Na^+ AND Cl^- REABSORPTION STUDIES IN THE RAINBOW TROUT URINARY BLADDER SAC

Na^+ AND Cl^- REABSORPTION STUDIES IN THE RAINBOW TROUT (*Oncorhynchus mykiss*) URINARY BLADDER SAC

By

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Abstract

The current study was designed to build upon earlier work on the rainbow trout (*O. mykiss*) urinary bladder (Miarczynski, 1997; Burgess *et al.*, 2000) in order to judge the suitability of the urinary bladder as a model for the freshwater teleost gill. The current study employed the bladder sac preparation in closed and perfused sac experiments, with physiological saline present on both mucosal and serosal surfaces.

Removal (replacement) of Na^+ or Cl^- from the mucosal saline caused no change in Cl^- or Na^+ influx or net flux rates, respectively. These results suggest that the *O. mykiss* urinary bladder exhibits independent Na^+ and Cl^- reabsorption under symmetrical saline conditions in the sac preparation. However, this result is contrary to that obtained in the Ussing chamber preparation of the *O. mykiss* urinary bladder which showed a partially coupled transport of Na^+ and Cl^- (Burgess *et al.*, 2000). Furthermore, Na^+ and Cl^- influx rates did not exhibit saturation kinetics in the sac preparation. This is again contrary to the findings using Ussing chambers (Burgess *et al.*, 2000), and suggests unidirectional influx rate, as measured by use of radiotracers, exhibits a large diffusive component that masks any active, transporter-mediated movement of Na^+ or Cl^-. Net flux rate measurements appeared to be more indicative of active transport, and therefore were employed in preference to influx rates in the subsequent pharmacological dissection of the transport mechanisms.

Mucosal application of 10^{-4} M bumetanide had no effect on influx or net flux rates of either Na^+ or Cl^-, ruling out the presence of $Na^+/2Cl^-/K^+$ co-transporters. Amiloride (10^{-4} M) was effective in the mucosal saline at reducing net flux rates (~45% reduction), but not influx rates, of Na^+ and Cl^-. DIDS, at a mucosal 10^{-4} M concentration, was only effective at reducing Cl^- net flux rates (35% reduction). Curiously, both drugs were ineffective in reducing flux rates at a 10^{-3} M concentration. Nevertheless, these results suggest the presence of apical Na^+ channels (or Na^+/H^+ anti-porters) and apical Cl^-/HCO_3^- anti-porters (or channels), respectively.

Mucosal 10^{-3} M chlorothiazide was ineffective at reducing both flux rates of Na^+ or Cl^-,

although simultaneous mucosal and serosal 10^{-3} M hydrochlorothiazide was responsible for ~75% reductions in Na^+ and Cl^- net flux rates (no change in influx rates). It is unclear whether the difference in thiazide treatment effectiveness was a result of mucosal versus serosal application or chlorothiazide versus hydrochlorothiazide effectiveness in the rainbow trout urinary bladder. Regardless, there is clearly some evidence for the presence of a Na^+/Cl^- co-transporter in the rainbow trout urinary bladder.

Acetazolamide (10^{-2} M) was applied separately at the mucosal and serosal surfaces. The only significant effect of acetazolamide was a 36% reduction in Na^+ influx rates during mucosal administration of the drug. This strange result eludes complete explanation at this time other than to show that carbonic anhydrase is present in the rainbow trout urinary bladder and may contribute to the transport of Na^+ and/or Cl^-.

Ouabain (10^{-3} M) was effective at inhibiting both Na^+ and Cl^- influx rates regardless of whether it was applied mucosally or serosally. Net flux rates were unaffected but influx rates were reduced by 40-49%. These results suggest that although the Na^+/K^+-ATPase is present it does not seem to contribute much to the establishment of the necessary Na^+ gradients to drive active (net) transport of Na^+ and Cl^-. Finally, metabolic inhibition by use of cyanide (10^{-3} M) was ineffective whether it was applied at the mucosal surface or simultaneously at the mucosal and serosal surfaces. The anaerobic ATP production blockers NaF (10^{-3} M) and iodoacetate ($5x10^{-3}$ M) were tested in the simultaneous presence of cyanide (10^{-3} M). The addition of NaF was ineffective, but iodoacetate was responsible for a >50% reduction in Na^+ and Cl^- net flux rates (influx rates unaffected). These results suggest that the energy supply for active reabsorption in the urinary bladder may be fueled mostly by anaerobic processes.

Therefore, it would appear that in the *O. mykiss* urinary bladder in the sac preparation under symmetrical saline conditions, Na^+ and Cl^- reabsorption occurs mainly by an independent process. The urinary bladder is an interesting model of transport but the mechanisms are not completely clear, therefore making it unsuitable as a gill model at the present time.

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Crystal, Collins, Greg, Cheryl and Sheryl, Christine, Mark Rheault (you still at Mac?), Nic, Gudrun and Gord McDonald. I just know I forgot to list some people, I apologize, but thanks to Chris there were so many. Contrary to how it seems we were not laughing all the time (but a lot of the time), it was just a very good place to work thanks to the people who accepted and returned my weird sense of humour.

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Thesis Format

This thesis is organized such that each chapter corresponds to the introduction, materials and methods, results and discussion. The appendix contains a manuscript accepted by the Journal of Experimental Zoology to be published in 2000; Burgess *et al.*, 2000. This manuscript contains work performed by myself (Maciej Miarczynski) during the completion of my M.Sc., but not directly part of it.

Chapter 1: **Introduction**

Chapter 2: **Materials and Methods**

Chapter 3: **Results**

Chapter 4: **Discussion**

Appendix: Burgess, Miarczynski, O'Donnell and Wood (2000). *J. Exp. Zoology.*

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Chapter 1

Introduction

Osmoregulation in fresh- and seawater

There is a high osmotic burden placed on fish living in freshwater or seawater. Typical freshwater has an osmolarity of <10 mOsm and seawater has an osmolarity of 1050 mOsm. Freshwater fish tend to have internal fluid osmolarities of 280-330 mOsm, and seawater fish internal fluids are between 300-400 mOsm (Withers, 1992). Although these differences somewhat reflect the environments in which the fish live, the freshwater fish are still highly hyperosmotic relative to their environment, and the seawater fish are highly hyposmotic to their environment. Since the entire surface of the fish is always exposed to the hypo- or hyperosmotic medium they inhabit, osmo- and ionoregulation are of paramount importance to survival. Structures involved in the process of osmoregulation include the gut, gills, kidney and urinary bladder. The gills represent a large, thin surface area permeable to O_2, CO_2, H_2O and ions. Although the skin is always exposed to the water, it is not as important to osmoregulation as the above mentioned organs.

The strategy of a seawater fish involves a conservation of water and active extrusion of ions from the body. The sources of water are mainly from drinking of seawater and to a lesser extent from the metabolism of food (Withers, 1992). The drinking strategy may seem counterintuitive in light of the high salt content of the seawater but, the organs function in such a way as to extract the most available water through the gut and expel ions through the gills, and to a lesser extent, through the urine. The gut is responsible for actively absorbing Na^+ and Cl^- to create an osmotic gradient favourable for water absorption from the lumen. Part of this process occurs in the esophagus and serves to remove a large amount of Na^+ and Cl^- but not water from

the ingested fluid (Loretz, 1995). Upon reaching the stomach and intestine, the Na^+ and Cl^- concentrations are similar to those of the body fluids and active Na^+ and Cl^- absorption causes absorption of water such that only 20-30% of the ingested fluid is not absorbed by the gut (Loretz, 1995). The process of extrusion of the ions gained through the gut occurs partially through the kidney, and to a larger extent through the gills. The kidney is unable to form a urine more concentrated than the blood and is mostly responsible for eliminating divalent ions. At the gills, active transport of Na^+ and Cl^- against a high concentration gradient allows the fish to remove a large amount of these monovalent ions from the blood and into the surrounding water. High levels of certain metals or toxicants in the water can be responsible for inhibition or disruption of ion movements across the gills, disrupting ionoregulation and ultimately causing death, demonstrating the importance of gill function in ionoregulation.

The freshwater fish strategy is opposite to the seawater strategy in that external water permeability or absorption is minimized and ion absorption is maximized. Freshwater fish drink only negligibly, but some water is inevitably swallowed during feeding and absorbed along with Na^+ and Cl^- (Loretz, 1995). The majority of water expulsion occurs through the kidney as water and ions are first filtered, followed by active reabsorption of Na^+ and Cl^- through increasingly water impermeable tubules (Withers, 1992). The already dilute urine leaves the kidney and enters the urinary bladder. The residence time of the urine in the urinary bladder is used to further reabsorb Na^+ and Cl^- to concentrations less than half those of the urine created by the kidney (Curtis and Wood, 1991). In the case of the freshwater fish, the gills serve as an area of ion absorption and not extrusion as in seawater fish. At the gills, ions are actively absorbed against a high concentration gradient and into the blood (Lin and Randall, 1995); toxicants which inhibit gill transport processes may cause death due to net NaCl loss.

The methods and organs for iono- and osmoregulation are quite obviously important for habitation of freshwater or seawater by fish. Knowledge of these mechanisms can be instrumental in developing theories of toxicity due to disruption of ionoregulation and how protection may be achieved.

The gill in iono- and osmoregulation

The primary cell for iono- and osmoregulation in the gill is known as a chloride cell (CC) or mitochondrial rich cell (MRC; Lin and Randall, 1995). As stated before, the gill is responsible for removal of Na^+ and Cl^- from the fish in seawater and absorption of those ions from the environment in freshwater. To accomplish these tasks the chloride cells are responsible for pumping Na^+ and Cl^- in the correct direction.

In seawater fish, basolateral Na^+/K^+-ATPase is thought to be the major mechanism responsible for the creation of electrochemical gradients for Na^+ and Cl^- expulsion against the high osmolarity of seawater (McCormick, 1995). The removal of Na^+ from the cell by Na^+/K^+-ATPase creates a Na^+ electrochemical gradient favourable for diffusion of Na^+ back into the cell from the blood plasma or extracellular fluid. This diffusion is accomplished through $Na^+/K^+/2Cl^-$ cotransporters on the basolateral membrane which drag K^+ and Cl^- into the cell in cotransport with Na^+; the K^+ exits through basolateral channels. The Cl^- leaves the negative interior of the cell by way of channels on the apical membrane of the chloride cell. Expulsion of Na^+ from the cell occurs by the Na^+/K^+-ATPase. Na^+ is expelled from the extracellular fluid through the paracellular pathway due to the negative potential of the external seawater relative to plasma

(McCormick, 1995). The simplified mechanisms and transporters involved in Na^+ and Cl^- extrusion in seawater gill are illustrated in Figure 1-1.

Na^+ and Cl^- conservation is a requirement for a freshwater fish and part of this process occurs as absorption by the gill from the surrounding water. It is still unclear what is the major motive force for Na^+ and Cl^- absorption and recent studies have suggested that Na^+ absorption may occur in the pavement cells of the gill rather than the chloride cells (McCormick, 1995; Sullivan *et al.* 1995). It is believed that one of the driving forces for ion absorption is the basolateral Na^+/K^+-ATPase that moves Na^+ into the plasma from the cell interior, as in the seawater gill. The electrochemical gradient created by Na^+/K^+-ATPase may cause an influx of Na^+ at the apical membrane through Na^+/H^+ antiporters. The apical uptake of Cl^- is thought to occur through Cl^-/HCO_3^- antiporters in response to movement of HCO_3^- out of the cell.

Although Na^+/K^+-ATPase represents one possibility, it has been found that ouabain may or may not have an effect on ion uptake in rainbow trout gills (Epstein *et al.*, 1967; Kerstetter and Keeler, 1976, respectively). An alternate, or additional, motive force for ion uptake in the freshwater gill may involve an apical H^+-ATPase (Lin and Randall, 1995). This model proposes an indirect Na^+/H^+ exchange by way of closely associated H^+-ATPase and Na^+ channel. The indirect exchange accomplishes the same function as the Na^+/K^+-ATPase motivated, direct Na^+/H^+ exchange through an antiporter. The apical H^+-ATPase is responsible for removing H^+ from the cellular fluid and into the surrounding water. The H^+ movement creates a local electrical gradient which energizes diffusion of Na^+ through the closely located Na^+ channel. The Na^+ is

then removed at the basolateral membrane by Na^+/K^+-ATPase. The creation of intracellular H^+ from CO_2 and H_2O, by carbonic anhydrase, also releases HCO_3^- which follows its electrochemical gradient through an apical Cl^-/HCO_3^- antiporter. The Cl^- leaves the cell through basolateral channels. In effect, the same movement of Na^+ and Cl^- against H^+ and HCO_3^- is effected as in the model involving the apical Na^+/H^+ antiporter, but the application of energy for these movements lies at the apical rather than basolateral membrane. Both mechanisms for freshwater gill ion absorption (simplified) are illustrated in Figure 1-2.

Osmoregulatory role of the urinary bladder

The role of the teleost urinary bladder in the movement of Na^+ and Cl^- has been examined extensively in various species that include rainbow trout (Fossat and Lahlou, 1977, 1979a, 1979b, 1982; Beyenbach and Kirschner, 1975; Harvey and Lahlou, 1986; Curtis and Wood, 1991, 1992), brook trout (Marshall, 1986, 1988; Marshall and Bryson, 1991), starry flounder (Demarest, 1984), flounder (Renfro, 1975, 1977; Demarest and Machen, 1984), goby (Loretz and Bern, 1980, 1983) and a variety of other species (Hirano *et al.*, 1973). In teleosts the urinary bladder is an extension of the mesonephric ducts (Hickman and Trump, 1969). It is a single layered epithelium surrounded by a contractile muscular wall (Fossat and Lahlou, 1977). For a freshwater fish, if osmoregulation occurs in the urinary bladder, the expected function would be to reabsorb Na^+ and Cl^- to preserve the ions and form a dilute urine. The urinary bladder would therefore function as an accessory organ to the kidney in osmoregulation. This is the case in rainbow trout urinary bladder which has been shown to modify the urine by reabsorbing Na^+ and Cl^- to final, excreted concentrations of 2.12 ± 0.41 mM and 3.35 ± 0.23 mM, respectively (Curtis and Wood,

1991). This is in comparison to Na^+ and Cl^- concentrations of 7.12±0.79 mM and 5.21±0.34 mM, respectively, if urine is siphoned from the ureters with no residence time in the bladder (Curtis and Wood, 1991).

Relatively recent studies on urinary bladders have involved the brook trout, *S. fontinalis*, which is a North American freshwater fish (Marshall, 1986 and 1988; Marshall and Bryson, 1991). The brook trout urinary bladder yielded results suggesting that Na^+ and Cl^- transport occur via independent processes. The independent model of Na^+ and Cl^- uptake was partially based on measurements of Na^+ and Cl^- unidirectional influx rates which were very similar, combined with the finding that substitution of either Na^+ or Cl^- did not inhibit the transport rates of the other ion (Marshall, 1986). Also, the brook trout urinary bladder exhibited no change in Na^+ influx in the absence of Cl^- but a secretion of acid was detected which was not present during control fluxes. These data seem to indicate that Na^+ influx is coupled with H^+ efflux and that Cl^- influx is coupled with HCO_3^- efflux (Marshall 1986). The independent model of transport involving apical Na^+/H^+ and Cl^-/HCO_3^- antiporters, and basolateral Na^+/K^+-ATPase is illustrated in Figure 1-3. Figure 1-4 shows how independent mechanisms may behave using the H^+-ATPase rather than the Na^+/H^+ antiporter. The purpose and mechanisms of these reabsorption processes are very similar to those of the gill, perhaps lending the bladder epithelium as a suitable substitute as a model for the gill.

Unlike the brook trout, the urinary bladder of a European strain of "rainbow" trout, *Salmo irideus*, exhibited coupled transport of Na^+ and Cl^- (Fossat and Lahlou, 1979b). As in the brook trout work the rainbow trout urinary bladder was tested by ion substitution. Cl^- substitution

caused a decrease in unidirectional Na^+ influx such that net flux (net uptake) was abolished (Fossat and Lahlou, 1979b). Similarly, removal of Na^+ from the mucosal saline reduced Cl^- influx such that net flux (net uptake) was abolished. The coupled model of Na^+ and Cl^- transport includes an apical Na^+/Cl^- cotransporter and basolateral Na^+/K^+-ATPase as illustrated in Figure 1-5.

Oncorhynchus mykiss urinary bladder

Initially the work on the *Oncorhynchus mykiss* urinary bladder started as an examination of the suitability of the bladder as a model for freshwater gill uptake of Na^+ and Cl^-, since both organs absorb Na^+ and Cl^- from a dilute medium. The urinary bladder is a tough, flat and large tissue, relative to the gill, and can easily be used in preparations such as the closed or perfused sac or in Ussing style chambers. The foundations for the *O. mykiss* studies were the work that had been done on urinary bladders of brook trout (*Salvelinus fontinalis*, Marshall 1986, 1988; Marshall and Bryson, 1991) and on the European strain of rainbow trout (*Salmo irideus*; Fossat and Lahlou, 1977, 1979a, 1979b, 1982).

Recent work, by our laboratory, has been performed on the urinary bladder of *O. mykiss* with results that indicate that the rainbow trout urinary bladder most likely contains a mix of mechanisms for Na^+ and Cl^- reabsorption (Miarczynski, 1997; Burgess *et al.*, 2000). This is not uncommon and has been described for winter flounder (*Pseudopleuronectes americanus*; Renfro, 1977), where coupled and independent transport are roughly 75% and 25%, respectively. Mixed transport has also been found in the urinary bladder of the starry flounder (*Platichthys stellatus*; Demarest, 1984) where coupled transport accounts for approximately 60% of total transport, with the balance presumably being independent.

Initially, experiments using *O. mykiss* urinary bladders in a closed, or sealed, sac preparation showed no inhibition in net flux (net uptake) rates of Na^+ or Cl^- during ion substitution experiments (Miarczynski, 1997). This suggested an independent transport of Na^+ and Cl^- in the rainbow trout urinary bladder similar to that observed in the brook trout.

At this point it appeared that the *O. mykiss* urinary bladder had independent Na^+ and Cl^- movement in the sealed sac preparation (symmetrical saline). These results were replicated in the Ussing chamber but only under low mucosal Na^+ or Cl^- concentrations (artificial urine; Burgess *et al.*, 2000). Under high mucosal concentrations of Na^+ or Cl^- (symmetrical saline) in the Ussing chambers, the urinary bladder seemed to react as if a coupled mechanism for Na^+ and Cl^- transport existed (Burgess *et al.*, 2000). These experiments were followed by kinetics studies employing unidirectional flux measurements with ^{22}Na and ^{36}Cl in Ussing chambers. These experiments showed that mucosal Cl^- removal caused a 80% decrease in unidirectional Na^+ influx at the highest tested concentration of Na^+ (Burgess *et al.*, 2000), rather different from the apparent situation in the closed sac preparations (Miarczynski, 1997). Similarly, Cl^- influx was reduced by 85%, at the highest Cl^- concentration, when Na^+ was removed from the mucosal bathing medium. These decreases in influx rates, of Na^+ or Cl^-, were noticeable only when mucosal concentrations of Na^+ and Cl^-, respectively, were above 40 mM. At mucosal concentrations similar to artificial urine (~2-3 mM and up to ~10 mM), inhibition of the unidirectional flux of one ion by removal of the other did not occur. The bladder kinetics results mimicked the Ussing chamber ion replacement results, which showed that ion replacement in the Ussing chambers under symmetrical saline conditions (resembling the kinetics curve treatment at high mucosal concentrations) caused an inhibition of the

influx of the other ion (Burgess *et al.*, 2000). Also, under mucosal artificial urine and serosal saline conditions, the ion replacement was seen to have no effect, as in the low concentrations of the kinetics curve. It appears that there were preparational differences between bladders in Ussing chambers compared to the bladder sac technique and that comparisons of fluxes between the two preparations should be made cautiously.

Burgess *et al.* (2000) also examined the effects of some common or expected transport inhibitors on the *O. mykiss* urinary bladder in the Ussing chamber. Bumetanide, amiloride and DIDS were expected to block any $Na^+/K^+/2Cl^-$ cotransporters, Na^+ channels/transporters or Cl^-/HCO_3^- antiporters, respectively, that may have been present. The inhibitors were completely ineffective under both symmetrical saline and mucosal artificial urine/serosal saline conditions. This strange result did not change when all three drugs were applied simultaneously in an attempt to eliminate the possibility that more than one mechanism existed and was "taking up the slack" when the other system was disabled (Burgess *et al.*, 2000).

The present study of *O. mykiss* urinary bladder

The present work was designed to build upon the data so far collected for the *O. mykiss* urinary bladder. However, the current work was performed using only the bladder sac technique in both sealed and perfused preparations. Initial experiments involved repetition of ion replacement experiments but this time using Na^+ and Cl^- isotopes to trace unidirectional influx rates as well as net flux rates. The perfused sac preparation was next employed in ion replacement experiments to decrease the backflux of Na^+ or Cl^-, which deposited a considerable concentration of the replaced ion back into the "ion-free" mucosal solution. Backflux occurred as a result of diffusion of Na^+ or Cl^- from the serosal compartment (full saline) to the mucosal, and Na^+- or Cl^--free, compartment.

By using a perfused sac preparation it is possible,with limits, to control the concentrations of specific ions, such as Na^+ or Cl^-, in the perfusate which in this case was the mucosal bathing solution. The perfusion was done in an attempt to reduce the likelihood that the replaced ion was able to re-establish a sufficiently high mucosal concentration to allow normal, or statistically deceptive, transport of the other ion. The perfused sac preparation was also used during the kinetics study to maintain mucosal Na^+ and Cl^- concentrations at the required low levels (5, 15, 45 and 95 mM) compared to the serosal concentrations (~135 mM).

The closed sac preparation was used in the present work in experiments where mucosal and serosal Na^+ and Cl^- concentrations were equivalent (symmetrical saline conditions). These experiments involved the use of inhibitors on mucosal and/or serosal sides of the urinary bladder. The first set of inhibitors examined were channel or transporter blockers. Amiloride, bumetanide and DIDS were employed for the first time in the sac preparation. As stated before, amiloride was expected to block Na^+ channels or the Na^+/H^+ antiporter (Benos, 1982; Epstein and Silva, 1985). Bumetanide is a Na^+/Cl^- or $Na^+/K^+/2Cl^-$ cotransporter inhibitor (Epstein and Silva, 1985) and DIDS blocks the Cl^-/HCO_3^- antiporter and Cl^- channels (Epstein and Silva, 1985; Stokes *et al.*, 1984). Amiloride and DIDS would likely affect Na^+ and/or Cl^- transport if independent transport were present whereas bumetanide might be expected to disrupt coupled transport. Chlorothiazide and hydrochlorothiazide are more specific Na^+/Cl^- cotransport blockers which were found to be quite effective in urinary bladder of winter flounder (Stokes *et al.*, 1984; Stokes, 1988). Hydrochlorothiazide was shown to significantly reduce Na^+ and Cl^- absorption in as little as 2-3 minutes at a concentration of 10^{-4} M (Stokes *et al.*, 1984). Chlorothiazide in 10^{-3} M

concentrations has been shown to completely inhibit Na^+ and Cl^- reabsorption in rat early distal tubule (Ellison *et al.*, 1987). Ouabain was expected to disrupt Na^+ and/or Cl^- transport if Na^+/K^+-ATPase (Marshall, 1995; Winder and Weiner, 1980; Renfro *et al.*, 1976) was the major motive force responsible for creating gradients for Na^+ and Cl^- reabsorption.

To further test the independent model of transport without specifically blocking a transporter, the carbonic anhydrase inhibitor acetazolamide (Lin and Randall, 1995; Stokes *et al.*, 1984) was introduced in an attempt to reduce H^+ and HCO_3^- production. Acetazolamide would be expected to disrupt the transport of Na^+ and Cl^- via the Na^+/H^+ and Cl^-/HCO_3^- antiporters, respectively, due to lack of the counter ions for the transporters.

Lastly, sodium cyanide (NaCN), an inhibitor of aerobic energy production (Winder and Weiner, 1980), was used in an attempt to abolish active reabsorption of Na^+ and Cl^-. The inhibition by cyanide should affect any active transporters relying on aerobic energy production that are responsible for creating the reabsorption gradients, which could include both Na^+/K^+-ATPases and H^+-ATPases. The final set of inhibition experiments involved the use of the anaerobic energy production inhibitors sodium fluoride (NaF) and iodoacetate (Cifarelli *et al.*, 1979; Webb, 1963 and 1966) in conjunction with cyanide. These inhibitors were used to examine the possibility that the energy consumed by any ATPases responsible for reabsorption gradients was obtained from anaerobic metabolism. The experiments were performed in conjunction with cyanide to see if active transport could be abolished completely by inhibiting aerobic and anaerobic energy production simultaneously.

In general, the present study's results show an independent mechanism of Na^+ and Cl^- reabsorption, which is supported by ion replacement studies, by reductions of net flux rates by

administration of amiloride and DIDS, and by reductions in influx rates when acetazolamide was applied. However, the reduction of net flux rates by hydrochlorothiazide, coupled with the very effective reduction of influx rates by ouabain, would seem to indicate that a model involving a Na^+/Cl^- co-transporter and using a Na^+/K^+-ATPase to provide the driving force is also a viable possibility. The metabolic inhibition results indicate that cyanide alone is not effective in reducing flux rates but when applied in conjunction with iodoacetate reductions in net flux rates are observed. This seems to indicate that the energy to support the Na^+ and Cl^- reabsorption process, in the urinary bladder, is obtained through anaerobic rather than aerobic pathways.

Figure 1-1. Model of Na^+ and Cl^- expulsion mechanisms in the seawater teleost gill. Basolateral Na^+/K^+-ATPase utilizes energy to remove Na^+, against its concentration gradient, from the cell interior. A basolateral co-transporter moves K^+ and Cl^- into the cell in conjunction with Na^+ using the above mentioned Na^+ electrochemical potential. Cl^- is removed at the apical side through channels, down its electrical gradient, and K^+ through basolateral channels. Na^+ is expelled from the extracellular fluild to the external seawater through paracellular pathways down its electrical gradient.

Figure 1-2. Model of Na^+ and Cl^- reabsorption mechanisms in the freshwater teleost gill. A basolateral Na^+/K^+-ATPase creates a driving force for Na^+ reabsorption through an apical Na^+/H^+ anti-porter. Cl^- leaves the cell through a basolateral channel due to its electrochemical gradient. Apical Cl^- reabsorption occurs through an anti-porter in exchange for HCO_3^- which is removed from the cell to balance H^+ loss. Alternately, the energy utilization may occur at the apical membrane through a H^+-ATPase which creates a local electrical gradient favourable for Na^+ reabsorption through apical channels.

Figure 1-3. Model of Na^+ and Cl^- reabsorption mechanisms in the brook trout urinary bladder (Marshall, 1986 and1988), via independent apical Na^+/H^+ and Cl^-/HCO_3^- anti-porters. This model is similar to the mechanisms seen in the freshwater gill.

Figure 1-4. An alternate model for trout urinary bladder reabsorption of Na^+ and Cl^- utilizing the apical Na^+/H^+-ATPase as in the freshwater gill model.

Figure 1-5. Model of Na^+ and Cl^- reabsorption in European rainbow trout (Fossat and Lahlou, 1977, 1979a and 1979b) via an apical Na^+/Cl^- co-transporter so Na^+ and Cl^- reabsorption are directly coupled, unlike the brook trout urinary bladder.

Figure 1-1

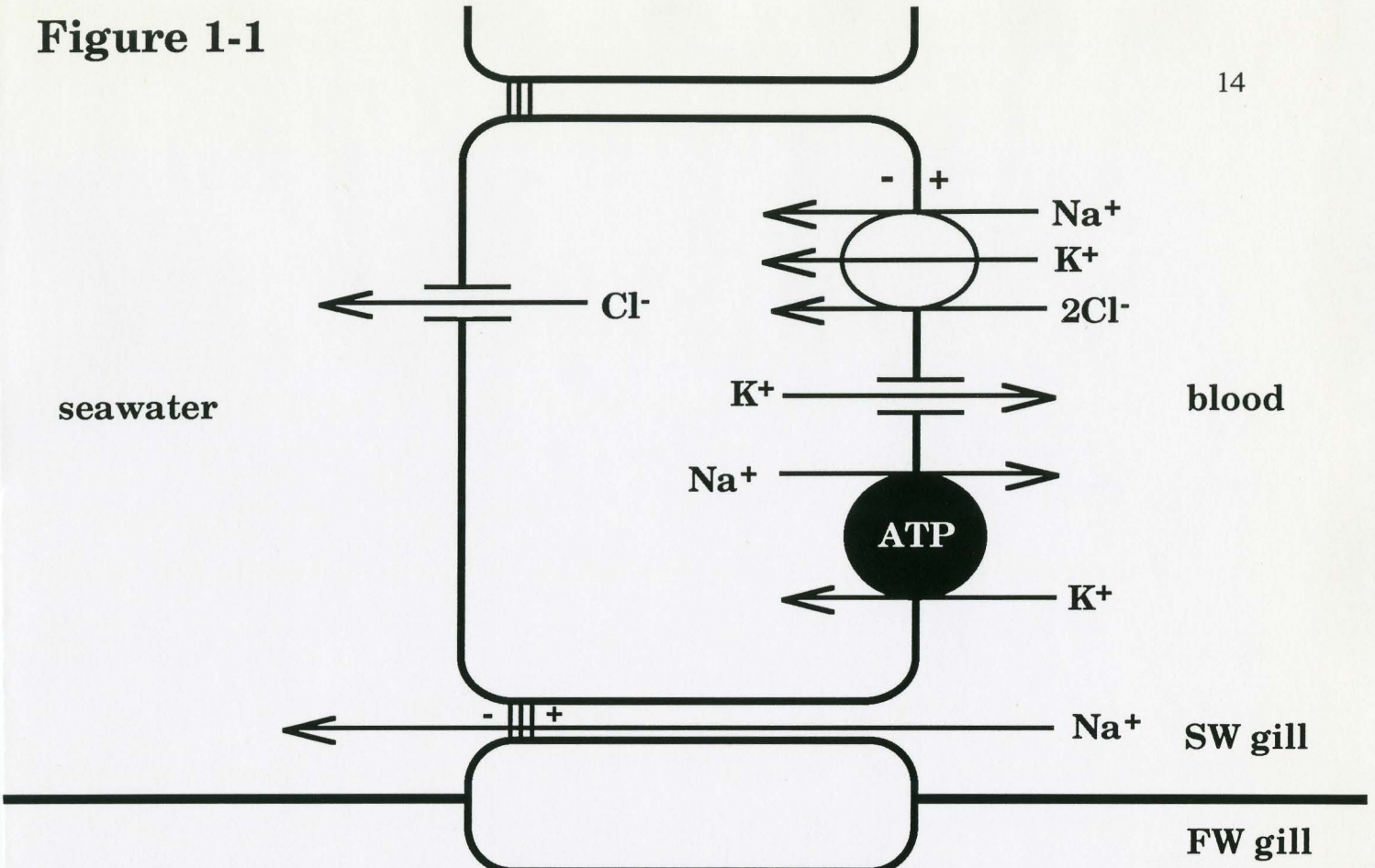


Figure 1-2

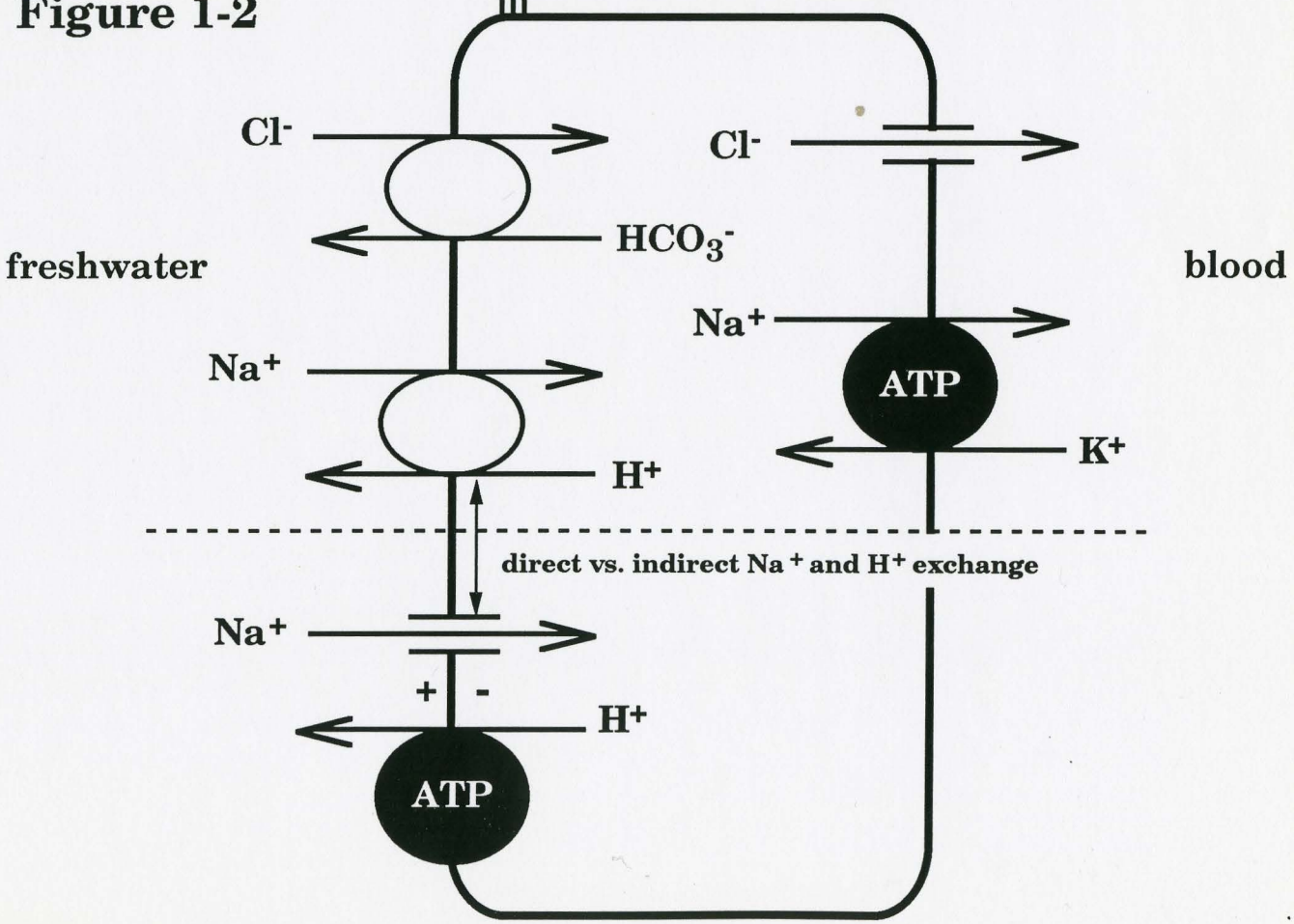
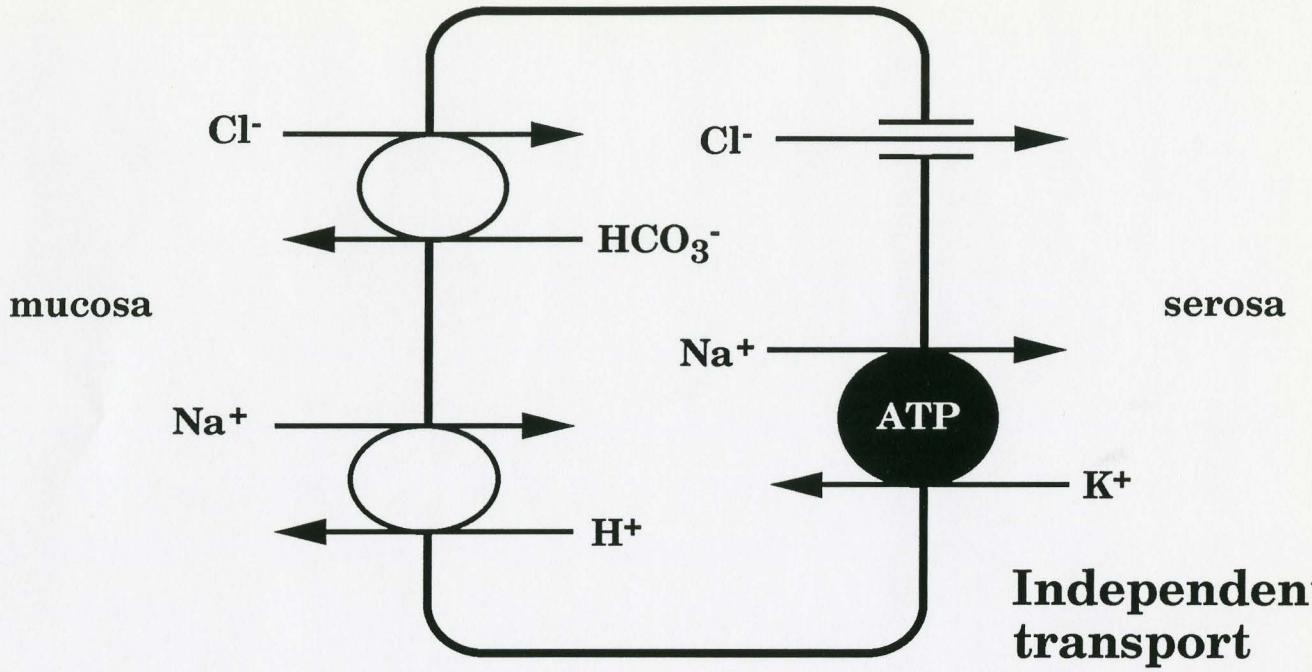


Figure 1-3



Independent transport

Salvelinus fontinalis **urinary bladder**

Figure 1-4

Independent transport with H^+-ATPase in urinary bladder

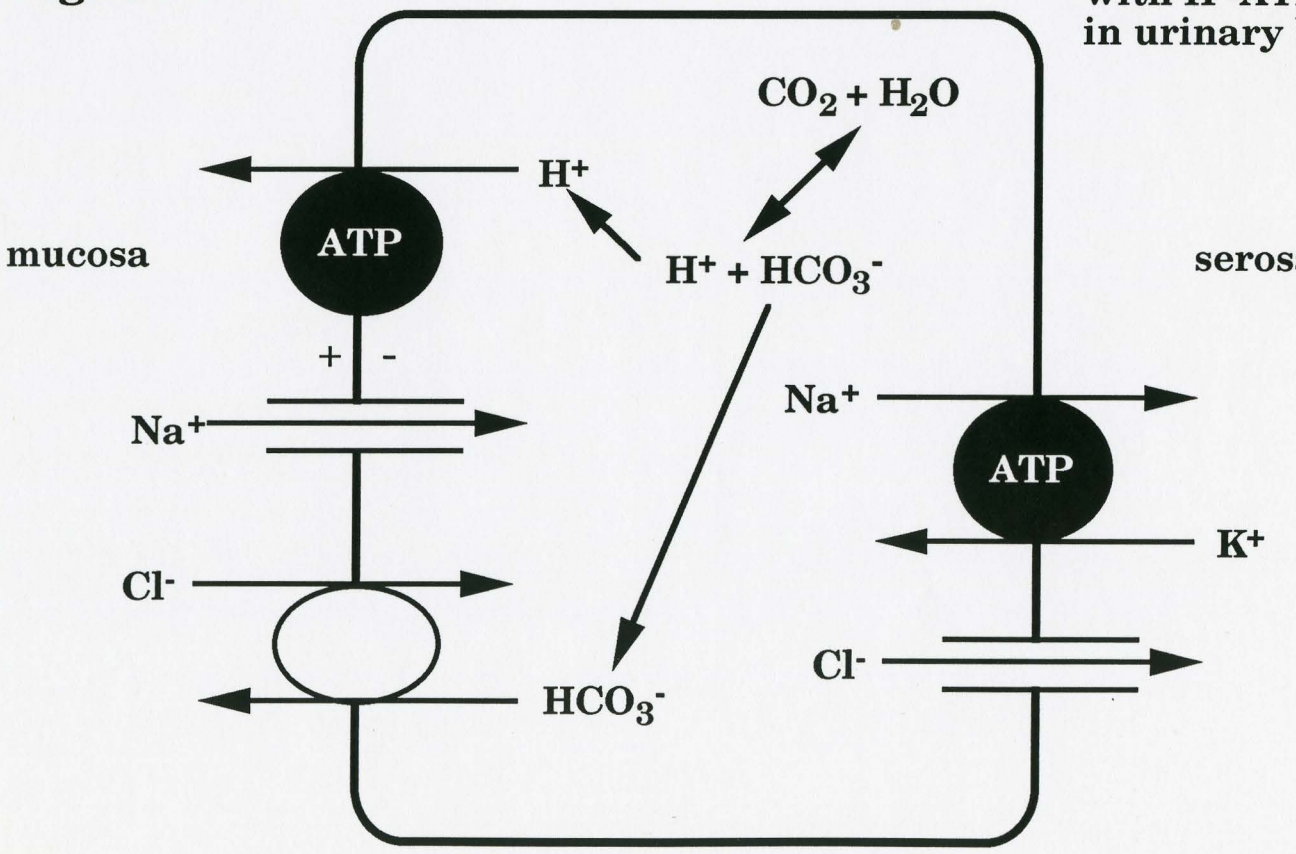
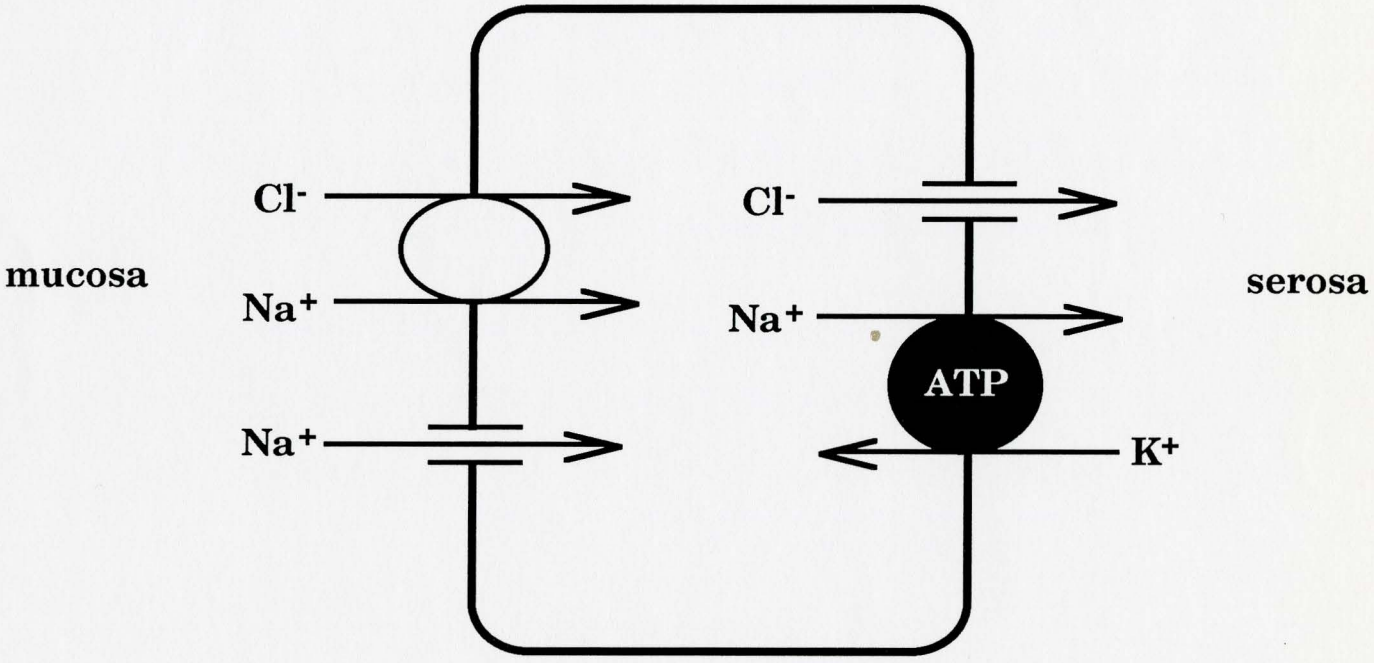


Figure 1-5

Co-transport

Salmo irideus
urinary bladder



Chapter 2

Materials and methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing approximately 250-600 g were obtained from Humber Springs Hatchery (Orangeville, Ontario). The trout were kept in 500 l tanks supplied with dechlorinated and aerated Hamilton tap water with a flow rate of approximately 900 $ml \cdot min^{-1}$. The approximate composition of the water was: 0.6 mM Na^+, 0.7 mM Cl^-, 1.05 mM Ca^{2+} with a pH of 7.5-8.0. A seasonal photoperiod was maintained and water temperature fluctuated according to seasonal ambient temperatures (5-16° C). Commercial trout pellets (Agribrands Purina Canada Inc., Ontario) were fed to the fish at 1% body mass per day.

Bathing solutions

Salts were obtained from Sigma Chemical Co., St. Louis, MO.

The trout saline used was a modified Cortland saline with concentrations of (mM): NaCl, 129.9; KCl, 2.55; $CaCl_2 \cdot H_2O$, 1.56; $MgSO_4 \cdot 7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4 \cdot H_2O$, 2.97; glucose, 5.55; NH_4Cl, 0.30. The saline was bubbled with 0.3% CO_2/balance O_2 to attain a pH of 7.8-7.9 (P_{CO_2}=2.3 torr). The saline was employed throughout in both the mucosal and serosal compartments with drugs added to one or both sides as indicated for each experiment.

During ion replacement experiments the Na^+ or Cl^- were replaced in the above saline. NaCl, $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were replaced with equal amounts of choline chloride ($C_5H_{14}NOCl$), choline bicarbonate ($C_5H_{14}NOHCO_3$) and KH_2PO_4, respectively, in Na^+-free

saline. Cl^--free saline used sodium gluconate ($NaC_6H_{11}O_7$), potassium gluconate ($KC_6H_{11}O_7$), hemicalcium gluconate ($1/2CaC_6H_{11}O_7$) and $(NH_4)_2SO_4$ to replace NaCl, KCl, $CaCl_2 \cdot H_2O$ and NH_4Cl, respectively.

The mucosal solutions used in the Na^+ and Cl^- uptake kinetics study were modifications of the regular saline in which all components except Na^+ and Cl^- were held at normal levels. Adjustments of the Na^+ and Cl^- concentrations were used to obtain mucosal solutions of approximately 5, 15, 45, 95 and 145 mM concentrations of Na^+ and of Cl^-. $NaHCO_3$ was replaced with choline bicarbonate as in the ion replacement solutions. The serosal bathing solution in the uptake kinetics study was regular saline.

Urinary bladder removal

The fish were anaesthetized using 1 g MS-222 per liter of water and placed in a V-shaped operation table while the gills were perfused with water containing the MS-222. Polyethylene tubing (PE-60, Becton Dickinson and Company, Sparks, MD) was flanged at one end, by heating, and inserted into the urinary papilla of fish up to 3 cm or until further entry was impeded due to the anterior wall of the bladder. The PE tubing was secured onto the urinary papilla using suture silk (size 2-0) and an incision was made starting at the anus. The incision was deep enough to cut through the muscle and open the cavity containing the swim bladder, urinary bladder and hindgut, and continued forward to the first set of ventral fins (~4 cm). The body walls were secured in an open position and the hindgut and swim bladder were removed. A holding ligation was made just posterior to the two ureters, but anterior to the body of the urinary bladder, and a second (main) ligation was made posterior to the first. The ureters were cut and the connective tissue holding the urinary bladder to the body walls was removed carefully to avoid damaging the bladder. A final

ligation was made at the posterior end of the urinary bladder, anterior to the area where urinary bladder and hindgut are connected. The remaining connective tissue was removed and the urinary bladder was lifted out of the body cavity. From this point on, different methods were used for closed bladder sac and open perfused bladder preparations.

Closed urinary bladder sac preparation

The PE tubing entering the urinary papilla was pulled as far out of the bladder as possible (until it was stopped by the posterior ligature) and the bladder was held upside down to remove as much urine as possible. The tubing was trimmed to approximately 2 cm and the bladder filled and emptied 3 times with the desired mucosal solution to clear any remaining urine. Mucosal salines contained ^{22}Na and/or ^{36}Cl, or DMSO (see **Pharmaceuticals** section) plus the radioisotopes, in the controls, and for the experimental series the mucosal solution contained the radioisotopes (+DMSO) and whichever drug was being tested. The mucosal bathing solutions were also bubbled with 0.3% CO_2/balance O_2 (Canadian Liquid Air Ltd., Montreal, Quebec) prior to injection into the urinary bladder. The bladder was then filled with the mucosal solution (0.1-0.3 ml) and the tubing stoppered with a small pin. The weight of the urinary bladder sac was recorded prior to the flux. Finally the bladder was placed in the serosal solution, 20 ml in a glass scintillation vial, which was bubbled with 0.3% CO_2/balance O_2 for the duration of the flux. The scintillation vials were held in a temperature controlled water bath at 15° C.

The bladder sacs were allowed to equilibrate for 0.5-1.5 hours, depending on the experimental series. After the equilibration period the flux measurement was run for 3-4 hours, again depending on the series.

Samples of the mucosal bathing solution were taken at the time of injection into the bladder and also collected at the end of the flux. Part of the sample was used for "cold" $[Na^+]$ and $[Cl^-]$

determination and part for the determination of "hot" Na^+ and Cl^- counts. Serosal samples were taken every 0.5 hours (100 μl) for the length of the flux period and "hot" Na^+ and Cl^- cpm determined. The change in serosal volume was incorporated into the calculation of flux rates.

At the conclusion of the flux period the bladder was weighed again, drained (thereby providing the mucosal sample mentioned above), weighed again without mucosal solution and finally the surface area was determined by spreading the preparation on graph paper. The data collected allowed calculation of net and unidirectional influx of both Na^+ and Cl^- as well as net water reabsorption rates.

The closed bladder sac preparation was initially employed for ion replacement experiments and subsequently for analysis of the effects of particular drugs.

Open, perfused urinary bladder preparation

After removal from the fish, the urinary bladder was cut near the ureteral ligations (anterior end) to gain access to the interior (mucosal) compartment. Another flanged piece of PE-60 was inserted into the anterior end and secured with suture silk. The PE tubing was connected to a peristaltic pump (either a Gilson Miniplus 2, Villiers Le Bel, France or a Cole Parmer Instrument Co. Model 7553-10, Chicago, IL) and the bladder placed in 20 ml serosal bathing saline in a glass scintillation vial. The bladder was then perfused with the mucosal perfusate at a rate of approximately 8 $ml \cdot h^{-1}$, which was determined to be the lowest speed necessary to ensure that concentrations of Na^+ or Cl^- remained at or below 3 mM, in the outflowing mucosal saline, for experiments where Na^+ or Cl^- were omitted from the inflowing mucosal saline but present in the serosal saline. Outflow was collected by placing all outflow tubing at the same height in the collection tubes during each experiment, to allow approximately 8 $ml \cdot h^{-1}$ perfusion rates and

consistent backpressure. Both mucosal and serosal solutions were continuously bubbled with 0.3% CO_2/balance O_2.

As in the closed sac preparation, the bladder was allowed 0.5-1 h for equilibration before the start of the measured flux period. The flux periods were 3-4 h in length and serosal samples were taken every 0.5 h (100 μl, as above). A mucosal sample was also obtained midway through the perfusion by sampling from the outflow tubing.

At the conclusion of the flux period the bladder surface area was measured using graph paper. The collected data allowed calculation of unidirectional influx rates of Na^+ and Cl^-. Water reabsorption rates were initially calculated by differences in weight between inflow and outflow perfusate, but were at least ten-fold higher than in the closed bladder sac preparation. Qualitatively, collecting the perfusate for water reabsorption rates proved to be difficult and the bladders displayed occasional rhythmic contractions which no doubt affected the amounts of perfusate collected. The water reabsorption rate measurements were discontinued.

The perfused sac preparation was used to minimize the effects of Na^+ and Cl^- backfluxes during ion replacement experiments and also to reduce backfluxes during Na^+ and Cl^- uptake kinetics experiments where mucosal Na^+ and Cl^- concentrations were maintained at approximately 5, 15, 45, 95 and 145 mM.

Pharmaceuticals

The initial drug experiments examined the channel blockers amiloride (Na^+ channel blocker and Na^+/H^+ exchange blocker), bumetanide ($Na^+/2Cl^-/K^+$ cotransporter blocker) and DIDS (4,4 diiso thiocyanato-stilbene-2,2 disulfonic acid, Cl^-/HCO_3^- exchanger and Cl^- channel blocker).

These drugs were applied in the mucosal saline at a concentration of 10^{-4} M and were dissolved in DMSO (dimethylsulphoxide) such that the DMSO made up only 0.1 % of the final saline solution (all chemicals from Sigma Chemical Co., St. Louis, MO). Later trials using amiloride (10^{-3} M) and DIDS (10^{-3} M) needed 0.15 % and 0.375 % final concentrations of DMSO, respectively, to dissolve the increased amount of drug. DMSO was used to dissolve only amiloride, bumetanide and DIDS; all other drugs were soluble in the salines.

The more selective Na^+/Cl^- cotransport blockers chlorothiazide (10^{-3} M), applied mucosally, and hydrochlorothiazide (10^{-3} M; Sigma Chemical Co.), applied simultaneously in the serosal and mucosal salines, were the next drugs tested. Acetazolamide (10^{-2} M; Sigma Chemical Co.), a carbonic anhydrase inhibitor, was used on the mucosal and serosal surface separately. The Na^+/K^+-ATPase inhibitor ouabain (Sigma Chemical Co.), was used at a concentration of 10^{-3} M and tested on both the mucosal and serosal surfaces separately. Metabolic inhibition was attempted using NaCN (sodium cyanide, 10^{-3} M; Sigma Chemical Co.) in the mucosal bathing saline as well as simultaneously in mucosal and serosal salines. Simultaneous inhibition of aerobic and anaerobic active transport was attempted using both NaCN (10^{-3} M) and NaF (sodium fluoride, 10^{-3} M) on the mucosal and serosal surfaces at the same time. This set of experiments was also repeated, in the same fashion, using NaCN (10^{-3} M) and iodoacetate ($5x10^{-3}$ M; Sigma Chemical Co.). The possibility of an access problem, coupled with the apparent lack of expected effects at lower concentrations, prompted the use of higher concentrations of most drugs than might be found in literature.

Analytical techniques

Na^+ concentrations in salines were measured using atomic absorption spectrophotometry (model AA-1275, Varian, Springvale, Australia). Cl^- concentrations were measured using a coulometric titration (model CMT10, Radiometer, Copenhagen) and/or using a colorimetric microassay (Zall *et al.*, 1956) read on a Molecular Devices Spectramax 340PC microtiter plate reader (Sunnyvale, CA).

Most flux experiments were double-labeled experiments where both ^{22}Na and ^{36}Cl were used simultaneously. Radioactive counts were measured in 4.0 ml of ACS fluor (Amersham Canada Ltd., Ontario). ^{22}Na counts per minute (CPM) were measured using a Minaxi Autogamma 5000 counter (Packard Instrument Co., Downers Grove, IL.) and ^{36}Cl CPM were measured on a Rackbeta 1217 liquid scintillation counter (LKB, Wallac, Turku, Finland). Since ^{22}Na is both a gamma and beta emitter, both types of its emissions were measured by scintillation counting, but only gamma emissions by gamma counting. ^{36}Cl however, emits only beta emissions which can be detected only by scintillation counting. Therefore, a subtraction procedure was used to separate the radioactivity of ^{22}Na and ^{36}Cl in experimental samples. A known amount of ^{22}Na was measured in both the gamma and scintillation counters and a ratio determined for the efficiency of ^{22}Na detection by gamma versus scintillation counting. This ratio was used to calculate the expected ^{22}Na CPM by scintillation counting, based on the measured CPM of the same sample by gamma counting. This value was subtracted from the total scintillation CPM of the sample to obtain ^{36}Cl beta radiation.

Flux calculations

Specific activity of the mucosal (radioactive or hot) compartment was calculated using:

$$\frac{\text{CPM of mucosal sample}}{\text{hot sample volume (ml) x [ion] on hot side (mM)}}$$

For perfused sacs the specific activity stayed constant since the mucosal solution was being refreshed by perfusion. In closed sacs, however, mucosal samples were taken only at the start and end of the flux. Serosal samples were used to calculate influxes for separate periods during the 3-4 hours of the experiment. Specific activities throughout the flux were calculated from the endpoint mucosal specific activities. This was accomplished by taking the natural log (*ln*) of both the start and end mucosal specific activities and fitting a linear regression over the time between the two samples. The times of the serosal samples, relative to the start mucosal sample, were then used to calculate the specific activity necessary to calculate the influx rate at that time (see below).

The unidirectional influx rates were calculated using:

$$\frac{\{CPM^2 \text{ x (cold vol./cold sample vol.)}\} - \{CPM^1 \text{ x (cold vol.-(cold sam. vol./cold sam. vol.))}\}}{\text{length of flux period (h) x specific activity of hot sample (CPM/}\mu\text{mol) x surface area (cm}^2\text{)}}$$

CPM^2 (and the associated volumes) refer to the cold sample (serosal) taken at the end of the period and CPM^1 to that taken at the start. The above equation produces a unidirectional flux rate with units $\mu mol \cdot cm^{-2} \cdot h^{-1}$.

Net flux rates were calculated using mucosal concentrations and volumes:

$$\frac{\{\text{end [ion] (mM) x end vol. (ml)}\} - \{\text{start [ion] x start vol.}\}}{\text{surface area (cm}^2\text{) x length of flux (h)}}$$

Volumes were obtained by weighing the filled bladder sac before the flux, after the flux (still filled) and after emptying. Negative flux rates indicate a net uptake from the mucosal solution. The net flux units obtained were $\mu mol \cdot cm^{-2} \cdot h^{-1}$.

Water net flux rates were calculated by:

$$\frac{\text{end volume (ml) - start volume (ml)}}{\text{surface area (cm}^2\text{) x flux length (h)}}$$

Negative water flux rates indicate a net uptake of water from the mucosal solution. Net water flux units were $\mu l \cdot cm^{-2} \cdot h^{-1}$.

Statistics

The data are presented as means ± SEM in all cases. Comparisons for significant changes between treatments (eg. control vs. 10^{-3} M NaCN treated sacs) were made using unpaired t-tests (two tailed) with a significance level of 0.05. Comparisons for flux rate changes within treatments (eg. control Na^+ influx vs. control Cl^- influx) were made using paired t-tests (two tailed) with a significance level of 0.05. For flux rates used in more than one comparison, a Bonferroni

correction was used. In practice, for a sample set involved in two, three or four comparisons alpha levels become 0.025, 0.017 and 0.013, respectively. For simplicity, these levels were then noted as P=0.05 and the corrected P values of the comparisons are shown as less than or greater than 0.05.

Chapter 3

Results

Ion replacement in closed and open, perfused bladder sacs

Ion replacement and control experiments were performed to determine net flux rates and unidirectional influx rates (using ^{22}Na and ^{36}Cl) under control conditions and under conditions where either Na^+ or Cl^- were removed from the mucosal bathing solutions of bladder sac preparations, under otherwise symmetrical conditions. Net flux rates were obtained by measuring the disappearance of Na^+ or Cl^- from the mucosal saline, while unidirectional influx rates were measured by the appearance of ^{22}Na and ^{36}Cl in the serosal saline and disappearance of the isotopes from the mucosal saline.

The viability of the sealed urinary bladder sac preparation is demonstrated in Table 3-1. The unidirectional Na^+ and Cl^- influx rates are shown over a 3.5 hour flux in 7 separate 0.5 hour periods, with a 1.0 hour equilibration period prior to flux measurement. Two typical closed bladder sac preparations are shown. The influx rates remain stable after 4.5 hours.

Under control, symmetrical saline conditions, net flux rates for Na^+ and Cl^- were in the mucosal to serosal direction (absorption) at 1.89±0.23 and 1.68±0.18 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively, and were not significantly different (Fig. 3-1). Replacement of Cl^- in the mucosal bathing medium with gluconate yielded a Na^+ net absorption rate of 1.77±0.21 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ which was not significantly different from the control value (Fig. 3-1). Likewise, replacement of Na^+ with choline did not significantly affect the Cl^- net absorption rate, 1.36±0.27 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-1).

Control unidirectional influx rates were slightly, but significantly, different between Na^+ (4.54±0.71 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) and Cl^- (5.86±0.94 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) under control, symmetrical saline conditions (Fig. 3-2). Replacement of the mucosal Cl^- caused no significant change in Na^+ influx rate (4.20±0.35 $\mu mol \cdot cm^{-2} \cdot h^{-1}$; Fig. 3-2). Similarly, Cl^- influx rate (4.93±0.40 $\mu mol \cdot cm^{-2} \cdot h^{-1}$)

was also unaffected by Na^+ removal from the mucosal bathing solution (Fig. 3-2).

Water reabsorption rates under Na^+ free (4.04±1.39 $\mu l \cdot cm^{-2} \cdot h^{-1}$) and Cl^- free (6.18±1.19 $\mu l \cdot cm^{-2} \cdot h^{-1}$) conditions also showed no significant change from control (3.10±1.66 $\mu l \cdot cm^{-2} \cdot h^{-1}$; Fig. 3-3), thereby reflecting the lack of effect of ion replacement on Na^+ and Cl^- flux rates.

The drawback of a sealed sac preparation, in studying ion replacement conditions, is that over the period of a 3-4 hour flux the replaced ion back flux can create a substantial concentration of the replaced ion in the mucosal bathing solution. Typical mucosal Na^+ concentrations at the end of a 3-4 hour flux ranged from 14-53 mM while mucosal Cl^- levels during mucosal Cl^- replacement reached 18-104 mM (Table 3-2). A method that reduces the back flux of the replaced ion would add validation to the ion replacement findings. For this reason, the open perfused bladder sac preparation was developed.

The viability of the perfused urinary bladder sac preparation is shown in Table 3-3. Typical Na^+ and Cl^- influx rates are shown for two perfused bladders over a 2.5 hour flux period with 1.5 hours of equilibration prior to flux measurement. As in the sealed bladder sac preparation, the influx rates appear to remain stable over the 4.0 hours of perfusion at 8 $ml \cdot h^{-1}$.

To complete the ion replacement experiments, bladder preparations were perfused under control, symmetrical saline conditions and under ion replaced conditions where the replaced ion was maintained at or below 3 mM ($[Na^+]$=1.77±0.24 mM or $[Cl^-]$=1.92±0.22 mM, outflow perfusate collected after passing through the bladder). The control Na^+ unidirectional influx rate was 3.84±0.52 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and removal of Cl^- yielded a Na^+ influx rate of 3.54±0.48 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-4). These rates were not significantly different. The Cl^- unidirectional influx rate after Na^+ removal was 4.32±0.62 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, which was not significantly different from the control Cl^- influx rate of 4.36±0.50 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-4). Under perfused conditions, as in the sacs, the control Cl^- influx was significantly higher than the control Na^+ influx. The nature of the perfused bladder preparation was such that the change in weight

measurements (weight at start and end, water flux) needed to calculate net flux were usually imprecise and variable. Therefore, no net flux measurements were obtained for Na^+, Cl^- and water in the perfused bladder (see **Methods**).

It is apparent that in both the sealed bladder sac preparation or the open perfused sac preparation, replacement of Na^+ or Cl^- has no effect on the unidirectional influx of the other ion.

Amiloride, bumetanide and DIDS treated bladder sacs

Amiloride, bumetanide and DIDS were initially employed, separately, in an attempt to dissect out the transporter types that may be present in the urinary bladder employing the sealed sac preparation. Initial experiments used mucosally applied drugs at a concentration of 10^{-4} M; ^{22}Na and ^{36}Cl were employed for unidirectional influx measurements.

The control (mucosal saline with 0.1% DMSO) Na^+ influx rate was 4.26±0.14 μmol•cm^{-2}•h^{-1} and the influx rates in the presence of 10^{-4} M amiloride (4.36±0.31 μmol•cm^{-2}•h^{-1}), 10^{-4} M bumetanide (4.54±0.86 μmol•cm^{-2}•h^{-1}) and 10^{-4} M DIDS (4.57±0.52 μmol•cm^{-2}•h^{-1}) were not significantly different from the control (Fig. 3-5). The Cl^- influx rates for control, amiloride, bumetanide and DIDS were: 5.78±0.45, 5.42±0.35, 6.01±1.13 and 6.57±0.63 μmol•cm^{-2}•h^{-1}, respectively (Fig. 3-5). None of the drug treatments had a significant effect on Cl^- influx rates. As in the ion replacement experiments, Cl^- influx rates were higher than Na^+ influx rate for all treatments including controls.

Na^+ net fluxes were not significantly different from control for both 10^{-4} M bumetanide and 10^{-4} M DIDS (Fig. 3-6). The net fluxes for bumetanide and DIDS were 2.21±0.45 and 1.43±0.20 μmol•cm^{-2}•h^{-1}, respectively. In the presence of 10^{-4} M amiloride, net Na^+ flux was 1.04±0.01 μmol•cm^{-2}•h^{-1} compared to a control net flux of 1.91±0.14 μmol•cm^{-2}•h^{-1}, a significant 46% reduction (Fig. 3-6). The control Cl^- net flux was 1.90±0.15 μmol•cm^{-2}•h^{-1} (Fig. 3-6). In this case the Cl^- net flux was significantly lower in the presence of both amiloride (45% reduction)

and DIDS (35% reduction) sacs (Fig. 3-6). Bumetanide treated sacs showed no difference in Cl^- net flux rates (1.67±0.37 $\mu mol \cdot cm^{-2} \cdot h^{-1}$).

The water reabsorption rates for the corresponding 10^{-4} M drug treated urinary bladder sacs were not significantly different from the control (8.63±1.02 $\mu l \cdot cm^{-2} \cdot h^{-1}$) for both bumetanide (8.43±2.17 $\mu l \cdot cm^{-2} \cdot h^{-1}$) and DIDS (6.51±1.09 $\mu l \cdot cm^{-2} \cdot h^{-1}$), although the DIDS treated sacs did show a decreasing trend (Fig. 3-7). The water reabsorption rate of the amiloride treated sacs was 5.29±0.89 $\mu l \cdot cm^{-2} \cdot h^{-1}$, a value significantly lower than the control.

Due to the apparent decreasing, but not always significant, trends when bladder sacs were treated with 10^{-4} M amiloride and 10^{-4} M DIDS, a problem with drug access was suspected. In an attempt to remedy the situation DIDS and amiloride were tested again in the bladder sac preparation using higher concentrations.

The amiloride and DIDS experiments were repeated but at a 10^{-3} M concentration, again applied on the mucosal surface only. To accomplish the higher drug concentrations, higher amounts of DMSO were required to dissolve the drugs so controls including 0.15%, 0.375%, 0.55% and 1% DMSO were also completed. This allowed comparison of the drug administered sacs back to a corresponding control with the same DMSO concentration.

Na^+ influx rates in the presence of mucosal 10^{-3} M amiloride and the corresponding control (0.15% DMSO) were not significantly different at 3.38±0.29 and 3.10±0.53 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively (Fig. 3-8). Amiloride treated Cl^- influx (3.62±0.41 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) was also not significantly different from the 0.15% DMSO control (2.95±0.37 $\mu mol \cdot cm^{-2} \cdot h^{-1}$; Fig. 3-9). The higher concentration of DIDS (10^{-3} M) caused an increase in Na^+ influx from 3.12±0.34 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (0.375% DMSO control) to 6.78±0.61 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-8). Cl^- influx rates followed the same trend. DIDS treated sacs showed an increased Cl^- influx (6.78±0.50 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) relative to the 0.375% DMSO control (3.63±0.41 $\mu mol \cdot cm^{-2} \cdot h^{-1}$; Fig. 3-9).

Na^+ net flux rates in both 10^{-3} M amiloride and 10^{-3} M DIDS treated sacs showed no

change from the corresponding controls (Fig. 3-10). The amiloride treated net flux rate was 1.59±0.20 μmol•cm^{-2}•h^{-1} and the corresponding control was 1.17±0.28 μmol•cm^{-2}•h^{-1} (Fig. 3-10). The DIDS treated sacs showed a Na^+ net flux of 1.62±0.18 μmol•cm^{-2}•h^{-1} compared to the corresponding control at 1.06±0.20 μmol•cm^{-2}•h^{-1} (Fig. 3-10).

Cl^- net flux rates were similar to Na^+ net flux rates in that neither 10^{-3} M amiloride nor 10^{-3} M DIDS treated sacs showed a difference in net flux rates from the corresponding controls (Fig. 3-11). The amiloride treated Cl^- net flux rate was 1.23±0.16 μmol•cm^{-2}•h^{-1} in comparison to 1.13±0.27 μmol•cm^{-2}•h^{-1} found in the 0.15% DMSO control (Fig. 3-11). The sacs treated with DIDS had a net Cl^- flux rate of 1.28±0.16 μmol•cm^{-2}•h^{-1} while the control was 0.83±0.18 μmol•cm^{-2}•h^{-1} (Fig. 3-11).

Water reabsorption rates followed the trends of Na^+ and Cl^- influx rates. Sacs treated with 10^{-3} M amiloride showed no change in water reabsorption rates (Fig. 3-12). The control (0.15% DMSO) water reabsorption rate was 5.55±1.86 μl•cm^{-2}•h^{-1} while the amiloride treated sac had a rate of 4.61±0.64 μl•cm^{-2}•h^{-1}. The DIDS treated sacs exhibited an increase in water reabsorption rates from 3.57±0.811 μmol•cm^{-2}•h^{-1} (0.375% DMSO control) to 6.18±0.61 μmol•cm^{-2}•h^{-1} (Fig. 3-12).

A bumetanide sensitive transport mechanism (traditionally $Na^+/K^+/2Cl^-$-co-transport) was obviously eliminated early from consideration due to the lack any effect at the 10^{-4} M concentration. The results of the DIDS and amiloride experiments in either the 10^{-4} M or 10^{-3} M concentrations were unclear and did not obviously suggest the presence of the independent antiport transport mechanisms traditionally sensitive to either of these drugs.

Chlorothiazide and hydrochlorothiazide treated bladder sacs

Chlorothiazide and hydrochlorothiazide were used in an attempt to specifically block any Na^+/Cl^- co-transporters. Both drugs were used in a 10^{-3} M concentration but chlorothiazide was

applied on the mucosal surface only while hydrochlorothiazide was applied both mucosally and serosally. Neither drug or method of application had any effect on Na^+ or Cl^- unidirectional influx rates. Control Na^+ influx rates (3.53±0.31 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) were no different than rates in chlorothiazide (3.80±0.53 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) or hydrochlorothiazide (2.79±0.36 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) treated sacs (Fig. 3-13). Cl^- influx rates were 3.40±0.56, 2.76±0.69 and 3.50±0.67 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ for control, chlorothiazide and hydrochlorothiazide treated sacs, respectively (Fig. 3-13).

Net flux rates for both Na^+ and Cl^- were also unaffected by mucosal chlorothiazide administration. Na^+ net flux rates were 1.12±0.16 and 1.16±0.20 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (control and chlorothiazide, respectively) and Cl^- net flux rates were 1.17±0.19 and 1.04±0.21 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (control and chlorothiazide; Fig. 3-14). However, the simultaneous mucosal and serosal administration of 10^{-3} M hydrochlorothiazide was responsible for a 76% reduction in net Na^+ flux and a 75% reduction in net Cl^- flux (Fig. 3-14).

Water reabsorption was also not affected by mucosal 10^{-3} M chlorothiazide but was reduced by 55% when sacs were exposed to simultaneous mucosal and serosal 10^{-3} M hydrochlorothiazide (Fig. 3-15).

It would appear that even with the apparent independent transport of Na^+ and Cl^-, from the ion replacement experiments, there may be an element of co-transport involved in the trout urinary bladder.

Acetazolamide treated bladder sacs

Acetazolamide was administered in both mucosal and serosal solutions, in separate sac experiments, at a concentration of 10^{-2} M in an attempt to determine if blocking carbonic anhydrase, and therefore H^+ and HCO_3^- supply, would affect Na^+ and Cl^- transport. The high acetazolamide concentration was used due to the lack of expected effects of more common transporter blockers (amiloride and DIDS) and the suspicion that there may be a problem with the

drug access into the tissue. Serosal application of acetazolamide did not elicit a significant response in either Na^+ or Cl^- influx rates. The Na^+ influx rate of control and serosally treated acetazolamide sacs were 2.93±0.30 and 2.51±0.57 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively, and control and serosally treated Cl^- influx rates were 2.10±0.51 and 1.30±0.80 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively. Although the Cl^- influx rate of mucosally treated sacs (1.54±0.48 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) was not significantly lower, the Na^+ influx rate after mucosal 10^{-2} M acetazolamide application was reduced by 36% (Fig. 3-16).

Net flux rates of both Na^+ and Cl^- were unaffected by the mucosal or serosal administration of acetazolamide (Fig. 3-17). The control net Na^+ flux rate (0.77±0.13 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) was no different than the net flux rates in mucosal acetazolamide sacs (0.67±0.21 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) or serosal acetazolamide sacs (0.94±0.12 $\mu mol \cdot cm^{-2} \cdot h^{-1}$). The control net Cl^- flux rate (0.74±0.12 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) was no different from the net flux rate of mucosal acetazolamide sacs (0.66±0.20 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) or serosal acetazolamide sacs (0.85±0.12 $\mu mol \cdot cm^{-2} \cdot h^{-1}$).

Water reabsorption rates for the acetazolamide treated sacs (mucosal, 6.18±1.52 $\mu l \cdot cm^{-2} \cdot h^{-1}$; serosal, 5.80±1.06 $\mu l \cdot cm^{-2} \cdot h^{-1}$) were also not significantly different from control values (4.41±0.87 $\mu l \cdot cm^{-2} \cdot h^{-1}$; Fig. 3-18).

Acetazolamide seems to have only a faint effect on influx rates when applied mucosally. It seems unlikely that at such a high concentration there would be a drug access issue.

Ouabain treated bladder sacs

The bladder sac preparation was treated with 10^{-3} M ouabain at the mucosal and serosal surfaces, separately, to determine if Na^+/K^+-ATPase contributes to the transport of Na^+ and Cl^-. Initial Na^+/K^+-ATPase measurements in the rainbow trout urinary bladder had shown that 0.120±0.022 μmoles of PO_4^- were liberated for every cm^2 of bladder per hour (also 0.82±0.21

μmoles PO_4^-/ μg protein/h; Burgess, 1997).

Na^+ influx rate measurements showed that application of 10^{-3} M ouabain in the mucosal and serosal bathing media was responsible for a 49% and a 40% significant reduction, respectively, relative to the control Na^+ influx rate of 5.57±0.76 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-19). Influx rates for Cl^- were also significantly reduced by mucosal and serosal ouabain application (48% and 43%, respectively) compared to the control value of 5.63±0.78 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-19).

Surprisingly, net flux rates for both Na^+ and Cl^-, under mucosal or serosal 10^{-3} M ouabain treatment, did not differ significantly from control values. Net Na^+ flux rates in the presence of mucosal and serosal ouabain were 0.95±0.19 and 1.21±0.42 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively, compared to a control value of 1.25±0.15 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-20). The mucosally treated sacs (0.97±0.11 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) and serosally treated sacs (0.86±0.43 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) did not show a significant difference in net Cl^- flux rates compared to the control (1.09±0.12 $\mu mol \cdot cm^{-2} \cdot h^{-1}$; Fig. 3-20).

Water reabsorption rates for controls (5.95±0.99 $\mu l \cdot cm^{-2} \cdot h^{-1}$), mucosal 10^{-3} M ouabain treated sacs (6.84±123 $\mu l \cdot cm^{-2} \cdot h^{-1}$) and serosal 10^{-3} M ouabain treated sacs (8.50±2.62 $\mu l \cdot cm^{-2} \cdot h^{-1}$) were not significantly different (Fig. 3-21).

It appears ouabain is an effective blocking agent with no access problems which works, equally well when applied at the mucosal or serosal surface, to reduce influx of both Na^+ and Cl^- in similar proportions.

Inhibition of energy supply mechanisms for active transport in bladder sacs

To follow up on the possibility of a drug access problem and to investigate the source of ATP supply for transport, 10^{-3} M NaCN was employed in an attempt to block the active transport of Na^+ and Cl^- that occurs in the trout urinary bladder. The high concentration was used in an

attempt to overcome any access issues since earlier CN^- was seen to require some time before effects were evident in bladder sac preparations mounted in Ussing chambers (Burgess *et al.*, 2000). The 10^{-3} M NaCN was tested in the mucosal bathing medium only, as well as in both the mucosal and serosal bathing media simultaneously. The bladder sacs were allowed 1.5 h for equilibration and the measured flux continued for a further 3 h, more time than was used in the Ussing chamber bladder sac preparation before cyanide effects were seen (Burgess *et al.*, 2000).

The Na^+ influx rates of the bladder sacs treated, both mucosally and simultaneously mucosally and serosally, with NaCN were not different from control influx rates. The bladder sacs with 10^{-3} M NaCN present at the mucosal surface had an Na^+ influx rate of 4.70±0.42 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, while the sacs treated with mucosal and serosal NaCN had an Na^+ influx rate of 3.80±0.26 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, compared with a control value of 3.96±0.45 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-22). Cl^- influx rates were also not affected where the control value was 3.50±0.71 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, mucosally treated sacs were 4.16±0.96 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and both mucosally and serosally treated sacs had a Cl^- influx rate of 3.08±0.62 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-22).

Net Na^+ flux rates showed a small, but insignificant, decreasing trend, under NaCN treatment, compared to the control value of 0.81±0.08 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-23). The 10^{-3} M NaCN mucosal only treated sacs had a net Na^+ flux of 0.57±0.12 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and the sacs treated on both mucosal and serosal surfaces had a net Na^+ flux of 0.63±0.09 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-23). Net Cl^- flux rates were similar to net Na^+ flux rates and also had a small but insignificant decreasing trend under both treatments. The Cl^- net flux rates were 0.78±0.09 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ for control, 0.61±0.11 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ for mucosally treated sacs and 0.55±0.07 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ for sacs with NaCN present in both mucosal and serosal bathing solutions (Fig. 3-23).

Like influx and net flux rates of Na^+ and Cl^-, there were no effects 10^{-3} M NaCN treatment seen in the water reabsorption rates. Water reabsorption rates were 2.84±0.47 $\mu l \cdot cm^{-2} \cdot h^{-1}$ for

control, 3.92±0.81 $\mu l \cdot cm^{-2} \cdot h^{-1}$ for mucosal NaCN treatment and 2.44±0.46 $\mu l \cdot cm^{-2} \cdot h^{-1}$ for simultaneous mucosal and serosal NaCN treatment (Fig. 3-24).

It was surprising to see the lack of effect at such a high concentration of NaCN in the urinary bladder, suggesting that anaerobic generation of ATP might be compensating for blockade of aerobic ATP generation. The next obvious step was to attempt to block anaerobic sources of energy, in conjunction with NaCN, in an attempt to inhibit active transport of Na^+ and Cl^-.

The next drugs tested were 10^{-3} M NaF and $5x10^{-3}$ M iodoacetate, blockers of anaerobic ATP supply. Each of these drugs were used in separate experiments but in conjunction with 10^{-3} M NaCN and applied on both the mucosal and serosal surfaces. In the following results only the use of NaF or iodoacetate is mentioned but NaCN is also present. NaCN was not present in the controls, only in the experimental sacs. The Na^+ influx rate in the presence of 10^{-3} M NaF was 5.80±0.83 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, not significantly different from the control influx rate of 3.96±0.45 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-25). The Na^+ influx rate when $5x10^{-3}$ M iodoacetate was used was 4.64±0.51 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, also not significantly different (Fig. 3-25). Unfortunately the Cl^- influx rates for the experimental treatments using NaF and iodoacetate were lost due to problems with scintillation counting.

There was no significant difference between the control sacs' net Na^+ flux rate of 0.81±0.08 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and the sacs treated with 10^{-3} M NaF, 0.88±0.16 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 3-26). The presence of $5x10^{-3}$ M iodoacetate, however, caused a reduction in the Na^+ net flux rate of 51% (Fig. 3-26). The Cl^- net flux rates followed the trend of Na^+ rates and control sacs and NaF treated sacs were not different (0.78±0.09 and 0.80±0.13 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively; Fig. 3-26). The presence of iodoacetate at the mucosal and serosal surfaces caused a reduction of 54%, of control, in the net Cl^- flux rate (Fig. 3-26).

Water reabsorption rates were not different between the control sacs (2.84±0.47 $\mu mol \cdot cm^{-2} \cdot h^{-1}$), 10^{-3} M NaCN and 10^{-3} M NaF treated sacs (5.06±0.87 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) and 10^{-3} M NaCN

and $5x10^{-3}$ M iodoacetate treated sacs (2.98±1.05 μmol•cm^{-2}•h^{-1}; Fig. 3-27).

These results suggest that not all the energy required for active transport is supplied through aerobic means in the urinary bladder of trout.

As a final look at active versus passive transport, reabsorbate concentrations (mM) of Na^+ and Cl^- were calculated for the various treatments by dividing net ion flux rates by net water flux rates. Control values were combined from all treatments (except controls with DMSO), and Na^+-free and Cl^--free treatments only show Cl^- or Na^+ reabsorbate concentrations, respectively. This was done because net flux of Na^+ in the Na^+-free series was negative, therefore producing a negative concentration. It is obvious, in most cases, that the reabsorbate concentrations were above saline concentrations, indicating an active transport of Na^+ and Cl^- (Table 3-4). Statistical analysis shows that control reabsorbate concentrations of Na^+ and Cl^- were significantly higher than those of saline. Some of the drug treatments appear to have also caused a decrease in reabsorbate concentrations, of Na^+ and Cl^-, below those observed in control bladder sacs (Table 3-4). Although it appears that some drugs decrease reabsorbate concentrations to near saline (passive) levels, in general the concentrations are quite variable making it difficult to establish clear trends.

Perfused urinary bladder kinetics

In an effort to further examine the mechanisms of Na^+ and Cl^- movement across the urinary bladder, concentration dependent kinetics of unidirectional Na^+ and Cl^- uptake were examined. Since low concentrations of Na^+ and Cl^- were employed in the mucosal bathing medium, the perfused sac preparation was used to reduce the backflux of Na^+ an Cl^- from the serosal saline. This allowed the mucosal Na^+ and Cl^- concentrations to be maintained at the intended levels.

The Na^+ influx rates are shown as a percentage of the maximum rate obtained at the highest

Na^+ concentrations and as a function of mucosal Na^+ concentration (Fig. 3-28). The Na^+ influx rates obtained in this study are shown as open squares and are compared to the filled circles denoting the Na^+ influx rates obtained in a similar study in Ussing chambers (Burgess *et al.*, 2000). The linear fit of the current data is obvious and supported by an r^2 of 0.998. Although the Ussing chamber data seems to follow the current data quite closely, there is an apparent arc to the Ussing influx rates which were found to support Michealis-Menten kinetics (Burgess *et al.*, 2000). Surprisingly, the current data seem to lack any saturation characteristics and Na^+ influx rates continue to increase with increasing mucosal concentrations of Na^+ up to a maximum rate of 13.52±6.13 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ at a mucosal Na^+ concentration of 131.3±2.4 mM. This compares to maximum values of 7.02±1.41 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ at 135.1±5.5 mM in the Ussing chamber preparation (Burgess *et al.*, 2000).

The influx kinetics for Cl^- were much the same as for Na^+ (Fig. 3-29). The current data supports a linear fit (r^2=0.995) and saturation is not evident. The influx rates of Cl^- in Ussing chambers seem to follow those of the current data closely but again show an arc indicating Michealis-Menten saturation characteristics (Burgess *et al.*, 2000). The absolute maximum influx of the current work was 15.30±6.53 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, found at a mucosal Cl^- concentration of 128.6±1.9 mM. These values are very similar to those obtained in the Ussing chamber preparation which were 13.56±3.11 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ at a mucosal Cl^- concentration of 136.4±2.3 mM.

It seems that unlike the Ussing chamber urinary bladder mount, the perfused urinary bladder sacs do not exhibit saturation kinetics and Na^+ and Cl^- influx rates continue to rise with increasing mucosal Na^+ and Cl^- concentrations.

Table 3-1. Typical Na^+ and Cl^- unidirectional influx rates ($\mu mol \cdot cm^{-2} \cdot h^{-1}$) in two closed urinary bladder sac preparations. The influx rates shown are relatively stable over a 3.5 h flux, measured in 0.5 h periods. A 1.0 h equilibration period was used before flux measurements, for a total of 4.5 h of incubation.

Time (h)	Sodium influx rates		Chloride influx rates	
	Sac 1	Sac 2	Sac 1	Sac 2
0.5	3.66	2.72	3.94	3.11
1.0	2.90	2.52	4.36	3.20
1.5	3.60	2.87	4.07	3.52
2.0	3.44	2.91	4.48	3.80
2.5	3.82	3.22	3.68	4.38
3.0	3.62	2.93	5.29	4.43
3.5	4.63	4.20	5.21	3.54

Table 3-2. Typical mucosal Na^+ and Cl^- concentrations at the end of ion replacement experiments (3-4 hours), in closed sacs, for four Na^+-free and four Cl^--free experiments, respectively.

Experiment	Sodium concentration (mM)	Chloride concentration (mM)
1	23.1	50.2
2	53.8	104.6
3	16.2	18.3
4	14.0	23.0

Table 3-3. Typical Na^+ and Cl^- unidirectional influx rates ($\mu mol \cdot cm^{-2} \cdot h^{-1}$) in two perfused urinary bladder sac preparations. The influx rates shown seem stable over a 2.5 h flux, measured in 0.5 h periods. A 1.5 h equilibration period was used before flux measurements, for a total of 4.0 h of incubation.

Time (h)	Sodium influx rates		Chloride influx rates	
	Sac 1	Sac 2	Sac 1	Sac 2
0.5	5.24	3.17	5.51	4.17
1.0	4.82	3.50	4.17	3.97
1.5	3.54	2.89	5.43	3.87
2.0	5.75	3.92	6.55	3.92
2.5	3.19	4.07	3.67	4.25

Table 3-4. Na^+ and Cl^- reabsorbate concentrations calculated as "net ion flux/net water flux". Some concentrations show large variability but, most are higher than saline levels of Na^+ or Cl^-. Control values were combined from all experiments, mucosal and serosal application are listed along with concentrations employed. Mean ± SEM, pound (#) indicates significantly different from saline, asterisk (*) indicates significantly different from controls.

Treatment	Reabsorbate Sodium concentration (mM)	Reabsorbate Chloride concentration (mM)	N =
Saline	145.9	135.9	-
Controls (combined)	363.8±54.2#	328.6±48.9#	45
Sodium free	-	182.4±91.4	6
Chloride free	474.7±193.2	-	6
Amiloride (muc. 10^-4 M)	201.6±23.9*	181.6±21.5*	10
DIDS (muc. 10^-4 M)	395.9±176.2	299.3±113.8	11
Bumetanide (muc. 10^-4 M)	313.4±52.2	223.1±33.3	7
Amiloride (muc. 10^-3M)	362.2±29.1	278.4±25.7	7
DIDS (muc. 10^-3 M)	278.0±41.5	226.1±43.2	11
Chlorothiazide (muc. 10^-3 M)	173.1±39.6*	145.4±26.0*	6
Hydrochlorothiazide (muc./ser. 10^-3 M)	163.1±64.6	453.3±355.6	7
Acetazolamide (muc. 10^-2 M)	94.0±24.5*	94.2±23.5*	6
Acetazolamide (ser. 10^-2 M)	188.3±34.0*	167.1±23.3*	6
Ouabain (muc. 10^-3 M)	124.6±23.5*	180.3±49.8	6
Ouabain (ser. 10^-3 M)	132.1±34.4*	51.0±42.3*	6
Cyanide (muc. 10^-3 M)	144.1±14.3*	157.6±12.5*	6
Cyanide (muc./ser. 10^-3 M)	774.5±542.9	662.5±459.2	6
NaF (muc./ser. 10^-3 M)	177.6±11.2*	164.8±9.3*	9
Iodoacetate (muc./ser. 5x10^-3 M)	145.0±30.4*	110.8±21.9*	9

Figure 3-1. Net flux rates of Na^+ in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control and mucosal Cl^--free conditions, and net Cl^- flux rates under control and mucosal Na^+-free conditions. Open bars represent control conditions and hatched bars represent experimental conditions. Means ± SEM. No significant decrease in Na^+ net flux rate or Cl^- net flux rate when the co-ion was removed (unpaired t-test, two-tailed, $P>0.05$). Also, no significant difference between control net flux rates of Na^+ and Cl^- (paired t-test, two-tailed, $P>0.05$).

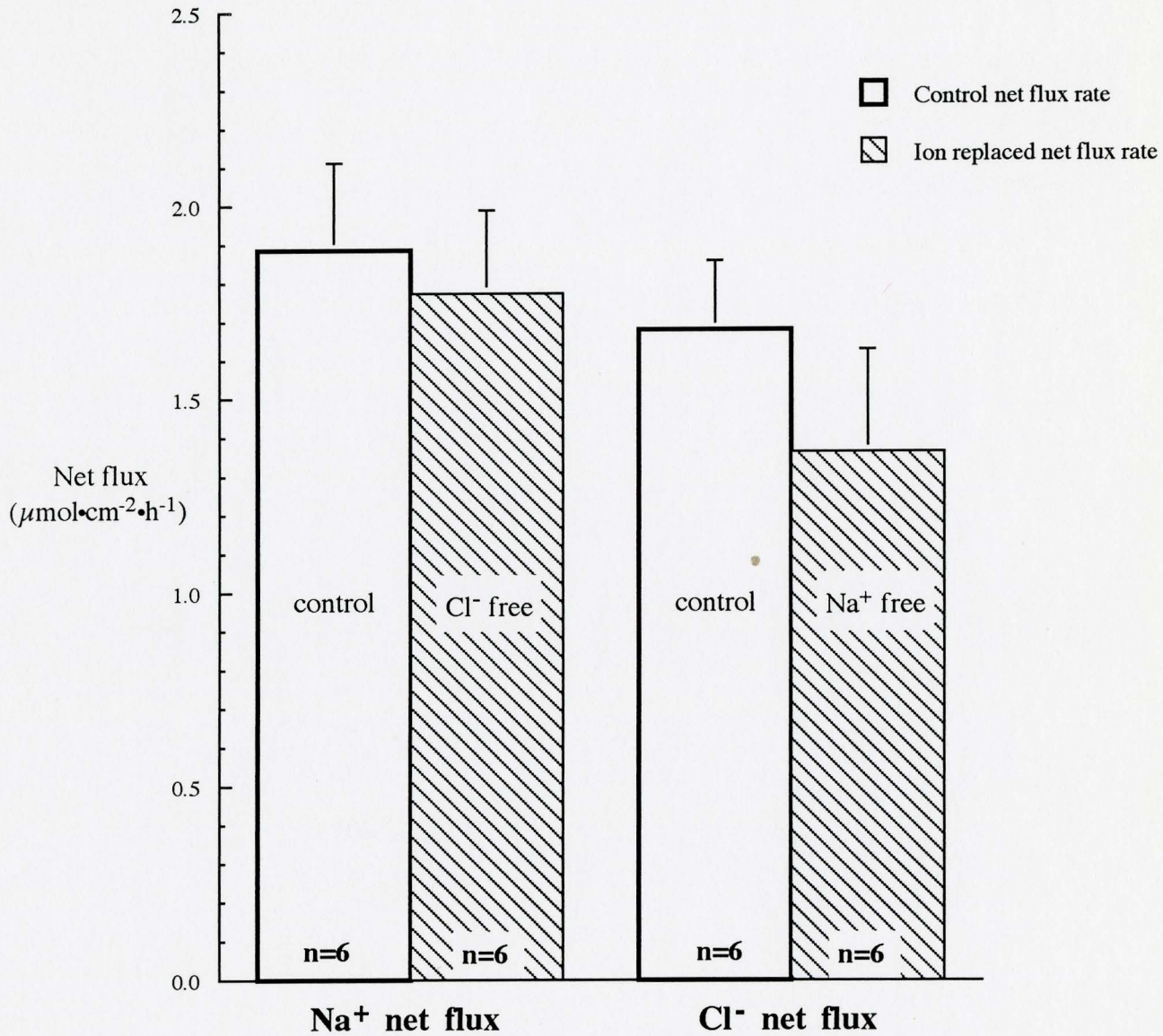

2.5
2.0
1.5
1.0
0.5
0.0
Net flux
(μmol•cm-2•h-1)
Control net flux rate
Ion replaced net flux rate
control
Cl- free
control
Na+ free
n=6
n=6
n=6
n=6
Na+ net flux
Cl- net flux

Figure 3-2. Influx rates of Na^+ in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control and mucosal Cl^--free conditions, and Cl^- influx rates under control and mucosal Na^+-free conditions. Open bars represent control conditions and hatched bars represent experimental conditions. Means ± SEM. No significant decrease in Na^+ influx rate or Cl^- influx rate when the co-ion was removed (unpaired t-test, two-tailed, $P>0.05$), indicating independent transport of each ion. Cross indicates significant difference between influx rates of Na^+ and Cl^- in the control sacs (paired t-test, two-tailed, $P<0.01$), supporting the independent transport of Na^+ and Cl^-.

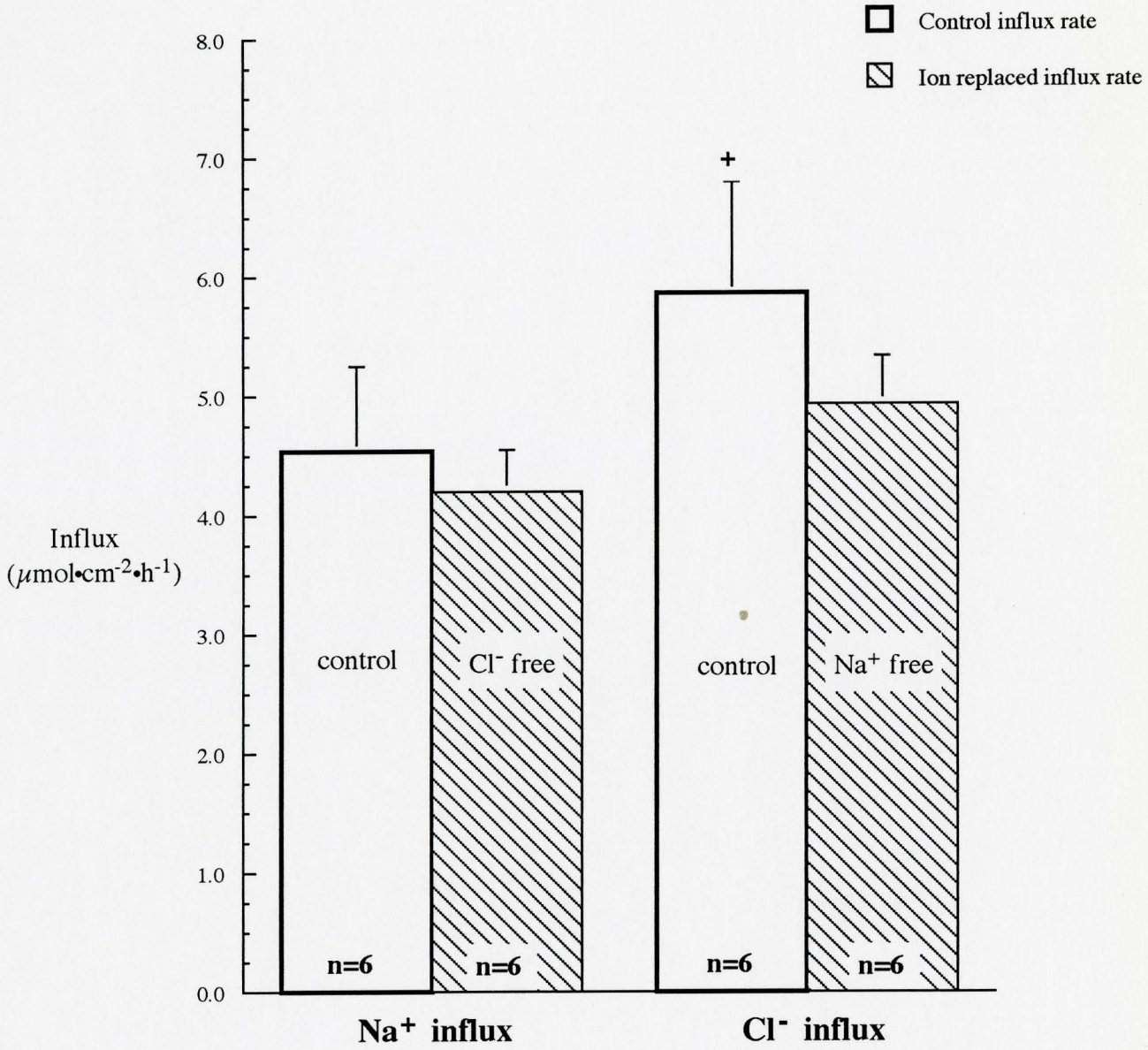

Control influx rate
Ion replaced influx rate
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Influx
(μmol•cm-2•h-1)
control
Cl- free
control
Na+ free
+
n=6
n=6
n=6
n=6
Na+ influx
Cl- influx

Figure 3-3. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal Cl^--free conditions and mucosal Na^+-free conditions. Open bar represents control, hatched bar represents mucosal Na^+-free and shaded bar represents mucosal Cl^--free conditions. Means ± SEM. No significant change in water reabsorption rates upon removal of Na^+ or Cl^- (unpaired t-test, two-tailed, $P>0.05$).

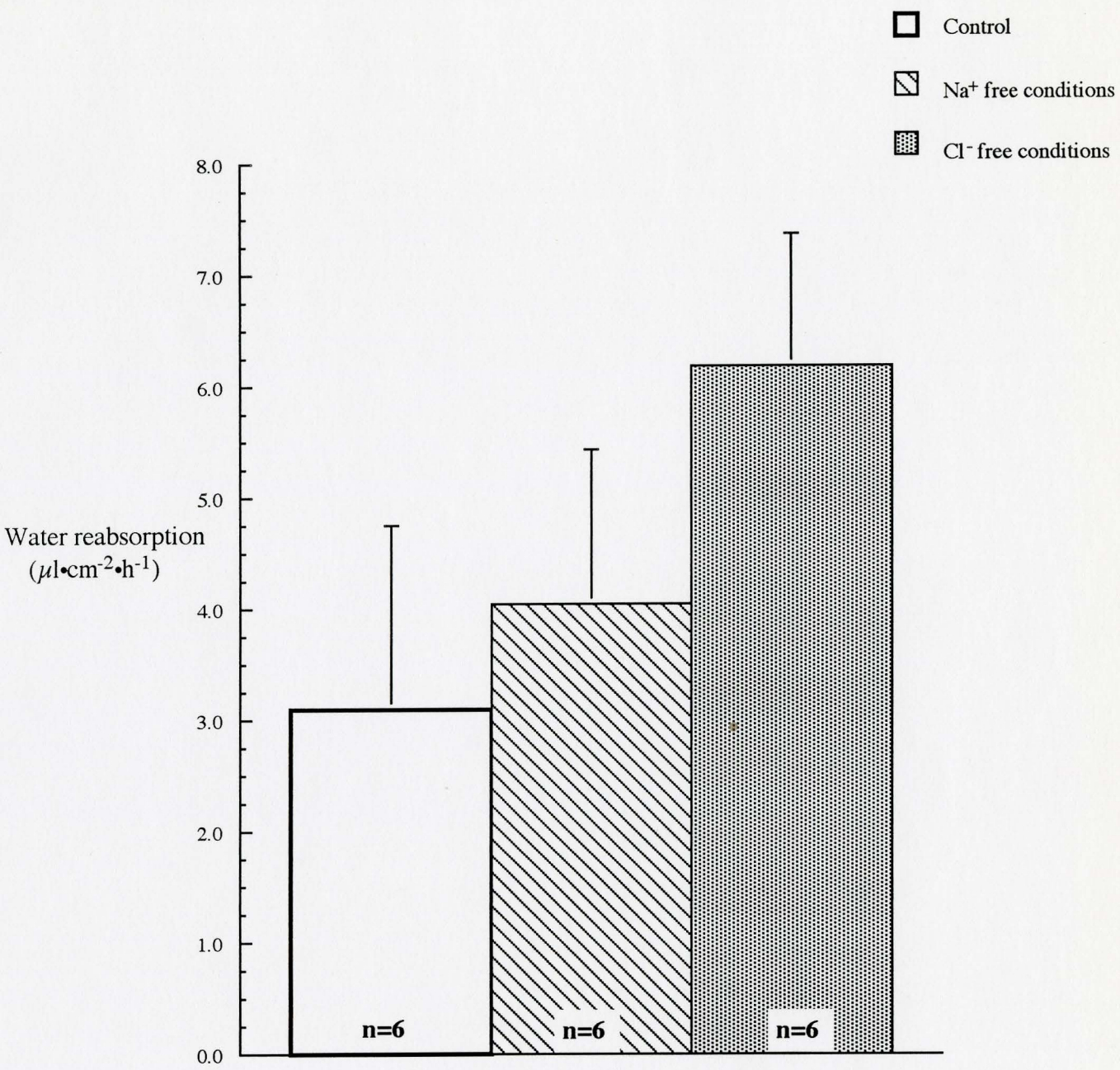

Control
Na+ free conditions
Cl- free conditions
Water reabsorption
(µl•cm-2•h-1)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
n=6
n=6
n=6

Figure 3-4. Influx rates of Na^+ in the perfused urinary bladder sac preparation of *Oncorhynchus mykiss* under control and mucosal Cl^--free conditions, and Cl^- influx rates under control and mucosal Na^+-free conditions. Open bars represent control conditions and hatched bars represent experimental conditions. Means ± SEM. No significant decrease in Na^+ influx rate or Cl^- influx rate when the co-ion was removed (unpaired t-test, two-tailed, $P>0.05$), indicating independent transport of each ion. Cross indicates significant difference between influx rates of Na^+ and Cl^- in the control sacs (paired t-test, two-tailed, $P<0.01$), further supporting the independent transport of Na^+ and Cl^-.

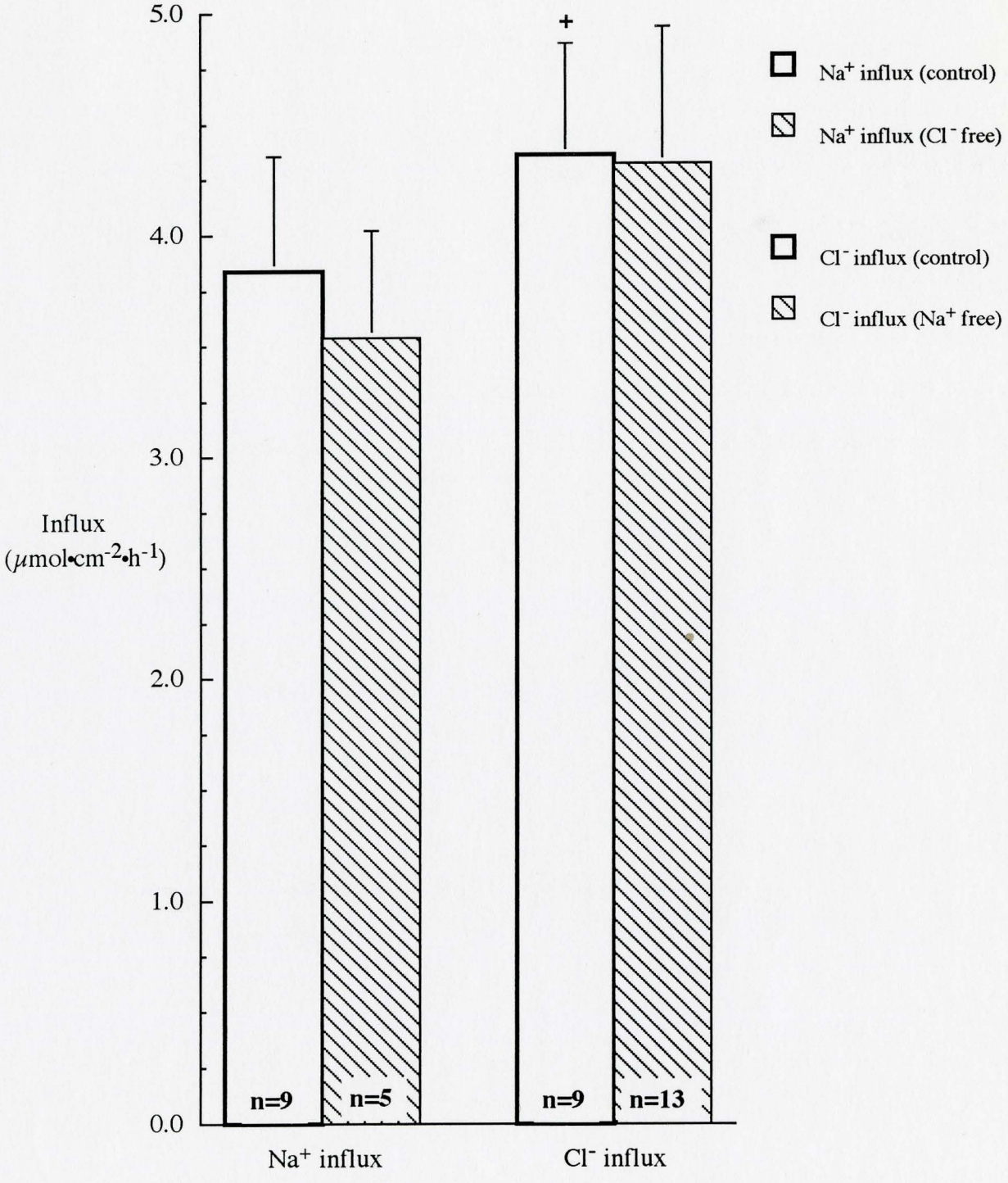

5.0
4.0
3.0
2.0
1.0
0.0
Influx
(μmol•cm-2•h-1)
+
n=9
n=5
n=9
n=13
Na+ influx
Cl- influx
Na+ influx (control)
Na+ influx (Cl- free)
Cl- influx (control)
Cl- influx (Na+ free)

Figure 3-5. Unidirectional influx rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-4} M amiloride, mucosal 10^{-4} M bumetanide and mucosal 10^{-4} M DIDS conditions. All mucosal salines (including controls) had 0.1% DMSO to dissolve the drugs. Open bars represent control conditions, light shaded bars represent 10^{-4} M amiloride conditions, medium shaded bars represent 10^{-4} M bumetanide and dark shaded bars represent 10^{-4} M DIDS conditions. Means ± SEM. None of the Na^+ or Cl^- influx rates were significantly affected by the drug treatments (unpaired t-test, two-tailed, $P>0.05$). Cross indicates a Cl^- influx rate different from Na^+ within the same treatment (paired t-test, two-tailed, $P<0.01$), supporting independent transport seen in ion substitution experiments.

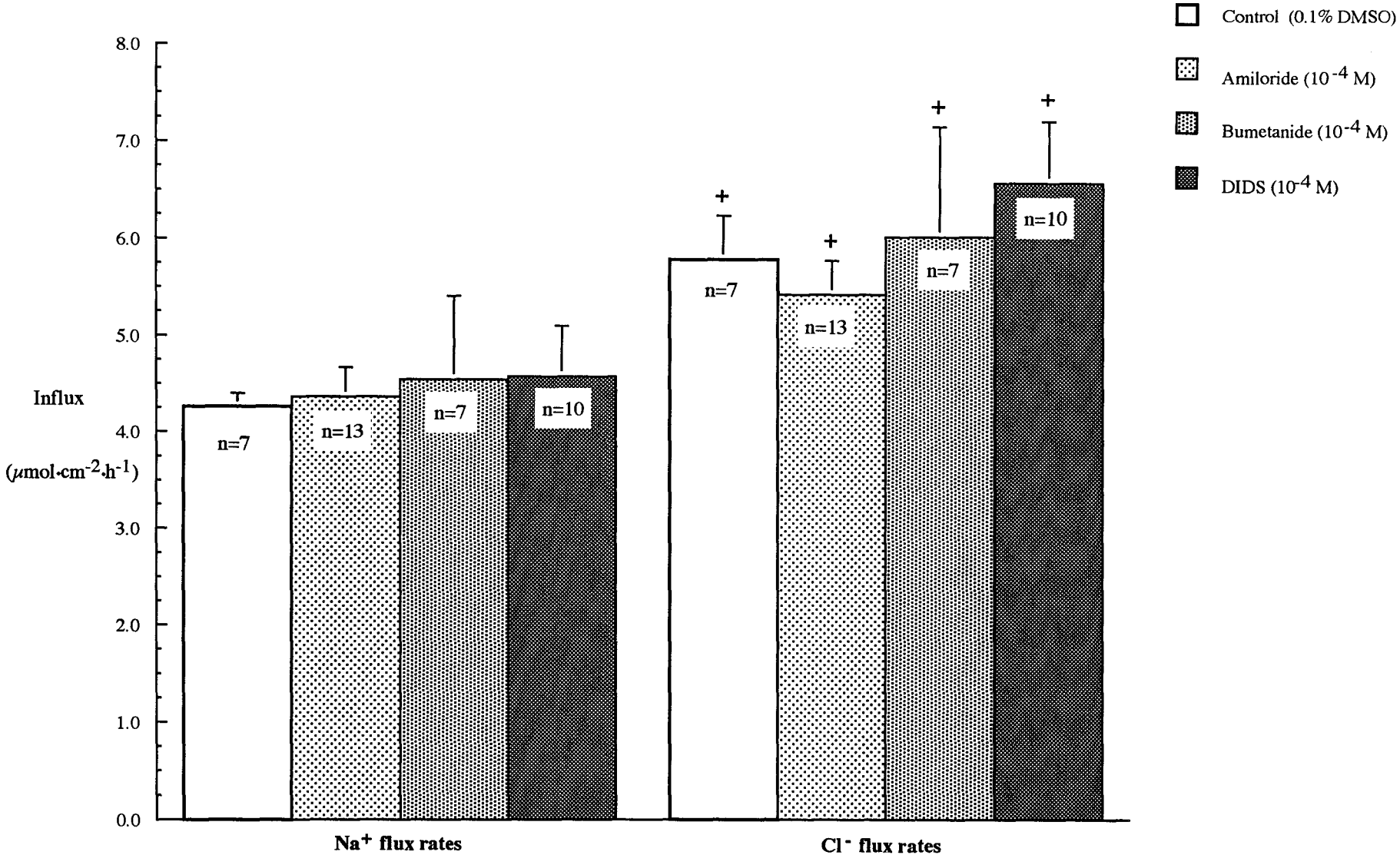

Control (0.1% DMSO)
Amiloride (10^{-4} M)
Bumetanide (10^{-4} M)
DIDS (10^{-4} M)
Influx
(μmol·cm^{-2}·h^{-1})
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
n=7
n=13
n=7
n=10
n=7
n=13
n=7
n=10
+
+
+
+
Na$^+$ flux rates
Cl$^-$ flux rates

Figure 3-6. Net flux rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-4} M amiloride, mucosal 10^{-4} M bumetanide and mucosal 10^{-4} M DIDS conditions. All mucosal salines (including controls) had 0.1% DMSO to dissolve the drugs. Open bars represent control conditions, light shaded bars represent 10^{-4} M amiloride conditions, medium shaded bars represent 10^{-4} M bumetanide and dark shaded bars represent 10^{-4} M DIDS conditions. Means ± SEM. Asterisks indicate a net, Na^+ or Cl^-, flux rate significantly different from controls (unpaired t-test, two-tailed, $P<0.05$).

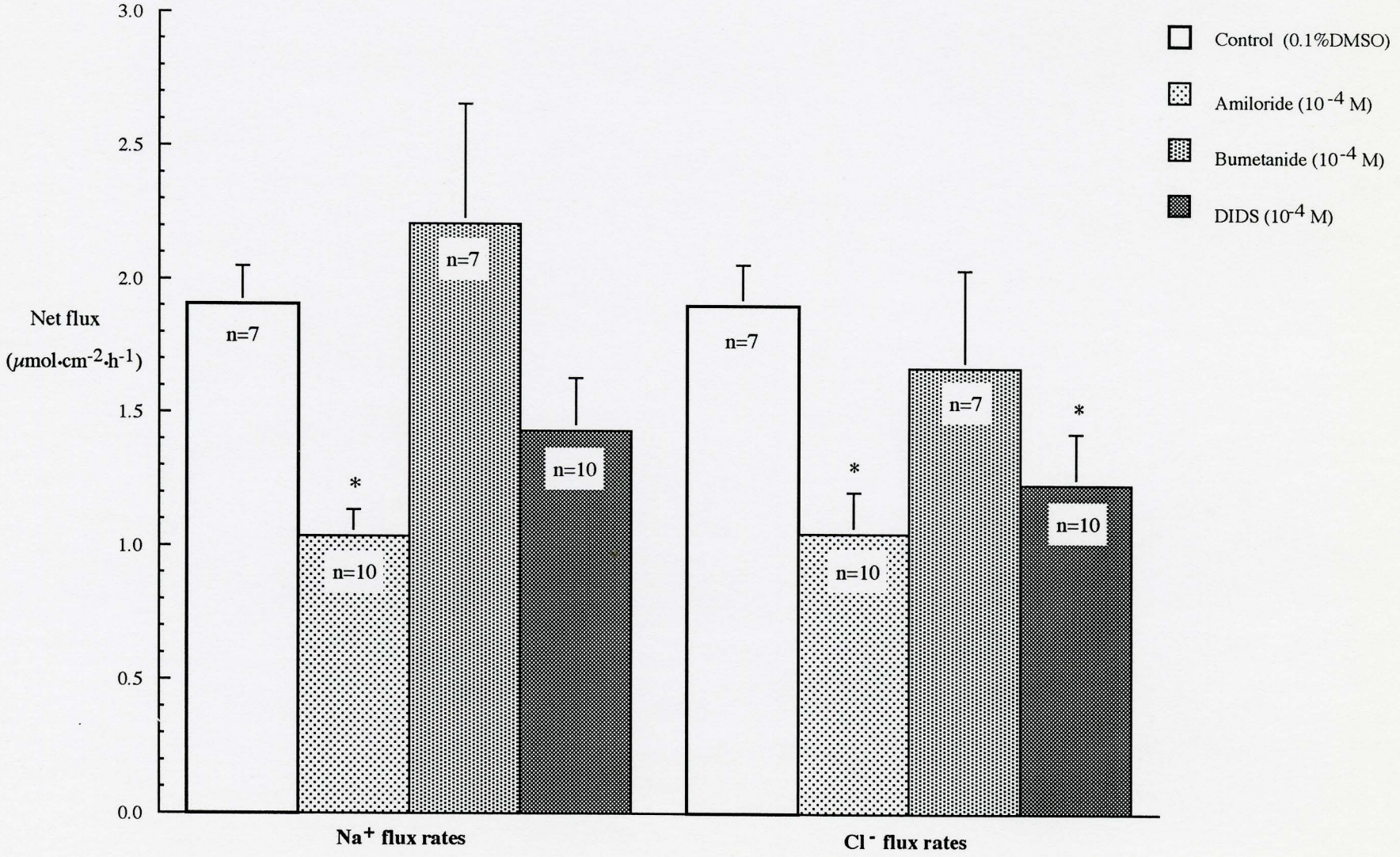

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Net flux
(μmol·cm-2·h-1)
n=7
*
n=10
n=7
n=10
n=7
*
n=10
n=7
*
n=10
Na+ flux rates
Cl- flux rates
Control (0.1%DMSO)
Amiloride (10-4 M)
Bumetanide (10-4 M)
DIDS (10-4 M)

Figure 3-7. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-4} M amiloride, mucosal 10^{-4} M bumetanide and mucosal 10^{-4} M DIDS conditions. All mucosal salines (including controls) had 0.1% DMSO to dissolve the drugs. Open bars represent control conditions, light shaded bars represent 10^{-4} M amiloride conditions, medium shaded bars represent 10^{-4} M bumetanide and dark shaded bars represent 10^{-4} M DIDS conditions. Means ± SEM. Asterisk indicates a water reabsorption rate significantly different from control sacs (unpaired t-test, two-tailed, $P<0.05$).

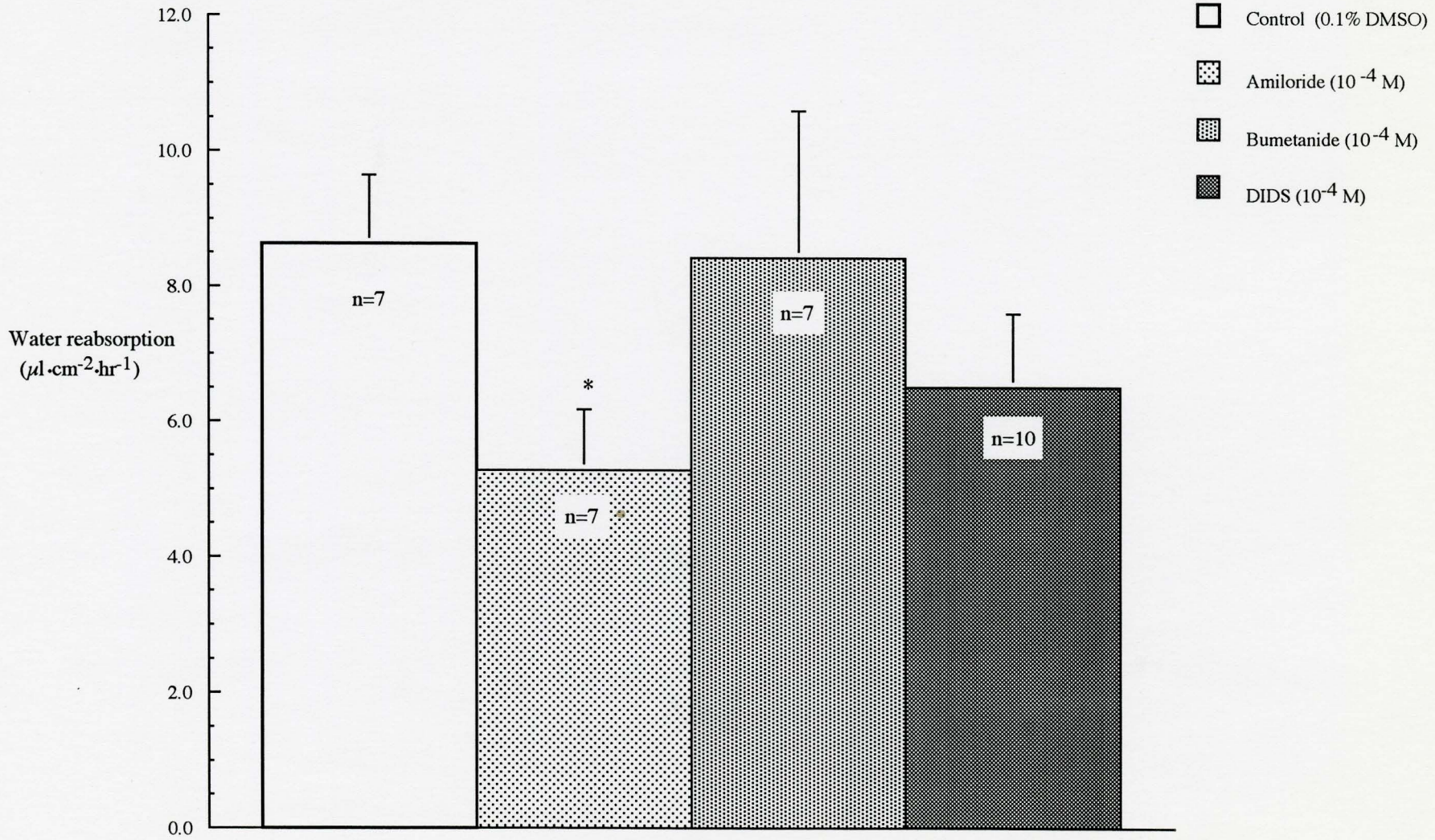

12.0
10.0
8.0
6.0
4.0
2.0
0.0
Water reabsorption
(μl·cm-2·hr-1)
n=7
*
n=7
n=7
n=10
Control (0.1% DMSO)
Amiloride (10 -4 M)
Bumetanide (10-4 M)
DIDS (10-4 M)

Figure 3-8. Unidirectional influx rates of Na^+ in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M amiloride and mucosal 10^{-3} M DIDS conditions. Treated sacs are compared to controls with the corresponding percentage of DMSO. Open and shaded bars on left represent control sacs, the light tiled bar represents 10^{-3} M amiloride treated sacs and the dark tiled bar represents 10^{-3} M DIDS treated sacs. Means ± SEM. Asterisk indicates a Na^+ influx different from control (unpaired t-test, two-tailed, $P<0.05$).

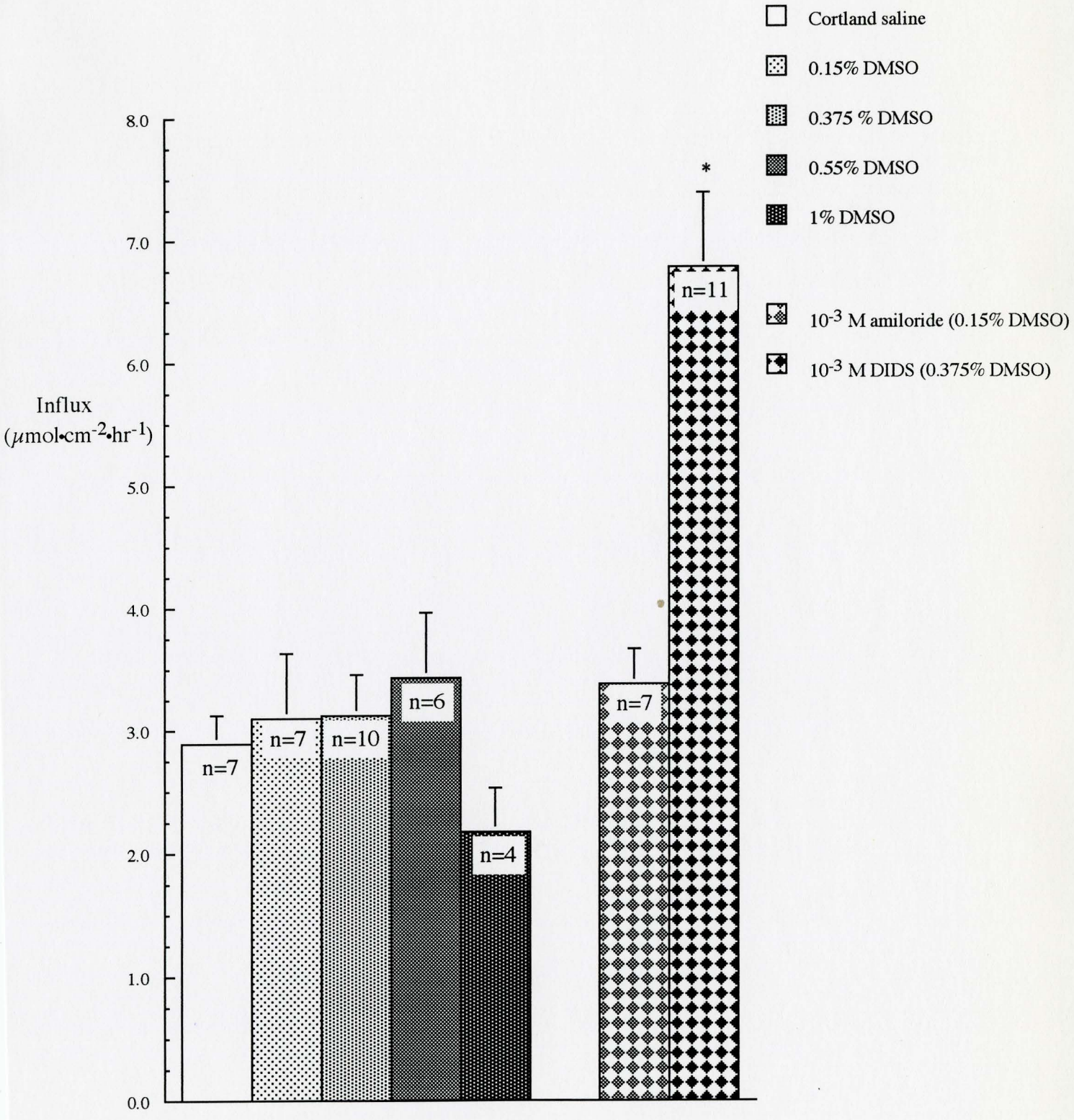

Cortland saline
0.15% DMSO
0.375 % DMSO
0.55% DMSO
1% DMSO
10-3 M amiloride (0.15% DMSO)
10-3 M DIDS (0.375% DMSO)
Influx
(μmol•cm-2•hr-1)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
n=7
n=7
n=10
n=6
n=4
n=7
n=11
*

Figure 3-9. Unidirectional influx rates of Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M amiloride and mucosal 10^{-3} M DIDS conditions. Treated sacs are compared to controls with the corresponding percentage of DMSO. Open and shaded bars on left represent control sacs, the light tiled bar represents 10^{-3} M amiloride treated sacs and the dark tiled bar represents 10^{-3} M DIDS treated sacs. Means ± SEM. Asterisk indicates a Cl^- influx different from control (unpaired t-test, two-tailed, $P<0.05$).

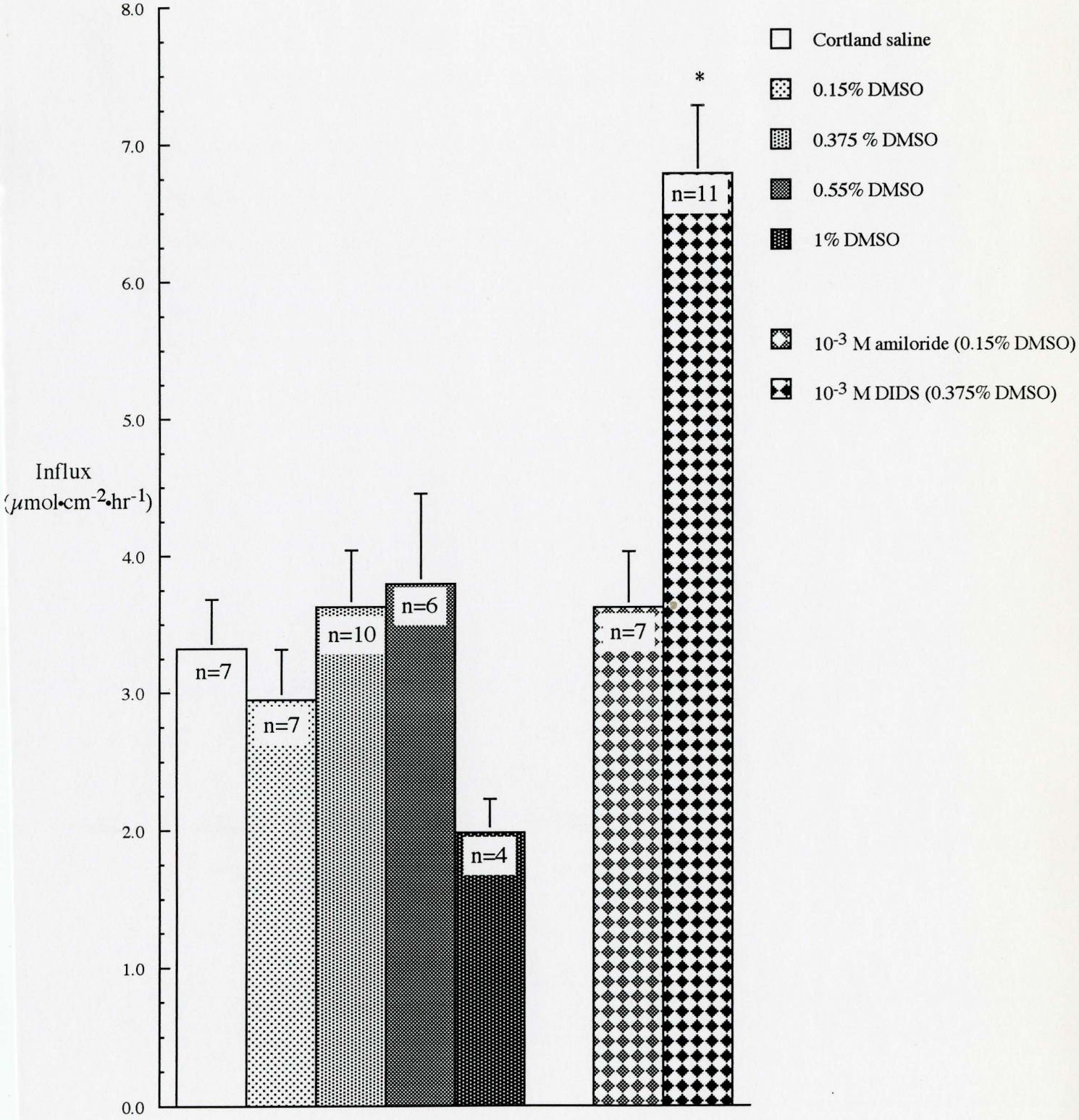

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Influx
(μmol•cm-2•hr-1)
n=7
n=7
n=10
n=6
n=4
n=7
n=11
*
Cortland saline
0.15% DMSO
0.375 % DMSO
0.55% DMSO
1% DMSO
10-3 M amiloride (0.15% DMSO)
10-3 M DIDS (0.375% DMSO)

Figure 3-10. Net flux rates of Na^+ in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M amiloride and mucosal 10^{-3} M DIDS conditions. Treated sacs are compared to controls with the corresponding percentage of DMSO. Open and shaded bars on left represent control sacs, the light tiled bar represents 10^{-3} M amiloride treated sacs and the dark tiled bar represents 10^{-3} M DIDS treated sacs. Means ± SEM. Neither 10^{-3} M amiloride or 10^{-3} M DIDS treated sacs showed a net Na^+ flux different from control (unpaired t-test, two-tailed, $P>0.05$).

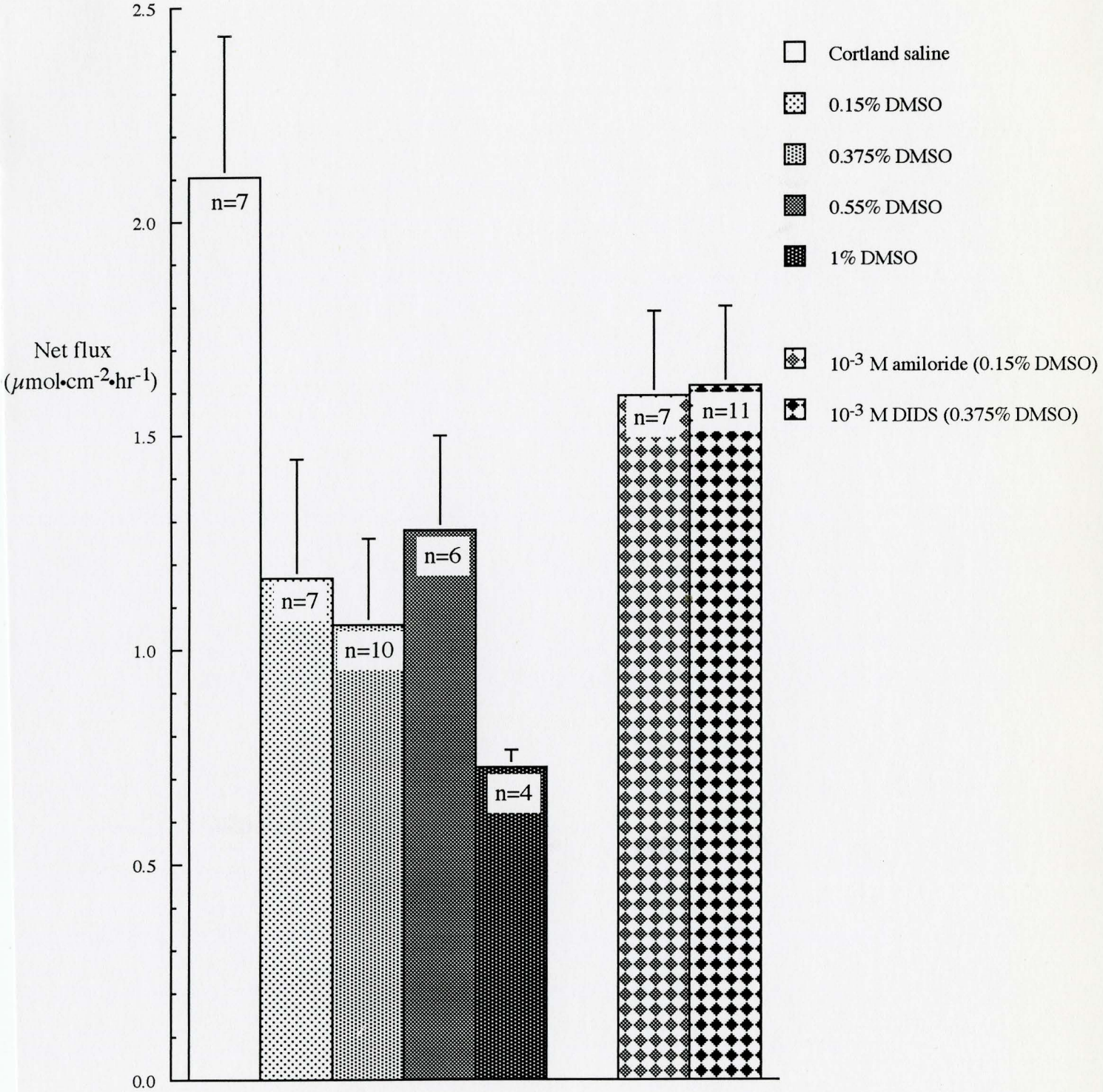

2.5
2.0
1.5
1.0
0.5
0.0
Net flux
(μmol•cm-2•hr-1)
n=7
n=7
n=10
n=6
n=4
n=7
n=11
Cortland saline
0.15% DMSO
0.375% DMSO
0.55% DMSO
1% DMSO
10-3 M amiloride (0.15% DMSO)
10-3 M DIDS (0.375% DMSO)

Figure 3-11. Net flux rates of Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M amiloride and mucosal 10^{-3} M DIDS conditions. Treated sacs are compared to controls with the corresponding percentage of DMSO. Open and shaded bars on left represent control sacs, the light tiled bar represents 10^{-3} M amiloride treated sacs and the dark tiled bar represents 10^{-3} M DIDS treated sacs. Means ± SEM. Neither 10^{-3} M amiloride or 10^{-3} M DIDS treated sacs showed a net Cl^- flux different from control (unpaired t-test, two-tailed, $P>0.05$).

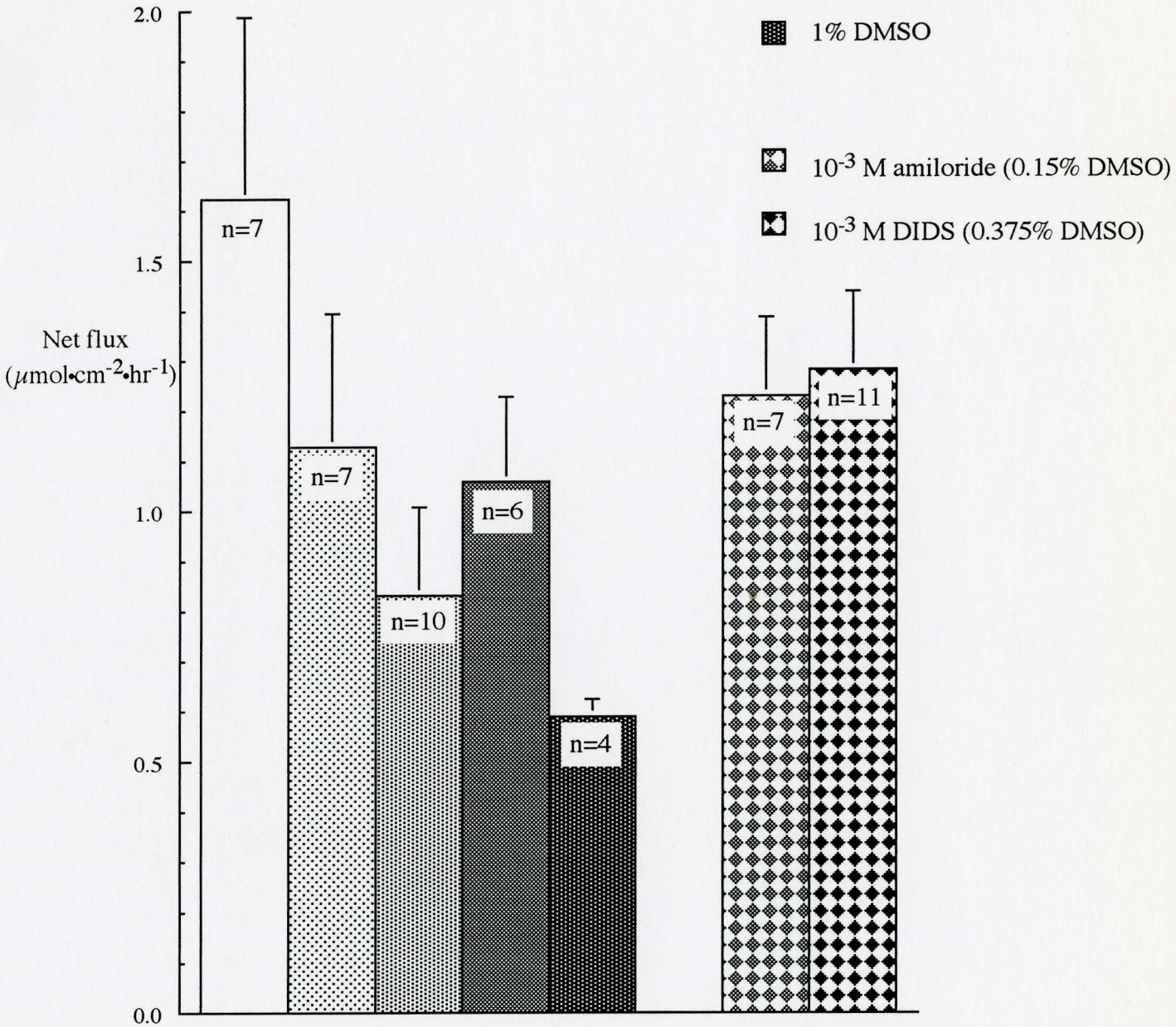

Cortland saline
0.15% DMSO
0.375% DMSO
0.55% DMSO
1% DMSO
10-3 M amiloride (0.15% DMSO)
10-3 M DIDS (0.375% DMSO)
Net flux
(μmol•cm-2•hr-1)
2.0
1.5
1.0
0.5
0.0
n=7
n=7
n=10
n=6
n=4
n=7
n=11

Figure 3-12. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M amiloride and mucosal 10^{-3} M DIDS conditions. Treated sacs are compared to controls with the corresponding percentage of DMSO. Open and shaded bars on left represent control sacs, the light tiled bar represents 10^{-3} M amiloride treated sacs and the dark tiled bar represents 10^{-3} M DIDS treated sacs. Means ± SEM. Asterisk indicates a water reabsorption rate different than control (unpaired t-test, two-tailed, $P<0.05$).

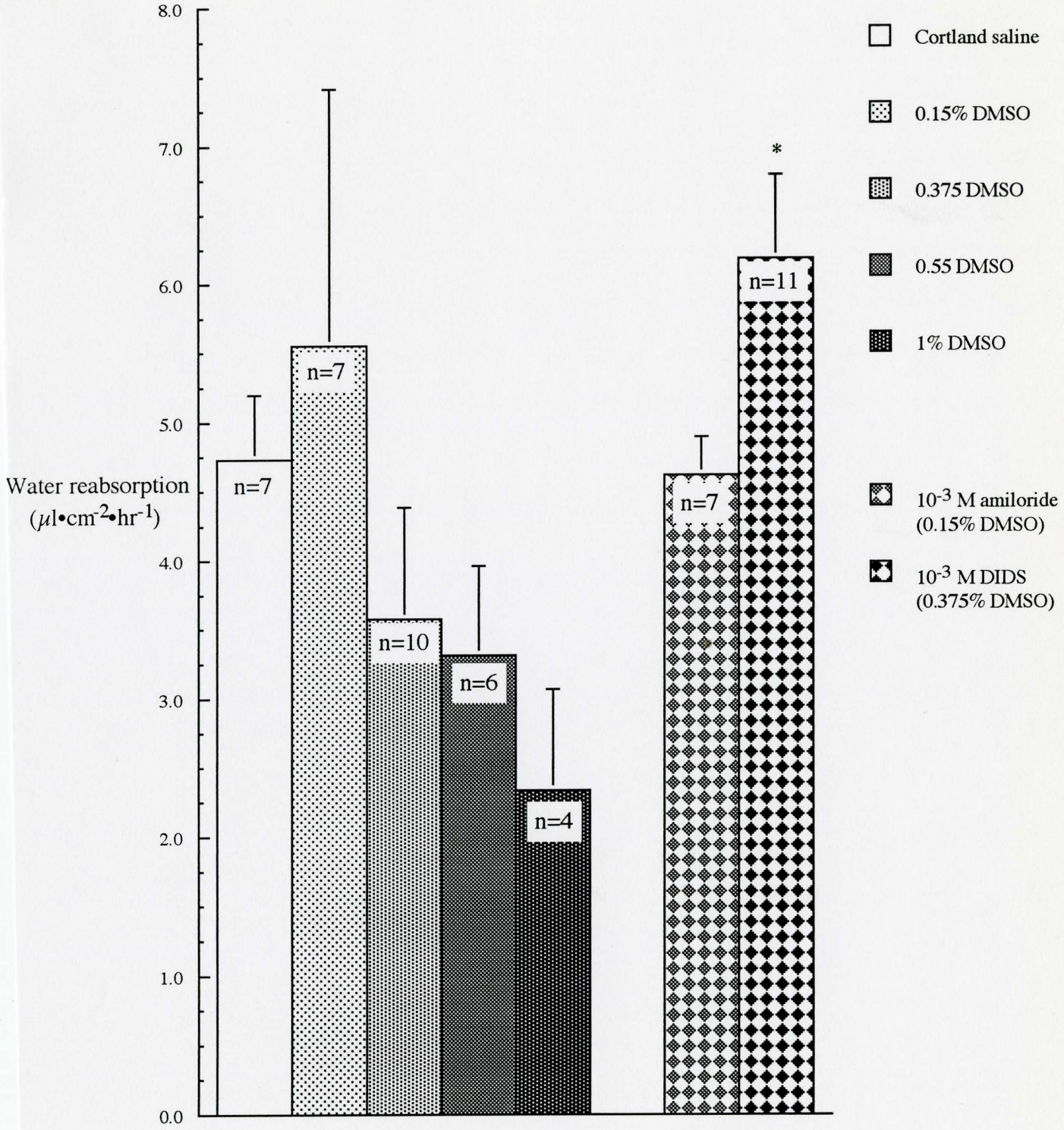

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Water reabsorption
(μl•cm-2•hr-1)
n=7
n=7
n=10
n=6
n=4
n=7
n=11
*
Cortland saline
0.15% DMSO
0.375 DMSO
0.55 DMSO
1% DMSO
10-3 M amiloride
(0.15% DMSO)
10-3 M DIDS
(0.375% DMSO)

Figure 3-13. Influx rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M chlorothiazide and simultaneous mucosal and serosal 10^{-3} M hydrochlorothiazide conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M chlorothiazide treated sacs and dark shaded bars represent mucosal/serosal 10^{-3} M hydrochlorothiazide treated sacs. Means ± SEM. Neither treatment caused a decrease in Na^+ or Cl^- influx rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

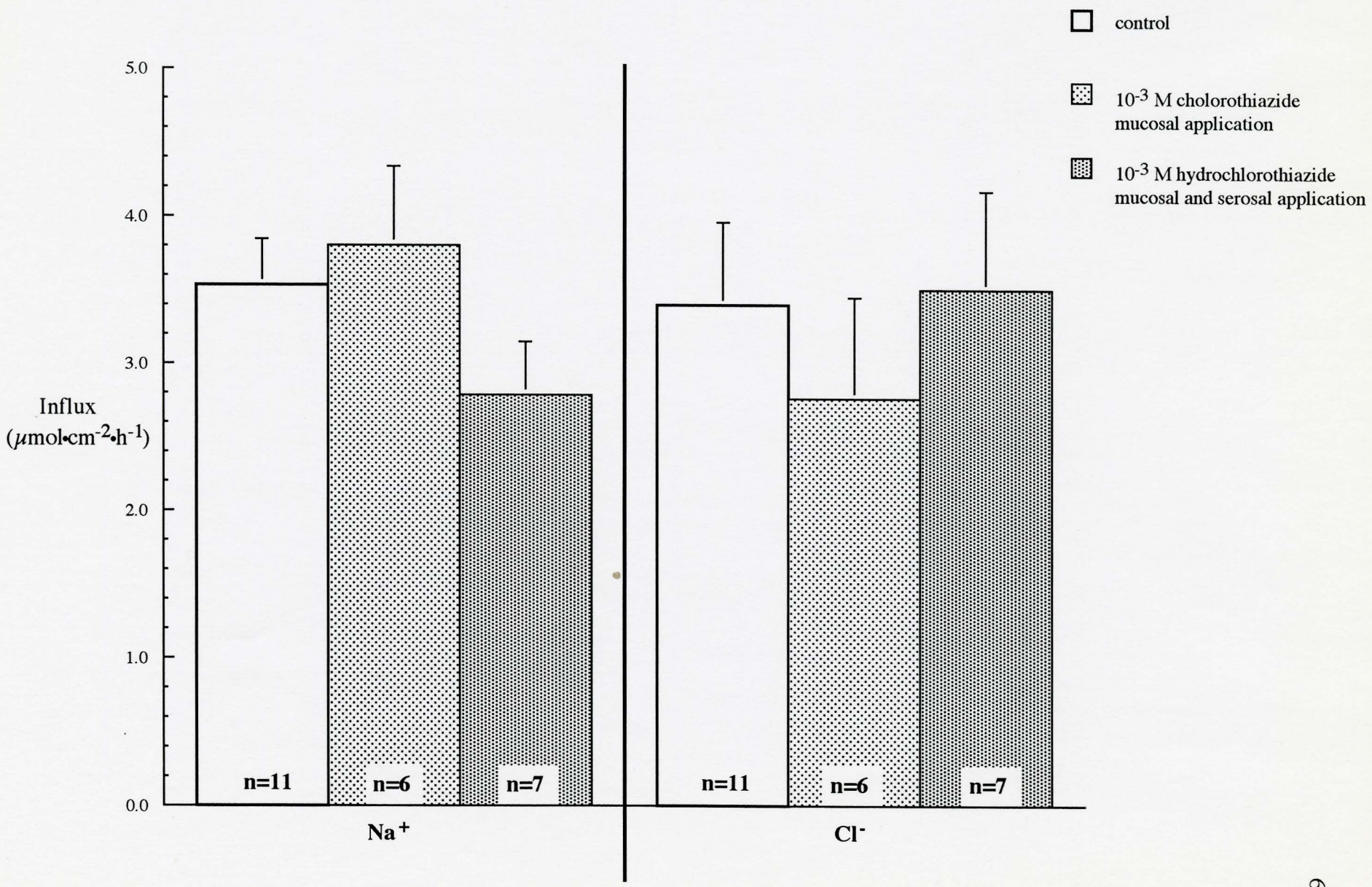

control
10-3 M cholorothiazide mucosal application
10-3 M hydrochlorothiazide mucosal and serosal application
5.0
4.0
3.0
2.0
1.0
0.0
Influx
(μmol•cm-2•h-1)
n=11
n=6
n=7
n=11
n=6
n=7
Na+
Cl-

Figure 3-14. Net flux rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M chlorothiazide and simultaneous mucosal and serosal 10^{-3} M hydrochlorothiazide conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M chlorothiazide treated sacs and dark shaded bars represent mucosal/serosal 10^{-3} M hydrochlorothiazide treated sacs. Means ± SEM. Asterisk indicates a decrease in net flux rates compared to control (unpaired t-test, two-tailed, $P<0.05$).

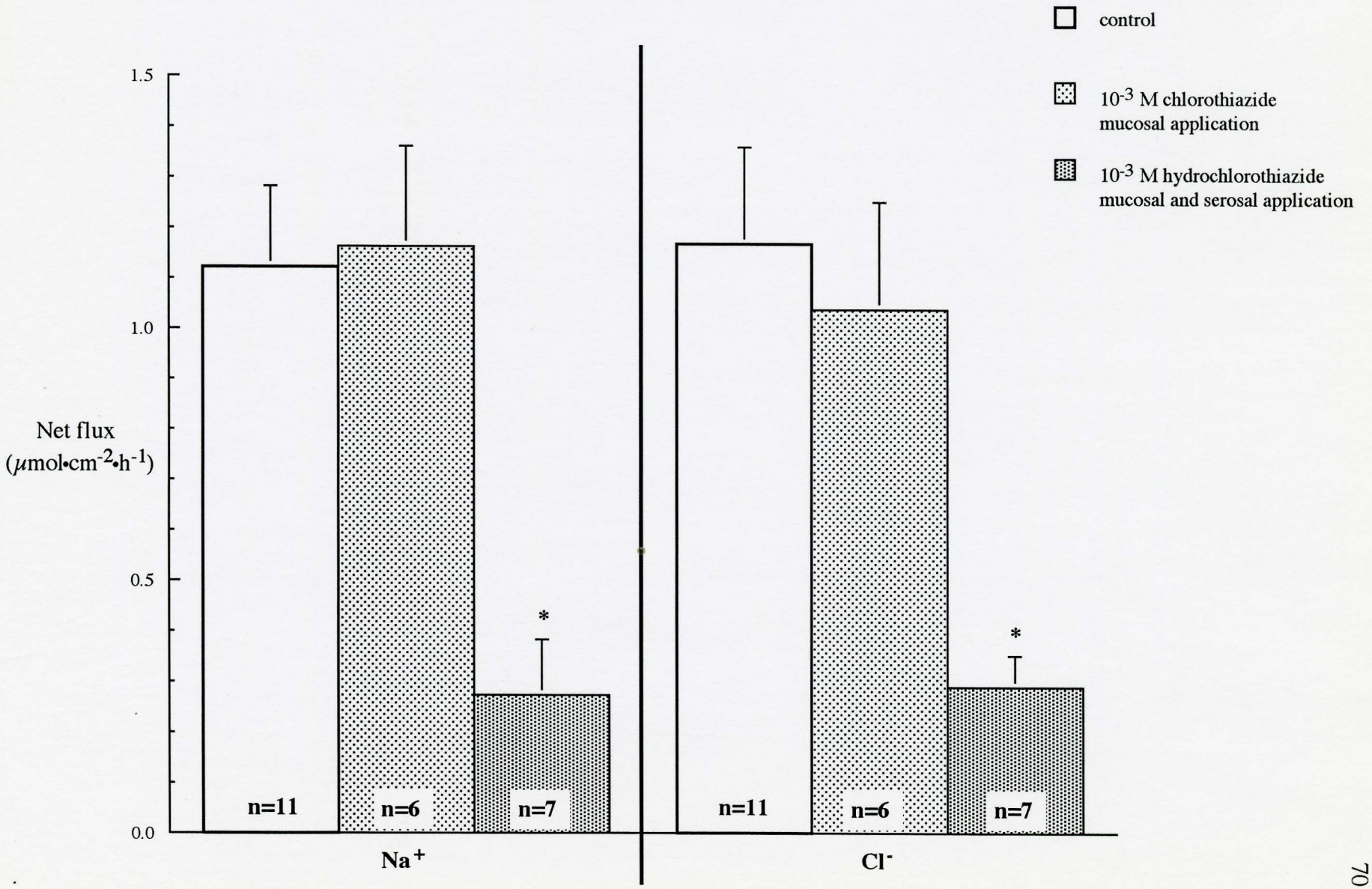

control
10-3 M chlorothiazide
mucosal application
10-3 M hydrochlorothiazide
mucosal and serosal application
Net flux
(μmol•cm-2•h-1)
1.5
1.0
0.5
0.0
n=11
n=6
n=7
*
n=11
n=6
n=7
*
Na+
Cl-

Figure 3-15. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M chlorothiazide and simultaneous mucosal and serosal 10^{-3} M hydrochlorothiazide conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M chlorothiazide treated sacs and dark shaded bars represent mucosal/serosal 10^{-3} M hydrochlorothiazide treated sacs. Means ± SEM. Asterisk indicates a significant difference in water reabsorption rates compared to control (unpaired t-test, two-tailed, $P<0.05$).

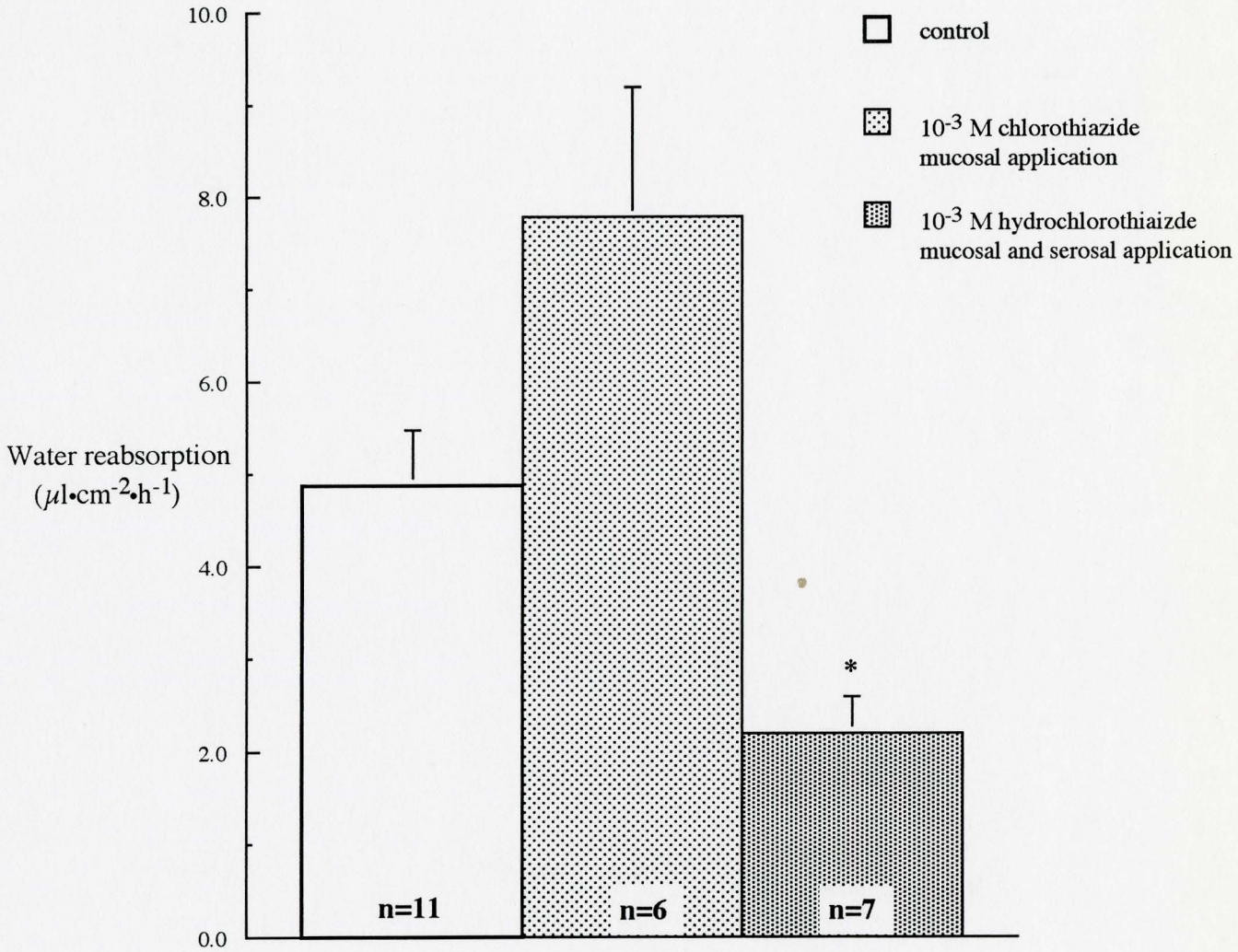

10.0
8.0
6.0
4.0
2.0
0.0
Water reabsorption
(μl•cm^{-2}•h^{-1})
control
10^{-3} M chlorothiazide
mucosal application
10^{-3} M hydrochlorothiaizde
mucosal and serosal application
*
n=11
n=6
n=7

Figure 3-16. Influx rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-2} M acetazolamide and serosal 10^{-2} M acetazolamide conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-2} M acetazolamide treated sacs and dark shaded bars represent serosal 10^{-2} M acetazolamide treated sacs. Means ± SEM. Asterisk indicates a decrease in influx rates compared to control (unpaired t-test, two-tailed, $P<0.05$).

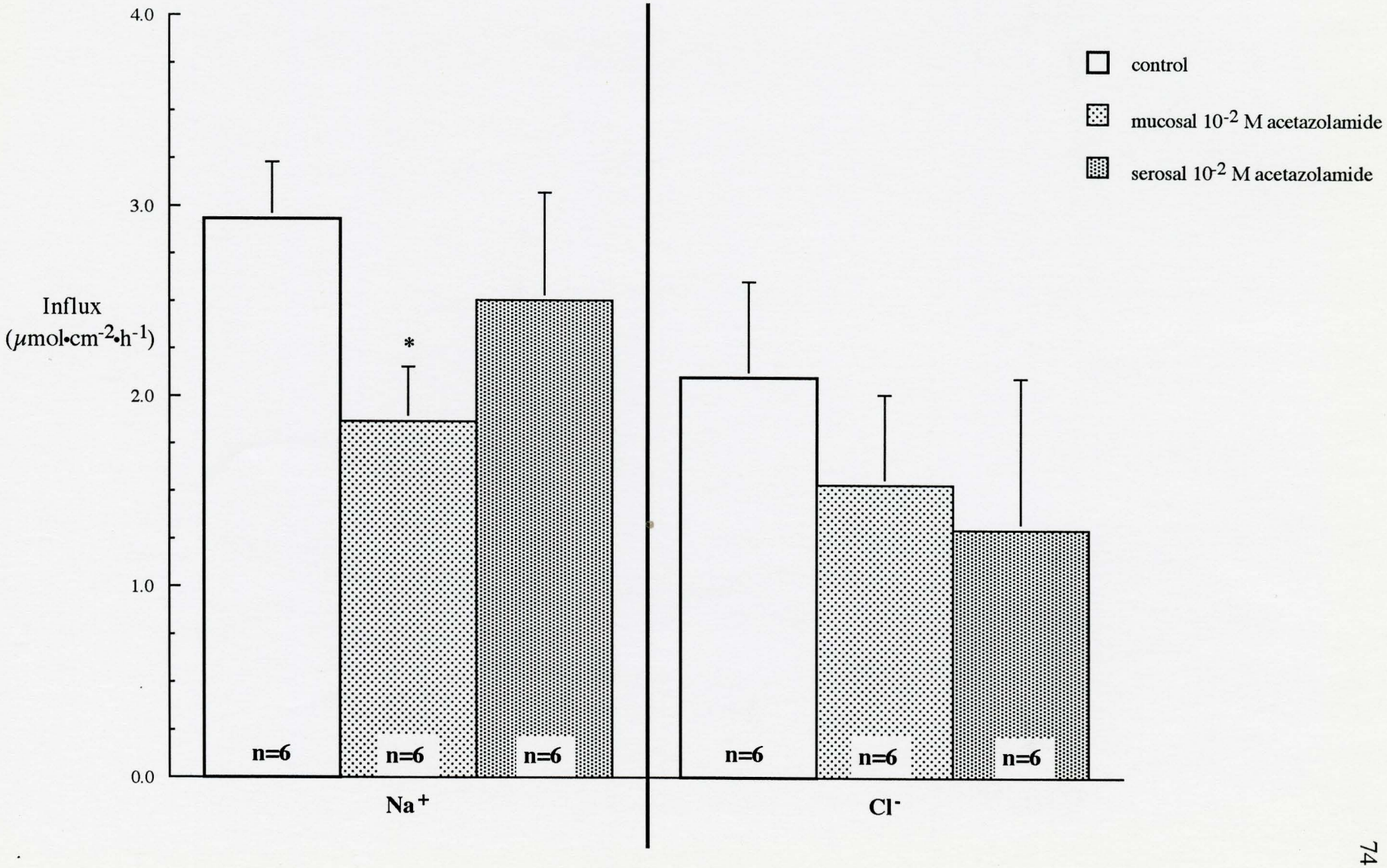

4.0
3.0
2.0
1.0
0.0
Influx
(μmol•cm-2•h-1)
*
n=6
n=6
n=6
n=6
n=6
n=6
Na+
Cl-
control
mucosal 10-2 M acetazolamide
serosal 10-2 M acetazolamide

Figure 3-17. Net flux rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-2} M acetazolamide and serosal 10^{-2} M acetazolamide conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-2} M acetazolamide treated sacs and dark shaded bars represent serosal 10^{-2} M acetazolamide treated sacs. Means ± SEM. There were no significant changes in net flux rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

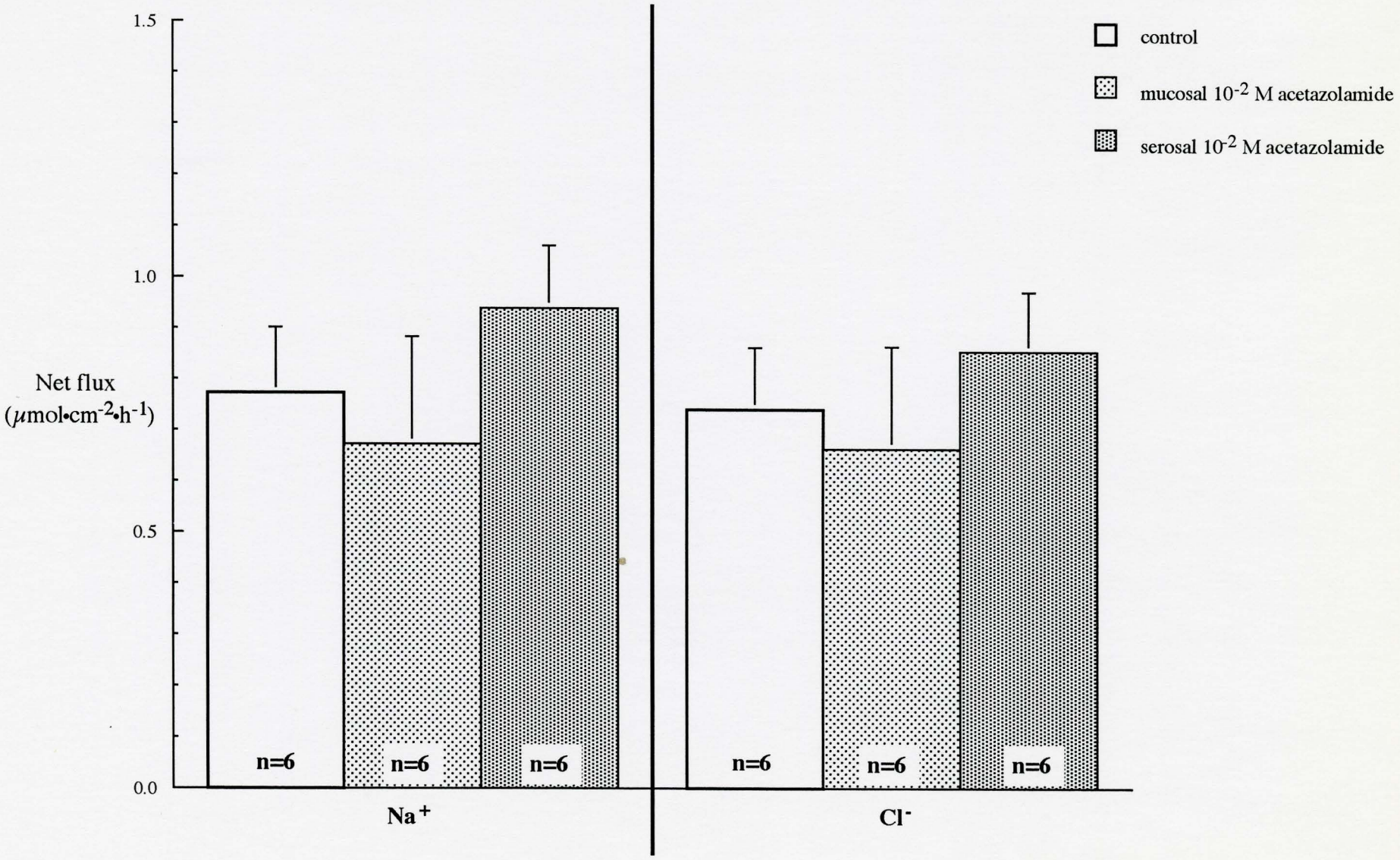

1.5
1.0
0.5
0.0
Net flux
(μmol•cm-2•h-1)
control
mucosal 10-2 M acetazolamide
serosal 10-2 M acetazolamide
n=6
n=6
n=6
n=6
n=6
n=6
Na+
Cl-

Figure 3-18. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-2} M acetazolamide and serosal 10^{-2} M acetazolamide conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-2} M acetazolamide treated sacs and dark shaded bars represent serosal 10^{-2} M acetazolamide treated sacs. Means ± SEM. There were no significant changes in water reabsorption rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

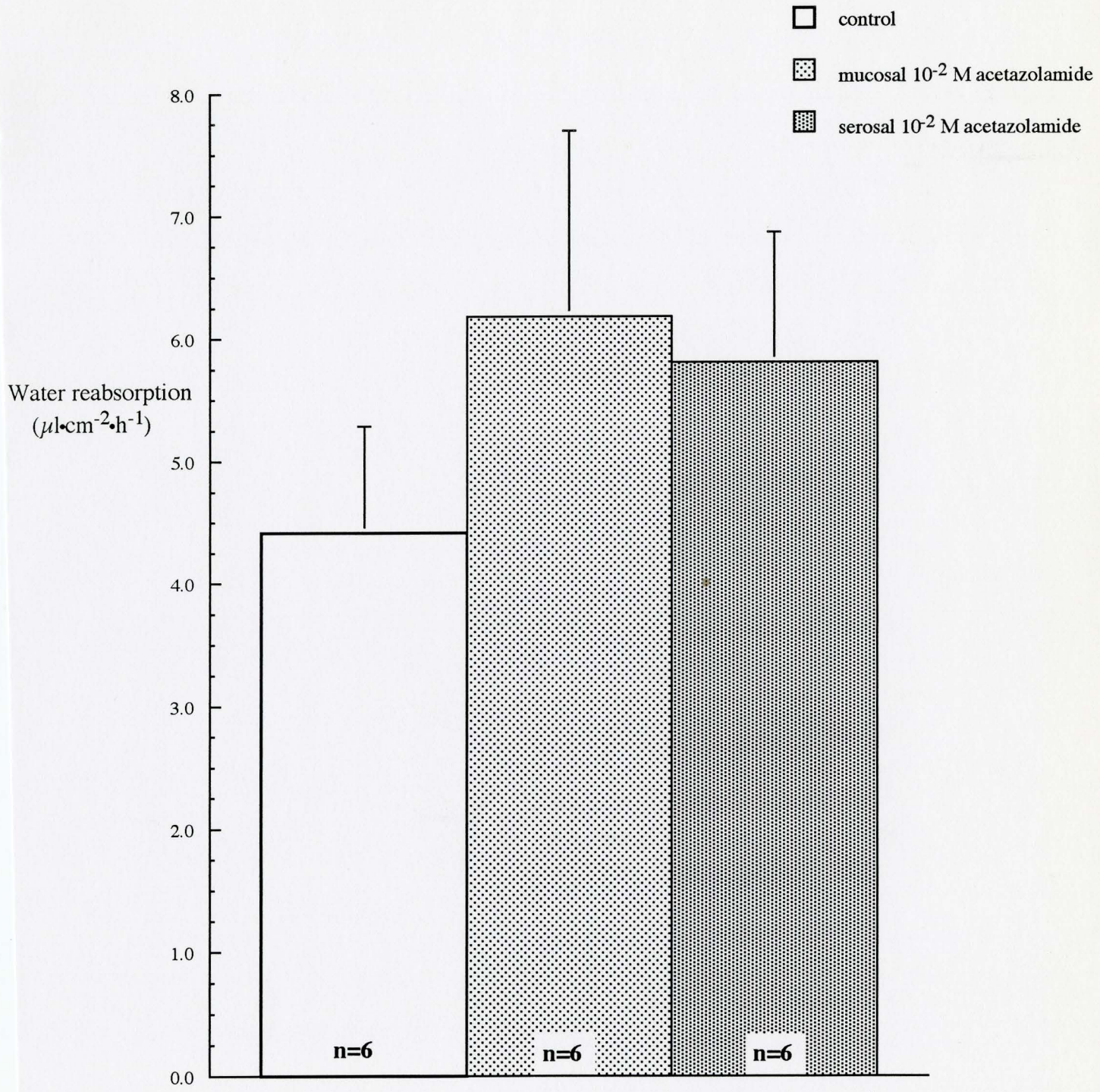

control
mucosal 10^{-2} M acetazolamide
serosal 10^{-2} M acetazolamide
Water reabsorption
($\mu l \cdot cm^{-2} \cdot h^{-1}$)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
n=6
n=6
n=6

Figure 3-19. Influx rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M ouabain and serosal 10^{-3} M ouabain conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M ouabain treated sacs and dark shaded bars represent serosal 10^{-3} M ouabain treated sacs. Means ± SEM. Asterisks indicate a significant decrease in influx rates compared to control (unpaired t-test, two-tailed, $P<0.05$).

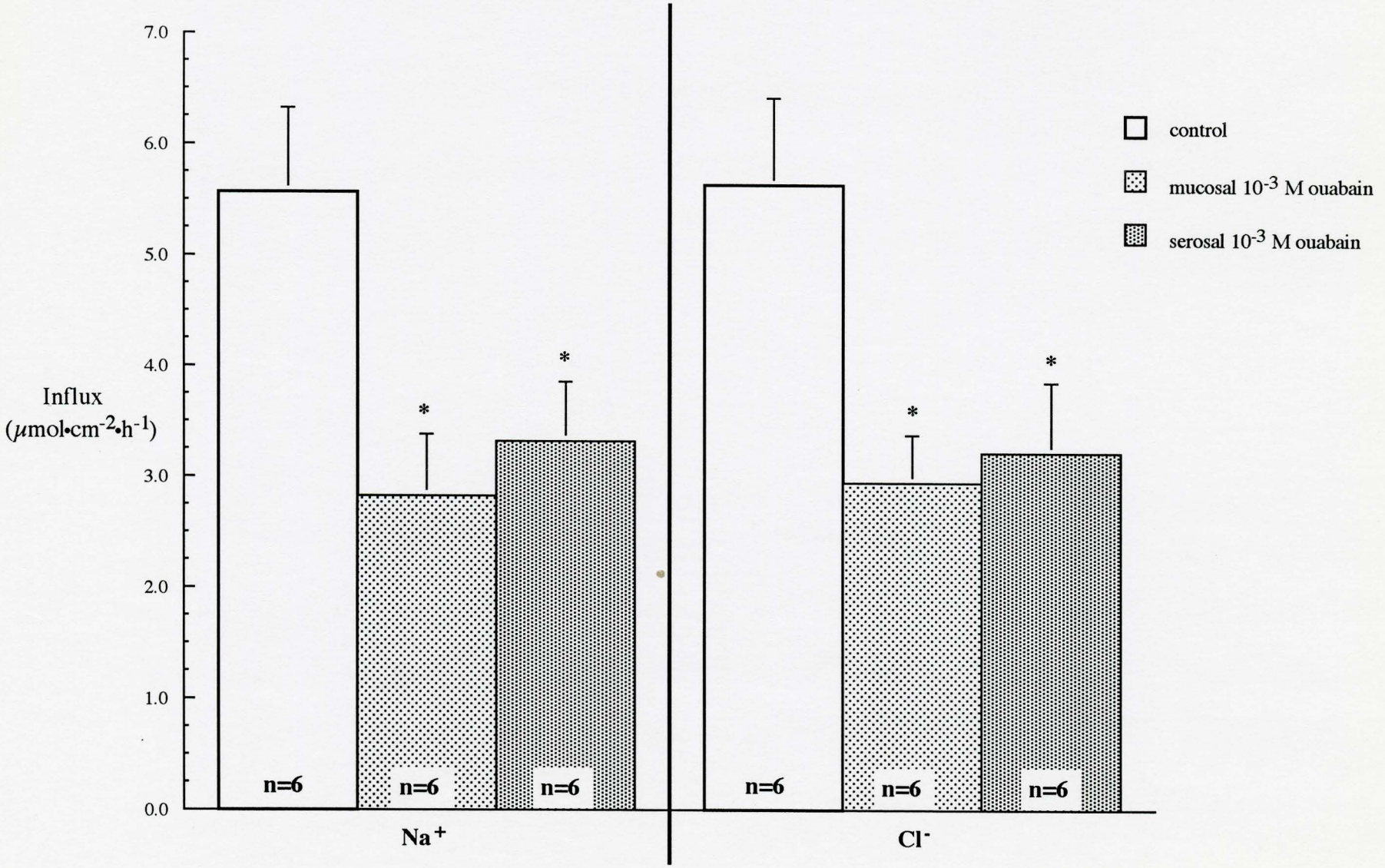

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Influx
(μmol•cm-2•h-1)
control
mucosal 10-3 M ouabain
serosal 10-3 M ouabain
*
*
*
*
n=6
n=6
n=6
n=6
n=6
n=6
Na+
Cl-

Figure 3-20. Net flux rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M ouabain and serosal 10^{-3} M ouabain conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M ouabain treated sacs and dark shaded bars represent serosal 10^{-3} M ouabain treated sacs. Means ± SEM. Neither of the treatments showed a significant decrease in influx rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

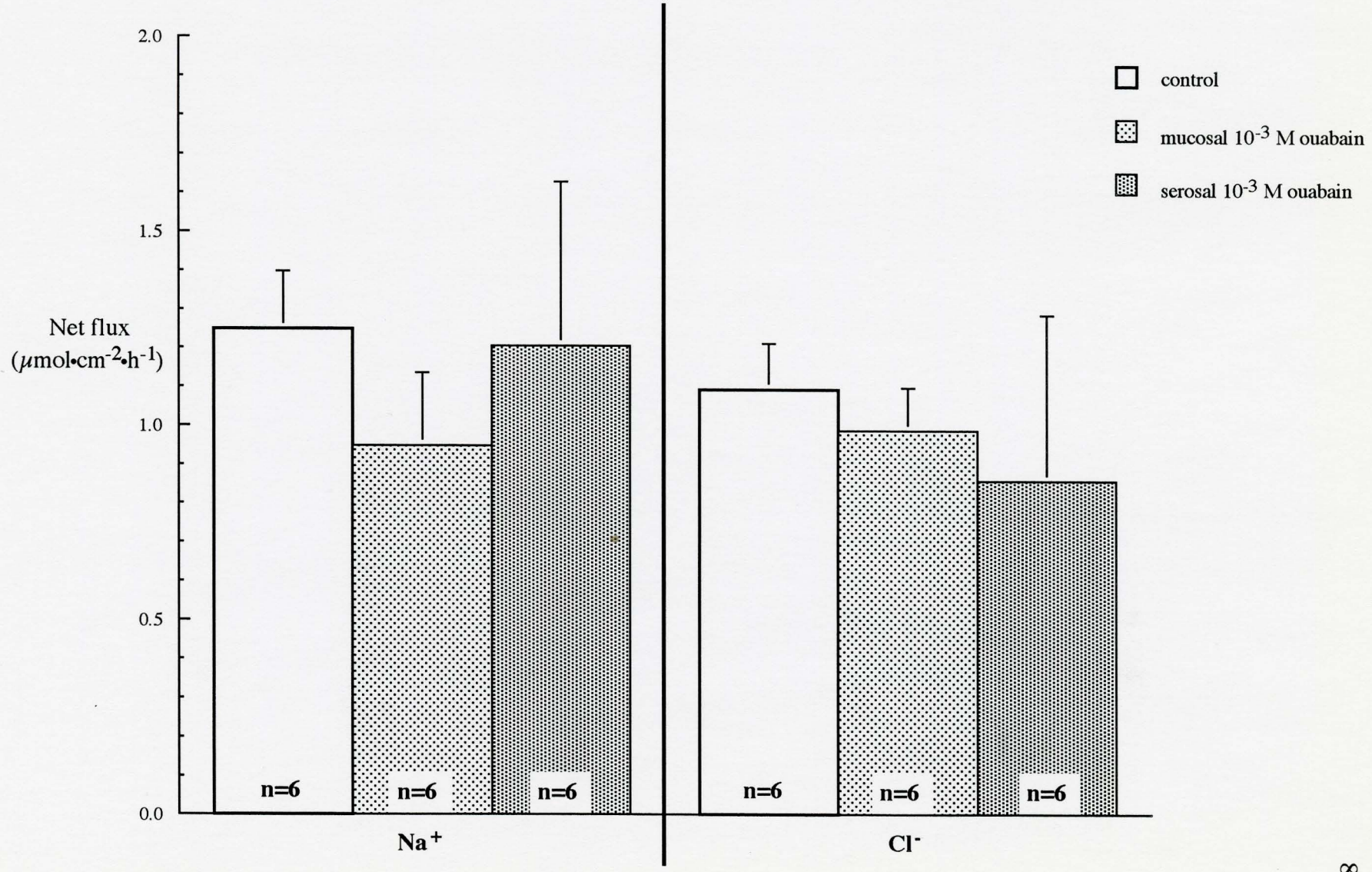
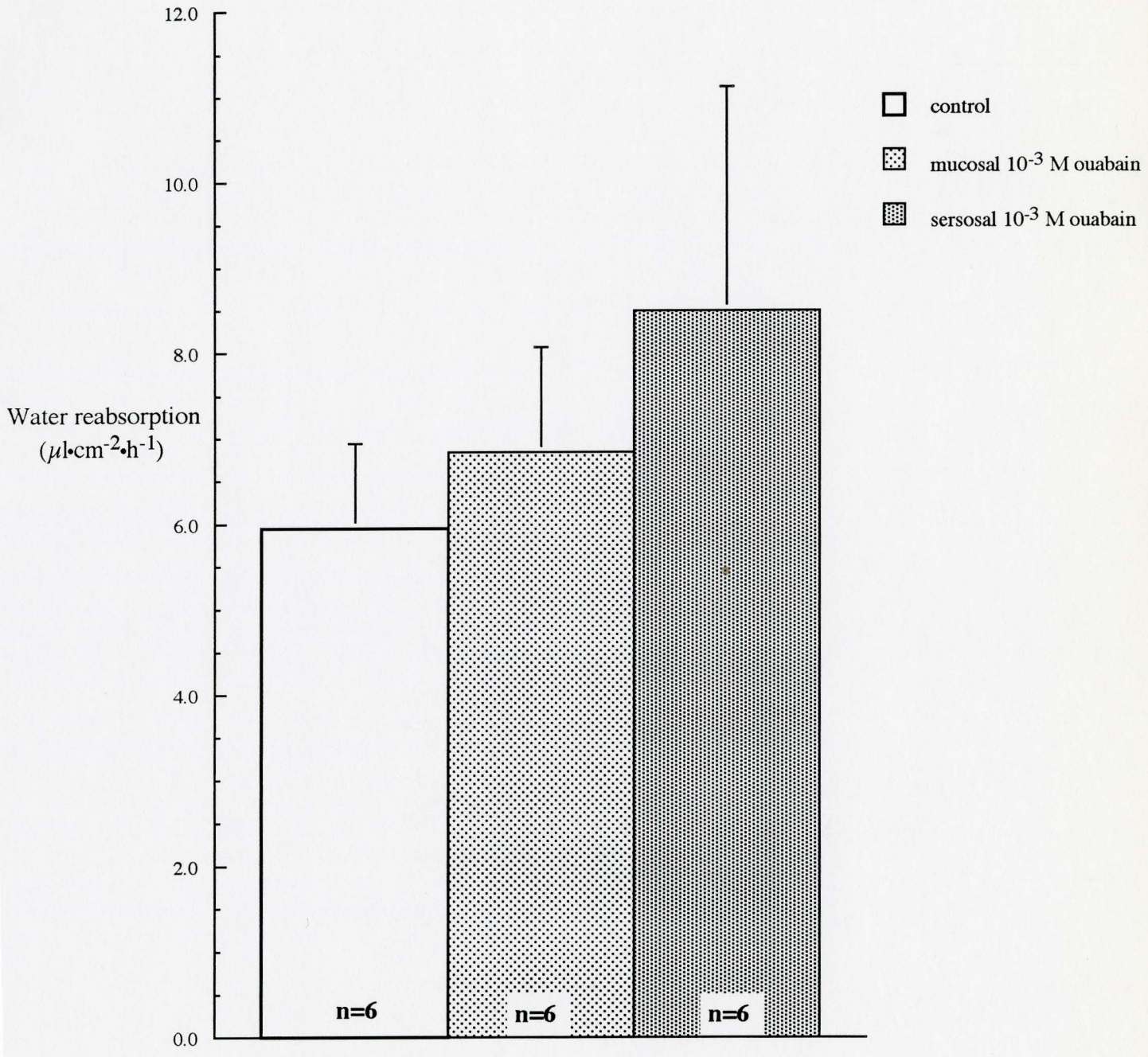

2.0
1.5
1.0
0.5
0.0
Net flux
(μmol•cm-2•h-1)
control
mucosal 10-3 M ouabain
serosal 10-3 M ouabain
n=6
n=6
n=6
n=6
n=6
n=6
Na+
Cl-

Figure 3-21. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M ouabain and serosal 10^{-3} M ouabain conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M ouabain treated sacs and dark shaded bars represent serosal 10^{-3} M ouabain treated sacs. Means ± SEM. There were no significant changes in water reabsorption rates compared to control (unpaired t-test, two-tailed, $P>0.05$).



NB no significant differences in water reabsorption rates

Figure 3-22. Influx rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M NaCN and simultaneous mucosal/serosal 10^{-3} M NaCN conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M NaCN treated sacs and dark shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN treated sacs. Means ± SEM. There were no significant changes in influx rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

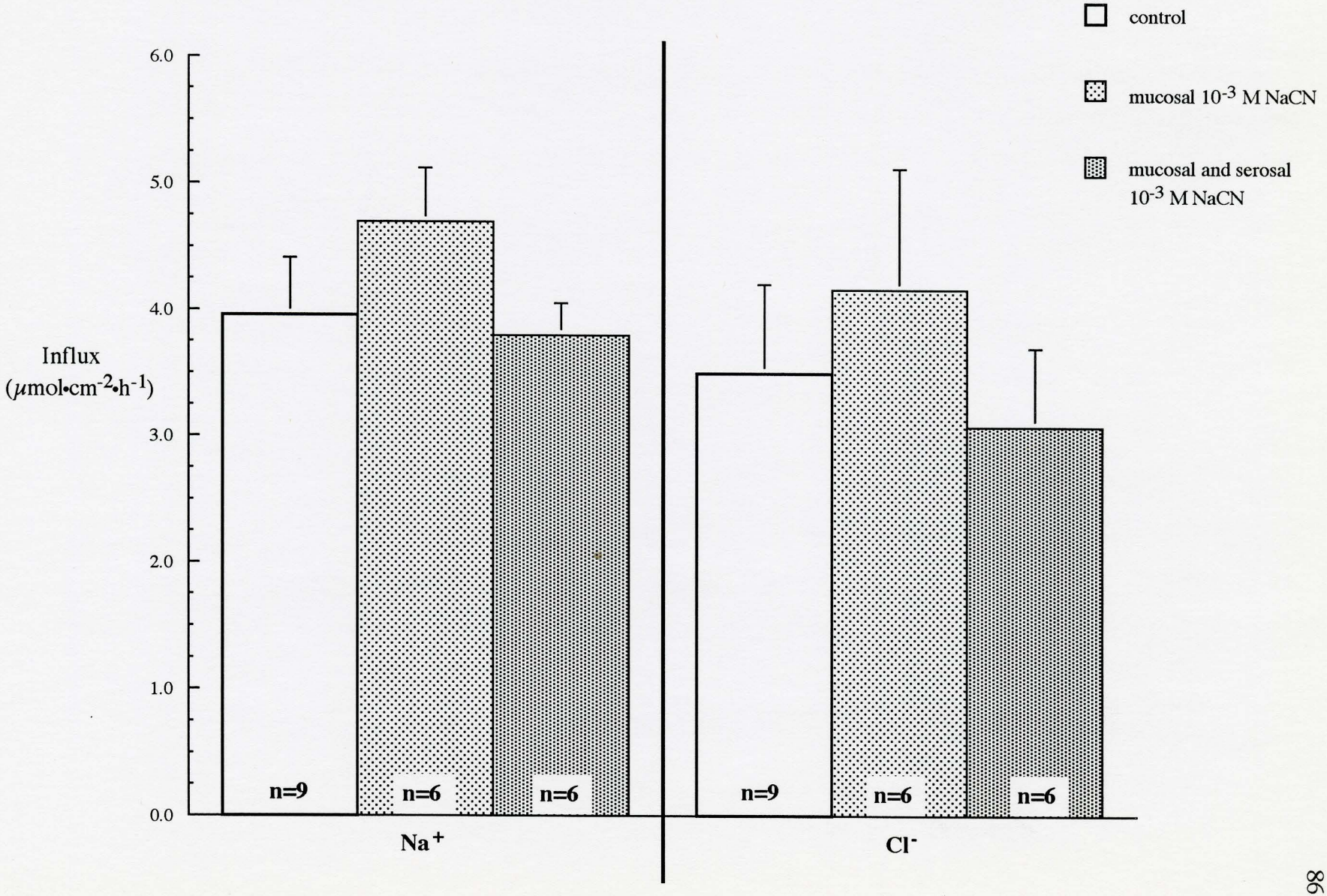

control
mucosal 10^{-3} M NaCN
mucosal and serosal 10^{-3} M NaCN
Influx
(μmol•cm^{-2}•h^{-1})
6.0
5.0
4.0
3.0
2.0
1.0
0.0
n=9
n=6
n=6
n=9
n=6
n=6
Na^+
Cl^-

Figure 3-23. Net flux rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M NaCN and simultaneous mucosal/serosal 10^{-3} M NaCN conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M NaCN treated sacs and dark shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN treated sacs. Means ± SEM. There were no significant changes in net flux rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

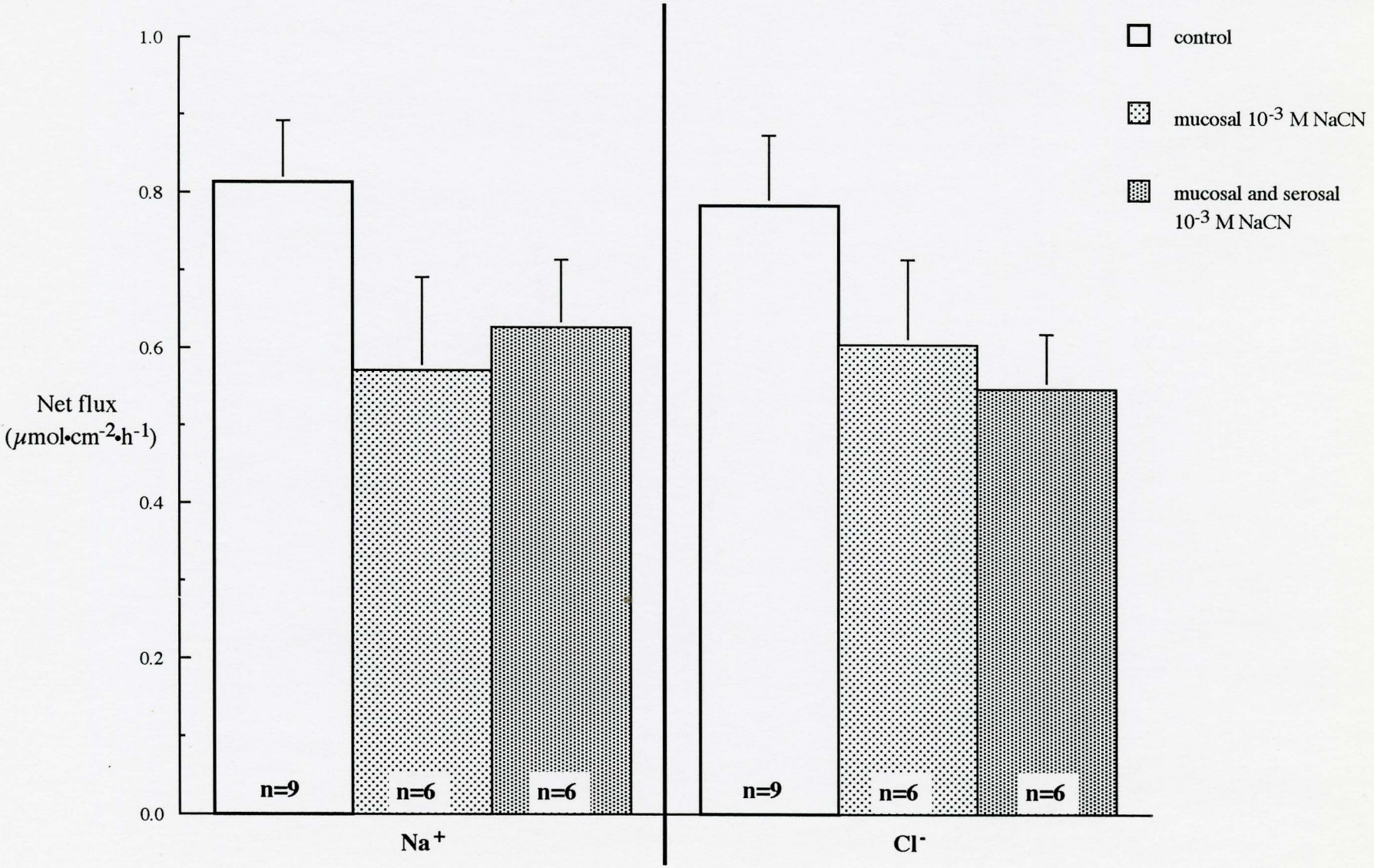

1.0
0.8
0.6
0.4
0.2
0.0
Net flux
(μmol•cm-2•h-1)
n=9
n=6
n=6
n=9
n=6
n=6
Na+
Cl-
control
mucosal 10-3 M NaCN
mucosal and serosal
10-3 M NaCN

Figure 3-24. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, mucosal 10^{-3} M NaCN and simultaneous mucosal/serosal 10^{-3} M NaCN conditions. Open bars represent control sacs, light shaded bars represent mucosal 10^{-3} M NaCN treated sacs and dark shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN treated sacs. Means ± SEM. There were no significant changes in water reabsorption rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

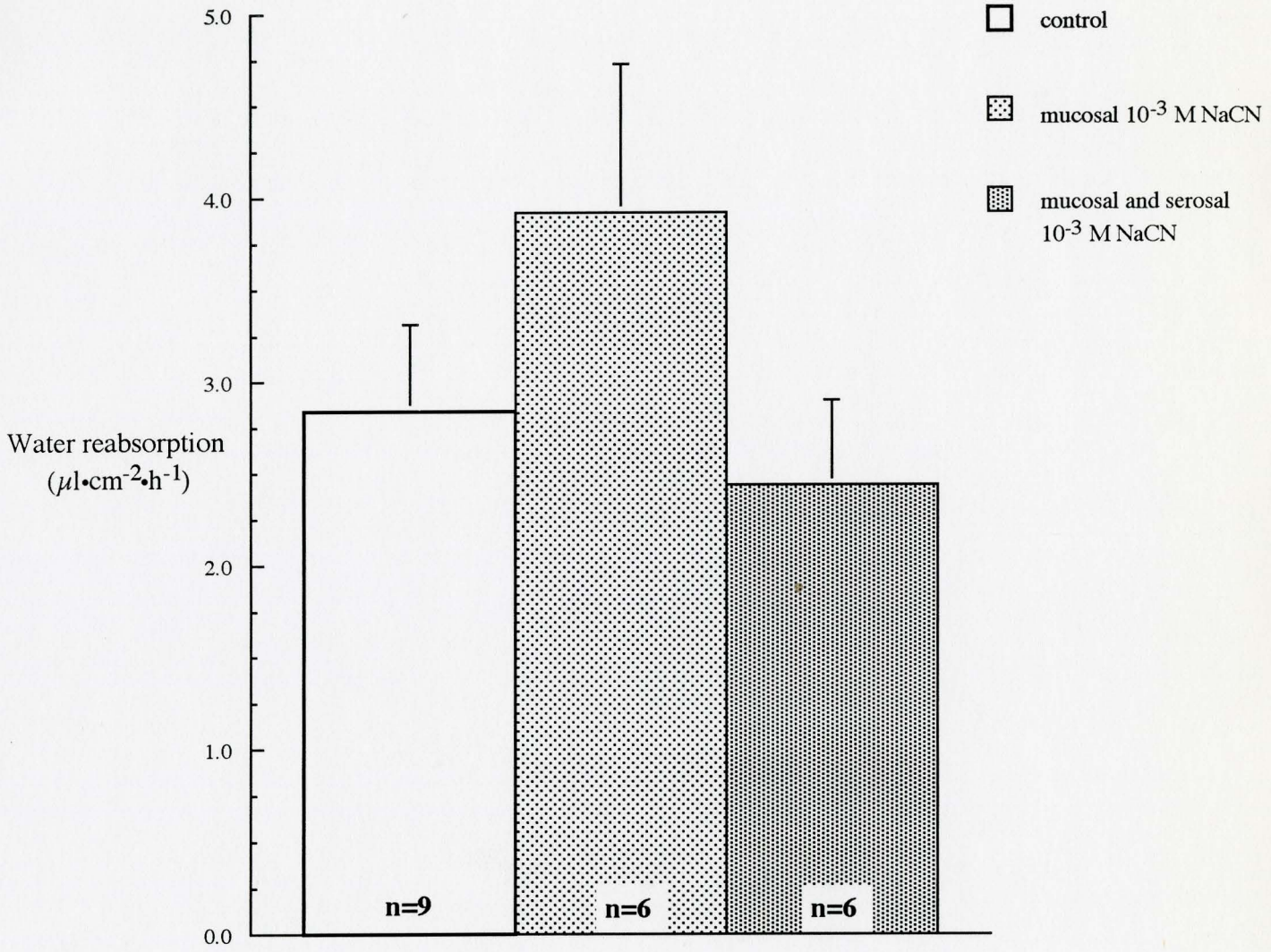

5.0
4.0
3.0
2.0
1.0
0.0
Water reabsorption
(μl•cm^{-2}•h^{-1})
n=9
n=6
n=6
control
mucosal 10^{-3} M NaCN
mucosal and serosal
10^{-3} M NaCN

Figure 3-25. Influx rates of Na^+ in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, simultaneous mucosal/serosal 10^{-3} M NaCN and 10^{-3} M NaF, and simultaneous mucosal/serosal 10^{-3} M NaCN and $5x10^{-3}$ M iodoacetate conditions. Open bars represent control sacs, light shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN and 10^{-3} M NaF treated sacs, and dark shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN and $5x10^{-3}$ M iodoacetate treated sacs. Means ± SEM. There were no significant changes in influx rates compared to control (unpaired t-test, two-tailed, $P>0.05$).

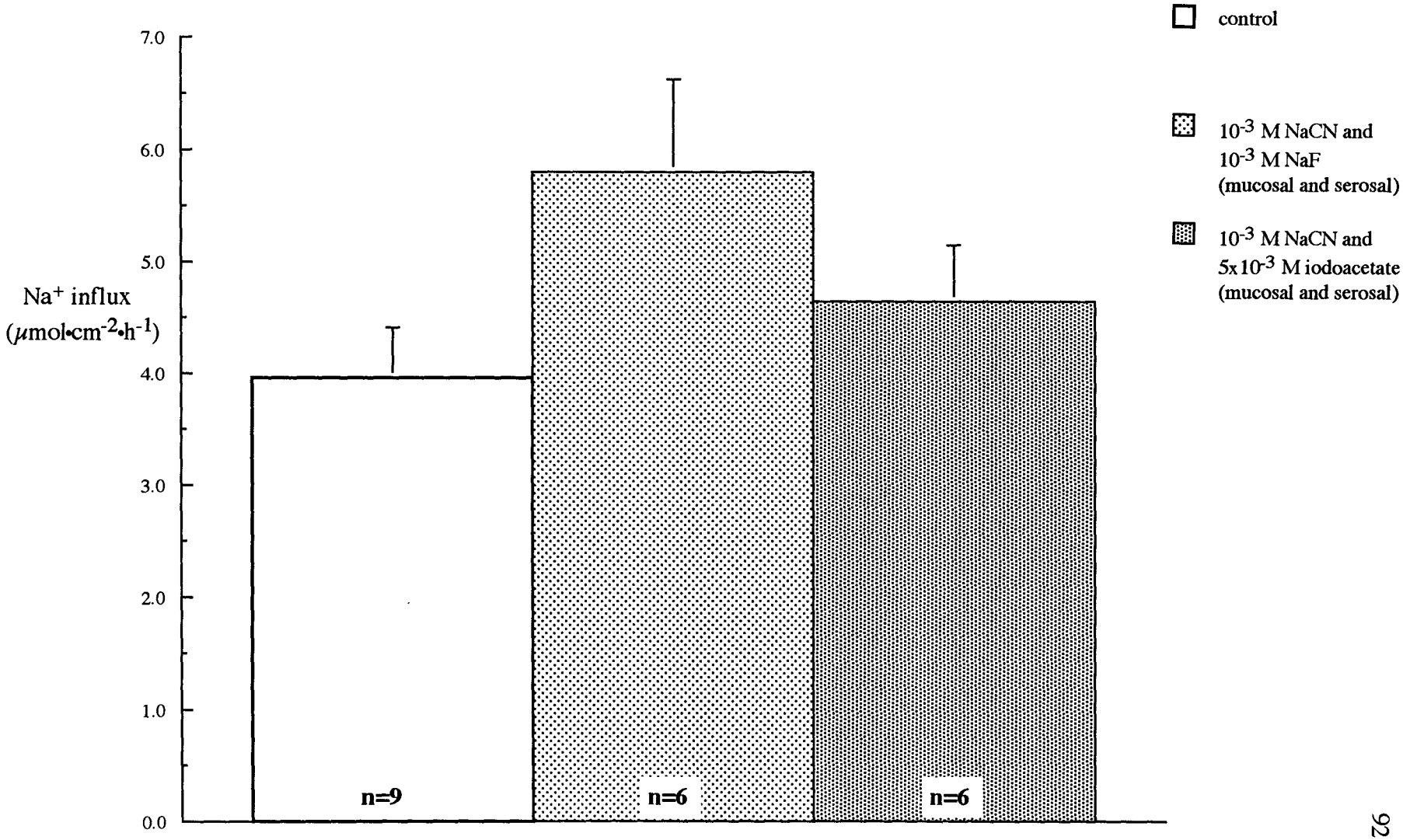

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Na+ influx
(μmol•cm-2•h-1)
n=9
n=6
n=6
control
10-3 M NaCN and
10-3 M NaF
(mucosal and serosal)
10-3 M NaCN and
5x10-3 M iodoacetate
(mucosal and serosal)

Figure 3-26. Net flux rates of Na^+ and Cl^- in the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, simultaneous mucosal/serosal 10^{-3} M NaCN and 10^{-3} M NaF, and simultaneous mucosal/serosal 10^{-3} M NaCN and $5x10^{-3}$ M iodoacetate conditions. Open bars represent control sacs, light shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN and 10^{-3} M NaF treated sacs, and dark shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN and $5x10^{-3}$ M iodoacetate treated sacs. Means ± SEM. Asterisk indicates a significant change in net flux rates compared to control (unpaired t-test, two-tailed, $P<0.05$).

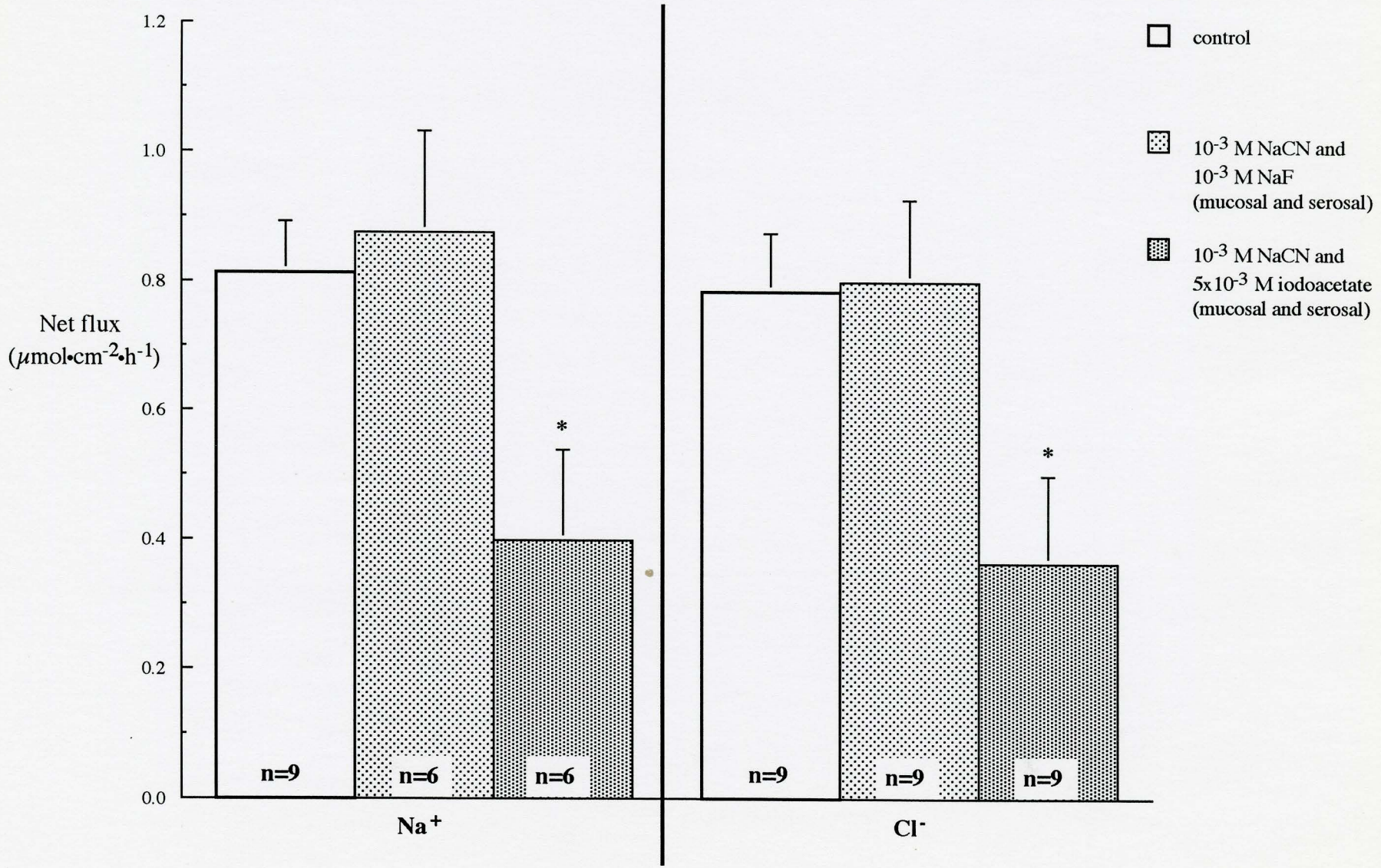

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Net flux
(μmol•cm-2•h-1)
n=9
n=6
n=6
n=9
n=9
n=9
*
*
Na+
Cl-
control
10-3 M NaCN and
10-3 M NaF
(mucosal and serosal)
10-3 M NaCN and
5x10-3 M iodoacetate
(mucosal and serosal)

Figure 3-27. Water reabsorption rates of the closed urinary bladder sac preparation of *Oncorhynchus mykiss* under control, simultaneous mucosal/serosal 10^{-3} M NaCN and 10^{-3} M NaF, and simultaneous mucosal/serosal 10^{-3} M NaCN and $5x10^{-3}$ M iodoacetate conditions. Open bars represent control sacs, light shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN and 10^{-3} M NaF treated sacs, and dark shaded bars represent simultaneous mucosal/serosal 10^{-3} M NaCN and $5x10^{-3}$ M iodoacetate treated sacs. Means ± SEM. No significant changes in water reabsorption rates were found compared to control (unpaired t-test, two-tailed, $P>0.05$).

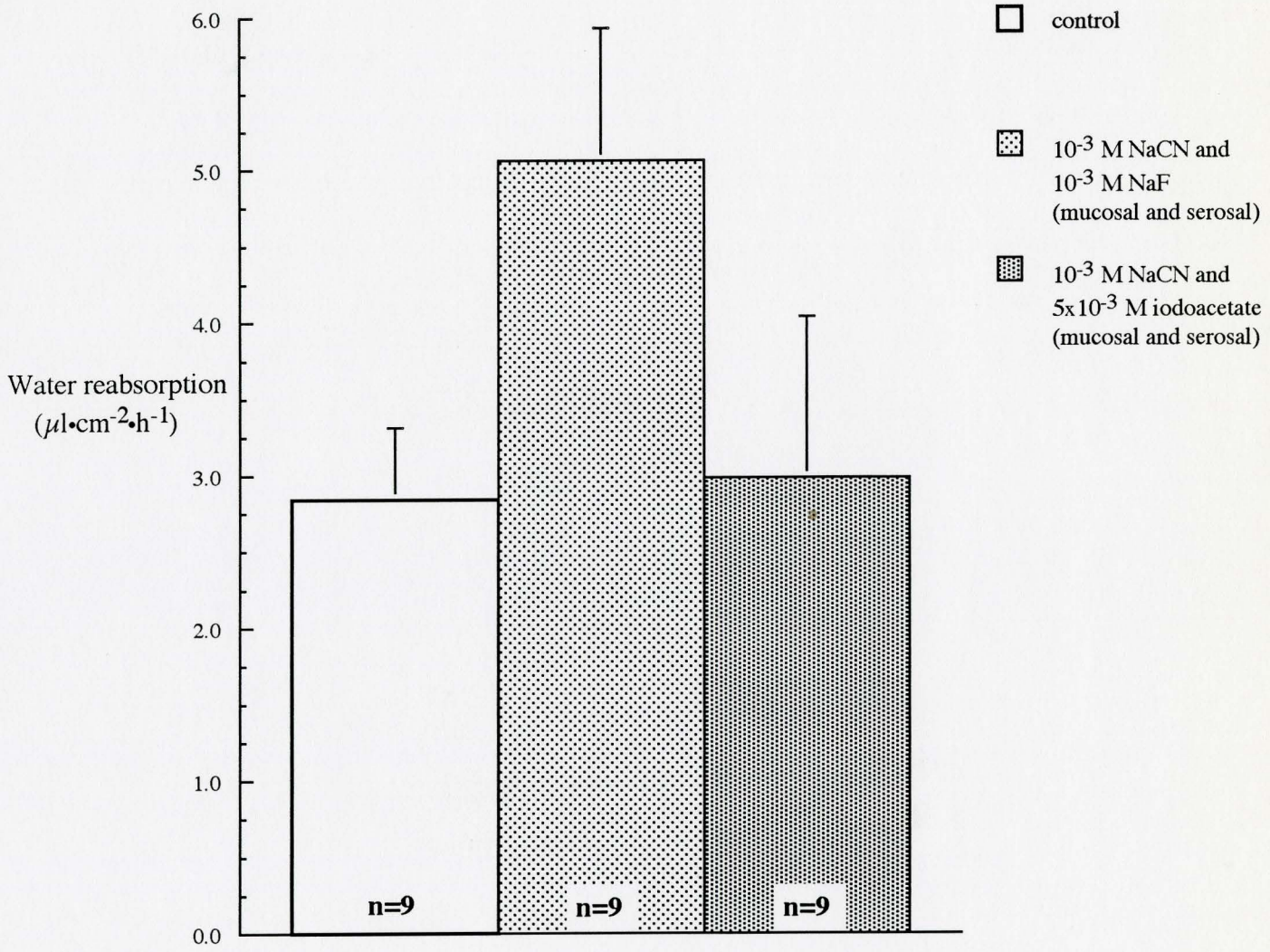

6.0
5.0
4.0
3.0
2.0
1.0
0.0
Water reabsorption
(µl•cm-2•h-1)
n=9
n=9
n=9
control
10-3 M NaCN and
10-3 M NaF
(mucosal and serosal)
10-3 M NaCN and
5x10-3 M iodoacetate
(mucosal and serosal)

Figure 3-28. Na^+ influx rate kinetics in open, perfused sacs and in the Ussing chamber preparation of the urinary bladder of *Oncorhynchus mykiss*. Open squares represent the current findings in the perfused urinary bladder sac and filled circles show the influx rates found in the Ussing chamber urinary bladder preparation (Burgess et al., 2000). Influx rates are shown as a percentage of the maximum rates found at the highest mucosal concentration of Na^+, as a function of mucosal Na^+ concentration, with SEM of both rates (y error) and concentrations (x error). A linear fit for the current findings is shown and has $r^2=0.998$.

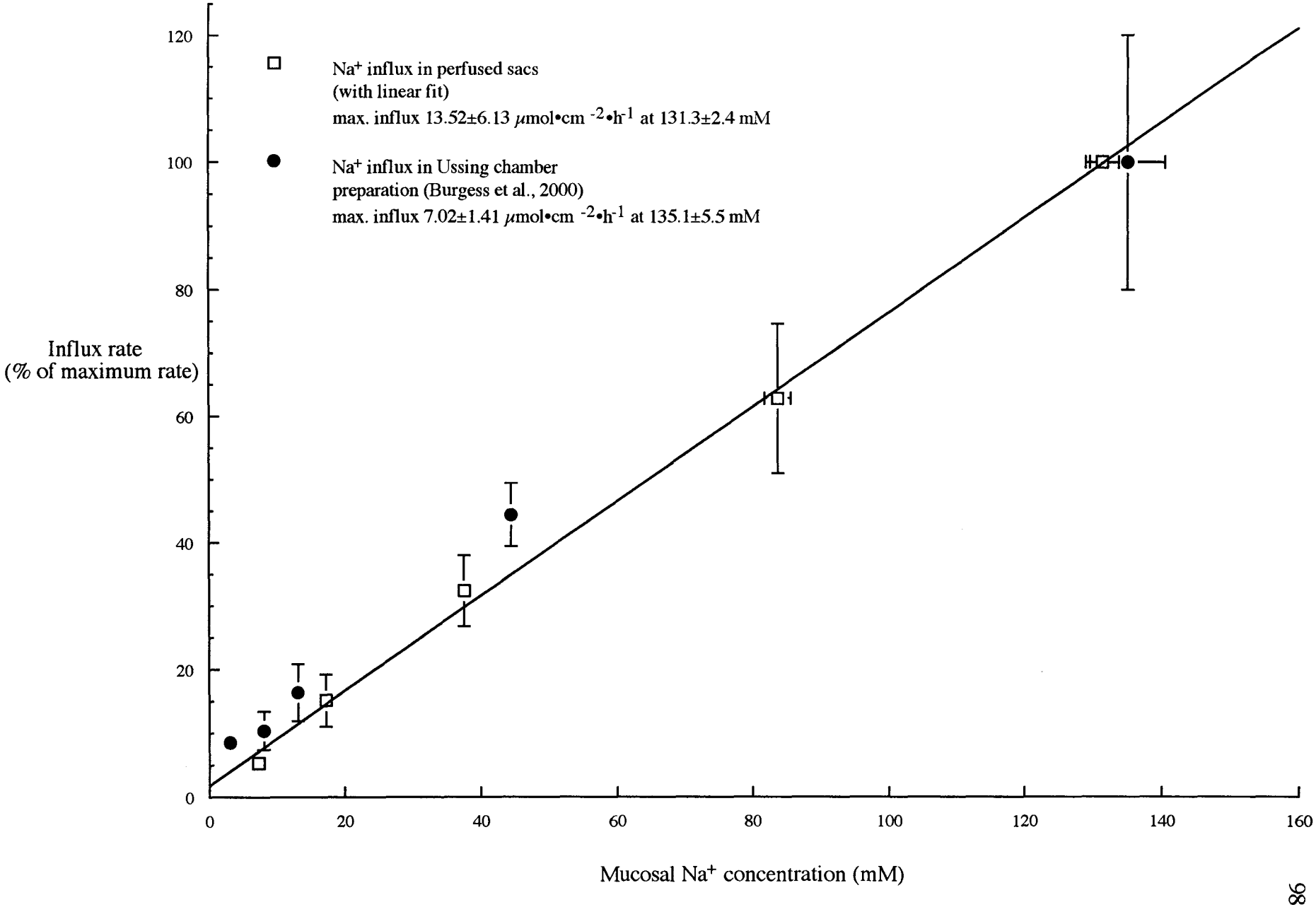
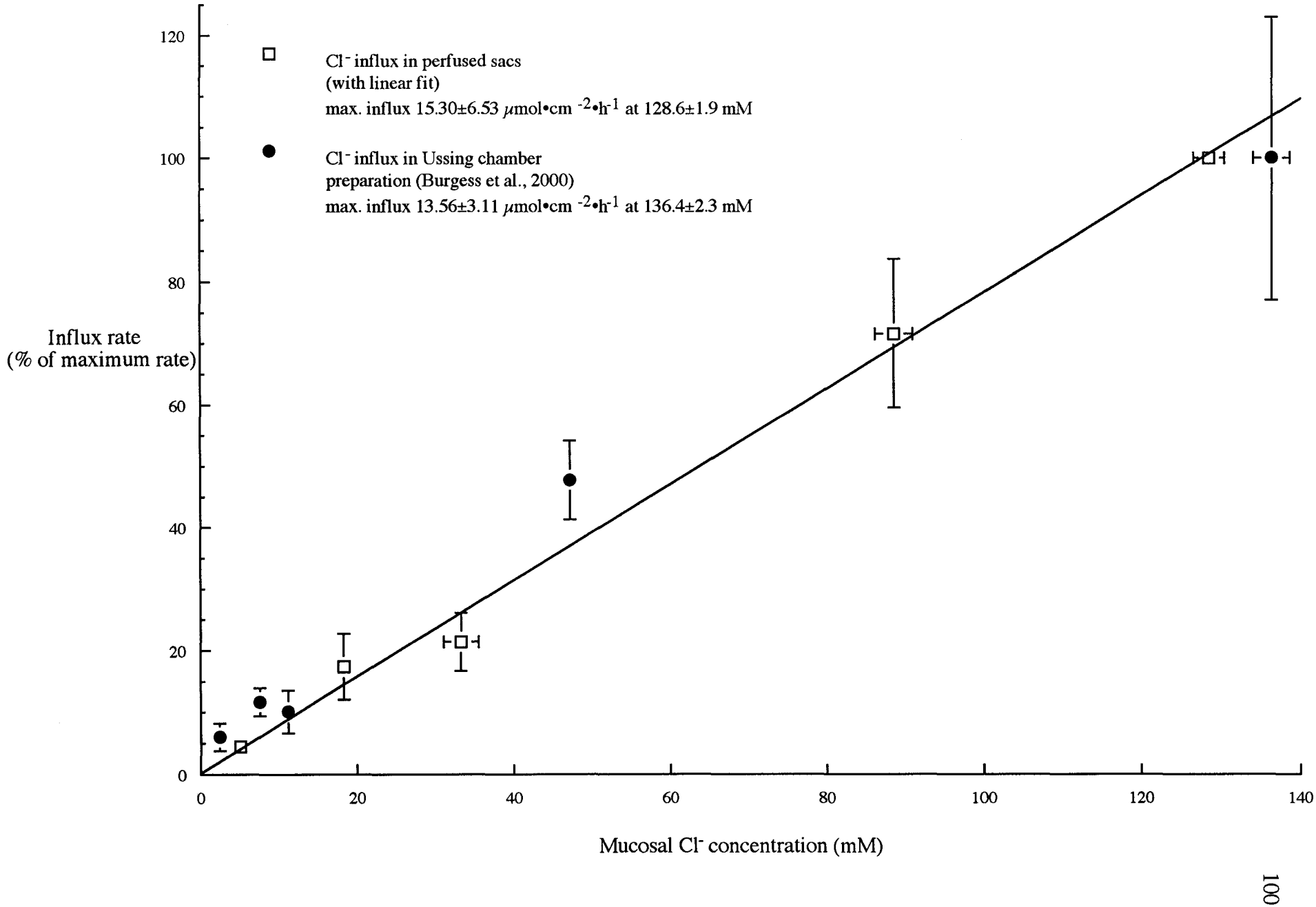

Na+ influx in perfused sacs
(with linear fit)
max. influx 13.52±6.13 μmol•cm -2•h-1 at 131.3±2.4 mM
Na+ influx in Ussing chamber
preparation (Burgess et al., 2000)
max. influx 7.02±1.41 μmol•cm -2•h-1 at 135.1±5.5 mM
Influx rate
(% of maximum rate)
0
20
40
60
80
100
120
0
20
40
60
80
100
120
140
160
Mucosal Na+ concentration (mM)

Figure 3-29. Cl^- influx rate kinetics in open, perfused sacs and in the Ussing chamber preparation of the urinary bladder of *Oncorhynchus mykiss*. Open squares represent the current findings in the perfused urinary bladder sac and filled circles show the influx rates found in the Ussing chamber urinary bladder preparation (Burgess et al., 2000). Influx rates are shown as a percentage of the maximum rates found at the highest mucosal concentration of Cl^-, as a function of mucosal Cl^- concentration, with SEM of both rates (y error) and concentrations (x error). A linear fit for the current findings is shown and has $r^2=0.995$.


Cl⁻ influx in perfused sacs
(with linear fit)
max. influx 15.30±6.53 μmol•cm -2•h-1 at 128.6±1.9 mM
Cl⁻ influx in Ussing chamber
preparation (Burgess et al., 2000)
max. influx 13.56±3.11 μmol•cm -2•h-1 at 136.4±2.3 mM
Influx rate
(% of maximum rate)
0
20
40
60
80
100
120
0
20
40
60
80
100
120
140
Mucosal Cl⁻ concentration (mM)

Chapter 4

Discussion

Ion replacement in the urinary bladder

The use of radiolabelled Na^+ and Cl^- allowed the examination of unidirectional influx rates, in conjunction with net flux rates, in the *O. mykiss* urinary bladder sac preparation. Although net flux rates can be informative it was felt that unidirectional influx rates would allow a clearer, more mechanistic view of the movement of Na^+ and Cl^-. However, based on an analysis of the ion replacement experiments andthe kinetic uptake experiments (presented below), it appears that unidirectional influx rates are largely reflective of a diffusive component, and that net flux rates may be more directly indicative of active transport. Therefore, complications often arose in trying to reconcile the two flux determinations. This was most apparent in the pharmacological experiments. However, this problem did not complicate matters in those ion replacement and kinetics studies which employed the perfused preparation and measured only unidirectional influx rates.

The replacement of Na^+ or Cl^- in the mucosal bathing medium of closed sacs was without effect on net flux rates of the "co"-ion (Fig. 3-1), supporting the view that Cl^- and Na^+ reabsorption occur via independent transporters as proposed in the model of the brook trout urinary bladder (Marshall, 1986, 1988; Marshall and Bryson, 1991). The net flux rates of both Na^+ and Cl^- were similar to those previously observed in the rainbow trout urinary bladder in the closed sac preparation (~1.8 $\mu mol \cdot cm^{-2} \cdot h^{-1}$; Miarczynski, 1997), although the previous study recorded an increasing trend (not significant) in Na^+ and Cl^- reabsorption when the co-ion was removed from the mucosal bathing medium. The observed net flux rates were also very similar to those

previously obtained in brook trout urinary bladders (Marshall, 1986 and 1988) and in European rainbow trout (Fossat and Lahlou, 1977), but slightly lower than those observed in rainbow trout by Marshall (1988). The current study shows no deviation above control for Na^+ or Cl^- reabsorption rates upon co-ion replacement (Fig. 3-1). The net flux rates of the current study are also similar to those observed *in vivo*, for *O. mykiss*, of 0.7-2.5 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Curtis and Wood, 1991).

Upon examination of unidirectional influx rates (from here onward called influx rates), the results also support transport via independent mechanisms since Na^+ or Cl^- removal from the mucosal bathing medium had no effect on the reabsorption of the co-ion (Fig. 3-2). It was also observed, in controls, that Cl^- influx was significantly higher than Na^+ influx, indicating a higher transport or permeability of Cl^- in the rainbow trout urinary bladder. This yielded a transport ratio of roughly 1 Na^+: 1.29 Cl^-. A trend for higher influx of Cl^- over Na^+ was previously reported in brook trout and North American rainbow trout bladders, where the transport ratio of Na^+:Cl^- was roughly 1:1.25 for rainbow trout and 1:1.51 for brook trout (Marshall, 1988).

The concern with Na^+ or Cl^- replacement in closed sacs is that over the length of the flux period, the backflux of the replaced ion may be substantial enough to reinstate near normal reabsorption of the ion under study. The mucosal concentrations of Na^+ or Cl^- required to achieve half maximal transport of the same ion (K_m), under control conditions, have been reported as approximately 8 mM in the European rainbow trout (Fossat and Lahlou, 1979b) and around 35 mM for both brook trout (Marshall, 1986) and North American rainbow trout (Burgess *et al.*, 2000). The backflux over a 4 hour flux period could deposit as much as 50 mM or more of the ion that was initially replaced (Table 3-2), possibly causing misleading results in ion replacement

experiments in the closed sac preparation. To counteract the backflux problem an open, perfused urinary bladder preparation was also used in ion replacement experiments. It was expected that perfusion at approximately 8 $ml \cdot h^{-1}$ would maintain mucosal levels of Na^+ or Cl^- near 0 mM. In actuality the levels were maintained at 3 mM or below, which was deemed sufficiently below any published or observed K_m values for brook trout or rainbow trout urinary bladders. The perfusion rate was also similar to those previously employed in perfusion experiments of winter flounder urinary bladders (Renfro, 1975).

The replacement of mucosal Na^+ or Cl^- in the perfused sac preparation did not cause any change in the influx of the co-ion and, as also seen in the closed sac preparation, there was a higher influx of Cl^- versus Na^+ in controls (Fig. 3-4). The influx rates were similar to those observed in the closed sac preparation. With the low concentrations of the replaced ion achieved by the perfusion method, it appears that Na^+ and Cl^- reabsorption does in fact occur via independent transporters. Surprisingly, previous studies using the *O. mykiss* urinary bladder in Ussing chambers, under symmetrical saline conditions, had shown that mucosal Na^+ or Cl^- replacement reduced the influx of the co-ion by up to 70%, although net flux was not completely abolished (Burgess *et al.*, 2000). The Ussing chamber results had suggested that although the influx was composed mostly of coupled transport of Na^+ and Cl^-, independent transport was also present. The current experiments seem to show the opposite, where independent transport is either responsible for 100% of the influxes of Na^+ and Cl^-, or a switch occurs during the ion replacement experiments such that independent transport is able to support the reabsorption rates observed in controls. Regardless, it is obvious from the sac experiments that reabsorption via independent transporters is present in *O. mykiss* urinary bladders. However, coupled with the Ussing chamber data, the *O. mykiss* urinary bladder is apparently able to employ reabsorption via either

independent and/or coupled transport mechanisms.

Urinary bladder sac kinetics

The surprising result of the urinary bladder sac kinetics study is that apparently neither Na^+ nor Cl^- influx rates exhibit saturation within the mucosal concentrations studied (Fig. 3-28 and 3-29, respectively). Both Na^+ and Cl^- influx rates increase in a linear fashion with increasing mucosal concentrations of Na^+ or Cl^-, respectively. Previously, when kinetics of Na^+ and Cl^- reabsorption were examined in the *O. mykiss* urinary bladder using the Ussing chamber preparation (Burgess *et al.*, 2000), at least a trend towards saturation was observed with K_m values similar to those observed by Marshall (1986) in the brook trout (~35 mM). These values differed from those found in European rainbow trout quite substantially (~8 mM; Fossat and Lahlou, 1979b), showing that the European rainbow trout Na^+ and Cl^- transporter(s) have a higher affinity for the ions. It is interesting to note that the findings of saturable transport in the brook trout (Marshall, 1986), European rainbow trout (Fossat and Lahlou, 1979b) and *O. mykiss* (Burgess *et al.*, 2000) urinary bladder were all obtained using the Ussing chamber preparation, not the sac preparation.

The current sac study shows no saturation and by default suggests a much higher maximum transport rate of both Na^+ and Cl^-, albeit at supra-physiological levels of Na^+ and Cl^- in the mucosal solution, and a lower affinity for both the ions than previously observed in trout urinary bladder. This lack of saturation suggests two possibilities, a) that the mucosal concentrations employed in this kinetics study were on the lower end of the saturation curve, therefore, only a linear relationship was observed or, b) that dual saturable and non-saturable components exist in the curve but the non-saturable component is larger or more apparent in the

mucosal concentrations studied, masking the saturable component. The case for the non-saturable component could involve passive movement of the ions through channels or paracellular pathways, either of which could have a high saturation limit (Christensen, 1975). The dual saturable and non-saturable component model was observed for the European rainbow trout where the saturable component was seen at low mucosal concentrations and the linear, non-saturable component was observed at higher concentrations (Fossat and Lahlou, 1979b). According to the current sac kinetics data, the likelihood is that dual high affinity and low affinity mechanisms of transport are present but the low affinity mechanism is much more prominent. In the current study, it is unlikely that much reabsorption is occurring through transporters (be they anti- or co-transporters) which would likely exhibit saturation at a much lower level, considering that the reabsorption must occur from a very dilute medium under normal circumstances. In reabsorption from a dilute medium, such as FW fish urine, the K_m values would be expected to be relatively low if efficient reabsorption were to take place. Further support is lent by the observation that although the current *O. mykiss* sac experiments do not show saturation and the Ussing chamber *O. mykiss* experiments did (Burgess *et al.*, 2000), the mean influx rates at specific mucosal concentrations are fairly similar between the two (see maximum observed influx rates on Fig. 3-28 and 3-29). The Ussing chamber kinetics measurements show influx rates slightly higher at the lower mucosal concentrations than in the current sac study, where the saturable component could be missing. This would seem to indicate that the Ussing chamber kinetics (Burgess *et al.*, 2000) could have a dual saturable/non-saturable nature as found by Fossat and Lahlou (1979b), but in the current study the saturable component is much less prominent.

It should be pointed out that the concentration of the reabsorbate from the bladder sac can be used as an indicator of active transport if the reabsorbate concentration is higher than that of the surrounding saline. The concentration can be attained by dividing the net Na^+ or Cl^- flux rate by

the net water flux rate for individual preparations. In general, reabsorbate concentrations of the control sacs were found to be higher than saline concentrations of Na^+ and Cl^- by about 200 mM (Table 3-4), indicating that Na^+ and Cl^- reabsorption was an active process. This certainly demonstrates that the observed active net flux rates of Na^+ and Cl^- could not be caused by solvent drag. As stated before, the bladder sac reabsorbate concentrations were highly variable (beyond 500 mM even) and did not allow clear conclusions to be drawn about drug action. However, some drug treated sacs did show significant reductions in reabsorbate concentrations which, for the most part, followed the observed reductions in Na^+ or Cl^- flux rates. Some treated sacs, such as those treated with chlorothiazide, acetazolamide, ouabain, cyanide, and cyanide and NaF, showed absolutely no reductions in flux rates or no reductions in net flux rates alone. But, these sacs did exhibit significant reductions in reabsorbate concentrations of Na^+ and/or Cl^- (Table 3-4). It should also be noted that reabsorbate values are obviously affected by net water flux rates which were only once significantly reduced as a result of drug treatment. This is similar to results observed by Fossat *et al.* (1974) where net Na^+ fluxes were significantly reduced by a treatment but net water flux rates were not. The high variability and reductions in reabsorbate concentrations where flux rates were unaffected, suggest that reabsorbate concentrations were not the most reliable measure for comparison purposes in this study, but they are certainly informative in establishing active transport and, with low variability, could be useful in supporting drug inhibition of ion transport.

The ion replacement and kinetics studies strongly support the idea that unidirectional influx measurements performed with the bladder sac preparation reflect mainly a prominent diffusive movement of Na^+ and Cl^- with only a small active component. This makes it difficult to differentiate effects of treatments such as drugs by examining the influx rates and makes the net

flux rate more credible for such purposes. Assuming that the diffusive components are roughly equal in the unidirectional influx and unidirectional efflux directions, it stands to reason that the net flux illustrates an active component which in the symmetrical saline urinary bladder sac preparation occurs as a reabsorption from the mucosal compartment.

Blocking of coupled mechanisms

It is expected that if the coupled model of Na^+ and Cl^- transport (involving a Na^+/Cl^- co-transporter, Fig. 1-5) were present, that the current study might see decreases in Na^+ and/or Cl^- flux rates when amiloride, bumetanide, chlorothiazide or hydrochlorothiazide were administered to the mucosal (or possibly serosal) bathing solutions. In the model of Fig. 1-5, amiloride would be expected to block Na^+ channel activity, bumetanide to block Na^+/Cl^- co-transporter activity (and $Na^+/K^+/Cl^-$ co-transporter activity which was not expected here), and the thiazides to more specifically block the Na^+/Cl^- co-transporter blockers. The drugs were all expected to work at the mucosal, or apical, membrane if the postulated mechanisms were present, but suspicions about lack of drug penetration demanded use of some drugs in both mucosal and serosal bathing solutions. Metabolic inhibitors and ouabain were grouped as drugs that should affect active transport regardless of whether it was via coupled or independent process, and as such are dealt with separately, not within each model.

The use of 10^{-4} M mucosal amiloride had no effect on Na^+ or Cl^- influx rates (Fig. 3-5). The same results were observed when 10^{-3} M mucosal amiloride was used to assure that drug penetration was not an issue (Fig. 3-8, 3-9). Although influx rates were not affected, 10^{-4} M mucosal amiloride did cause a 46% decrease in Na^+ net flux rates and a 45% reduction in Cl^- net

flux rates (Fig. 3-6). Surprisingly, net flux rates were not affected at 10^{-3} M mucosal concentrations of amiloride (Fig. 3-10, 3-11). The results are similar to those in brook trout (Marshall, 1986), where a 35% reduction in Na^+ net flux was observed with a 10^{-4} M concentration of amiloride. However, the brook trout urinary bladder also exhibited a 32% reduction in Na^+ influx and no effects on Cl^- fluxes were reported for amiloride (Marshall, 1986). Amiloride (alone or in conjunction with DIDS and bumetanide) was shown to be ineffective in the *O. mykiss* urinary bladder when using the Ussing preparation under symmetrical saline conditions, as employed in the current sac study (Burgess *et al.*, 2000). The Burgess *et al.* (2000) study also showed coupling of Na^+ and Cl^- transport under symmetrical saline conditions. Similarly, the coupled system of Na^+ and Cl^- transport in winter flounder urinary bladder has been found to be insensitive to amiloride application (Stokes *et al.*, 1984). Current results point to amiloride sensitivity whereas results from past studies have shown that coupled systems tend toward amiloride insensitivity. This seems to suggest that although Na^+ channels may be present in a coupled model (Fig. 1-5), it is likely that the Na^+ channels do not contribute much to Na^+ transport under normal conditions.

Bumetanide (10^{-4} M) was quite obviously not effective in reducing net flux rates or influx rates of either Na^+ or Cl^- (Fig. 3-5, 3-6). These results correspond to those of Marshall (1986) where it was found that 10^{-5} M and 10^{-4} M bumetanide had no significant effect on Na^+ or Cl^- transport. Marshall (1986), also points out that both Na^+/Cl^- and $Na^+/K^+/Cl^-$ co-transport are usually quite sensitive to bumetanide. However, in the urinary bladder tissue of the winter flounder, 10^{-4} M bumetanide was again ineffective whereas hydrochlorothiazide (deemed a more

specific blocker for Na^+/Cl^- co-transporters) did reduce influx rates of both Na^+ and Cl^- (Stokes *et al.*, 1984).

As with other drug treatments, 10^{-3} M mucosal chlorothiazide or 10^{-3} M mucosal and serosal hydrochlorothiazide had no effect on influx rates of either Na^+ or Cl^- (Fig. 3-13). The net flux rates, however, did respond to the mucosal and serosal hydrochlorothiazide treatment with 76% and 75% reductions in Na^+ and Cl^- rates, respectively (Fig. 3-14). This is an interesting result suggesting either that hydrochlorothiazide is much more effective in blocking Na^+/Cl^- co-transporters in trout urinary bladder than chlorothiazide, or that the thiazide diuretics must be applied serosally to be effective. Stokes *et al.* (1984) showed that mucosal hydrochlorothiazide (10^{-4} M) was responsible for an 85% reduction in Na^+ net flux and a 64% reduction in Cl^- net flux in the flounder urinary bladder where the coupled mechanism of Na^+ and Cl^- transport exists. Hydrochlorothiazide effects were not seen, however, when applied serosally (Stokes *et al.*, 1984). Since the ion replacement experiments seem to support a prominent independent mechanism, it is surprising to see such a large reduction in net flux rates by blocking Na^+/Cl^- co-transport.

Blocking of independent mechanisms

Amiloride may be an "ambiguous" drug because the coupled model proposed in Fig. 1-5 is thought to contain Na^+ channels. The proposed independent mechanism of Na^+ and Cl^- transport (Fig. 1-3 and 1-4) is thought to contain either apical Na^+/H^+ exchangers or apical H^+-ATPases closely linked with Na^+ channels, both of which can be sensitive to amiloride (Benos, 1982).

Because of this, amiloride itself is not too informative on the mechanisms present in the rainbow trout urinary bladder. As mentioned before, amiloride was effective in reducing net flux rates of both Na^+ and Cl^- at a 10^{-4} M concentration. The lack of effect at 10^{-3} M concentrations is rather surprising although Stokes (1988) does mention that at 10^{-4} M amiloride is expected to block channels and at 10^{-3} M to block Na^+/H^+ exchangers (also, Benos, 1982). If this is indeed the case, it is still difficult to explain the lack of effect at 10^{-3} M, but it could also rule out the presence of Na^+/H^+ exchangers. The present results run contrary to the complete lack of effect of amiloride in the rainbow trout urinary bladder in the Ussing preparation which seemed to exhibit largely coupled mechanisms at mucosal saline levels of Na^+ and Cl^-(Burgess *et al.*, 2000). The flounder urinary bladder exhibited a significant, but small, reduction in net Na^+ flux when 10^{-4} M amiloride was used (Renfro, 1977). Interestingly, amiloride in the flounder bladder seemed to more clearly separate the dual coupled/independent mechanisms because Cl^--free saline coupled with amiloride caused a greater reduction in net Na^+ flux than either of the treatments alone. Also, Cl^- net flux was not affected by amiloride, indicating that in the flounder bladder amiloride specifically blocks the Cl^- independent component of Na^+ reabsorption (Renfro, 1977).

It is odd that use of DIDS (Cl^-/HCO_3^- exchanger blocker) shows stimulatory effects on Na^+ and Cl^- influx rates at 10^{-3} M and inhibitory effects on Cl^- net flux rates at 10^{-4} M concentrations. This is somewhat similar to the lack of effect and inhibitory effect of amiloride on Na^+ and Cl^- net flux rates at the 10^{-3} M and 10^{-4} M concentrations, respectively. When used mucosally at a 10^{-4} M concentration, DIDS significantly reduced Cl^- net flux rate by 35% , but did

not significantly reduce Na^+ net flux rates (Fig. 3-6). As with amiloride, there were no changes in either Na^+ or Cl^- influx rates (Fig. 3-5). When DIDS was used at a 10^{-3} M concentration both Na^+ and Cl^- influx rates increased (Fig. 3-8 and 3-9) but net flux rates were unaffected (Fig. 3-10 and 3-11). This suggests that at 10^{-3} M, DIDS is responsible for increasing the permeability of the bladder to both Na^+ and Cl^- but does not affect the active component of transport which is reflected in the net flux rates. The fact that at a 10^{-4} M concentration, DIDS significantly reduced Cl^- net flux indicates that Cl^-/HCO_3^- exchangers may be present but it appears that all of the carrier-mediated transport does not occur strictly via these exchangers. Much like amiloride, there was no effect of DIDS in the Ussing chamber preparation of the rainbow trout urinary bladder (Burgess *et al.*, 2000), further supporting the observation that independent mechanisms seem to dominate in the sac preparation while coupled transport is quite prominent in the Ussing chamber preparation.

Acetazolamide, employed to examine the importance of carbonic anhydrase in Na^+ and Cl^- reabsorption, was one of only two drugs to affect influx rates but not net flux rates. Mucosal application of 10^{-2} M acetazolamide caused a reduction in Na^+ influx rates by 36% (Fig. 3-16). However, serosal application did not significantly reduce either Na^+ or Cl^- influx rates, and mucosal application did not decrease Cl^- influx rates. None of the treatments with acetazolamide changed net flux rates (Fig. 3-17). The small reduction of Na^+ influx suggests that carbonic anhydrase (and therefore the H^+ which is created) may be important for Na^+ transport through either Na^+/H^+ exchangers or H^+-ATPases with closely associated Na^+ channels.

It is apparent from the combined amiloride and DIDS data, and the ion replacement studies,

that independent transport is present in the rainbow trout urinary bladder. The acetazolamide results are more difficult to interpret, but certainly do not oppose the possibility of independent Na^+ and Cl^- transport mechanisms dependent on formation of H^+ and possibly HCO_3^-.

Inhibition of ATP dependent transport

As noted earlier, only two drugs affected influx rates without affecting net flux rates, and the second of those was 10^{-3} M ouabain. Ouabain was applied both mucosally and serosally in two separate sets of experiments, but the surface of application was not important as either one reduced Na^+ and Cl^- influx rates by 40-50% (Fig. 3-19). To reduce the influx rates of Na^+ or Cl^- would require that the appearance of radiotracer on the serosal side was reduced or, that the disappearance of radiotracer from the mucosal side was reduced. However, from the lack of reduction in net fluxes (Fig. 3-20), it is obvious that reduction of uptake of Na^+ or Cl^-, hot or cold, from the mucosal solution was not occurring meaning that ouabain had blocked basolateral membrane movement of Na^+ or Cl^- into the plasma, or extracellular fluid, from the cell. This was expected since ouabain was meant to block basolateral Na^+/K^+-ATPases. However, the fact that net fluxes continued without significant reductions, indicates that Na^+/K^+-ATPase may not be important in creating a driving force for active reabsorption of Na^+ and Cl^- from the mucosal solution, at least not under symmetrical saline conditions. This explanation may not be satisfactory since it would place the basolateral Na^+/K^+-ATPase as part of the diffusive pathway (most unlikely) if influx does truly reflect a prominent passive movement of Na^+ and Cl^-. These findings would be quite contrary to those observed in winter flounder urinary bladder where ouabain was

seen to remove the active component of Na^+ and Cl^- reabsorption and the remaining unidirectional influx was thought to represent passive movement (Stokes *et al.*, 1984). Other studies on flounder also demonstrated a basolateral Na^+/K^+-ATPase responsible for creating the driving force for Na^+ reabsorption, however, unlike the current study, mucosal application of ouabain caused lower inhibition than if ouabain was applied serosally (Renfro *et al.*, 1976). The European rainbow trout also exhibited ~27-65% reductions in net Na^+ fluxes when 10^{-4} M ouabain was applied at the serosal surface only (Fossat *et al.*, 1974), showing that the Na^+/K^+-ATPase was likely creating the driving force for Na^+ reabsorption, although no mention of Cl^- flux rates was made. Such strong evidence for reductions in net Na^+ flux rates by administration of ouabain in these other species, and the current study's lack of such effects, further strengthens the argument for another source for the gradient driving Na^+ uptake, possibly the H^+-ATPase.

Cyanide is used to block aerobic production of ATP which can fuel active transport mechanisms (Winder and Weiner, 1980). Administration of 10^{-3} M cyanide on the mucosal or simultaneous mucosal/serosal had no effect on influx (Fig. 3-22), which was to be expected in our case if influx really does represent mainly diffusive movement of Na^+ and Cl^-. However, cyanide also caused no significant reduction in net flux rates of either Na^+ or Cl^-, although non-significant decreasing trends were observed under mucosal and mucosal/serosal cyanide application (Fig. 3-23). These results suggest that the prominent form of ATP production in the urinary bladder may be through anaerobic pathways. NaF and iodoacetate were employed, in conjunction with cyanide, to test these theories. Use of 10^{-3} M NaF or $5x10^{-3}$ M iodoacetate simultaneously on mucosal and serosal sides (in conjunction with 10^{-3} M NaCN), caused no change in Na^+ influx

rates (Fig. 3-25). When examining the Na^+ and Cl^- net flux rates it appears that NaF was ineffective but iodoacetate caused an approximately 50% reduction in both Na^+ and Cl^- net flux rates (Fig. 3-26). It is unclear whether NaF was not effective and iodoacetate was effective in reducing net flux rates due to a problem with drug penetration or as a matter of concentration. Iodoacetate was more comfortably employed in higher concentrations because it would not affect the Na^+ concentration of the mucosal or serosal concentration. It was thought that iodoacetate concentrations above 10^{-3} M may confuse interpretation of results but some circumstances (such as the lack of effect of cyanide in the present study) may warrant higher concentrations (Webb, 1963 and 1966). Although cyanide in conjunction with iodoacetate reduced the net flux rates of Na^+ and Cl^-, they were still not completely effective as net flux rates continued at about 0.4 $\mu mol \cdot cm^{-2} \cdot h^{-1}$. This suggests, if net flux rates truly reflect the active reabsorption component of unidirectional influx, that active reabsorption was not completely abolished by the metabolic inhibitors, even in combination. It might be interesting to see what effect cyanide, iodoacetate and ouabain might have together even though ouabain affected influx and not net flux.

Further conclusions

Due to the apparent prominence of diffusive transport in the influx rate measurements, the net flux rate measurements were taken as more indicative of any drug actions on the urinary bladder. Although influx rates are still considered, the net flux rates were taken as the more reliable of the two for interpretation purposes.

The drug and ion replacement experiments support Na^+ and Cl^- transport via a prominent independent pathway with a smaller portion contributed by a coupled mechanism. Amiloride and

DIDS at 10^{-4} M concentration indicate the presence of Na^+ channels and Cl^-/HCO_3^- exchangers, respectively. The effects of ouabain suggest that Na^+/K^+-ATPase is present at the basolateral membrane, however, it appears to affect the diffusive pathway demonstrated by the influx and does not affect the active, net flux rates! This, coupled with the 10^{-4} M amiloride reductions in net flux, may indicate that an apical H^+-ATPase with associated Na^+ channels may be the major motivating force for active Na^+ and Cl^- reabsorption. Recent evidence points to the presence of H^+-ATPases in the rainbow trout kidney (Perry *et al.*, 2000). Therefore, it seems likely that the urinary bladder which is thought to function as an extension of the kidney, could also make use of H^+-ATPases for creating Na^+ reabsorption gradients. This could be further examined by administration of specific H^+-ATPase blockers such as bafilomycin (Lin and Randall, 1995). Hydrochlorothiazide effects suggest that Na^+/Cl^- co-transporters are present although how the interaction between independent and coupled mechanisms actually may work remains unclear at this time. The acetazolamide results are also puzzling at this time. The metabolic inhibition clearly shows that a major energetic supply is obtained through anaerobic means with small portions possibly being contributed by aerobic energy production.

A proposed model of the mechanisms involved in Na^+ and Cl^- reabsorption in the urinary bladder sac preparation under symmetrical saline conditions is presented in Fig. 4-1. The apical Na^+ and Cl^- transport is handled by both independent (Na^+ channel, Cl^-/HCO_3^- antiporter) and coupled (Na^+/Cl^- co-transporter) mechanisms. A basolateral Na^+/K^+-ATPase is present but the apical H^+-ATPase seems to create the motivating force for apical Na^+ reabsorption. A dual system has also been observed in flounder urinary bladder where the coupled mechanism is prominent

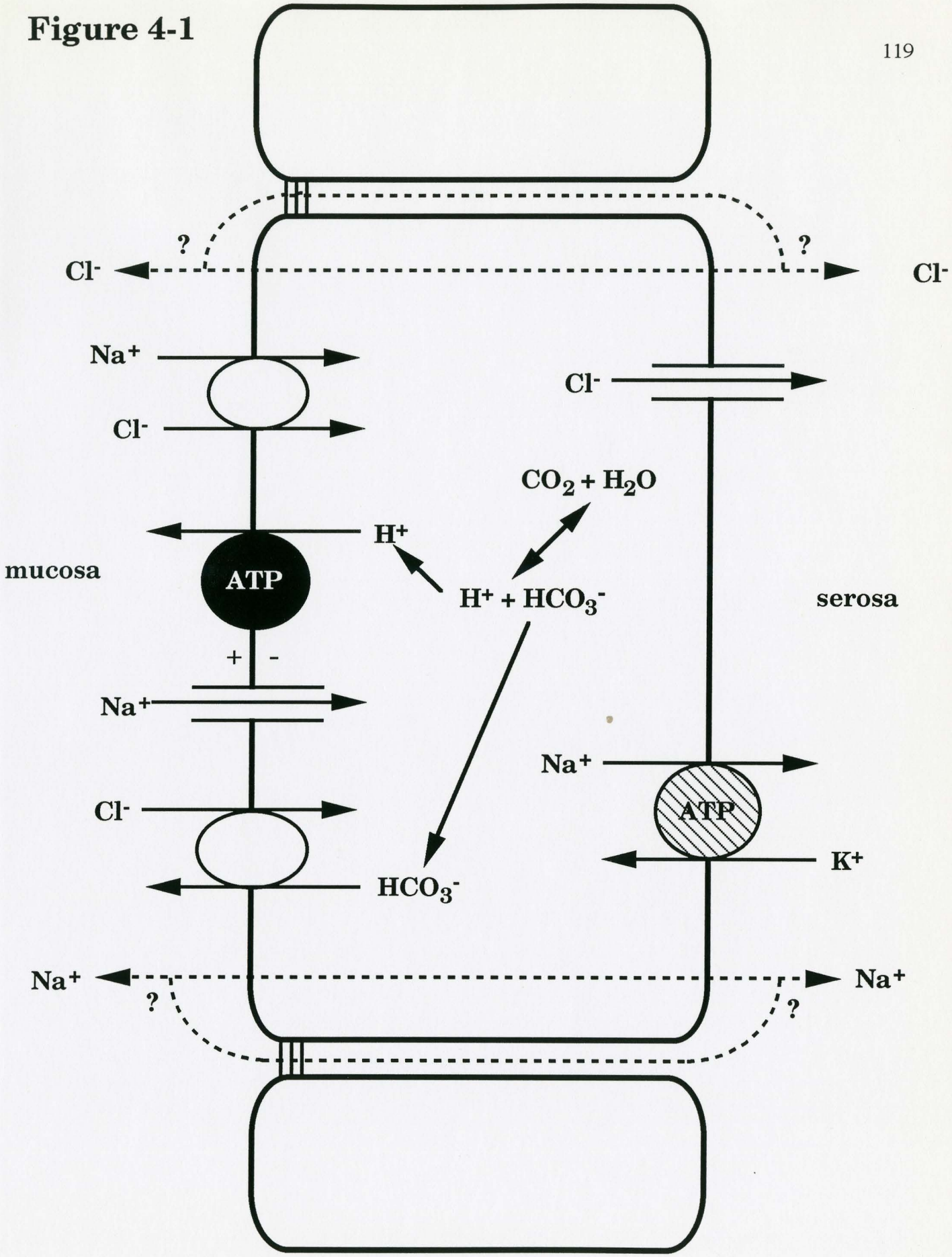
when both Na^+ and Cl^- are present (Renfro, 1977).

An obvious future direction would involve more thorough testing of hydrochlorothiazide to determine whether it was more effective on the apical versus basolateral surfaces. As previously mentioned, ouabain could be examined in the presence of cyanide and iodoacetate to see if Na^+/K^+-ATPase does contribute somewhat to the driving force for Na^+ and Cl^- reabsorption. Alternately, vanadate could be used as a more general ATPase inhibitor (Lin and Randall, 1995) in further efforts to abolish active reabsorption. Amiloride and DIDS might be tested after replacement of Na^+ and Cl^- in the perfused sac preparation, respectively, in an attempt to inhibit Na^+ and Cl^- reabsorption in a situation where co-transport could not possibly be occurring. Further studies could involve a mucosal solution more closely approximating the urine that enters the urinary bladder before the urine is processed by the bladder (~7 mM; Curtis and Wood, 1991). To date studies in our lab have examined the bladder as a sac under symmetrical saline conditions (current study; Miarczynski, 1997) and in Ussing chambers under symmetrical saline and mucosal artificial urine, serosal saline conditions (Burgess *et al.*, 2000; Burgess, 1997). Under symmetrical saline conditions both preparations show a net reabsorption from the mucosal compartment with large unidirectional flux components. Under artificial urine conditions the bladders exhibit a net loss of Na^+ and Cl^- with much smaller unidirectional components (Burgess *et al.*, 2000). It would be interesting to see how the bladder behaves when confronted by more typical, unprocessed urine. Regardless, caution must be exercised when comparing effects of treatments on the trout urinary bladder between the Ussing and sac preparation. In the Ussing chamber preparation the urinary bladder is cleaned of excess surrounding tissue more thoroughly, and it is usually stretched to a greater extent than a bladder in the sac preparation. This may cause the preparational differences observed between the current study and that of Burgess *et al.* (2000).

The urinary bladder seems to react by employing opposite mechanisms in the sac versus the Ussing chambers. Under symmetrical saline conditions the bladder exhibits a very prominent independent transport whereas in Ussing chambers the main mechanism appears to be coupled (Burgess *et al.*, 2000).

Figure 4-1. Proposed model of Na^+ and Cl^- reabsorption mechanisms in the *O. mykiss* urinary bladder under symmetrical saline conditions in the sac preparation. Transporter inhibition suggests apical Na^+ channels, apical Cl^-/HCO_3^- antiporters, most likely apical Na^+/Cl^- co-transporters and basolateral Na^+/K^+-ATPase. The majority of active transport occurs via the independent pathways, most likely fueled by an apical H^+-ATPase (solid ATP), because ouabain inhibition of the basolateral Na^+/K^+-ATPase (hatched ATP) did not reduce net, or active, reabsorption. A large diffusive movement (dotted lines) of Na^+ and Cl^- was observed in the unidirectional influx where the active component was not readily discernible.

Figure 4-1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Cl-
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Na+
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CO2 + H2O
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serosa
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Na+
Cl-
ATP
K+
HCO3-
Na+
Na+
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Appendix

The following manuscript (Burgess *et al.*, 2000) was submitted and accepted for publication in the year 2000 by *The Journal of Experimental Zoology*. It was co-authored by M. Miarczynski, M.J. O'Donnell and C.M. Wood and contains experiments performed by D.W. Burgess as well as M. Miarczynski. In particular, the kinetics data, combined amiloride, bumetanide and DIDS data, chlorothiazide data, and cyanide data were obtained by M. Miarczynski during the completion, but not as part of, the current thesis.

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

by

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ION TRANSPORT IN TELEOST URINARY BLADDER

Index Terms: trout, freshwater, urinary bladder, Na^+, Cl^-, Ussing chamber, ion transport

Abbreviations: FW: freshwater, SW: seawater, G_t: transepithelial conductance, V_t: transepithelial potential, J_{ms}: influx, J_{sm}: efflux, J_{net}: J_{ms}-J_{sm}

Abstract

Freshwater (FW) rainbow trout (*Oncorhynchus mykiss*) urinary bladders mounted *in vitro* under symmetrical saline conditions displayed electroneutral active absorption of Na^+ and Cl^- from the mucosal side; the transepithelial potential (Vt) was 0.1 mV and the short-circuit current was less than $1\mu A \cdot cm^{-2}$. Removal of Na^+ from mucosal saline decreased Cl^- absorption by 56% and removal of Cl^- decreased Na^+ absorption by 69%. However, active net absorption of both Na^+ and Cl^- were not abolished when Cl^- or Na^+ were replaced with an impermeant ion (gluconate or choline respectively). Under physiological conditions with artificial urine ($[Na^+] = 2.12$ mM, $[Cl^-] = 3.51$ mM) bathing the mucosal surface and saline bathing the serosal surface, transepithelial potential (V_t) increased to a serosal positive ~+7.6 mV. Unidirectional influx rates of both Na^+ and Cl^- were 10-20 fold lower but active absorption of both ions still occurred according to the Ussing flux ratio criterion. Replacement of Na^+ with choline, or Cl^- with gluconate, in the mucosal artificial urine yielded no change in unidirectional influx of Cl^- or Na^+, respectively. However, kinetic analyses indicated a decrease in maximum Na^+ transport rate (Jmax) of 66% with no change in affinity (Km) in the low Cl^- mucosal solution relative to the control solution. Similarly, there was a 79% decrease in Jmax values for Cl^-, again with no change in Km, in the low-Na^+ mucosal bathing. The mucosal addition of DIDS, amiloride or bumetanide (10^{-4} M) had no effect on either Na^+ or Cl^- transport, under either symmetrical saline or artificial urine/saline conditions. Addition of the three drugs simultaneously (10^{-4} M), or chlorothiazide (10^{-3} M), under symmetrical saline conditions also had no effect on Na^+ or Cl^- transport rates. Cyanide (10^{-3} M) addition to

mucosal artificial urine caused a slowly developing decrease of Na^+ influx to 59% and Cl^- influx to 50% in the period after drug addition. Na^+ and Cl^- reabsorption appears to be a partially coupled process in the urinary bladder of *O. mykiss*; transport mechanisms are both dependent upon and independent of the other ion.

Introduction

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

The mechanism of Na^+ and Cl^- transport in the urinary bladder of *Salmo irideus* has been characterized as electroneutral co-transport (Fossat and Lahlou, '79a). Thus the removal of either ion from the mucosal side induced the disappearance of the net flux of the other ion. This coupled NaCl transport is associated with an undetectable transepithelial potential difference (V_t) and short-circuit current (I_{sc}). The urinary bladder is termed a 'leaky' epithelium due to its low transepithelial resistance Rt (~200

$\Omega \cdot cm^{-2}$) and the paracellular pathway does not exhibit cation selective properties, in contrast to other low resistance epithelia (Fossat and Lahlou, '79b).

In urinary bladder of brook trout, *Salvelinus fontinalis*, by contrast, uptake of NaCl is again electroneutral, but Na^+ or Cl^- transport continue when Cl^- or Na^+-free solutions, respectively, are placed on the mucosal surface (Marshall, '86, '88). There is also evidence of independent Cl^-/HCO_3^- and Na^+/H^+ exchange mechanisms, and there is no response of Na^+ or Cl^- movement to the mucosal addition of the cotransporter antagonist bumetanide (Na^+-K^+-$2Cl^-$ transport blocker). This "independent model" of mucosa to serosa absorption appears very similar to that normally presented for the freshwater teleost gill (Wood, '91; Perry, '97).

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

The aim of the present study was to establish the mechanism(s) of Na^+ and Cl^- transport *in vitro* in the urinary bladder of the North American strain of freshwater rainbow trout (*Oncorhynchus mykiss*). Isolated bladders were set up in Ussing chambers under open- or short-circuit conditions and Na^+ and Cl^- transport was measured under a variety of conditions. In particular we wished to determine whether the transport of these ions was coupled or independent. First, the urinary bladder was bathed in symmetrical saline, either with both ions present, or with one removed, and flux rates were measured using radiolabelled Na^+ and/or Cl^-. Secondly, artificial urine was used to more closely

mimic the conditions found *in vivo* and similar experiments were performed as above. Thirdly, with saline or artificial urine bathing the mucosal surface, a number of drugs known to affect Na^+ and Cl^- transport in other transporting epithelia were applied. Finally, electrophysiological and kinetic transport properties of the epithelium were determined. Our results suggest that Na^+ and Cl^- transport in the urinary bladder of *O. mykiss* is a partially coupled process, where uptake processes are both dependent on and independent of Cl^- and Na^+, respectively.

Materials and Methods

Animals

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

Bathing solutions

All salts were obtained from Sigma Chemical Co., St. Louis, MO. The Ringer's solution used for dissection and for the basolateral bathing solution was a modified Cortland saline composed of (in mmol l^{-1}) NaCl, 129.9; KCl, 2.55; $CaCl_2 \cdot H_2O$, 1.56;

$MgSO_4 \cdot 7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4 \cdot H_2O$, 2.97; glucose, 5.55; NH_4Cl, 0.30. Saline pH was 7.8-7.9 when equilibrated with a 0.3% CO_2, balance O_2.

The artificial urine solution for the mucosal surface of the membrane was formulated from the results of "spot-sampling" of urine from the bladder of undisturbed, non-cannulated rainbow trout, as reported by Curtis and Wood (1991). Artificial urine consisted of (in mmol l^{-1}) KCl, 0.81; $CaCl_2 \cdot H_2O$, 1.35; $MgSO_4 \cdot 7H_2O$, 0.79; $NaHCO_3$, 1.66; $NaH_2PO_4 \cdot H_2O$, 0.46; urea, 0.55; $Ca(NO_3)_2 \cdot 4H_2O$, 0.045; $(NH_4)_2SO_4$, 0.235. The Ringer's and artificial urine solutions were each modified for ion replacement experiments. In the Cl^--free Ringer's, sodium-, potassium- and calcium gluconate and $(NH_4)_2SO_4$ were substituted for NaCl, KCl, $CaCl_2 \cdot 2H_2O$ and NH_4Cl, respectively. The Na^+-free Ringer's substituted choline chloride, choline bicarbonate, and KH_2PO_4 for NaCl, $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ respectively. The osmolality of Ringer's, Cl^- free Ringer's and Na^+-free Ringer's were 288, 279 and 281 mosmol/kg respectively. In Cl^--free artificial urine, KCl and $CaCl_2 \cdot 2H_2O$ were replaced with K^+ gluconate and Ca^{2+} gluconate, respectively. In Na^+-free artificial urine, $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were replaced with choline bicarbonate and KH_2PO_4, respectively.

For the kinetic experiments, in the initial mucosal "urine", Cl^- was adjusted to equal 2.16 mM and Na^+ was set initially to 2.12 mM, so that Cl^- and Na^+ concentrations were roughly equal. The Na^+ and Cl^- concentrations in artificial urine were then adjusted to approximately 5, 10, 50, and 150 mM by addition of NaCl, Na^+ gluconate or choline chloride as appropriate.

The pH of artificial urine when equilibrated with 0.3% CO_2, balance O_2, was 7.2-7.5. Prior to the start of the experiment and at the end of each 60 minute flux period (see

below) except in the kinetic experiment, the mucosal solution was rinsed extensively to maintain the original ion composition of the artificial urine (Na^+ = 2.12, Cl^- = 3.51 and Ca^{2+} = 1.4 mmol l^{-1}).

Isolated urinary bladder preparation

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

The Ussing membrane apertures (0.125 cm^2) were coated with stopcock grease (Dow Corning, Midland, MI) and a thin vinyl mesh was placed over each aperture to support the tissue. Each portion of urinary bladder was stretched gently (to remove macroscopic folds) and carefully pinned mucosal side up to the aperture; the second half of the aperture then sandwiched the epithelium. After mounting, the mucosal surfaces were thoroughly rinsed with the appropriate solution to remove the saline and any mucus that had accumulated during the dissection. The aperture sandwich was then placed between the two hemi-chambers and tightened into place. Hemi-chambers were filled with the appropriate solutions: Ringer's or artificial urine or ion replacement solution for

the mucosal side, and saline for the serosal side. Each hemi-chamber was filled at an equal rate to prevent damage by unequal hydrostatic pressure. Membranes were then given a 30 min. period to adjust to the *in vitro* conditions. Appropriate gases were passed across the surface of each hemi-chamber's media and mucosal and serosal solutions were mixed by magnetic stirrers.

Electrophysiology

Polyethylene 4% agar/Ringer bridges were used to measure transepithelial potential (V_t, mucosal side grounded) and membrane conductance (G_t). Each bridge was connected to the voltage/current clamp (DVC-1000, WP Instruments, New Haven, CT) by Hg/HgCl calomel half-cells. Membrane conductance was corrected for solution resistances. In asymmetrical solutions, corrections for liquid junction potentials were accomplished by measurements against a free flowing 3 M KCl half-cell. Ussing chambers were maintained at 15°C using water jackets. In flux experiments with saline bathing the mucosal surface, the preparation was short-circuited to negate any effect of V_t on ion transport. G_t was determined by clamping the membrane to a set voltage every 10 min. and V_t was recorded at the beginning and end of each 60 min. short-circuited flux period.

Experimental protocols

One chamber was set up for each portion of the urinary bladder for independent measurements of either influx or efflux. Therefore either the anterior or posterior portion yielded a mucosal to serosal unidirectional influx (J^{ms}) measurement while the other

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

Dual flux experiments were performed with ^{36}Cl ($Na^{36}Cl$ from I.C.N. Radiochemicals, Irvine, California) and ^{22}Na ($^{22}NaCl$ from NEN-Dupont, Boston, MA) which were added to either the mucosal side (final specific activity of 300 000 CPM μmol^{-1}, artificial urine) for unidirectional influx or serosal side (8 000 CPM μmol^{-1}, Ringer) to monitor unidirectional efflux. Upon first addition of the radioisotope(s), a 45 min equilibration period was employed. Each experiment consisted of three 60 min. periods; within each period samples were taken from the unlabelled bath every 20 min. for radioactivity analyses. Flux values for each 20 min. period were averaged to produce a final flux rate for each 60 min. experimental period. At the start and end of each 60 min. period, samples were taken from the labeled side to determine the specific activity and ionic concentrations and the solutions were replaced at the end. Unidirectional fluxes were determined by measuring the specific activity on the labeled side and the appearance of isotope on the unlabeled side. Three 60 min. periods were employed to check whether steady-state conditions were achieved, and to compare the effects of various drugs applied in period 2 against control treatments in periods 1 and 3.

For amiloride, DIDS and bumetanide experiments in Ussing chambers, only the solvent (DMSO) used to dissolve the drugs was present for the first 60 min. control

period. DMSO was added at the same concentration as in the following drug period. The chambers were then emptied, new isotope along with the drug and DMSO solution was added, and allowed to equilibrate for at least 45 min. The drug experimental flux period was 60 min. in duration and was followed by a final 60 min. control period (DMSO alone again present). Prior to the final control period, the chambers were emptied and the membrane was removed. The hemi-chambers were flushed extensively with distilled H_2O, the membrane was then replaced and new solutions with isotope were added and equilibrated for 45 min. The procedure for chlorothiazide and cyanide experiments was similar except that DMSO was not employed.

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

The observed flux ratio (J^{ms}/J^{sm}) was compared to the predicted flux ratio using the Ussing flux ratio equation (Ussing, '49). Disagreement between the observed and predicted value indicated the presence of non-diffusive transport. The predicted flux ratio equation was calculated as follows:

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

The activities of ion *i* are ai^s and ai^m on the serosal and mucosal sides respectively; valency of the ion is Z_i, V_t is the transepithelial potential and F, R and T have their usual thermodynamic meanings. The ionic activity of Na^+ (109.5 mmol l^{-1}) in Cortland saline was taken from measurements with microelectrodes filled with the appropriate ionophore (Steiner et al. '79), while Cl^- was predicted to have the same relative activity (on a % basis) as Na^+ from theory for a solution of this ionic strength (Lee, '81). The activities of Na^+ and Cl^- in artificial urine were taken as equal to their measured concentrations.

Pharmaceuticals

DIDS (4,4 diiso thiocyanato-stilbene-2,2 disulfonic acid) was dissolved in dimethylsulphoxide (DMSO, 20 $\mu l \cdot mg^{-1}$) and added to the mucosal side at a final concentration of 10^{-4} M. Amiloride (10^{-4} M) and bumetanide (10^{-4} M) were dissolved using the same protocol as DIDS. DMSO concentrations in each drug experiment were $\leq$0.1 %. The same DMSO concentration was added to the mucosal side in both control periods. Chlorothiazide was dissolved in Cortland saline at a concentration of 10^{-3} M in the mucosal bathing medium. NaCN (sodium cyanide) was dissolved in artificial urine at a concentration of 10^{-3} M on the mucosal side. All drugs were obtained from Sigma Chemical Co., St. Louis, MO.

Analytical techniques

Saline Cl^- concentrations were measured by coulometric titration (model CMT10, Radiometer, Copenhagen) and artificial urine Cl^- concentrations were determined by

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

Samples of 40µl from the labeled side and 250µl from the unlabelled side of the Ussing chamber were added to 4.0 ml of Readysafe fluor (Beckman, Fullerton, CA). Radioactivity of ^{22}Na was determined on a Minaxi Autogamma 5000 counter (Packard Instrument Co., Downers Grove, IL), of ^{22}Na and ^{36}Cl on a Rackbeta 1217 liquid scintillation counter (LKB, Wallac, Turku, Finland) and of ^{36}Cl by a count subtraction procedure (see Wood et al, '84).

Data are presented as means ± 1 standard error unless indicated otherwise. Comparisons within and between treatments were analyzed by paired or un-paired *t*-tests (two-tailed and one tailed), as appropriate, at $P<0.05$ after checking for homogeneity of variance using F-tests with standard transformations applied as needed. In cases where these transformations were unsuccessful in normalizing the variance, the non-parametric Wilcoxon signed rank sum test was employed to test for significance. Regressions were performed by the method of least squares, and correlation evaluated by Pearson's linear

correlation coefficient. Kinetic values of Jmax (maximum transfer rate) and Km (substrate concentration at 50% of Jmax) were determined by Eadie-Hofstee plots, and kinetic plots were generated according to the Michaelis-Menten relationship.

Results

Mucosal saline/serosal saline condition

V_t and G_t of the isolated urinary bladder bathed in symmetrical saline were 0.10 ± 0.04 mV, serosa-positive and 9.15 ± 1.50 $mS \cdot cm^{-2}$, respectively, under open-circuit conditions. Short-circuit current (I_{sc}) of the preparation was <1 $\mu A \cdot cm^{-2}$. Comparison of both Na^+ and Cl^- observed flux ratios with their respective predicted flux ratios indicated active uptake of both ions under symmetrical conditions ($P<0.05$) (Fig. 1). Both unidirectional flux values and net flux values (all periods) of Na^+ were less than those for Cl^- ($P<0.05$).

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

When Cl^- was removed from the mucosal saline by replacement with gluconate, V_t increased to 11.6 ± 0.2 mV ($P<0.05$) and G_t decreased to 3.26 ± 0.4 mS·cm^{-2} ($P<0.05$) from their respective control values (see above). Net mucosa→serosa flux of Na^+ remained positive but was smaller ($P<0.05$; 2 of 3 periods) in the Cl^- free treatment group compared to control values (Fig. 3). Na^+ influx was also significantly lower ($P<0.05$) than control values by about 69% in all three periods of the Cl^- free treatment; the accompanying decrease in efflux was not significant (Fig. 3). Observed flux ratios for Na^+ in the Cl^- free solution remained different from the predicted ratios ($P<0.05$) as in the control treatment, indicating continued active transport of Na^+. For each of the one hour flux periods, Na^+ flux ratios in the Cl^- free solution were not significantly different from those in the control treatment ($P>0.05$).

The addition of either DIDS (10^{-4} M) or amiloride (10^{-4} M) or bumetanide (10^{-4} M) to the mucosal saline had no effect on Cl^- or Na^+ observed flux ratios or unidirectional flux of either ion (Table 1). There were no effects on V_t or G_t with any of the drug treatments. Treatment of the bladder with the three drugs simultaneously, under mucosal saline/serosal saline conditions, also had no effect on flux rates of Na^+ or Cl^- (data not shown; n = 4). Unidirectional influx of Na^+ was 10.8 ± 1.8 μmol·cm^{-2}·h^{-1} in the presence of the three drugs simultaneously, relative to control values of 10.7 ± 1.6 and 9.5 ± 3.1 μmol·cm^{-2}·h^{-1}. For Cl^- the unidirectional influx was 7.4 ± 1.4 μmol·cm^{-2}·h^{-1} in the presence of the three drugs, relative to control values of 7.7 ± 1.5 and 8.1 ± 2.3 μmol·cm^{-2}·h^{-1}. Chlorothiazide (10^{-3} M) treatment caused no change in unidirectional influx rates (data not shown; n = 6) of either Na^+ (Hour 1: 6.71 ± 2.36 and Hour 3: 5.59 ± 2.19 μmol·cm^{-2}·h^{-1} controls, Hour 2: 6.25 ± 2.17 μmol·cm^{-2}·h^{-1} chlorothiazide), or Cl^-

(Hour 1: 8.64 ± 2.96 and Hour 3: 7.69 ± 2.82 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ controls, Hour 2: 7.80 ± 2.59 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ chlorothiazide).

Mucosal urine/serosal saline conditions

When the isolated urinary bladder was bathed on the mucosal side with artificial urine, the V_t increased to 7.6 ± 0.4 mV and the G_t decreased to 1.47 ± 0.1 $mS \cdot cm^{-2}$, both significant relative to the saline/saline condition ($P<0.05$). Net fluxes of Cl^- and Na^+ were serosa→mucosa and unidirectional influx rates were reduced to 5-10% of the rates under saline/saline conditions.

With artificial urine on the mucosal surface, the net Cl^- flux rates were similar to net Na^+ flux rates except in Hour 1 ($P<0.05$) (Fig. 4). Cl^- unidirectional influx was larger than Na^+ influx ($P<0.05$) in two of the three hours and Cl^- unidirectional efflux was greater than Na^+ efflux in all periods ($P<0.05$). Of interest was the significant increase in the observed Cl^- flux ratio over time; the observed Na^+ flux ratio did not display any trend over time. The Na^+ and Cl^- observed flux ratios were significantly different ($P<0.05$) from each of their respective predicted flux ratios suggesting non-diffusive uptake of both ions.

With Na^+-free artificial urine bathing the mucosal surface, V_t (9.7 ± 0.4 mV) and G_t (1.1 ± 0.1 $mS \cdot cm^{-2}$) were not different from control, Na^+-replete artificial urine ($P>0.05$). Unidirectional influx and efflux of Cl^- were also the same in both treatments ($P>0.05$) (Fig. 5). The net movement of Cl^- was still serosa→mucosa, and there was no difference between the net flux rates in the two treatments. In all three periods, observed Cl^- flux ratios in the Na^+ free treatment were not significantly different ($P>0.05$) from the

observed ratios in the control treatment. Cl^- observed flux ratios were different from their respective predicted values for each bathing solution ($P<0.05$) indicating that non-diffusive transport was maintained. Again observed Cl^- flux ratios increased significantly over time.

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

With artificial urine bathing the mucosal surface of the urinary bladder, net NH_4^+ flux was 0.56 ± 0.87 μmol·cm^{-2}·h^{-1} mucosa→serosa. Net titratable acid flux was 0.91 ± 0.40 μmol·cm^{-2}·h^{-1}, mucosa→serosa. When Cl^- free or Na^+ free artificial urine bathed the mucosal side, net NH_4^+ flux was -0.60 ± 0.20 and -0.22 ± 0.40 μmol·cm^{-2}·h^{-1} respectively (serosa→mucosa), and net titratable acid flux was 0.22 ± 0.30 and -1.96 ± 0.90 μmol·cm^{-2}·h^{-1} (mucosa→serosa and serosa→mucosa) respectively. None of the above net acid or net NH_4^+ fluxes were significantly different from zero ($P>0.05$).

The addition of either DIDS (10^{-4} M) or amiloride (10^{-4} M) or bumetanide (10^{-4} M) to the mucosal artificial urine had no effect on Cl^- or Na^+ observed flux ratios or unidirectional flux of either ion (Table 2). There were no effects on V_t or G_t with any of the drug treatments.

There were no immediate, significant decreases in either Na^+ or Cl^- influx (m→s) rates upon addition of 10^{-3} M NaCN (CN^-) to the mucosal bathing solution (Hour 2, Fig. 7). However, there was a decreasing trend after the addition of CN^- and both Na^+ and Cl^-

influx rates were significantly reduced in the second control period (Hour 3, $P<0.05$). Cl^- influx was reduced by 50% from 0.55 ± 0.15 to 0.28 ± 0.03 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, and Na^+ influx by 41% from 0.37 ± 0.10 to 0.22 ± 0.05 $\mu mol \cdot cm^{-2} \cdot h^{-1}$. Concomitant with the reduced influx, G_t decreased in Hour 3 (2.13 ± 0.36 to 1.93 ± 0.30 $mS \cdot cm^{-2}$, $P<0.05$), and V_t increased in both Hour 2 and 3 (4.09 ± 0.51 mV to 4.94 ± 0.51 and 6.14 ± 0.50 mV, respectively, $P<0.01$). Note that while CN^- caused a significant reduction in the observed flux ratios of Cl^- in both Hours 2 and 3 ($P<0.05$), the observed ratios for both Na^+ and Cl^- remained significantly higher than the predicted values, indicating that active transport persisted to some degree.

Na^+ influx rates (ie "Na^+ kinetics") in response to increases in Na^+ concentration in the artificial urine (control) and low Cl^- artificial urine solutions bathing the mucosal surface are shown in Fig. 8. Analysis of the kinetic curves by Eadie-Hofstee plots indicated Jmax and Km under control conditions were 6.1 ± 2.3 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and 34 ± 20 mM respectively. The solution low in Cl^- decreased ($P<0.05$) the Jmax (2.1 ± 0.56 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) of the Na^+ influx but did not change Km (27 ± 12 mM) ($P>0.05$). Comparison of Na^+ influx rates between the two mucosal solutions demonstrated significant differences in the 40-50 and 110-150 mM range ($P<0.05$). Unidirectional influx of Na^+ decreased 80% and net uptake was abolished (not shown) in the 110-150 mM range when the solution low in Cl^- bathed the mucosal surface.

Cl^- influx rates in response to increases in Cl^- concentration (ie "Cl^- kinetics") in the artificial urine and low Na^+ mucosal solutions are shown in Figure 9. The control and low Na^+ curves had different ($P<0.05$) Jmax values of 11.4 ± 5.1 and 2.4 ± 0.16 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ and similar ($P>0.05$) Km values of 37 ± 26 and 16 ± 2 mM respectively.

Control Cl^- influx rates were significantly greater than those in the low Na^+ treatment at mucosal Cl^- concentration ranges of 5-10, 40-50 and 110-150 mM ($P<0.05$). In the range of 110-150 mM of Cl^- in the mucosal low Na^+ artificial urine solution, unidirectional influx of Cl^- decreased 85% from control values and the net uptake of Cl^- was abolished (not shown). There were no significant differences between the Km and Jmax values of the Na^+ and Cl^- control curves.

Discussion

Urinary bladder Na^+ and Cl^- transport

The negligible transepithelial potential (~0.1 mV) and apparent active absorption of Na^+ and Cl^- (Fig. 1) suggests an electrically neutral uptake mechanism for these ions in the rainbow trout urinary bladder. Electro-neutral transport is a well documented characteristic of many fish transporting epithelia such as the flounder intestine (Field et al., '78), sculpin intestine (House and Green, '65), marine eel intestine (Skadhauge, '74), SW-adapted (Renfro, '77) and FW-adapted flounder urinary bladder (Demarest and Machen, '84), and the urinary bladder of the European strain of rainbow trout (Lahlou and Fossat, '71).

Replacement of either Na^+ or Cl^- in the mucosal saline significantly decreased influx of the co-transport ion by 56 and 69% respectively (Figs. 2 and 3). In the kinetics experiments, the absorption of Na^+ and Cl^- appeared saturable at high mucosal concentrations of substrate. However, under these conditions, removal of the Cl^- or Na^+ from the mucosal bath caused an 80-85% inhibition of the absorption rate of the other ion (Na^+ or Cl^-) (Figs. 8 and 9). These results suggest that a Na^+-Cl^-dependent, carrier-

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

Under more realistic conditions representative of those occurring in the animal *in vivo*, with artificial urine bathing the mucosal surface, the active unidirectional influx of Na^+ and Cl^- continued, though the net balance was negative (Fig. 4). However, unlike

results reported under symmetrical saline conditions, there was no effect on the rate of transport of Na^+ or Cl^- upon the removal of the Cl^- or Na^+, respectively (Figs. 5 and 6). These data suggest that at lower mucosal concentrations of Na^+ and Cl^-, coupled transport did not occur. These results are more consistent with data reported by Marshall ('86) which indicated active independent transport of Na^+ and Cl^- in the urinary bladder of brook trout (*Salvelinus fontinalis*). However unlike results from Marshall's ('86) study, the removal of either Na^+ or Cl^- at higher levels of Na^+ and Cl^- substantially reduced the absorption rates of Cl^- and Na^+, respectively. Furthermore, analyses of net acid and NH_4^+ fluxes in the present study argue against acid-base linkage of an independent transport mechanism.

Under these "more realistic" conditions, bladder epithelia were also in negative balance (net loss of Na^+ and Cl^-) (Fig. 4). While this might indicate that the bladder was not behaving as efficiently as *in vivo*, alternately it is possible that the bladder may be "poised" kinetically to transport at the concentrations of Na^+ and Cl^- present in ureteral urine (7.12 mM and 5.21 mM, respectively; Curtis and Wood, '91, '92) and not for the concentrations in the final "polished" urine (2.12 mM and 3.51 mM, respectively) which we supplied. Indeed, comparison of the kinetic curves for influx (Figs. 8, 9) with the unidirectional flux data of Fig. 4, suggests that both Na^+ and Cl^- influx would be markedly higher at 5.2-7.1 mM than at 2.1-3.5 mM, and that influx and efflux would be in approximate balance at these mucosal concentrations.

Effects of pharmaceuticals

Mucosal addition of DIDS (Cl^-/HCO_3^- exchange blocker), amiloride (Na^+/H^+ exchange and Na^+ channel inhibitor) or bumetanide (Na^+-Cl^- and Na^+-K^+-Cl^- co-transport blocker) all had no effect on the transport (absorption/extrusion) of Na^+ and/or Cl^- (Tables 1 and 2) in either symmetrical saline or mucosal artificial urine conditions. A combination of the three drugs was also ineffective in blocking Na^+ or Cl^- transport under symmetrical saline conditions. Non-diffusive, apparent active absorption of Na^+ and Cl^- continued in the presence of each drug, which reinforces the suggestion of a dual mechanism type of transport. Thus, for example, if two transport systems were operating in parallel (Na^+ and Cl^- independent and dependent mechanisms), addition of DIDS would impair anion exchange but co-transport mechanisms would be unaffected. In fact in saline/saline conditions, DIDS decreased Cl^- absorption and bumetanide decreased Cl^- and Na^+ absorption, but not significantly. However, in each case active transport persisted. The use of the diuretic chlorothiazide at 10^{-3} M was also ineffective in blocking Na^+ and/or Cl^- influx. The thiazide diuretics (hydrochlorothiazide, chlorothiazide) are known Na^+/Cl^- cotransporter blockers as well as inhibitors of carbonic anhydrase (Weiner, '90). Winter flounder urinary bladder studies have shown that 10^{-4} M hydrochlorothiazide or metolazone (also a diuretic) were sufficient to inhibit Na^+ and Cl^- cotransport (Stokes, '84). Both diuretics were equally effective in reducing Na^+ and Cl^- absorption except that full hydrochlorothiazide effects were witnessed in 2-3 min. whereas metolazone appeared to take up to 60 min. (Stokes, '84). The present studies have shown that 10^{-3} M chlorothiazide was ineffective in inhibiting cotransport after a 45 min. pre-incubation period (see Methods), even though the same concentration of

chlorothiazide was responsible for the complete inhibition of Na^+ and Cl^- absorption in rat early distal tubule (Ellison et al., '87). The carbonic anhydrase inhibition by thiazide diuretics could complicate interpretation, but lack of any effect of the chlorothiazide made this an immaterial consideration in the present study.

The existence of a dual dependent/independent transport mechanism is a possible explanation for the lack of effect with use of amiloride, bumetanide, chlorothiazide and DIDS, but the results of the combination of amiloride, bumetanide and DIDS experiment make this difficult to accept. However, physical explanations may be responsible for the lack of change in Na^+ or Cl^- transport. The bladder preparation may be such that effective penetration by the drugs does not occur. This would occur if the sites that are primarily acted on by the drugs were obscured or not readily accessible to the drug molecules. The results of the cyanide experiment were informative in this regard, and indicate that any conclusions based on pharmacological results must be tempered by recognition of this slow penetration problem.

Cyanide was employed as a means to establish that active transport did indeed occur under asymmetric artificial urine and saline conditions, and to evaluate the time course of drug penetration. Cyanide was tested under the worst case scenario of asymmetric conditions, rather than under saline/saline conditions. In the latter, the evidence for active transport was obvious – *i.e.* net mucosal-to-serosal transport of both Na^+ and Cl^- in the absence of any driving gradients. However, under artificial urine/saline conditions, the evidence for the occurrence of active transport based solely on the Ussing flux ration equation might be considered weaker, since concentration, electrical, and osmotic gradients all existed across the preparation, and net mucosal-to-

serosal transport was not occurring. Although cyanide was not immediately effective, there were decreasing trends in the influx rates of both Na^+ and Cl^- which became significant during the final control period, even though cyanide had been removed from the mucosal bath by this time. Cyanide, therefore, appeared to cause a slowly developing inhibition of Na^+ and Cl^- unidirectional influx at low mucosal concentrations of Na^+ and Cl^- when net efflux was occurring. This, in conjunction with the Ussing flux ratio criterion (Fig. 7), indicates that even during low mucosal Na^+ and Cl^- concentrations, the low, unidirectional influx is indeed active. Although the influx of both ions was reduced by cyanide, the Ussing flux ratio shows that active transport was not completely abolished during the second and third hour (cyanide and control/post cyanide periods, respectively). The length of time for cyanide effectiveness along with the retention of some active transport seems to indicate that accessibility may indeed be hampering the action of the other drugs. It must also be remembered, however, that anaerobic ATP supply to transport systems can persist in the presence of cyanide.

Further conclusions

The present study observed electroneutral co-transport of Na^+ and Cl^- in the urinary bladder of *O. mykiss* under symmetrical saline conditions, thus reinforcing previous studies performed on the urinary bladder of the closely related European strain of rainbow trout (*Salmo irideus*) (Fossat and Lahlou '79a, '82). However, there was not 100% coupling of Na^+ and Cl^- transport as reported in *S. irideus* (Fossat and Lahlou '79a), and relevant pharmacological agents (bumetanide, chlorothiazide) did not provide supporting evidence for the presence of a co-transport system. Experiments performed on

S. irideus did not use an artificial urine, and Na^+ and Cl^- transport measured at these more realistic levels of mucosal NaCl in the present study appeared to be independent processes. Therefore, the NaCl transport process in *O. mykiss* appears to be only partially coupled; transport mechanisms are both dependent upon and independent of the other ion. Electrical parameters of *S. irideus* (Fossat and Lahlou '79a, '82) and *O. mykiss* (present study) were very similar under symmetrical saline conditions (negligible V_t, I_{sc} $<1 \mu A \cdot cm^{-2}$ and G_t = 3-4 $mS \cdot cm^{-2}$). In contrast, the urinary bladder of the brook trout (*Salvelinus fontinalis*), exhibited independent transport of Na^+ and Cl^- at both higher and lower levels of mucosal NaCl (Marshall '86). The transport of NaCl, under saline/saline conditions, was reported to be electroneutral, however V_t (6.7 mV) and G_t (0.23 $mS \cdot cm^{-2}$) were rather different from those of the present study. It appears, therefore, that the brook trout transports Na^+ and Cl^- in a very different manner than the rainbow trout (*O. mykiss*).

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

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

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

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

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

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

Table 2. Effect of the 10^{-4}M mucosal addition of DIDS, amiloride or bumetanide on unidirectional efflux (Jsm), influx (Jms) and flux ratios of Cl^- and Na^+ and on V_t and G_t in urinary bladder of *Oncorhynchus mykiss* (n = 4) bathed on mucosal side with artificial urine under open circuit conditions.

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

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

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

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

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

Table 3. Effect of the 10^{-3}M mucosal addition of NaCN on unidirectional efflux (Jsm), influx (Jms) and flux ratios of Cl^- and Na^+ and on V_t and G_t in urinary bladder of *Oncorhynchus mykiss* (n = 6) bathed on mucosal side with artificial urine under open circuit conditions.

Experimental Period	Vt (mV)[a]	Gt ($mS \cdot cm^{-2}$)[b]	Jsm ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	Jms ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	Jms/Jsm Observed	Jms/Jsm Predicted
Cl^-						
Control (Hour 1)	-6.41 ± 0.53	2.13 ± 0.36	3.00 ± 0.77	0.55 ± 0.15	0.35 ± 0.17	0.03 ± 0.007
CN^- (Hour 2)	-5.56 ± 0.54[f]	2.14 ± 0.30	3.40 ± 0.61	0.37 ± 0.06[d]	0.14 ± 0.04	0.03 ± 0.004
Control (Hour 3)	-4.36 ± 0.52[f]	1.93 ± 0.30[g]	3.50 ± 0.80	0.28 ± 0.03[e]	0.14 ± 0.06	0.03 ± 0.003
Na^+						
Control (Hour 1)			2.48 ± 0.50	0.37 ±0.10	0.27 ± 0.10	0.04 ± 0.006
CN^- (Hour 2)			2.72 ± 0.63	0.27 ± 0.07[d]	0.17 ± 0.09	0.04 ± 0.002
Control (Hour 3)			2.14 ± 0.58	0.22 ± 0.05[e]	0.30 ± 0.21	0.03 ± 0.000

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

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

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

[d] Paired *t*-test, one-tailed, Hour 2 *vs.* Hour 1, $P>0.05$.

[e] Paired *t*-test, one-tailed, Hour 3 *vs.* Hour 1, $P<0.05$.

[f] Paired *t*-test, two-tailed, *vs.* Hour 1, $P<0.01$.

[g] Paired *t*-test, one-tailed, *vs.* Hour 1, $P<0.05$.

FIGURE LEGENDS

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

Figure 2. Observed and predicted flux ratios for the unidirectional movements of Cl^- with control or Na^+-free saline bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Cl^- in control and in Na^+-free mucosal saline respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- in control and in Na^+-free mucosal saline respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant differences from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- in control and in Na^+-free mucosal saline. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar) or net

flux (beside bar) ($P<0.05$, un-paired t-test, two-tailed) of Cl^- between the two treatment groups.

Figure 3. Observed and predicted flux ratios for the unidirectional movements of Na^+ with control or Cl^--free saline bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Na^+ in control and in Cl^--free mucosal saline respectively, hatched and crossed bars represent the predicted flux ratios for Na^+ in control and in Cl^--free mucosal saline respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Na^+ in control and in Cl^--free mucosal saline. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar) or net flux (beside bar) ($P<0.05$, un-paired t-test, two-tailed) of Na^+ between the two treatment groups.

Figure 4. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *Oncorhynchus mykiss* bathed with artificial urine on the mucosal surface. Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+ respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+ respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out})

and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar) or net flux (beside bar) ($P<0.05$, paired t-test, two-tailed) between Cl^- and Na^+.

Figure 5. Observed and predicted flux ratios for the unidirectional movements of Cl^- with control or Na^+-free artificial urine bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Cl^- in control and in Na^+-free mucosal artificial urine respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- in control and in Na^+-free mucosal artificial urine respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- in control and in Na^+-free mucosal artificial urine. There were no significant differences ($P>0.05$) between the two treatment groups.

Figure 6. Observed and predicted flux ratios for the unidirectional movements of Na^+ with control or Cl^--free artificial urine bathing the mucosal surface of urinary bladder epithelia of FW *Oncorhynchus mykiss*. Open bars and solid bars represent the observed flux ratios for Na^+ in control and in Cl^--free mucosal artificial urine respectively, hatched and crossed bars represent the predicted flux ratios for Na^+ in control and in Cl^--free mucosal artificial urine respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio

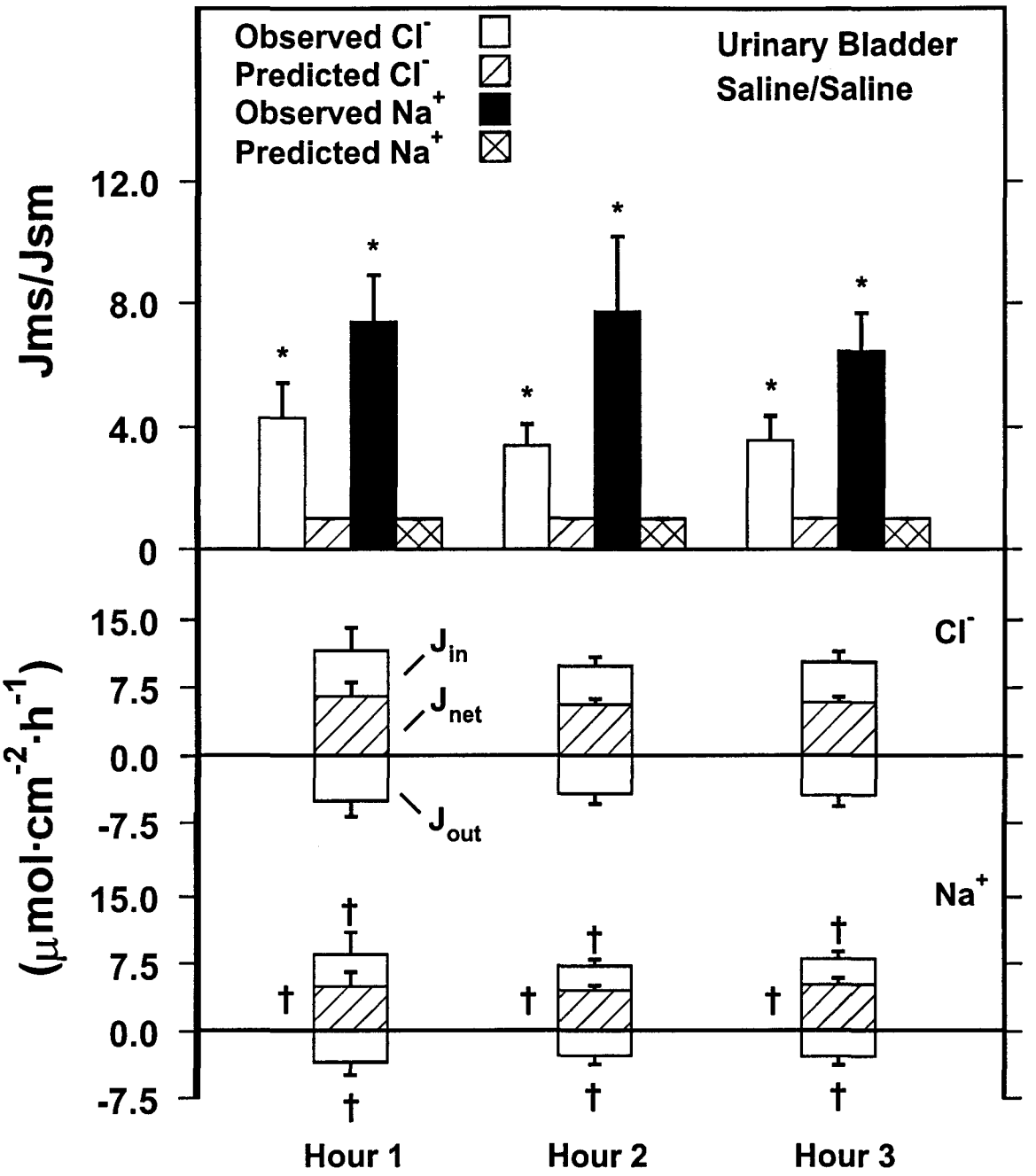
($P<0.05$, paired t-test, two-tailed) implying evidence for non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Na^+ in control and in Cl^--free mucosal artificial urine. There were no significant differences ($P>0.05$) between the two treatment groups.

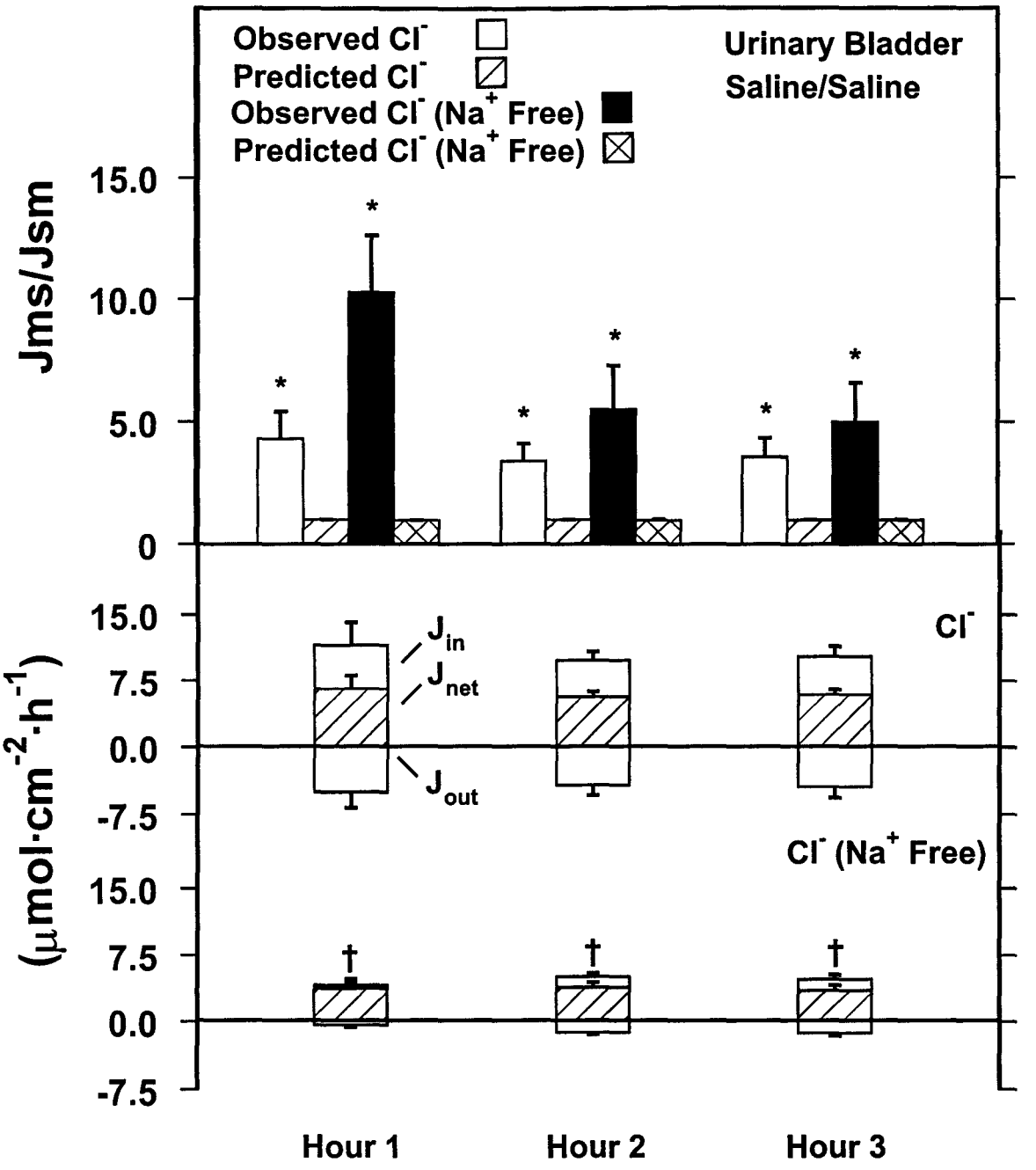
Figure 7. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *Oncorhynchus mykiss* bathed with artificial urine on the mucosal surface with addition of 10^{-3} M cyanide (NaCN) during the second period (Hour 2). Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+ respectively, hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+ respectively based on the Ussing flux ratio criterion. Means ± 1SEM (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P<0.05$, Wicoxon signed rank sum test), indicating non-diffusive transport. Pound sign (#) indicates observed flux ratio significantly lower than Hour 1 ($P<0.05$, Wilcoxon signed rank sum test). Unidirectional influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant difference from unidirectional influx of Hour 1 ($P<0.05$, paired t-test, one-tailed).

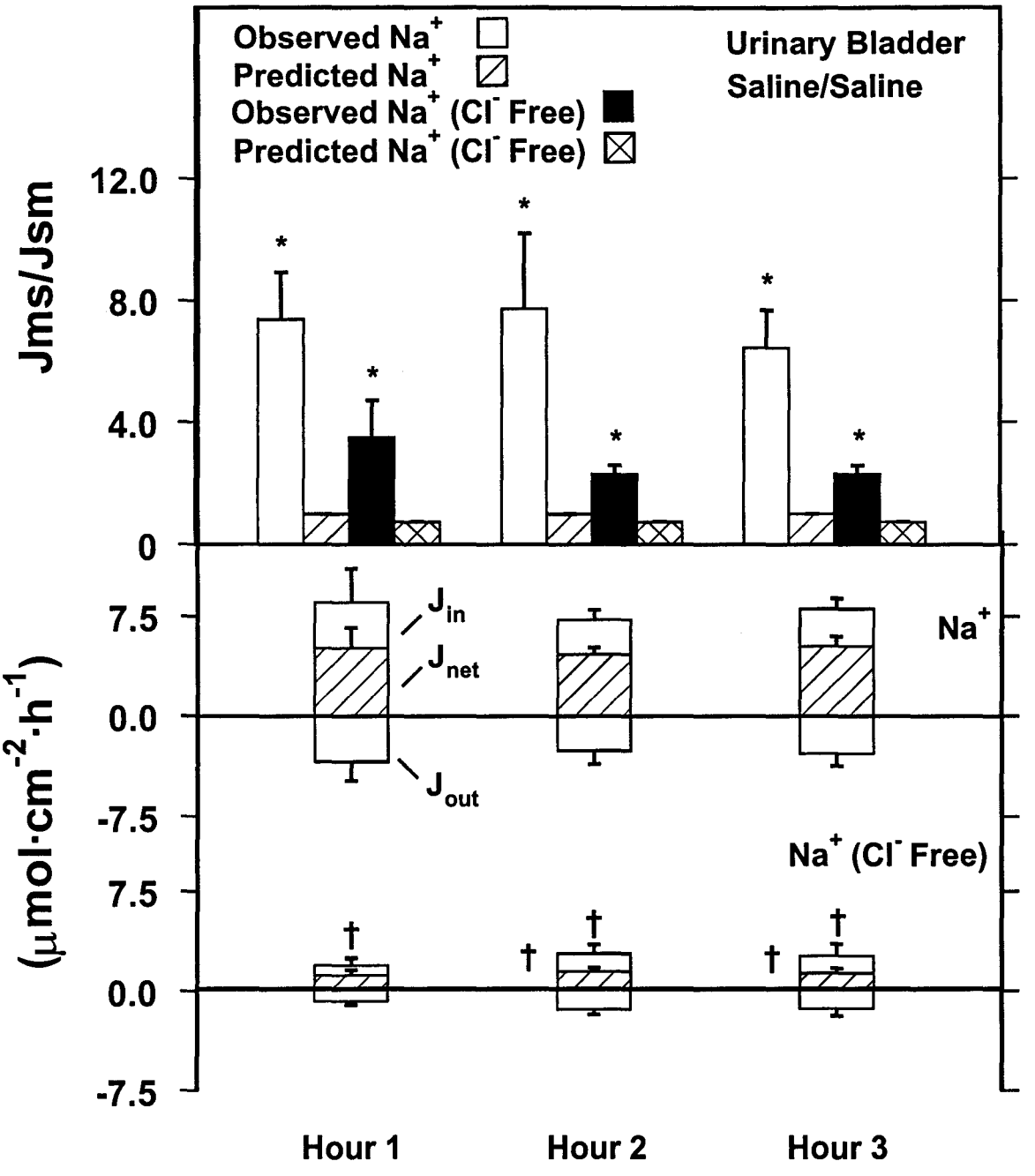
Figure 8. Relationship of mucosal to serosal Na^+ flux (Na^+ influx) to the concentration (mM) of Na^+ in control solutions (closed circles) and low Cl^- solutions (open circles). Serosal medium was Cortland's saline and mucosal medium was artificial urine with varying concentrations of Na^+ and/or Cl^-. Vertical and horizontal lines represent ± the standard error of mean; n = 4-8 for each concentration range of (0-5, 5-10, 10-20, 40-50

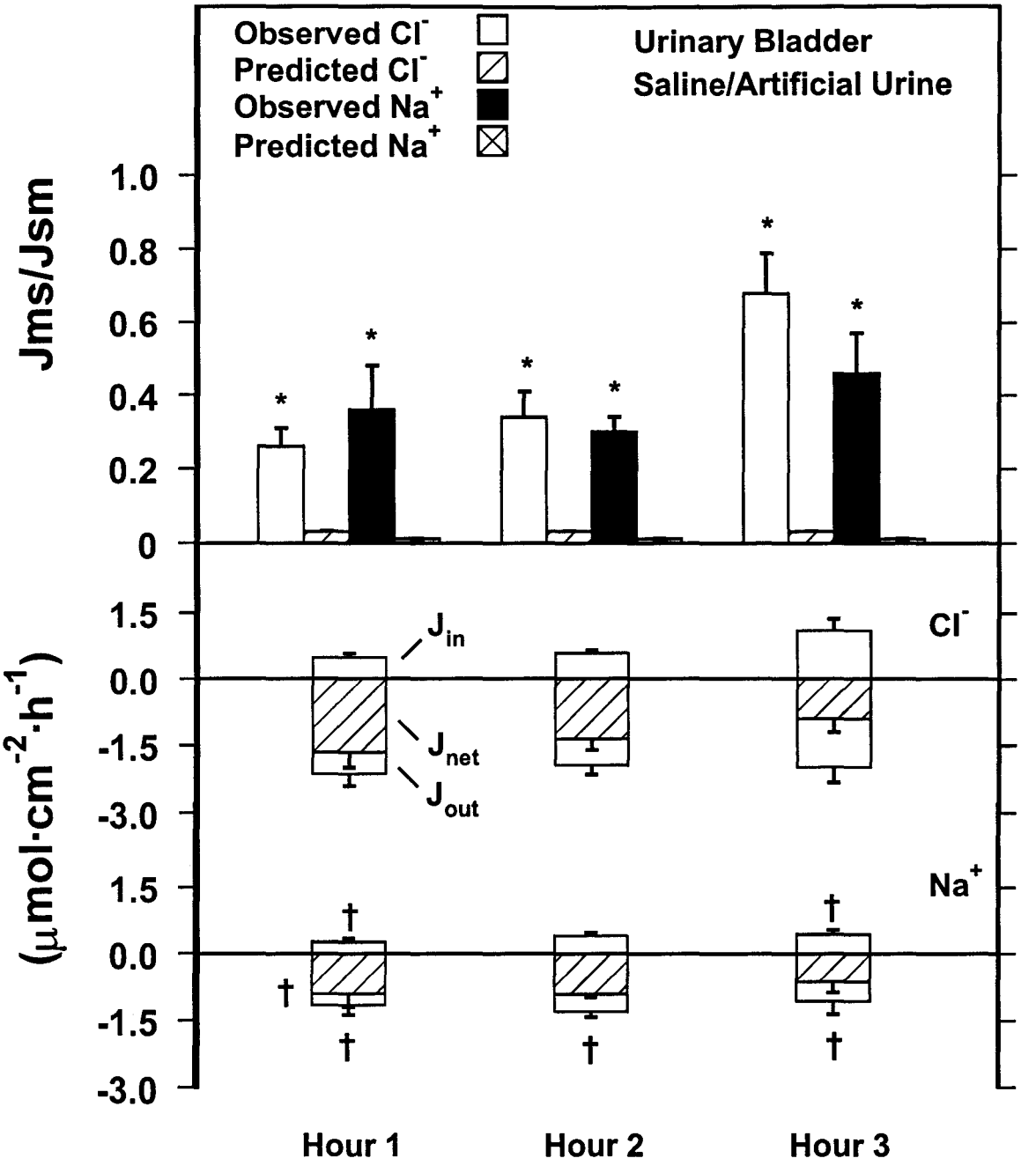
and 110-150 mM). Curves were best fit for Michaelis-Menten kinetics. Asterisk indicates significant difference between concentration range of each treatment ($P<0.05$, un-paired t-test, two-tailed).

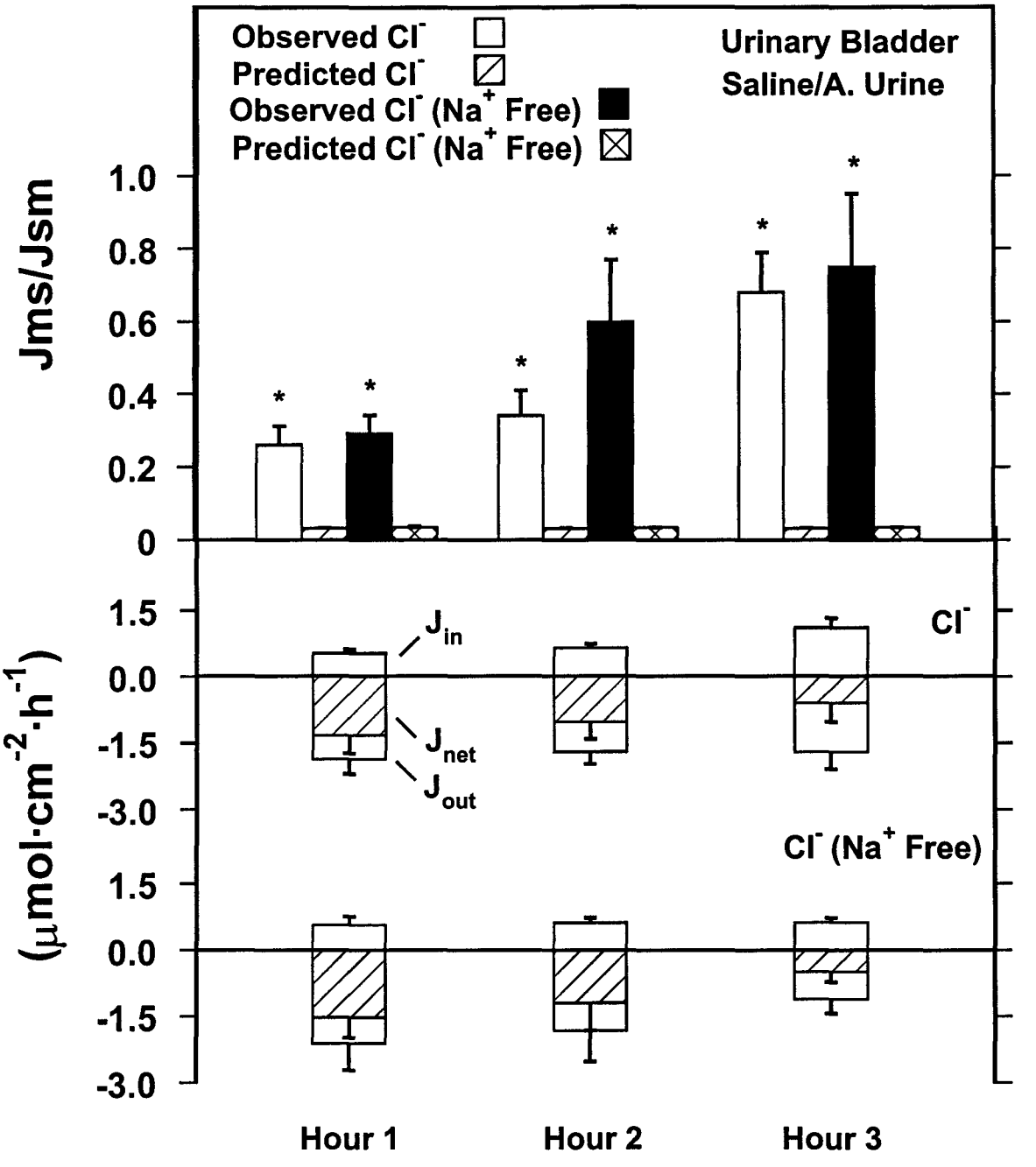
Figure 9. Relationship of mucosal to serosal Cl^- flux to the concentration (mM) of Cl^- in control solutions (closed circles) and low Na^+ solutions (open circles). Serosal medium was Cortland's saline and mucosal medium was artificial urine with varying concentrations of Na^+ and/or Cl^-. Vertical and horizontal lines represent ± the standard error of mean; n = 4-8 for each concentration range of (0-5, 5-10, 40-50 and 110-150 mM, excluding 10-20 mM range for low Na^+ artificial urine where no observations were recorded). Curves were best fit for Michaelis-Menten kinetics. Asterisk indicates significant difference between concentration range of each treatment ($P<0.05$, un-paired t-test, two-tailed).

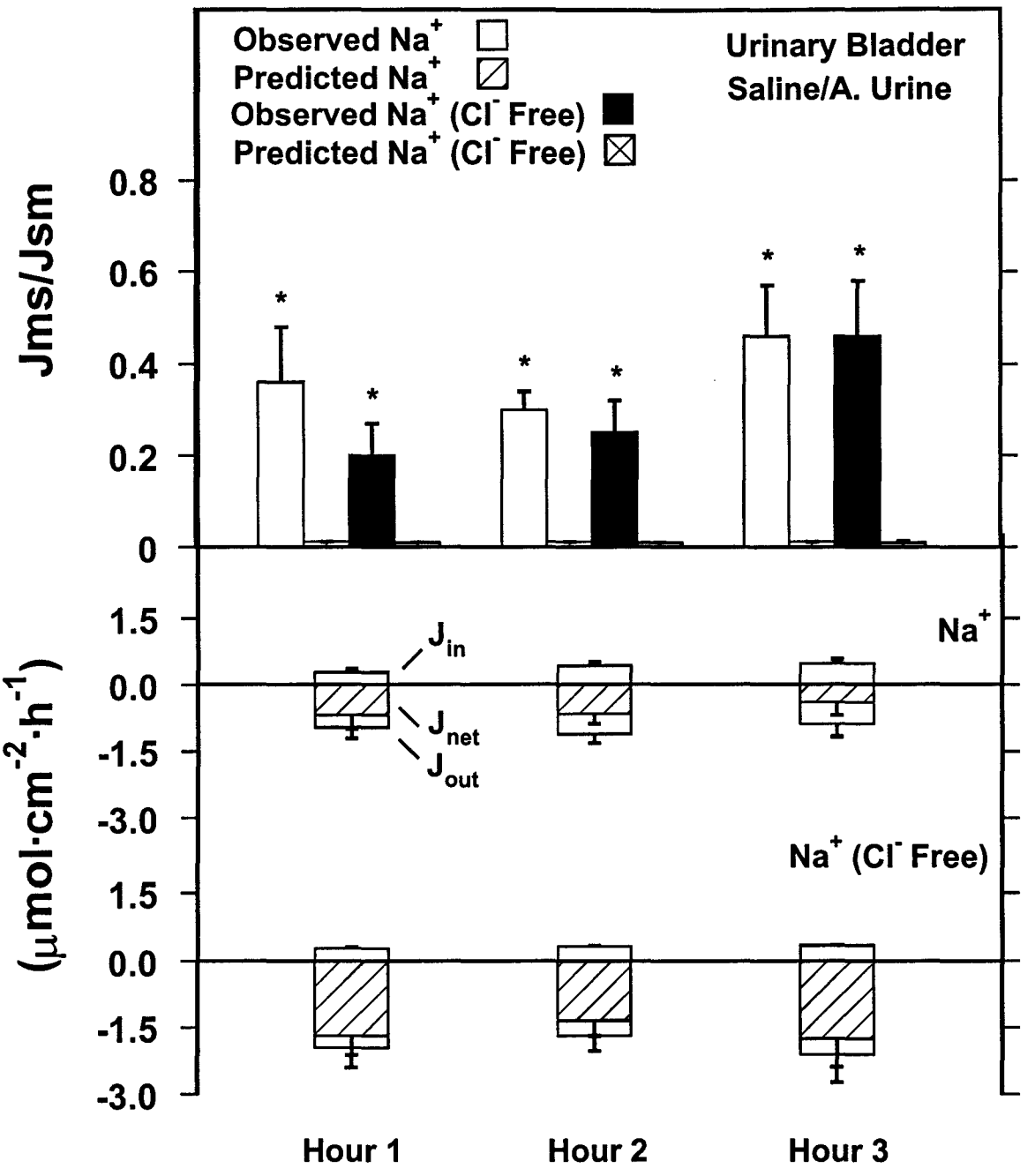

Observed Cl-
Predicted Cl-
Observed Na+
Predicted Na+
Urinary Bladder
Saline/Saline
Jms/Jsm
12.0
8.0
4.0
0
15.0
7.5
0.0
-7.5
(μmol·cm-2·h-1)
Jin
Jnet
Jout
Cl-
Na+
Hour 1
Hour 2
Hour 3

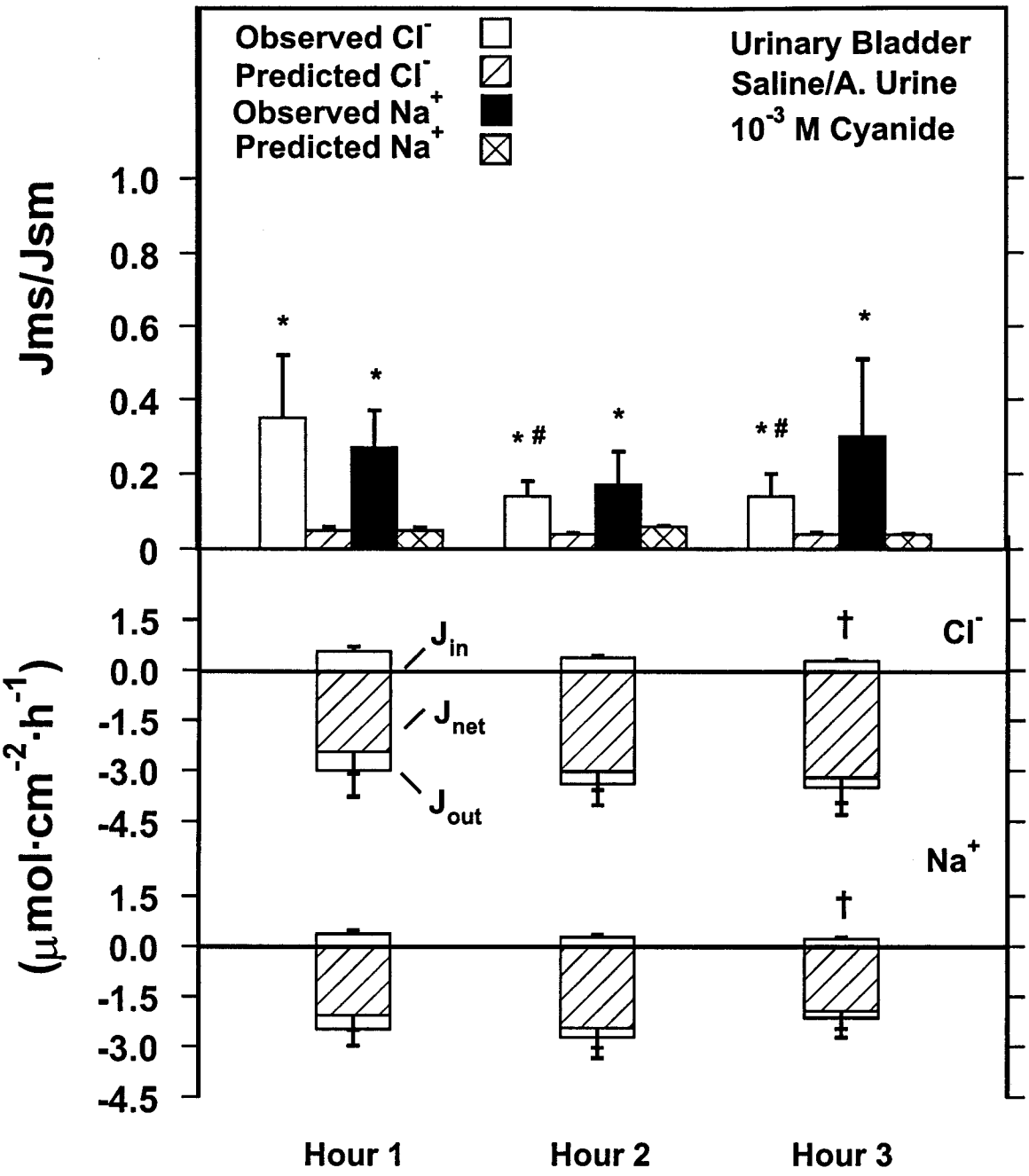

Observed Cl-
Predicted Cl-
Observed Cl- (Na+ Free)
Predicted Cl- (Na+ Free)
Urinary Bladder
Saline/Saline
Jms/Jsm
15.0
10.0
5.0
0
*
15.0
7.5
0.0
-7.5
Jin
Jnet
Jout
Cl-
Cl- (Na+ Free)
(μmol·cm-2·h-1)
†
Hour 1
Hour 2
Hour 3


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

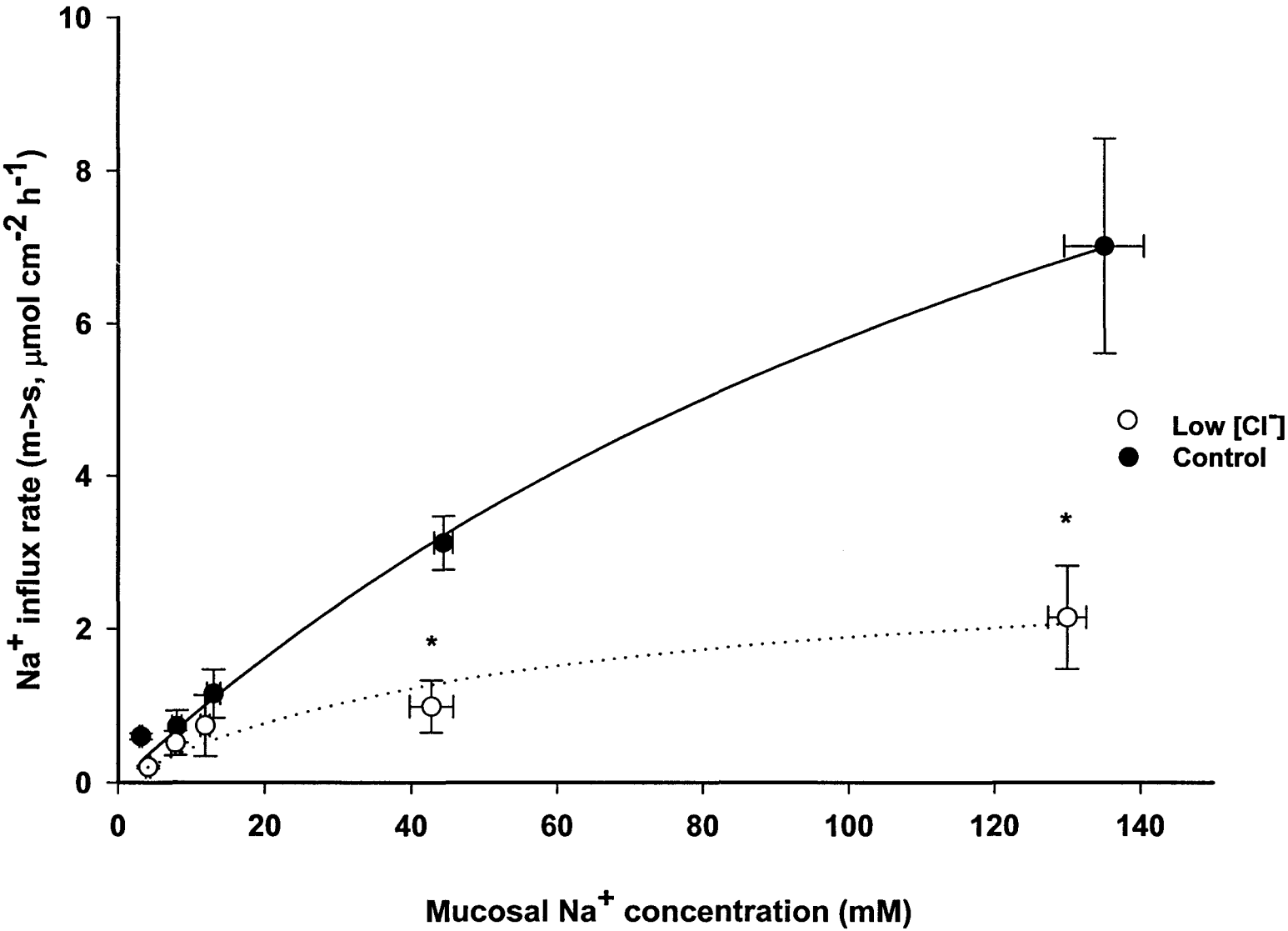

Observed Cl⁻
Predicted Cl⁻
Observed Na⁺
Predicted Na⁺
Urinary Bladder
Saline/Artificial Urine
Jms/Jsm
(μmol·cm⁻²·h⁻¹)
1.0
0.8
0.6
0.4
0.2
0
1.5
0.0
-1.5
-3.0
J_in
J_net
J_out
Cl⁻
Na⁺
*
†
Hour 1
Hour 2
Hour 3

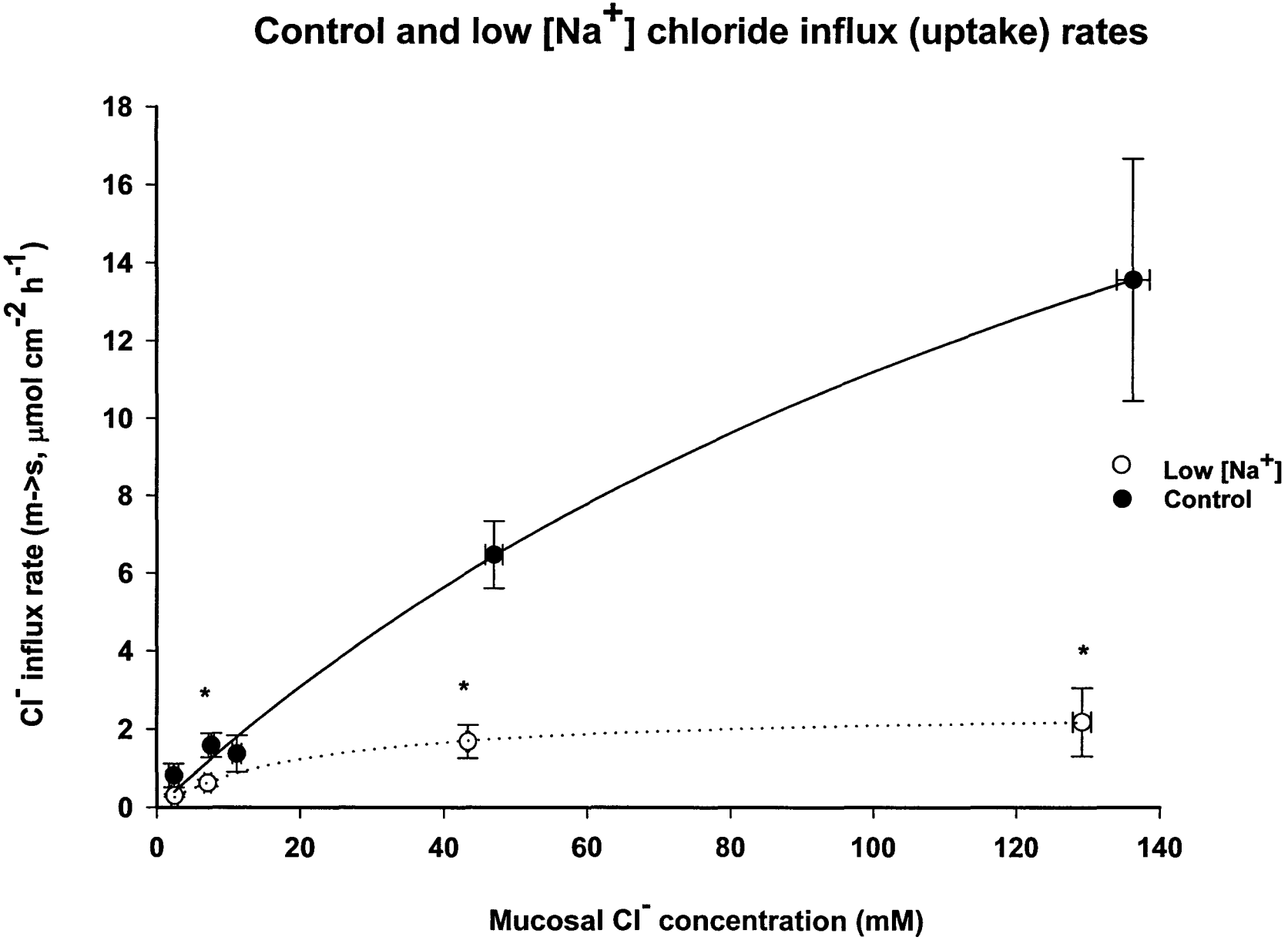

Observed Cl⁻
Predicted Cl⁻
Observed Cl⁻ (Na⁺ Free)
Predicted Cl⁻ (Na⁺ Free)
Urinary Bladder
Saline/A. Urine
Jms/Jsm
(μmol·cm⁻²·h⁻¹)
1.0
0.8
0.6
0.4
0.2
0
1.5
0.0
-1.5
-3.0
J_in
J_net
J_out
Cl⁻
Cl⁻ (Na⁺ Free)
Hour 1
Hour 2
Hour 3


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


Observed Cl-
Predicted Cl-
Observed Na+
Predicted Na+
Urinary Bladder
Saline/A. Urine
10-3 M Cyanide
Jms/Jsm
(μmol·cm-2·h-1)
1.0
0.8
0.6
0.4
0.2
0
1.5
0.0
-1.5
-3.0
-4.5
Jin
Jnet
Jout
Cl-
Na+
Hour 1
Hour 2
Hour 3

Control and low [Cl^-] sodium influx (uptake) rates




Control and low [Na+] chloride influx (uptake) rates
Cl- influx rate (m->s, µmol cm-2 h-1)
Mucosal Cl- concentration (mM)
Low [Na+]
Control
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
*
*
*