IONOREGULATION OF FRESHWATER-ADAPTED *FUNDULUS HETEROCLITUS*

THE IONOREGULATORY PHYSIOLOGY

OF

FRESHWATER-ADAPTED MUMMICHOG

(*FUNDULUS HETEROCLITUS*)

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1994) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario

TITLE: The Ionoregulatory Physiology of Freshwater-Adapted Mummichog (*Fundulus heteroclitus*)

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SUPERVISOR: Dr. C.M. Wood

NUMBER OF PAGES: xii, 219

ABSTRACT

This thesis examined the ionoregulatory physiology of freshwater-adapted mummichog (*Fundulus heteroclitus) in vivo* and its interaction with acid-base regulation. Under control conditions (water [NaCl] ≈ 0.7-1.0 $mmol \cdot L^{-1}$, $[Ca^{2+}] \approx$ 0.1 or 1.0 $mmol \cdot L^{-1}$), Na^+ turnover was vigorous with a positive Na^+ balance maintained, whereas unidirectional Cl^- influx was virtually zero resulting in a slight negative Cl^- balance. Michaelis-Menten analysis revealed a low affinity, high capacity Na^+ uptake mechanism that was independent of both Na^+ efflux and ammonia excretion. Cl^- uptake started at higher water [NaCl] levels (> 2 $mmol \cdot L^{-1}$) but did not saturate within the freshwater [NaCl] range, indicating a completely different uptake mechanism, independent from Na^+ influx. Using both internal and environmental manipulation, Na^+ uptake was found <u>not</u> to be coupled to ammonia excretion. Instead, a link with acid excretion (Na^+/H^+ exchange or Na^+ channel/H^+-ATPase coupling) remains possible but could not be confirmed. There was no evidence for the presence of a Cl^-/HCO_3^- exchange mechanism in the gills. However, mummichog were capable of differentially manipulating Na^+ and Cl^- efflux components as an additional response to an internal acid-base disturbance. This ability and the suggested Na^+ uptake/acid excretion coupling indicate that mummichog resemble other

freshwater fish in that an iono/acid-base relationship exists. In these studies, the use of the Strong Ion Difference Theory as a means of assessing acid-base balance through the measurement of differential Na^+ and Cl^- fluxes proved to be acceptable and practical alternative to the measurement of acid-base fluxes by traditional titration methodology. Finally, whole-body Ca^{2+} uptake was investigated using a recently developed technique for small fish. Ca^{2+} uptake by the mummichog involves a carrier-mediated step as revealed by saturation of uptake as external [Ca^{2+}] increased. Inhibition of uptake by external La^{3+} but not Mg^{2+} suggested that apical Ca^{2+} channels are involved in the uptake process but are not voltage-gated. Chronic exposure to low Ca^{2+} water resulted in a stimulated Ca^{2+} uptake, most likely in response to depletion of internal Ca^{2+} levels whereas chronic exposure to high Ca^{2+} did not elicit any changes in uptake.

This thesis revealed that the freshwater-adapted mummichog does share certain ionoregulatory qualities with other freshwater fish but at the same time possesses unique characteristics which may reflect its euryhaline nature.

ACKNOWLEDGEMENTS

I CAN'T BELIEVE I MADE IT!!!

First and foremost, I would like to express my fortune in having Chris Wood as a mentor. I am indebted to Chris for his constant support and encouragement throughout this roller coaster ride called graduate school. Not only does he possess a vast wealth of knowledge in the field of fish physiology (and beyond), but his unwavering drive to push his body to its limit is also highly commendable. May there be many more pints raised together in the name of research and good friends. I thank you.

Next I would like to thank the many members of the Wood-McDonald conglomerate starting with Gord McDonald for the continous use of his lab and equipment and also his input during my research. At this point, I will mention that ABBA will be in my memory for all time. Moving right along, I would like to thank Mike Wilkie (that's Dr. now) for the many, many stimulating conversations which at times, did have scientific merit. Thanks Mikie for the help with the SEM work and we got a publication to boot (?). I am grateful to several Woodsters for many fond memories that I will carry with me: Dr. Rod "G&T's" Wilson, Yuxiang "big skinny" Wang, Steve "#$%*" Munger, Tyler "hillbilly" Linton, Dr. Scott "don't make me laugh" Reid, Dr. Peter Pärt(y), Dr. Christer "soon-to-be-hillbilly" Hogstrand, Jacqui "poopy" Dockray, Dr. Annika Salamaa (I won't touch this one), Rod "Chewbacka MacGyver" Rhem, Russ "I'm too sexy for myself" Ellis, and Emma "Marj that's really gross" Postlethwaite and all the others that have passed through room 203 and 202. Thanks to Randy Lauff for caring for my mummies while I was away and also making graduate life just a little more bearable (excluding one of the 5 senses!).

For the calcium aspect of my thesis, I want to thank Dr. Bill Marshall and Sharon Bryson for the use of their facilities during my research at St. Francis Xavier University and allowing us (Mikie and I) to contribute to one of the mummichog manuscripts. Together, I think we will help others to appreciate our friend, the mummi.

I would also like to thank my third committee member Dr. Mike O'Donnell for his helpful comments and his neverending tolerance to flux figures! Some day, I'm quite confident you'll come to appreciate them.

Now, the accolades for the non-participants. I want to thank my Mom and Dad and the rest of my immediate family for just being there and trying to understand why someone would want to go through so much anxiety over some little fish!

Finally, a big, BIG thanks to my husband, Dr. Rick Gonzalez for his love, patience, support, and encouragement throughout my entire thesis. Without those wonderful getaways to Chicago and San Diego, I would not have maintained my sanity during the past 3 years. Without you Pook, I would not have survived!

This research was supported by an NSERC grant to CMW.
This thesis was made possible by Tim Horton's Canada.

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CHAPTER 1

General Introduction

Natural History of Fundulus heteroclitus

The Cyprindontidae are distributed world wide, with the exception of the Australian continent, and comprise 45 genera and 300 species. The greatest speciation has occurred within the southeastern region of North America. The mummichog or common killifish *Fundulus heteroclitus* ranges from northeastern Florida to the Gulf of the St. Lawrence and can be located in regions where the salinity fluctuates rapidly. The two subspecies of *Fundulus heteroclitus*, *macrolepidotus* and *heteroclitus* can be readily categorized as northern and southern subspecies respectively as the border between the two approximates 40 °N, near the coast of New Jersey (Scott & Crossman, 1973).

F. heteroclitus inhabits estuaries and salt marshes along the Atlantic coast where water salinity ranges from brackish to more than twice full strength sea water. Populations of mummichogs residing in fresh water have also been documented in Digby Neck, Nova Scotia (Klawe, 1957), Bronx river, New York (Samaritan & Schmidt, 1982) and the Susquehanna river, Pennsylvania (Denoncourt *et al.* 1978). Some populations were introduced by bait bucket (Samaritan & Schmidt, 1982), others through natural migration (Denoncourt *et al.* 1978). These reports suggest that the mummichog can readily adapt to a wide range of water qualities, with Griffith (1974) providing evidence that in fact

this species of *Fundulus* can survive salinities of 0‰ (*i.e.* freshwater) to 120 ‰ (almost 4-fold normal strength sea water). In addition, mummichogs can tolerate daily abrupt transfers from full strength sea water to freshwater and vice versa with incredible ease as indicated by a lack of distress symptoms and unchanged appetite (Griffith, 1972). This tolerance has been determined to exist in all life stages as both embryos and newly hatched killifish survive indefinitely in a wide range of salinities and osmotic pressures (Atz, 1986).

Griffith (1972) attempted to determine salinity preference of several *Fundulus* species including the mummichog. Using a Salinity Preference Index (SPI), based on frequency of occurrence of the fish in 3 salinities (FW, 50% SW, and SW), the results did not demonstrate a strong preference but suggested a brackish water choice. Fritz & Garside (1974) determined that the mummichog shows a modest preference for intermediate salinity, approximately 20‰, regardless of prior acclimation. It was noted in this paper that 20‰ salinity is the value for maximal hatching for mummichog, and perhaps this salinity choice by adults is a means to ensure reproductive success.

Salinity preference may be governed by the need to minimize energy requirements. Residing in isosmotic water would reduce the need to either actively secrete or take up vital electrolytes such as Na^+ and Cl^-, thus diminishing the energy demand for ion balance. However, both Griffith (1972) and Touchton & Bulger (1980) found no correlation between O_2

and salinity in the mummichog as there was no difference amongst freshwater, isosmotic and seawater-adapted animals.

Reproduction occurs within the intertidal zone, usually during night high tide, with fertilized eggs placed in empty mussel shells or on the marsh grass *Spartina alterniflora*. During subsequent low tide and day high tide (which is lower than night), the leaves and stems of the marsh grass and mummichog eggs become stranded above the water level (Di Michele & Taylor, 1980; Taylor *et al.* 1977; Kneib, 1986). This phenomena may be advantageous in improving survival as it would reduce predation during the 7-9 day incubation period. Hatching of mummichog eggs is dependent upon low dissolved oxygen concentration and submergence in water, irrespective of salinity (Di Michele & Taylor, 1980). This lack of salinity requirement would allow *F. heteroclitus* to reproduce and establish populations in fresh water. No research has been published revealing the reproductive behaviour in freshwater though landlocked populations do exist as mentioned above.

Mummichogs are quite abundant and play important roles as both predator and prey within salt marsh ecosystems, making them important in the trophic structure. If either population density or predation pressure were to increase, this may force this opportunistic teleost to search out alternate habitat without salinity being a restriction. Perhaps reports of mummichogs in fresh water tributaries some distance from the coast present cases of such pressures (Denoncourt *et al.* 1978).

The basis for the geographic distribution of *F. heteroclitus* is likely a combination of many factors, including the ones mentioned above. *F. heteroclitus* readily adapts to a wide range of environments and this plasticity is evident throughout every life stage.

Control of Osmoregulation

The ability of *F. heteroclitus* to inhabit estuaries where salinity changes are rapid and daily, and the establishment of populations in freshwater and hypersaline environments has made the killifish a focus for studies on osmoregulatory mechanisms. The first investigations of the possible role of pituitary influence on ion balance were those of Burden (1956) and Pickford & Phillips (1959). Hypophysectomized killifish were unable to survive in fresh water but survived indefinitely in sea water. The exact cause of death in fresh water was unclear but a correlation between progressive asthenia (lack of energy) and hypochloremia was noted (low plasma Cl^-) (Pickford & Phillips, 1959). Serum sodium levels were reduced to the same extent, accompanied by a large sodium loss to the water. Radiotracer-based measurements of unidirectional sodium fluxes revealed that hypophysectomized SW-adapted *F. heteroclitus* placed in fresh water showed elevated sodium effluxes with no change in uptake (Potts & Evans, 1966), or a slight reduction in uptake (Maetz *et al.* 1967*a*). This suggests that the pituitary must regulate permeability when mummichogs enter fresh water. Burden (1956) was the first to inject *Fundulus*

pituitary brei into FW hypophysectomized killifish and reported that it allowed the mummichogs to survive indefinitely.

Pickford and Phillips (1959), by the process of elimination, discovered that the hormone prolactin was necessary for the survival of hypophysectomized mummichogs in fresh water. Ovine prolactin (Pickford & Phillips, 1959; Pickford & Pang, 1966; Pickford *et al.* 1970; Potts, 1970; Griffith, 1972) as well as bovine prolactin (Potts & Evans, 1966) were commonly employed in early studies. In the past 10 years, prolactin from several teleost species such as chum and chinook salmon have been isolated and purified allowing researchers to use teleost prolactin (Grau *et al.* 1984; Hasegawa *et al.* 1986). *Fundulus* prolactin has yet to be isolated, but injection of chum salmon prolactin into *F. heteroclitus*, demonstrated an 100 times greater sodium-retaining activity in comparison to mammalian preparations (Hasegawa *et al.* 1986).

The extensive studies involving hypophysectomy and prolactin therapy have revealed that *F. heteroclitus* is strongly dependent upon prolactin to maintain serum ion levels while in fresh water. A balance is achieved by reducing passive ion loss but prolactin therapy does not correct the reduced ion uptake caused by the hypophysectomy (Maetz *et al.* 1967*a*). Mummichogs acclimated to 50% sea water and injected with prolactin developed hypernatremia confirming that sodium efflux is reduced which in this case, impairs ionoregulation (Hasegawa *et al.* 1986).

Other euryhaline teleosts such as the European eel, *Anguilla anguilla*

(Maetz *et al.* 1967*b*) and tilapia, *Oreochromis mossambicus* (Dharmamba & Maetz, 1972) have demonstrated the same prolactin requirement as the *F. heteroclitus*. Prolactin appears to be a freshwater hormone which certain species require in order to osmoregulate effectively in dilute media.

The function of prolactin suggested originally was to increase the mucous cell population, thereby increasing mucus production which could reduce ion loss at the gill by thickening the non-stirred layer (Potts & Evans, 1966). Burden (1956) observed mucous cells in freshwater-adapted mummichog, including both intact and hypophysectomized fish which had been injected with pituitary brei. Mucous cells were most numerous on the intact fresh water gills with hypophysectomized fish possessing far fewer mucous cells which appeared emptied and atrophied. This condition seemed to be reversed with pituitary brei.

The second important finding was the effect of prolactin on branchial and renal Na^+/K^--ATPase. This enzyme's activity was measured in hypophysectomized *F. heteroclitus*, with and without prolactin therapy. With prolactin injection, branchial ATPase activity was greatly curtailed while kidney activity was stimulated. This would likely reduce sodium loss at the gill (*i.e.* reduce active secretion) and increase reabsorption in the kidney thereby minimizing sodium loss overall (Epstein *et al.* 1969; Pickford *et al.* 1970).

The role of other pituitary hormones in fresh water survival have been investigated in both *F. heteroclitus* and *F. kansae*, again with variable

responses. Injections of ACTH produced variable responses, and in general, did not augment Na^+ retention in hypophysectomized *F. kansae* (Stanley & Fleming, 1967). ACTH also failed to improve survival of hypophysectomized *F. heteroclitus* in fresh water (Burden, 1956; Pickford & Phillips, 1959). Tests with other pituitary hormones (TSH, GH, and posterior lobe preparations), and products of the adrenal (cortisol, aldosterone) suggested that these were not required for fresh water survival of *F. heteroclitus.* However they may be required while inhabiting sea water (Pickford & Phillips, 1959). The only other candidate considered is the corpuscles of Stannius (CS). CS are responsible for the release of a hypocalcemic substance (*i.e.* stanniocalcin) which regulates calcium metabolism in teleosts as reviewed by Pang & Pang (1986). Interestingly, hypophysectomized mummichogs became hypocalcemic and subsequent injections of pituitary homogenates corrected this, which suggests that CS may be regulated by the pituitary (Pang, 1981). Whether or not CS influence sodium and chloride balance is yet to be determined.

Ionoregulation in various salinities

Whether in sea water, freshwater, or salinities in between, teleosts are faced with ionoregulatory challenges. Serum electrolyte measurements in *F. heteroclitus* have shown that Na^+ and Cl^- are maintained at approximately the same concentration regardless of salinity, in fully acclimated animals, demonstrating the flexibility of the ionoregulatory mechanisms of the

mummichog (Burden, 1956; Epstein *et al.* 1969; Pickford *et al.* 1970). There are discrepancies between studies on this point as some have found body Na^+ and Cl^- levels much higher in SW killifish, but this can be attributed to the high salt content of the gut (Potts & Evans, 1967).

In sea water, there is an inward diffusion of ions and a continuous loss of water, both of which are compensated by active excretion of ions and drinking sea water. This drinking of seawater induces an additional large NaCl load to the body fluids. The situation is reversed in fresh water as body fluids are hyperosmotic to the medium resulting in ion loss and water gain, even though drinking has halted (Bergeron, 1956). Ions must be actively taken up from the dilute medium at an equal or greater rate in order to alleviate loss and maintain electrolyte levels. In both scenarios, fish must expend energy to attain rates of removal or uptake that balance or exceed the unfavourable passive movement of each ion. In addition, body permeability can be manipulated, thereby reducing energy requirements for active ion transport. For instance, freshwater-adapted *F. heteroclitus* exhibit reduced body permeability which attenuates ion efflux and water gain. This adaptation is essential for fresh water survival; otherwise ion losses would be 10 times greater than the losses actually measured in the freshwater-adapted mummichog (Potts & Evans, 1967).

Sea water to fresh water transfer

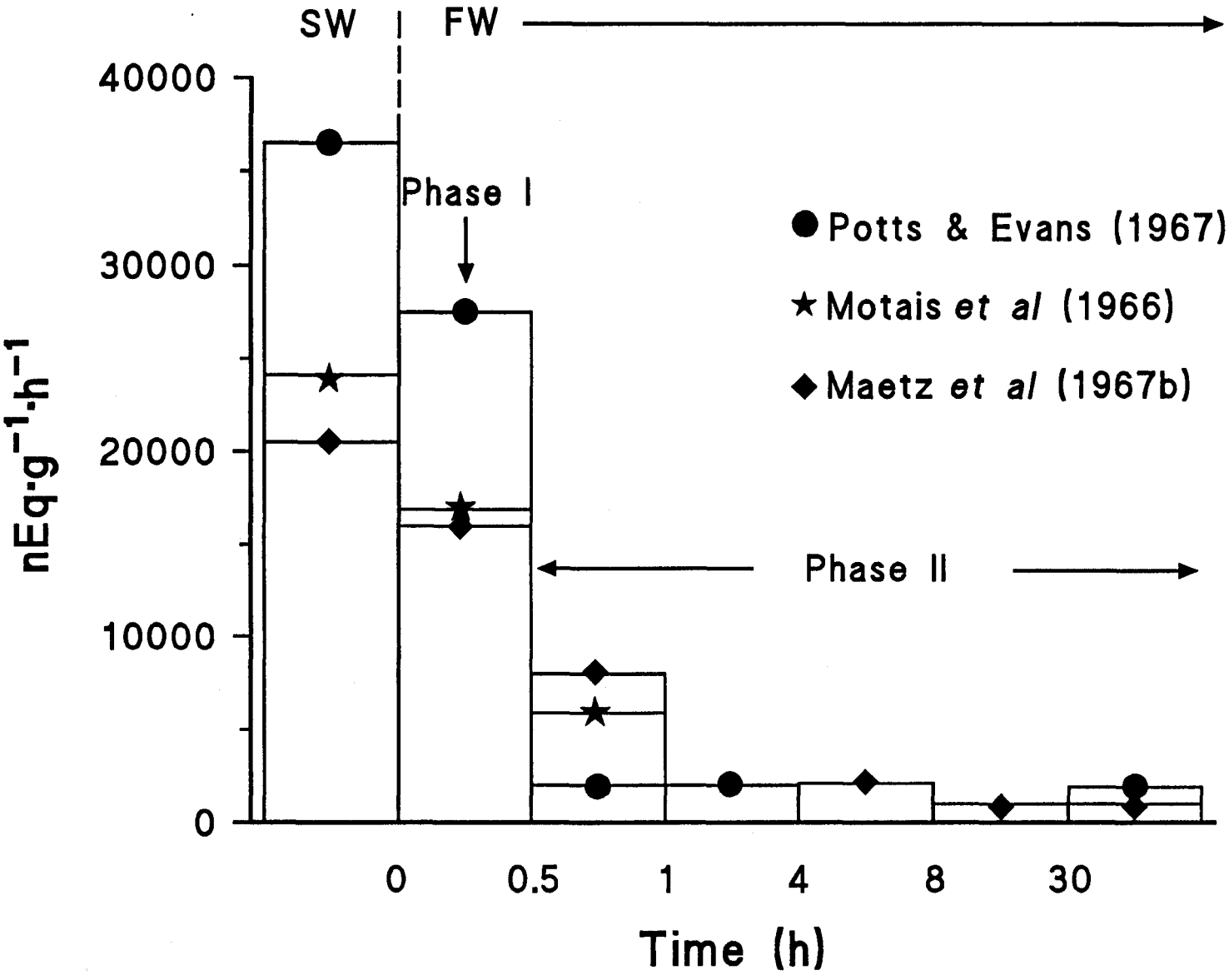
During the transfer from sea water to fresh water, killifish initially suffer

great depletions in both Na^+ and Cl^- plasma levels but return to normal levels within 2-3 days. This slow response to salinity change and subsequent ion disturbance is due to a delayed reduction in unidirectional Na^+ and Cl^- efflux. Several studies have measured sodium efflux in *F. heteroclitus* during transfer to fresh water and have observed two phases of reduction (Figure 1.1). First, there is a small drop (30%) within the first half hour which is followed by a second larger and progressive decrease in which efflux is reduced to less than ¼ of seawater values and approximates freshwater effluxes within about 24 hours (Motais *et al.* 1966; Maetz *et al.* 1967*a*; Potts & Evans, 1967). In comparison to other euryhaline teleost such as flounder *Platichthys flesus* and eel *Anguilla anguilla* (Motais *et al.* 1966), the rates of both phases appear somewhat slower in the killifish.

Exchange diffusion was originally suggested as the mechanism responsible for the initial drop in efflux during SW-FW transfer (Motais *et al.* 1966): some portion of efflux would be directly linked to influx and if either component were altered, the other would follow. During sea water to fresh water transfer, external NaCl concentration is reduced which by exchange diffusion would slow the rate of Na^+ and Cl^- entry (*i.e.* influx) and exit (*i.e.* efflux).

More recently, the existence of exchange diffusion has been questioned and other explanations for the rapid changes in ion effluxes during SW-FW transfer have been put forth. Pic (1978) measured the gill potential difference

Figure 1.1. Changes in Na^+ efflux (J_{out}) in *F. heteroclitus* over a period of time following transfer from seawater to freshwater. During phase I, the instantaneous reduction in J^{Na+}_{out} is a result of the lower external Na^+ concentration and J^{Na+}_{in} whereas the greater reduction observed in phase II is believed to be under endocrine control. Data were recalculated from three separate studies by Potts & Evans (1967) [●], Motais *et al* (1966) [★], and Maetz *et al* (1967*a*) [◆]. See text for details.


SW
FW
40000
30000
20000
10000
0
Phase I
Phase II
Potts & Evans (1967)
Motais et al (1966)
Maetz et al (1967b)
nEq·g⁻¹·h⁻¹
0
0.5
1
4
8
30
Time (h)

($PD = V_{ext} - V_{int}$), of seawater-adapted mummichog to be in the order of -20 mV. Upon transfer to fresh water, PD inverted to +20 mV. The rapid change in sign could explain the instantaneous reduction of Na^+ efflux but not Cl^- efflux (Pic, 1978). A third possible mechanism is based on *in vitro* experiments with *F. heteroclitus* opercular epithelia. In this tissue, the rapid reductions in efflux observed during transfer can be explained by a direct regulatory effect of external salinity on the conductance of the Cl^- channels and paracellular pathway for Na^+ (Zadunaisky, 1984). As yet, the relative contribution of these three possible explanations to the Phase I effect in the intact killifish remain undecided.

Phase II of SW-FW transfer is clearly due to reduction in permeability of the body surface, specifically the gills. The delay in this largest reduction of efflux (*i.e.* beyond 30 minutes), suggests that the regulating factor is endocrine in nature. The most obvious choice is prolactin. As described earlier, prolactin injections reduced Na^+ and Cl^- effluxes in fresh water by modulating Na^+/K^+ ATPase in gill and kidney (Pickford *et al.* 1970). This is in agreement with Jacob & Taylor (1983) who measured Na^+/K^+ ATPase activity in mummichog gills and found decreases in ATPase activity within 24 hours after transfer to freshwater. Other actions of prolactin during this phase are unknown but may include reductions in the "leakiness" of ion transporting epithelium via increased mucous secretion (Burden, 1956; Pickford & Pang, 1966) and/or direct modulation of conductive properties by tightening paracellular junctions with

increased Ca^{2+} binding (Potts & Fleming, 1971).

Chloride cells

Keys and Wilmer (1932) used a heart-gill perfusion of seawater-adapted eel to discover that salt secretion occurs across the branchial epithelia, and probably through a particular type of cell. These columnar, acidophilic cells, first discovered on gill filaments, were believed to be involved in Cl^- secretion in sea water teleosts and so were named "chloride cells". Chloride cells seemingly altered their morphology with varying salinity (Copeland,1948, 1950; Doyle & Gorecki, 1961; Philpott & Copeland, 1963). An elaborate baso-lateral tubular system, large mitochondria population and an apical crypt are characteristic of the salt water chloride cell. In the FW chloride cell, the two former structures are retained but the crypt becomes more shallow or even disappears as the apical surface becomes exposed (Kessel & Beams, 1960; Philpott & Copeland, 1963; Hossler *et al.* 1985; Karnaky, 1986; Marshall *et al.* 1994). Almost 45 years after the Keys and Wilmer discovery, Karnaky *et al.* (1976), using gill perfusion and [^{3}H]-ouabain autoradiography, determined that chloride cells are the primary site of Na^+/K^+-ATPase and are most probably responsible for NaCl secretion in seawater-adapted *F. heteroclitus.* An additional important finding was that Na^+/K^+-ATPase is located on the elaborate baso-lateral tubular system. The greater ouabain binding that occurred in the SW-adapted fish suggested that the ATPase population is diminished upon adaptation to FW. Silva *et al.*

(1977) used intact seawater adapted eels (*Anguilla rostrata*) that were either injected or bathed with ouabain. Their results indicated that ouabain could gain access to Na^+/K^+-ATPase only via the blood or interstitial fluid, not from the external water, thereby providing more evidence for the baso-lateral location of the enzyme.

What is surprising is that chloride cells make up only 6% of the total cell population in the gill (Karnaky *et al.* 1976); the rest are respiratory, mucous and non-differentiated cells. Meanwhile, the opercular membrane, a flat epithelium which lines the opercular bone in *Fundulus* and a few other teleosts such as *Oreochromis*, is comprised of 50-70% chloride cells (Karnaky *et al.* 1976). This epithelium was first examined by Burns & Copeland (1950) who were searching for areas in *F. heteroclitus* that might contribute to salt secretion, in addition to the gills. They noticed that a substantial part of the epithelia lining the operculum was well vascularized and within that region, there resided a large population of chloride cells. Further morphological studies revealed that the opercular epithelium was very similar to the gill epithelium with respect to cell types but lacked respiratory lamellae (Karnaky, 1986).

Opercular epithelia as a model-system

Though gill perfusions have been very useful in localizing Na^+/K^+-ATPase in chloride cells, it is extremely difficult to obtain any electrical or thermodynamic measures because of the complex anatomy of the epithelium

and blood supply of the gill. The opercular epithelium of *F. heteroclitus*, with its similarity to gill and planar construction, made it ideal for *in vitro* investigations of ion transport; this particular model system has now contributed greatly to our knowledge of ionoregulation in teleosts, especially in sea water.

Several papers have described similar procedures for *in vitro* studies of the opercular epithelium (Degnan *et al.* 1977; Karnaky *et al.* 1977; Degnan & Zadunaisky, 1980) and comparable preparations such as the jaw epithelium of the goby *Gillichthys mirabilis* (Marshall, 1977). Further discussion and detailed description of this methodology can be found in Zadunaisky (1984).

This skin can be readily lifted off the opercular bone and mounted in an Ussing-style chamber. Certain electrochemical properties of the epithelia can then be surveyed within this setup. Using radio-labelled ions, unidirectional fluxes in the serosal to mucosal direction (*i.e.* efflux; J_{sm}) and vice versa (*i.e.* influx; J_{ms}) can be measured. The ratio of two flux rates can be compared against the predicted ratio calculated by the Ussing-Teorell equation (Zadunaisky, 1984) which is based on passive, inert membrane electrical properties. If the observed and predicted flux ratios are in agreement, ion movement is passive whereas disagreement indicates that the membrane must be actively transporting the ion in question. Transepithelial potential (TEP, mV), the voltage difference across the membrane, is developed by the differential ion movement due to selective membrane permeability. When both sides of the epithelia are bathed with the same saline and an external current (I_{sc}) is applied

which "clamps" the TEP to 0 mV, both electrical and chemical gradients are eliminated. This is called the short circuit current method and combined with measurements of isotopic fluxes, the contribution of the different ions to the I_{sc} can be determined. Open circuit conditions are used when simulating *in vivo* conditions. Seawater or freshwater are placed on the mucosal side of the epithelium which sets the chemical gradients, and electrical gradients become established (*i.e.* TEP $\neq$ 0). Other characteristics measured *in vitro* are the conductance (G) and the inverse of this, resistance (R), which quantifies the ease or difficulty of ion passage across the membrane.

Studies using seawater epithelia

Using the procedures as described above, several investigators have laid the foundations for an ion transport model for the seawater gill of teleosts in general based on the function of the opercular epithelium of the seawater-adapted *F. heteroclitus* (Figure 1.2).

Under open circuit conditions, epithelia from SW mummichogs display a TEP of +20 mV, serosal positive, relative to mucosal as zero with physiological saline on both sides. Unidirectional flux measurements have revealed a substantial serosal to mucosal Cl^- movement with a small net flux in the same direction. The observed flux ratio is much larger than the predicted ratio (see above) suggesting active Cl^- secretion (Pequeux *et al.* 1988). When a short-circuit current (SCC) is introduced so that the TEP is held at 0 mV, Cl^- fluxes

are unchanged with the flux ratios still in disagreement. Na^+ fluxes have been measured under SCC revealing no net Na^+ movement. Observed and predicted Na^+ flux ratios are similar, suggesting passive diffusion (Degnan & Zadunaisky, 1980). It should be noted that TEP of +20 mV is close to the Nernst equilibrium potential for Na^+ (Degnan & Zadunaisky, 1980) which would also suggest a passive distribution. Sea water placed in the mucosal chamber increased TEP to +36 mV and enhanced Cl^- unidirectional fluxes as a result of greater conductance (G_t). Degan *et al.* (1977) calculated that net Cl^- flux was not different from I_{sc} indicating that most of the current across the epithelia is Cl^- secretion. Combined results from several studies (Degnan & Zadunaisky, 1979 1980*a*, *b*) can be interpreted as showing a membrane potential that is a Na^+ diffusion potential overlaying the electrogenic contribution of Cl^- secretion (Pequeux *et al.* 1988).

Evidence in favour of the chloride cells as the site of Cl^- secretion was obtained by Ussing chamber studies, in which the fluorescent dye DASPEI (diethylaminostyrylethylpyridinium) was utilized to stain the mitochondria-rich chloride cells at the end of the experiment. The size of the I_{sc} correlated not only with the magnitude of net Cl^- secretion (Degnan *et al.* 1977) but also with the absolute number of DASPEI-stained chloride cells (Marshall & Nishioka, 1980; Karnaky *et al.* 1984).

The chloride cell as the site of Cl^- secretion was confirmed by means of the vibrating probe technique which detects changes in current density over a

voltage clamped epithelia. The tilapia (*Oreochromis mossambicus*) opercular membrane was used as it is a salt secretory epithelia and contains a large number of chloride cells. By observing through a microscope as the probe was passed across the surface of the epithelium, the negative, high current density, indicating Cl^- secretion, was localized over the apical crypt of the chloride cell (Scheffey *et al.* 1983).

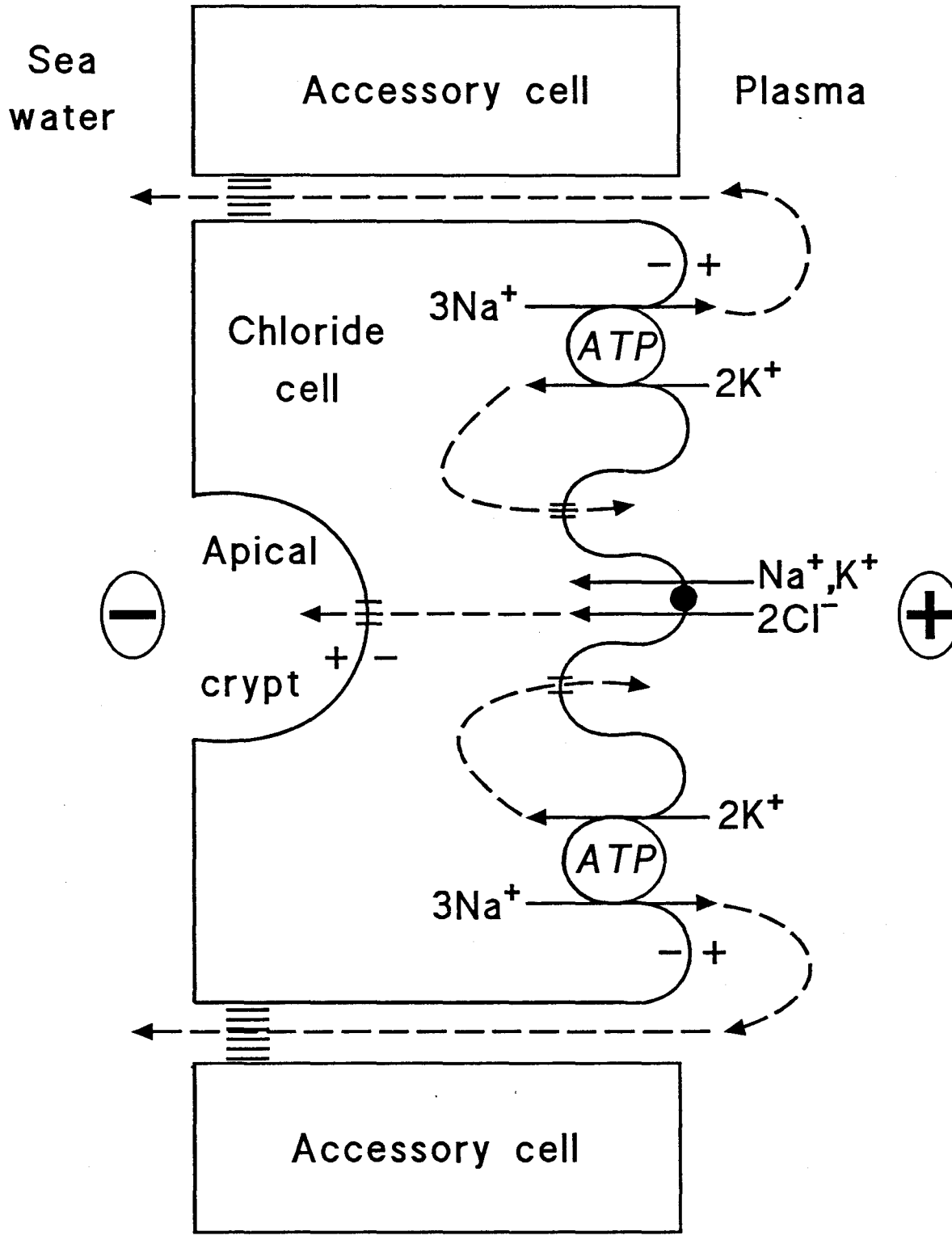
Investigation of neural and hormonal regulation of ion transport in SW opercular epithelium has been extensive and is reviewed by Foskett *et al.* (1983) and Zadunaisky (1984). The primary control of Cl^- secretion in the opercular epithelium is believed to be inhibitory via α_2-adrenergic receptors and muscarinic-cholinergic receptors whereas β-adrenergic control is stimulatory. The hormone prolactin which is necessary for freshwater survival in *F. heteroclitus* (Pickford & Phillips, 1959), not only inhibits Cl^- secretion in seawater epithelium but it also induces morphological changes in chloride cells which could be interpreted as dedifferentiation (Foskett *et al.* 1981). Cortisol injections into the whole animal prior to the *in vitro* preparation did not influence the electrophysiological parameters but increased the density of DASPEI-staining cells (Foskett *et al.* 1981) and stimulated differentiation of chloride cells in tilapia (*Oreochromis mossambicus*) (McCormick, 1990).

Seawater model of ion transport

The seawater model of ion transport as illustrated in Figure 1.2 was initially proposed by Silva *et al.* (1977) based on whole animal experiments with intact eel and the details have been confirmed and elaborated almost entirely by experiments with the opercular epithelium *in vitro* (see reviews by Zadunaisky, 1984; Karnaky, 1986).

The primary source of energy for active Cl^- secretion is the large Na^+ gradient set up across the baso-lateral membrane by Na^+/K^+-ATPase. Intracellular $[Na^+]$ is kept low due to this pumping so there is a strong electrochemical gradient for Na^+ entry. This movement is believed to occur through a Na^+, $K^+/2Cl^-$ cotransporter which is driven by the Na^+ gradient (and therefore secondarily by the Na^+/K^+-ATPase). Cl^- and K^+ are dragged into the cell by the movement of Na^+ down its gradient. The exact stoichiometry is uncertain but other NaCl carrier systems that are also loop diuretic-sensitive are 1 Na^+: 1K^+: 2 Cl^- (Karnaky, 1986). Within the cell, levels of Cl^- rise above electrochemical equilibrium allowing Cl^- to passively exit at the apical surface. This was confirmed by Potts & Oates (1983) with measurements of ionic concentrations by X-ray emission analysis. The accumulation of Cl^- within the cell could possibly alter the conductance of the apical membrane or activate anion channels/carriers allowing Cl^- to exit. Na^+ extrusion occurs through "leaky" paracellular channels between adjacent chloride cells or chloride cell - accessory cell junctions (Sardet *et al.* 1979; Ernst *et al.* 1980). The driving

Figure 1.2. The current model for NaCl transport in ionoregulating epithelia, including the gills and opercular epithelium, for seawater-adapted teleosts as described by Silva *et al* (1977). Further confirmation of this model was obtained from *in vitro* experiments utilizing *F. heteroclitus* opercular epithelium preparations. Cl^- extrusion occurs across the chloride cell whereas Na^+ movement is believed to be paracellular, between adjacent chloride cells and/or chloride cell-accessory cell junctions. Carrier-mediated transport is indicated by solid arrows, diffusive movement by dashed lines. See text for further details.


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

force of the TEP (+35-40 mV), which is established by Cl^- movement, appears large enough to explain passive Na^+ extrusion through this route (Silva *et al.* 1977; Karnaky, 1986; Wood & Marshall, 1994).

One limitation of this current seawater ion transport model is that it provides no linkage to acid-base regulation. Evans *et al.* (1982) have described several lines of evidence which suggest that Na^+ and Cl^- transport may in some way be keyed to acid-base regulation in seawater teleosts *in vivo*. Indeed Na^+/acid and Cl^-/base exchange have been found in several seawater species but this has not been addressed in *F. heteroclitus* or in any opercular epithelium-based models. Some of the earlier studies (Degnan *et al.* 1977; Degnan & Zadunaisky 1979, 1980*b*) did not properly mimic the *in vivo* physiological acid-base conditions for *F. heteroclitus* and obtained atypical results. Pequeux *et al.* (1988) did indicate that the epithelial electrochemical status is sensitive to changes in $[HCO_3^-]$ of the serosal bathing solution. Suggestions of an HCO_3^--permeable anion channel are under investigation (W.S. Marshall, personal communication) in the mummichog as these channels have been detected in other Cl^- secreting tissue and are involved in transporting HCO_3^- (Foskett *et al.* 1983). Carbonic anhydrase (CAH) has been detected in high concentrations in the chloride cells of both SW and FW killifish opercular epithelia (Lacy, 1983) but its role in ionoregulation is unknown, as addition of acetazolamide (CAH inhibitor) to the serosal aspect of SW opercular membrane did not alter the short circuit current or TEP (Zadunaisky, 1984). In contrast,

CAH plays a pivotal role in the coupling of acid-base and iono- regulation in many freshwater teleosts (see below).

Freshwater opercular epithelium

In light of the great advances made in understanding chloride cell function in seawater through isolated opercular epithelial preparations, it is surprising that there has been so little work on comparable freshwater preparations. However, early studies were disappointing for they indicated that Cl^- secretion still occurred under short circuit conditions in epithelia from 1-5% SW adapted fish (Degnan *et al.* 1977, Karnaky, 1986). This suggested that the opercular chloride cells were retaining their seawater character and that ion uptake in freshwater must be occurring elsewhere, perhaps at the gills. However, recently there has been a revival of interest in using freshwater-adapted *F. heteroclitus* opercular epithelium.

Wood & Marshall (1994) and Marshall *et al.* (1994) have recently reported results with opercular epithelia of freshwater-adapted *F. heteroclitus* that conflict with the earlier *in vitro* killifish investigations mentioned above. With freshwater on the mucosal side and saline on the serosal side, the observed unidirectional Cl^- fluxes were in disagreement with the predicted ratio indicating an active Cl^- uptake whereas the two ratios for Na^+ were comparable meaning a passive distribution across the membrane. Unidirectional fluxes of Na^+ were larger than Cl^- suggesting a greater membrane permeability which also coincides with the

large negative TEP (-30 to -70 mV). The substantial Na^+ efflux in fresh water could reflect the inability to close the cation-selective paracellular pathways that are prominent in SW killifish (Sardet *et al.* 1979; Ernst *et al.* 1980). When the external solution was changed to saline isotonic to the internal saline, a net Cl^- efflux did not develop, in contrast to previous studies. In summary, while this preparation shows some promise as a "freshwater gill surrogate model", it will need a lot more validation work.

Chloride cells of the FW killifish opercular membrane are similar to the SW-adapted species with respect to shape and population. Recent TEM and SEM work (Marshall *et al.* 1994) revealed larger chloride cells with a more shallow apical crypt and microvilli on the apical surface, perhaps an adaptation to increase surface area for ion transport. This is in agreement with earlier ultrastructural studies of gill filaments (Kessel & Beams, 1960; Philpott & Copeland, 1963; Hossler *et al.* 1985) and opercular epithelium (Karnaky, 1986). Adjacent chloride cells or chloride cell-accessory cell arrangements are typically found sharing the same apical crypt, an arrangement frequently observed in SW with shallow tight junctions of simple construction. Tight junctions located between chloride cells and pavement cells are deeper and more complex, suggesting a less likely path for diffusive ion movement (Sardet *et al.* 1979). Marshall *et al.* (1994) found adjacent chloride cells sharing apical crypts in opercular epithelium of FW-adapted killifish. In addition to this, Lacy (1983) reported that accessory cells were also present within the apical crypts of FW

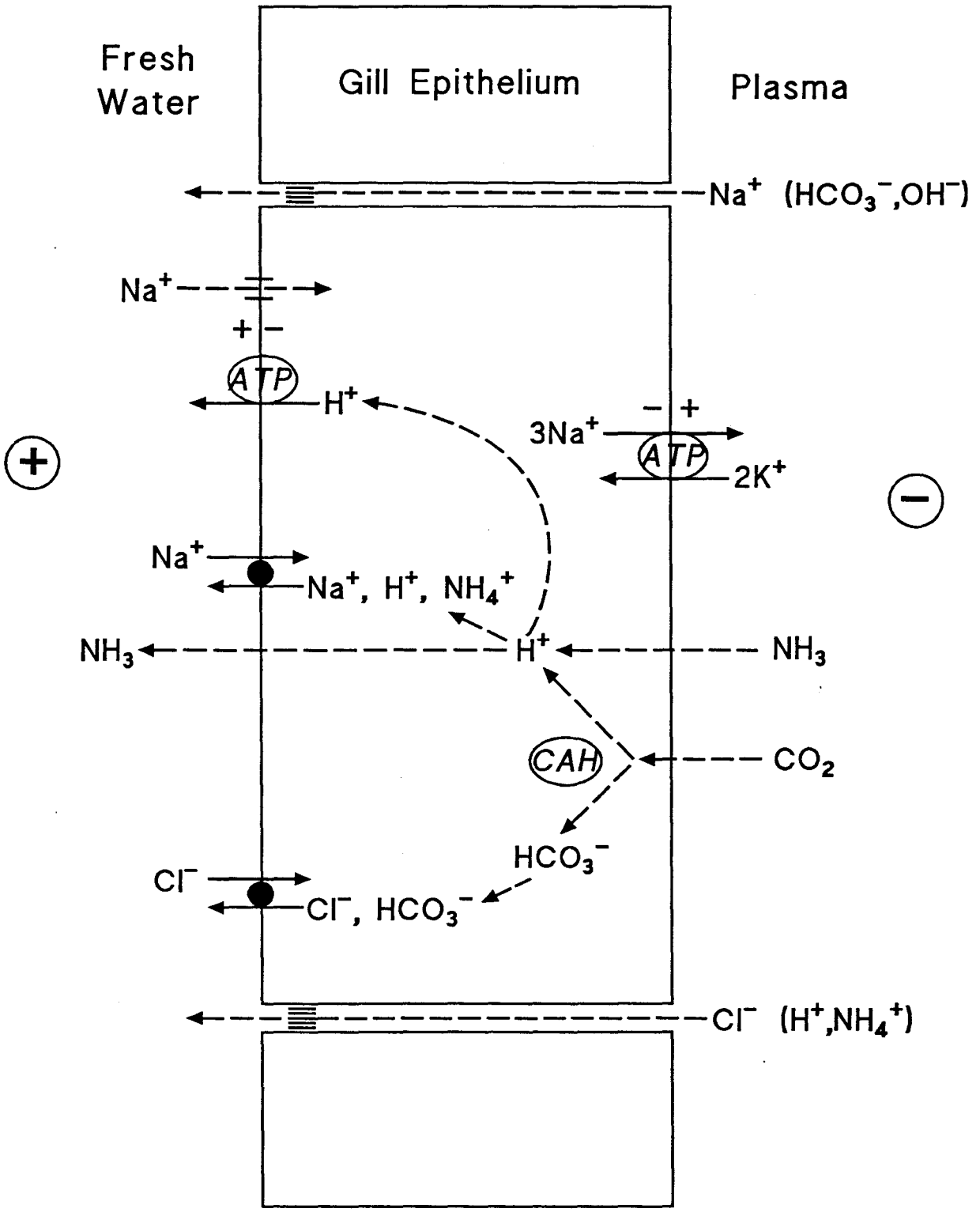
opercular epithelium. Both of these studies suggest that *F. heteroclitus* retains certain seawater characteristics even after acclimation to freshwater. An intriguing discovery in the opercular membrane of freshwater killifish was that some chloride cells were completely covered by pavement cells as detected by TEM and were only perceivable as bumps under the epithelium by SEM (Marshall *et al.* 1994). This suggests that though the number of chloride cells remains the same in FW as in SW in the killifish membrane, a certain population may be paved over and non-functional in FW. How this affects ionoregulation *in vivo* is yet to be determined.

From the *in vitro* work, it appears that the killifish is designed for life mainly in sea water and brackish water, but appears only to cope in fresh water. But the establishment of freshwater populations (see above) and their ability to thrive within the very dynamic environment of an estuary, suggests otherwise.

Fresh water model of ion transport

The present model of ion transport in the gills of freshwater fish is derived from flux measurements on both intact fish and gill perfusions. No stable *in vitro* preparations of FW gills, and no good surrogate models comparable to the seawater opercular epithelium have yet been developed. Teleosts most utilized in fresh water studies have been the euryhaline salmonids, most commonly the rainbow trout (*Oncorhynchus mykiss*) as well as *Salmo* and *Salvelinus*, as well as the stenohaline goldfish (*Carassius auratus*), channel catfish (*Ictalurus*

Figure 1.3. The current model of Na^+ and Cl^- uptake across the branchial epithelium in freshwater teleosts showing the direct coupling to acid-base regulation. The model is based upon experiments utilizing both intact fish and perfused gill preparations. Two proposed mechanisms for Na^+ uptake are presented. Carrier-mediated transport is indicated by solid arrows, passive diffusion by dashed lines. The paracellular diffusion of HCO_3^-, OH^-, H^+, and NH_4^+ are shown in parentheses as their movement is contingent upon differential strong cation (Na^+) and anion (Cl^-) efflux. The cell types responsible for uptake are yet to be confirmed and are not labelled. See text for details.


Fresh Water
Gill Epithelium
Plasma
Na^+ (HCO_3^-, OH^-)
Na^+
+ −
ATP
H^+
$3Na^+$
− +
ATP
$2K^+$
+
−
Na^+
Na^+, H^+, NH_4^+
NH_3
H^+
NH_3
CAH
CO_2
HCO_3^-
Cl^-
Cl^-, HCO_3^-
Cl^- (H^+, NH_4^+)

punctatus) and the European carp (*Cyprinus carpio*). To date, almost no information has been gleaned from intact freshwater mummichog. The model (Figure 1.3) is far from complete as some major aspects are yet to be resolved (see reviews by McDonald *et al.* 1989; Goss *et al.* 1992; Kirschner, 1994; Potts, 1994). Those points under debate are both presented and discussed below.

I. Ionic uptake in freshwater fish

Krogh (1939) and Garcia-Romeu & Maetz (1964) first reported that Na^+ and Cl^- were independently extracted from the external medium as replacement of either Na^+ or Cl^- with an impermeant ion did not disrupt the uptake of the permeant ion. Maetz & Garcia-Romeu (1964) through a series of exposures and injections of ammonium and bicarbonate ion, provided the first evidence of electroneutral Na^+/NH_4^+ and Cl^-/HCO_3^- exchange in the goldfish (*Carassius auratus*). The actual mechanism by which Na^+ is transported remains uncertain but three possibilities are included in the FW model (Figure 1.3). The counterion for the Na^+ uptake has been defined as an acidic equivalent which could be either H^+ or NH_4^+. The acidic equivalents could be electroneutrally exchanged for Na^+, or protons could be actively extruded via a H^+-ATPase which would establish an electrochemical gradient across the apical membrane allowing passive inward Na^+ movement through cation-selective channels. The energy required to drive ion absorption is believed to be provided by the Na^+/K^+-ATPase located on the basolateral membrane. Low intracellular [Na^+] is

maintained by this pumping and may be sufficient to allow the passive Na^+ entry at the apical surface. As for Cl^- absorption, it is unclear where the energy source is derived from unless the Na^+/acidic equivalent coupling sets up a pH gradient driving HCO_3^- extrusion via Cl^-/HCO_3^- exchange.

The occurrence of exchange diffusion or self-exchange (*i.e.* Na^+/Na^+, Cl^- /Cl) in some studies (Maetz, 1972; DeRenzis & Maetz, 1973; Wood & Randall, 1973; DeRenzis, 1975; Goss & Wood, 1990*a*, *b*) is believed to be mediated through $Na^+/H^+(NH_4^+)$ and Cl^-/HCO_3^- antiports as intracellular Na^+ and Cl^- would displace the corresponding acid-base equivalent from the carrier on the inner apical membrane. Goss & Wood (1990*a*) were able to provide strong evidence for the occurrence of exchange diffusion in rainbow trout by demonstrating that diffusional efflux in NaCl-free water (rather than deionized) was less than efflux measured by radioisotopic methods. This discrepancy was ascribed to the absence of exchange diffusion in NaCl-free, water as Na^+ uptake was eliminated whereas, under normal conditions, exchange diffusion contributes to the efflux component. Regardless of whether or not exchange diffusion occurs, there is a general agreement that most if not all of Na^+ and Cl^- efflux by simple diffusion occurs through the paracellular pathways (McDonald *et al.* 1989).

II. Mechanisms of ammonia excretion in freshwater fish

There is still an ongoing debate as to what mechanism of ammonia

excretion is present and how is it coupled to Na^+ uptake in the gill epithelium. Avella & Bornancin (1989) suggested that all ammonia excretion must occur in the unionized form (NH_3) whereas Maetz (1972, 1973) stated that in the goldfish (*Carassius auratus*) both NH_3 diffusion and Na^+/NH_4^+ exchange could exist. More recently, evidence for this exchanger has been presented by the 1:1 correlation between Na^+ uptake and ammonia excretion (McDonald & Prior, 1988) and the concurrent inhibition of J^{Na}_{in} and J^{Amm} with low pH exposure (Wright & Wood, 1985). Maetz (1972, 1973) did not find this correlation in goldfish and proposed a Na^+/H^+ exchanger in addition to the Na^+/NH_4^+ exchanger because the correlation between Na^+ uptake and ammonia excretion was only revealed when H^+ flux was considered. Both Na^+/H^+ exchange and the newly proposed Na^+ channel/H^+-ATPase configuration (Lin & Randall, 1991) could be coupled to passive NH_3 diffusion and would produce the same end result as the Na^+/NH_4^+ exchanger. The recent detection of H^+-ATPase activity in the rainbow trout gill (Lin & Randall, 1993) and experiments presenting evidence of acidification of water as it passes over the gill (Playle & Wood, 1989; Wright *et al.* 1989; Lin & Randall, 1991) suggest that ammonia excretion is passive (as NH_3) and dependent upon protons that are either extruded by the H^+-ATPase or are derived from the hydration of metabolic CO_2 by external carbonic anhydrase (CAH). In both cases, protons would acidify the region adjacent to the gill with the latter event (hydration of CO_2) being independent of the Na^+ uptake mechanism. NH_3 entering this acidified gill boundary layer

would become protonated thereby maintaining a blood-to-water ammonia diffusion gradient (ΔP_{NH_3}).

Recent high external ammonia (HEA) studies, have brought forth more evidence for passive NH_3 excretion in the rainbow trout (Wilson *et al.* 1994). Initially, J^{Amm} was inhibited by HEA but gradually resumed to near control rates as ΔP_{NH_3} was reestablished by the elevated plasma ammonia levels. Once this new steady state had been reached, the experimental addition of HEPES buffer to the water to prohibit acidification of the boundary layer resulted in a blockade of ammonia excretion. Therefore, it seems unlikely that Na^+/NH_4^+ exchange is functioning in rainbow trout as this form of ammonia excretion would not be dependent upon the acidification of the gill boundary layer.

III. Ionic and acid-base regulation coupling and the Strong Ion Difference Theory (SID)

Recently, more insight into the coupling of iono- and acid-base regulation has been revealed by the use of two-substrate kinetic analysis in rainbow trout (Goss & Wood, 1991). Prior to this, all kinetic uptake studies relied on Michaelis Menten one-substrate theory to characterize Na^+ and Cl^- uptake in freshwater (*e.g.* Kerstetter *et al.* 1970; Maetz, 1972, 1973; DeRenzis & Maetz, 1973; DeRenzis, 1975; Goss & Wood, 1990*a*, *b*). In these kinetic studies, both Na^+ and Cl^- uptake typically follow a hyperbolic curve over an external [NaCl] range indicating that both transport mechanisms are saturable (*i.e.* the rate of

uptake is limited). But Kirschner (1988) postulated that this apparent saturation, first found in amphibians, was limited by the H^+ efflux. As it turns out, in rainbow trout, both Na^+ and Cl^- uptake appear to be limited by the availability of internal counterions (*i.e.* H^+, HCO_3^-). Using experimental acid and base loading and two-substrate analysis, Goss & Wood (1991) determined that the *true* J_{max} for both ions is 4 to 5 times greater than measured using the one-substrate analysis.

Exposure to either low pH (as reviewed by McDonald, 1989; Wood, 1989) or high pH in the environment (Wilkie & Wood, 1994) inhibits both Na^+ and Cl^- uptake . These inhibitions are not a result of internal acid-base disturbances or compensation thereof. Rather, they appear to reflect the direct influence of either H^+ or OH^-/HCO_3^- at the transport sites, either by competitive inhibition, or by polarity changes within the cation or anion channels (Motais & Garcia-Romeu, 1972; Wood, 1989).

Respiratory acid-base disturbances induced by environmental hyperoxia (Wood *et al.* 1984; Goss & Wood, 1990*a*) and hypercapnia (Perry *et al.* 1987; Goss *et al.* 1992) stimulate Na^+ uptake and inhibit Cl^- uptake. This differential modulation appears to involve morphological changes in the gill epithelium that would reduce the number of available uptake sites for Cl^- (see below). Upon recovery from hyperoxia and hypercapnia, an alkalosis ensues with a corresponding reversal of trends in Na^+ and Cl^- uptake (*i.e.* reduced J^{Na}_{in}; elevated J^{Cl}_{in}). In this case, the availability of internal counterions (H^+, HCO_3^-)

appears to be the major component influencing the differential adjustments in Na^+ and Cl^- uptake, though a reversal of the morphological changes occurs also. Metabolic acid-base disturbances induced by infusions of either HCl or $NaHCO_3$ have demonstrated that both morphological manipulations (*i.e.* changes in apical surface area) which determine transport site numbers, and changes in internal substrate levels, are involved in modulating Na^+ and Cl^- uptake in response to acid-base imbalance (Goss *et al.* 1994).

Stewart (1983) first introduced the Strong Ion Difference Theory (SID) as an alternative method for quantifying acid-base status within a compartment such as a cell. SID is calculated from the difference between the mathematical sum of strong anion concentration and the sum of strong cation concentration (the term strong means these ions are derived from strong acids or bases, fully dissociated at physiological pH). The difference between the two totals determines the concentration of acidic equivalents and hence, the acid-base status of the compartment. The SID concept has been applied to *in vivo* studies of freshwater teleosts as a means of describing the extracellular acid-base regulation that is performed through differential modulation of the ion efflux component (Hyde & Perry, 1987; McDonald *et al.* 1989; Goss & Wood, 1990*a*, *b*; Goss *et al.* 1992). Na^+ and Cl^- are the strong ions used in estimating extracellular SID *in vivo*. K^+ is not usually considered in these studies as K^+ is present in only very low concentrations in the ECF (but K^+, at high concentrations inside cells plays a pivotal role in intracellular SID). Essentially,

the difference between net Cl^- and Na^+ fluxes indicates a charge imbalance which must be accounted for by another permeable ion. For the FW teleost investigations, this ion is equated to the net flux of acidic equivalents across the gill (*i.e.* $J^{H+}_{net} = J^{Cl}_{net} - J^{Na}_{net}$). For instance, if net Cl^- loss exceeded net Na^+ loss, this would result in a negative J^{H+}_{net} which represents a net acid loss or base uptake whereas a positive J^{H+}_{net} indicates a base loss/acid uptake. Recently, this differential adjustment of ion efflux has been found to be particularly important in correcting systemic alkalosis (Goss & Wood, 1990*a*, *b*). The mechanism of diffusive efflux control in fish has yet to be revealed though paracellular channels are the suspected route for passive ion outflux (McDonald *et al.* 1989) and regulation of the tight junctions within these channels has been demonstrated in other epithelia (Madara, 1988).

IV. Pharmacological characterization of gill ion transport mechanisms

Various ion transport blockers have been used to assess which uptake mechanisms are present in freshwater gills. Amiloride, which is known to block both Na^+/H^+ exchange and Na^+ channels in other systems, is effective not only in blocking Na^+ uptake (Perry & Randall, 1981) but also reduces ammonia excretion (Wright & Wood, 1985), an effect which may be attributed to the reduced proton excretion into the gill boundary layer (Avella & Bornancin, 1989). The decrease in ammonia excretion is generally not as large as the decrease in Na^+ uptake. SITS, a disulfonic stilbene derivative, is known to

inhibit anion transport in many systems. Perry & Randall (1981) found SITS effective in inhibiting J^{Cl}_{in} in rainbow trout but also reported a concurrent J^{Na}_{in} reduction. This co-inhibition was ascribed to the rise of epithelial cell pH and reduction of exchangeable H^+. Acetazolamide, a carbonic anhydrase inhibitor, has been widely used and has shown the importance of CAH in coupling acid-base regulation and branchial ion uptake. By inhibiting the production of branchial H^+ and HCO_3^- from the hydration of CO_2 inside the gill cells, Na^+ and Cl^- uptake should be effectively eliminated as demonstrated in rainbow trout (*O.mykiss*) (Kerstetter *et al.* 1970, Kerstetter & Kirschner, 1972; Perry & Randall, 1981), goldfish (*C. auratus*) (Maetz, 1956, 1973; Maetz & Garcia-Romeu, 1964) and channel catfish (*I. punctatus*) (Henry *et al.* 1988). At the same time, net H^+ excretion was reduced by ~ 50% but ammonia excretion was unchanged.

V. Sites of ion transport

The chloride cell has been implicated as the site of Na^+ and Cl^- uptake in freshwater teleosts. The large population of mitochondria in this cell type suggests an abundant energy source which could supply the active ion transport processes. Carbonic anhydrase (CAH), the enzyme which mediates the production of H^+ and HCO_3^-, has been detected in both pavement and chloride cells. This finding does not aid in distinguishing the chloride cell as the transporting site (Perry & Laurent, 1990). Recent EM studies have revealed

changes in the chloride cell fractional surface area (CCFA) during various acid-base disturbances. Using rainbow trout (*O. mykiss*), Goss *et al.* (1994) reported a decrease in CCFA with a respiratory acidosis (induced by environmental hyperoxia) and an increase in CCFA with metabolic alkalosis ($NaHCO_3$ infusion or recovery from hyperoxia). This modulation of the exposed apical surface of the chloride cell likely plays a role in regulating the availability of branchial Cl^-/HCO_3^- exchangers as reflected in the correlation with changes in maximum Cl^- uptake capacity (*i.e.* J_{max}). During hypercapnia, the apical surface of the chloride cells became covered with numerous erect microvilli. Upon return to normoxic conditions, these microvilli decreased in number and were less erect (Justesen *et al.* 1993), suggesting that the H^+ extruding activity that was enhanced during hypercapnia was occurring through the chloride cell. In the same study, proton-transporting ATPase activity was detected, confirming the findings of Lin & Randall (1993) of the existence of a proton pump in trout gill epithelium. On the otherhand, the brown bullhead (*Ictalurus nebulosus*) during hypercapnia showed increases in microvilli density on the pavement cells suggesting that in this particular freshwater fish, the Na^+ uptake/acidic equivalent excretion mechanism was located on the pavement cell whereas changes $J^{Cl^-}_{in}$ were closely associated with CCFA (Goss *et al.* 1992*a*). What seems to be apparent, is that Cl^- uptake occurs across the chloride cell yet the location of Na^+ uptake and acid excretion remains debatable.

F. heteroclitus is one of many fresh water inhabitants that have not been

considered in the model. As more and more species of fish are examined, this model will be a tool of comparison, as it was for this research thesis.

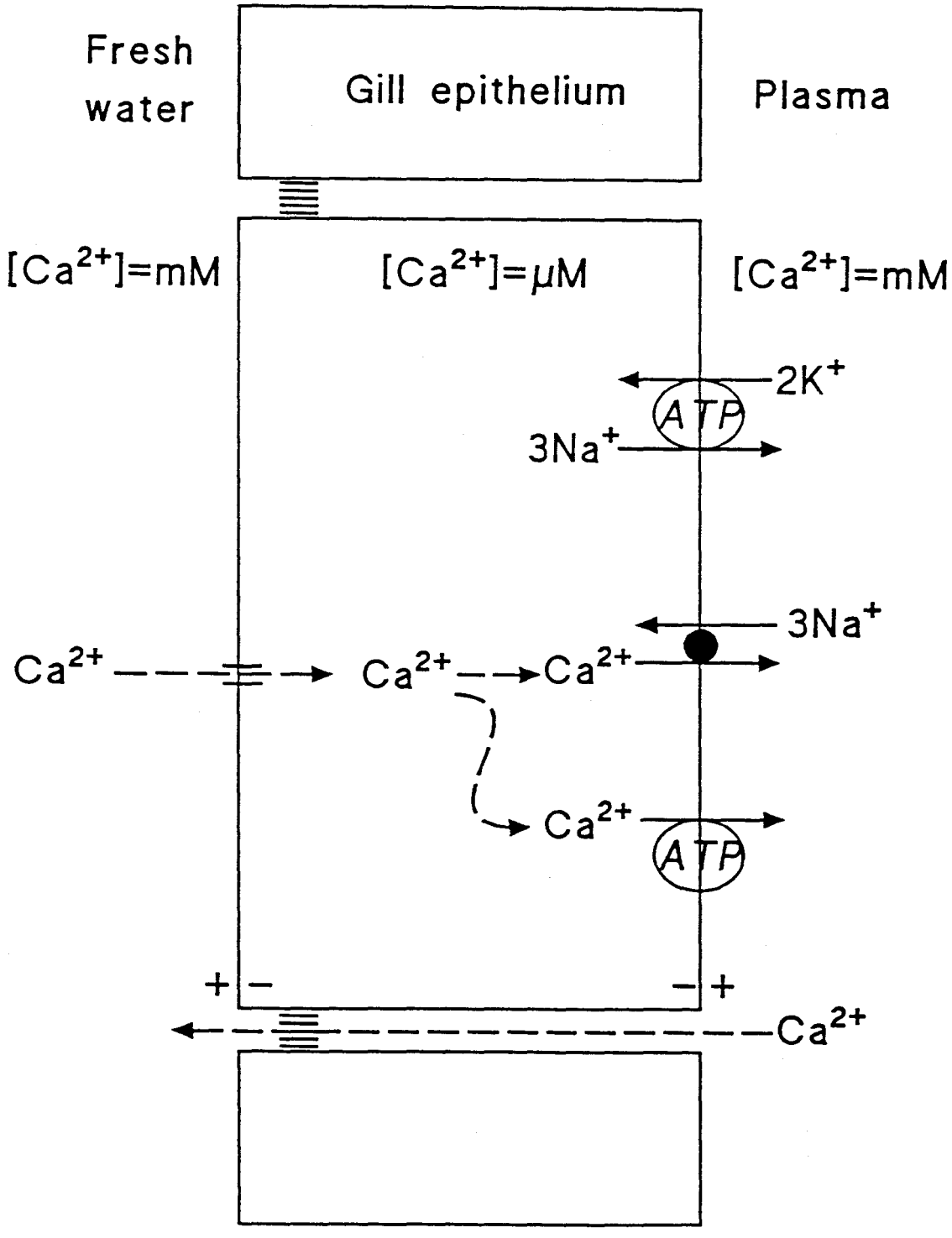
Model for Calcium Transport

Like the model for NaCl regulation, the mechanisms underlying branchial Ca^{2+} transport in freshwater fish are yet to be completely revealed. Many studies have been performed on several freshwater-adapted fish including: trout (Payan *et al.* 1981; Flik & Perry, 1989; Hogstrand *et al.* 1994; Marshall *et al.* 1992; Perry & Flik, 1988; Perry & Wood, 1985; Verbost *et al.* 1987, 1989), eel (*Anguilla anguilla* and *rostrata*) (Milet *et al.* 1979; Perry *et al.* 1989; So & Fenwick, 1979), channel catfish (*Ictalurus punctatus*) (Bentley, 1992), and tilapia (*Oreochromis mossambicus* and *niloticus*) (Flik *et al.* 1985, 1986, 1993*a*, *b*; McCormick *et al.* 1992; Verbost *et al.* 1993). Ca^{2+} uptake in *Fundulus heteroclitus* adapted to freshwater has been examined in only a few earlier studies (Pang *et al.* 1980; Mayer-Gostan *et al.* 1983).

In general, these studies have employed a variety of techniques for investigating Ca^{2+} uptake mechanisms, including measurement of Ca^{2+} fluxes in intact fish, in *in vitro* preparations of opercular and gill epithelium, in plasma membrane vesicles as well as enzymatic determinations to characterize specific Ca^{2+} transporting enzymes. Figure 1.4 is based upon the findings from the studies listed above but excludes current ideas on the regulation of Ca^{2+} uptake.

Transcellular Ca^{2+} influx is generally thought to occur across the chloride cell of the branchial epithelium but this is yet to be confirmed. First, Ca^{2+} enters through the apical, voltage-independent channels down an electrochemical gradient. Ca^{2+} transport across the basolateral membrane requires energy as cytosolic [Ca^{2+}] is substantially lower (~1μM) than plasma (~1.5mM) and also the potential across the basolateral membrane does not favour passive Ca^{2+} movement. Until recently, basolateral Ca^{2+} transport was believed to occur exclusively via a calmodulin-dependent, high-affinity Ca^{2+}-ATPase, which has been demonstrated in trout (Perry & Flik, 1988), tilapia (Flik *et al.* 1985) and eel (Flik *et al.* 1984). However, a Na^+-dependent Ca^{2+} transporter has been now been characterized in tilapia branchial (Verbost *et al.* 1993) and intestinal epithelium (Flik *et al.* 1993*b*) and is believed to be a $Ca^{2+}/3Na^+$ exchanger with the driving force supplied by the Na^+ electrochemical gradient (*i.e.* Na^+/K^+-ATPase). Recent studies suggest that Ca^{2+}-ATPase is the primary transporter in branchial epithelium when cytosolic Ca^{2+} levels approximate 1μM whereas $Ca^{2+}/3Na^+$ exchange is secondary and may become predominant at concentrations exceeding 1μM (Flik *et al.* 1993*b*, Verbost *et al.* 1993). Ca^{2+} efflux occurs between adjacent branchial cells, specifically chloride-pavement cell or pavement-pavement cell junctions (Perry & Flik, 1988)

Figure 1.4. The current model for Ca^{2+} uptake across the branchial epithelium in freshwater teleosts based upon studies utilizing intact fish and *in vitro* opercular and branchial epithelium preparations. Two proposed mechanisms for basolateral Ca^{2+} transport are presented. The cell type involved in transepithelial Ca^{+} transport is believed to be the chloride cell but is not labelled. Carrier-mediated transport is indicated by solid arrows, passive diffusion by dashed arrows. See text for further details.


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

Although Ca^{2+} metabolism in *F. heteroclitus* has been extensively investigated, these studies have focussed more so on the endocrine control (see Pang *et al.* 1980; Pang & Pang, 1986 for reviews) and have contributed less to elucidating the actual transport mechanisms.

Purpose of Present Thesis

The great majority of studies on the euryhaline teleost *Fundulus heteroclitus* have focussed on seawater-adapted animals. Thanks to the exceptionally successful yet simple *in vitro* method utilizing the opercular epithelium, a comprehensive model has been developed to describe transport pathways and regulatory mechanisms involved in salt secretion in seawater, a model (Figure 1.2) which seems of general acceptability to all seawater teleosts. In comparison, a model for freshwater-adapted mummichog is far from reaching the drawing board as very little data are available to indicate if *F. heteroclitus* conforms to the current freshwater ion transport model (Figure 1.3). Recent *in vitro* studies of the freshwater opercular membrane have indicated an active Cl^- uptake with a passive Na^+ distribution but the contribution of this tissue to whole body ion uptake is unknown (Wood & Marshall, 1994; Marshall *et al.* 1994). It is astonishing that so little is known about freshwater ionoregulation in a species which has been so important in elucidating the mechanisms of seawater ionoregulation.

Therefore the main goal of this thesis is to characterize, *in vivo*, the mechanism of Na^+ and Cl^- uptake in freshwater-adapted *Fundulus heteroclitus* and determine if uptake is coupled to acid-base regulation. Essentially, two components were assessed: ionic exchange and acid-base exchange and the SID approach was used to evaluate possible relationships between the two.

The first objective of chapter 2 was to characterize the transporters involved in the uptake of Na^+ and Cl^- using one-substrate Michaelis-Menten analysis to describe affinity (*i.e.* K_m) and maximum transport capacity (*i.e.* J_{max}). Na^+ and Cl^- efflux rates were monitored during the experiments to ascertain the presence or absence of exchange diffusion. To assess the possible presence of a Na^+/NH_4^+ exchange mechanism, ammonia excretion was followed throughout the range of $[NaCl]_{ext}$, and therefore the range of Na^+ influx rates, used in the uptake kinetic studies. The second objective of chapter 2 was to discern possible linkages between ion uptake and acid-base regulation by inducing internal acid-base disturbance via intra-peritoneal injections of acid (HCl) or base ($NaHCO_3$). Unidirectional Na^+ and Cl^- fluxes and net acid-base fluxes were measured for several hours post-injection to detect any disturbances by or compensation for the systemic acidosis and alkalosis. The final aspect of this chapter is an SEM study of both the opercular epithelium and gill filament epithelium of freshwater-adapted mummichogs. Part of this morphological study has been submitted (Marshall *et al.* 1994).

Chapter 3 continues the investigation of connections between ion uptake and acid-base status using environmental disturbances (low pH, high pH, high ammonia exposure) and pharmacological tools (amiloride, acetazolamide). The injection studies of chapter 2 involved handling of the fish and induction of internal acidosis or alkalosis. Manipulation of environmental factors not only minimizes non-specific responses to handling (a potential problem with the injection studies of Chapter 2) but also provides a more environmentally realistic means of disturbing acid-base equilibrium. Amiloride, a potent inhibitor of Na^+/H^+, Na^+/NH_4^+, Na^+ channel systems, was added to the water under control conditions to test for a linkage between Na^+ uptake, ammonia excretion, and acidic equivalent excretion. Carbonic anhydrase (CAH) inhibition was attempted by injections of acetazolamide, the carbonic anhydrase inhibitor described above. CAH produces H^+ and HCO_3^-, which are exchange counterions for Na^+ and Cl^- uptake, at least in those freshwater fish conforming the model illustrated in Figure 1.3. Elimination of the necessary counterions for exchange mechanisms could potentially disrupt ion balance as was evident in other studies (see review by Perry & Laurent, 1990).

The main purpose for Chapter 4 was to characterize *in vivo* Ca^{2+} uptake in *F. heteroclitus* utilizing a new method of measuring whole-body uptake in small fish that was developed by Hogstrand *et al.* (1994). The initial studies of Mayer-Gostan *et al.* (1983) on this topic in *Fundulus heteroclitus* had produced rather unusual results, including an absence of saturation kinetics for Ca^{2+}

uptake. First, one substrate Michaelis-Menten analysis was used to determine if saturation kinetics occurred and to characterize the affinity (K_m) and maximum capacity (J_{max}) of the calcium transport system. The second component of this study looked at the influence of magnesium, a competitive inhibitor of voltage-gated Ca^{2+} channels (Hartzell & White, 1989) and the trivalent cation, lanthanum which is a general Ca^{2+} channel blocker (Verbost *et al.* 1987; Perry & Flik, 1988), on whole body calcium uptake rates of *F. heteroclitus*. Recently, the above two inhibiting cations were tested on freshwater mummichog opercular epithelium *in vitro* (Burghardt, 1993), allowing comparison with the present *in vivo* studies. In the final study of this chapter, mummichogs were acclimated to water with either high or low [Ca^{2+}], then subsequently exposed to low and high [Ca^{2+}] respectively in an attempt to determine how Ca^{2+} uptake rates are affected by external calcium levels; the results of such tests in the original study of Mayer-Gostan *et al.* (1983) were unclear.

It should be emphasized again that there is very little information about *F. heteroclitus* in fresh water. Discussion in subsequent chapters is strongly comparative in nature. Because rainbow trout studies have contributed greatly to the fresh water model of ion transport, much of the comparison will be with this particular salmonid.

CHAPTER 2

Sodium and Chloride Uptake Kinetics, Responses to Intraperitoneal Acid and Base Injections and SEM Examination of Gill and Opercular Epithelium.

It has been stated that measuring the affinity of an ion uptake system will provide insight into a given species' ability to adapt to a defined medium (Evans, 1973). Na^+ and Cl^- uptake kinetics are used to characterize both affinity (K_m) and maximum transport rate (J_{max}) of the uptake mechanisms and have been applied to both seawater (Motais *et al.* 1966; Mayer & Nibelle, 1970; Evans, 1973) and freshwater-adapted teleosts (Motais *et al.* 1966; Kerstetter *et al.* 1970; Kerstetter & Kirschner, 1972; Maetz 1972, 1973; Evans, 1973; DeRenzis, 1975; Bornancin *et al.* 1977; Stiffler *et al.* 1986; Frain, 1987; Goss & Wood, 1990*a*, 1990*b*, 1991; Postlethwaite & McDonald, 1994). Overall, it seems that most freshwater-adapted species tend to have a high affinity (low K_m) but a small uptake capacity (J_{max}) for both ions, but there are a few exceptions (Evans, 1973; Frain, 1987). Interestingly, the one report on *Fundulus heteroclitus in vivo* (Potts & Evans, 1967) also suggests a low affinity for Na^+ absorption, though J_{max} was not measured.

Another common trait amongst freshwater-adapted teleosts is the coupling of Na^+ and Cl^- uptake with the extrusion of acidic (H^+, NH_4^+) or basic equivalents (HCO_3^-) as illustrated in Figure 1.3 of Chapter 1. This relationship becomes

apparent when compensatory shifts in ion absorption take place during an acid-base disturbance. In addition to this, diffusive effluxes of Na^+ and Cl^- appear to be differentially manipulated allowing for the appropriate acid or base net movement according to the Strong Ion Difference Theory (SID) (see Chapter 1). There is no information to date about the acid-base regulatory abilities of *F. heteroclitus*. Even the well-defined seawater model of ion transport for the mummichog does not address this issue (see Chapter 1).

The morphology of the opercular epithelium has been examined in freshwater-adapted *F. heteroclitus* demonstrating that it possesses a large population of mitochondria-rich cells, generally termed chloride cells (see review by Karnaky, 1986). In the opercular epithelium from seawater-adapted mummichogs, chloride cells are responsible for the active Cl^- secretion *in vitro* . In freshwater opercular epithelium there is an active Cl^- uptake component *in vitro* but it is uncertain if these chloride cells are responsible for Cl^- or Na^+ absorption (Wood & Marshall, 1994). However, gill morphology and the contribution of the gills to whole body ion uptake is relatively uninvestigated in freshwater-adapted mummichogs.

The aim of this present chapter was to characterize Na^+ and Cl^- uptake mechanisms using Michaelis-Menten one-substrate kinetic analysis in order to reveal the affinity constant K_m and capacity of each transport system (J_{max}). Second, systemic acidosis and alkalosis were induced via intraperitoneal injections of HCl and $NaHCO_3$ in order to investigate which compensatory

mechanisms, if any, are available to *F. heteroclitus* and whether Na^+ and/or Cl^- ionoregulatory fluxes are involved. Finally, using scanning electron microscopy (SEM), a surface morphology study of both opercular and gill epithelium of freshwater-adapted mummichogs was performed to determine which cell types are present in order to provide insight into the ionoregulatory functions of these two epithelia.

Materials and Methods

Experimental Animals

Mummichogs (*Fundulus heteroclitus*), weighing 2.44 - 7.22g, of both sex, were collected from a brackish estuary located near Antigonish, Nova Scotia and were held at St Francis Xavier University, Antigonish or were air-shipped to McMaster University, Hamilton, Ontario. At both locations, the fish were held indoors in 500 L fibreglass tanks containing 10% seawater at ambient temperature (18-25°C). The water was well aerated and continuously filtered by percolation through polyester fibre and charcoal. Fish were fed a 1:2 mixture Tetramin/Tetramarin daily.

Acclimation Conditions

The acclimation and experimental water was a defined freshwater medium and had the following composition: Na^+, 1.0; Cl^-, 1.0; Ca^{2+}, 0.1; Mg^{2+}, 0.06; K^+, 0.02; titration alkalinity to pH=4.0, 0.28, all in $mmol \cdot L^{-1}$; pH=6.8-7.2. In Antigonish, this was attained by supplementing tap water with NaCl. In Hamilton, dechlorinated tap water was diluted 10 fold using reverse osmosis product followed by addition of NaCl.

Ten to fourteen days prior to the experiment, 10-12 fish were placed in a white plastic tank containing 60 L of acclimation water and a percolating filter consisting of polyester fibre and charcoal. Aeration was provided plus a replacement flow of 3L/hour. Fish were fed Tetramin daily up until 4 days

before the experiment. For the HCl and one NaCl injection trials performed in Antigonish, mummichogs were acclimated to freshwater for 28 days under the same acclimation conditions.

The night prior to experiments, fish were weighed and placed in individual darkened 250 ml Nalgene beakers with lids. Mummichogs used in uptake kinetic studies received exchange flow (200 ml/hour) and aeration overnight, whereas the fish used in the injection study were held in a static 250 ml volume with vigorous aeration for no longer than 6 hours prior to experimentation.

Ion Uptake Kinetics

The primary goals of these experiments were to determine if sodium and chloride uptakes are concentration-dependent within a freshwater [NaCl] range and if the nature of transport conformed to Michaelis-Menten first order kinetics. A secondary goal was to determine whether ammonia excretion varied with Na^+ influx. Sodium (N=11) and chloride (N=6) uptake kinetic experiments were run independently but were performed using identical protocols. Mummichogs in individual containers were exposed to each [NaCl] by increments. Flux periods varied in time according to the [NaCl] exposure: 500 and 800 $\mu mol \cdot L^{-1}$, 1.0 hour; 1300 and 2300 $\mu mol \cdot L^{-1}$, 1.5 hour; 4200 $\mu mol \cdot L^{-1}$, 2 hours; 8000 $\mu mol \cdot L^{-1}$, 3 hours. The defined freshwater medium (see above) was made initially without the addition of NaCl.

For each new flux, water was removed by siphon from the container holding the fish and 105 ml of defined medium was added, together with an aliquot of 1.0 M NaCl stock, to set the required [NaCl]. The NaCl stock solution contained either 6 μCi/ml ^{22}Na for the sodium uptake kinetics or 10 μCi/ml ^{36}Cl for the chloride uptake kinetics. To ensure thorough mixing of the isotope solution, the water was stirred for several minutes. The fish were allowed 10 minutes to settle. Water samples (20 ml) were taken at the beginning and end of each flux period and were frozen immediately for subsequent analysis of ammonia, Na^+, Cl^-, ^{22}Na, and ^{36}Cl. Once the flux period had ended, the water was siphoned out of each container and the cycle repeated with the next higher [NaCl].

The Cl^- kinetics results indicated that J_{max} was not reached within the range of external [NaCl] tested. There was therefore a need to test higher external [NaCl] levels. However, due to specific activity limitations, at such high external [NaCl] concentrations, it was not feasible to measure Cl^- uptake by disappearance of ^{36}Cl cpm from the water. Instead, a range finder experiment to test for saturation of Cl^- uptake at higher [NaCl] was performed by measuring the appearance of ^{36}Cl cpm in the fish.

Water [NaCl]'s of 6200, 15000, and 25000 $\mu mol \cdot L^{-1}$ were used with ^{36}Cl added to make a water specific activity of 46.3 cpm/nmol Cl^-. Three or four fish each were placed in one of three darkened 250 ml Nalgene flasks that contained the three $[NaCl]_{ext}$ solutions in defined freshwater medium. After 2

hours, the fish were removed from the beaker and rinsed briefly in 100 mmol·L^{-1} [NaCl] solution to remove any surface-bound ^{36}Cl. The bodies were frozen in liquid nitrogen, placed in plastic bags then broken into small pieces. These fragments were then transferred to a liquid nitrogen-cooled mortar and ground to a uniform powder. Triplicate 0.5 g portions were weighed out into glass scintillation vials. Each sample was then digested using 2.0 ml tissue solubilizer (NCS, Amersham) for 24 hours at 45°C. The samples were then neutralized with 20µl of glacial acetic acid following 10 ml addition of scintillation cocktail (OCS, Amersham) and counted in a scintillation counter (LKB 1217 Rack Beta, Pharmacia-LKB AB). Water samples (1-2 ml) were counted in the standard fashion (see below) in 10 mL ACS (Amersham). In order to correct for the reduced efficiency of whole body relative to water activity measurements (see above), two additional samples were prepared: i) 0.4 ml of 6200 µmol·L^{-1} NaCl/ ^{36}Cl experimental water in 0.6 ml distilled water plus 10 ml ACS; ii) 0.4 ml of 6200 µmol·L^{-1} NaCl/ ^{36}Cl experimental water plus 0.5 g blank fish followed by the NCS/OCS protocol. The ratio of i:ii was used as the efficiency correction for the tissue samples in order to equate tissue counts to the water counts.

Injection Studies

The purpose of this series was to observe changes in the unidirectional fluxes of sodium, chloride, ammonia and acid-base equivalents in response to

an intraperitoneal injection of a mineral acid or base. Four injection studies were performed, three of which consisted of 1000 $nEq \cdot g^{-1}$ intraperitoneal injections of *i*)NaCl, *ii*) HCl and *iii*) $NaHCO_3$. These three were performed using the defined freshwater medium described in the kinetics studies above. A fourth consisted of 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ injection and was performed in Hamilton dechlorinated tapwater (Na^+, 0.6; Cl^-, 0.8; Ca^{2+}, 1.0; Mg^{2+}, 0.25; K^+, 0.04; titration alkalinity to pH=4.0, 1.2 $mmol \cdot L^{-1}$; pH=8.0) the medium to which the fish had been acclimated, as in Chapter 3. The first two series utilized ^{22}Na and ^{36}Cl to measure unidirectional Na^+ and Cl^- fluxes with each injection study run twice, once for each radio-labelled ion. Both $NaHCO_3$ (*iii*, *iv*) injection experiments were run only once with using ^{24}Na and ^{36}Cl concurrently. The amount of isotope solution used in experiments with ^{22}Na or ^{36}Cl was 2 µCi and with ^{24}Na, 16.0 µCi.

After the overnight settling period, the flux containers were siphoned and flushed, and 250 ml fresh water added 90 minutes before the beginning of the experiment. Next, two-1.5 hour control fluxes were performed. For these, water was siphoned from the containers and replaced with 105 ml fresh water (200 ml in *iv*). Isotope solution (essentially carrier-free) was added to each beaker and allowed to mix via aeration for 10 minutes. Water samples (20 ml) were taken at 0, 1 and 2 hour intervals. Water analysis followed the same pattern as described for the kinetics studies with the additional measurement of titration alkalinity in all series, and urea in series *iv*. Only one control flux was

performed in series *iv*. Following the control periods, the flux containers were siphoned and the fish removed, blotted dry and given an intraperitoneal injection of 1000 $nEq \cdot g^{-1}$ *i*) NaCl, *ii*) HCl, *iii*) $NaHCO_3$, or *iv*) 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ using a 50 µl gas-tight Hamilton syringe. A 140 $mmol \cdot L^{-1}$ solution of each injectate was employed, with the volume injected determined by the weight of the fish. A 420 $mmol \cdot L^{-1}$ $NaHCO_3$ solution was used for the 3000 $nEq \cdot g^{-1}$ injection. The mummichog was blotted again, inspected for visual signs of leakage (leaking fish were rejected), then put back into its container with 250 ml fresh water and allowed to recover for 15-20 minutes and to permit any leakage of the injectate from the puncture to be complete. Containers were then flushed, followed by the addition of 105 ml fresh water and isotope. For *i*, *ii*, and *iii*, four, 1 hour flux periods were performed following injection. 20 ml water samples were taken at 0, 1, and 2 hours, then water was changed and sampling continued at 2, 3, and 4 hours. Series *iv* involved four, 2 hour fluxes with the water changed after each flux period. Water was vigorously aerated throughout each experiment. It should be noted that the HCl injection and one set of NaCl injection study were performed by C.M.Wood in Antigonish. Additional NaCl injection studies were performed at Hamilton with the same results, so the data were combined in the final analysis.

SEM study of gill and opercular epithelium.

Mummichogs acclimated to dechlorinated Hamilton tapwater (see composition above) were studied. At the time of sampling, the mummichogs were sacrificed with an overdose of MS 222 anaesthetic (Syndel; 1.5 $g \cdot l^{-1}$ buffered to pH 7.8 with $NaHCO_3$). Methods for fixation of the gill opercular epithelia closely followed those used for fish gills in previous studies (*e.g.* Goss *et al.*, 1992, Wilkie and Wood, 1994). First, the entire opercular bone with attached epithelia was excised from each side of the fish. The operculum was then briefly rinsed in the same dechlorinated tapwater and fixed in ice cold 2.5% glutaraldehyde solution (buffered with 0.15 $mol \cdot l^{-1}$ sodium cacodylate) for approximately 70 - 80 minutes. Immediately after the operculum was removed, the entire gill basket was excised, rinsed in water and placed in a petri dish with paraffin wax melted to form a flat surface. A small amount of 2.5% glutaraldehyde solution was added to the dish. Using a razor blade, the gill basket was cut longitudinally. Each individual gill arch was separated from the bone using fine scissors and forceps, taking care not to damage the filaments. A fine paint brush was used to gently remove any debris or blood clots that may have adhered to the gill filaments. The arches were then immediately placed in a vial containing the same fixing solution as mentioned above. After fixation, both the opercula and gill arches were washed 3 times with ice cold buffer and stored at 4°C overnight. The opercula and gill samples were then partially dehydrated via an ethanol series (15 minutes each in 30% and 50%

EtOH, 3 times 10 minutes in 70% EtOH) and stored at 4°C in 70% EtOH. The gill arches were further dissected at this point by placing them in a petri dish containing 70% EtOH, and using a razor blade to separate 2-3 pairs of gill filaments with a small section of the gill arch attached. All samples were then dehydrated to completion (10 minutes in 95% ethanol, 10 and 20 minutes in absolute ethanol) and taken through 2 successive (2 minutes each) baths of 1,1,1,3,3,3-hexamethlydisilazane (Aldrich) and air dried for approximately 1 hour. The operculi were then mounted, epithelia side up, on aluminum stubs with epoxy glue. Gill filament sections were mounted so that both filament pairs were facing up, providing a side view. To prevent rehydration, the mounted tissues were held in a desiccator that was kept at 60°C for several hours. Immediately prior to viewing, the samples were sputtered coated with a 10nm layer of gold and subsequently viewed on a ISI-DS130 dual stage scanning electron microscope at 15 kV.

Analytical Methods and Calculations

Ammonia concentrations (T_{Amm})were determined by a micro-modification of the salicylate-hypochlorite assay of Verdouw *et al.* (1978). Urea concentrations were determined using a diacetyl monoxime colormetric urea assay (Rahmatullah & Boyd, 1980) as modified by T.P. Mommsen (personal communication) to detect low urea concentrations (0-80μM urea). Titratable alkalinity (TA) was determined by titration of 10 ml water samples with

standardized HCl to pH=4.0 as described by McDonald & Wood (1981). The difference between initial and final TA measurements represents the net base flux which includes ammonia excreted as NH_3. The difference between net ammonia and TA excretion measures the net acidic equivalent movement. As pointed out in McDonald & Wood (1981), this procedure does not distinguish between a net excretion of acid and a net uptake of base, or vice versa, but fortunately this does not matter in terms of net acid-base balance of the fish. All measurements were made using a Radiometer GK2041C pH electrode coupled to a PHM82 pH meter. Titration acid (0.005 N or 0.02 N in different experiments) was dispensed using a Gilmont microburette . All water samples were measured within 12 hours of sampling and aerated 10 minutes prior to titration and another 15 minutes after titration to pH 4.2 to remove respiratory CO_2 before the titration to pH 4.0 was completed.

Water Na^+ concentrations were measured using an atomic absorption spectrophotometer (Varian AA-1275) and Cl^- levels were determined by coulometric titration (Radiometer CMT-10). Duplicate 2 ml water samples were prepared with 10 ml cocktail scintillant (ACS, Amersham) and counted on a scintillation counter (LKB 1217 Rack Beta, Pharmacia-LKB AB) to measure activities (cpm/ml) for ^{22}Na and ^{36}Cl. In experiments involving simultaneous use of ^{24}Na and ^{36}Cl, the ^{24}Na was allowed to decay for 2 weeks before the ^{36}Cl cpm were measured. A Packard Minaxi 5000 autogamma counter detected ^{24}Na activity alone (^{36}Cl does not emit gamma cpm) of 5 ml water samples, with

automatic decay correction. Samples were measured immediately after the final flux period as $t_{½}$ for ^{24}Na is only 15 hours. Unidirectional fluxes of Na^+ and Cl^- (in $nEq \cdot g^{-1} \cdot h^{-1}$), as measured by disappearance of radioactivity from the water, were calculated from the following equations:

$$J_{in} = \frac{\text{weight}}{\text{volume}} \cdot \frac{1}{\text{time}} \cdot (cpm_1 - cpm_2) \cdot \frac{1}{SA}$$

where: cpm is activity of the isotope (cpm/ml) and SA is the mean specific activity of the water calculated from the $cpm_{1,2}$ divided by $[ion]_{1,2}$.

$$J_{out} = J_{net} - J_{in}$$

where J_{net} is calculated by:

$$J_{net} = \frac{\text{weight}}{\text{volume}} \cdot \frac{1}{\text{time}} \cdot ([ion]_1 - [ion]_2)$$

where [ion] is the water concentration at the start and end of flux period. Net titratable alkalinity and ammonia excretion rates were calculated in a similar fashion to J_{net} for ion fluxes by substituting [ion] with initial and final TA and T_{Amm} concentrations.

The calculation of J_{in} for Cl^- using the appearance of ^{36}Cl cpm in the fish during the high [NaCl] range experiment followed this formula:

$$J_{in} = \frac{cpm/g}{SA \cdot time}$$

where: cpm/g is the mean cpm/g tissue of the triplicate samples and was corrected for counting efficiency (see above), SA is the mean specific activity of the water calculated from the cpm divided by $[Cl^-]$.

The relationship between $[NaCl]_{ext}$ and Na^+ uptake was examined using Michaelis-Menten analysis for first-order kinetics. Na^+ uptake followed a distinctive saturation curve which was well described by the one-substrate equation:

$$J_{in} = \frac{J_{max} \cdot [Na^+]_{ext}}{K_m + [Na^+]_{ext}}$$

where J_{in} is the uptake rate at a particular $[Na^+]_{ext}$. J_{max} is the maximum uptake rate and K_m is the $[Na^+]_{ext}$ at which uptake is 50% of J_{max}, and is an index for the inverse of affinity of the transport system. Values of K_m and J_{max} for each individual fish were calculated via Eadie-Hofstee regression analysis, and then grand means were calculated (N=11). It should be noted that Cl^- uptake rates over the range of $[NaCl]_{ext}$ tested did not exhibit saturation kinetics, and therefore this type of analysis could not be employed for Cl^- kinetics.

Statistical Analysis

All values are presented as means $\pm$ SEM. Comparisons between control and post injection means were tested by multiple Student's paired *t*-test with the significance level ($p \leq 0.05$) adjusted according to the Bonferroni table (Nemenyi *et al.* 1977).

Results

Na^+ and Cl^- uptake kinetics

Figure 2.1 presents the Na^+ and Cl^- uptake kinetic results for *F. heteroclitus* and compares them uptake kinetic curves determined by Goss & Wood (1991) for the freshwater rainbow trout (*Oncorhynchus mykiss*). For the mummichog, Na^+ uptake rate (J^{Na}_{in}) followed a distinctive hyperbolic path over the range of $[NaCl]_{ext}$ indicating that transport becomes saturated (Figure 2.1*a*). In contrast, Cl^- uptake was slow to increase. The first $[NaCl]_{ext}$ concentration at which J^{Cl}_{in} was significantly different from zero was 2300 $\mu mol \cdot L^{-1}$. However by 8000 $\mu mol \cdot L^{-1}$ $[NaCl]_{ext}$, J^{Cl}_{in} did approximate J^{Na}_{in} at 8000 $\mu mol \cdot L^{-1}$ $[NaCl]_{ext}$ but did not level off (Figure 2.1*b*). In comparison, trout Na^+ and Cl^- uptake curves were similar to one another as both became saturated within a much lower range of $[NaCl]_{ext}$. J^{Na}_{in} plateaued at a higher level than J^{Cl}_{in} in the trout.

J^{Cl}_{in} calculated from ^{36}Cl activity of mummichogs placed in $[NaCl]_{ext}$ ranging between 6200 and 25000 $\mu mol \cdot L^{-1}$ are listed in Table 2.1. This technique (appearance of ^{36}Cl in the fish) yielded a lower J^{Cl}_{in} than measured at 8000 $\mu M \cdot L^{-1}$ $[NaCl]_{ext}$ (by disappearance of ^{36}Cl cpm from the water) but showed a continuing gradual increase within the higher $[NaCl]_{ext}$ range, suggesting that saturation of uptake still did not occur. The difference in absolute values for J^{Cl}_{in} between Table 2.1 and Figure 2.1 may be due to the different techniques used or the use of different batches of fish at a different time of year.

Table 2.1: Cl^- uptake rates calculated by whole body ^{36}Cl measurements of *F heteroclitus*. $[NaCl]_{ext}$ concentrations used were greater than range used in the kinetic studies of Figure 2.1. Mean ± SEM (N).

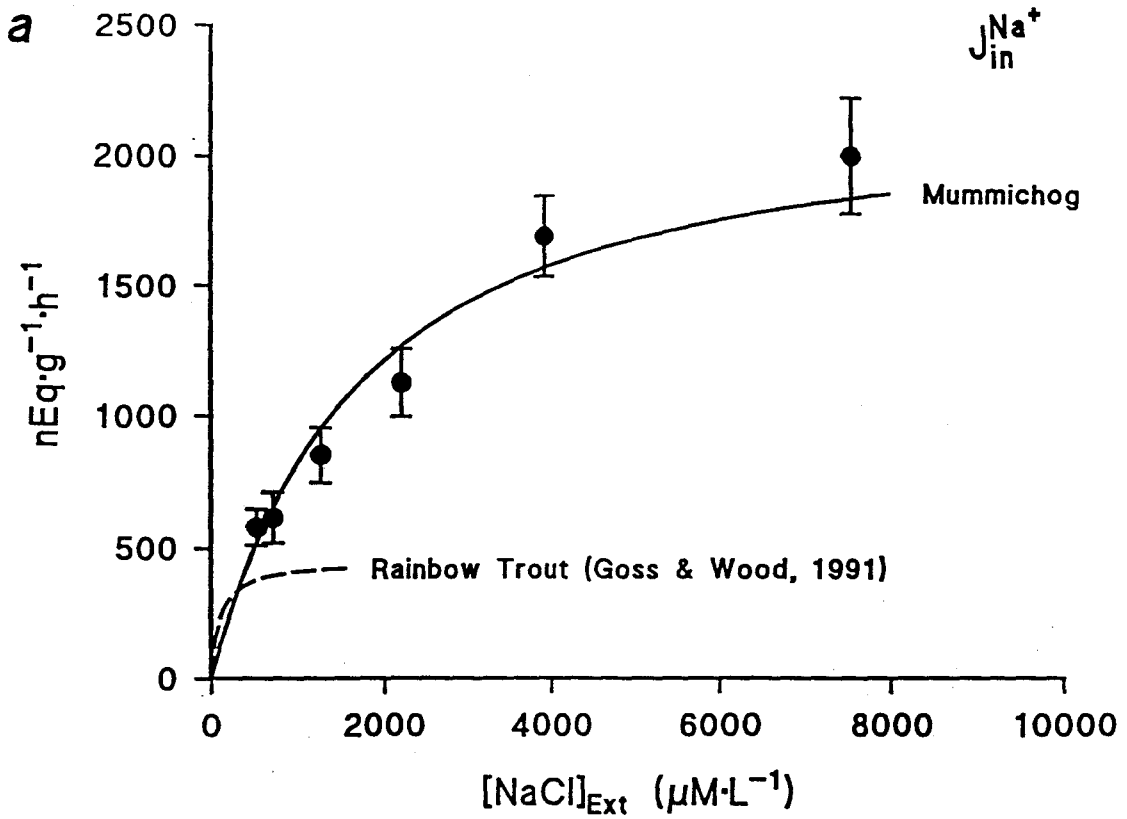
$[NaCl]_{ext}$ ($\mu mol \cdot L^{-1}$)	J^{Cl}_{in} ($nEq \cdot g^{-1} \cdot h^{-1}$)
6200	585.6 ± 88.1 (4)
15000	786.9 ± 59.6 (3)
25000	964.4 ± 24.1 (3)

Table 2.2: K_m and J_{max} values obtained by one-substrate Michaelis-Menten analysis for Na^+ and Cl^- uptake mechanisms in several freshwater-adapted teleosts. Mean ± SEM (N)

	K_m Na^+ ($\mu Eq \cdot L^{-1}$)	K_m Cl^- ($\mu Eq \cdot L^{-1}$)	J_{max} Na^+ ($nEq \cdot g^{-1} \cdot h^{-1}$)	J_{max} Cl^- ($nEq \cdot g^{-1} \cdot h^{-1}$)
Mummichog (*Fundulus heteroclitus*)[1]	1723 ± 223 (11)	>4700 (6)	2258 ± 288 (11)	>2000 (6)
Mummichog (*Fundulus heteroclitus*)[2]	2000 (8-10)	----	----	----
Trout (adult 250-400g) (*Oncorhynchus mykiss*)[3]	99 ± 15 (8)	127 ± 20 (8)	420 ± 52 (8)	286 ± 27 (8)
Trout (juvenile 8 g)[4] (*Oncorhynchus mykiss*)	138 ± 56 (8-10)	152 ± 46 (8-10)	560 ± 55 (8-10)	524 ± 81 (8-10)
Sailfin molly (*Poecilia latipinna*)[5]	3000 (15-18)	----	3800 (15-18)	----
Minnow (*Phoxinus phoxinus*)[6]	3800 (6)	----	12000 (6)	----
Goldfish (*Carassius auratus*)	300[7] (8-12)	100[8] (12-17)	650[7] (8-12)	500[8] (12-17)

[1] present study
[2] Potts & Evans (1967)
[3] Goss & Wood (1991)
[4] Postlethwaite & McDonald (1994)
[5] Evans (1973)
[6] Frain (1987)
[7] Maetz (1972, 1973)
[8] De Renzis (1975)

Figure 2.1. The influence of external [NaCl] on whole-body Na^+ (*a*) and Cl^- (*b*) uptake (J_{in}) of mummichog (solid lines) in comparison to rainbow trout values (dashed lines), the latter redrawn from Goss & Wood (1991). Mean ± 1 SEM. N = 11 for Na^+, N = 6 for Cl^-.


a
2500
2000
1500
1000
500
0
$J_{in}^{Na^+}$
Mummichog
Rainbow Trout (Goss & Wood, 1991)
nEq·g⁻¹·h⁻¹
0
2000
4000
6000
8000
10000
[NaCl]Ext (μM·L⁻¹)

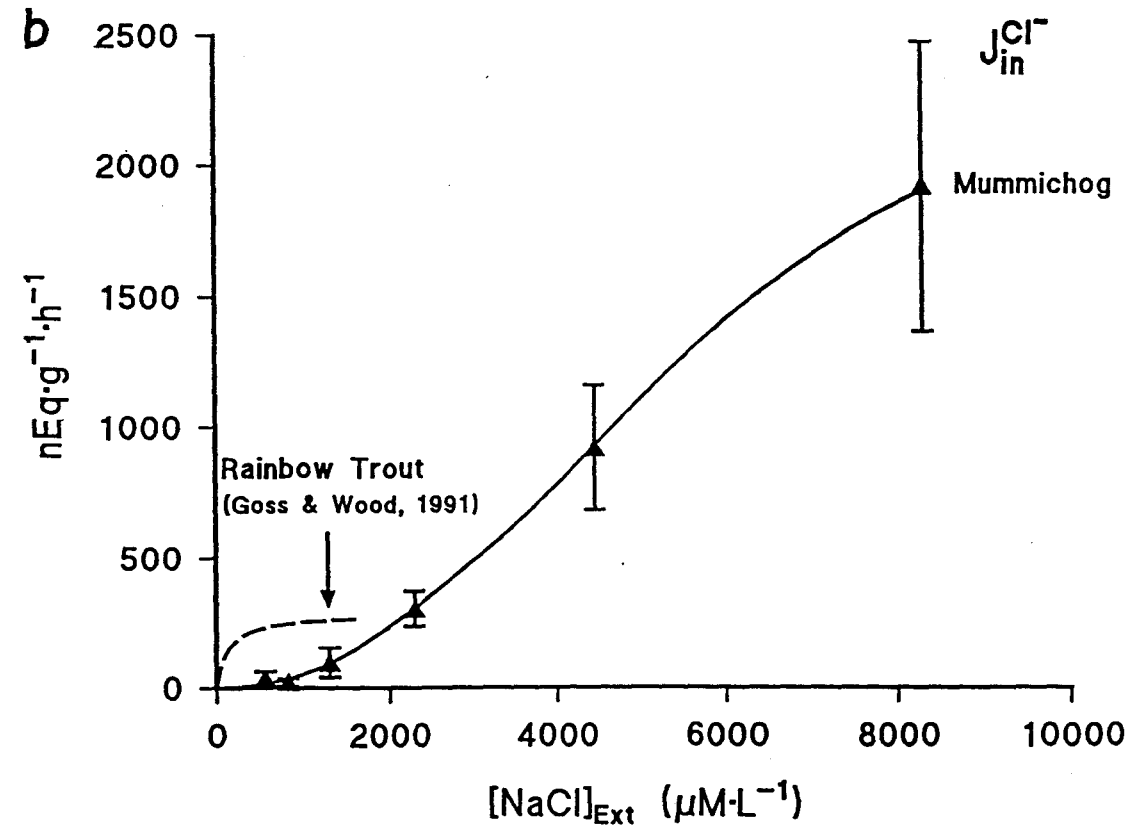

b
2500
2000
1500
1000
500
0
$J_{in}^{Cl^-}$
Mummichog
Rainbow Trout
(Goss & Wood, 1991)
nEq·g⁻¹·h⁻¹
0
2000
4000
6000
8000
10000
[NaCl]Ext (μM·L⁻¹)

Table 2.2 lists J_{max} and K_m calculated by one-substrate Michaelis-Menten analysis for Na^+ (N=11) and Cl^- (N=6) uptake in *F. heteroclitus* and also presents values measured in adult freshwater rainbow trout (Goss & Wood, 1991). The absence of saturation for Cl^- uptake restricted the analysis to an estimate of J_{max} greater than the highest mean J^{Cl}_{in} observed and an estimate of K_m greater than the $[Cl^-]_{ext}$ providing 50% of that rate. This distinction in kinetic properties demonstrates a difference between the uptake of Na^+ and Cl^- in the mummichog. K_m for both ions approximate each other in the trout but J_{max} for Na^+ is two fold greater than J_{max} for Cl^-. K_m for both ions was much higher for the mummichog indicating a much lower affinity uptake system, whereas the capacity of the system, J_{max}, was greater in comparison to trout.

Unidirectional Na^+ and Cl^- effluxes (J_{out}) were unchanged throughout the range of water [NaCl] used (Figure 2.2) suggesting that there was no coupling of J_{out} to uptake, and efflux was not influenced by external [NaCl] (*i.e.* no exchange diffusion). As $[NaCl]_{ext}$ increased, J_{net} became more positive, indicating a net gain strictly due to the large stimulation of J_{in}. This was true for both Na^+ and Cl^-. Note however, that J^{Na}_{out} (~800 $nEq \cdot g^{-1} \cdot h^{-1}$) was much larger than J^{Cl}_{out} (~200 $nEq \cdot g^{-1} \cdot h^{-1}$).

The acclimation [NaCl] was 1000 $\mu M \cdot L^{-1}$. At this point in Figure 2.1, there was a substantial J^{Na}_{in} but J^{Cl}_{in} was not significantly different from zero. J^{Na}_{out} was approximately equal to uptake suggesting that *F. heteroclitus* was in positive Na^+ balance. The actual balance point, where influx equals outflux,

Figure 2.2. The influence of external [NaCl] on whole-body Na^+ (solid line) and Cl^- (dashed line) efflux (J_{out}) of mummichog. Mean ± 1 SEM. N = 11 for Na^+, N = 6 for Cl^-.

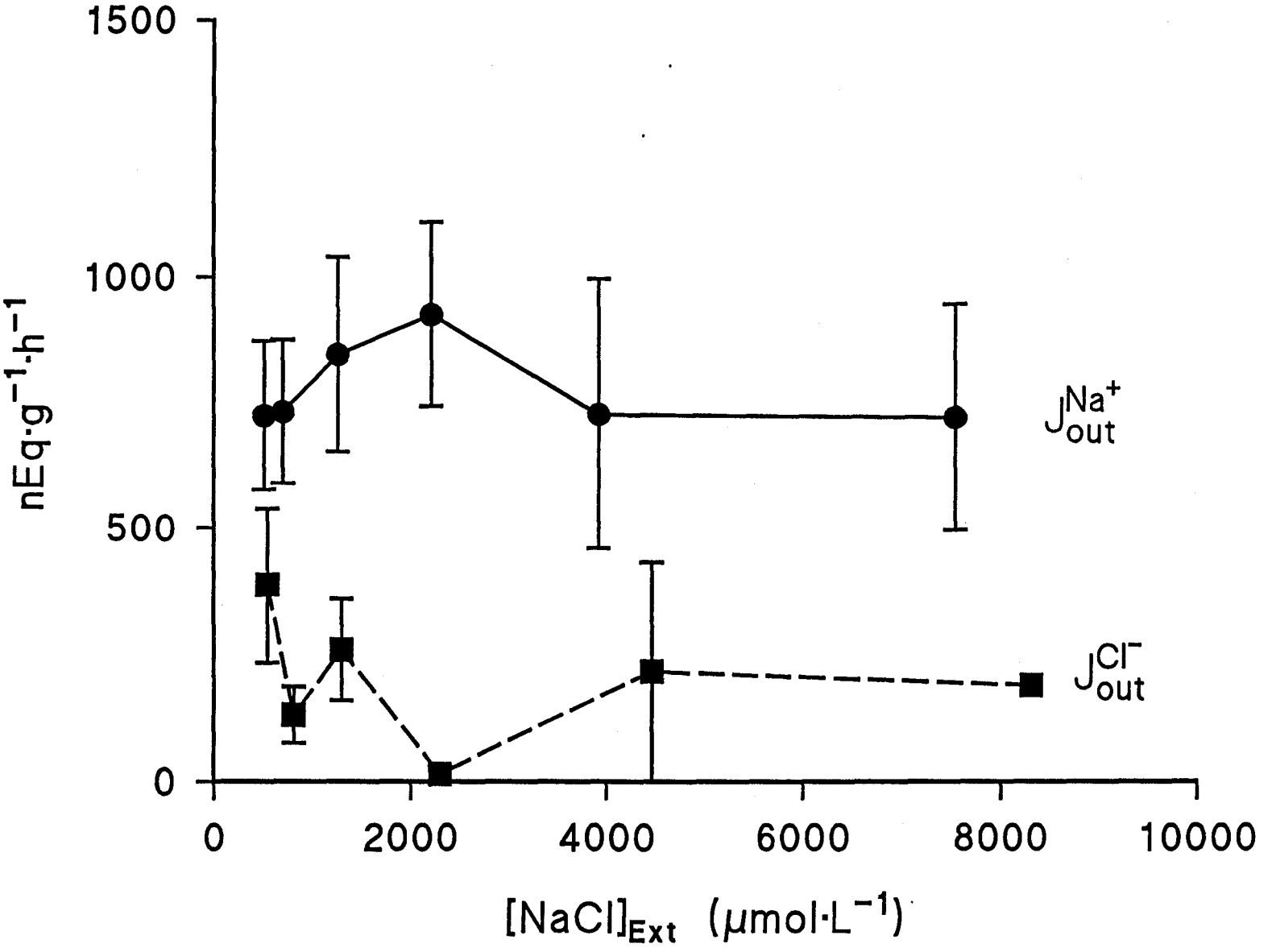
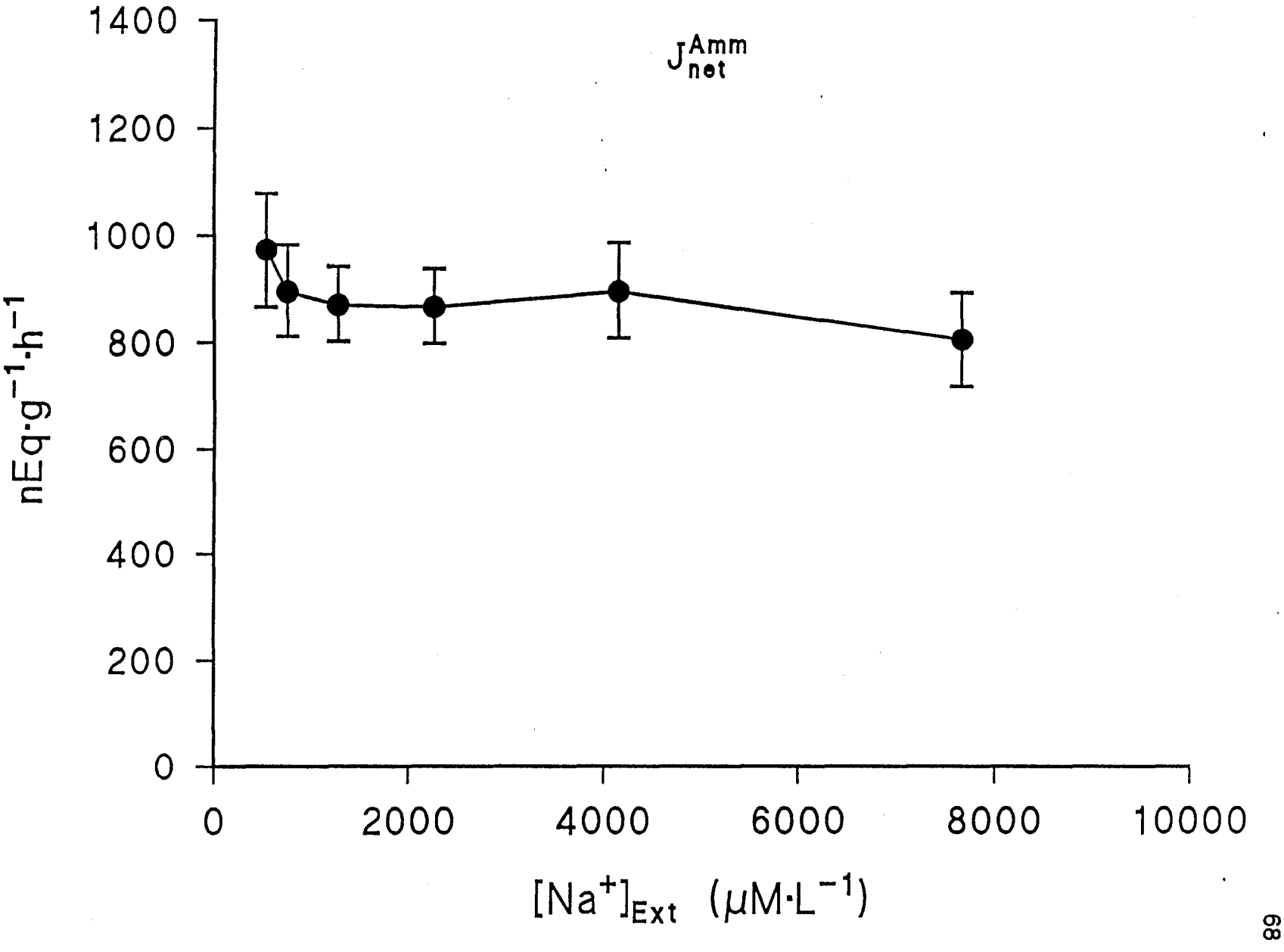

1500
1000
500
0
nEq·g⁻¹·h⁻¹
0
2000
4000
6000
8000
10000
[NaCl]Ext (µmol·L⁻¹)
J Na+ out
J Cl− out

Figure 2.3. The influence of external [NaCl] on net ammonia excretion (J^{Amm}) of mummichog. Mean ± 1 SEM. N = 16.


J_{net}^{Amm}
1400
1200
1000
800
600
400
200
0
nEq·g^{-1}·h^{-1}
0
2000
4000
6000
8000
10000
[Na$^+$]$_{Ext}$ (μM·L^{-1})

was 980 $\mu Eq \cdot L^{-1}$ $[Na^+]$, which is very similar to the acclimation level. For Cl^-, the balance point was 1620 $\mu Eq \cdot L^{-1}$ $[Cl^-]$ which is substantially higher than the acclimation [NaCl], thus the animal would have been in negative Cl^- balance due to a continuing small Cl^- loss while uptake was virtually zero.

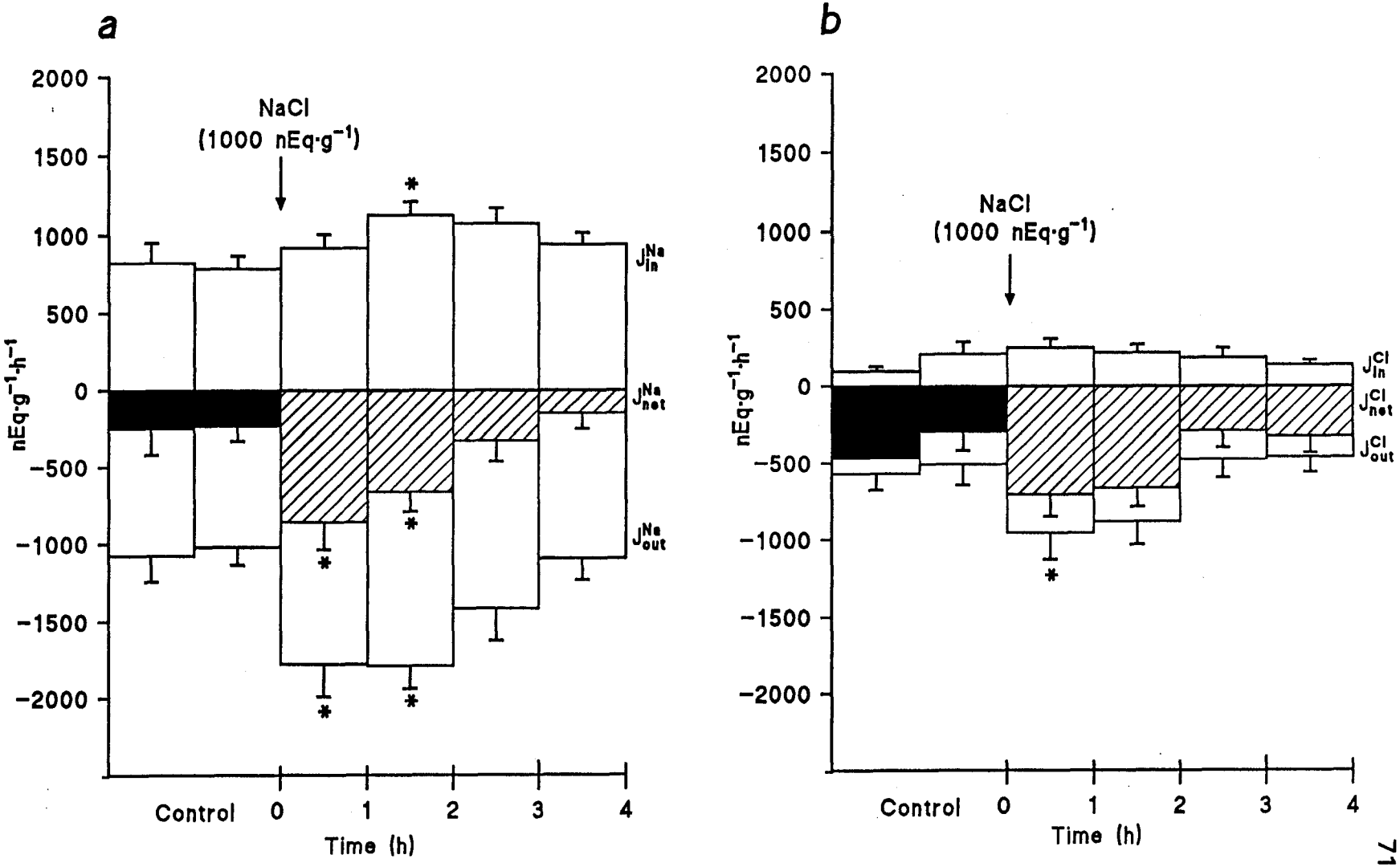
Net ammonia excretion (J_{Amm}) remained unchanged throughout the kinetic experiment (Figure 2.3) indicating that it was not influenced by $[NaCl]_{ext}$ and was not coupled to any Na^+ or Cl^- uptake mechanism, at least under these experimental conditions. Water titrations were not performed in this study therefore effects, if any, on net H^+ movement could not determined.

Injection studies

Na^+ and Cl^- unidirectional fluxes for 1000 $nEq \cdot g^{-1}$ injection of *i*) NaCl, *ii*) HCl, *iii*) $NaHCO_3$ and *iv*) 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ are presented in Figures 2.4, 2.5, 2.6 and 2.7 respectively. Note that three trials of NaCl injections were combined as there was no significant difference between the results (N=14-16). The control fluxes from all treatments (*i.e.* pre-injection) exhibited considerable differences in the amplitude between unidirectional fluxes of Na^+ and Cl^-. The sizable Na^+ outflux was compensated by a large uptake component, maintaining Na^+ balance as indicated by a small J_{net} which was not significantly different from zero. Cl^- efflux, approximately one fifth of J^{Na}_{out}, was not counterbalanced by an equal J_{in}. Therefore a larger net Cl^- loss resulted. Indeed, in several trials (*e.g.* Figure 2.4-2.7), J^{Cl}_{in} was not significantly different from zero. These

Figure 2.4. The effect of an intraperitoneal injection of 1000 $nEq \cdot g^{-1}$ NaCl on whole-body influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*). Pre-injection fluxes are represented by the solid bars and the fluxes measured following injection are indicated by cross hatching. Mean ± 1 SEM. N = 16 for Na^+, N = 14 for Cl^-.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
2000
1500
1000
500
0
−500
−1000
−1500
−2000
NaCl
(1000 nEq·g⁻¹)
nEq·g⁻¹·h⁻¹
J^{Na}_{in}
J^{Na}_{net}
J^{Na}_{out}
Control
0
1
2
3
4
Time (h)
b
2000
1500
1000
500
0
−500
−1000
−1500
−2000
NaCl
(1000 nEq·g⁻¹)
nEq·g⁻¹·h⁻¹
J^{Cl}_{in}
J^{Cl}_{net}
J^{Cl}_{out}
Control
0
1
2
3
4
Time (h)

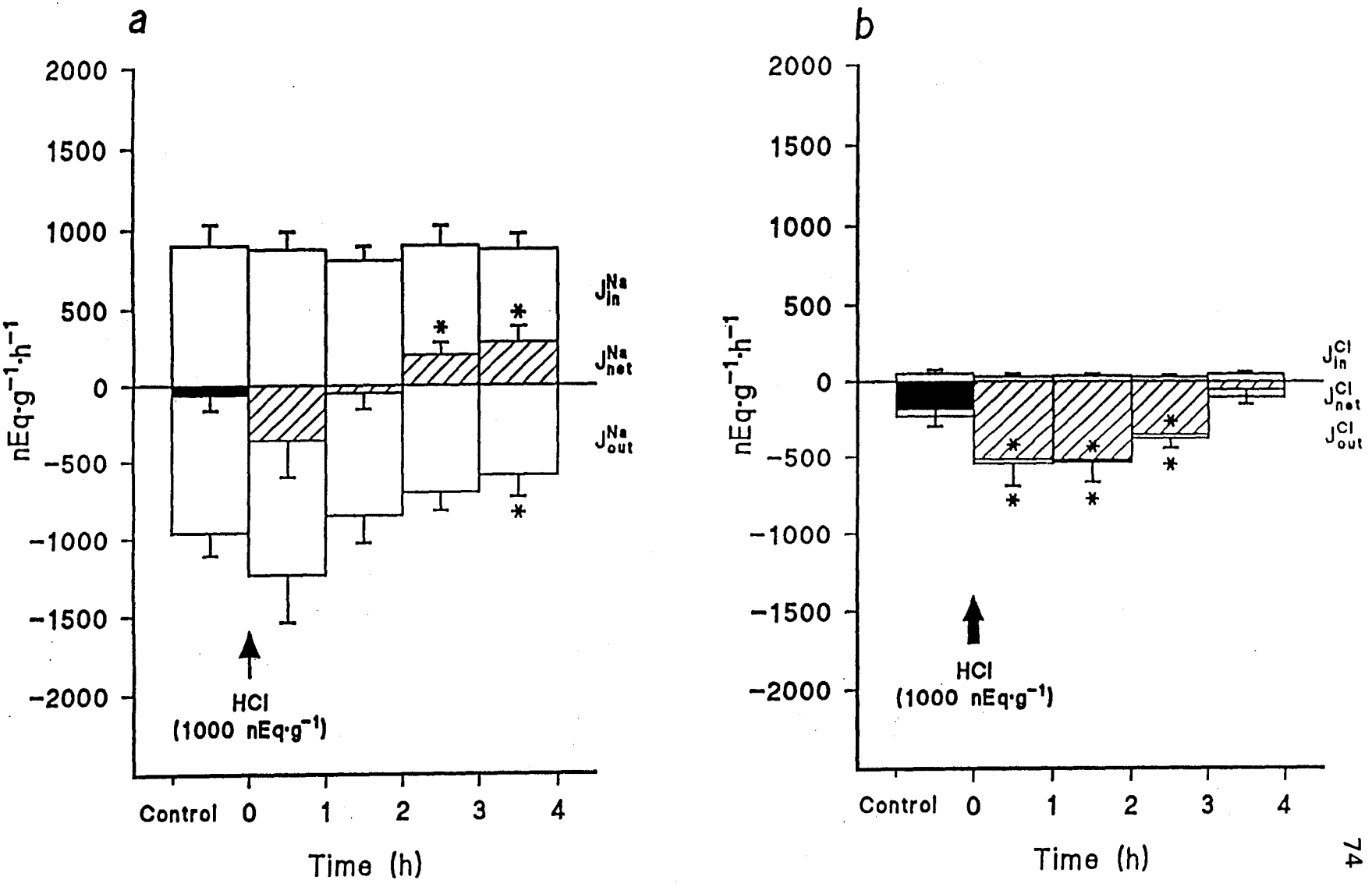
observations are in accord with the balance point analysis in the kinetic experiment.

NaCl injections induced significant increases in J_{out} for both Na^+ and Cl^- in the first 2 hours, which elevated net losses for Na^+ and Cl^-, with the increased with Na^+ net flux being significant (Figure 2.4). J_{in} for Cl^- remained unchanged throughout the 4 hours whereas Na^+ uptake was slightly but significantly stimulated at 2 hours post-injection. It seems that the isotonic salt load was removed by the increasing both Na^+ and Cl^- efflux without any contribution of decreased uptake.

Systemic acidosis was induced via intraperitoneal HCl injection as revealed by blood pH and HCO_3^- measurements in a parallel series (C.M. Wood, personal communication). In contrast to NaCl injection, Na^+ and Cl^- unidirectional effluxes were differentially adjusted (Figure 2.5*a*, *b*). Cl^- efflux was immediately stimulated resulting in a significant Cl^- loss which prevailed throughout the first 3 hours post-injection. In contrast, Na^+ efflux remained unchanged until hour 4 when it was significantly reduced. By this time, Cl^- efflux and net loss had returned to control values. The attenuated Na^+ outflux enhanced the net gain which had become significant at hour 3. Over the 4 hours, there was a net Na^+ gain and net Cl^- loss which was accomplished by differential modulation of the efflux component. Both Na^+ and Cl^- uptake remained constant.

Figure 2.5. The effect of an intraperitoneal injection of 1000 $nEq \cdot g^{-1}$ HCl on whole-body influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*). Mean ± 1 SEM. N = 9 for Na^+, N = 7 for Cl^-.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
2000
1500
1000
500
0
-500
-1000
-1500
-2000
nEq·g⁻¹·h⁻¹
J_{in}^{Na}
J_{net}^{Na}
J_{out}^{Na}
HCl
(1000 nEq·g⁻¹)
Control
0
1
2
3
4
Time (h)
b
2000
1500
1000
500
0
-500
-1000
-1500
-2000
nEq·g⁻¹·h⁻¹
J_{in}^{Cl}
J_{net}^{Cl}
J_{out}^{Cl}
HCl
(1000 nEq·g⁻¹)
Control
0
1
2
3
4
Time (h)

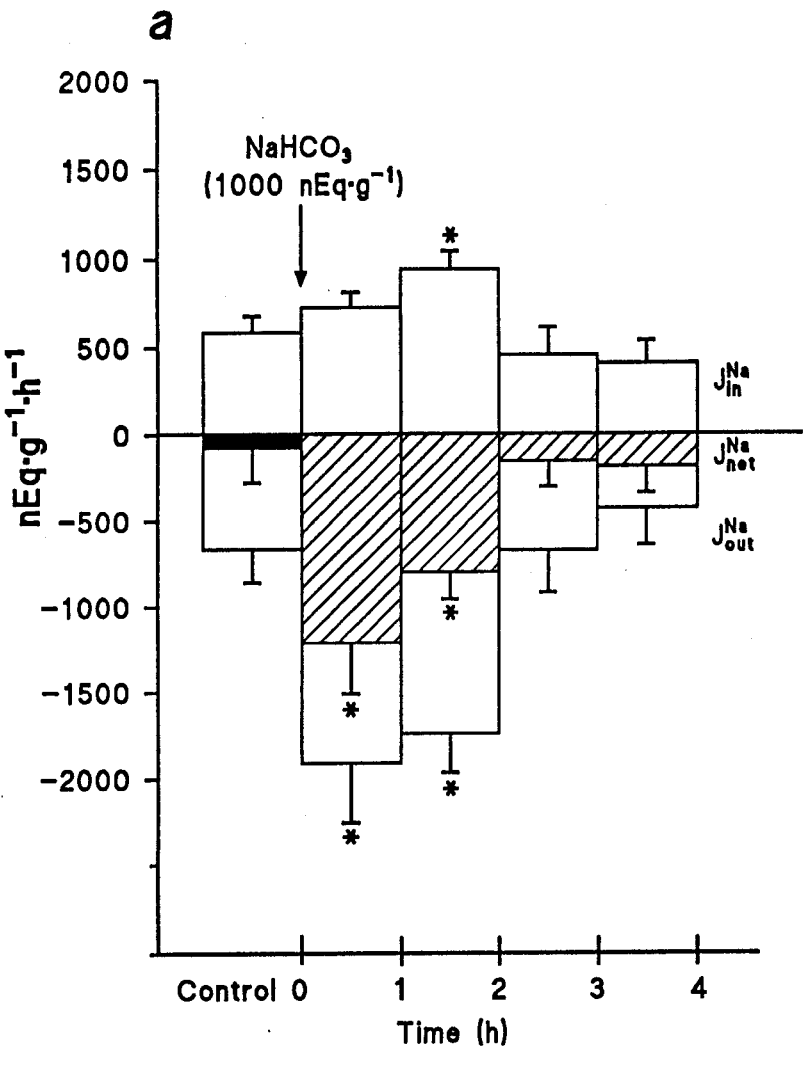
Unidirectional ion fluxes following the 1000 nEq·g^{-1} $NaHCO_3$ injection are exhibited in Figure 2.6. Na^+ and Cl^- efflux and net loss were significantly elevated in the first two hours with Cl^- continuing to be elevated into hour 3. Again, Cl^- uptake remained unchanged whereas Na^+ uptake did become stimulated at hour 2, but decreased to below control values in hours 3 and 4. As in the HCl injection, the efflux components were the sole contributors to the significant net losses of Na^+ and Cl^- but in contrast, there was no differential adjustment of Na^+ and Cl^- outflux.

The $NaHCO_3$ injection study was repeated but the base load was tripled (*i.e.* 3000 nEq·g^{-1}), an experiment performed in water with a ten-fold higher $[Ca^{2+}]$ and a slightly lower $[Na^+]$ and $[Cl^-]$ than the previous three experiments. Under control conditions (*i.e.* pre-injection), unidirectional ion fluxes of experiment *iv* (Figure 2.7) were similar to *i, ii* and *iii* (Figure 2.4, 2.5, 2.6). After injection of 3000 nEq·g^{-1} $NaHCO_3$, only the Na^+ efflux and net flux were significantly stimulated during the first hour, and these were much smaller than the changes seen with 1000 nEq·g^{-1} injection in Figure 2.6. In fact, these were the only notable changes with the larger base load. Perhaps the greater $[Ca^{2+}]_{ext}$ may attenuate the mummichog's ability to modulate the efflux component by reducing permeability across the body surface.

Acid-base (*a*) and net Na^+ and Cl^- (*b*) fluxes are presented in Figures 2.8, 2.9, 2.10 and 2.11 for injections *i* - *iv* respectively. Note for experiments *i* - *iii*, acid-base fluxes are the combined results of all trials for each injection series

Figure 2.6. The effect of an intraperitoneal injection of 1000 $nEq \cdot g^{-1}$ $NaHCO_3$ on whole-body influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*). Mean ± 1 SEM. N = 8.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
2000
1500
1000
500
0
−500
−1000
−1500
−2000
NaHCO3
(1000 nEq·g−1)
nEq·g−1·h−1
Jin Na
Jnet Na
Jout Na
Control 0
1
2
3
4
Time (h)

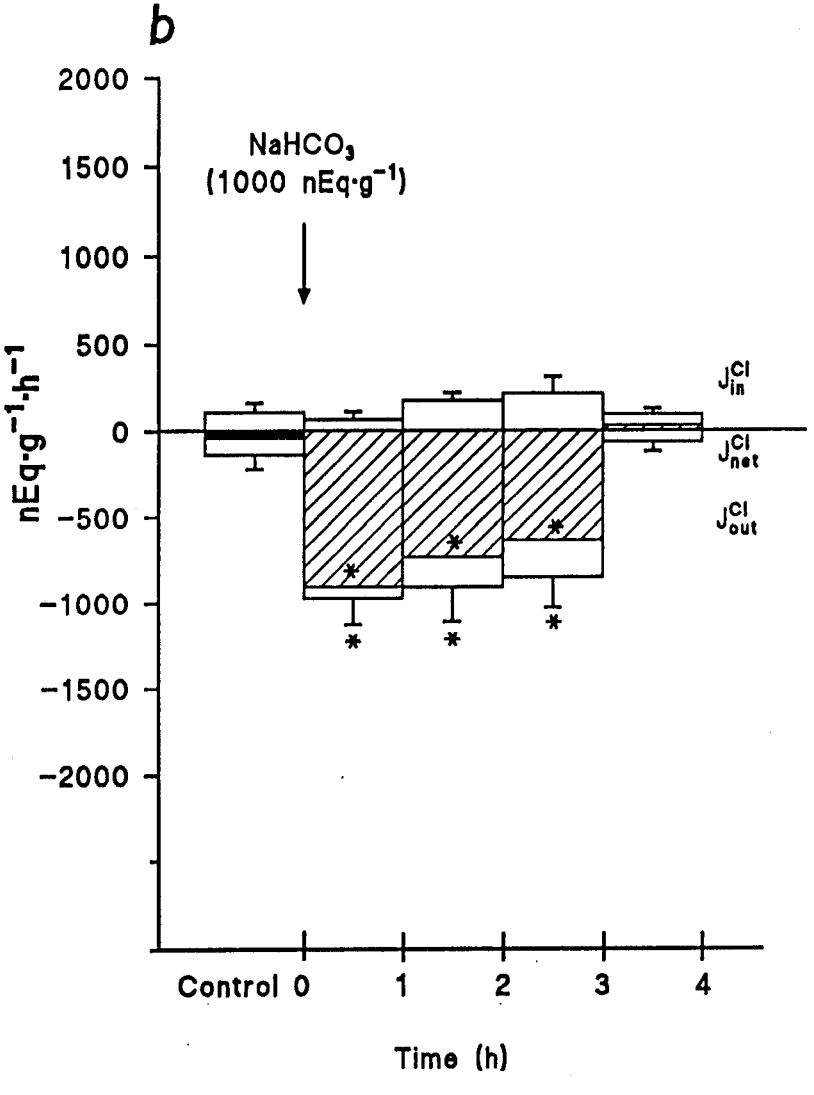
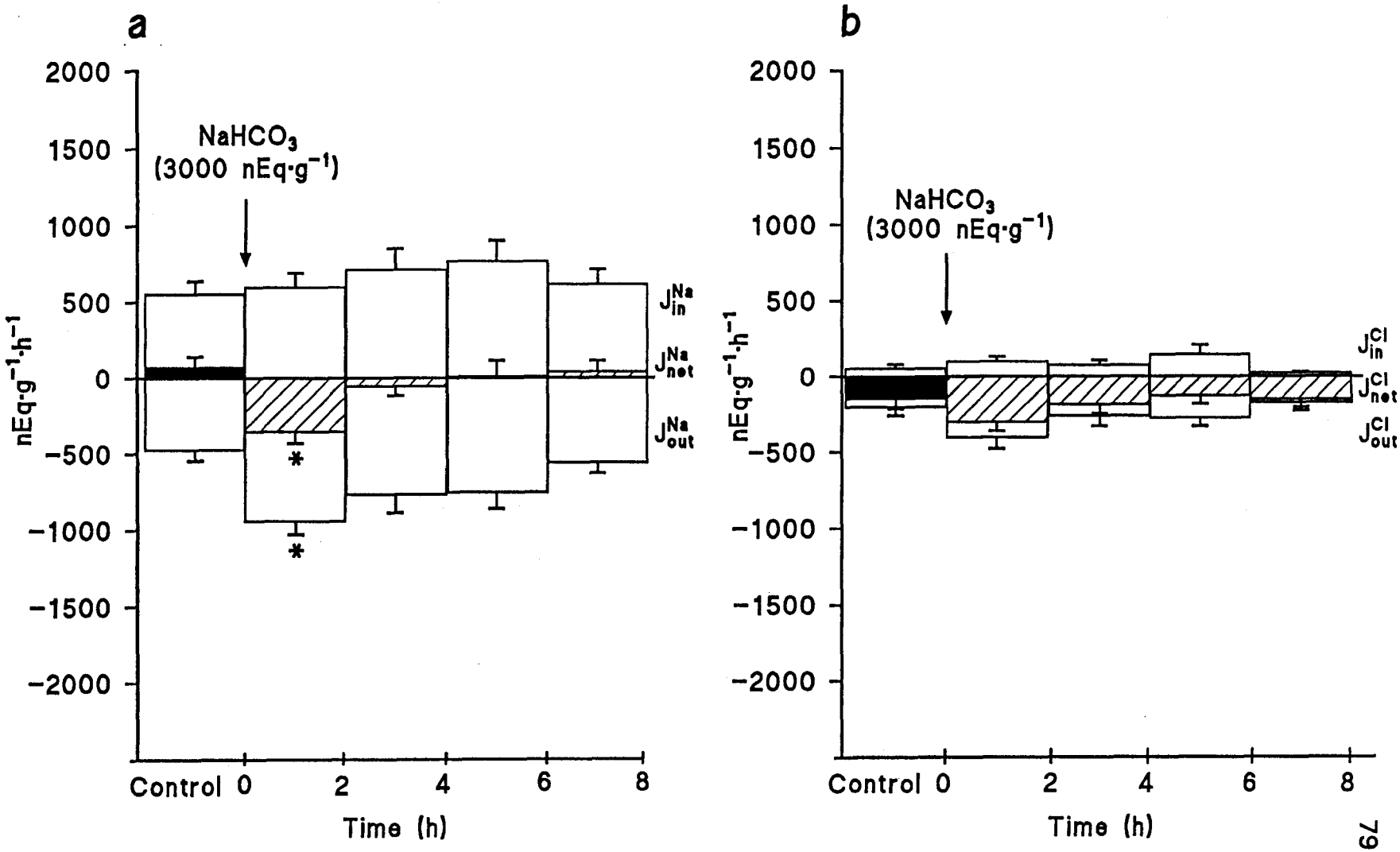

b
2000
1500
1000
500
0
−500
−1000
−1500
−2000
NaHCO3
(1000 nEq·g−1)
nEq·g−1·h−1
Jin Cl
Jnet Cl
Jout Cl
Control 0
1
2
3
4
Time (h)

Figure 2.7. The effect of an intraperitoneal injection of 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ on whole-body influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*). Mean ± 1 SEM. N = 8. Note that the time scale is 8 hours for this experiment.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
2000
1500
1000
500
0
−500
−1000
−1500
−2000
NaHCO3
(3000 nEq·g−1)
nEq·g−1·h−1
Jin Na
Jnet Na
Jout Na
*
*
Control 0
2
4
6
8
Time (h)
b
2000
1500
1000
500
0
−500
−1000
−1500
−2000
NaHCO3
(3000 nEq·g−1)
nEq·g−1·h−1
Jin Cl
Jnet Cl
Jout Cl
Control 0
2
4
6
8
Time (h)

Comparison of net ion and acid-base fluxes requires the use of the Strong Ion Difference Theory (SID) as described by Stewart (1983). The difference between total strong anion and cation concentrations is an important determinant of acid-base status within a compartment such as a cell. Chapter 1 of this thesis explains how this theory has been applied to net ion movement across the teleost body *in vivo*. Na^+ and Cl^- are considered to be the major strong cations and anions respectively. If this is true, then an excess net Na^+ loss over Cl^- loss dictates a net base loss or acid uptake whereas an excess of Cl^- loss over Na^+ loss signifies a net acid loss or base uptake. (*i.e.* $J^H = J^{Cl} - J^{Na}$, in terms of net fluxes). The SID flux thereby provides an independent measure of the net acidic equivalent flux for comparison with that measured by titration.

Looking at control acid-base fluxes in all of the treatments, net H^+ excretion was essentially zero because net ammonia excretion (J^{Amm}) was counterbalanced by an equal net base excretion (J^{TA}). From this, one can postulate that most of the titratable alkalinity measured was likely ammonia in its free-base form (NH_3).

NaCl injection (Figure 2.8*a*) caused significant increases in ammonia excretion in the first and third hour and a slight but not significant elevation in net H^+ excretion in the third hour. The lack of a persistent acid-base disturbance is also supported by a negligible difference between net Na^+ and Cl^- losses throughout the 4 hours following injection (Figure 2.8*b*).

Figure 2.8. The effect of an intraperitoneal injection of 1000 $nEq \cdot g^{-1}$ NaCl on whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net fluxes of Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) (*b*). Mean ± 1 SEM. N = 16 for figure *a*; Na^+: N = 16, Cl^-: N = 14 for figure *b*.

* denotes a significant difference from the control rates ($p \leq 0.05$).

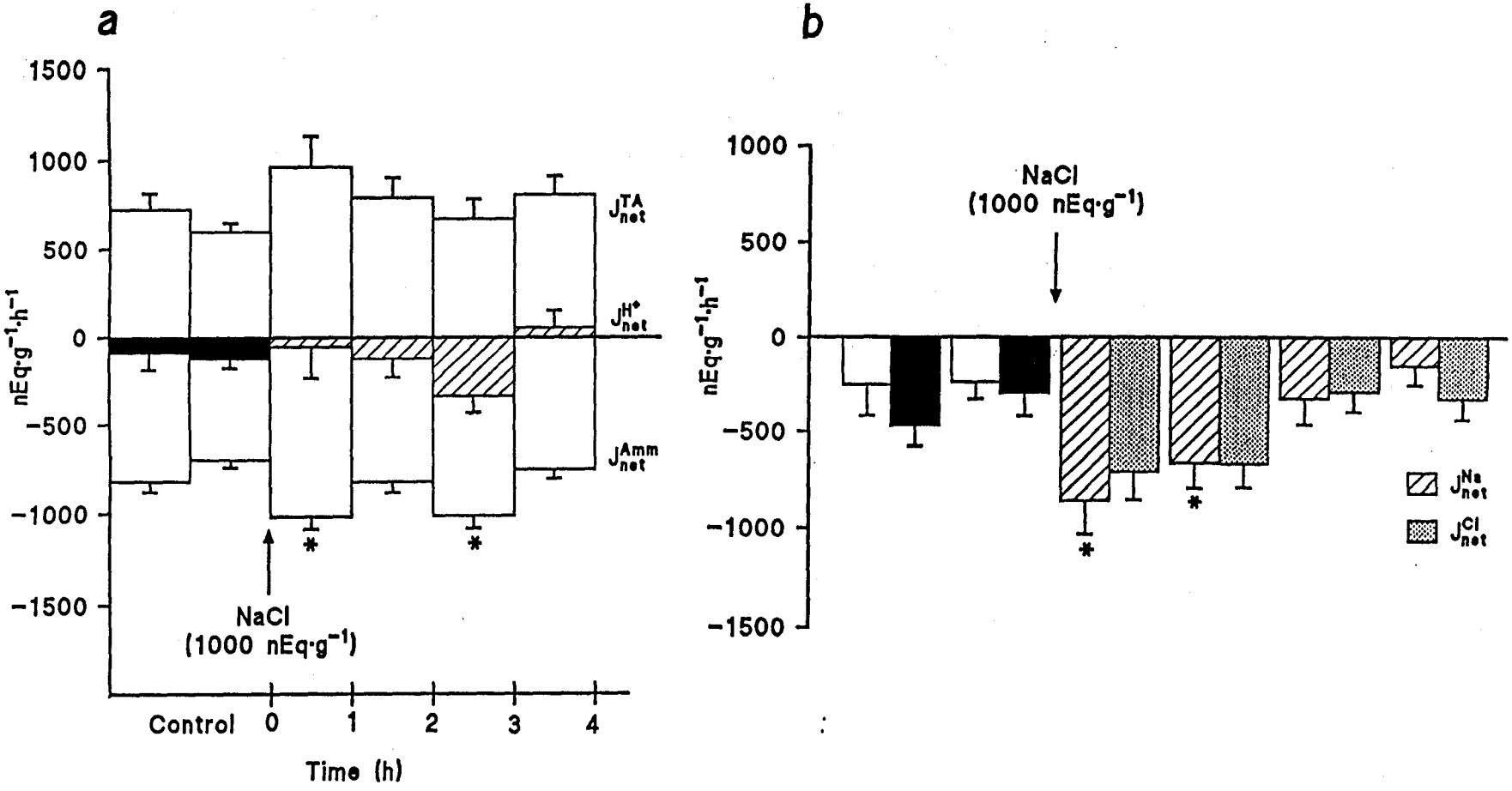
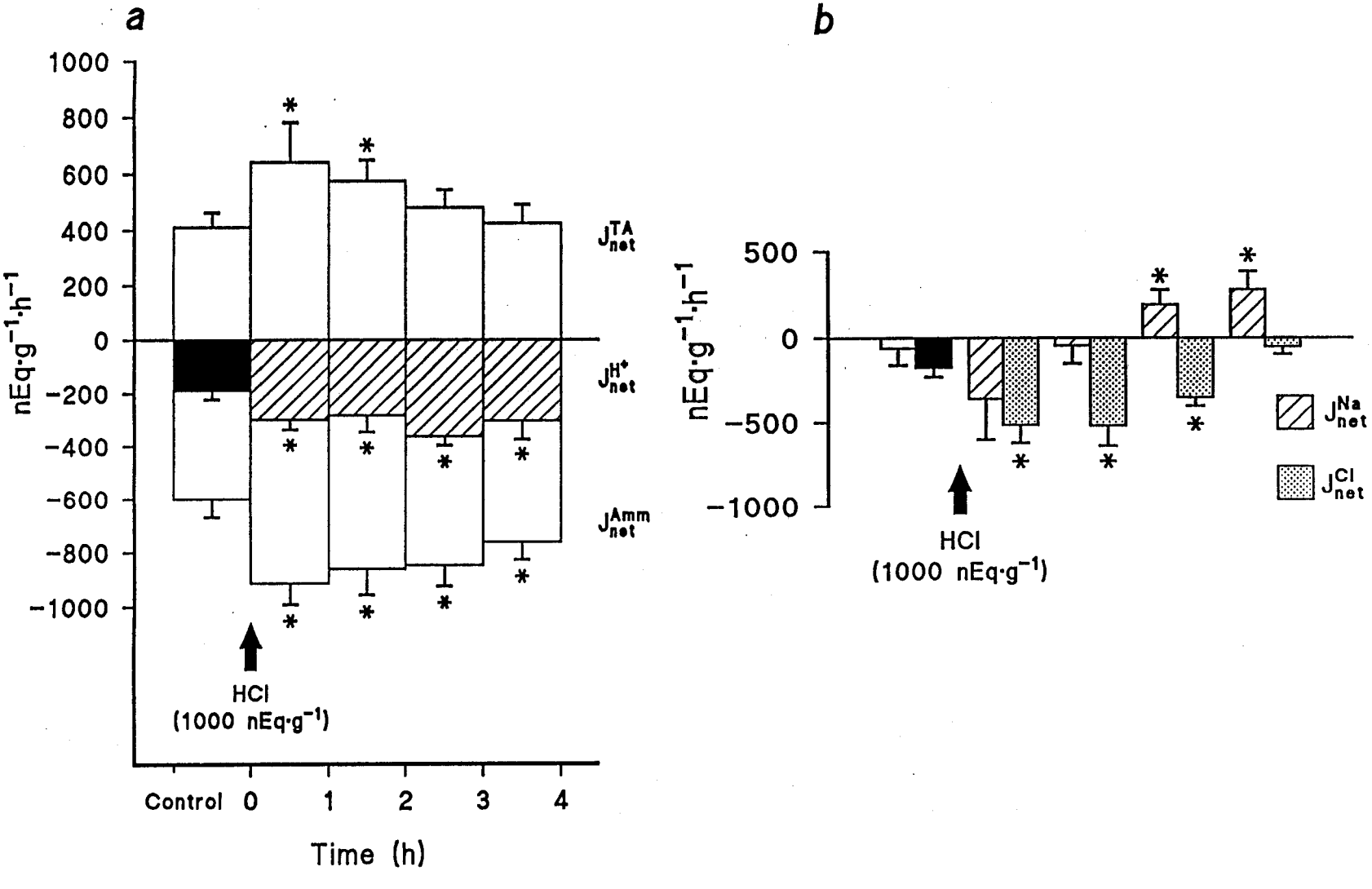

a
1500
1000
500
0
−500
−1000
−1500
nEq·g⁻¹·h⁻¹
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
NaCl
(1000 nEq·g⁻¹)
Control
0
1
2
3
4
Time (h)
b
1000
500
0
−500
−1000
−1500
nEq·g⁻¹·h⁻¹
NaCl
(1000 nEq·g⁻¹)
J^{Na}_{net}
J^{Cl}_{net}

Figure 2.9. The effect of an intraperitoneal injection of 1000 nEq·g^{-1} HCl on whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net fluxes of Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) (*b*). Mean ± 1 SEM. N = 9 for figure *a*; Na^+: N = 9, Cl^-: N = 7 for figure *b*.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
b
1000
800
600
400
200
0
−200
−400
−600
−800
−1000
nEq·g⁻¹·h⁻¹
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
HCl
(1000 nEq·g⁻¹)
Control
0
1
2
3
4
Time (h)
500
0
−500
−1000
J^{Na}_{net}
J^{Cl}_{net}

A very different story emerges from the acid injection (Figure 2.9*a*). A significant increase in net H^+ excretion was initiated within the first hour after injection and remained at the same elevated level through 4 hours. Ammonia excretion followed the same pattern whereas J^{TA} initially increased but returned to a control rate by the third hour. With respect to SID, the greater net Cl^- loss over Na^+ (Figure 2.9*b*) also implies a net acid loss. By the fourth hour, there was a net Na^+ gain while net Cl^- flux resumed its pre-injection level. By calculating the total amount of H^+ excreted, above the control rate, over the 4 hours, it seems that the mummichog was able to excrete almost half of the acid load (*i.e.* ~475 $nEq \cdot g^{-1}$). In the same manner, the difference between net Cl^- and Na^+ fluxes above the control value, over the 4 hours, shows that this divergence (*i.e.* ~800 $nEq \cdot g^{-1}$) could easily account for all of the acid excreted.

Neither 1000 nor 3000 $nEq \cdot g^{-1}$ injection of $NaHCO_3$ resulted in any disruption or adjustment in the measured acid-base fluxes (Figure 2.10*a*, 2.11*a*). Net H^+ excretion was unchanged in both experiments even with the flux periods extended to 8 hours post-injection (Figure 2.11*a*). There appeared to be a slight but not significant net acid excretion with the 3000 $nEq \cdot g^{-1}$ base load and zero net acid excretion with 1000 $nEq \cdot g^{-1}$. Net acid flux, as independently estimated by net Na^+ and Cl^- fluxes also confirmed the absence of an acid-base disturbance. These results including the unidirectional ion fluxes (Figures 2.6, 2.7) suggest that either the injection did not induce a substantial alkalosis or that the mummichog may cope with a base load by internal mechanisms. An

Figure 2.10. The effect of an intraperitoneal injection of 1000 $nEq \cdot g^{-1}$ $NaHCO_3$ on whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net fluxes of Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) (*b*). Mean ± 1 SEM. N = 8 for figure *a*; N = 8 for figure *b*.

* denotes a significant difference from the control rates ($p \leq 0.05$).

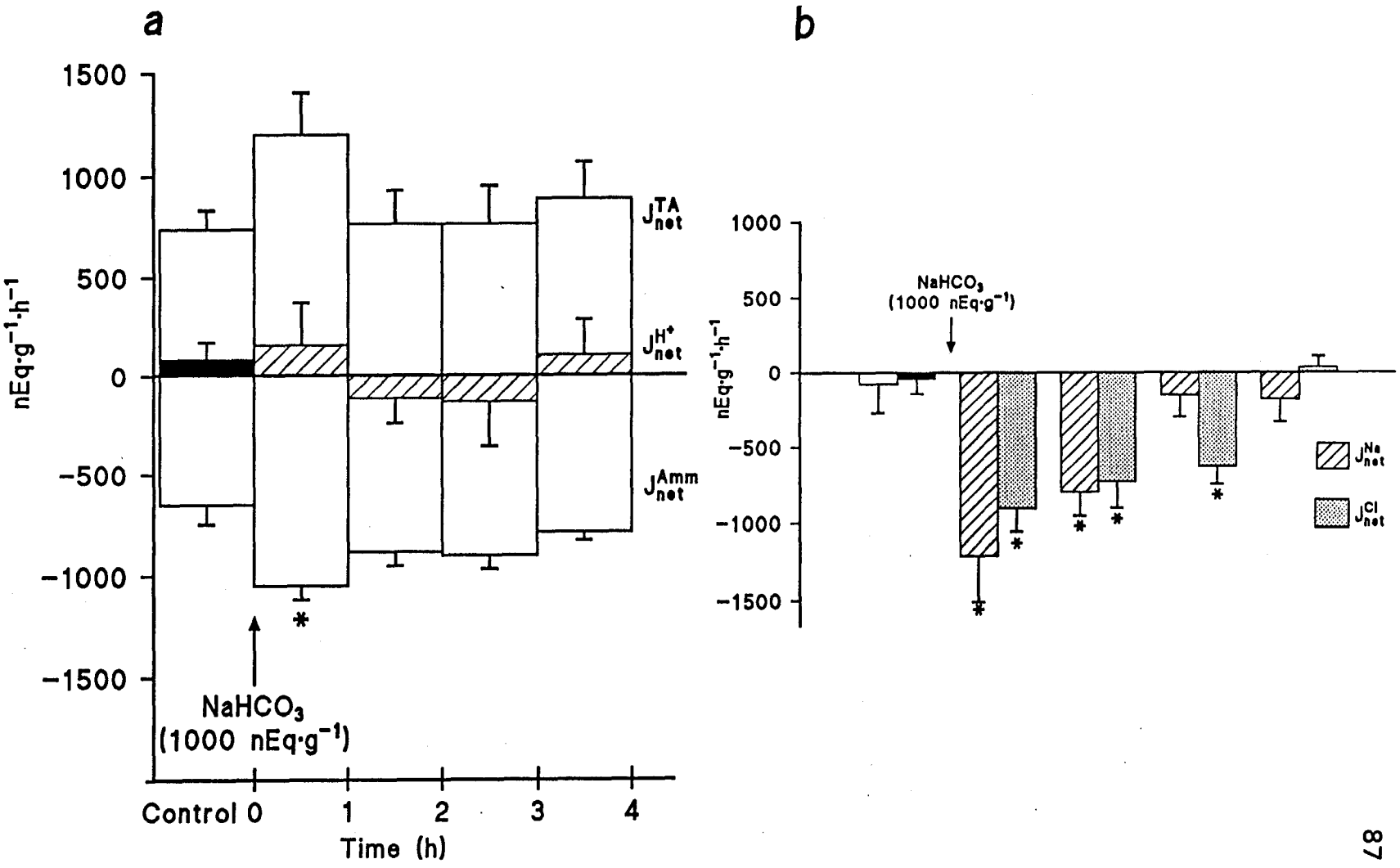

a
b
1500
1000
500
0
−500
−1000
−1500
nEq·g⁻¹·h⁻¹
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
NaHCO₃
(1000 nEq·g⁻¹)
Control 0
1
2
3
4
Time (h)
*
J^{Na}_{net}
J^{Cl}_{net}

Figure 2.11. The effect of an intraperitoneal injection of 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ on whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net fluxes of Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) (*b*). Mean ± 1 SEM. N = 8 for figure *a*; N = 8 for figure *b*. Note the time scale is 8 hours for this experiment.

* denotes a significant difference from the control rates ($p \leq 0.05$).

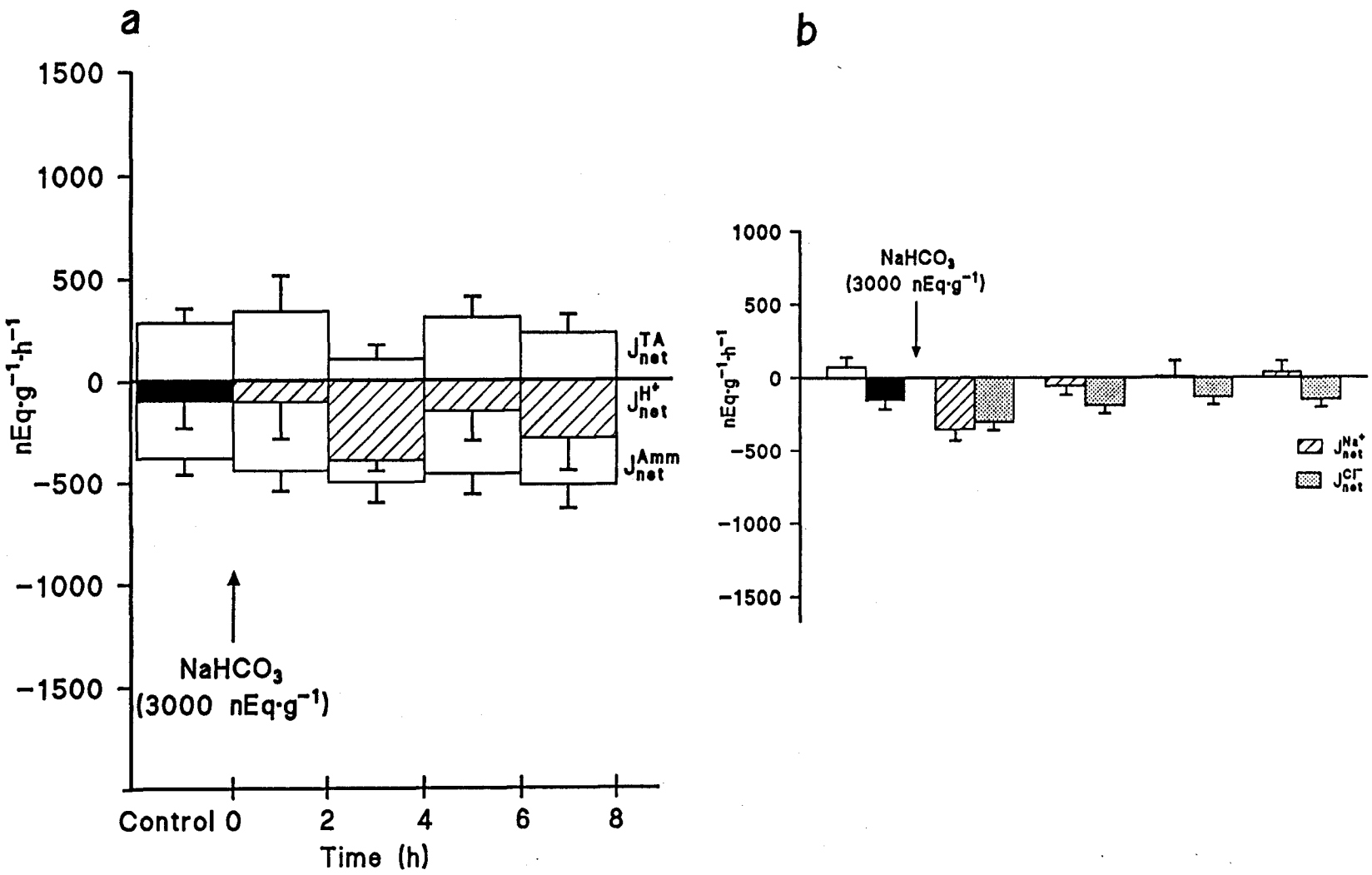

a
b
1500
1000
500
0
−500
−1000
−1500
nEq·g⁻¹·h⁻¹
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
NaHCO₃
(3000 nEq·g⁻¹)
Control 0
2
4
6
8
Time (h)
$J^{Na^+}_{net}$
$J^{Cl^-}_{net}$

Table 2.3: Urea excretion rates (nmol $N \cdot g^{-1} \cdot h^{-1}$) from the 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ injection experiment (series *iv*). Mean ± SEM, N = 7.

	Injection ⇓			
Control	0-2 h	2-4 h	4-6 h	6-8 h
50.32 ± 21.52	20.32 ±16.01	21.38 ±11.42	11.08 ± 5.52	22.42 ± 9.40

alternative route for HCO_3^- removal in some teleosts may be via urea excretion. However, urea excretion rate measured in the 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ injection study was unchanged throughout 8 hours following injection (Table 2.3)

SEM studies

Both gill and opercular epithelia from *F. heteroclitus* were examined using SEM. It should be noted that the purpose of this investigation was not to perform morphometry and quantify cell types and size but rather observe any physical differences between the two tissues. Figure 2.12 *a* and *b* are photomicrographs of the leading and trailing edge respectively of the gill filament at different magnifications. The leading edge (Figure 2.12*a*) is composed mostly of pavement cells which are characterized by the microridges that form a fingerprint pattern. Mucous cells are located at the intersection between four adjacent pavement cells and are very few in number. In contrast, the trailing edge (Figure 2.12*b*) possesses more mucous cells which can be clustered in groups of 2 or 3. In addition to mucous and pavement cells are what appear to be holes or pits that are found between adjacent pavement cells or the junction between pavement and mucous cells. These pits are less numerous than the mucous cells which are in turn fewer than the pavement cells.

Figure 2.12. Scanning electron micrographs of the leading edge of *F. heteroclitus* gill filament showing secondary lamellae (*a*) and the trailing edge epithelium (*b*). In micrograph *a*, the black arrows point to mucous cells; *b*, white arrows point to the mucous cells and black arrows point to the openings of apical crypts of chloride cells. Note that no apical crypts were present on the leading edge. pvc = Pavement cell; bar = 10 micrometers.
Magnification: 1870 X (*a*), 2820 X (*b*).

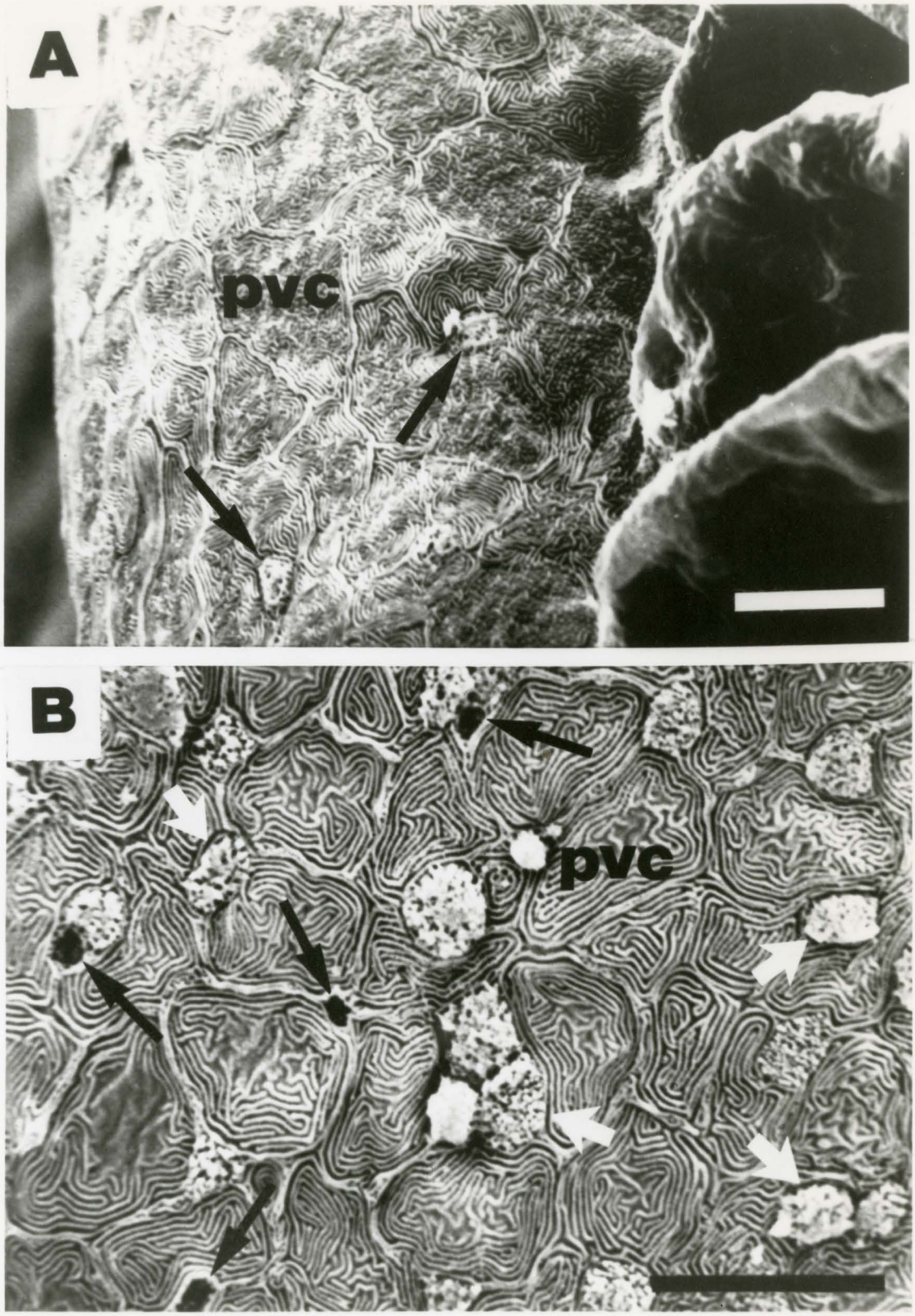

A
pvc
B
pvc

Figure 2.13. Scanning electron micrographs of the mucosal aspect of opercular epithelium of *F. heteroclitus.* The black arrows indicate openings of apical crypts of chloride cells and white arrows, mucous cells. Note the asterisks (*) in *b* bring attention to the "bumps" underlying the epithelium. pvc = Pavement cell; bar = 10 micrometers.

Magnification: 2950 X (*a*), 2740 X (*b*).

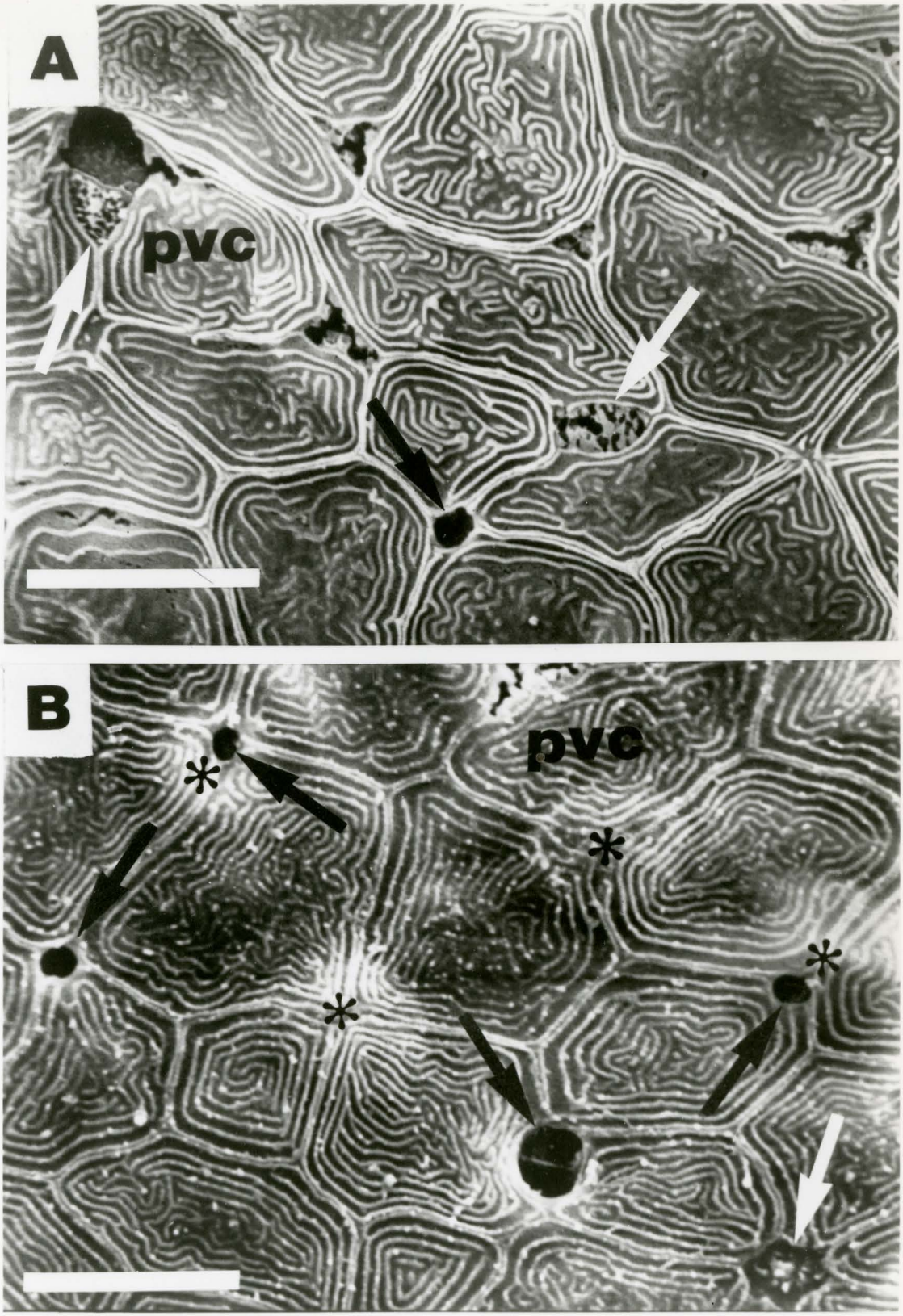

A
pvc
B
pvc

Figure 2.13 *a* and *b* are SEM shots of *F. heteroclitus* opercular epithelium (OE), specifically the apical surface which is normally exposed to water. The cell types are more or less the same as found on the gill epithelia (Figure 2.12*a*, *b*). Pavement cells predominate with mucous cells interspersed. The mucous cells are not identical in surface structure as on the gill epithelia. This may be due to the difference in the geometry of the epithelia on gills and operculum. The opercular epithelium is flat and perhaps may have become taut during the fixation or dehydration steps. In any case, mucus is apparent on the apical surface of the gill epithelia, but not on the OE. Pits that were noticed on the trailing edge of the gill filament are also present on the OE and are approximately the same diameter (~1.42μ for gill; ~1.73μ for OE). One characteristic that was not evident in the gill (Figure 2.12.*b*) but is in Figure 2.13*b* are bumps underlying the opercular epithelia which occur consistently at the junctions of pavement cells. Some are located directly under the pits. These bumps may in fact be chloride cells, and these apical pits may be their only contact with the external medium. Comparison to other ultrastructural studies of the gills and OE of freshwater-adapted teleosts including *F. heteroclitus* will be pursued in the discussion.

Discussion

Na^+ and Cl^- fluxes under control conditions

It is evident from the uptake kinetics (Figure 2.1, 2.2) and all four injection series (Figure 2.4, 2.5, 2.6, 2.7) that under control conditions ([NaCl] = 1000 $\mu M \cdot L^{-1}$), Na^+ unidirectional fluxes are substantial whereas Cl^- fluxes are much smaller, with influx completely absent. The large Na^+ efflux component may be a residual effect of the mummichogs' strategy for ionoregulation in seawater in which cation permeable paracellular pathways allow Na^+ to move passively outward (see Chapter 1, Figure 1.2). Mummichogs may not be able to abolish this "leakiness" in fresh water, thus constraining a large uptake component to remain in Na^+ balance. This is not the case for Cl^- as there is virtually no uptake component and Cl^- efflux is a small but finite value. Therefore the fish is in negative Cl^- balance. Mummichogs must rely on diet as a singular Cl^- source. Euryhaline eels (*Anguilla anguilla* and *A. rostrata*) also lack Cl^- uptake while in freshwater, an effect which is reflected in the disparity between Na^+ and Cl^- plasma levels (~148 vs 105 $mEq \cdot L^{-1}$) (Farrell & Lutz, 1975; Bornancin *et al.* 1977; Hyde & Perry, 1987). Hannah & Pickford (1981) reported a similar deficit of 40 mEq Cl^- in *F. heteroclitus*. The question is what anion is present to balance charge within the blood. Eels have unusually high plasma HCO_3^- levels (~11$mEq \cdot L^{-1}$) but this does not completely eliminate the charge difference and makes for an alkalotic blood pH (Bornancin *et al.* 1977). Plasma [HCO_3^-] is much lower (4.7 $mEq \cdot L^{-1}$) for FW mummichogs and would not contribute greatly

to overall anion charge (C.M.Wood, personal communication). Unfortunately, plasma Na^+ and Cl^- concentrations were not measured in this study, consequently the actual plasma charge difference for these fish is unknown. Lactate is another anion but would only be prevalent after vigorous exercise and thus is not a probable candidate (Hannah & Pickford, 1981).

Na^+ and Cl^- uptake kinetics

The kinetic parameters determined for *F. heteroclitus* indicate a high capacity but low affinity (high K_m) Na^+ uptake system. Cl^- uptake parameters could only be estimated, and were indicative of a greater capacity and a lower affinity than Na^+ (*i.e.* higher K_m). Table 2.1 and 2.2 demonstrates that Cl^- uptake, if it saturates at all, does so at a much higher external salt concentration which is well beyond the freshwater range (*i.e.* > 2% sea water). Cl^- uptake was still increasing at 25000 $\mu mol \cdot L^{-1}$ NaCl (5% seawater). This discrepancy between Na^+ and Cl^- kinetic properties suggests that entirely different mechanisms may be responsible for Na^+ and Cl^- uptake. In adult rainbow trout, despite being independent processes, Na^+ and Cl^- uptakes have identical K_m values (Table 2.2) and are both saturated within a lower $[NaCl]_{ext}$ range at similar J_{max} values (Figure 2.1). Postlethwaite & McDonald (1994) reported K_m values that approximated those of the adult trout but J_{max} for Cl^- uptake in juveniles was almost twice that found in adult trout (Goss & Wood, 1991). The goldfish (*Carassius auratus*), also displays a relatively high affinity

and low capacity for Na^+ (Maetz, 1972, 1973) and Cl^- (DeRenzis, 1975; DeRenzis & Maetz, 1973) uptake although the K_m values for Na^+ are larger than for Cl^-. In comparison to the rainbow trout and goldfish, both of which have contributed immensely to the development of the FW ion transport model (Figure 1.3), it is evident that mummichog ion absorption properties depart from what has been established for other freshwater-adapted fish.

It has been suggested that the affinity for ion uptake is correlated with the adaptability of a particular species to freshwater (Potts & Parry, 1964; Evans, 1973). Potts & Evans (1967) were the only other group to measure Na^+ uptake in *F. heteroclitus* in freshwater using kinetic analysis (Table 2.1). The K_m value which they reported closely approximates the present study, confirming that Na^+ uptake is a low affinity transport mechanism in the mummichog. This finding suggests that *F. heteroclitus* is not ideally equipped for fresh water life. Table 2.1 lists two other teleosts which can be found in fresh water and possess K_m values that are even higher than mummichog. The sailfin molly (*Poecilia latipinna*), a normally stenohaline marine teleost, has been known to reside in fresh water bodies in Florida (Evans, 1973) whereas the minnow (*Phoxinus phoxinus*) is a stenohaline fresh water species (Frain, 1987). Perhaps ion affinity is not a reliable indicator for "adaptedness" to fresh water. Na^+ transport capacity (J_{max}) for all three are much higher than that measured in rainbow trout and goldfish but again, this does not necessarily indicate proficiency of ion uptake. Kinetic studies of several species cannot be used as a scale of

ionoregulating ability in fresh water but can only characterize the mechanisms at work within each particular species.

Saturable ion uptake is suggestive of a carrier-mediated process which may be the case for Na^+ absorption in *F. heteroclitus*. Because Cl^- uptake did not reach a maximum within freshwater [NaCl] values, a mechanism other than carrier transport is probable. A very similar curve for Cl^- uptake has been reported in amphibian epithelium *in vitro*, with saturation occurring at ~ 60 $mmol \cdot L^{-1}$ Cl^- (Harck & Larsen, 1986). Alvarado *et al.* (1975) found that measuring kinetics of Cl^- in amphibians is complicated because uptake occurs passively through anion-selective channels located in the apical membrane. Cl^- conductance (*i.e.* permeability) is very low when the skin is bathed in dilute [Cl^-] and is attributed to closed anion channels. When $[Cl^-]_{ext}$ is increased, these channels open allowing passive influx, hence increasing permeability of the skin. In toad (*Bufo bufo*) skin, at [NaCl] greater than 5000 $\mu mol \cdot L^{-1}$ and a TEP of 20-40 mV, inside positive, these channels will open and under *in vivo* conditions, these vital [NaCl] and TEP values exist (Harck & Larsen, 1986). Similar apical channels may very well exist in *F. heteroclitus* and could account for the results of the present study. For instance, apical Cl^- channels would be closed under acclimation conditions ([NaCl] = 1000 $\mu mol \cdot L^{-1}$) as indicated by zero uptake in Figure 2.1*b*. At 4000 $\mu mol \cdot L^{-1}$ NaCl, Cl^- uptake is stimulated which could signify the opening of the channels, allowing the inward movement of Cl^-. The only drawback with this idea is both transepithelial potential across

the opercular epithelium (Marshall *et al.* 1994) and transgill potential (Pic, 1978) are inside negative (by 30-70 mV) for FW mummichogs, which would not favour passive, inward Cl^- movement.

A carrier-mediated, self exchange of ions (Na^+/Na^+ and Cl^-/Cl^-), known as exchange diffusion, is believed to occur to a certain extent in those freshwater teleost which conform to the present freshwater ion transport model (Figure 1.3). Ion absorption is a transcellular process and assuming that exchange diffusion occurs, then a proportion of the total ion efflux could also be transcellular. However, paracellular channels are believed to be the main site of ion efflux in freshwater teleosts (McDonald *et al.* 1989). The goldfish (*Carassius auratus*) demonstrates coupling for both Na^+ and Cl^- when external [NaCl] is increased as both J_{in} and J_{out} increase and become saturated at 1000 $\mu M \cdot L^{-1}$ [NaCl]. The efflux curve follows the same hyperbolic curve as uptake but the maximum rate is lower (Maetz, 1972; DeRenzis, 1975). Wood & Randall (1973) reported a similar Na^+/Na^+ exchange diffusion linkage in rainbow trout. This finding was later supported by Goss & Wood (1990*a*, *b*) who suggested that the discrepancy between Na^+ (and Cl^-) diffusive efflux measured in NaCl-free water and the efflux measured radioisotopically was a result of exchange diffusion occurring in the latter. However, in *F. heteroclitus*, the efflux components of both Na^+ and Cl^- remained unchanged throughout the range of $[NaCl]_{ext}$ (Figure 2.2). The lack of correlation between efflux and influx shows that exchange diffusion is not functioning in *F. heteroclitus*, and therefore

suggests that efflux occurs via paracellular routes.

Even though ammonia, in its free-base form (NH_3) can readily diffuse across membranes, one of the several proposed mechanisms for ammonia excretion involves the exchange of the protonated form (NH_4^+) for Na^+. In support of this idea, McDonald & Prior (1988) found a 1:1 correlation between Na^+ and net ammonia excretion when $[NaCl]_{ext}$ was varied, suggesting a Na^+/NH_4^+ exchange for rainbow trout. However Maetz, (1973) using goldfish (*Carassius auratus*) and Kerstetter *et al.* (1970) using rainbow trout varied $[Na^+]_{ext}$ and found no change in ammonia excretion. The discrepancy may lie within the length of exposure to each of the $[Na^+]_{ext}$, as the trout fluxes of McDonald & Prior (1980) were 20 minutes whereas in the goldfish study, the fluxes were 1.5 to 2 hours. Perhaps the acute response to $[Na^+]$ involves modulation of Na^+/NH_4^+ exchange but for long term regulation, other mechanisms become more important to keep ammonia excretion constant. In *F. heteroclitus*, Figure 2.3 presents evidence that ammonia excretion is unaltered by rapid changes in external [NaCl], and is therefore not coupled to Na^+ uptake. Perhaps the flux duration (*i.e.* $\geq$ 1 hour) was adequate to allow these long term regulatory mechanisms to obscure changes that may have occurred in Na^+/NH_4^+ exchange or ammonia excretion was occurring by transcellular diffusion as NH_3 or paracellularly as NH_3 or NH_4^+. Both mechanisms could be totally independent of Na^+ uptake (McDonald & Prior, 1988) .

Because Na^+ uptake is independent of anion movement (*i.e.* Cl^-), a second cation must move out in order to maintain charge balance. In frog skin, under freshwater conditions, this counterion is a proton (H^+) which is actively pumped out, creating a electrochemical gradient for Na^+ to passively move inward though an apical channel (Ehrenfeld *et al.* 1985). As mentioned above, ammonia can be protonated and may be excreted as the counterion in order to maintain neutrality in a freshwater teleost. The saturation of Na^+ influx observed in many freshwater animals, including *F. heteroclitus*, can be attributed to the transporter reaching its maximum carrying capacity but Kirschner (1988) recently postulated that the apparent saturation of Na^+ influx in amphibians is actually limited by the H^+ efflux. This idea was supported by recent studies using two-substrate kinetics analysis of Na^+ and Cl^- uptake in rainbow trout gills with H^+ and HCO_3^- being the second, internal substrates. According to this idea, any changes in acid-base status (*i.e.* internal H^+ or HCO_3^- levels) will affect the kinetic properties of Na^+ and Cl^- uptake, specifically J_{max} (Goss & Wood, 1991). Therefore the use of one-substrate kinetic analysis, as in this study, limits the characterization of transport properties if in fact ion uptake is directly coupled to internal acid-base regulation. The next section deals with attempting to determine if this link exists in *F. heteroclitus*.

Injection studies

The goal of the injection series was to induce systemic acid-base

disturbances in *F. heteroclitus* in an attempt to elucidate the mechanisms responsible for restoring acid-base balance and to determine if ionoregulation is directly involved in this process.

The mummichog did respond to the isosmotic 1000 $nEq \cdot g^{-1}$ HCl load but not to the 1000 or 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ injections. Specifically, half of the acid load was excreted within the four hours monitored following HCl injection (Figure 2.9). Net ammonia and H^+ excretion were elevated without a concurrent increase of Na^+ uptake (Figure 2.5), again suggesting that these two processes are not coupled (*i.e.* Na^+/NH_4^+ exchange). Wright & Wood (1985) concluded that Na^+/NH_4^+ exchange and NH_3 diffusion are both functioning in rainbow trout under normal conditions. If this is also true for *F. heteroclitus*, then Na^+/NH_4^+ exchange is clearly not involved in stimulation of ammonia excretion noted in Figure 2.9. This implies that NH_3 diffusion must have been elevated, perhaps due to an increase in the ammonia diffusion gradient across the gill due to local ammonia trapping by H^+ excretion in the gill boundary layer. Another possibility is the passive diffusion of NH_4^+ via the paracellular route (McDonald *et al.* 1989) as mummichogs maintain a rather large cation permeability as evident in the large Na^+ effluxes (Figure 2.5). This may be sufficient to allow NH_4^+ diffusion.

The question remains as to how 50% of the acid load was excreted within four hours. Because water titrations cannot distinguish between acid excretion or base uptake or vice versa (see methods), it is difficult to conclude which process occurred *in vivo*. But additional evidence of acid or base movement

between the animal and water is provided by measurement of Strong Ion Difference (SID) (Stewart, 1983). Essentially, the difference between the net Na^+ and Cl^- fluxes indicates a charge deficit that must be attributed to either a cation (acid) or anion (base) being excreted or absorbed. Acid injection resulted in a greater Cl^- over Na^+ loss (Figure 2.9*b*) and is interpreted as an acid loss which agrees with the elevated net H^+ excretion depicted in the acid-base fluxes (Figure 2.9*a*).

The disparity between the net Na^+ and Cl^- movement found only in the acid injection study originated from differential manipulation of the efflux components. Other researchers have agreed that this can be an additional device to compensate for an acid-base disturbance but that the major contributor is the influx mode, specifically for those fish conforming to the FW model (Wood *et al.* 1984; McDonald *et al.* 1989; Goss and Wood, 1990*a*, *b*; Goss *et al.* 1992). The present study suggests that *F. heteroclitus* may rely solely on differential adjustment of efflux to correct a systemic acidosis. The mechanisms for control of diffusive efflux of Na^+ and Cl^- are yet to be defined. However Madara (1988) reported that tight junctions of leaky epithelia can alter charge selectivity and structure which in turn varies the resistance to paracellular ion flow. Therefore selective regulation of the tight junctions in gill epithelia would allow for the appropriate ion to diffuse through more easily (Goss *et al.* 1992).

It would appear that *F. heteroclitus* cannot utilize Na^+ or Cl^- uptake

mechanisms as means to reduce an acid-base imbalance. As Cl^- uptake is virtually zero in this species, internal HCO_3^- levels must be regulated in a manner different from that in rainbow trout. This became obvious as neither 1000 nor 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ injections resulted in any dramatic changes in Cl^- unidirectional fluxes (Figure 2.6*b*, 2.7*b*) or acid-base fluxes (Figure 2.10*a*, 2.11*a*). In contrast, $NaHCO_3$ infusion of rainbow trout caused a significant stimulation of Cl^- uptake with a coinciding elevation of net H^+ uptake (base excretion) demonstrating the direct coupling of the two activities (Goss & Wood, 1990*b*). Similar injection studies revealed that Cl^-/HCO_3^- exchange exists in goldfish (*Carassius auratus*) (Maetz & Garcia Romeu, 1964; DeRenzis & Maetz, 1973; DeRenzis, 1975). Two species of euryhaline eel (*A. anguilla*, *A. rostrata*) are the only other teleosts without detectable Cl^- uptake. Their ability to regulate acid-base status is limited not only due to the lack of Cl^-/HCO_3 exchange but because Cl^- efflux must be reduced in order to minimize loss (Hyde & Perry, 1987). In the same eel study, stimulation of J^{Na}_{in} was the greatest contributor to J^{SID} elevation in compensating for the acidosis following air exposure. This demonstrates that the eel can modulate Na^+ uptake as a means of regulating internal acidic equivalent levels (*i.e.* H^+). With HCl injection, Na^+ uptake was not utilized in *F. heteroclitus*. Instead, adjustments of diffusive efflux were performed to compensate for this induced acidosis.

The lack of response to the either 1000 or 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ is puzzling. The greater stimulation of both Na^+ and Cl^- net fluxes with the smaller

(isosmotic) base load (Figure 2.6) in comparison to the three-fold larger load (Figure 2.7) may demonstrate the influence of $[Ca^{2+}]_{ext}$ on ion loss. Series *iv* injections were performed in Hamilton tapwater which contains 1 $mmol \cdot L^{-1}$ Ca^{2+} versus the 0.1 $mmol \cdot L^{-1}$ of the defined medium for series *iii* (see methods). Control unidirectional ion fluxes were not different between the two experiments, but in the event of stress (*i.e.* injection), tight junctions within the paracellular channels are thought to become "leaky" and may also lose their selective nature allowing ions to move out, regardless of charge. With higher water $[Ca^{2+}]$, the extent of "leakiness" may be attenuated. This may explain relatively smaller net losses of Na^+ and Cl^- after the reduction of net Na^+ and Cl^- flux following injection in the latter experiment.

Another possibility is that the induced alkalosis was corrected by internal metabolic processes. Atkinson (1992) has suggested that acid-base disturbances, specifically alkalosis, could be rectified by urea synthesis which consumes ammonium ions and HCO_3^- in equal amounts. If this were the case, base excretion would not necessarily occur, but urea excretion should be elevated and ammonia excretion reduced. Urea and ammonia excretion remained unchanged with the 3000 $nEq \cdot g^{-1}$ $NaHCO_3$ injection suggesting that this route was not utilized by the mummichog. The only reasonable conclusion is that the mummichog retained the additional HCO_3^- within the body over the time course of the experiment and was unable to excrete it due to the absence of an exchange mechanism with Cl^- influx. Future studies are planned to

measure blood acid-base parameters of base loaded mummichogs to reveal the time course of internal alkalosis, specifically whether it is corrected within the same time frame as the present studies.

Scanning electron microscopy

Though this was only a qualitative study, some interesting points can be raised with respect to ionoregulating mechanisms present in freshwater-adapted *F. heteroclitus*. First, it would seem that both gill and opercular epithelia contain similar cell types with pavement cells dominating, followed by mucous cells and what appear to be apical crypts of chloride cells. There does not appear to be any substantial differences between the two epithelia except that mucus was present only on the gill filamental epithelium (Figure 2.12*a*, *b*) (Figure 2.13*a*, *b*) which could be attributed to the complex geometry of the gill epithelium as compared to the planar opercular epithelium.

The apical crypts noted in Figures 2.12*a*, *b* and 2.13*b* are generally considered to be characteristic of seawater adaptation but were also described in a surface ultrastructure study of the gill arches of both SW and FW killifish (Hossler *et al.* 1985), Lake Magadi tilapia (*Oreochromis alcalicus grahami* Boulenger), which live in a very extreme environment of intermediate salinity but high alkalinity and temperature (Maina, 1990; Laurent *et al.* 1994) and a second FW-adapted tilapia (*Oreochromis mossambicus*) held in less alkaline water (Perry *et al.* 1992). The α and β chloride cells which are SW and FW

type chloride cells respectively have been reported in several species of euryhaline fish including the above noted (Pisam *et al.* 1987, 1993; Maina, 1990; Laurent *et al.* 1994). It is uncertain if either of these cell types are present by viewing Figures 2.12 and 2.13 but recent TEM study of FW-adapted *F. heteroclitus* demonstrated that the chloride cells are much larger but less numerous in the opercular epithelia than found in the SW killifish. Whether these chloride cells could be either α or β was not addressed (Marshall *et al.* 1994).

It is difficult at this point to suggest which cell type is responsible for which ion absorption in FW-adapted mummichogs. *In vitro* opercular epithelium studies have measured a small but active Cl^- influx while Na^+ uptake was passive (Wood & Marshall, 1994). If one calculates a whole body J^{Cl}_{in} for the mummichog from the largest *in vitro* OE Cl^- influx value reported (90 $nmol \cdot cm^{-2} \cdot h^{-1}$; Wood & Marshall, 1994) and assume a 5g body mass and a opercular epithelium surface area of 0.5 cm^2 (*i.e.* aperture size), it is apparent that the chloride cells of the opercular epithelium could be solely responsible for the almost negligible *in vivo* J^{Cl}_{in} measured in the present study. But this is complicated by the fact that chloride cell apical crypts were observed on the trailing edge of the gill filament (Figure 2.12*b*) suggesting that these chloride cells are also in communication with the external milieu and could very well be functioning. The sites of Na^+ uptake, which is far more vigourous, could be either these gill chloride cells or the gill pavement cells.

Two or more chloride cells or adjacent accessory and chloride cells can share an apical crypt. These groupings are common in SW-adapted teleosts (Karnaky, 1986) but have also been noted in FW-adapted *F. heteroclitus* (Lacy, 1983, Marshall *et al.* 1994). The junctions between adjacent chloride cells and accessory cells are shallow and simple in construction allowing for passive movement of ions, specifically Na^+ efflux during SW adaptation (Sardet *et al.* 1979; Pisam & Rambourg, 1991). The retention of this association of chloride-accessory cells in freshwater could explain the continued permeability to Na^+ in FW-adapted mummichogs (see Figures 2.1-2.7).

A more rigorous EM study must be pursued to quantify the number of MR cells that are indeed functioning in both the opercular and gill epithelium and if these cells are involved in whole body ion transport or acid-base regulation in the freshwater-adapted mummichog.

CHAPTER 3

The influence of environmental pH, ammonia, amiloride, and acetazolamide on iono- and acid-base regulation.

With the onset of the acid rain phenomenon, a surge of research revealed the debilitating effects of low pH water on aquatic organisms including fish (see reviews by Wood & McDonald, 1982; McDonald, 1983, Wood, 1989). Alkalinization of lakes caused by eutrophication (Barica & Mathias, 1979) and anti-eutrophication liming (Koschel *et al.* 1983), in addition to pH surges due to photosynthetic activity of algae, has become a growing concern in western Canada and the U.S. Oddly enough, very little is known about the impact on the aquatic organisms residing in these lakes except for the seasonal fish kills which are attributed to the alkaline pH (Jordan & Lloyd, 1964; Wilkie & Chow-Fraser, 1992). Several populations of fish have been reported residing in lakes with pH ranges between 9.4 and 10.0. In one case, the reduced water flow into Pyramid Lake, Nevada could expose the resident Lahontan cutthroat trout to further increases in alkalinity which could prove lethal (Wilkie *et al.* 1993).

In addition to its relevance to serious environmental disturbances in the wild, manipulation of water pH has proven to be a useful tool in elucidating the linkage between acid-base balance and Na^+ and Cl^- uptake in freshwater teleosts. The responses by salmonids to acidic and alkaline environments are

at least superficially quite similar in that Na^+ uptake is inhibited by both; depending upon the duration and severity of exposure, a certain level of recovery occurs. For Cl^- uptake, the responses are variable for low pH (McDonald, 1983) whereas high pH immediately inhibits J^{Cl}_{in} followed by a rapid recovery (Wilkie & Wood, 1994). Blood acidosis and alkalosis are commonly induced by low and high pH respectively, with the former condition dependent upon the environmental calcium levels. Soft water (low $[Ca^{2+}]$) acclimated trout did not experience an acidosis whereas hard water trout are susceptible (McDonald *et al.* 1980). Lactic acidosis may be initiated to attenuate the systemic alkalosis during high pH (Wilkie & Wood, 1991).

Ammonia is a common agricultural and industrial pollutant, and high external ammonia (HEA) can be toxic to aquatic organisms (Thurston *et al.* 1984). Sublethal ammonia levels which are sufficient to reverse the ammonia diffusion gradients from blood to water have been used to study the relationship between ammonia excretion and Na^+ uptake in fish. To date, the nature of this coupling is disputed as ammonia excretion can be explained by a) diffusion of NH_3 or NH_4^+, b) NH_3 diffusion coupled to a Na^+/H^+ exchanger or Na^+ channel/H^+-ATPase arrangement, c) a Na^+/NH_4^+ exchanger, or d) a combination of a, b, c (Wood, 1993). Recent HEA studies have provided more evidence for b) in the rainbow trout, *Oncorhynchus mykiss* (Wilson & Taylor, 1992; Wilson *et al.* 1994).

Amiloride, a potent Na^+ uptake inhibitor, also affects ammonia and/or acid

excretion (Wright & Wood, 1985; Avella & Bornancin, 1989; Lin & Randall, 1991; Wilson *et al.* 1994) in rainbow trout, indicating that acid-base regulation is linked with ion uptake. Another tool useful in elucidating the link between the two regulatory systems is acetazolamide (ACZ), which inhibits carbonic anhydrase (CAH) in both erythrocytes and more importantly branchial epithelial cells. Reduced CO_2 hydration reduces the availability of counterions (H^+ and HCO_3^-) for Na^+ and Cl^- uptake (see review by Perry & Laurent, 1990). ACZ has revealed the dependence of ionoregulation upon CAH in many freshwater species including goldfish (*Carassius auratus*; Maetz, 1973), channel catfish (*Ictalurus punctatus*; Henry *et al.* 1988), rainbow trout (Kerstetter *et al.* 1970; Avella & Bornancin, 1989; Lin & Randall, 1991) and amphibian skin *in vitro* (Ehrenfeld *et al.* 1985). Lacy (1983) reported measurable levels of CAH in the opercular epithelium of both SW and FW-adapted *F. heteroclitus* and its activity was blocked by ACZ. However *in vitro* ion transport by SW killifish opercular epithelium was unperturbed by the presence of ACZ (Zadunaisky, 1984) suggesting that ionoregulation of *F. heteroclitus* may not be dependent upon CAH activity.

The goal of this chapter was to determine if Na^+ and Cl^- uptake in *Fundulus heteroclitus* are coupled to ammonia excretion and/or acid-base regulation, and if the nature of this relationship is similar to the present ion transport model for freshwater teleosts (Figure 1.3). Acute exposures to high and low pH, HEA and amiloride with subsequent recovery periods, and

acetazolamide injections were performed in the hope that responses, or lack thereof, to each of these treatments would provide insight into the regulatory mechanisms of the mummichog.

Materials and Methods

Experimental Animals

Mummichogs (*Fundulus heteroclitus*), weighing 2.61-10.61g, of both sex, were collected from a brackish estuary located near Antigonish, Nova Scotia and were held at St. Francis Xavier University, Antigonish for several weeks before being air-shipped to McMaster University, Hamilton, Ontario. The fish were held indoors in 500 L fiberglass tanks containing 10% seawater (Hamilton dechlorinated tapwater and Forty Fathoms Instant Ocean) at ambient temperature (18-20°C). The water was well aerated and continuously filtered by percolation through polyester fibre and charcoal. Fish were fed a 1:2 mixture Tetramin/Tetramarin daily.

Acclimation Conditions

All acclimation and experiments were performed in Hamilton dechlorinated tapwater (see Chapter 2 for composition). Twelve to fourteen days prior to experiment, 10-12 fish were placed in a plastic tank, containing 60 L of acclimation water and supplied with aeration and a percolating filter consisting of polyester fibre and charcoal. The entire water volume was replaced every six days with fresh water. Fish were fed daily Tetramin only.

The night prior to experiments, fish were weighed and placed in individual darkened 400 ml plastic flux containers with lids. Each container was filled with water and supplied with vigorous aeration. Overnight holding was no

longer than 6 hours prior to experimentation.

Experimental Protocols

The primary goal of these studies were to determine the effects of environmental disturbances and pharmacological agents on unidirectional Na^+ and Cl^- fluxes, nitrogenous waste (ammonia, urea) excretion and acid-base fluxes.

After the overnight settling period, the flux containers were drained, flushed and 300 ml fresh water was added. Next, two-1.5 hour control fluxes were performed. The volume of water used in each study varied (see values in each series below) but water was changed every 3 hours throughout the experimental period. All series of experiments (*i-v*) utilized ^{24}Na and ^{36}Cl concurrently except for one low pH trial where the latter was not available. The amount of isotope used during each flux period was 16 µCi of ^{24}Na and 3 µCi of ^{36}Cl. Immediately following the water change, aliquots of both isotope stocks were added to each flux container and allowed to mix via aeration for 10 minutes. Water samples (20 ml) were taken at 0, 1.5 and 3 hour intervals for subsequent analysis of titratable alkalinity, ammonia, Na^+, Cl^-, ^{24}Na, and ^{36}Cl. Urea was measured in series *ii* and *iii* only.

Series i: Low pH. Water volume used throughout control, experimental and recovery was 300 ml. The night prior to the experiment, 5 L of dechlorinated

tap water was titrated to pH=4.0 using 1.0 N H_2SO_4. Water pH was measured using a Radiometer GK2401C pH electrode coupled to a PHM82 meter. Once titrated, the water was vigorously aerated to remove CO_2 and kept at room temperature until time of use, when it was readjusted to pH 4.0. Following the control periods at circumneutral pH, the flux containers were drained, flushed and 300 ml of pH=4.0 water added, followed by isotope addition. After 10 minutes of mixing, water samples (20mL) were taken at 0 and 1.5 hours. Following the 1.5 hour sample, water pH was measured using the setup described above. A volume of 0.1 N H_2SO_4 sufficient to return the pH to 4.0 was added to each flux container. The volume of acid required to return the water pH to 4.0 was calculated on the basis of the known titration curve for Hamilton dechlorinated tapwater to pH 4.0 using 0.1 N H_2SO_4. The additional acid was allowed to mix for 10 mins followed by water samples (20 ml) taken at 1.7 and 3.2 hours (nominally 1.5 and 3 hours). At the end of 3 hours of low pH exposure, the containers were drained, flushed and filled with 300 ml fresh control water at circumneutral pH followed by isotope addition. Water samples (20 ml) were taken at 0, 1.5 and 3 hours constituting the recovery period. It should be noted that two trials of low pH experiments (N=15) were performed with results for all parameters except Cl^- unidirectional fluxes combined as they were similar. However Cl^- unidirectional fluxes were measured in only one trial (N=8). The average pH over the 3 hour experimental period was 4.51.

Series ii: High pH. Because of the unusual water chemistry at high pH, several tests were performed to determine if titratable alkalinity could be reliably measured at high pH. Briefly, 2 separate containers of 200 mL of Hamilton dechlorinated tapwater were titrated to pH 10 and left aerating overnight. The following morning, additional KOH was added to return pH to 10. One beaker was aerated with compressed air while the second one was bubbled with a 1.5% CO_2 gas mixture (balanced with nitrogen). This second test was used to simulate the CO_2 that is excreted by a fish during an experiment. Water samples (20 ml) and pH measurements were taken from both containers at 0 and 1.5 hours. TA and [Ca^{2+}] were subsequently measured in the water samples. Titration of Hamilton dechlorinated tapwater to pH 10 resulted in an overnight reduction of [Ca^{2+}] from 1.0 to 0.39 mmol·L^{-1}. At high pH, there is virtually no free CO_2 in solution but rather a co-existence of HCO_3^- and CO_3^{2-} with the latter precipitating out as $CaCO_3$, hence the lower calcium levels (M. Wilkie, personal communication). Aeration of this alkaline medium alone over 1.5 hours resulted in reductions in pH (10.013 to 9.911), TA (Δ=116 µmol·L^{-1}) and Ca^{2+} (Δ=60 µmol·L^{-1}). It seems that the reduction of TA is accounted for by the additional titration by calcium precipitation. The addition of CO_2 by the fish was mimicked by bubbling the medium with 1.5% CO_2 over 1.5 hours and demonstrated a large reduction in pH (10.067-7.588) but an increase in TA (Δ=+100 µmol·L^{-1}) and [Ca^{2+}] (Δ=+30 µmol·L^{-1}) suggesting that as pH drops, titration of base by Ca^{2+} is prevented. The possibility of Na^+ precipitation

titrating the added CO_2 was ruled out as water [Na^+] remained unchanged from control values in both tests. From these results it seems that TA measurements performed at high pH in this experiment would be complicated by the titration of base by water Ca^{2+} and the influence of CO_2 on this reaction. Because of this, TA fluxes were excluded during the 3 hour high pH treatment.

The protocol was the same as for low pH with some modifications. Flux water volume was reduced to 200 ml and the experimental water (3 L) was titrated to pH=10.0 using 1N KOH. The water was left to aerate overnight at room temperature; the next morning, additional KOH was added to return the pH to 10 as $CaCO_3$ had precipitated overnight. At the 1.5 hour mark of the experimental period, water pH was measured and a volume of 0.1 N KOH was added to each container (see above). A titration curve was constructed in the same manner as in series *i* only using 0.1N KOH to a pH of 10.0. The average pH over the 3 hour experimental period was 9.36. N=8.

Series iii: High external ammonia. The experimental period was extended from 3 to 6 hours for this study. Consequently, recovery fluxes were not performed due to the length of the experiment. A 200 ml water volume was used throughout with water changes following control and at 0 and 3 hours during the experimental flux period. The day prior to the experiment, 3 L of 0.5 $mmol \cdot L^{-1}$ $(NH_4)SO_4$ experimental water was made with pH remaining close to 7.9. The water was aerated and left overnight at ambient temperature. N=8.

Series iv: Amiloride. Amiloride (10^{-4}M) was added to Hamilton tapwater and sonicated for 2 hours to ensure complete dissolution. After sonication, water was aerated overnight and left at room temperature. Water pH remained close to 7.9. The flux protocol followed the same control, experimental and recovery periods as in series *i* and *ii* (sampling at 0, 1.5, 3.0 hours in each). The water volume used was 200 mL/container. N=8.

Series v: Acetazolamide injections. A stock solution of 1 mg/ml acetazolamide (Sigma), was made using Cortland saline. Due to the low solubility, the solution was warmed and sonicated for 3 hours to ensure complete dissolution. Water volume for each container was 200 ml. Once the control fluxes were performed, the containers were drained and the fish removed, blotted dry and given an intraperitoneal injection (0.01 mg/g, *i.e.* 10 µL/g) of acetazolamide using a 50 µL gas-tight Hamilton syringe. This volume is similar to that used in the control injection of Chapter 2 (7.1µL/g). This dose of acetazolamide, if distributed in a body fluid volume of 0.70 mL/g, would have produced a concentration of 6.29×10^{-4} M internally. The mummichogs were blotted again and placed back into their containers with fresh water added. After 20 minute recovery, the containers were flushed and 200 ml fresh water and isotope were added. 10 minutes later, a water sample started the flux period with sampling (20 mL) at 1.5, 3, 4.5, and 6 hours. N = 7.

Analytical Methods and Calculations

All water analysis techniques used in these experiments were the same as described in Chapter 2. Water urea concentrations were measured only in series *ii* and *iii*.

In series *iii*, ammonia concentrations during the HEA exposure were measured using a modification of the salicylate-hypochlorite assay (Verdouw *et al.* 1978) described in Chapter 2 to improve the resolution with which small changes in T_{Amm} could be measured against high background levels. Because T_{Amm} of the water was 1.0 mmol·L^{-1}, the lowest standard (800 µmol·L^{-1}) was used to set the spectrophotometer absorbance reading to zero. To improve accuracy of this assay, standards that bracketed 1.0 mmol·L^{-1} [T_{Amm}] were used and each sample and standard were run in triplicate.

Statistical Analysis

Results were expressed as means ± SEM (N) throughout. For multiple comparisons, analysis of variance (ANOVA) was applied, followed by Fisher's test of Least Significant Difference (LSD) in cases where the F value indicated significance ($p \leq 0.05$).

Results

Na^+ and Cl^- unidirectional fluxes

Series i: pH 4.5.

The three hour exposure to low pH caused a significant inhibition of J^{Na}_{in} (Figure 3.1*a*) whereas the much lower level of J^{Cl}_{in} remained unchanged (Figure 3.1*b*). J^{Na}_{out} was not modulated during the pH change but net Na^+ loss was significantly elevated as direct result of reduced uptake. Net Cl^- loss became significant by 1.5-3 hours of low pH due to the elevated J^{Cl}_{out}. Upon return to circumneutral water, J^{Na}_{in} was significantly elevated and this response alone was responsible for a substantial net uptake. Both uptake and net gain of Na^+ continued to increase at three hours recovery. In contrast, J^{Cl}_{out} continued to be elevated during the first 1.5 hour of recovery but was reduced in the second 1.5 hour resulting in a net Cl^- gain. It is apparent that low pH exposure and subsequent recovery have the greatest influence on J^{Na}_{in}.

Series ii: pH 9.3.

Responses to alkaline pH were similar to those of low pH in that both inhibited J^{Na}_{in} without altering J^{Cl}_{in}. Na^+ and Cl^- effluxes were not effected by high pH but a negative Na^+ balance did develop during the 3 hours exposure (Figure 3.2*a*). During the first 1.5 hours of recovery, J^{Na}_{net} became positive and was stimulated above control values. This large net Na^+ gain is unlike that seen during recovery from low pH (Figure 3.1*a*), in that

Figure 3.1. The influence of low pH (pH = 4.5) and subsequent return to circumneutral water upon whole-body influx (J_{in}), outflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*). Mean ± 1 SEM; N = 15 for Na^+, N = 8 for Cl^-.

* denotes a significant difference from the control rates measured at pH = 8.0 ($p \leq 0.05$).

† denotes a significant difference from the low pH treatment ($p \leq 0.05$).

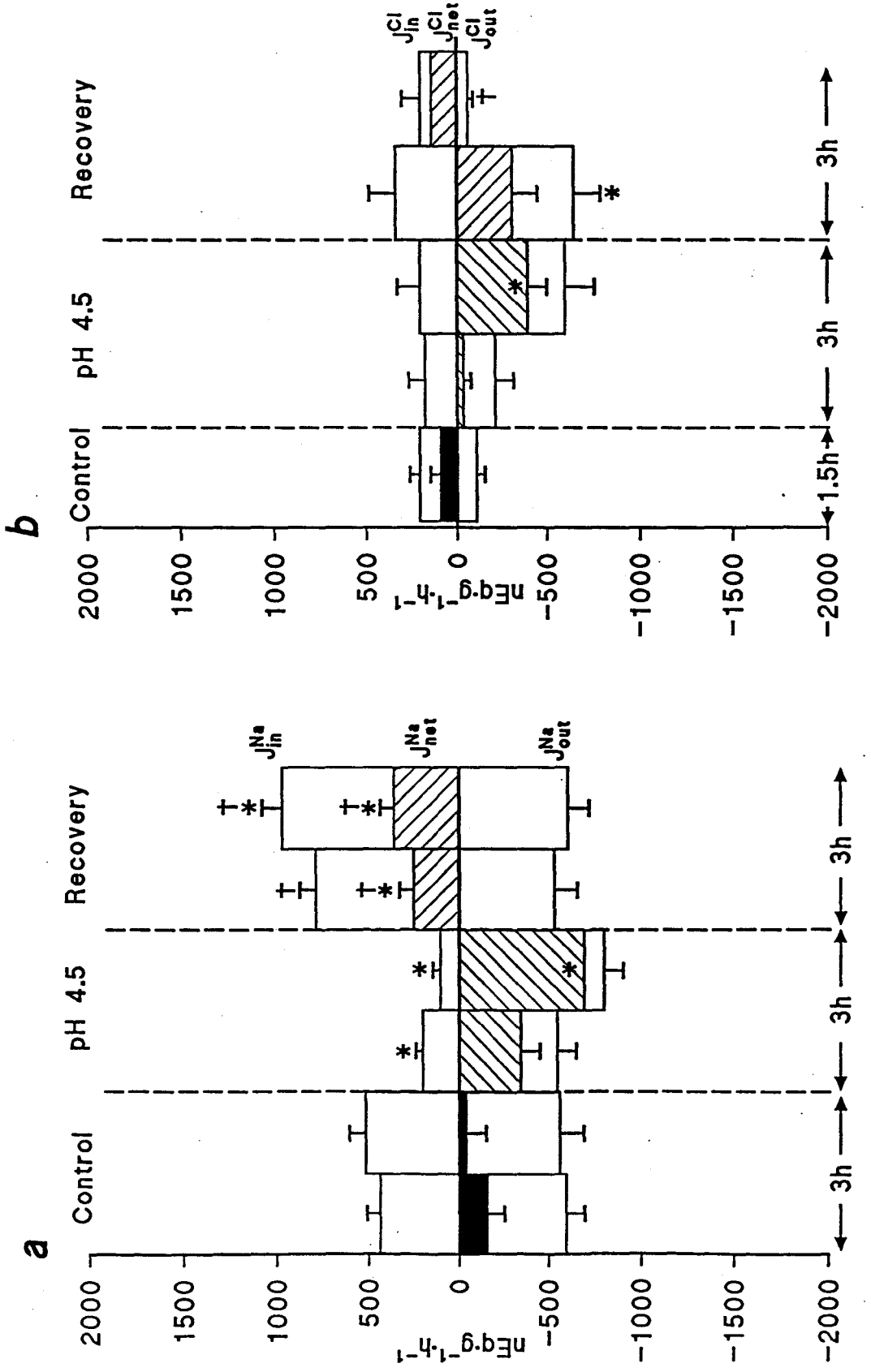
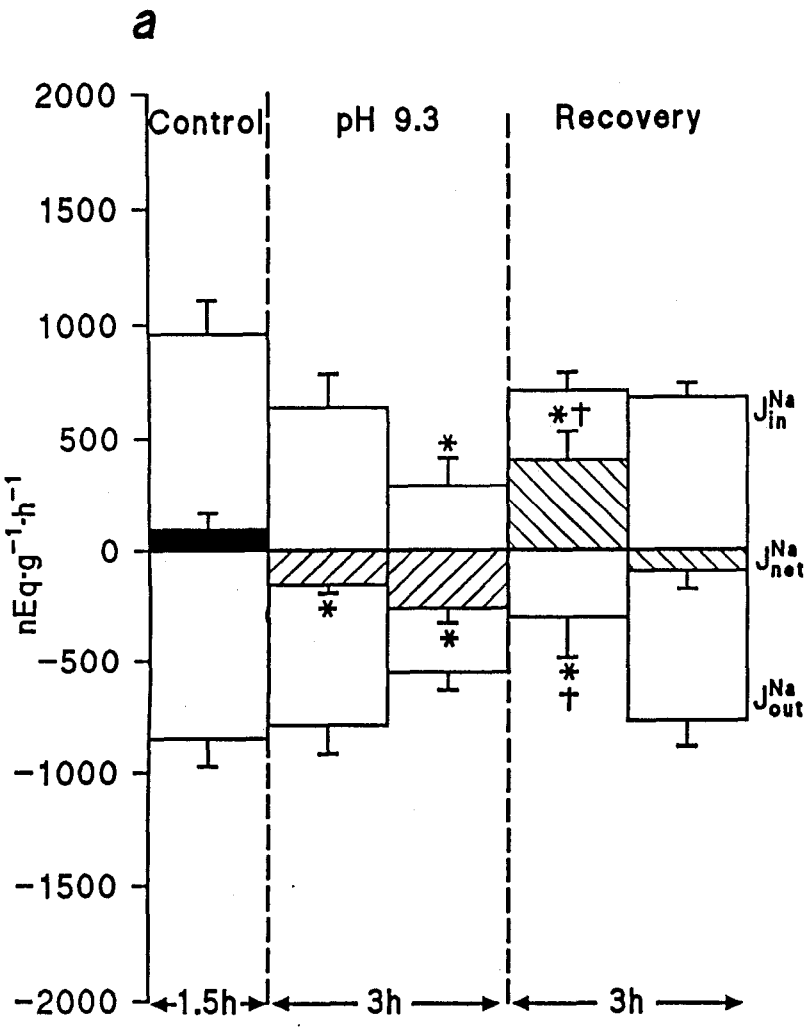
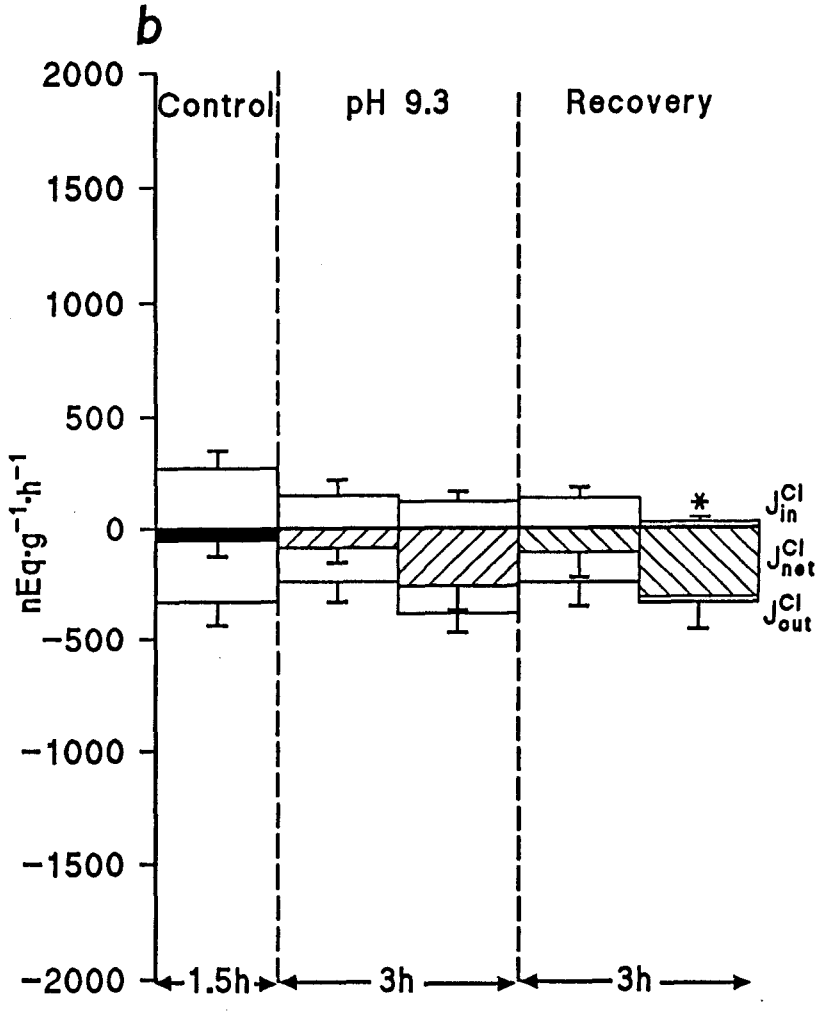

a
b
Control
pH 4.5
Recovery
3h
1.5h
nEq·g⁻¹·h⁻¹
2000
1500
1000
500
0
-500
-1000
-1500
-2000
J^{Na}_{in}
J^{Na}_{net}
J^{Na}_{out}
J^{Cl}_{in}
J^{Cl}_{net}
J^{Cl}_{out}

Figure 3.2. The influence of high pH (pH = 9.3) and subsequent return to circumneutral water upon whole-body influx (J_{in}), outflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*) fluxes. Mean ± 1 SEM; N = 8.

* denotes a significant difference from the control rates measured at pH = 8.0 ($p \leq 0.05$).

† denotes a significant difference from the high pH treatment ($p \leq 0.05$).


a
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g⁻¹·h⁻¹
Control
pH 9.3
Recovery
1.5h
3h
3h
J^{Na}_{in}
J^{Na}_{net}
J^{Na}_{out}


b
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g⁻¹·h⁻¹
Control
pH 9.3
Recovery
1.5h
3h
3h
J^{Cl}_{in}
J^{Cl}_{net}
J^{Cl}_{out}

recovery from high pH was accomplished largely through the significant reduction of J^{Na}_{out} (Figure 3.2*a*). By the second 1.5 hour of recovery, all Na^+ flux components had returned to control values. Unlike pH 4.5 exposure (Figure 3.1*b*), Cl^- unidirectional fluxes were unperturbed by high pH exposure (Figure 3.2*b*).

Series iii: High external ammonia.

Exposure to water [T_{amm}] of 1.0 mmol·L^{-1} lasted 6 hours and over that time, neither Na^+ (Figure 3.3*a*) nor Cl^- (Figure 3.3*b*) unidirectional fluxes varied from control values. These results suggest that Na^+ and Cl^- balance are not sensitive to external ammonia at least at levels which are known to reverse the diffusive gradients of both NH_3 and NH_4^+ across the gills.

Series iv: Amiloride exposure.

Amiloride (10^{-4}M) reduced J^{Na}_{in} to a level approximately 40% of the control value (Figure 3.4*a*). J^{Na}_{out} was not affected by amiloride, hence a significant net Na^+ loss ensued. When the mummichogs were returned to control water, both J^{Na}_{in} and J^{Na}_{net} returned to pre-exposure levels with a net gain of Na^+. Amiloride had no effect whatsoever on the unidirectional Cl^- fluxes (Figure 3.4*b*).

Figure 3.3. The influence of high external ammonia (T_{Amm} = 1.0 mmol·L^{-1}) upon whole-body influx (J_{in}), outflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*) fluxes. Mean ± 1 SEM; N = 8.

* denotes a significant difference from the control rates ($p \leq 0.05$).

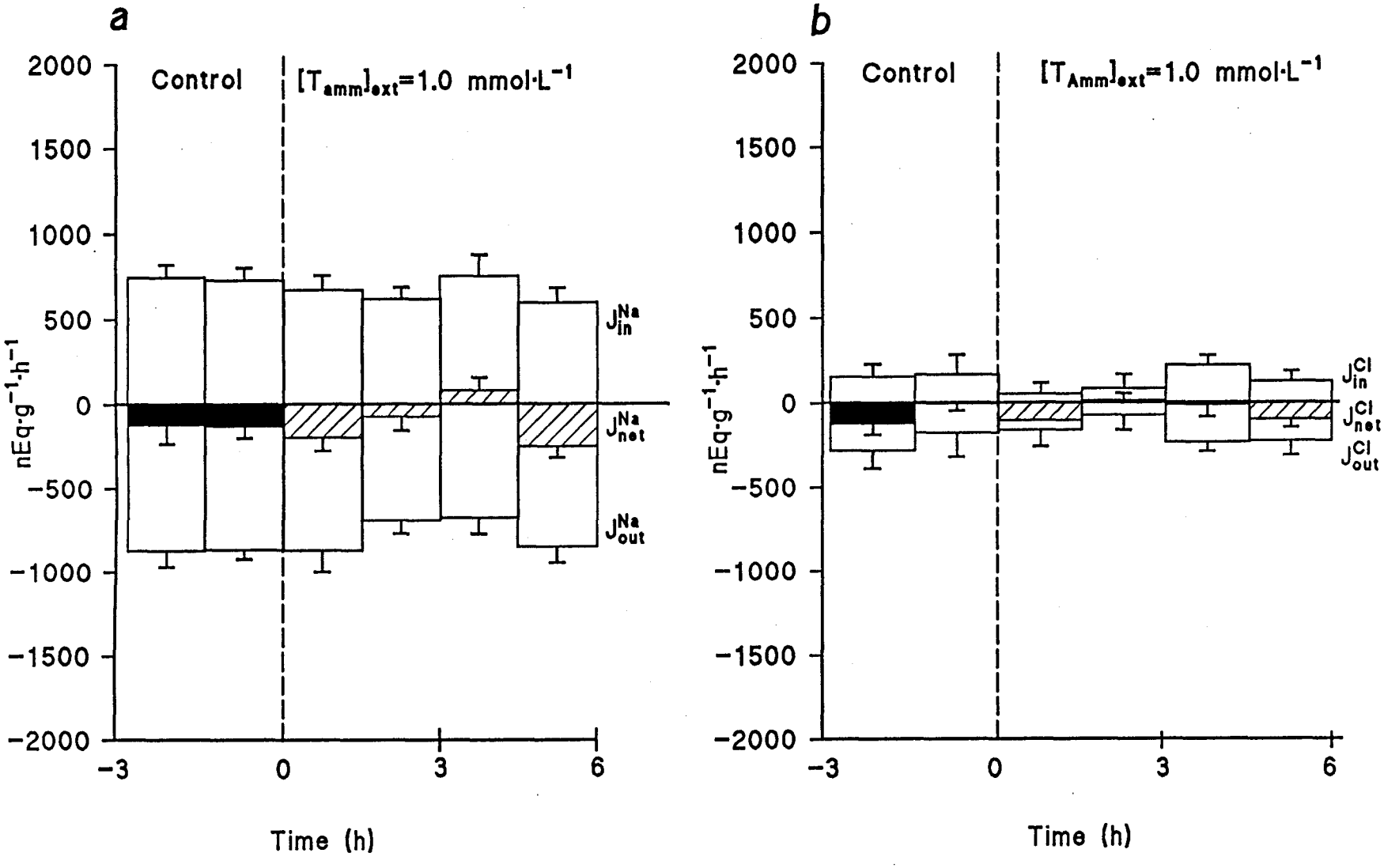

a
Control
$[T_{amm}]_{ext}$=1.0 mmol·L^{-1}
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g^{-1}·h^{-1}
J_{in}^{Na}
J_{net}^{Na}
J_{out}^{Na}
−3
0
3
6
Time (h)
b
Control
$[T_{Amm}]_{ext}$=1.0 mmol·L^{-1}
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g^{-1}·h^{-1}
J_{in}^{Cl}
J_{net}^{Cl}
J_{out}^{Cl}
−3
0
3
6
Time (h)

Figure 3.4. The effect of amiloride treatment (10^{-4} M) and subsequent removal thereof upon whole-body influx (J_{in}), outflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*) fluxes. Mean ± 1 SEM; N = 8.

* denotes a significant difference from the control rates ($p \leq 0.05$).

† denotes a significant difference from the amiloride treatment ($p \leq 0.05$).

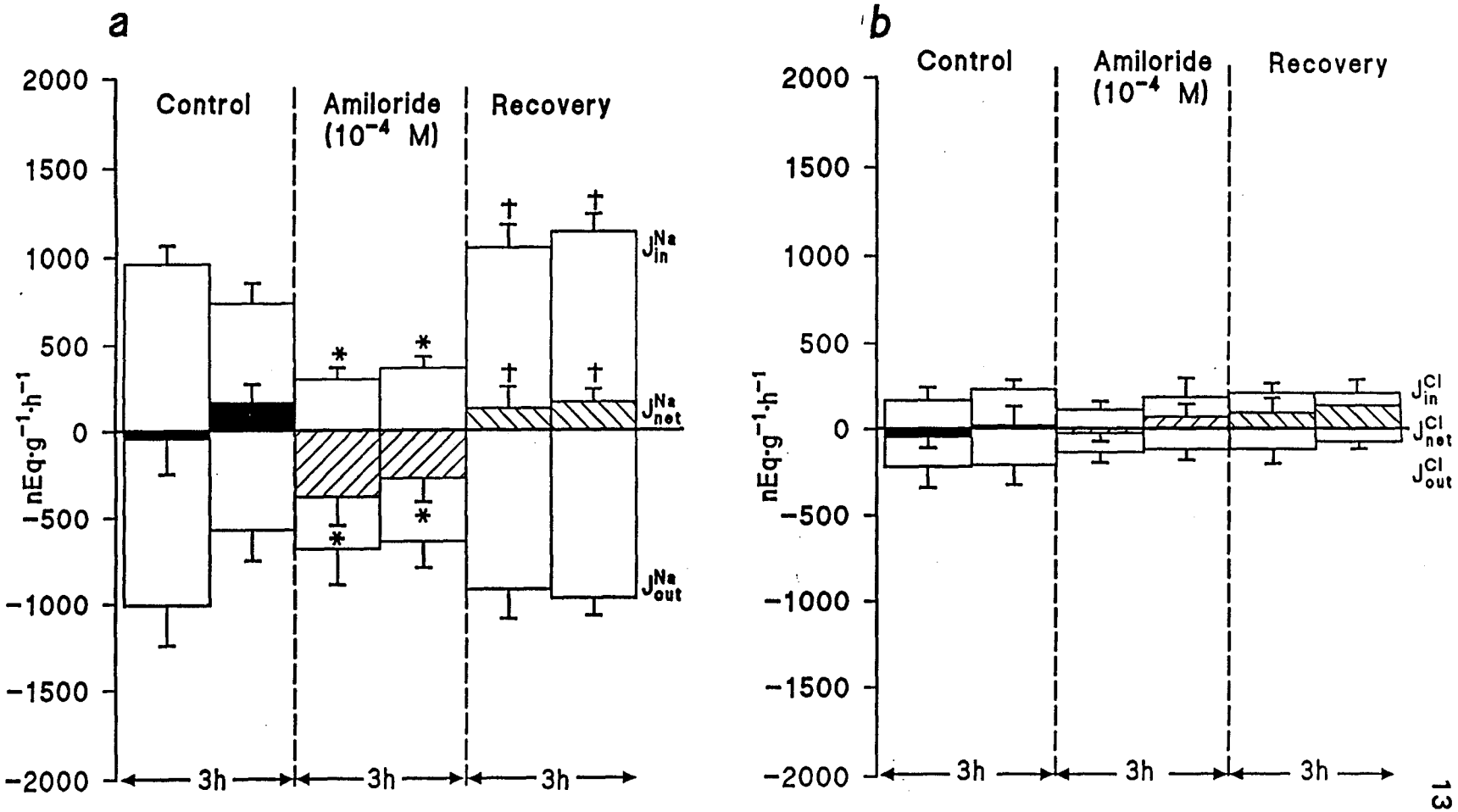
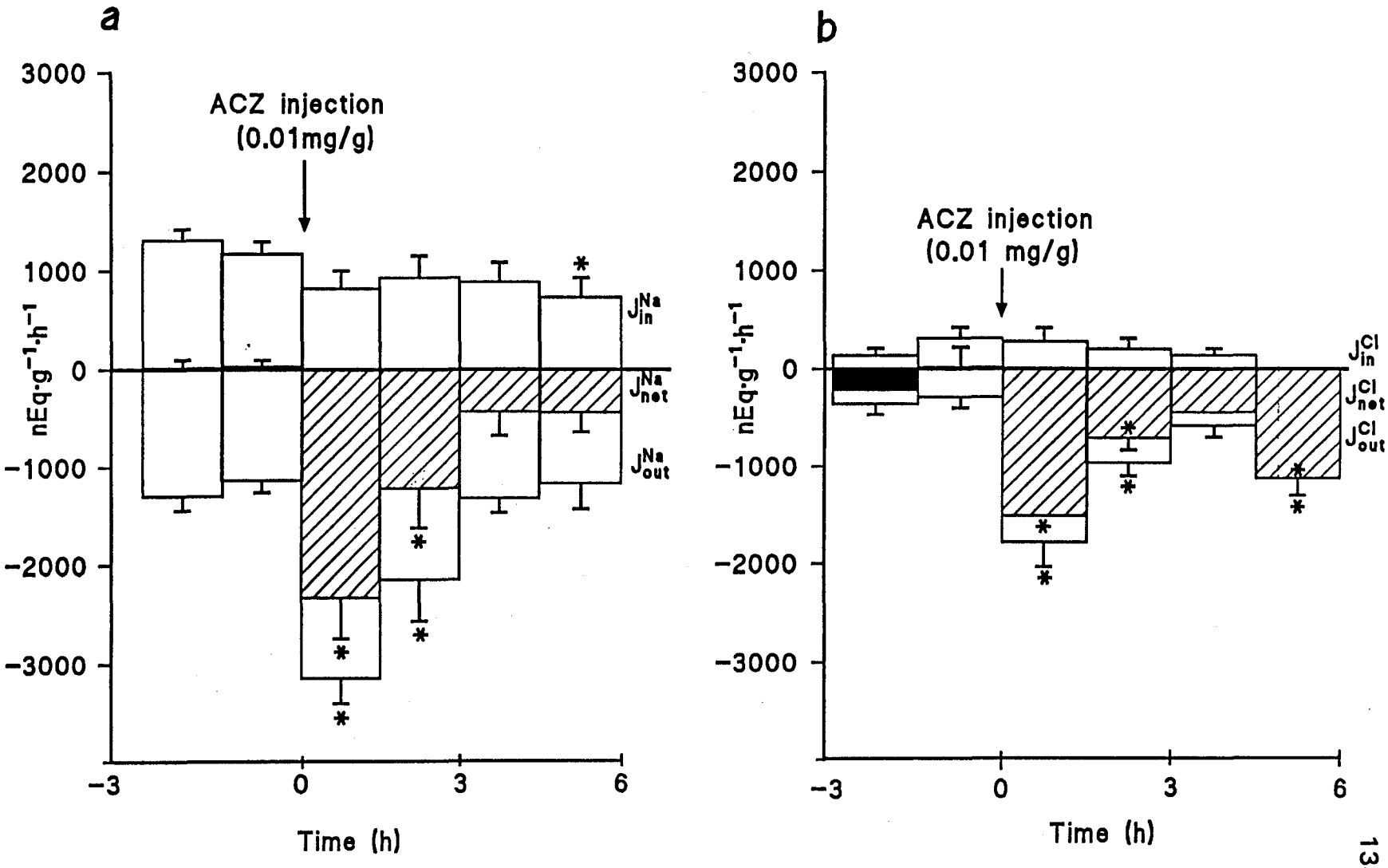

a
Control
Amiloride
(10⁻⁴ M)
Recovery
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g⁻¹·h⁻¹
3h
3h
3h
J_in^Na
J_net^Na
J_out^Na
b
Control
Amiloride
(10⁻⁴ M)
Recovery
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g⁻¹·h⁻¹
3h
3h
3h
J_in^Cl
J_net^Cl
J_out^Cl

Figure 3.5. The effect of intraperitoneal injection of acetazolamide (ACZ) upon whole-body influx (J_{in}), outflux (J_{out}), and net flux (J_{net}) of Na^+ (*a*) and Cl^- (*b*) fluxes. Injection dosage = 0.01 mg/g. Mean ± 1 SEM; N = 7.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
3000
2000
1000
0
−1000
−2000
−3000
nEq·g⁻¹·h⁻¹
ACZ injection
(0.01mg/g)
J_{in}^{Na}
J_{net}^{Na}
J_{out}^{Na}
−3
0
3
6
Time (h)
b
3000
2000
1000
0
−1000
−2000
−3000
nEq·g⁻¹·h⁻¹
ACZ injection
(0.01 mg/g)
J_{in}^{Cl}
J_{net}^{Cl}
J_{out}^{Cl}
−3
0
3
6
Time (h)

Series v: Acetazolamide injections.

Immediately following injection, J_{out} for both Na^+ and Cl^- dramatically increased, approximately 3-fold (Figure 3.5*a*, *b*). Large net losses of both ions occurred over the first three hours post-injection with Na^+ outflux reduced to control values by 4.5 hours (Figure 3.5*a*) whereas J^{Cl}_{out} and J^{Cl}_{net} were reduced slightly at 3-4.5 hours but resumed substantial losses in the last flux period (Figure 3.5*b*). J^{Na}_{in} was unaltered until hour 6 when a slight reduction occurred. J^{Cl}_{in} also showed a slight decrease and was essentially zero by hour 6.

Acid-base fluxes and net Na^+ and Cl^- fluxes.

Series i: low pH.

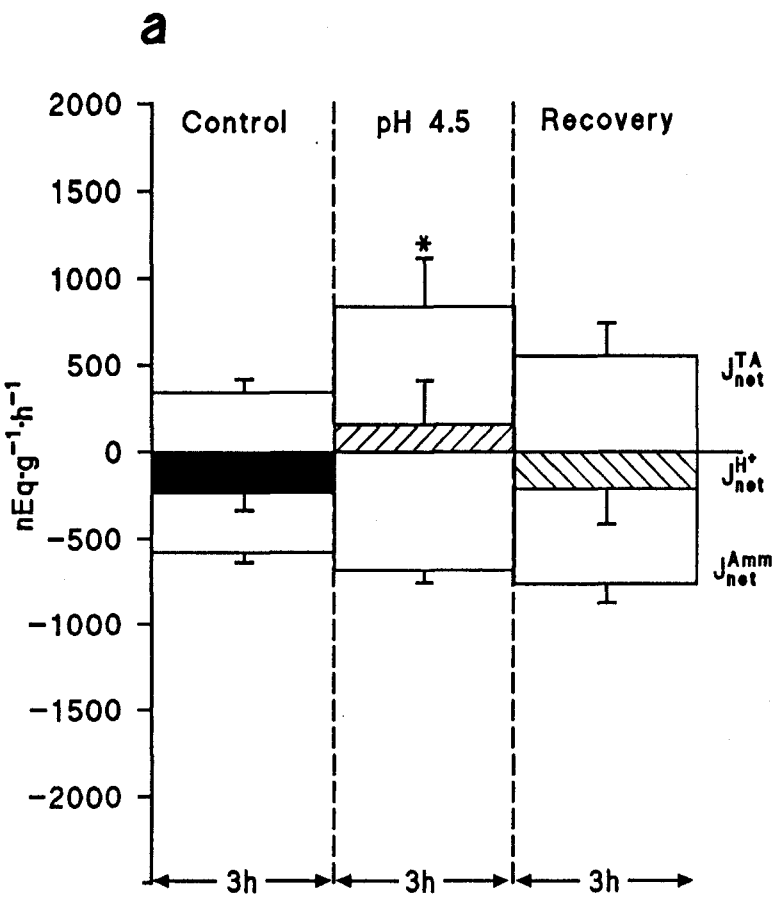
It should be noted that the beginning and end points were 0 and 3 hours for the titratable alkalinity flux (J^{TA}) during the control and recovery periods but during the acid exposure, where the pH was returned to 4.0 after 1.5 hours, J^{TA} was calculated for each of the 2 fluxes and was averaged for the entire 3 hour exposure (Figure 3.6*a*). This was performed because the large water volume used in the flux containers (*i.e.* 300 mL) reduced the accuracy of the delta value for the water titrations. J^{Amm} values were averaged over the 2 flux periods of each treatment.

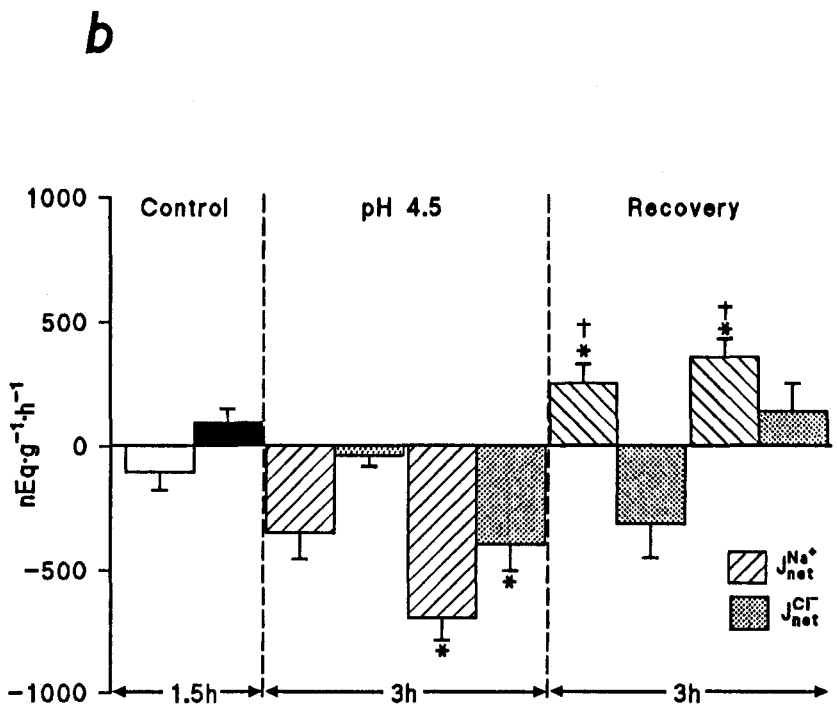
Net ammonia excretion (J^{Amm}) remained unchanged throughout exposure and recovery whereas J^{TA} increased significantly during the 3 hour low pH treatment (Figure 3.6*a*). In consequence, J^{H+} became positive (*i.e.* net acid

Figure 3.6. The influence of low pH (pH = 4.5) and subsequent return to circumneutral water upon whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) fluxes (*b*). Mean ± 1 SEM; N = 8 for (*a*), N = 15 for Na^+, N = 8 for Cl^-.

* denotes a significant difference from the control rates measured at pH = 8.0 ($p \leq 0.05$).

† denotes a significant difference from the low pH treatment ($p \leq 0.05$).


a
Control
pH 4.5
Recovery
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g⁻¹·h⁻¹
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
3h
3h
3h


b
Control
pH 4.5
Recovery
1000
500
0
−500
−1000
nEq·g⁻¹·h⁻¹
$J^{Na^+}_{net}$
$J^{Cl^-}_{net}$
1.5h
3h
3h

uptake) although the change was not statistically significant. When returned to control water, J^{H+} excretion resumed pre-exposure rates. This change in net acid excretion agrees with the estimated J^{H} using SID which was calculated from net Na^+ and Cl^- fluxes (Figure 3.6*b*). During low pH exposure, the greater Na^+ than Cl^- loss constrained a net base excretion or net acid uptake. When the mummichog was returned to circumneutral pH, a net Na^+ gain developed while a net Cl^- loss continued. The discrepancy between the two net fluxes indicates a net acid loss or base uptake. It should be noted that the net Na^+ gain continued to increase during recovery and was significantly higher than the control net movement. Cl^- net movement also became positive in the second recovery period, but was not as large because J^{Cl}_{in} was not substantial.

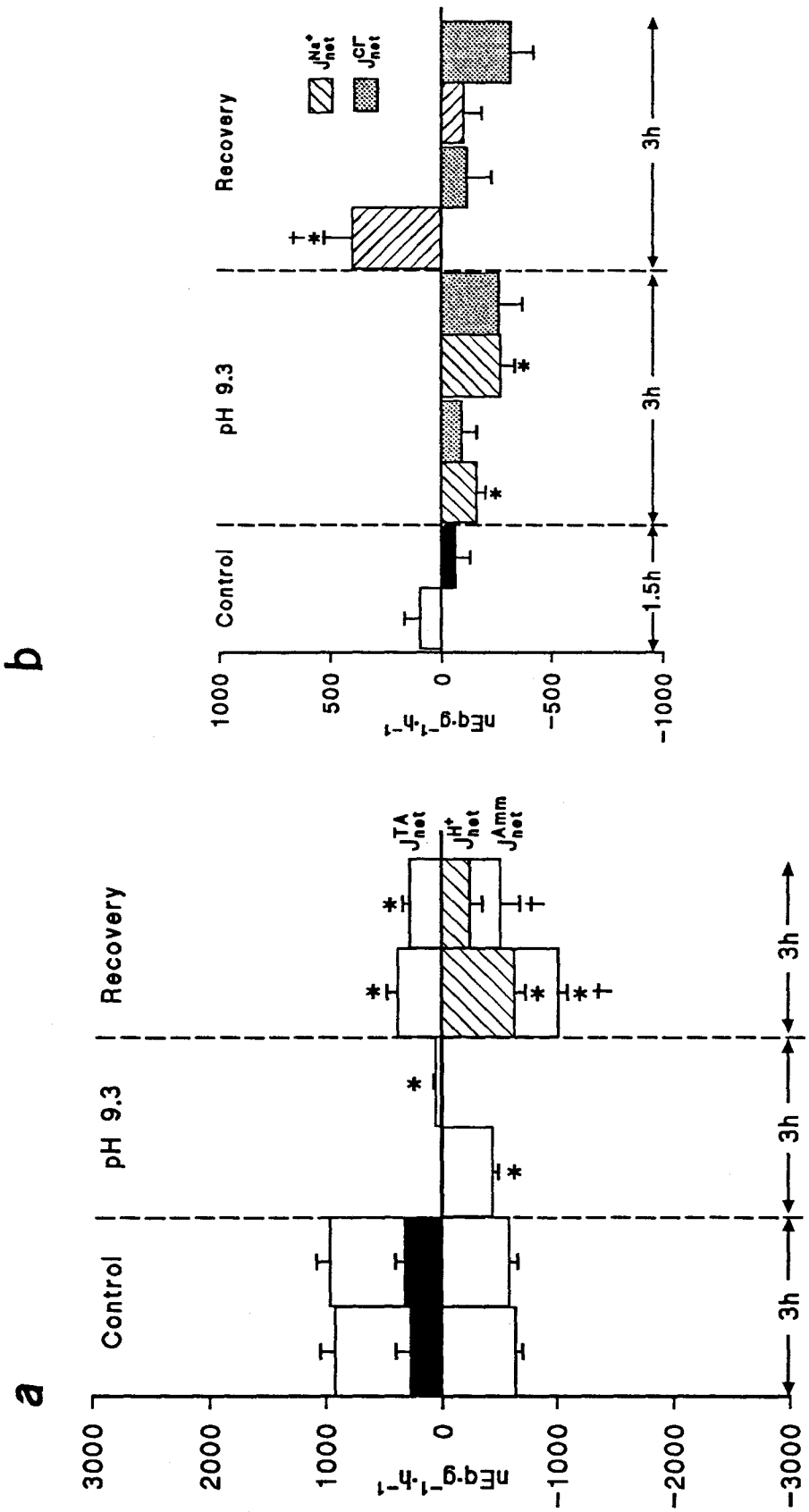
Series ii: high pH.

The net base fluxes (J^{TA}) were not plotted for the pH 9.3 exposure due to the unusual water chemistry at this pH and the resulting unreliability of this measurement as indicated by the tests performed (see Methods). J^{Amm} was significantly reduced by 1.5 hours but was completely inhibited by the second flux period. With regard to SID, the net Na^+ and Cl^- fluxes (Figure 3.7*b*) did not indicate that there was any net acid-base movement during pH 9.3 exposure and in fact the net fluxes were equimolar. During recovery, the net Na^+ gain and net Cl^- loss constrained a substantial net acid loss which agrees with the

Figure 3.7. The influence of high pH (pH = 9.3) and subsequent return to circumneutral water upon whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) fluxes (*b*). Note that J^{TA} and J^{H+} were not measured during high pH exposure (see Methods). Mean ± 1 SEM; N = 8.

* denotes a significant difference from the control rates measured at pH = 8.0 ($p \leq 0.05$).

† denotes a significant difference from the high pH treatment ($p \leq 0.05$).


a
Control
pH 9.3
Recovery
3h
3h
3h
3000
2000
1000
0
-1000
-2000
-3000
neq·g⁻¹·h⁻¹
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
b
Control
pH 9.3
Recovery
1.5h
3h
3h
1000
500
0
-500
-1000
neq·g⁻¹·h⁻¹
$J^{Na^+}_{net}$
$J^{Cl^-}_{net}$

significant net H^+ excretion which was measured in the first 1.5 hours (Figure 3.7*a*). This net acid loss resulted from the continued depression of J^{TA} over the three hours of recovery and the substantial increase in J^{Amm} which rapidly returned to pre-exposure levels by hour 3. The net Na^+ gain was short-lived as a net loss occurred by hour 3 which was still less than the net Cl^- loss (Figure 3.7*b*).

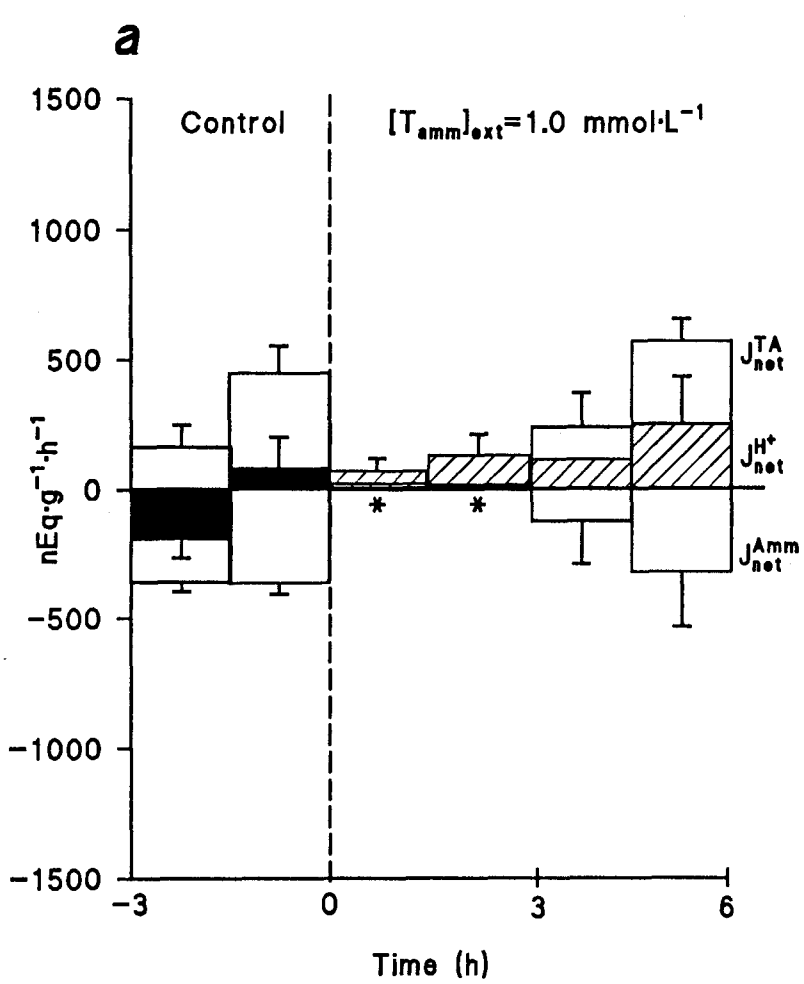
Urea excretion (Table 3.1) paralleled ammonia excretion (Figure 3.7*a*) in that it was inhibited at high pH and stimulated upon return to control water. It would seem that exposure to high alkalinity does not stimulate urea production as an alternate route of nitrogen excretion when ammonia excretion is blocked.

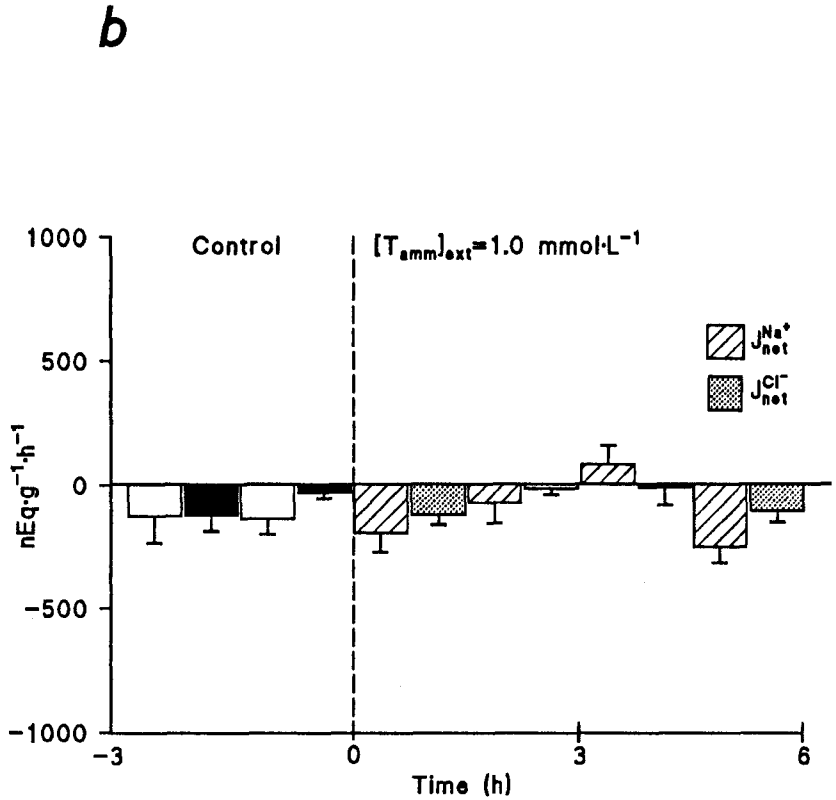
Series iii: high external ammonia.

Both J^{TA} and J^{Amm} were completely abolished within the first 1.5 hours of exposure to 1.0 $mmol \cdot L^{-1}$ T_{amm}. Both started to recover after three hours (Figure 3.8*a*). By hour 6, both J^{TA} and J^{Amm} had returned to approximately control values. Over the entire exposure period, J^{H+} gradually became more positive indicating a net base excretion/acid uptake and approximated J^{Amm} in amplitude of recovery over the 6 hours. A net base loss/acid uptake was also determined by SID in the final flux period as net Na^+ loss exceeded Cl^- loss (Figure 3.8*b*). The large external ammonia levels were sufficient to inhibit J^{Amm} by reversing both NH_3 and NH_4^+ gradients and also appeared to block acid excretion (or stimulate base excretion).

Figure 3.8. The influence of high external ammonia (T_{Amm} = 1.0 mmol·L^{-1}) upon whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) fluxes (*b*). Mean ± 1 SEM; N = 8.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
Control
$[T_{amm}]_{ext}$=1.0 mmol·L^{-1}
1500
1000
500
0
−500
−1000
−1500
nEq·g^{-1}·h^{-1}
J^{TA}_{net}
$J^{H^+}_{net}$
J^{Amm}_{net}
*
*
−3
0
3
6
Time (h)


b
Control
$[T_{amm}]_{ext}$=1.0 mmol·L^{-1}
1000
500
0
−500
−1000
nEq·g^{-1}·h^{-1}
$J^{Na^+}_{net}$
$J^{Cl^-}_{net}$
−3
0
3
6
Time (h)

Urea excretion was immediately stimulated and continued to rise throughout the ammonia exposure (Table 3.1). Urea-N, or the total amount of nitrogen excreted as urea as (2 mol N/ mol urea) had reached almost the same rate of excretion as ammonia (Figure 3.8*a*). The ratio of J^{Amm} to J^{Urea} changed from 4:1 under control conditions to 1.28:1 after 6 hours of high external ammonia exposure. Based on an estimate of the rise of internal ammonia (*i.e.* in the whole body fluids) over the 6 hours due to the inhibition of J^{Amm}, the total urea-N excreted could effectively remove 25% of the buildup of internal ammonia! This contrasts with the high pH exposure in which both J^{Amm} and J^{Urea} were inhibited and subsequently both were stimulated during recovery (Figure 3.7*a*).

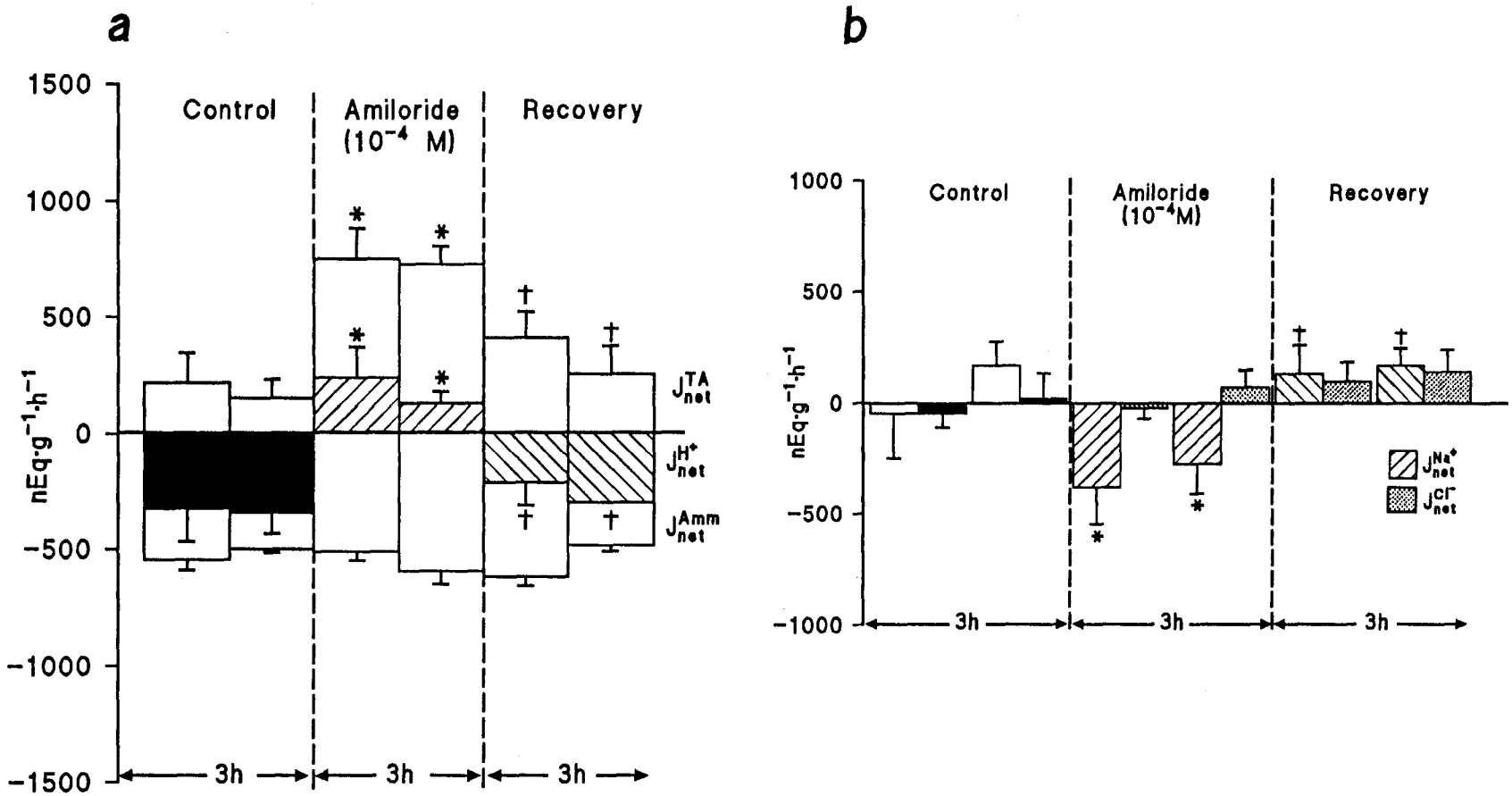
Series iv: amiloride.

Not only did amiloride inhibit Na^+ uptake (Figure 3.4*a*) but it also blocked net acid excretion, an effect which was significant (Figure 3.9*a*). Despite the dramatic reversal of net acid movement, net ammonia excretion remained unchanged throughout the amiloride treatment. Upon return to control water, J^{H+} resumed pre-exposure levels of excretion. This blockade of acid excretion was also demonstrated by the greater net Na^+ loss over Cl^- loss meaning a net base loss/acid uptake (Figure 3.9*b*). Equimolar net Na^+ and Cl^- gains were measured during recovery which approximated control values. J^{H+} returned to a negative value indicating a net acid excretion which was also the case prior to the amiloride treatment (Figure 3.9*a*).

Figure 3.9. The effect of amiloride treatment (10^{-4} M) and subsequent removal thereof upon whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (*a*); and net Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) fluxes (*b*). Mean ± 1 SEM; N = 8.

* denotes a significant difference from the control rates ($p \leq 0.05$).

† denotes a significant difference from the amiloride treatment ($p \leq 0.05$).


a
Control
Amiloride
(10⁻⁴ M)
Recovery
1500
1000
500
0
−500
−1000
−1500
nEq·g⁻¹·h⁻¹
3h
3h
3h
J^TA net
J^H+ net
J^Amm net
b
Control
Amiloride
(10⁻⁴M)
Recovery
1000
500
0
−500
−1000
nEq·g⁻¹·h⁻¹
3h
3h
3h
J^Na+ net
J^Cl− net

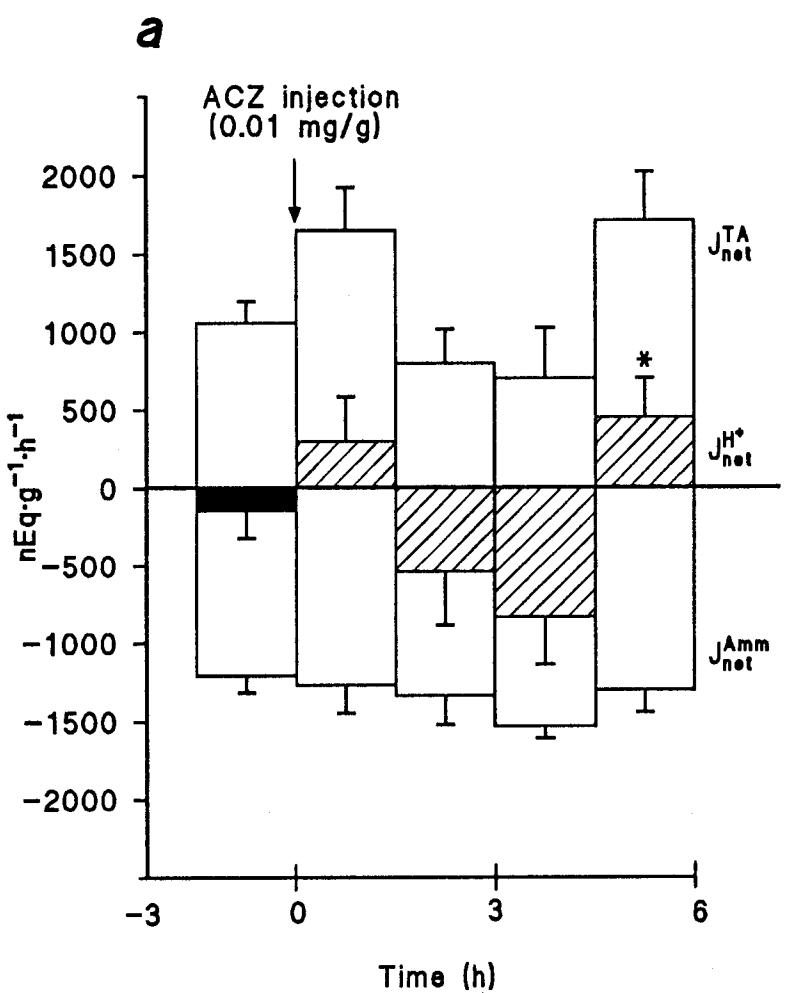
Series v: Acetazolamide injection.

Overall, there did not appear to be an obvious effect of ACZ on acid-base fluxes except for the marked inhibition of net acid excretion at hour 6 (Figure 3.10*a*). Ammonia excretion remained unchanged following injection whereas titratable alkalinity fluctuated. The net Na^+ and Cl^- fluxes in Figure 3.10*b* did not follow the pattern of net acid movement found in Figure 3.10*a*. According to SID, a net base excretion took place in the first three hours following ACZ injection then reversed at hour 6 to constrain a net acid loss. Interestingly, the significant and positive J^{H+} (*i.e.* acid uptake) parallels the significant inhibition of J^{Na}_{in} (Figure 3.5*a*).

For environmental disturbances involving changes in pH (*i*, *ii*), ammonia levels (*iii*) and application of the Na^+ uptake inhibitor, amiloride (*iv*), strong ion difference estimates of J^H, measured by differences in Na^+ and Cl^- net fluxes, generally agreed with the acid-base fluxes measured for these experiments. However, during the application of the carbonic anhydrase inhibitor, ACZ (*v*), this relationship broke down.

Figure 3.10. The effect of intraperitoneal injection of acetazolamide (ACZ) upon whole-body net titratable alkalinity flux (J^{TA}), net acid movement (J^{H+}), and net ammonia excretion (J^{Amm}) (a); and net Na^+ (J^{Na+}) and Cl^- (J^{Cl-}) fluxes (*b*). Injection dosage (0.01 mg/g). Mean ± 1 SEM; N = 7.

* denotes a significant difference from the control rates ($p \leq 0.05$).


a
ACZ injection
(0.01 mg/g)
2000
1500
1000
500
0
−500
−1000
−1500
−2000
nEq·g⁻¹·h⁻¹
J_{net}^{TA}
$J_{net}^{H^+}$
J_{net}^{Amm}
*
−3
0
3
6
Time (h)

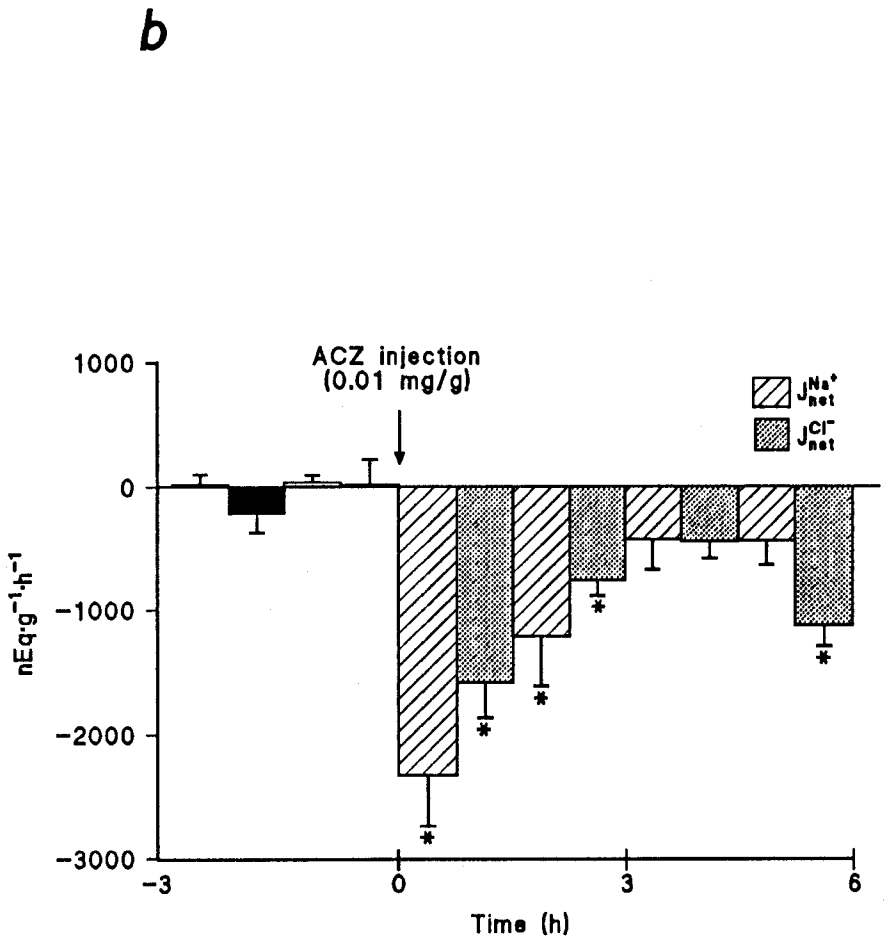

b
ACZ injection
(0.01 mg/g)
1000
0
−1000
−2000
−3000
nEq·g⁻¹·h⁻¹
$J_{net}^{Na^+}$
$J_{net}^{Cl^-}$
*
−3
0
3
6
Time (h)

Table 3.1: Urea excretion rates from the high pH experiment (series *ii*) and the high external ammonia experiment (series *iii*). Mean ± SEM (N).

		Urea excretion rates (N nmol·g·h^{-1})		
		Series *ii*		Series *iii*
Control flux	1	49.5 ± 14.8 (8)		94.1 ± 5.1(8)
	2	70.4 ± 11.6		84.8 ± 5.4
pH 9.3 flux	1	38.6 ± 21.1	HEA flux 1	137.0 ± 10.8*
	2	-25.0± 20.2*	2	127.6 ± 11.0*
Recovery flux	1	100.7± 14.4†	3	163.0 ± 14.3*
	2	89.8 ± 12.8†	4	234.1 ± 26.0*

* significantly different from control excretion rates ($p \leq 0.05$)

† significantly different from the rates at pH 9.3 ($p \leq 0.05$).

Discussion

Low pH

The main cause of mortality of fish at low pH is the depletion of plasma Na^+ and Cl^- (McDonald, 1983; Wood, 1989). The significant reduction of J^{Na}_{in} with acute exposure to pH 4.5 demonstrates this inhibitory nature of H^+ in freshwater-adapted *Fundulus heteroclitus* (Figure 3.1*a*), an effect which has been reported in earlier low pH studies involving rainbow trout (McDonald *et al.* 1983; Wright & Wood, 1985), brown trout (McWilliams & Potts, 1978), brook trout (McDonald *et al.* 1983), goldfish (Maetz, 1973), and also in amphibian skin (Garcia Romeu *et al.* 1969). At a pH of 4.0, rainbow trout and brook trout show an immediate and complete inhibition of J^{Na}_{in} (Packer & Dunson, 1970; Wright & Wood, 1985) whereas *F. heteroclitus* experienced a 50% reduction at an average pH of 4.5 (Figure 3.1*a*) which may suggest a greater resilience to acidity. It is commonly believed that protons compete for the same transport site as Na^+ thereby reducing J^{Na}_{in}. However J^{Na}_{in} inhibition in goldfish was similar (*i.e.* 60%) regardless of $[Na^+]_{ext}$ indicating that H^+ and Na^+ do not compete for the same uptake site (Maetz, 1973). Another explanation is the titration of negative charges by H^+ within the Na^+ channels, or the channels that lead to the transporter (Motais & Garcia Romeu, 1972). This is a strong possibility but the recently proposed Na^+ channel/H^+-ATPase configuration may provide the best answer (Lin & Randall, 1991). In this situation, the actual Na^+ channel may or may not be directly blocked. However more importantly, the

H^+-ATPase would be inhibited by the high $[H^+]_{ext}$ which would reduce the inward voltage gradient across the apical membrane, thereby slowing Na^+ entry. The very low level of Cl^- uptake was unchanged throughout low pH exposure (Figure 3.1*b*). According to Motais & Garcia Romeu (1972), external H^+ would have little or no effect on the already positively charged Cl^- channels.

By the third hour of low pH, significant losses of both Na^+ and Cl^- had occurred (Figure 3.1*a*, *b*) with the Na^+ loss being a consequence of inhibited uptake whereas stimulated Cl^- efflux contributed greatly to the Cl^- loss. Net Na^+ loss is a common response by acid-intolerant freshwater teleosts (Freda & McDonald, 1988) but studies have reported either reduced Cl^- losses (McWilliams, 1980), no change (Maetz, 1973) or large Cl^- losses (McDonald *et al.* 1983). Low pH alters the permeability of epithelia. Specifically, H^+ ions are believed to displace Ca^{2+} ions from the paracellular channels thus allowing for greater passive diffusion of both Na^+ and Cl^- (*i.e.* a nonselective permeability effect). Gonzalez *et al.* (1989) determined that *F. heteroclitus* have a considerable acid tolerance (*i.e.* incipient lethal pH 3.75-4.0) compared to rainbow trout in soft water which survived at pH 4.6 for only 6.5 hours (Freda & McDonald, 1988). This tolerance is surprising as estuarian habitats are rarely acidic. Even so, *F. heteroclitus* are not immune from the effects of low pH as significant ion loss did occur within the short 3 hour exposure.

In contrast to HCl injection (see Chapter 2), where Na^+ and Cl^- efflux were manipulated to compensate for the systemic acidosis, exposure to

environmental acidity induced changes in the Na^+ uptake component, with inhibition by external H^+.

Upon recovery, J^{Na}_{in} was greatly stimulated and this was solely responsible for the significant net Na^+ gain (Figure 3.1*a*). In contrast, J^{Cl}_{out} remained significantly elevated during the first 1.5 hours recovery but was returned to control values in the second 1.5 hour recovery resulting in a net gain of Cl^- (Figure 3.1*b*). This would indicate that *F. heteroclitus* can adjust J^{Na}_{in} to modulate net Na^+ movement but can only modify J^{Cl}_{out} to reduce a Cl^- loss.

Evidence against a coupling between Na^+ uptake and ammonia excretion was provided by this experiment. While J^{Na}_{in} was significantly inhibited by low pH and subsequently stimulated during recovery (Figures 3.1*a*), J^{Amm} remained unchanged throughout the entire experiment (Figure 3.6*a*). This differs from the significant reductions of both J^{Na}_{in} and J^{Amm} in rainbow trout exposed to low pH (McDonald *et al.* 1983; Wright & Wood, 1985; Lin & Randall, 1990), an effect which was attributed to the coupling between these two processes (*i.e.* Na^+/NH_4^+ exchange). This may not be the case for *F. heteroclitus* which may excrete ammonia entirely in the form of NH_3. However this hypothesis is not completely supported by the results, because low pH should have enhanced NH_3 efflux due to the improved ΔP_{NH_3} gradient and limitless ammonia trapping by environmental H^+ (see Chapter 1). While enhanced J^{Amm} did not occur, a plausible explanation is outlined below.

Concurrent with the diminished J^{Na}_{in}, net acid flux (J^{H+}) became positive by

hour 3 of low pH exposure (Figure 3.6*a*) suggesting an inhibition of acid excretion. This provides more support for a Na^+/H^+ association in *F. heteroclitus.* Whether or not the link is via a Na^+/H^+ exchanger or a Na^+ channel/H^+-ATPase configuration, the amount of diffusion trapping provided by the external H^+ may have been sufficient to compensate for the blockage of H^+ excretion, thereby maintaining the same degree of diffusion trapping and unchanged J^{Amm} (Figure 3.6*a*).

The estimate of a net base excretion/acid uptake during low pH using SID (Figure 3.6*b*) agreed with the actual acid-base fluxes (Figure 3.6*a*). Acid excretion did resume during recovery but did not surpass the control rate (Figure 3.6*b*) whereas a much larger gap between net Na^+ and Cl^- fluxes did imply a greater acid efflux (Figure 3.6*a*). This discrepancy could be a result of 3 hour acid-base fluxes measurements versus the 1.5 hour Na^+ and Cl^- fluxes.

High pH

Few studies have looked at the effects of high pH on ionoregulation as more focus has been directed towards the disturbances caused by acidity in freshwater fish (McDonald, 1983). Of the few, Wright & Wood (1985) performed a similar 3 hour, high pH exposure using rainbow trout and found inhibition of J^{Na}_{in} resulting in a significant Na^+ loss while J^{Na}_{out} remained unchanged. This is identical to the results in the present study (Figure 3.2*a*). However in contrast to pH 4.5 exposure (Figure 3.1*a*), the inhibition of J^{Na}_{in} was

not as great and there was virtually no change in Cl^- unidirectional fluxes. Recently, Na^+ and Cl^- unidirectional fluxes of rainbow trout exposed to high pH for a longer period high pH have been measured (Wilkie & Wood, 1994). Within the first 3 hours, both J^{Na}_{in} and J^{Cl}_{in} were immediately inhibited with the latter experiencing the greatest inhibition. After 48 hours, J^{Cl}_{in} resumed control values while exposure to pH 10 continued. Plasma Na^+ and Cl^- concentrations stabilized at reduced levels by this time. Wilkie & Wood (1994) hypothesized that the recovery of J^{Cl}_{in} was due to the observed increase in chloride cell fractional surface area (CCFA) (see Chapter 2) thus exposing more Cl^- uptake sites whereas J^{Na}_{in} did not recover. Because *F. heteroclitus* does not have an appreciable J^{Cl}_{in}, it is unlikely that a tremendous depletion of plasma Cl^- would occur unless J^{Cl}_{out} was greatly stimulated. In the present study, this did not occur.

In contrast to recovery from low pH, J^{Na}_{out} was significantly reduced and was solely responsible for the large net Na^+ gain following high pH exposure (Figure 3.2*a*). The positive J^{Na}_{net} was short lived as the unidirectional Na^+ fluxes resumed pre-exposure levels. Surprisingly, J^{Cl}_{in} was significantly reduced in the latter part of recovery, the first time that this component has ever been modulated in this study (Figure 3.2*b*). The reason is unknown.

Figure 3.7*a* demonstrates that highly alkaline water dramatically inhibited J^{Amm} and by 3 hours, it was completely blocked. This is in agreement with the trout response to high pH (Wright & Wood, 1985; Wilkie & Wood, 1991; Wilkie

et al. 1993) keeping in mind that the time reference to these studies is the initial 3-8 hours. Beyond the first 8 hours of high pH, J^{Amm} does recover to control rates in trout. The mechanism of inhibition is believed to be the elimination of the acidified boundary layer next to the gill epithelium thereby prohibiting ammonia trapping. The elevated internal T_{Amm} over this time period is sufficient to reestablish ΔP_{NH_3}, thereby allowing for a restoration of passive ammonia diffusion. It would be difficult to predict if *F. heteroclitus* would follow the same trend as rainbow trout during long term exposure.

Upon recovery, J^{Amm} was initially stimulated above control levels but resumed control rates by 3 hours (Figure 3.7*a*). This suggests a build-up of internal ammonia occurred during high pH exposure that was removed upon return to circumneutral pH. J^{TA} resumed but at a rate approximately half of the control value whereas net acid excretion remained significantly elevated until 1.5 hours. Both the rainbow (Wilkie & Wood, 1991) and the Lahontan cutthroat trout (Wilkie *et al.* 1993) compensated for the alkalosis of high pH exposure (induced by both respiratory and metabolic components) by production of lactic acid. The elevated J^{H+} during recovery could imply that a similar internal compensation was performed in *F. heteroclitus* (Figure 3.7*a*).

J^{H+} determined by the net Na^+ and Cl^- fluxes (Figure 3.7*b*) agreed with the net acid excretion (Figure 3.7*a*) during recovery as reflected by the large difference between the net Cl^- loss and net Na^+ gain. Because J^{TA} could not be reliably measured during pH 9.3 exposure, the only indication of acid-base

status is given by the net Na^+ and Cl^- fluxes which were virtually equimolar at pH 9.3 (Figure 3.7*b*). This suggests that an alkalosis was not induced. Again the possibility of a compensating endogenous acidosis via lactate production could be implicated.

Environmental alkalinity did not stimulate J^{Urea} even though J^{Amm} was significantly diminished (Table 3.1). Surprisingly, J^{Urea} exhibited the same pattern of inhibition as J^{Amm} at high pH followed by a significant stimulation in the first 1.5 hours of recovery. This contrasts with the longer term response to pH 10 exposure in both rainbow (Wilkie & Wood, 1991) and Lahontan cutthroat trout (Wilkie *et al.* 1993) where J^{Urea} became greatly elevated over 48 hours apparently by a stimulation of the uricolysis pathway of the liver. In the present study, the high pH exposure was acute hence, plasma T_{Amm} may not have risen enough to induce a rapid urea production. On the other hand, the higher J^{Urea} following high pH may imply that indeed urea production was stimulated but excretion was blocked. The reason for urea excretion inhibition at high pH is unknown as urea is a small uncharged molecule that presumably diffuses out of the fish (Wood, 1993). Perhaps mucification of the gill epithelium during exposure prevented its simple diffusion.

The idea that urea production in teleosts is a mechanism of acid-base regulation, specifically for the removal of excess HCO_3^- (Atkinson, 1992), does not apply to *F. heteroclitus* as both $NaHCO_3$ injection (see Chapter 2) and high pH exposure (Table 3.1) did not stimulate J^{Urea}.

High External Ammonia

In agreement with the low pH series of this chapter plus the Na^+ kinetics and HCl injection study of Chapter 2, J^{Na}_{in} and J^{Amm} do not appear to be directly linked. Exposure to high external ammonia (HEA) disrupted J^{Amm} (Figure 3.8*a*) whereas J^{Na}_{in} remained undisturbed (Figure 3.3*a*). In contrast to this, Maetz & Garcia Romeu (1964) using an addition of only 16-40 $\mu mol \cdot L^{-1}$ T_{Amm} (as ammonium sulfate) to the external medium, caused significant inhibition of J^{Na}_{in} and J^{Amm} in the goldfish (*Carassius auratus*). Later, Maetz (1973), using $[T_{Amm}]$ ~ 7 $mmol \cdot L^{-1}$ also inhibited J^{Na}_{in}. Both of these studies claimed that J^{Na}_{in} was inhibited by NH_4^+ competition and that ammonium ion was being transported inward while Na^+ was excreted during HEA. This provided evidence for Na^+/NH_4^+ exchange in goldfish and suggested that this transport could reverse directions. Apparently, this is not the case for *F. heteroclitus*.

Wilson & Taylor (1992) reported that an external $[T_{Amm}]$ of 1.0 $mmol \cdot L^{-1}$ would suffice in reversing both NH_3 and NH_4^+ gradients in freshwater rainbow trout. This may also hold true for *F. heteroclitus* as J^{Amm} was abolished initially, but J^{Amm} resumed near-control rates by hour 6 of exposure (Figure 3.8*a*). J^{TA} followed a similar path of recovery as J^{Amm} which suggests that ammonia excretion had resumed in the unionized form, NH_3, perhaps due to a restoration of the ΔP_{NH_3} gradient as $[T_{Amm}]_{pl}$ increased. The positive J^{H+} indicates a net acid uptake/base excretion which argues against ammonia excretion occurring via the protonated form (NH_4^+). In the rainbow trout exposed to the same HEA,

J^{Amm} became negative initially indicating a substantial ammonia entry but recovery and return to control excretion rates occurred within the same time frame as found in the present study (Wilson *et al.* 1994).

In addition to the apparent NH_3 diffusion against the large concentration gradient (Figure 3.8*a*), the lack of J^{Na}_{in} modulation (Figure 3.3*a*) during HEA was another significant piece of evidence against Na^+/NH_4^+ exchange in *F. heteroclitus*. Na^+/NH_4^+ exchange was used to explain the re-establishment of J^{Amm} in rainbow trout (Cameron & Heisler, 1983) and goldfish (Maetz & Garcia Romeu, 1964) in the face of chronically elevated water [T_{Amm}]. However, recently, Wilson *et al.* (1994) have presented evidence against the idea in trout, inasmuch as amiloride completely inhibited J^{Na}_{in}, but only slightly reduced the recovery of J^{Amm} during HEA exposure. Wilson *et al.* (1994) suggested that recovery of J^{Amm} was largely due to enhanced boundary layer acidification for NH_3 diffusion trapping. H^+/NH_4^+ exchange has also been proposed as a possible mechanism of J^{Amm} in trout (Wilson & Taylor, 1992) and channel catfish (Cameron, 1986) at HEA. However since H^+/NH_4^+ exchange is acid-base neutral, the present finding of net H^+ uptake during HEA does not fit the theory (Figure 3.8*a*).

Na^+ and Cl^- net fluxes (Figure 3.8*b*) agree with the elevated net acid uptake (Figure 3.8*a*) at hour 6 of HEA as Na^+ net loss predominated over Cl^- loss.

According to Table 3.1, urea excretion was stimulated only when J^{Amm} was inhibited by high external ammonia (Figure 3.8*a*). In fact, the increase of J^{Urea} paralleled the recovery of J^{Amm} and by hour 6, urea-N excretion approximated 80% of control ammonia excretion (see Results) and could account for the removal of 25% of the built up ammonia within the body fluids. The Magadi tilapia (*O. alcalicus grahami*) (Randall *et al.* 1989) and the gulf toadfish (*O. beta, O.tau*) (Walsh & Mommsen, 1989) both possess functional ornithine-urea cycle (OUC) enzymes with the former excreting all nitrogenous waste as urea (Randall *et al.* 1989). Both the Magadi tilapia and gulf toadfish (*O. beta*) respond to HEA by rapidly elevating J^{Urea} suggesting that (*i*); urea provides an alternate pathway of nitrogen excretion in the face of elevated internal T_{Amm} and (*ii*); OUC enzymes must be readily active (Wood, 1993). This suggests that *F. heteroclitus* may be another of the few species of fish possessing the OUC pathway as stimulation of J^{Urea} during HEA was immediate (Table 3.1). Clearly, this is a topic worthy of future investigation.

Amiloride

This study provided more evidence against Na^+/NH_4^+ coupling as J^{Na}_{in} (Figure 3.4*a*) was significantly inhibited (~60%) by 10^{-4} M amiloride whereas J^{Amm} was undisturbed (Figure 3.9*a*). Amiloride is thought to competitively block Na^+ channels, exchangers and co-transporters (Kleyman & Cragoe, 1990) which evidently, are not responsible for J^{Na}_{out} as it remained unchanged during

exposure. What did dramatically change with amiloride exposure was J^{H+} which switched from a negative value (loss) to a positive value (gain) (Figure 3.9*a*). The implication of this result is a concurrent inhibition of Na^+ uptake and net acid excretion which suggests a coupling of Na^+ uptake and H^+ extrusion. The same amiloride concentration used on rainbow trout completely blocked J^{Na}_{in}, while J^{Amm} was only slightly inhibited (23%) suggesting either that a small contribution from Na^+/NH_4^+ exchange was blocked and/or that NH_3 diffusion coupled to Na^+ uptake via H^+ diffusion trapping was inhibited. Thus ammonia excretion would continue only in the unionized form (NH_3) (Wright & Wood, 1985; Wilson *et al.* 1994). However, J^{H+}_{net} was not measured in these studies. Lin & Randall (1991) used the same amiloride dose but reported no changes in either net proton excretion or ammonia excretion. The explanation presented was an amiloride-insensitive electrogenic proton pump presumably functioning in the trout gill epithelium. However, this conclusion must be tempered by the fact that the method which they used for measuring net proton excretion was unconventional and theoretically questionable. Using *in vitro* techniques, Ehrenfeld *et al.* (1985) measured net H^+ excretion correctly and Na^+ unidirectional fluxes in amphibian epithelium with respect to various treatments, including amiloride. In agreement with the trout study by Lin & Randall (1991), J^{H+}, via the electrogenic H^+ pump, was not altered by amiloride doses less than 5×10^{-4} M while J^{Na}_{in} was completely inhibited with subsequent net Na^+ losses at doses half that used in the present study (10^{-4} M). Perhaps the H^+ extrusion

mechanism in *F. heteroclitus* is more sensitive to amiloride.

Upon recovery from amiloride, J^{Na}_{in} resumed pre-exposure rates with a net Na^+ gain demonstrating the reversibility of the amiloride effects (Figure 3.4*a*). This follows the same pattern of recovery as in rainbow trout (Wright & Wood, 1985).

Figure 3.4*b* shows that unidirectional Cl^- fluxes in *F. heteroclitus*, including the very low J^{Cl}_{in}, were unaffected by amiloride. This finding, in addition to the results from high and low pH (Figure 3.1*b*, 3.2*b*), high external ammonia (Figure 3.3*b*), and both the uptake kinetics and injection series (see Chapter 2) provide very strong evidence for a Cl^- uptake mechanism completely independent of Na^+ uptake in *F. heteroclitus*. It would be instructive to test whether SITS (4-acetamido-4'-isothiocyanatostilbene-2-2'-disulphonic acid), a known Cl^- uptake inhibitor, would block the low J^{Cl}_{in}, and whether it would alter J^{Na}_{in}. Perry & Randall (1981) reported that SITS completely inhibited J^{Cl}_{in} over 3 hours in rainbow trout and also blocked J^{Na}_{in} by 65% within the same time period. The mechanism of this "crossover effect" in trout remains unknown.

The significant inhibition of net acid excretion (Figure 3.9*a*) was also predicted using SID as net Na^+ loss surpassed net Cl^- loss (Figure 3.9*b*) during amiloride exposure. Upon recovery, both the acid-base net fluxes (Figure 3.9*a*) and net ion fluxes (Figure 3.9*b*) resumed pre-amiloride values demonstrating the reversibility of amiloride effects.

Acetazolamide

ACZ has been utilized in several studies involving: goldfish (*Carassius auratus*); (Maetz, 1956; Maetz & Garcia Romeu, 1964), rainbow trout (Kerstetter *et al.* 1970; Kerstetter & Kirschner, 1972) and channel catfish (*Ictalurus punctatus*); (Henry *et al.* 1988). All demonstrated large and immediate reductions of Na^+ and Cl^- uptake. Maetz (1956) noted an initial negative Na^+ uptake and substantial Na^+ loss followed by a slow return to control values 24 hours following intramuscular injection. This indicates that the effects of ACZ do diminish with time but the effect is very rapid even when intramuscular injections are performed. In contrast, *F. heteroclitus* injected intraperitoneally with ACZ, exhibited an unchanged J^{Na}_{in} except for a slight fall at 6 hours (Figure 3.5a). Maetz & Garcia Romeu (1964) injected ACZ intraperitoneally and found an immediate and complete block of J^{Cl}_{in} in goldfish, indicating a dependence of Cl^- uptake upon the HCO_3^- supplied by CAH. In the present study, J^{Cl}_{in} was not significantly altered, though it did decline at hour 6 (Figure 3.5*b*). These results may imply that neither Na^+ nor Cl^- uptake in *F. heteroclitus* rely upon the counterions (H^+ or HCO_3^-) produced by CAH, in contrast to the situation in teleosts which conform to the standard freshwater model (Figure 1.3). However this conclusion must be tempered by the equivocal results at 6 hours. Longer term studies are required in the future.

Lacy (1983) was able to measure substantial amounts of CAH in both FW and SW-adapted *F. heteroclitus* opercular epithelium and the application of ACZ

reduced a histochemical reaction product indicative of HCO_3^- production suggesting that ACZ was effective *in vitro*. Though CAH appears to be active and plentiful in this epithelium, Zadunaisky (1984) did not observe disruption of either the short circuit current (I_{sc}) or the transepithelial potential (TEP) of the SW opercular membrane with serosal addition of ACZ. Thus CAH may not be directly involved in ionoregulation in *F. heteroclitus* in either freshwater or seawater. Perhaps instead it serves to facilitate CO_2 diffusion, as in the shark rectal gland (C.M. Wood & T.J. Shuttleworth, personal communication).

The tremendous stimulation of both Na^+ and Cl^- efflux components after ACZ injection, resulting in large net losses of both ions (Figure 3.5*a*, *b*), was perplexing. These losses were much larger than seen after control saline injections in Chapter 2 (Figure 2.4 *a*, *b*). It is unlikely that acetazolamide would influence paracellular permeability but Perry & Laurent (1990) did allude to the fact that there are inherent side effects with ACZ use, though this point was not elaborated. Ehrenfeld *et al.* (1985) reported large effluxes of Na^+ when ethoxzolamide, a lipophilic CAH inhibitor, was applied to the serosal surface of amphibian skin *in vitro*. This effect was attributed to hyperpolarization of the epithelium. This is a possible explanation for the large stimulation of Na^+ and Cl^- efflux in the present study.

Due to the variability in the data (Figure 3.10*a*), it is difficult to determine if ACZ injection has any effect on net acid-base fluxes. There was little clarification using SID as net Na^+ and Cl^- fluxes were also variable and the

apparent response at 4.5-6 hours was directly opposite to the response in J^{H+}_{net} (Figure 3.10*b*). While net acid movement was highly variable, J^{Amm} remained constant (Figure 3.10*a*) suggesting that carbonic anhydrase blockage did not interfere with ammonia excretion. Kerstetter *et al.* (1970), Avella & Bornancin (1989) and Lin & Randall (1991), using rainbow trout (*Oncorhynchus mykiss*), also noted that acetazolamide did not affect ammonia excretion. Again these results argue against the presence of a Na^+/NH_4^+ exchange.

CHAPTER 4

Calcium uptake in the freshwater-adapted *Fundulus heteroclitus*

Mummichogs (*F. heteroclitus*) not only cope with large fluctuations of NaCl in their natural habitat but environmental calcium as well. Calcium levels can range from soft, fresh water levels (< 0.2 $mEq \cdot L^{-1}$) to full strength sea water (20 $mEq \cdot L^{-1}$). Regardless of external calcium concentrations, like many other teleosts, mummichogs are able to maintain plasma calcium levels within a narrow range (~2.0-2.5 mM Ca^{2+}) (Pang *et al.* 1980; Mayer-Gostan *et al.* 1983). This requires fine tuned regulation which is why much research has focussed on the endocrine control of calcium homeostasis. Prolactin, termed the freshwater hormone, possesses hypercalcaemic properties and modulates the activity of basolateral Ca^{2+}-ATPase (Flik & Verbost, 1993) but is slow in its action (*i.e.* several days) (Pang & Pang, 1986). Stanniocalcin, a hypocalcaemic factor, provides minute-to-minute regulation of branchial permeability to calcium, specifically through the non-voltage gated apical Ca^{2+} channels (Verbost *et al.* 1993; Flik & Verbost, 1993). Elevated plasma cortisol has been shown to increase calcium uptake in freshwater rainbow trout by stimulating proliferation of the chloride cells on the gill epithelium (Perry & Wood, 1985; Flik & Perry, 1989). This cell type has been determined to be the site of calcium absorption in freshwater teleosts (Payan *et al.* 1981; Perry & Wood, 1985; Ishihara &

Mugiya, 1987; Flik & Perry, 1989; Perry *et al.* 1992).

In view of the extensive body of research on the hormonal control of calcium metabolism in the mummichog (reviewed by Pang & Pang, 1986), it is surprising that so few *in vivo* investigations have attempted to characterize calcium uptake in *Fundulus heteroclitus*. The problem with measuring calcium uptake in fish is that calcium adsorbs to the body surface, to excreted mucus, and to the walls of the flux container once they become coated with organic material. One cannot use the disappearance of radio-labelled calcium (^{45}Ca) from the water as a reliable indicator of true calcium uptake by the fish. Rather it is necessary to monitor ^{45}Ca appearance in the fish, after correcting for displaced ^{45}Ca adsorbed to the body surface. Only a single terminal flux measurement (influx only) is obtained for each animal, and the procedure is tedious and more time-consuming than the water-based approach used for Na^+ and Cl^-. Nevertheless, it has been utilized in the few studies investigating calcium uptake in *F. heteroclitus* (Pang *et al.* 1980; Mayer-Gostan *et al.* 1983). These investigators argued that calcium uptake was modulated in response to changing external Ca^{2+} concentrations and that it reflected a change in permeability of the outer branchial membrane.

Recent *in vitro* studies using opercular epithelium from freshwater-adapted *F. heteroclitus* have indicated this membrane possesses Ca^{2+} transporting properties (Burghardt, 1993). Previously, the gill epithelium was thought to be the predominant site of ionoregulation in freshwater fish. The contribution of

opercular and gill epithelium to whole-body Ca^{2+} uptake has never been addressed experimentally.

In the present study, Ca^{2+} uptake kinetics were performed using freshwater-adapted mummichogs to determine if the change in J^{Ca}_{in} over the range of external [Ca^{2+}] tested conforms to one-substrate Michaelis-Menten kinetics. The new protocol for juvenile trout developed by Hogstrand *et al.* (1994*a*) to measure the kinetics of unidirectional Ca^{2+} uptake *in vivo* was used in the present study. Further, two known inhibitors of calcium transport were used in an attempt to determine the nature of Ca^{2+} movement across the outer aspect of the branchial and/or opercular epithelium. La^{3+} is specific in blocking apical Ca^{2+} channels thereby inhibiting influx quite rapidly and irreversibly in freshwater rainbow trout (Verbost *et al.* 1987, 1989; Perry & Flik, 1988). It is unknown if La^{3+} is more effective against voltage or voltage-insensitive Ca^{2+} channels whereas Mg^{2+} is a known competitive inhibitor of voltage-gated Ca^{2+} channels in many systems (*e.g.* frog cardiac myocytes) (Hartzell & White, 1989). Finally, chronic low and high Ca^{2+} exposure (50 and 20 000 $\mu Eq \cdot L^{-1}$ respectively) and acute exposure to low Ca^{2+} were performed to determine how uptake would be modulated by external [Ca^{2+}] and to determine if mechanisms could be implemented within the first few hours of exposure to low Ca^{2+}. In each test (except the kinetic study), net Ca^{2+} and Na^{+} movement were measured in order to detect any disruptions in net Ca^{2+} and Na^{+} balance incurred by the various treatments. Net acid (J^{H+}), titratable alkalinity (J^{TA})

fluxes and ammonia excretion (J^{Amm}) were measured to detect disturbances in acid-base balance. These variables were analyzed to determine if the treatments did have effects on other processes other than calcium uptake.

Materials and Methods

Experimental animals

Mummichogs (*Fundulus heteroclitus*), weighing 2.68-11.72g, of both sexes, were collected from a brackish estuary located near Antigonish, Nova Scotia and were held at St. Francis Xavier University. The fish were held indoors in 500 L fiberglass tanks containing 10% seawater at ambient temperature (18-25°C). The water was well aerated and continuously pumped through a filter comprised of polyester fiber and charcoal. Fish were fed daily a 1:2 mixture of Tetramin/Tetramarin.

Acclimation conditions

Ten days prior to the experiment, a maximum of 25 fish were placed in 80 L glass aquariums equipped with a percolating filter consisting of polyester fiber and charcoal containing the appropriate acclimation medium (see below). Aeration was provided and water was replaced every 3 days. Fish were fed Tetramin up until 4 days before the experiment.

For series *i* and *ii*, the acclimation water was a defined freshwater medium with the following composition: Na^+, 1000; Cl^-, 1000; Ca^{2+}, 200; Mg^{2+}, 120; K^+, 20; titration alkalinity to pH=4.0, 280 all in $\mu Eq \cdot L^{-1}$; pH=6.8-7.3. This was attained by supplementing dechlorinated Antigonish tapwater with NaCl.

For series *iii*, the low Ca^{2+} medium was prepared from distilled water and supplemented with NaCl, $Ca(NO_3)_2 \cdot 4H_2O$, KCl and $NaHCO_3$ to approximate the above composition except $[Ca^{2+}] = 50\ \mu Eq \cdot L^{-1}$. High Ca^{2+} medium was

achieved by supplementing the defined freshwater medium with $Ca(NO_3)_2 \cdot 4H_2O$ to raise the $[Ca^{2+}]$ to 20 000 $\mu Eq \cdot L^{-1}$

For series *i*, the point of transfer of acclimated fish to the flux containers marked the beginning of the experiment. For series *ii* and *iii* ,the fish were placed in individual darkened flux containers the night prior to experiment. During this period, the fish were held in a static 300 ml volume of the defined medium with vigorous aeration for no longer than 6 hours before initiating experimentation.

Experimental Methodology

The defined freshwater medium lacking Ca^{2+} was prepared in the same manner as described for the low Ca^{2+} medium but without $Ca(NO_3)_2 \cdot 4H_2O$ addition. This Ca^{2+}-free water was utilized in all experiments with the $[Ca^{2+}]$ adjusted accordingly with the $^{45}Ca/Ca(NO_3)_2$ stock solution defined below. In all experiments, unidirectional Ca^{2+} influx from the water was measured by monitoring ^{45}Ca appearance in the fish.

Series i: calcium uptake kinetics

The primary goal of this study was to determine if whole body Ca^{2+} uptake by *F. heteroclitus* was concentration-dependent and if Ca^{2+} transport conformed to Michaelis-Menten first order kinetics.

Prior to addition of the fish, 1.5 L of Ca^{2+}-free water was added to each of

eight, 3 L black polypropylene flux bags. Next, an aliquot of a $^{45}Ca/Ca(NO_3)_2$ stock solution (608 $mEq \cdot L^{-1}$; specific activity = 2.0 $\mu Ci \cdot mEq^{-1}$) was added and allowed to mix for 15 minutes via aeration which was supplied to each flux bag. The approximate Ca^{2+} concentrations in each of the seven bags were: 50, 100, 200, 400, 800, 1600, 3200 $\mu Eq \cdot L^{-1}$.

The experiment was initiated by placing eight fish in each of the individual flux bags with the differing Ca^{2+} concentrations. Water samples (30 ml) were taken at 0, 2 and 4 hours for subsequent analysis of ^{45}Ca and $[Ca^{2+}]$. After 4 hours, fish were placed in a container of 20, 000 $\mu Eq \cdot L$ $Ca(NO_3)_2$ with 1.0 g/L MS-222 to remove any surface bound ^{45}Ca and Ca^{2+} and concurrently kill the fish by an overdose with anaesthetic. After one minute, the fish were removed, blotted dry, weighed and wrapped in aluminum foil. Unless processed immediately, the fish were frozen and kept at -20°C.

Series ii: The effects of Mg^{2+}, and La^{3+}.

This study was performed to determine if the addition of 100 $\mu mol \cdot L^{-1}$ $MgSO_4$ or $LaCl_3$ equimolar to Ca^{2+} would modulate Ca^{2+} influx and if net Ca^{2+}, Na^+, ammonia, and acid-base fluxes were affected by the presence of either cation.

Control, 100 $\mu mol \cdot L^{-1}$ $[MgSO_4]$ and 100 $\mu mol \cdot L^{-1}$ $[LaCl_3]$ water were made up in 3 L volumes. $MgSO_4$ and $LaCl_3$ were dissolved in the Ca^{2+}-free medium to achieve 100 $\mu mol \cdot L^{-1}$ concentration. An aliquot of the $^{45}Ca/Ca(NO_3)_2$ stock

solution was added to each batch of experimental water to achieve a [Ca^{2+}] of 200 $\mu Eq \cdot L^{-1}$ and vigorously aerated to ensure mixing of isotope.

Following the overnight settling period in the defined freshwater medium, each flux container was drained, flushed and 300 ml of fresh medium was added. After 90 minutes, the containers were drained and flushed again, followed by the addition of 300 ml experimental water with continued aeration. For the control group, N=6; $MgSO_4$ treatment, N=8 and $LaCl_3$ treatment, N=8. Prior to changing the water in the containers, the 0 hour water sample (30 ml) was taken from the three media. Once the experiment had started, samples (30 ml) were taken at 2 and 4 hours for later analysis of ^{45}Ca, Ca^{2+}, Na^+, titratable alkalinity, and ammonia. The procedure for killing and preparing the fish for subsequent analysis of ^{45}Ca was identical to that described for series *i*.

Series iii: exposure to high and low Ca^{2+} water.

The purpose of this series was to investigate the effects of chronic high and low Ca^{2+} exposure. The chronic low Ca^{2+} exposed mummichogs (50 $\mu Eq \cdot L^{-1}$ Ca^{2+}) were fluxed in the medium to which they were chronically exposed (10 days) in order to determine the endogenous rate of Ca^{2+} uptake in a low calcium environment (*i.e.* 50 $\mu Eq \cdot L^{-1}$). This was not possible for the high Ca^{2+} experiment (20 000 $\mu Eq \cdot L^{-1}$) as the amount of isotope required to maintain a water specific activity (cpm/μEq) similar to the other experiments was prohibitive. Instead, Ca^{2+} uptake rates for the chronic high Ca^{2+} exposed

mummichogs were determined in 200 $\mu Eq \cdot L^{-1}$ Ca^{2+} defined medium (*i.e.* control). The group of mummichogs exposed to 50 $\mu Eq \cdot L^{-1}$ Ca^{2+} (the kinetic study in series *i*) was used as acute low Ca^{2+} comparison.

The Ca^{2+}-free medium was supplemented with the $Ca(NO_3)_2$ to make up the prescribed [Ca^{+}] for acclimation: 50 $\mu Eq \cdot L^{-1}$ for low Ca^{2+} medium and 20 000 $\mu Eq \cdot L^{-1}$ for high Ca^{2+} medium.

Aliquots of the $^{45}Ca/Ca(NO_3)_2$ stock solution were added to the Ca^{2+}-free medium to make up either 50 $\mu Eq \cdot L^{-1}$ or 200 $\mu Eq \cdot L^{-1}$ Ca^{2+} water for the flux measurements. The fish were placed in their individual flux containers and left undisturbed overnight. At the beginning of the test, the containers were drained and flushed with fresh acclimation water added (300 ml). After 90 minutes, the flux containers were drained and 300 ml of the experimental water was added to each flux container (N=8 for high and low Ca^{2+}). Water samples (30 ml) were taken at 0, 2 and 4 hours for the same analysis as in series *ii*, and the killing and preparation of the fish followed the same protocol as stated for series *i*.

Analytical Methods and Calculations

Whole body calcium uptake

While still frozen, individual fish were cut into small pieces and placed in ceramic crucibles. The crucibles were covered with individual lids and were transferred to a muffle furnace. The fish were ashed at 750°C for a minimum of

4 hours. Following a cooling period, the crucibles were removed and the ash was transferred to glass scintillation vials. Next, ash was dissolved with 2N HCl. The volume of HCl used ranged from 3.0 to 3.6 ml depending on the amount of ash (*i.e.* size of fish) as complete dissolution was necessary. The acid was not neutralized in order to avoid further dilution of the sample. Duplicate 0.5 or 1.0 ml aliquots of the dissolved fish were prepared with 10 ml ACS (aqueous counting scintillant) and counted on a Packard 2000 CA liquid scintillation counter.

A test was run to check if the amount of ash or the HCl used quenched the Ca^{2+} activity (cpm) in the samples. Several fish that were not used in ^{45}Ca experiments were ashed. A range of ash quantities were weighed out and subsequently dissolved with HCl as described above. A known volume of the $^{45}Ca/Ca(NO_3)_2$ stock solution was added to each sample and mixed thoroughly. Next, duplicate 1.0 ml samples of 2 N HCl and distilled water together with an aliquot of the $^{45}Ca/Ca(NO_3)_2$ stock solution were prepared to test for possible quenching by the acid. The results indicated that neither the amount of ash nor acid induced colour or chemical quenching.

Water Ca^{2+} concentrations were measured using an atomic absorption spectrophotometer (Varian 375 AA). The samples were prepared by the appropriate dilution to read within the 0 to 200 $\mu Eq \cdot L^{-1}$ Ca^{2+} range. Both samples and standards contained 5mM KCl in order to eliminate Na^{+} interference.

J^{Ca}_{in} ($nEq \cdot g^{-1} \cdot h^{-1}$) was determined by the following equation:

$$J_{in} = \frac{CPM}{SA \cdot t}$$

where CPM is the average counts in the tissue samples ($cpm \cdot g^{-1}$) based on the volume of ash/HCl solution counted, the amount of acid added to dissolve the ash and the weight of the fish prior to ashing, SA is the specific activity of ^{45}Ca in the water ($cpm \cdot nEq^{-1}$) and t is time (hours).

The relationship between $[Ca^{2+}]_{ext}$ and Ca^{2+} uptake in series *i* was examined using Michaelis-Menten analysis for first-order kinetics. Ca^{2+} uptake followed a distinctive saturation curve which was well described by the one-substrate equation:

$$J_{in} = \frac{J_{max} \cdot [Ca^{2+}]_{ext}}{K_m + [Ca^{2+}]_{ext}}$$

where J_{in} is the uptake rate at a particular $[Ca^{2+}]_{ext}$. J_{max} is the maximum uptake rate and K_m is the $[Ca^{2+}]_{ext}$ at which uptake rate is 50% of J_{max}. K_m is an index for the inverse of affinity of the transport system. K_m and J_{max} values for J^{Ca}_{in} were determined from Eadie-Hofstee plots.

Water analysis

Measurement of titratable alkalinity (TA), ammonia, and Na^+ concentrations and the calculation of J^{Na}_{net} were the same as described in Chapter 2 but J^{Cl}_{net} was not measured because of the small sample volume left following all other

analyses. J^{Ca}_{net} was calculated using water [Ca^{2+}] values and J^{ion}_{net} equation (Chapter 2). J^{TA} and J^{H+} were measured in the $LaCl_3$ treatment but not reported as further tests indicated that the water titration values were not reliable due to the presence of $LaCl_3$. The 100 $\mu mol \cdot L^{-1}$ La^{3+} defined medium used in series *ii* was titrated before and after the addition of ammonium hydroxide (~100 $\mu mol \cdot L^{-1}$) which simulated the excretion of NH_3 by fish during the flux experiments. A titration of the control defined medium was performed alongside the La^{3+} medium. The test revealed a discrepancy between the control and La^{3+} titratable alkalinity values following the addition of the base indicating that $LaCl_3$ somehow interfered with the titration of the base added. The source of this interference is unknown.

Statistical analysis

All values are presented as mean $\pm$ 1 SEM. Multiple comparisons between treatment groups were performed by analysis of variance (ANOVA) followed by Fisher's test of least significant difference (LSD) in cases where the F value indicated significance ($P \leq 0.05$).

Results

Calcium uptake kinetics

The results of the Ca^{2+} uptake kinetics (Figure 4.1) indicate that saturation did occur within the range of external [Ca^{2+}] tested. J^{Ca}_{in} followed a hyperbolic curve typical of saturable transport. The affinity constant, K_m and the maximum uptake rate, J_{max} were 125 ± 36 $\mu Eq \cdot L^{-1}$ and 31 ± 4 $nEq \cdot g^{-1} \cdot h^{-1}$ respectively (Table 4.1). The K_m was slightly lower than the acclimation Ca^{2+} concentration, 200 $\mu Eq \cdot L^{-1}$.

The effects of Mg^{2+} and La^{3+}.

The equimolar addition of Mg^{2+} (*i.e.* 100 $\mu mol \cdot L^{-1}$) to the medium did not influence J^{Ca}_{in} whereas the same amount of La^{3+} significantly inhibited uptake by approximately 67% (control, 11.13 ± 4.08; La^{3+}, 3.64 ± 0.40 $nEq \cdot g^{-1} \cdot h^{-1}$) (Figure 4.2). While J^{Ca}_{in} was inhibited by La^{3+}, net Ca^{2+} movement was not significantly altered although J^{Ca}_{net} for both treatments were lower than the control group (Table 4.2) which was in positive Ca^{2+} balance (*i.e.* significantly different from zero). Notably, J^{Ca}_{net} was greater than J^{Ca}_{in} in the control group, likely reflecting adsorptive loss of Ca^{2+} from the water.

Figure 4.1. The influence of external calcium on whole-body Ca^{2+} uptake (J^{Ca}_{in}) of the freshwater-adapted mummichog. Mean ± 1 SEM. N = 8 at each [Ca^{2+}].

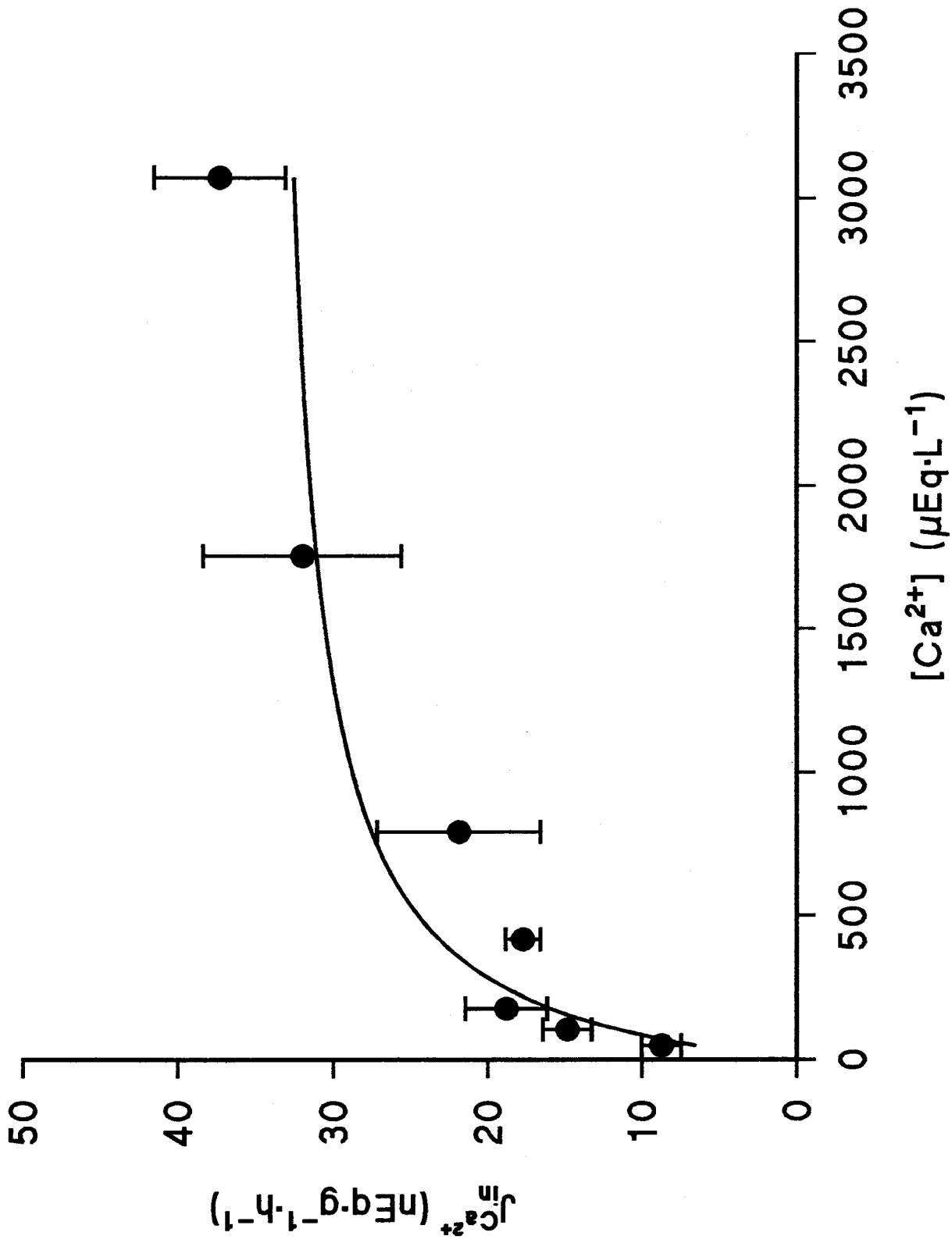

$J_{in}^{Ca^{2+}}$ (nEq·g^{-1}·h^{-1})
0
10
20
30
40
50
0
500
1000
1500
2000
2500
3000
3500
[Ca^{2+}] (μEq·L^{-1})

Table 4.1: The acclimation calcium concentration, affinity constant, K_m and maximum transport rate, J_{max} for calcium uptake measured in freshwater-adapted mummichog (*Fundulus heteroclitus*); a comparison with earlier measurements in this species and in rainbow trout (*Oncorhynchus mykiss*). Mean ± 1 SEM.

	$[Ca^{2+}]$ ($\mu Eq \cdot L^{-1}$)	K_m ($\mu Eq \cdot L^{-1}$)	J_{max} ($nEq \cdot g^{-1} \cdot h^{-1}$)
Mummichog (*in vivo*)[1]	200	125 ± 36	31 ± 4
Mummichog (*in vivo*)[2]	200	≤100	~100
Mummichog (*in vitro*)[3]	200	696	296[†]
Rainbow trout (21 g)[4]	2000	184 ± 16	106 ± 5
Rainbow trout (244 g)[5]	2000	280 ± 70	12 ± 2
Rainbow trout[5]	50	230 ± 10	66 ± 13

[1] present study; (N=7 groups of 8 fish).
[2] Mayer-Gostan *et al.* (1983); estimate based on extraintestinal rates (N=4-7)
[3] Burghardt (1993) and Marshall (personal communication); (N=6-8 opercular epithelia). [†]Estimate based on J_{max} = 740 $nEq \cdot cm^{-2} \cdot h^{-1}$ and assuming a 2 cm^2 total area (2 opercular epithelia) for an average fish weighing 5g .
[4] Hogstrand *et al.* (1994); (N=6 groups of 8 fish)
[5] Perry & Wood (1985); (N=7 for 2000 $\mu Eq \cdot L^{-1}$ acclimated, N=6 for 50 $\mu Eq \cdot L^{-1}$ acclimated for 7 days).

In addition to inhibiting Ca^{2+} uptake, La^{3+} exposure also induced a significant net Na^+ loss (Table 4.2). Mg^{2+} treatment did not affect J^{Na}_{net}. Ammonia (J^{Amm}), titratable alkalinity (J^{TA}) and net acid movement (J^{H+}) were not affected by Mg^{2+} exposure, and J^{Amm} was not altered by La^{3+} treatment (J^{TA} and J^{H+} are not reported - see Methods).

The major outcome of this series was that Ca^{2+} uptake was blocked and Na^+ balance disrupted by the presence of La^{3+} in the water and Mg^{2+} did not.

Low and high Ca^{2+} acclimation

The endogenous Ca^{2+} uptake rate for mummichogs chronically exposed to low Ca^{2+} water (50 $\mu Eq \cdot L^{-1}$) and fluxed in low Ca^{2+} water (Figure 4.3*a*) was approximately 3 fold greater than the endogenous rate measured under control conditions (200 $\mu Eq \cdot L^{-1}$ Ca^{2+}) (Figure 4.3*b*). Control fish that were acutely exposed to the low Ca^{2+} water and fluxed in low Ca^{2+} water exhibited a Ca^{2+} uptake rate that was significantly less than the chronically exposed group (chronic, 33.29 ± 3.6 $nEq \cdot g^{-1} \cdot h^{-1}$; acute, 8.75 ± 1.28 $nEq \cdot g^{-1} \cdot h^{-1}$) (Figure 4.3*a*). Mummichogs that were chronically exposed to high Ca^{2+} (20 000 $\mu Eq \cdot L^{-1}$) and fluxed in control water exhibited a J^{Ca}_{in} similar to control (Figure 4.3*b*).

Chronic high Ca^{2+} exposure caused a significant net Ca^{2+} loss as displayed in Table 4.2. For the chronic high Ca^{2+} group, J^{Ca}_{net} was determined from the final 2 hour flux period to avoid possible contamination of the experimental medium with high Ca^{2+} water carried over by the fish.

Figure 4.2. The influence of external $MgSO_4$ (N = 8) and $LaCl_3$ (N = 8) on calcium uptake (J^{Ca}_{in}) in comparison to the control rate (N = 7). Concentration was 100 $\mu mol \cdot L^{-1}$ for both treatments. Mean ± 1 SEM.

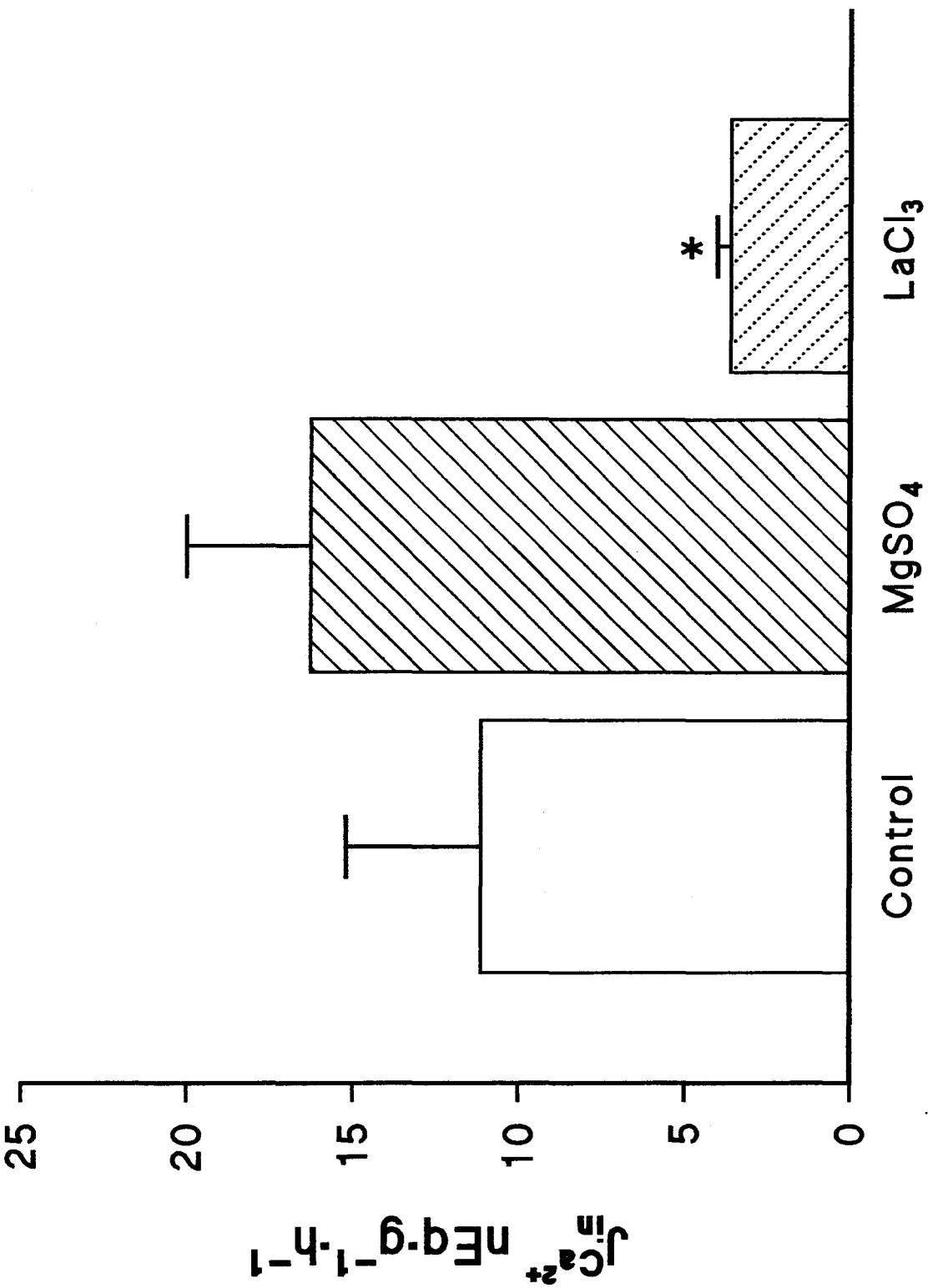
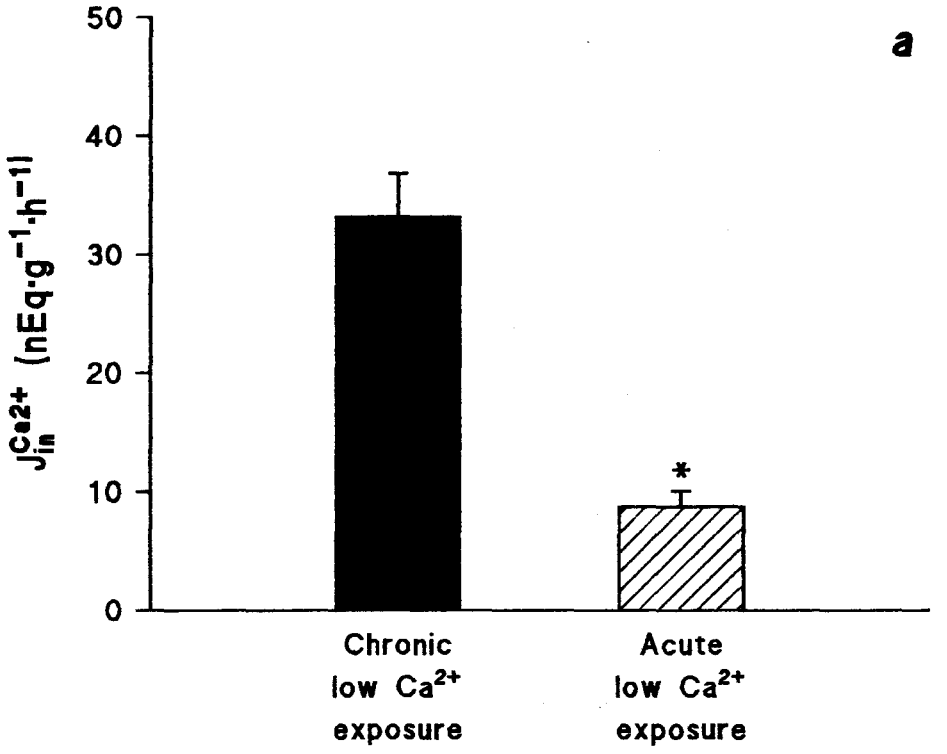

$J_{in}^{Ca^{2+}}$ nEq·g^{-1}·h^{-1}
25
20
15
10
5
0
Control
$MgSO_4$
$LaCl_3$
*

Figure 4.3. The influence of chronic and acute low calcium exposure (*a*) and chronic high calcium exposure (*b*) on calcium uptake (J^{Ca}_{in}). Low calcium concentration = 50 $\mu Eq \cdot L^{-1}$; high calcium concentration = 20 000 $\mu Eq \cdot L^{-1}$. N = 8 for acute low Ca^{2+}, chronic low Ca^{2+} and chronic high Ca^{2+} treatments, N = 7 for control.


a
50
40
30
20
10
0
J_{in}^{Ca2+} (nEq·g^{-1}·h^{-1})
*
Chronic low Ca^{2+} exposure
Acute low Ca^{2+} exposure

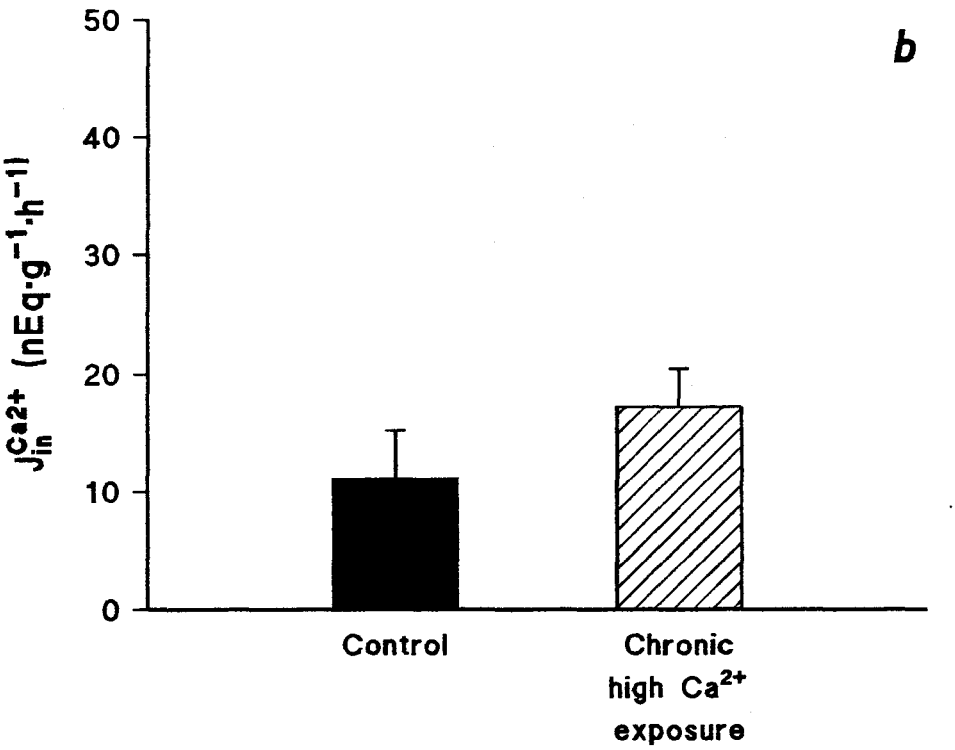

b
50
40
30
20
10
0
J_{in}^{Ca2+} (nEq·g^{-1}·h^{-1})
Control
Chronic high Ca^{2+} exposure

Table 4.2: Net Ca^{2+}, Na^{+}, titratable alkalinity (TA), ammonia (Amm), and acidic equivalent (H^{+}) fluxes ($nEq \cdot g^{-1} \cdot h^{-1}$) measured during various exposures. No values were determined for acute low Ca^{2+} exposure. Net TA and H^{+} flux rates were not reported for 0.1 $mmol \cdot L^{-1}$ $LaCl_3$ exposure as La^{3+} interfered with the water titrations. See text for details. Mean $\pm$ 1 SEM (N). N number for J^{TA} is the same for J^{Amm} and J^{H+}.

	J^{Ca2+}_{net}	J^{Na+}_{net}	J^{TA}	J^{Amm}	J^{H+}
Control	40.5 ± 6.64 (6)	159.8 $\pm$ 76.2 (7)	694.7 ± 75.6(5)	638.8 ± 55.1	55.8 ± 91.1
0.1 $mmol \cdot L^{-1}$ $MgSO_4$	2.6 ± 27.1 (8)	34.8 ± 91.1 (8)	983.9 ± 120.0(4)	838.7 ± 148.4	145.2 ± 228.5
0.1 $mmol \cdot L^{-1}$ $LaCl_3$	1.1 ± 17.4 (8)	-185.9 $\pm$ 69.9*(8)	-----	470.8 ± 79.1(5)	-----
Chronic high Ca^{2+}	-77.7 ± 38.2*(8)	67.8 $\pm$ 51.4 (8)	503.3 ± 93.4(7)	639.9 ± 33.2	-134.5 ± 83.0
Chronic low Ca^{2+}	-20.5 ± 5.7 (8)	-327.2 ± 142.2*(8)	777.0 ± 96.1 (8)	625.3 ± 91.3	151.6 ± 97.9

* indicates significant difference from control ($p \leq 0.05$).

A significant net Na^+ loss was noted during chronic low Ca^{2+} exposure whereas J^{Na}_{net} for the high Ca^{2+} group was slightly lower than the control group, but the difference was not significant. Ammonia excretion and net acid-base fluxes were not affected by either exposure (Table 4.2).

Discussion

Although many *in vivo* studies investigating the hormonal control of calcium homeostasis have utilized freshwater-adapted *F. heteroclitus*, only a very few have reported rates of calcium uptake from the external medium. Pang *et al.* (1980) reported a J^{Ca}_{in} of 65 $nEq \cdot g^{-1} \cdot h^{-1}$ for mummichogs chronically exposed to 1300 $\mu Eq \cdot L^{-1}$ Ca^{2+}. Mayer-Gostan *et al.* (1983) chronically exposed two groups of fish to 3000 and 200 $\mu Eq \cdot L^{-1}$ Ca^{2+} which in both cases had Ca^{2+} uptake rates of between 80 and 100 $nEq \cdot g^{-1} \cdot h^{-1}$. Control Ca^{2+} uptake rate determined in the present study was much lower, 11.13 $\pm$ 4.08 $nEq \cdot g^{-1} \cdot h^{-1}$ (Figure 4.2) at an acclimation $[Ca^{2+}]$ of 200 $\mu Eq \cdot L^{-1}$. The variability in Ca^{2+} uptake rates amongst the three studies cannot be attributed to one factor alone. Acclimation conditions were quite different as well as the means of determining J^{Ca}_{in}. Both Mayer-Gostan *et al.* (1983) and Pang *et al.* (1980) reported extraintestinal J^{Ca}_{in} which differs from the present study in that ^{45}Ca activity trapped in the gut was subtracted from the whole body count. However the gut would not likely be a major route of Ca^{2+} entry for freshwater-adapted fish as drinking is minimal (Potts & Evans, 1967), therefore the gut count would likely be very small. In any event, this correction would lower rather than raise the calculated J^{Ca}_{in} value.

Differences among estimates could simply reflect normal fluctuations in Ca^{2+} uptake rates. Similar fluctuations have been observed in trout (Wagner *et al.* 1993; Hogstrand *et al.* 1994*a*, *b*), most likely due to cycling of regulating

hormones The most likely candidate responsible for this phenomenon is stanniocalcin which rapidly controls branchial apical membrane permeability to calcium. In juvenile rainbow trout, J^{Ca}_{in} has been known to vary as much as 5 fold over several weeks, most likely due to fluctuating levels of stanniocalcin (Wagner *et al.* 1993). In the present study, all experiments were performed within 3 weeks to minimize any natural fluctuation in uptake rates. With this in mind, comparisons between studies must be made with caution.

Calcium uptake kinetics

Figure 4.1 illustrates the saturation of Ca^{2+} uptake within the range of $[Ca^{2+}]$ tested indicating carrier-mediated transport in freshwater-adapted *F. heteroclitus*. Saturation of J^{Ca}_{in} was also observed by Mayer-Gostan *et al.* (1983) utilizing freshwater/low Ca^{2+}-acclimated (FW-Ca) *F. heteroclitus* which were acclimated to the same $[Ca^{2+}]$ as the present study (*i.e.* 200 $\mu Eq \cdot L^{-1}$). Curiously, a second freshwater group (FW) acclimated to high calcium concentration (3000 $\mu Eq \cdot L^{-1}$) did not demonstrate saturation of J^{Ca}_{in} in the same study. Mayer-Gostan *et al.* (1983) suggested that the FW-Ca group could have attained the maximum capacity of the active transport of Ca^{2+} but felt that the curve was reflecting changes in apical membrane permeability. Further evidence for this idea was provided when J^{Ca}_{in} continued to increase for both FW and FW-Ca groups when external $[Ca^{2+}]$ reached 24 000 $\mu Eq \cdot L^{-1}$.

In contrast to these findings, Perry & Wood (1985), using rainbow trout,

showed that at both high and low Ca^{2+} acclimation (*i.e.* 2000 and 50 $\mu Eq \cdot L^{-1}$ Ca^{2+} respectively) saturation of J^{Ca}_{in} occurred. The transport capacity (J_{max}) varied depending upon the length of acclimation to low Ca^{2+} suggesting that the number of transporters available could be modulated.

It is difficult to ascertain whether the changes in J^{Ca}_{in} in Figure 4.1 are a result of changes in apical membrane permeability. Although Mayer-Gostan *et al.* (1983) found no correlation between Ca^{2+}-ATPase activity and the changes in extraintestinal Ca^{2+} uptake over the range of external [Ca^{2+}] tested and argued that the apical membrane permeability was responsible for the increase in J^{Ca}_{in}, they did hint at active transport becoming important during low Ca^{2+} acclimation. Perhaps the saturation noted in both the present and the abovementioned study for the mummichogs acclimated to 200 $\mu Eq \cdot L^{-1}$ Ca^{2+} indicates that the active transport capacity of the system, not the membrane permeability was the limiting factor.

Table 4.1 compares the acclimation [Ca^{2+}], K_m and J_{max} values for *F. heteroclitus* in the present study with values for the FW-Ca group of Mayer-Gostan *et al.* (1983), values from *in vitro* studies utilizing opercular epithelium from freshwater-adapted *F. heteroclitus* (Burghardt, 1993; W.S. Marshall, personal communication), an *in vivo* study using juvenile rainbow trout (Hogstrand *et al.* 1994*a*) and *in vitro* perfused head preparations of adult rainbow trout acclimated to high and low Ca^{2+} (Perry & Wood, 1985). Michaelis-Menten analysis was not applied by Mayer-Gostan *et al.* (1983) but

an estimate based on their reported data gives a J_{max} that is several fold greater and a K_m less than the present study (Table 4.1). The source for the two disparities between these studies is unknown although differences in fish stocks, acclimation conditions, and the hormonal fluctuations discussed earlier could all contribute. *In vitro* Ca^{2+} uptake characterized in the opercular epithelium of *F. heteroclitus* (Burghardt, 1993) had a much lower affinity and a much larger transport capacity than estimated *in vivo*, yet a similar stock of fish and acclimation conditions were used as in the present study (W.S. Marshall, personal communication). The estimate of whole body rate based on the opercular epithelium preparation was based on our assumption that the entire opercular membrane could potentially transport Ca^{2+}. If the actual functioning surface area were smaller, than the J_{max} estimate would be proportionately reduced. The marked discrepancy in K_m (much higher *in vitro*, *i.e.* lower affinity) is independent of the assumed active surface area. It may reflect the absence of neural or hormonal activity in the *in vitro* preparation.

Relative to both adult and juvenile rainbow trout (Table 4.1), intact *F. heteroclitus* has a greater affinity for Ca^{2+} (*i.e.* lower K_m) and a transport capacity that is slightly higher than that determined for adult rainbow trout acclimated to 2000 $\mu Eq \cdot L^{-1}$ Ca^{2+} but much lower than for both juvenile and low Ca^{2+}- acclimated adult trout (Table 4.1). It is conceivable that young trout would have a large transport capacity for Ca^{2+} because of their accelerated growth rates and requirement for Ca^{2+} in bone development (Hogstrand *et al.* 1994*a*).

Effects of Mg^{2+} and La^{3+}

The addition of 100 $\mu mol \cdot L^{-1}$ Mg^{2+} to the external medium did not affect Ca^{2+} uptake in the mummichog (Figure 4.2). In contrast to this, Burghardt (1993), using opercular epithelium from freshwater-adapted mummichog, demonstrated inhibition of Ca^{2+} uptake *in vitro* using 1000 $\mu mol \cdot L^{-1}$ Mg^{2+}. Perhaps a Mg^{2+} concentration greater than ambient [Ca^{2+}] (*i.e.* 100 $\mu mol \cdot L^{-1}$) would be necessary to observe inhibition *in vivo*, which would suggest that magnesium is not a potent inhibitor of the Ca^{2+} channels involved in Ca^{2+} uptake. This idea is supported by the finding that external magnesium can inactivate voltage-gated Ca^{2+} channels in frog cardiac myocytes but not to the same magnitude as calcium because magnesium binds to negative charges on the cell surface with a lesser affinity (Hartzell & White, 1989). Acid-base fluxes and net Na^{+} movement were also undisturbed by the presence of external magnesium (Table 4.2).

The addition of 100 $\mu mol \cdot L^{-1}$ La^{3+} to the external medium caused a significant inhibition of Ca^{2+} uptake (Figure 4.2). This is in agreement with findings that lanthanum inhibited unidirectional Ca^{2+} uptake in rainbow trout perfused head preparations (Verbost *et al.* 1987, 1989), intact trout (Perry & Flik, 1988), trout cleithrum skin *in vitro* (Marshall *et al.* 1992) and also in mummichog opercular epithelium *in vitro* (Burghardt, 1993). The specific action of lanthanum has been proposed to be a that of a Ca^{2+} antagonist as it

possesses an ionic radius similar to Ca^{2+} and can bind to Ca^{2+} sites, but in a less reversible manner, thereby blocking Ca^{2+} binding (Weiss, 1974). As a consequence, lanthanum could easily inactivate an apical Ca^{2+} channel thereby, blocking Ca^{2+} entry. Using transmission electron microscopy, Perry & Flik (1988) observed a greater density of bound lanthanum on the chloride cell apical surface (versus pavement cells) of the trout gill epithelium indicating that Ca^{2+} entry into the fish was occurring through this cell type. Apical crypts of chloride cells have been observed in gill and opercular epithelium of *F. heteroclitus* (see Chapter 2). It is very likely that Ca^{2+} entry is via these cell types as Burghardt (1993) found a correlation between J^{Ca}_{in} and chloride cell number in the opercular epithelium of mummichog, just as in the cleithrum skin of the trout (Marshall *et al.* 1992) and the opercular epithelium of the tilapia (McCormick *et al.* 1992).

Although J^{Ca}_{in} was reduced by 67% (Figure 4.2), the mummichogs remained in Ca^{2+} balance as net Ca^{2+} flux was essentially zero (Table 4.2) suggesting that the Ca^{2+} efflux component remained undisturbed in the presence of lanthanum, a finding that was also reported in intact trout (Perry & Flik, 1988). This lack of change is surprising as lanthanum is believed to displace Ca^{2+} from the paracellular junctions which in itself, would increase J_{out}. This Ca^{2+} displacement and increase in efflux was observed in the mummichog opercular epithelium (Burghardt, 1993) and trout cleithrum skin (Marshall *et al.* 1992). However J^{Ca}_{net} measurements may be complicated by adsorptive losses

of Ca^{2+} from the water, and therefore may not be reliable. The idea that lanthanum bound to the paracellular calcium sites increases paracellular permeability to ions was supported by the significant net Na^+ loss noted in Table 4.2. Unidirectional Na^+ influx was not measured in the present study but a recent study revealed that lanthanum did not inhibit J^{Na}_{in} in trout (Hogstrand & Wood, personal communication). Eddy & Bath (1979) also reported significant losses of both Na^+ and Cl^- with lanthanum treatment and found only transient increases in uptake which were difficult to explain.

Chronic exposure to high and low Ca^{2+}

The endogenous Ca^{2+} uptake rate for mummichogs chronically exposed to low Ca^{2+} (50 $\mu Eq \cdot L^{-1}$) was substantially higher than the rate determined for fish acutely exposed to low Ca^{2+} or for fish acclimated and tested under control conditions (200 $\mu Eq \cdot L^{-1}$) (Figure 4.3 *a*, *b*). Mayer-Gostan *et al.* (1983) reported no difference in uptake rates between the high and low Ca^{2+} acclimated mummichogs but stated that the low external Ca^{2+} level must somehow facilitate the uptake mechanism in order to maintain the same rate. Stimulation of J^{Ca}_{in} under low Ca^{2+} conditions has been directly observed in freshwater rainbow trout *in vivo* (Perry & Wood, 1985) and in freshwater tilapia (*Oreochromis mossambicus*) both *in vivo* (Flik *et al.* 1986) and *in vitro* (McCormick *et al.* 1992).

Internal calcium depletion due to Ca^{2+} loss during the chronic low Ca^{2+}

exposure could be the cause for the observed stimulation of Ca^{2+} uptake. Flik *et al.* (1986) suggested that Ca^{2+} efflux might be stimulated under low Ca^{2+} conditions as the electrochemical gradient for Ca^{2+} is outward (Flik & Verbost, 1993). It is well known that fish placed in low Ca^{2+} water display increases in monovalent ion losses due to the increase permeability of the paracellular pathways (Dharamba & Maetz, 1972). This was evident in the present study as J^{Na}_{net} became significantly negative during chronic low Ca^{2+} exposure (Table 4.2). Unidirectional Ca^{2+} effluxes were not measured in this study but even with elevated Ca^{2+} uptake, mummichogs were in negative Ca^{2+} balance following 10 days at low Ca^{2+} (Table 4.2). Acute low Ca^{2+} exposure did not stimulate J^{Ca}_{in} (Figure 4.3*a*) indicating that the need to elevate uptake (*i.e.* Ca^{2+} loss) was not prevalent within the first 4 hours at low Ca^{2+}.

One way in which J^{Ca}_{in} could be elevated would be by an increase in the transport capacity (J_{max}). Perry & Wood (1985) demonstrated an increase in J_{max} (no change in K_m) with acclimation to low Ca^{2+}. Concurrent with the change in transport capacity (J_{max}) was an elevation of J^{Ca}_{in} although J^{Ca}_{in} did start to return towards control values by 30 days of low Ca^{2+} acclimation. Another means of modulating Ca^{2+} uptake is via apical permeability of branchial epithelium. Recent studies have shown that the hypocalcaemic hormone stanniocalcin can rapidly modulate the apical membrane permeability in branchial epithelium (see review by Flik & Verbost, 1993). Prolactin, a pituitary hormone, was discovered to be hypercalcaemic in a study utilizing *F.*

heteroclitus (Pang *et al.* 1971). The manner in which prolactin may modulate Ca^{2+} metabolism (uptake and/or efflux) is completely unknown but its action takes several days. A third candidate for stimulating J^{Ca}_{in} during low Ca^{2+} exposure is cortisol. Perry & Wood (1985) and Flik & Perry (1989) showed that plasma cortisol levels become elevated in rainbow trout over several days at 50 $\mu Eq \cdot L^{-1}$ Ca^{2+}, a response which paralleled the increase in Ca^{2+}-ATPase activity, J_{max}, and chloride cell proliferation. Similar changes in these three qualities were observed in fish injected with cortisol and maintained at higher ambient Ca^{2+}.

Chronic high Ca^{2+} exposure did not modulate J^{Ca}_{in} (Figure 4.3*b*). However inasmuch as the normal Ca^{2+} uptake system appears to be saturated under control conditions (Figure 4.1), there is likely no need to decrease the transport capacity of the system during chronic high Ca^{2+} exposure. Assuming that most Ca^{2+} entry moves through the transport system, Ca^{2+} loading may not be greatly increased. The significant net Ca^{2+} loss noted in this treatment (Table 4.2) suggests that efflux rather than influx mechanisms are adjusted.

Final consideration

The present study did not separate the contribution of opercular and branchial epithelium to whole body Ca^{2+} uptake, and indeed this has never been done. However using the *in vitro* opercular membrane Ca^{2+} influx rate of 40 $nEq \cdot cm^{-2} \cdot h^{-1}$ at $[Ca^{2+}]$ = 200 $\mu Eq \cdot L^{-1}$ (Burghardt, 1993), an estimate of whole

body J^{Ca}_{in} of 16 nEq·g^{-1}·h^{-1} (see Table 4.1 for assumption of the calculation) approximates the control value of the present study (11 nEq·g^{-1}·h^{-1}). This demonstrates that the opercular epithelium could be responsible for most if not all of whole body Ca^{2+} uptake in *F. heteroclitus*. In future studies, it seems pertinent to the investigation of whole body Ca^{2+} uptake and the regulation thereof to confirm whether or not the opercular epithelium is a major contributor to calcium homeostasis.

CHAPTER 5

General Summary and Conclusions

This thesis examined the ionoregulation of the freshwater-adapted mummichog (*Fundulus heteroclitus*) *in vivo*. The primary focus was to determine if mummichog shares ion transport mechanisms similar to those of other freshwater fish which have contributed to the current models of ion transport (Figure 1.3, 1.4).

Under control conditions (water [NaCl]≈ 1 mmol·L^{-1}), freshwater-adapted mummichog exhibited a very large Na^+ turnover while remaining in positive Na^+ balance. A slight negative Cl^- balance existed because of the lack of an appreciable Cl^- uptake component to compensate for Cl^- loss. A disparity between plasma Na^+ and Cl^- levels has been noted in *F. heteroclitus* (Hannah & Pickford, 1981) which could be explained by the above findings and perhaps an insufficient dietary source of Cl^-. The Michaelis-Menten kinetic study (Chapter 2) revealed a low affinity, high capacity Na^+ uptake system that was independent of both Na^+ efflux and ammonia excretion. Cl^- influx started at higher water [NaCl] levels (> 2 mmol·L^{-1}) but did not saturate within the freshwater [NaCl] range, which implies that two different mechanisms are responsible for Na^+ and Cl^- uptake in mummichog. Although these findings are atypical of a strictly freshwater fish, euryhaline eels (*Anguilla anguilla* and *A.*

rostrata) have also demonstrated the same negligible Cl^- uptake and a discrepancy between Na^+ and Cl^- plasma levels (Farrell & Lutz, 1975; Bornancin *et al.* 1977; Hyde & Perry, 1987). This may suggest that freely euryhaline fish utilize Na^+ and Cl^- uptake mechanisms that differ from the proposed mechanisms depicted in Figure 1.3. For example, the Cl^- uptake kinetics results from this thesis were similar to uptake by frog skin which has been proposed to occur through anion-selective channels which open when external [Cl^-] exceed 5 $mmol \cdot L^{-1}$.

The studies described in Chapter 2 and 3 quite convincingly ruled out a link between Na^+ uptake and ammonia excretion suggesting that ammonia movement is purely diffusional (*i.e.* NH_3). The possibility of a Na^+ uptake/acid excretion coupling was revealed and in particular was evident in the low pH exposure and amiloride treatments. Traditionally, this has been considered to be a Na^+/H^+ exchanger, but recently the Na^+ channel/H^+-ATPase coupling has been gaining more acceptance. It cannot be concluded as to which mechanism is active in the mummichog but this issue is yet to be resolved in the current model for freshwater fish. The lack of Cl^- influx under control conditions, and the absence of consistent changes in Cl^- influx in response to acid-base disturbances suggest that Cl^-/HCO_3^- exchange is of negligible importance in acid-base regulation in this species. Unfortunately, the well-developed seawater model for ion transport based on *in vitro* mummichog work does not include an acid-base component and does not provide insight into these

relationships for the freshwater species.

Na^+ and Cl^- efflux components cannot be dismissed simply as inevitable losses, but rather provide an additional means to maintain acid-base balance. Intraperitoneal injection of HCl demonstrated that mummichog could differentially modulate Na^+ and Cl^- effluxes to excrete over 50% of the acid load within 4 hours. This ability to adjust Na^+ and Cl^- efflux as a mechanism for acid-base regulation has been noted in other freshwater fish.

The application of the Strong Ion Difference Theory (SID) utilizing net Na^+ and Cl^- fluxes in this thesis proved to be a practical means of assessing acid-base status. SID flux approximated the measured acidic/basic equivalent movements in all of the studies performed and in one case (high pH exposure) was the only available estimate of acid-base movement between the fish and the external medium as water alkalinity titrations were not reliable. Although controversial, SID theory does deserve consideration as a method for *estimating* acid-base status as ionoregulation is intimately involved.

This thesis also elucidated certain characteristics of Ca^{2+} uptake in mummichog. The Michaelis-Menten kinetic analysis suggested that uptake has an carrier-mediated transport step. This contrasts to an earlier study by Mayer-Gostan *et al.* (1983) who reported a lack of saturation and suggested that this reflected changes in the apical permeability to Ca^{2+}. La^{3+} was effective in blocking Ca^{2+} uptake, in accord with current ideas that the first step in the uptake pathway is Ca^{2+} entry through an apical channel. The Mg^{2+} treatment did

not inhibit unidirectional Ca^{2+} uptake which further agrees with the current theory that the apical channel involved is of the voltage-independent type (Figure 1.4). Chronic exposure to low Ca^{2+} stimulated Ca^{2+} uptake whereas chronic high Ca^{2+} exposure did not alter uptake. The underlying cause for uptake stimulation in the former exposure could be a depletion of internal Ca^{2+} stores.

Overall, the freshwater-adapted mummichog adheres to current ideas of ionoregulation for freshwater fish though the Cl^- uptake system appears atypical. To further understand freshwater ionoregulation of this fish, certain pertinent questions need to be addressed. First, what are the contributions of the gill and opercular epithelium to whole-body Na^+, Cl^- and Ca^{2+} uptake? Second, is the chloride cell the site for uptake of these three ions and what is the absolute population of these cells in the two epithelia mentioned above? Third, what is the exact nature of the link between Na^+ uptake and acid movement? And finally, what is the reason for minimal Cl^- uptake component in freshwater? All of these queries and certainly many others could prove to be extremely beneficial in furthering our knowledge of ionoregulation in freshwater fish.

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