to a specified length, and a soldering iron was used to shape the bridges into a

functioning U-shape. The tips of the voltage and current agar bridges were cut to face the direction of the epithelium once in the Ussing chamber. To conduct voltage and current, the agar bridges were filled with 4% agar/Cortland's saline. A mixture of agar/saline/trypan blue was gently stirred in a beaker and brought to a boil for at least 5 minutes. The trypan blue (Sigma, St. Louis, MO) was used as a visual indicator to determine if any bubbles or breaks of agar/saline occurred once injected into the PE tubing. The agar/saline was injected into the bridge, using a well heated 10cc syringe and 21 gauge needle. Between injections the syringe was submerged in a beaker of hot water to ensure the agar/saline would not harden during the procedure.

Correction for junction potentials

Junction potentials are caused when at any one point in a circuit a charge separation (liquid potential difference) occurs. In our case, corrections were made for junction potentials when the agar/saline voltage measuring bridges were immersed in dilute media such as freshwater or artificial urine.

To measure and eventually correct for the junction potential created by the bridge, the following procedure was (Fig. A.2). Initially, two separate beakers were filled with Cortland's saline and then the circuit was completed with one agar/saline bridge (Fig. A.2 A). One beaker contained a Hg/HgCl calomel half-cell and the other beaker contained a 3M KCl free flowing half-cell (see below). Using the DVC-1000 (WPI New Haven, CT) the potential was 'zeroed'. Once the test solution was introduced, the assumption was that there was no change in the composition of the test solution (freshwater, artificial urine) during junction potential measurements, and to ensure this a large volume (1000

ml) of test solution was used. Under well stirred conditions the agar/saline bridge and 3M KCl free flowing half-cell were inserted into the test solution and measurements were recorded on a dual channel chart recorder (Linear Instruments, Reno, NV) (Fig A.2 B).

The 3M KCl free flowing half-cell consisted of a glass microelectrode (no filament, 1 mm diameter; W.P.I. Inc., Sarasota, FL) and a Ag/AgCl electrode. By stretching and heating the middle of the glass microelectrode, an extremely fine tip was made (Narishige, Tokyo, Japan). Using a napkin, the tip of the glass microelectrode was carefully broken to yield an aperture (10-20 μm) which gradually leaks 3M KCl. To fill the glass microelectrode with 3M KCl, the tip of a 1 cc syringe was heated and stretched until the tip was able to insert into the glass microelectrode. The syringe was then used to inject 3M KCl into the glass microelectrode and the Ag/AgCl electrode. All air bubbles were removed from both electrodes. The glass microelectrode was inserted into the Ag/AgCl electrode to form a 3M KCl free flowing half-cell.

"Cold" versus "hot" net fluxes

Measurement of net fluxes of Na^+ and Cl^- by chemical techniques was especially pertinent when the mucosal solution contained low levels of Na^+ and/or Cl^- (e.g. freshwater or artificial urine). This was to determine the "rinse-off effect" of the epithelium over the duration of the flux period. Thus, net radio-labelled measurements tended to differ from the net "cold", chemically made measurements.

"Cold" net fluxes were determined by a subtraction procedure ($[ion]_i$ - $[ion]_f$) and ionic concentrations for Cl^- and Na^+ were measured by colorimetric assay (Zall *et al.*,

1956) and by atomic absorption spectrophotometry (model AA-1275, Varian, Springvale, Australia) respectively. "Hot" net fluxes were determined by subtraction of unidirectional efflux from unidirectional influx ($J_{ion}^{ms} - J_{ion}^{sm}$), measured by the use of ^{22}Na and ^{36}Cl. Radioactivities of ^{36}Cl were determined by counting on a Rackbeta 1217 liquid scintillation counter (LKB, Wallac, Turku, Finland), and ^{22}Na radioactivity was counted on Minaxi Autogamma 5000 counter (Packard Instrument Co., Downers Grove, IL). ^{22}Na emits both gamma and beta radiation, therefore scintillation counts collected from a ^{22}Na and ^{36}Cl dual flux experiment were from both ^{22}Na and ^{36}Cl. ^{36}Cl counts were determined by a count subtraction procedure. This was accomplished by measuring the CPM of a known concentration of ^{22}Na in both the scintillation and gamma counters and then determining the relative efficiency of the two counters for detecting ^{22}Na. The CPM from the gamma counter was then multiplied by this ratio and subtracted from the CPM of the scintillation counter to yield the beta emission of ^{36}Cl only.

Flux equations

Specific activity was determined by:

$$\frac{\Sigma(\text{hot chamber CPM for all samples in period})}{(\#\text{hot samples in period}) \times (\text{hot sample volume (ml)}) \times ([\text{ion}] \text{ hot side (mM)})}$$

Unidirectional flux was determined by:

$$\frac{\{[cCPM' \times (\text{cold vol.(ml)}/\text{cold sam. vol.(ml)})] - cCPM[(\text{cold vol.(ml)} - \text{cold sam. vol.(ml)}/\text{cold sam. vol.(ml)})]\}}{(\text{time of sampling period(min)}) \times (\text{specific activity for period(CPM}/\mu\text{mol)}) \times (\text{aperture area(cm)})}$$

The above equation is multiplied by 60 min./hr to obtain a flux rate in $\mu mol \cdot cm^{-2} \cdot h^{-1}$. CPM is counts per minute (cCPM' = latest cold sample; cCPM = previous sample). Cold vol. represents the chamber volume where the appearance of isotope is being

monitored. Cold and hot sam. vol. denotes samples periodically taken from cold and hot chambers respectively.

Ohm's law

Ohm's law was used to calculate the transepithelial potential (V_t), transepithelial resistance (R_t), conductance ($G_t = R_t^{-1}$) or membrane current (I_m) when two of the three variables were known. Ohm's law is as follows:

$$V_t = R_t \times I_m.$$

Activity measurements

Ionic activity of a given ion is referred to as the concentration of ion available to the membrane for transport and not bound to another molecule (ie protein) or precipitated out of solution. The ionic activities of Na^+ and Ca^{2+} in Cortland's saline and Na^+ in 10% SW were taken from measurements with microelectrodes filled with the appropriate ionophore (Steiner et al., 1979), while Cl^- was predicted to have the same relative activity (on a % basis) as Na^+ from theory for solutions of these ionic strengths (Lee, 1981). The FW and artificial urine ionic activities of Na^+, Cl^- and Ca^{2+} were taken as equal to their measured concentrations because the activity coefficients of these ions are close to one at these lower concentrations.

For Na^+ activity measurements, the electrodes were back-filled using 100 mM NaCl and then front-filled with the Na^+ ionophore (Sodium Ionophore 11-Cocktail A, 71178, Fluka Chemical). The electrodes were calibrated using three different concentrations of solutions (0.1 mM, 1.0 mM and 10.0 mM NaCl).

For Ca^{2+} activity measurements, the electrodes were back-filled using 100 mM $CaCl_2$ and then front-filled with the Ca^{2+} ionophore (Calcium Ionophore I- Cocktail A, 21048, Fluka Chemical). The electrodes were calibrated using three different concentrations of solutions (0.1 mM, 1.0 mM and 10.0 mM NaCl).

Ussing flux ratio criterion

The observed flux ratio (J^{ms}/J^{sm}) was compared to the predicted flux ratio using the Ussing flux ratio equation (Ussing, 1949). Disagreement between the observed and predicted value indicated the presence of non-diffusive transport. The predicted flux ratio equation was as follows:

$$J_i^{ms}/J_i^{sm} = (a_i^m/a_i^s)e^{(z_iFV_t/RT)}.$$

The activities of ion *i* are a_i^s and a_i^m on the serosal and mucosal sides respectively; valency of the ion is z_i, V_t is the transepithelial potential and F, R and T have their usual thermodynamic meanings.

Opercular epithelia dissection

The fish were killed by pithing and the opercular epithelia were immediately dissected carefully from its underlying opercular bone. The fish head was halved using bone-cutting scissors (Fine Science Tools, Vancouver, BC) and each half was gently stretched and pinned down. Pins (Grand and Toy, Toronto, ON) were inserted between the second and third brachio-stegal rays and through the ocular cavity. This way the preparation was held steady as the membrane was carefully teased off the opercular bone. Prior to dissection, mucus was removed from the surface of the opercular epithelium by

carefully pinching and then moving the mucus with the forceps over the surface of the membrane. The mucus was rather viscous and would collect quite readily. A scalpel was used to separate the membrane between the first and second branchio-stegal rays and also along the dorsal-anterior portion of the opercular bone. The first incision of the scalpel cut the pectoral fin muscle thereby enabling the use of opthalmic forceps (Fine Science Tools, Vancouver, BC) to hold onto the edge of the opercular epithelium throughout the dissection. As the membrane was gently teased from the opercular bone using #5 biologie forceps (Fine Science Tools, Vancouver, BC), the portion already removed was being held by the opthalmic forceps. The opercular epithelium was removed in a ventral-anterior to a dorsal-posterior direction and each would yield an approximate area of 1-2 cm^2. Throughout the dissection the preparation was kept moist by the addition of saline.

The Ussing membrane adaptors were prepared with stopcock grease (Dow Corning, Midland, MI) and a thin vinyl mesh was placed over each aperture's opening to support the tissue. The opercular epithelium was carefully pinned down to the adaptor mucosal side up and the second half of the adaptor then 'sandwiched' the epithelium. After mounting, the mucosal surfaces were rinsed with fresh water to remove the saline and any mucus that had accumulated during the dissection. The adaptor 'sandwich' was then placed between the two hemi-chambers and tightened into place.

Urinary bladder dissection

The fish were anaesthetized using 0.1 g MS-222/l FW and the gills were irregated externally with this solution on an operating table. Prior to insertion of the catheter the

urinary papilla was dilated with a blunt probe. To ensure that the catheter would not catch the lumenal border of the urinary bladder once inserted, a flanged PE-tubing (made by heating the tip and pressing it onto a hard surface) was inserted into the bladder and tied to the urinary papilla. The flanged PE-60 was connected to a 21 gauge needle and 1cc syringe filled with Cortland's saline. The PE-tubing was also marked in 1cm increments for reference points. To ensure the catheter passed the two sphincters located posterior to the urinary bladder and not the gonadal duct, the PE-tubing was initially inserted in a downward (vertical) direction. The bladder was also filled with Ringer's (~0.25 ml) to aid in differentiation of its structure from the peritoneum during dissection. A longitudinal cut beginning at the anal opening ended just below the pectoral fins. The large intestine and air bladder were removed, exposing the urinary bladder. Both ureters just anterior to the urinary bladder were ligated with surgical thread and the urinary bladder was separated from surrounding tissues. Connective tissue around the bladder was carefully teased away using blunt forceps working in a posterior direction towards the end of the body cavity without causing damage to the urinary bladder; at this point surgical thread was used to tie the bladder. Following careful removal of remaining fat and connective tissue under the dissecting microscope, the bladder was cut into equal anterior and posterior portions and then both portions were opened by a longitudinal cut with extra fine scissors (Fine Science Tools, North Vancouver, BC). Each portion of the bladder yielded an approximate area of 1-2 cm^2. Throughout the dissection, the preparation was kept moist by the addition of saline. Using a dissection scope, the bladder epithelia were pinned mucosal side up to an adaptor. This adaptor eventually

formed a 'sandwich' with another adaptor and was placed between two hemi-chambers and tightened into place.

Figure A.1 A schematic representation of the Ussing-style chamber (view from above).

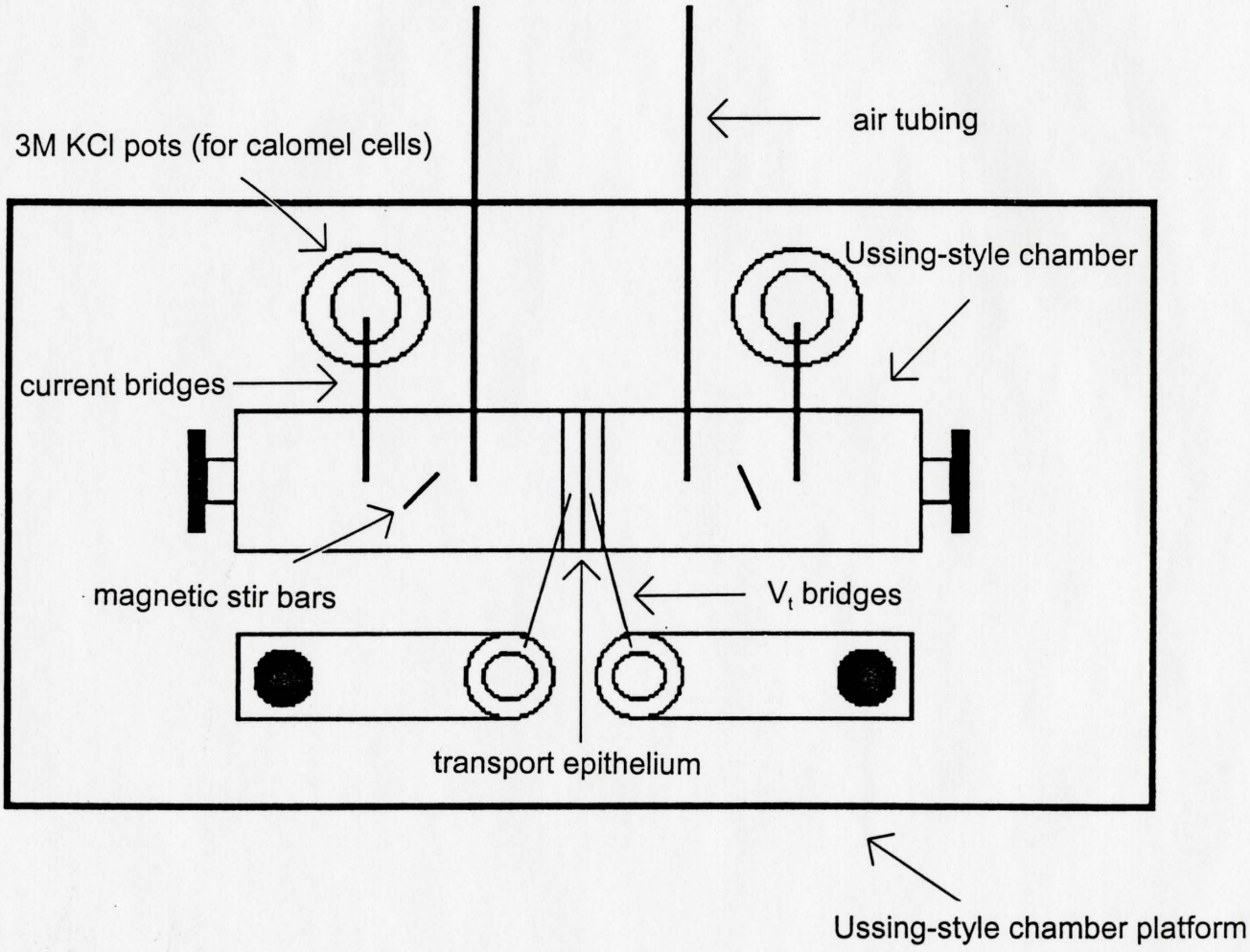
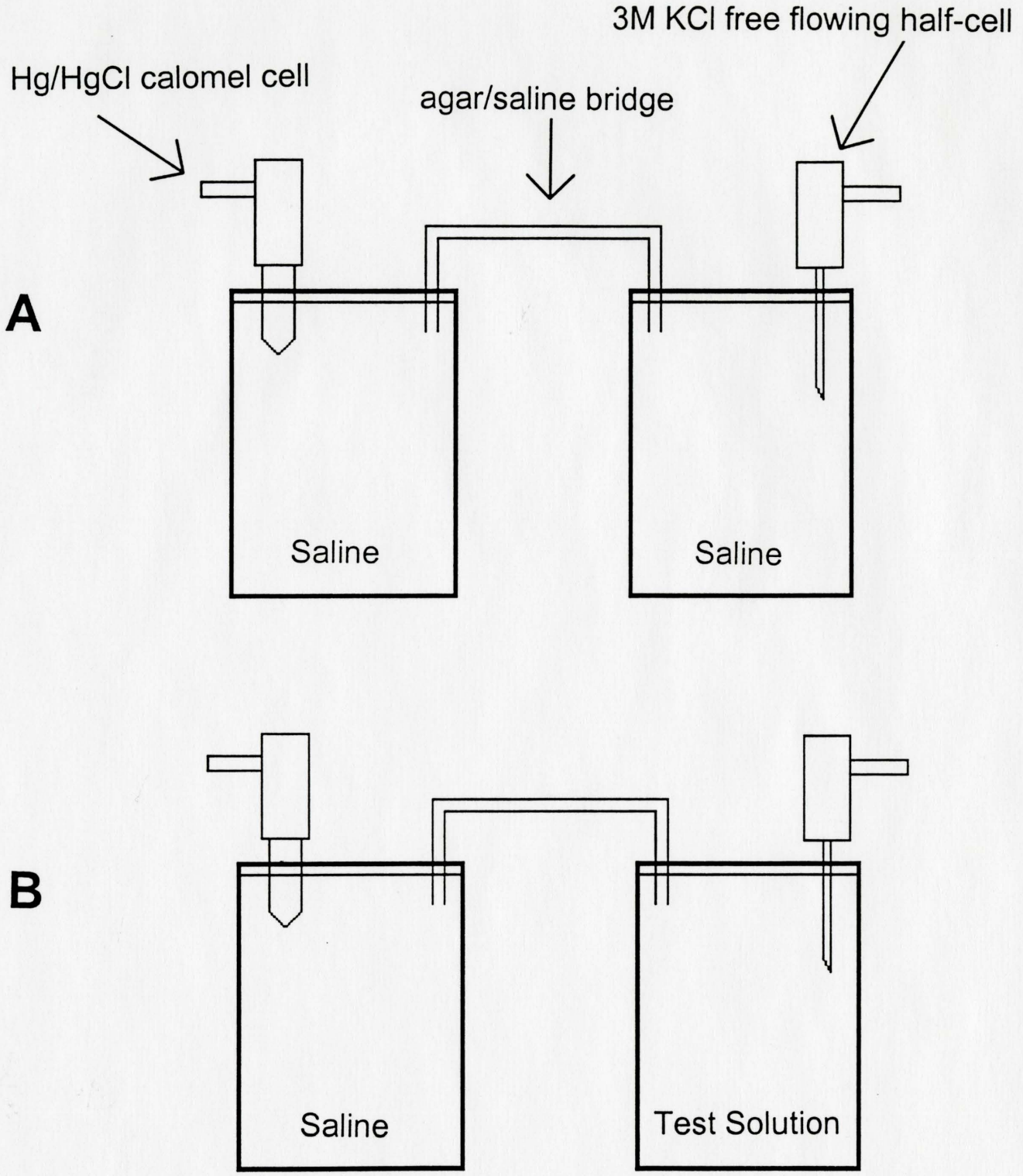

air tubing
3M KCl pots (for calomel cells)
Ussing-style chamber
current bridges
magnetic stir bars
V_t bridges
transport epithelium
Ussing-style chamber platform

Figure A.2 Procedure used in the determination of junction potential corrections. A. Agar/saline bridge is initially zeroed in symmetrical saline. B. The tip of the 3M KCl free flowing half-cell is submerged in the test solution (e.g. freshwater or artificial urine) along with the tip of the saline/agar bridge. The junction potential is then observed on the voltmeter.


3M KCl free flowing half-cell
Hg/HgCl calomel cell
agar/saline bridge
A
Saline
Saline
B
Saline
Test Solution

General References

Bently, P.J. 1992. Some observations on the calcium metabolism of channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol. **102**: 259-261.

Bornancin, M., Derenzis, S., and Maetz, J. 1977. Branchial Cl^- transport, anion stimulated ATPase, and acid-base balance in *Anguilla anguilla* adapted to freshwater: Effects of hyperoxia. J. Comp. Physiol. **117**: 313-322.

Curtis, B.J. 1990. Rainbow trout urinary bladder function *in vivo*. MSc. Thesis, McMaster University, Ontario.

Curtis, B.J., and Wood, C.M. 1991. The function of the urinary bladder *in vivo* in the freshwater rainbow trout. J. Exp. Biol. **155**: 567-583.

Curtis, B.J., and Wood, C.M. 1992. Kidney and urinary bladder responses of freshwater rainbow trout to isosmotic NaCl and $NaHCO_3$ infusion. J. Exp. Biol. **173**: 181-203.

Degnan, K.J., Karnaky, K.J., and Zadunaisky, J.A. 1977. Active chloride transport in the in vitro opercular skin of a teleost (*Fundulus heteroclitus*), a gill-like epithelium rich in chloride cells. J. Physiol. Lond. **271**: 155-191.

Degnan, K.J., and Zadunaisky, J.A. 1979. Open-circuit Na^+ and Cl^- fluxes across isolated opercular epithelium from the teleost *Fundulus heteroclitus*. J. Physiol. Lond.

294: 483-495.

Degnan, K.J., and Zadunaisky, J.A. 1980. Ionic contributions to the potential and current across the opercular epithelium. Am. J. Physiol. **238**: R231-R239.

Demarest, J.R. 1984. Ion and water transport by the flounder urinary bladder: salinity dependence. Am. J. Physiol. **246**: F395-F401.

Elger, B., and Hentschel, H. 1983. Effects of adrenergic blockade with bretylium and phentolamine on glomerular filtration rate in the rainbow trout, *Salmo gairdneri* Rich., adapting to saline water. Comp. Biochem. Physiol. **75**: 253-258.

Elger, B., Neukirch, M., and Hentschel, H. 1986. The kidney of rainbow trout, *Salmo gairdneri* Richardson, in the acute phase of viral haemorrhagic septicaemia: *in vivo* experiments on the renal excretion of fluid, electrolytes and protein. J. Fish. Dis. **9**: 381-392.

Erickson, D.A., and Gingerich, W.H. 1986. Effect of injected rotenone on the production and composition of urine from the rainbow trout (*Salmo gairdneri*). Aquatic Tox. **9**: 263-274.

Ernst, S.A., Dodson, W.C., and Karnaky, K.J. 1980. Structural diversity of occluding

junctions in the low resistance chloride-secreting opercular epithelium of seawater-adapted killifish (*Fundulus heteroclitus*). J. Cell Biol. **87**: 488-497.

Evans, D.H., Claiborne, L., Farmer, L., Mallery, C., and Krasny, E. 1982. Fish gill ionic transport: Methods and models. Biol. Bul. **163**: 108-130.

Flik, G., Van Rijs, J.H., Wendelaar Bonga, S.E. 1985. Evidence for high affinity Ca^{2+}-ATPase activity and ATP-driven Ca^{2+}-transport in membrane preparations of the gill epithelium of cichlid fish *Oreochromis mossambicus*. J. Exp. Biol. **119**: 335-347.

Flik, G., Fenwick, J.C., Kolar, Z., Mayer-Gostan, N., and Wendelaar Bonga, S.E. 1986. Whole body calcium flux rates in the cichlid teleost fish *Oreochromis mossambicus* adapted to freshwater. Am. J. Physiol. **249**: R432-R437.

Flik, G., and Perry, S.F. 1989. Cortisol stimulates whole body calcium uptake and the branchial calcium pump in freshwater rainbow trout. J. Endocrinol. **120**: 75-82.

Flik, G., Van Der Veldon, J.A., Dechering, K.J., Verbost, P.M., Schoenmakers, J.M., Kolar, Z.I., and Wendelaar Bonga, S.E. 1993. Ca^{2+} and Mg^{2+} transport in gills and gut of tilapia, *Oreochromis mossambicus*: A review. J. Exp. Zool. **265**: 356-365.

Foskett, J.K., Logsdon, 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.

Foskett, J.K., and Scheffey, C. 1982. The chloride cell: definitive identification as the salt-secretory cell in teleosts. Science. **215**: 164-166.

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., and Machen, T.E. 1985. Vibrating probe analysis of teleost opercular epithelium: Correlation between active transport and leak pathways of individual chloride cells. J. Mem. Biol. **85**: 23-35.

Fossat, B., Lahlou, B., and Bornancin, M. 1974. Involvement of a Na-K-ATPase in sodium transport by fish urinary bladder. Experientia. **30**: 376-377.

Fossat, B., and Lahlou, B. 1977. Osmotic and solute permeabilities of isolated urinary bladder of the trout. Am. J. Physiol. **233**: F525-F531.

Fossat, B., and Lahlou, B. 1979a. The mechanism of coupled transport of sodium and chloride in isolated urinary bladder of the trout. J. Physiol., Lond. **294**: 211-222.

Fossat, B., and Lahlou, B. 1979b. Failure of 2,4,6-triaminopyrimidine (TAP) to block sodium pathways in a 'leaky' epithelium. Pflugers Arch. **379**: 287-290.

Fossat, B., and Lahlou, B. 1982. Ion flux changes introduced by voltage clamping or by amphotericin B in the isolated urinary bladder of the trout. J. Physiol., Lond. **325**: 111-123.

Frizzell, R.A., Dugas, M.C., and Shultz, S.G. 1975. Sodium chloride transport by rabbit gall-bladder. Direct evidence for a coupled NaCl influx process. J. Gen. Physiol. **65**: 769-795.

Gerzeli, G., De Piceis Polver, P., and Cucchi, M.L. 1973. Ricerche istofunzionali sulla vesica urinaria di alcuni teleostei. Arch. Biol. (Bruxelles). **84**: 1-17.

Goss, G.G., Perry, S.F., Wood, C.M., and Laurent, P.L. 1992. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. J. Exp. Biol. **106**: 143-159.

Hirano, T., Johnson, D.W., Bern, H.A., and Utida, S. 1973. Studies on water movements in the isolated urinary bladder of selected freshwater, marine and euryhaline teleosts. Comp. Biochem. Physiol. **45A**: 529-540.

Hoffman, E.L., and Butler, D.G. 1979. The effect of increased metabolic rate on renal function in the rainbow trout, *Salmo gairdneri*. J. Exp. Biol. **82**: 11-23.

Hogstrand, C., Wilson, R.W., Polgar, D., and Wood, C.M. 1994. Effects of zinc on the

kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. J. Exp. Biol. **186**: 55-73

Holmes, W.N., and Stainer, I.M. 1966. Studies on the renal excretion of electrolytes by the trout (*Salmo gairdneri*). J. Exp. Biol. **44**: 33-46.

Hunn, J.B. 1969. Chemical composition of rainbow trout urine following acute hypoxic stress. Trans. Am. Fish. Soc. **98**: 20-22.

Hunn, J.B., and Willford, W.A. 1970. The effect of anaesthetization and urinary bladder catheterization on renal function of rainbow trout. Comp. Biochem. Physiol. **33**: 805-812.

Jürss, K., and Bastrop, R. 1995. The function of mitochondria-rich cells (chloride cells) in teleost gills. Rev. Fish Biol. Fish. **5**: 235-255.

Kamalaveni, S. 1961. The urinary bladder of some Indian teleostean fishes. Rec. Indian. Mus. **59**: 119-130.

Karnaky, K.J., and Kinter, W.B. 1977. Killifish opercular skin: A flat epithelium with a high density of chloride cells. J. Exp. Biol. **199**: 355-364.

Karnaky, K.J., Degnan, K.J., and Zadunaisky, J.A. 1977. Chloride transport across

isolated opercular epithelium of killifish: A membrane rich in chloride cells. Science. **195**: 203-205.

Karnaky, K.J. 1980. Ion-secreting epithelia: chloride cells in the head region of *Fundulus heteroclitus*. Am. J. Physiol. **238**: R185-198.

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

Karnaky, K.J. 1991. Teleost osmoregulation: changes in the tight junction in response to the salinity of the environment. *In* The Tight Junction. *Edited by* M. Cereijido. CRC Press, Boca Raton. pp. 175-185.

Kerstetter, T.H., Kirschner, L.B., Rafuse, D.D. 1970. On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout *Salmo gairdneri*. J. Gen. Physiol. **56**: 342-359.

Kültz, D., and Onken, H. 1993. Long-term acclimation of the teleost *Oreochromis mossambicus* to various salinities: two different strategies in mastering hypertonic stress. Mar. Biol. **117**: 527-533.

Lacy, E.R. 1983. Histochemical and biochemical studies of carbonic anhydrase activity in the opercular epithelium of the euryhaline teleost *Fundulus heteroclitus*. Am. J.

Anat. **166**: 19-39.

Lahlou, B., and Fossat, B. 1971. Mécanisme du transport de l'eau et du sel à travers la vessie urinaire d'un poisson téléostéen en eau douce, la truite arc-en-ciel. C.R. hebd. Séanc Acad. Sci., Paris. **273**: 2108-2110.

Lahlou, B., and Fossat, B. 1984 Transport properties of fish urinary bladders in relation to osmoregulation. *In* Osmoregulation in Estuarine and Marine Animals. *Edited by* A. Pequex, R. Gilles and L. Bolis. Springer Verlag, Berlin. pp. 93-106.

Lee, C.O. 1981. Ionic activities in cardiac muscle cells and application of ion-selective microelectrodes. Am. J. Physiol. **241**: H459-H478.

Lin, H., and Randall, D. 1991. Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. J. Exp. Biol. **161**: 119-134.

Loretz, C.A., and Bern, H.A. 1980. Ion transport by the urinary bladder of the gobiid teleost, *Gillichthys mirabilis*. Am. J. Physiol. **239**: R415-R423.

Maetz, J., Mayer, N., and Chartier-Baraduc, M.M. 1967a. La balance minérale du sodium chez *Anguilla anguilla*en eau de mer, en eau douce et au cours de transfert d'un milieu à l'autre: Effets de l'hypophysectomie et de la prolactine. Gen. Comp. Endocrinol. **8**: 177-188.

Maetz, J., Sawyer, W.H., Pickford, G.E., and Mayer, N. 1967b. Evolution de la balance minérale du sodium chez *Fundulus heteroclitus* au cours du transfert d'eau de mer d'eau douce: Effets du hypophysectomie et de la prolactine. Gen. Comp. Endocrinol. **8**: 177-188.

Marshall, W.S. 1977. Transepithelial potential and short-circuit current across the isolated skin of *Gillichthys mirabilis* (Teleostei: Gobiidae) acclimated to 5% and 100% seawater. J. Comp. Physiol. **114**: 157-165.

Marshall, W.S., and Nishioka, R.S. 1980. Relation of mitochondria-rich chloride cells to active chloride transport in the skin of a marine teleost. J. Exp. Zool. **214**: 147-156.

Marshall, W.S. 1981. Sodium dependency of active chloride transport across isolated fish skin (*Gillichthys mirabilis*). J. Physiol. Lond. **319**: 165-178.

Marshall, W.S. 1986. Independent Na^+ and Cl^- active transport by the urinary bladder epithelium of the brook trout. Am. J. Physiol. **250**: R227-R234.

Marshall, W.S. 1988. Passive solute and fluid transport in brook trout (*Salvelinus fontinalis*) urinary bladder epithelium. Can. J. Zool. **66**: 912-918.

Marshall, W.S., and Bryson, S.E. 1991. Intracellular pH regulation in trout urinary bladder epithelium: Na^+-H^+(NH_4^+) exchange. Am. J. Physiol. **261**: R652-R658.

Marshall, W.S., Bryson, S.E., and Wood, C.M. 1992. Calcium transport by isolated skin of rainbow trout. J. Exp. Biol. **166**: 297-316.

Marshall, W.S. 1995. Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. *In* Cellular and Molecular Approaches to Fish Ionic Regulation. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, London. pp. 1-23.

Marshall, W.S., Bryson, S.E., Burghardt, J.S., and Verbost, P.M. 1995. Ca^{2+} transport by opercular epithelium of the freshwater adapted euryhaline teleost, *Fundulus heteroclitus*. J. Comp. Physiol. B. **165**: 268-277.

Marshall, W.S., Bryson, S.E., Darling, P., Whitten, C., Patrick, M., Wilkie, M., Wood, C.M., and Buckland-Nicks, J. 1997. NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. J. Exp. Zool. **277**: 23-37.

McCormick, S.D., Hasegawa, S., Hirano, T. 1992. Calcium uptake in the skin of a freshwater teleost. Proc. Nat. Acad. Sci. U.S.A. **89**: 3635-3638.

McCormick, S.D. 1995. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. *In* Cellular and Molecular Approaches to Fish Ionic Regulation. *Edited by* C.M. Wood and T.J. Shuttleworth. Academic Press, London. pp. 285-315.

McDonald, D.G., Tang, Y., and Boutilier, R.G. 1989. Acid and ion transfer across the gills of fish: Mechanisms and regulation. Can. J. Zool. **67**: 3046-3054.

Motais, R., Garcia-Romeu, F., and Maetz, J. 1966. Exchange diffusion effect and euryhalinity in teleosts. J. Gen. Physiol. **50**: 391-442.

Nagahama, Y., Bern, H.A., Doneen, B.A., and Nishioka, R.S. 1975. Cellular differentiation in the urinary bladder of a euryhaline marine fish, *Gillichthys mirabilis*, in response to environmental salinity change. Dev. Growth. Differ. **17**: 367-381.

Nellans, H.N., Frizzell, R.A., and Schultz, S.G. 1973. Coupled sodium chloride influx across the brush border of rabbit ileum. Am. J. Physiol. **225**: 467-475.

Nellans, H.N., Frizzell, R.A., and Schultz, S.G. 1974. Brush border processes and transepithelial Na and Cl transport by rabbit ileum. Am. J. Physiol. **226**: 1131-1141.

Nishimura, H., Imai, M., and Ogawa, M. 1983. Sodium chloride and water transport in the renal distal tubule of the rainbow trout. Am. J. Physiol. **244**: F247-F254.

Oikari, A.O.J., and Rankin, J.C. 1985. Renal excretion of magnesium in a freshwater teleost, *Salmo gairdneri*. J. Exp. Biol. **117**: 319-333.

Perry, S.F., Haswell, M.S., Randall, D.J., Farrell, A.P. 1981. Branchial ionic uptake and acid-base regulation in the rainbow trout, *Salmo gairdneri*. J. Exp. Biol. **92**: 289-303.

Perry, S.F., and Wood, C.M. 1985. Kinetics of branchial calcium uptake in the rainbow trout: Effects of acclimation to various external calcium levels. J. Exp. Biol. **116**: 411-433.

Perry, S.F. 1997. The chloride cell: structure and function in the gills of freshwater fishes. Annu. Rev. Physiol. **59**: 325-347.

Pisam, M., Caroff, A., and Rambourg, A. 1987. Two types of chloride cells in the gill epithelium of a freshwater-adapted euryhaline fish: *Lebistes reticulatus*; their modifications during adaptation to saltwater. Am. J. Anat. **179**: 40-50.

Pisam, M., Prunet, P., Boeuf, F., and Rambourg, A. 1988. Ultrastructural features of chloride cells in the gill epithelium of the Atlantic salmon, *Salmo salar*, and their modifications during smoltification. Am. J. Anat. **183**: 235-244.

Pisam, M., Boeuf, F., Prunet, P., and Rambourg, A. 1990. Ultrastructural features of mitochondria-rich cells in stenohaline freshwater and seawater fishes. Am. J. Anat.

187: 21-31.

Pisam, M., and Rambourg, A. 1991. Mitochondria-rich cells in the gill epithelium of teleost fishes: an ultrastructural approach. Int. Rev. Cytol. **130**: 191-232.

Pisam, M., Auperin, B., Prunet, P., Rentier-Delrue, F., Martial, J., and Rambourg, A. 1993. Effects of prolactin on α and β chloride cells in the gill epithelium of the saltwater adapted tilapia "*Oreochromis niloticus*". Anat. Rec. **235**: 275-284.

Potts, W.T.W., and Evans, D.H. 1967. Sodium and chloride balance in the killifish *Fundulus heteroclitus*. Biol. Bul. **133**: 411-425.

Potts, W.T.W. 1984. Trans-epithelial potentials in fish gills. *In* Fish Physiology. Vol. **10B**. *Edited by* W.S. Hoar and D.J. Randall. Academic Press, Orlando, Florida. pp. 105-128.

Renfro, J.L. 1977. Interdenpendence of active Na^+ and Cl^- transport of the isolated urinary bladder of the teleost, *Pseudopleuronectes americanus*. J. Exp. Zool. **199**: 383-390.

Scheffey, C., Foskett, J.K., and Machen, T.E. 1983. Localization of ionic pathways in the teleost opercular membrane by extracellular recording with a vibrating probe. J. Membrane. Biol. **75**: 193-203.

Silva, P., Solomon, R., Spokes, K., and Epstein, F.H. 1977. Ouabain inhibition of gill Na^+-K^+-ATPase: Relationship to active chloride transport. J. Exp. Zool. **199**: 419-426.

Smith, N.F., Talbot, C., and Eddy, F.B. 1989. Dietary salt intake and its relevance to ionic regulation in freshwater salmonids. J. Fish Biol. **35**: 749-753.

Steiner, R.A., Oehme, M., Ammann, D., and Simon, W. 1979. Neutral carrier ion selective microelectrodes for intracellular studies. Analyt. Chem. **51**: 351-353.

Ussing, H.H. 1949. The distinction by means of tracers between active transport and diffusion. Acta. Physiol. Scand. **19**: 43-56.

Verbost, P.M., Flik, G., Lock, R.A.C., and Wendelaar Bonga, S.E. 1987. Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. Am. J. Physiol. **253**: R216-R221.

Verbost, P.M., Van Rooij, J., Flik, G., Lock, R.A.C., and Wendelaar Bonga, S.E. 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. J. Exp. Biol. **145**: 185-197.

Verbost, P.M., Flik, G., Fenwick, J.C., Greco, A., Pang, P.K.T., and Wendelaar Bonga, S.E. 1993. Branchial calcium uptake: possible mechanisms of control by

stanniocalcin. Fish Physiol. Biochem. **11**: 1-6.

Wheatley, M.G., Hobe, H., and Wood, C.M. 1984. The mechanisms of acid-base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. II. The role of the kidney. Resp. Physiol. **55**: 155-173.

Wood, C.M. 1988. Acid-base and ionic exchange at gills and kidney after exhaustive exercise in the rainbow trout. J. Exp. Biol. **136**: 461-481.

Wood, C.M. 1991. Branchial ion and acid-base transfer in freshwater teleost fish: Environmental hyperoxia as a probe. Physiol. Zool. **64**: 68-102.

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.

Wood, C.M., Gilmour, K.M., and Pärt, P. 1997. Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A. In press.

Wood, C.M., and Pärt, P. 1997. Cultured branchial epithelia from freshwater fish gills. J. Exp. Biol. **200**: 1047-1059.

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

Zall, D.M., Fisher, M.D., and Garner, Q.M. 1956. Photometric determination of chlorides in water. Analyt. Chem. **28**: 1665-1678.