BRANCHIAL AND RENAL

UREA TRANSPORT MECHANISMS IN THREE TELEOST FISH

By

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A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

McMaster University

UREA TRANSPORT MECHANISMS IN TELEOST FISH

DOCTOR OF PHILOSOPHY (2002) McMaster University

(Biology) Hamilton, Ontario

TITLE: Branchial and renal urea transport mechanisms in three teleost fish

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NUMBER OF PAGES: xxii, 276

Abstract

Physiological and pharmacological approaches were used to characterize the pulsatile, facilitated diffusion urea transport mechanism (tUT) known from molecular evidence to be present in the gills of the facultatively ureotelic gulf toadfish (*Opsanus beta*). Using those findings, potential urea transport mechanisms in the kidney of the toadfish and in both excretory organs of the ammoniotelic plainfin midshipman (*Porichthys notatus*), closely related to the toadfish, and the ammoniotelic freshwater rainbow trout (*Oncorhynchus mykiss*), distantly related to the toadfish, were investigated.

With respect to the gulf toadfish, the measured branchial permeabilities to urea and the urea analogues, acetamide and thiourea, were similar in the periods between pulses when tUT was not activated. During pulsatile excretion (when tUT was activated), the permeabilities of all three substances increased, however urea permeability was significantly greater than analogue permeability. This translated to a branchial clearance of urea > acetamide >> thiourea. Midshipmen and trout displayed a similar pattern of branchial urea and analogue handling, suggesting a facilitated diffusion urea transport mechanism like that of the gulf toadfish.

In all three species, the movement of urea into the kidney tubule was against an apparent concentration gradient, suggesting the presence of an active transport mechanism. The secretion clearance rate (SCR) of urea, acetamide and thiourea in toadfish and midshipmen displayed a pattern where $SCR_{thiourea} > SCR_{urea} > SCR_{acetamide}$. In contrast, the pattern of renal handling of these substances in trout was $SCR_{urea} =$

$SCR_{acetamide} > SCR_{thiourea}$. The difference between trout and batrachoidid fish is likely a combination of different urea transport mechanisms and different kidney morphology; the handling of urea in batrachoidid fish was not correlated with Na^+ as observed in trout, and the kidneys of toadfish and midshipmen are aglomerular while the kidney of the trout is glomerular. Based on the different patterns of urea and analogue handling observed in the kidney and gill in all three species, plus the fact that urea is moving against a concentration gradient in the kidney, it is unlikely that the urea transport mechanisms in the two organs are the same.

When infused with consecutive exogenous urea loads, initial plasma and urine urea concentrations steadily increased in toadfish and midshipmen. In the toadfish, there was no change in pulse frequency and only a very weak relationship between pulse size (measured as branchial excretion during pulsatile excretion of urea) and plasma urea concentration, suggesting that the branchial excretion mechanism was already saturated at normal plasma urea concentrations. In contrast, the branchial excretion rate of urea in midshipmen was linearly correlated with plasma urea concentration with no observable transport maximum. In both toadfish and midshipmen, renal urea secretion rate maintained a strong linear relationship to plasma urea levels with no observable transport maximum, suggesting that the renal secretory transport mechanism could not be saturated even at plasma levels well above normal.

Further characterization of the branchial urea excretion mechanism in the trout was achieved by using an *in vitro* membrane vesicle preparation, isolating the basolateral membrane of the gill. Basolateral membrane vesicles (BLMV) demonstrated urea uptake

with a saturable component (K_m = 1.17 ± 0.43 mmol·l^{-1}; V_{max} = 0.42 ± 0.08 μmol·mg protein^{-1}·h^{-1}) at low, physiologically relevant urea concentrations and a non-saturable component at urea concentrations of up to 17.5 mmol·l^{-1}. Uptake of urea by BLMV was inhibited by the presence of the urea analogue, thiourea, was sensitive to phloretin, a potent blocker of UT-type urea transport mechanisms, and altered by temperatures above and below the temperature of acclimation.

Cortisol and glucocorticoid receptors appear to be involved in the regulation of urea production in the ammoniotelic rainbow trout (*Oncorhynchus mykiss*). Fish implanted with cortisol alone had 3-fold greater plasma and urine urea concentrations relative to fish of all other groups, including fish with similarly high cortisol levels but implanted with RU486. In addition, a positive correlation was observed between plasma cortisol and plasma urea concentration in cortisol-implanted fish that was abolished in fish implanted with cortisol + RU486. Compared to sham-implanted fish, cortisol-treated fish had significantly elevated branchial (two-fold higher) and urinary (three-fold higher) excretion rates of urea but not ammonia. However, as branchial and renal urea clearance were unaffected, there appears to be no stimulation or inhibition of urea excretion mechanisms in the gill or kidney separate from effects due to changes in plasma urea concentrations.

Acknowledgements

"If I have seen further than others, it is by standing on the shoulders of giants."
--Sir Isaac Newton

The obscene length of time I have spent at McMaster University multiplied by the turnover rate in Chris Wood's lab could feasibly result in an acknowledgement "chapter." I will try to be short and sweet. I guess the best place to start is the beginning.

Thank-you to those around when I was just starting out: Ian Morgan, Jim Keiffer, Tyler Linton, Jackie Dockray, Katie Gilmour, Nathan Webb, Jim "MacGyver" McGeer, Lydia Hollis (bat woman), Derek Alsop and Mary Fletcher (a couple that never stops laughing), Bob, Yuxiang Wang and Mike Wilkie (we survived!!), Gudrun DeBoeck and Nic Bury; all of you were a huge influence on me.

Thanks to the most recent gang: Sara Croke, Wendy McFarlane, Lisa Taylor (tines, migs and other film clips), Colin and Crystal Brauner, Collins Kamunde, Tammie Morgan, Carrie Ho, Christine Guadagnolo (the best hugger in the world), Eric and Mich Pane and baby Kobe, Sean Gregory, Joe Rodgers, Reinier Mann, and, unforgettably, Kath Sloman, who will go down in history as the only Woodite (to date) to leave the Phoenix in an ambulance; life in the Wood lab wouldn't have been the same without you.

Thanks to Kathy G., Marg, Hannah and especially Barb Reuter and Pat Hayward for their smiles, encouragement, advice and endless hugs when needed.

Thanks to John Lott for his help during the last few months of experiments.

Thanks to Pat Walsh who has taught me there is more to life than fish.

Thank-you to my supervisory committee: Patricia Wright, Mike O'Donnell and especially Colin Nurse for surviving my committee meetings and actually coming back year after year.

Thanks to Scott Kelly, Angel Sing and Jeffie Richards for just being there.

Thanks to my girls: Sheri Coghill, Nancy Douglas, Lynn Brodie, Kristin Bebbington and Andrea James--without your love, support and laughs, nothing would be possible.

To Chris Wood for taking a chance and seeing it through even though I was impossible at times, for the endless opportunities, for the laughs and gossip and for being the best supervisor a girl could ask for.

To Martin Grosell for all the things I miss when we are apart.

And most of all to my parents, Betty and Egan McDonald, who have given me the confidence to do anything I've set out to do and have never believed that I would be anything but successful.

Bye MAC, I'll miss you.

Thesis Organization and Format

The present thesis is in a "closed-faced" format approved by McMaster University. Therefore, this dissertation consists of a total of 8 chapters plus an appendix. Chapter 1 provides a general introduction and statements on the objectives of the thesis. Chapters 2 through 7 are manuscripts that have been published or intend to be submitted for publication in peer-reviewed journals. Chapter 8 provides a summary of the findings and conclusions.

CHAPTER 1		**General introduction and thesis objectives**
CHAPTER 2		**Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish, *Opsanus beta*: evidence for two transport mechanisms.**
	Authors:	M. Danielle McDonald, Chris M. Wood, Yuxiang Wang, Patrick J. Walsh
	Date Accepted:	December 1, 1999
	Journal:	*Journal of Experimental Biology* 203: 1027-1037 (2000)

	Comments:	This study was conducted by MDM under the supervision of CMW in the lab of PJW. YW provided technical assistance.
CHAPTER 3		**The branchial and renal handling of urea in the gulf toadfish, *Opsanus beta*: the effect of exogenous urea loading.**
	Authors:	M. Danielle McDonald, Chris M. Wood, Martin Grosell, Patrick J. Walsh
	Date Submitted:	To be submitted September 2002
	Journal:	*Comparative Biochemistry and Physiology*
	Comments:	This study was conducted by MDM under the supervision of CMW in the lab of PJW. MG provided technical assistance.
CHAPTER 4		**Branchial and renal excretion of urea and urea analogues in the plainfin midshipman, *Porichthys notatus.***
	Authors:	M. Danielle McDonald, Patrick J. Walsh, Chris M. Wood
	Date Accepted:	July 2002

	Journal:	*Journal of Comparative Physiology In press*
	Comments:	This study was conducted by MDM under the supervision of CMW. PJW provided technical assistance.
CHAPTER 5		**The branchial and renal handling of urea and its analogues in the freshwater rainbow trout (*Oncorhynchus mykiss*).**
	Authors:	M. Danielle McDonald and Chris M. Wood
	Date Submitted:	To be submitted September 2002
	Journal:	*Physiological Zoology*
	Comments:	This study was conducted by MDM under the supervision of CMW.
CHAPTER 6		**Evidence for facilitated diffusion of urea by the gill of the freshwater rainbow trout, *Oncorhynchus mykiss*.**
	Authors:	M. Danielle McDonald and Chris M. Wood
	Date Submitted:	To be submitted September 2002
	Journal:	*Journal of Biological Chemistry*
	Comments:	This study was conducted by MDM under the supervision of CMW.

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Chapter 1

General Introduction

Up until a few decades ago, urea had been described as a small, neutral molecule that diffused easily across cell membranes. Recently however, a series of major discoveries using mammalian models has led to a greater understanding of urea transport. The commonly accepted simple movement of urea has been proven, in many cases, to be carrier-mediated and both active and facilitated diffusion mechanisms have been identified.

Active urea transport mechanisms have now been described in many organisms spanning mammals, amphibians and elasmobranchs. Na^+-linked, active urea reabsorption was originally described in the renal tubule of the spiny dogfish, *Squalus acanthias*, where urea was reabsorbed in a 1:1.6 ratio with Na^+ (Schmidt-Nielsen and Rabinowitz, 1969; Schmidt-Nielsen et al. 1972). Recent studies have identified Na^+-dependent, secondary active urea reabsorptive and secretory mechanisms in rat inner medullary collecting ducts (Isozaki et al. 1993, 1994a,b; Sands et al. 1996; Kato and Sands, 1998a,b; reviewed by Sands, 1999). In amphibians, Na^+-independent and/or phloretin-sensitive active urea transporters have been described in the skins of *Bufo bufo, Rana esculenta, Bufo viridis* and *Bufo marinus* (Ussing and Johnansen, 1969; Garcia-Romeu et al. 1981; Lacoste et al. 1991; Rappoport et al. 1988).

In contrast to active urea transport mechanisms, facilitated diffusion urea transport mechanisms are unable to move urea against a concentration gradient and the movement of urea is electroneutral and is not coupled to Na^+ (You et al. 1993; Olives et al. 1994). To date, all of the known cloned facilitative UT proteins are products of one of two genes, either UT-A (Fig. 1-1) or UT-B, both found in a variety of organs (reviewed by Sands et al. 1997; Smith and Rousselet, 2001). Many cDNAs encoding UT-A and UT-B proteins have been isolated from vertebrates including rabbit (You et al. 1993), human (Olives et al. 1994), rat (Smith et al. 1995), frog (Couriaud et al. 1999), shark (Smith and Wright, 1999), mouse (Fenton et al. 2000), toadfish (Walsh et al. 2000), tilapia (Walsh et al. 2001b) and eel (Mistry et al. 2001) and the proteins they encode show a high degree of amino acid conservation (reviewed by Smith and Rousselet, 2001).

Facilitated diffusion of urea is inhibited by phloretin and certain urea analogues (You et al. 1993) and is stimulated by arginine vasopression (AVP; Chou and Knepper, 1989) in higher vertebrates. In both mammals and aquatic organisms, facilitated diffusion of urea is regulated by glucocorticoids (Wood et al. 1997, 1998; Naruse et al. 1998; Peng et al. 2002). In mammals, glucocorticoids have been shown to regulate urea transport by significantly inhibiting the activity of the UT-A promoter region resulting in a decrease in mRNA expression (Peng et al. 2002). In the gulf toadfish, an elevation in circulating endogenous cortisol appears to inhibit the activation of tUT, a facilitated difffusion mechanism found in the gill of this fish (Wood et al. 1997, 2001).

UT transporters are highly selective for urea and are generally held not to transport water (Sidoux-Walker et al. 1999). Similarly, the aquaporine water channels

(AQPs) are typically highly specific for water (Borgnia et al. 1999), although several do transport urea.

The kinetic variables K_m and V_{max} for cloned urea transporters have not been established in mammals, due to complications associated with the high amounts of urea needed to saturate these mechanisms (Chou et al. 1990; reviewed by Smith and Rousselet, 2001). The estimated turnover number of urea molecules through UT-A protein is higher than would be expected if urea was carrier-mediated in the classical sense, thus, the consensus is that UT proteins may be mechanistically more like ion channels which typically handle millions of molecules per second (Kishore et al. 1997). However, UT proteins in lower vertebrates may act more like carriers, since the kinetic parameters for urea transport have been reported for facilitated diffusion UT-type mechanisms (Sphun and Katz, 1989, 1990; Pilley and Wright, 2000).

Urea analogues are often used to identify urea transport mechanisms. Being of similar molecular weight and structure, analogues (*i.e.* thiourea, acetamide, N-methylurea) interact competitively with urea at transport sites, thereby inhibiting urea transport (Chou and Knepper, 1989; Gillan and Sands, 1992). Depending on the transport mechanism, different analogues display varying degrees of competitive inhibition. This variability reflects the specificity of the transporter, since the analogues themselves are not always transported as effectively as urea (Brahm, 1983; Mayrand and Levitt, 1983; Chou et al. 1990). To illustrate, the transport capacity through the mammalian UT-A carrier is much lower for thiourea than it is for urea (Chou et al. 1990). Likewise, the human red blood cell permeability for urea was reported to be 2.4×10^{-5}

$cm \cdot sec^{-1}$, while it was only 0.07×10^{-5} $cm \cdot sec^{-1}$ for thiourea through UT-B transporters (Naccache and Sha'afi, 1973).

In fact, studies characterizing urea transport mechanisms in other organisms have shown that urea transporters not only move analogues less effectively than urea but can often distinguish between analogues. Early work on the kidney of the frog *Rana catesbeiana* demonstrated the active secretion of thiourea but the lack of acetamide transport (Schmidt-Nielsen and Shrauger, 1963). Classic work on the kidney of the spiny dogfish (*Squalus acanthias*) showed that acetamide and methylurea were reabsorbed nearly as well as urea but thiourea was not (Schmidt-Nielsen and Rabinowitz, 1964). At present, it has not been determined whether this differentiation was by the recently cloned facilitated diffusion urea transporter (ShUT; Smith and Wright, 1999), or by the hypothesized Na^+-dependent secondary active transporter (Schmidt-Nielsen et al. 1972). More recent work has demonstrated a high specificity for urea compared with acetamide, thiourea and N-methylurea through the phloretin-sensitive urea transporter in hepatocytes of the gulf toadfish (Walsh et al. 1994b) and the branchial facilitated diffusion mtUT in the gills of the Lake Magadi tilapia (Walsh et al. 2001b).

Urea is mainly excreted by terrestrial organisms that are required to detoxify two molecules of ammonia into one molecule of urea at the expense of 4 ATP, in order to rid themselves of nitrogenous wastes. Aquatic organisms are able to excrete nitrogen directly through the gills as ammonia, since it is diluted by the surrounding environment, and thus bypass the energy-consuming detoxification steps that are necessary for land-dwelling animals. However, there are some aquatic organisms that employ alternative methods to

excrete their nitrogenous waste. A few fish have evolved mechanisms to excrete ammonia more effectively, such as the mudskipper, *Periophthalmodon schlosseri*, that has an active NH_4^+ branchial excretory mechanism (Randall et al. 1999), and the mangrove killifish, *Rivulus marmoratus* that volatilizes NH_3 during air exposure (Frick and Wright, 2002). Other aquatic organisms have the ability to detoxify ammonia into urea *via* the ornithine-urea cycle (OUC). Elasmobranch fishes produce and retain high endogenous concentrations of urea (~300 $mmol \cdot l^{-1}$) in order to osmoconform to a marine environment. A handful of teleost fishes will excrete primarily urea when environmental conditions are unfavorable for ammonia excretion (Saha and Ratha, 1987; Randall et al. 1989; Mommsen and Walsh, 1989; Wood et al. 1989). For instance, the Lake Magadi tilapia (*Alcolapia grahami*) can excrete only urea across the gills (obligately ureotelic) since its highly alkaline habitat makes ammonia excretion unfeasible. The gulf toadfish (*Opsanus beta*) has the ability to excrete primarily urea but only does so occasionally (facultatively ureotelic), mainly when exposed to environmental stressors such as crowding, confinement, air emersion or ammonia (Walsh et al. 1990; Walsh et al. 1994a; Walsh and Milligan, 1995). Urea transport mechanisms have been identified and cloned in the gills of both the Lake Magadi tilapia (mtUT; Walsh 2001b) and the gulf toadfish (tUT; Walsh. 2000), showing high homology to each other (75% homology at the amino acid level) and to mammalian UT-A transporters (56-61% and 62%, respectively).

The existence of urea transport mechanisms in the handful of teleosts that have a fully functional OUC stands to reason, however, there is evidence that urea transport mechanisms exist in ammoniotelic organisms as well. In both the ammoniotelic plainfin

midshipman, (*Porichthys notatus)*, closely related to the gulf toadfish, and the ammoniotelic rainbow trout (*Oncorhynchus mykiss*), an extensively studied aquatic model, there is evidence of carrier-mediated transport of urea. Unlike the gulf toadfish, midshipmen and adult rainbow trout do not express the ornithine urea cycle and do not have the ability to switch to ureotelism when under stressful conditions (Wilkie and Wood, 1991; Wright et al. 1995a; Wang and Walsh, 2000; Walsh et al. 2001a). Nonetheless, both fish do excrete some (< 15%) of their nitrogenous waste as urea but branchial urea excretion is continuous and not pulsatile as in the toadfish (reviewed by Walsh, 1997, McDonald and Wood, 1998). However, when a ^{32}P-labelled cDNA probe based on the toadfish branchial urea transporter (tUT) was used to examine gill mRNA by Northern blot analysis, the relative signal strength was strong for the plainfin midshipman (Walsh et al. 2001a). Similarly, in the rainbow trout, preliminary Northern blot analysis and sequencing revealed a mRNA from trout gill with approximately 2000 bases homologous to the mRNA of the toadfish tUT (CM Pilley, PW Wright and PJ Walsh, unpublished data).

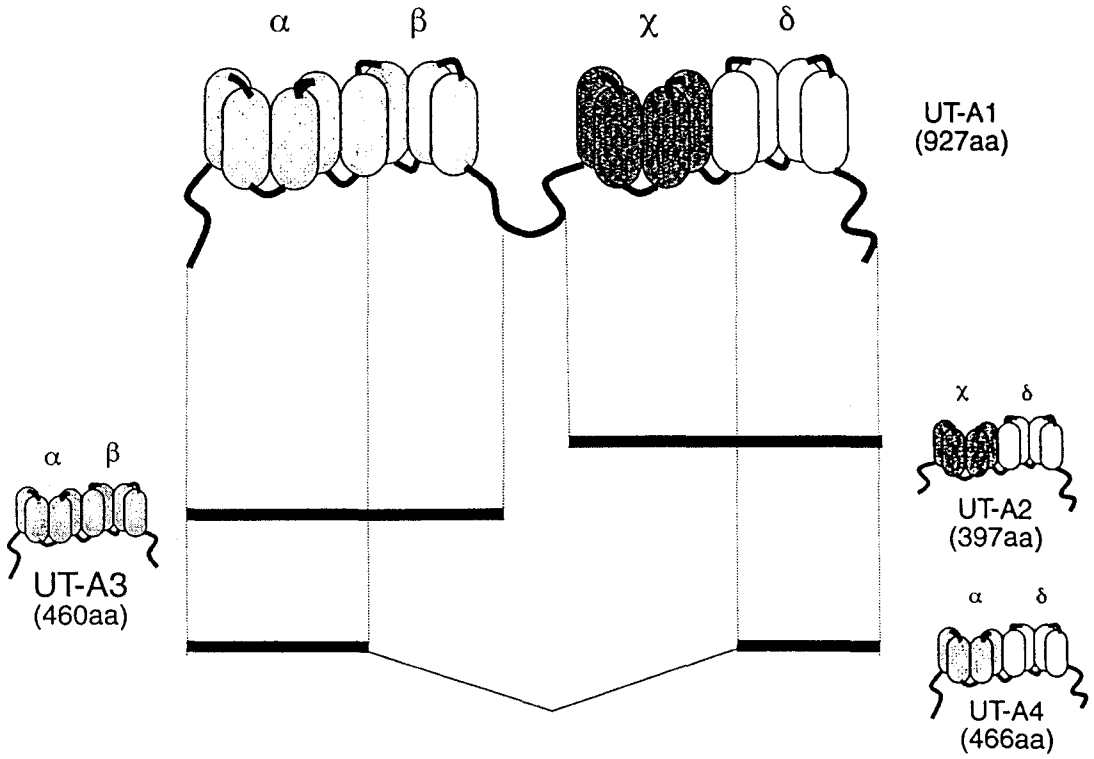
The gills are the primary site of nitrogenous waste excretion for aquatic organisms and are the most likely site for specialized urea transport mechanisms. However, the kidney in freshwater and marine teleosts plays an important role in the osmoregulation. Living in a hypotonic environment, freshwater fish are continuously plagued with water gain and thus have a well-developed glomerulus that filters fluid from the blood into the kidney tubule to be excreted in the urine. In order to maintain osmotic balance, freshwater fish excrete a very dilute urine, consisting mostly of water, and have high

glomerular filtration rates averaging approximately 6 ml kg^{-1} h^{-1} (Hickman and Trump, 1969; Curtis and Wood, 1992). At least in the rainbow trout, the kidney may be involved in the homeostatic regulation of endogenous urea concentrations. Evidence indicates that circulating plasma urea concentrations are greater than those in the urine of the trout, suggesting the presence of an active urea reabsorption mechanism (reviewed by Wood, 1993; Korsgaard et al. 1995; McDonald and Wood, 1998).

In contrast to the freshwater kidney, marine teleosts are in a hypertonic environment and are consequently challenged with constant water loss. Since there is no need to filter water from the blood as in freshwater fish, in many marine teleosts the filtration area of the glomerulus is reduced. Thus, the marine nephron ranges from one that has a functional but under-developed glomerulus (pauciglomerular) to a nephron completely lacking a glomerulus (aglomerular; Hickman and Trump, 1969). The aglomerular kidney of the oyster toadfish, *Opsanus tau*, has been well studied in this respect, using both physiological and microscopic methods (Marshall, 1929; Bulger, 1965). The gulf toadfish and the plainfin midshipman are among those marine teleosts considered to be aglomerular, although this notion is based solely on their close relation to *Opsanus tau*. In the toadfish, evidence suggests the possibility of active urea secretion in the kidney, since urine urea levels exceed plasma urea levels by at least 30% and urea is believed to enter the kidney tubule solely by secretion (Wood et al. 1995).

Thus, the first goal of the following research has been to physiologically and pharmacologically characterize the urea transport mechanism (tUT) that is known from molecular evidence to be present in the gills of the toadfish. Using those findings,

potential urea transport mechanisms in the kidney of the toadfish and in both excretory organs of the ammoniotelic plainfin midshipman, closely related to the toadfish, and the ammoniotelic freshwater rainbow trout, distantly related to the toadfish, were investigated. Based on the defining features of tUT, there is evidence of carrier-mediated transport of urea in the kidney of the toadfish and branchial and renal urea transporters in both midshipmen and trout. The characteristics of branchial urea excretion are similar among the three teleost species. However, the renal excretion of urea shows similar qualities between the midshipman and toadfish, but differs from that in the rainbow trout.


α
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UT-A1
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δ
UT-A2
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UT-A4
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Chapter 2

Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish, *Opsanus beta*: evidence for two transporters

Abstract

The possible presence of a urea transporter in the kidney of the gulf toadfish (*Opsanus beta*) and further characterization of the pulsatile facilitated transporter previously identified in its gills were investigated by comparing the extra-renal and renal handling of two urea analogues with the handling of urea. Toadfish were fitted with indwelling caudal artery and urinary ureteral catheters and injected with an isosmotic dose of [^{14}C]-labeled urea analogue (acetamide or thiourea) calculated to bring plasma analogue concentrations close to plasma urea concentrations. Branchial permeabilities to urea, acetamide and thiourea were similar during non-pulsing periods and all increased during pulse events although urea permeability was greater than analogue permeability during pulses. The incidence and magnitude of acetamide and urea pulses at the gills were significantly correlated, acetamide pulses being 35-50 % the size of urea pulses. However, thiourea and urea pulses at the gills were only weakly correlated, thiourea being less than 16 % the size of urea pulses. Thiourea inhibited branchial urea excretion by reducing the pulse frequency. The renal handling of thiourea and urea were similar in that both substances were more concentrated in the urine than in the plasma, whereas acetamide was found in equal concentrations in the urine and plasma. Urea and thiourea

were secreted into the kidney tubule 2-3 times more effectively than Cl^- and water, whereas acetamide was secreted at a similar relative rate. The differential handling of the urea analogues by the gills and kidney indicates the presence of a different, possibly unique transporter in the kidney. The movement of thiourea and urea into the renal tubule against an apparent concentration gradient suggests the presence of an active transport mechanism.

Introduction

Classically, urea was believed to move passively through lipoprotein cell membranes. Over the past few decades, this prevailing doctrine has been refuted by the discovery of various urea transport mechanisms, including facilitated and active carrier mediated transport. Urea analogues such as thiourea (H_2NCSNH_2) and acetamide (H_3CCONH_2) are useful tools for determining the presence of a transport mechanism. Since their structure is similar to urea (H_2NCONH_2), thiourea and acetamide interact competitively at transport sites, thereby inhibiting urea transport (Chou and Knepper, 1989; Gillan and Sands, 1992).

Despite the inhibitory effects, analogues are not always transported as effectively as urea, on account of the specificity of the carrier. The vasopressin (arginine vasopressin; AVP)-sensitive facilitated diffusion carrier (originally considered to be UT-2 but now considered to be UT-A1; You et al.1993; Smith et al. 1995; Shayakul et al. 1996; Karakashian et al. 1999) is characteristically difficult to saturate, even at urea concentrations well beyond the physiological range (Chou et al. 1990). However, urea transporters can often be saturated at substantially lower thiourea concentrations, demonstrating that the transport capacity is much lower for thiourea than for urea (Brahm, 1983; Mayrand and Levitt, 1983; Chou et al. 1990).

Studies involving the characterization of renal urea transport mechanisms have shown not only that transporters move analogues less effectively than urea, but they can often distinguish between analogues. In the kidney of the spiny dogfish shark, *Squalus acanthias,* the urea transporter reabsorbed urea by apparent secondary active transport

also reabsorbed acetamide, albeit to a lesser extent than urea. However, thiourea was not reabsorbed (Schmidt-Nielsen and Rabinowitz, 1964). Very recently, Smith and Wright (1999) have cloned a urea transporter from shark kidney which appears related to the mammalian UT-A family of facilitated diffusion transporters, but its role in urea reabsorption remains unknown. In contrast to shark kidney, a study by Schmidt-Nielsen and Shrauger (1963) on the kidney of the frog, *Rana catesbeiana*, demonstrated the apparent active secretion of thiourea and the lack of acetamide transport. In mammalian studies, it has been demonstrated that thiourea has the ability to pass through the vasopressin-sensitive urea transport mechanism in rat inner medullary collecting ducts (IMCD), although the permeability of thiourea was much lower than the permeability of urea (Chou et al. 1990). Naccache and Sha'afi (1973) reported human erythrocyte permeability for urea to be 2.4×10^{-5} cm·sec^{-1} while it was only 0.07×10^{-5} cm·sec^{-1} for thiourea.

Only recently has investigation into the existence of urea transport mechanisms been initiated in teleost fish. The gulf toadfish, *Opsanus beta*, among the best studied in this regard, is one of only a few adult teleosts with an active ornithine-urea cycle (for others refer to Saha and Ratha, 1987; Randall et al. 1989; Wood et al. 1989, 1994). It also has the remarkable ability to switch from ammoniotelism to ureotelism when placed under stressful circumstances such as ammonia exposure, air exposure, crowding and/or confinement (Walsh et al. 1990; Walsh et al. 1994a; Walsh and Milligan, 1995). The primary route of urea excretion is *via* the gills (> 90% total urea excretion), where excretion is not continuous but occurs in distinct 0.5-3 hour pulses, on average once or

twice every 24 hours (Wood et al. 1995, 1997). Of late, the cDNA for a hormonally-controlled facilitated diffusion mechanism related to the mammalian UT-A urea transporters has been isolated in the gills (Smith et al. 1998). The toadfish transporter appears to be functional only when plasma cortisol is low (Walsh, 1997; Wood et al. 1997, 1998) and may be specifically activated by arginine vasotocin (AVT-the piscine analogue of mammalian AVP; Gilmour et al. 1998; Perry et al. 1998). During pulsing periods, the activation of this transporter gives rise to at least a 35x increase in urea permeability (Wood et al. 1998). At the same time, there is only a 5x increase in thiourea permeability during pulses, significantly lower than the observed urea permeability (Wood et al. 1998).

A secondary route of excretion is the kidney (< 10% total urea excretion). The closely related oyster toadfish (*Opsanus tau*) is generally believed to have an aglomerular kidney, although indirect evidence by Lahlou et al. (1969) suggested minimal glomerular filtration (Marshall, 1929; Hickman and Trump, 1969). Regardless, urine is formed primarily by secretion. Although minor, the kidney may play a role in the regulation and maintenance of blood urea concentrations. Toadfish urine urea concentrations generally exceed plasma levels by at least 30%, and both urine and plasma concentrations increase when the fish activate ureotelism (Wood et al. 1995). Thus, the movement of urea into the kidney tubule may occur against a concentration gradient, suggesting the presence of an active transport mechanism.

There were two main objectives of the present study. The first objective was to quantitatively compare the handling of urea and two urea analogues, acetamide and

thiourea, by the branchial, hormonally-controlled, facilitated diffusion carrier. Permeabilities for the three substances during pulsing and non-pulsing periods were determined, and the effect of analogues on pulse frequency and size was monitored. The second goal was to investigate the possibility of a renal urea transporter by characterizing the renal handling of urea relative to analogues and other substance such as Na^+, Cl^- and water. The handling of urea, acetamide and thiourea by each respective organ was compared.

Our results indicate that there exist two distinct transport mechanisms involved in urea excretion in the gulf toadfish, found in the gill and the kidney, each of which differentially handles urea, acetamide and thiourea.

Materials and Methods

Experimental Animals

Gulf toadfish (*Opsanus beta*) were caught by commercial shrimpers in Biscayne Bay, Florida in November and December, 1998. The toadfish (70-300 g) were held in an outdoor tank at the shrimpers' holding facility with running sea water (ambient seasonal conditions) for no longer than 24 h following capture, then transferred to the laboratory. Fish were treated with a dose of malachite green (final concentration 0.05 $mg \cdot l^{-1}$) in formalin (15 $mg \cdot l^{-1}$) (AquaVet, Hayward, CA, USA) on the day of transfer to the laboratory in order to prevent infection by the cilate, *Cryptocaryon irritans* (Stoskopf, 1993). Initially the fish were kept in 50 L Rubbermaid containers with flowing, aerated seawater. Fish were maintained in this relatively crowded environment (> 10 fish per

tank) in order to initiate a switch to ureotelism (Walsh et al. 1994a). In order to ensure full expression of ureotelic behaviour, the fish were transferred in groups of four to plastic tubs (6 L) served with flowing seawater at least 48 h prior to surgery. The temperature in these tanks was 26°C. Fish were fed weekly throughout the experimental period.

Experimental Protocol

Caudal artery and ureteral catheterization were performed simultaneously on fish anaesthetized with MS-222 (0.5 $g \cdot l^{-1}$; Sigma-Aldrich Canada) and wrapped with wet towels. Caudal artery catheters were inserted as described by Wood et al. (1997). The caudal vertebrae were exposed by a 1.5-2.0 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with Clay-Adams PE50 tubing filled with toadfish saline and 50 $i.u \cdot ml^{-1}$ sodium heparin (Sigma-Aldrich Canada). A heat-flared PE 160 sleeve glued in place with cyanoacrylate tissue cement (Vetbond; 3M Corporation) and sutured at the site of exit in order to secure the catheter. The wound was treated with oxytetracycline powder, in order to prevent infection, and sutured securely with 2-0 silk.

The technique for inserting indwelling ureteral catheters was modified from the protocol described by Howe and Gutknecht (1978) and Lahlou et al. (1969). Our goal was to bypass the urinary bladder thereby eliminating any reabsorptive/secretory roles of the bladder epithelium so as to examine the function of the kidney alone (see Baustain et al. 1997 for a discussion of this problem). The end of a catheter, made of approximately

60 cm of Clay Adams PE 50 tubing, was filled with distilled water and advanced dorsally 1.5-2.0 cm through the urogenital papilla into the urinary sinus. The catheter was held in place by three 2-0 silk ligatures around the papilla and was attached to the body of the fish by four cutaneous ties. A ventral incision approximately 1.5-2.0 cm in length was then made just anterior to the urogenital papilla. Both urinary bladders were isolated and ligated close to the ureter end with 2-0 silk, hence the storage function of the bladders was bypassed, and the urine drained directly from the ureters into the urinary sinus where the catheter tip was located. Oxytetracycline powder was dusted into the body cavity and the ventral musculature and skin were then sutured securely. During recovery and experimentation, urine was collected continuously with the catheter emptying into a vial approximately 3.0 cm below the water level of the box. The urine flow rate (UFR) was determined gravimetrically. Following surgery, fish were kept in darkened individual containers that were continually aerated, supplied with flowing water (200 $ml \cdot min^{-1}$) and had a minimum volume of 1.5 L.

Following the procedure outlined by McDonald and Wood (1998), [^{3}H]-polyethylene glycol (PEG, Mr4000) was used as a marker for glomerular filtration rate (GFR) in order to detect whether there were any functional glomeruli in this supposedly aglomerular kidney. PEG 4000 was chosen because it is considered a more accurate and conservative indicator of GFR in fish than other commonly used markers (*e.g.* inulin derivatives), as it undergoes minimal radioautolysis, metabolic breakdown, or post-filtration reabsorption across tubules and bladder (Beyenbach and Kirschner, 1976; Erickson and Gingrich, 1986; Curtis and Wood, 1991). Prior to injection of PEG, the fish were allowed to

recover from surgery for at least 12 hours, a period during which the patency of the arterial and urinary bladder catheters was confirmed. A dose of 5 μCi·100g body weight^{-1} of [^{3}H]-PEG 4000 (New England Nuclear) was injected *via* the caudal arterial catheter followed by an additional 0.3 ml of saline in fish where both catheters were deemed successful. The [^{3}H]-PEG 4000 was then allowed to equilibrate throughout the extracellular space for 12h before sampling commenced.

The pattern of gill urea excretion, UFR, GFR, urine composition, plasma composition and urinary urea and ion excretion rates of the toadfish were monitored. Two series were performed: 5 fish treated with acetamide and 5 fish treated with thiourea. Mean weights of fish in the acetamide series were 0.264 ± 0.035 kg ranging from 0.133 to 0.328 kg. Mean weights of fish in the thiourea series were 0.365 ± 0.038 kg ranging from 0.256 to 0.462 kg. Following the [^{3}H]-PEG 4000 injection and equilibration period, blood, urine and water samples were taken and water flow to the box was then stopped and the volume set to at least 1.5 L. Thereafter, water samples (5 ml) were taken every two hours for the remainder of the experiment, the water being changed at 24 h intervals. Blood and urine samples were taken every 12 hours, making a total of approximately 30 water samples (at 2 h intervals), 6 blood samples (at 12 h intervals) and 6 urine samples (each spanning a 12 h collection period). Twenty four hours after the [^{3}H]-PEG 4000 injection, the fish were injected with a dose of 5μCi·100g body weight^{-1} of [^{14}C]-labeled thiourea or acetamide in 420 μmol·100g body weight^{-1} of isosmotic cold urea analogue (concentration = 320 mmol·l^{-1}) in order to render internal analogue concentrations approximately equal to internal urea concentrations. Blood samples were

immediately centrifuged (10, 000g for 2 min.). Plasma and urine were stored at -20°C for later analysis of Na^+, Cl^-, urea, [^{14}C]-analogue and [^{3}H]-PEG 4000 concentrations. Water samples were analyzed for urea, [^{3}H]-PEG 4000 and [^{14}C]-analogue only.

In order to determine the normal composition of bladder urine, separate urine samples were taken directly from the bladder of 72 toadfish and analysed for urea, Na^+ and Cl^- concentration. These fish were part of a separate study and were killed by a rapid blow to the head, opened in the ventral midline and sampled by direct puncture of the exposed bladder with a # 22 needle and 1 cm^3 syringe.

Analytical Techniques and Calculations

Urea concentrations in blood, urine and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in the three media and correction for the presence of thiourea and acetamide. This correction was done by adding the calculated concentration of analogue present in the urine, plasma or water (see Equation 1 and 2) in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. Ammonia concentrations in the water were measured by the method of Ivancic and Degobbis (1984). Na^+ and Cl^- concentrations in plasma and urine were measured using a Varian 1275 Atomic Absorption Spectrophotometer and a Radiometer CMT10 Chloridometer respectively.

For measurements of [^{3}H]-PEG 4000 and [^{14}C]-analogue, blood and urine samples (25 μl plus 1 ml of seawater) or water samples (1 ml) were added to 10 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 Counter using an onboard quench correction program to separate [^{3}H] and [^{14}C] counts.

The concentration of thiourea or acetamide in the plasma, urine and water was determined from the specific activity (cpms·μmol^{-1}) of the injected solution and then converting the radioactivity found in the samples into concentration (mmol·l^{-1}):

$$\text{Specific Activity} = \frac{\text{cpm}_{in}}{[\text{analogue}]_{cold}} \quad \frac{(\text{cpm}\cdot\text{ml}^{-1})}{(\mu\text{mol}\cdot\text{ml}^{-1})} \qquad (1)$$

$$[\text{analogue}] = \frac{\text{cpm}_s}{\text{Specific Activity}} \quad \frac{(\text{cpm}\cdot\text{ml}^{-1})}{(\text{cpm}\cdot\mu\text{mol}^{-1})} \qquad (2)$$

where cpm_{in} indicates the radioactivity of the injected solution, and cpm_s is the radioactivity in the sample. The branchial clearance (C_B; ml kg^{-1} h^{-1}) of any substance (X) was calculated taking the concentration of the substance in the water $[X]_w$ corrected for fish body weight (wt) and plasma concentration $[X]_p$ and calculated as:

$$C_{BX} = \frac{[X]_w \times V_f}{\text{wt} \times [X]_p} \quad \frac{(\mu\text{mol})}{(\mu\text{mol}\cdot\text{kg}\cdot\text{ml}^{-1})} \qquad (3)$$

where V_f is the volume of water surrounding the fish. A pulse was identified as a sudden increase in urea, acetamide or thiourea appearance in the surrounding water of at least 20 μmol kg^{-1}.

The branchial permeability (P; cm sec^{-1}) of any substance (*X*) was determined as:

$$P_X = \frac{C_{BX}}{T \times SA_B} \quad \frac{(cm^3 \cdot kg^{-1})}{(sec \cdot cm^2 \cdot kg^{-1})} \qquad (4)$$

using the calculated values of branchial clearance (C_B) and branchial surface area for toadfish (SA_B) (1920 cm^2 kg^{-1}; Hughes and Grey, 1972), where T is the period duration.

All the following rates were related to fish body weight by expressing urinary flow rate (UFR) in $ml \cdot kg^{-1} \cdot h^{-1}$. Urinary excretion rates (U; μmol kg^{-1} h^{-1}) of any substance (*X*) were calculated as:

$$U_X = [X]_u \times UFR \qquad (5)$$

using measured values of urine flow rates (UFR) and urine concentrations $[X]_u$. Glomerular filtration rates (GFR) were calculated as the clearance of [^{3}H]-PEG 4000 - *i.e.* the excretion of radioactivity in the urine (cpm_u) relative to its concentration in the blood plasma (cpm_p):

$$GFR = \frac{cpm_u \times UFR}{cpm_p} \qquad (6)$$

The filtration rate (FR; μmol kg^{-1} h^{-1}) of a substance *X* at the glomeruli was calculated as:

$$FR_X = [X]_p \times GFR \qquad (7)$$

and consequently the tubular secretion rate (TS; μmol kg^{-1} h^{-1}) of *X* was calculated as:

$$TS_X = U_X - FR_X \qquad (8)$$

The renal clearance rate by secretion (C_R; ml kg^{-1} h^{-1}) of *X* was calculated as:

$$C_{RX} = \frac{TS_X}{[X]_p} \qquad (9)$$

Statistics

Data were reported as means ± 1 S.E.M (N = number of fish). Regression lines were fitted by the method of least squares, and the significance ($P < 0.05$) of the Pearson's correlation coefficient r assessed. The significance of differences between means was evaluated using Student's paired or unpaired, two-tailed or one-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977).

Results

Branchial Excretion

The toadfish used in this study were predominantly ureotelic, excreting 80% urea-N (81.7 ±11.1 μmol-N·kg^{-1}·h^{-1}) and 20% ammonia-N (23.2 ±3.3 μmol-N·kg^{-1}·h^{-1}) when averaged over the experimental period of 60 hours. The gills were the major route of urea excretion, averaging 56.9 ± 9.9 (10) μmol·kg^{-1}·h^{-1} relative to a urinary excretion rate of only 1.91 ± 0.07 (10) μmol·kg^{-1}·h^{-1}. More than 90% (92.5 ± 2.4 (10) %) of the branchial excretion occurred in discrete pulses, with a mean frequency of 1.20 ± 0.20 (10) pulses per 12 h and a mean size of 596 ± 107 (10) μmol·kg^{-1}·pulse^{-1} during the 12h control period. The permeability of the gills to urea was significantly greater during pulse events ((7.79 ± 1.68) x 10^{-6} (10) cm·sec^{-1}) than during non-pulsing periods ((0.26 ± 0.10) x 10^{-6} (10) cm·sec^{-1}). Loading the fish with acetamide had no effect on these patterns, but loading with thiourea caused a greater than 50% reduction in branchial urea

excretion rate (Table 2-1). This effect was entirely due to a significant reduction in pulse frequency; mean pulse size was not affected (Table 2-1).

Overall, 94.8 ± 0.0 (5) % of acetamide excretion occurred through the gills and 72.9 ± 12.6 (5) % of this occurred in pulse events. Pulses of acetamide excretion through the gills always occurred simultaneously with those of urea (18 pulse events in 5 fish over 48 h; *e.g.* Fig. 2-1A), and there was a strong quantitative correlation between acetamide and urea pulses. When expressed as plasma clearance ($ml \cdot kg^{-1}$), acetamide clearance exhibited a linear proportional relationship ($r = 0.85$, $P < 0.001$) to urea clearance during pulses with a slope of 0.35, and an intercept not significantly different from zero (Fig 2-2). When expressed as clearance ratio for individual pulses (acetamide/urea), the ratio was 0.50 ± 0.09 (18). Thus, branchial acetamide clearance during pulses was 50-65 % less than urea clearance. The permeability of the gills to acetamide during pulse events was 18-fold greater than during non-pulsing periods, but was approximately 55% less than the permeability of the gills to urea during the same pulse events (Table 2-2).

The relationship of thiourea excretion to urea excretion was much weaker. Only 63.8 ± 0.0 (5) % of thiourea excretion occurred through the gills, and 56.2 ± 10.5 (5) % of this occurred in pulse events. Pulses of thiourea excretion occurred simultaneously with those of urea excretion (9 pulse events in 5 fish over 48h; *e.g.* Fig. 2-1B), but there were two additional thiourea pulses that did not appear to be associated with urea pulses. Furthermore, when expressed as plasma clearances, there appeared to be no quantitative relationship between thiourea and urea pulses ($r = -0.19$, n.s; slope not significantly

different from zero; Fig. 2-2). Note that urea clearances occurred in an overall lower range in the thiourea-treated fish, reflecting higher plasma urea concentrations than in the acetamide treated fish, despite similar absolute urea pulse sizes. When expressed as clearance ratio for individual pulses (thiourea/urea), the ratio was 0.16 ± 0.04 (9), significantly lower than the value for acetamide (above). Thus, thiourea clearance during pulses was at best only 16 % of the clearance of urea. The permeability of the gills to thiourea was 5x greater during pulse events than during non-pulsing periods, but was approximately 9-fold less than the permeability of the gills to urea during these same pulse events (Table 2-2). Thus, substantial differences in permeability (urea > acetamide > thiourea) were seen during pulse events, whereas all three analogues exhibited similar low permeabilities during non-pulsing periods.

Urinary Excretion

Our ureteral catheterization technique was designed to avoid modification of the urine by residence in the bladder. Nevertheless, the concentration of urea in ureteral urine (7.96 ± 0.36 (10) $mmol \cdot l^{-1}$) was not significantly different than that in bladder urine (8.60 ± 0.55 (72) $mmol \cdot l^{-1}$) collected from a separate set of fish (see Methods). Notably both bladder urine and ureteral urine urea concentrations were much greater than plasma urea concentrations (4.47 ± 0.59 (10) $mmol \cdot l^{-1}$). Ion levels were lower in bladder urine (Na^+: 11.9 ± 2.0 (39) $mmol \cdot l^{-1}$; Cl^-: 80.7 ± 4.4 (59) $mmol \cdot l^{-1}$) compared to concentrations in urine that had only been modified by the kidneys (Na^+: 27.9 ± 4.8 (10) $mmol \cdot l^{-1}$; Cl^-:

106.0 ± 10.7 (10) $mmol \cdot l^{-1}$. Plasma levels were: Na^+ = 154.0 ± 2.9, Cl^- = 126.4 ± 2.8 (10) $mmol \cdot l^{-1}$.

Based on the renal clearance of the glomerular filtration rate marker [^{3}H]-PEG 4000, the toadfish had a glomerular filtration rate that was significantly greater than zero (0.05 ± 0.01 (10) $ml \cdot kg^{-1} \cdot h^{-1}$), suggesting the presence of a small number of functional glomeruli. However, the urine flow rate (0.30 ± 0.11 (10) $ml \cdot kg^{-1} \cdot h^{-1}$) was substantially greater than the glomerular filtration rate indicating that the urine was largely formed by secretion. Consistent with the relationship between UFR and GFR was a net secretion of urea (1.56 ± 0.46 (10) $\mu mol \cdot kg^{-1} \cdot h^{-1}$) that was approximately 10-fold greater than its net filtration (0.15 ± 0.04 (10) $\mu mol \cdot kg^{-1} \cdot h^{-1}$).

Fish treated with acetamide demonstrated relatively stable urine urea concentrations (mean: 8.35 ± 0.61 (5) $mmol \cdot l^{-1}$) that were significantly greater than plasma concentrations (mean: 3.37 ± 0.06 (5) $mmol \cdot l^{-1}$) throughout the experimental period (Fig. 2-3A). Plasma and urine acetamide concentrations were not significantly different from one another (means: 3.48 ± 1.17 (5) $mmol \cdot l^{-1}$ and 2.52 ± 0.77 (5) $mmol \cdot l^{-1}$ respectively) and had a tendency to decrease throughout the experiment as acetamide was cleared from the gills (Fig. 2-3B). If anything, acetamide levels were greater in the plasma than in the urine, although this difference was not significant.

As in the acetamide series, urea concentrations in the urine of fish treated with thiourea (8.84 ± 0.98 (5) $mmol \cdot l^{-1}$) were significantly greater than plasma levels (5.01 ± 0.98 (5) $mmol \cdot l^{-1}$), although urine levels were not as stable as those seen in the acetamide

series (Fig. 2-4A). Thiourea concentrations in the urine and plasma showed the same trend as urea concentrations, in that thiourea was much greater in the urine (mean: 12.22 ± 1.68 (5) $mmol \cdot l^{-1}$) than the plasma (mean: 3.81 ± 0.22 (5) $mmol \cdot l^{-1}$), suggesting that the kidney handles thiourea in the same way that it handles urea (Fig. 2-4B), and very differently from the way it handles acetamide (Fig. 2-3B).

Fish treated with thiourea exhibited substantially lower secretion clearance rates of urea through the kidney (Fig. 2-5A,B) than did those treated with acetamide, suggesting that thiourea may have played an inhibitory role at the kidney comparable to that at the gills (Table 2-1). In simultaneous secretion clearance measurements, relative urea secretion (0.83 ± 0.29 (5) $ml \cdot kg^{-1} \cdot h^{-1}$) was almost 4-fold greater than relative acetamide secretion (0.22 ± 0.18 (5) $ml \cdot kg^{-1} \cdot h^{-1}$; Fig. 2-5A). In contrast, in the presence of thiourea, the secretion clearance of urea (0.18 ± 0.03 (5) $ml \cdot kg^{-1} \cdot h^{-1}$) was less than that of thiourea (0.35 ± 0.02 (5) $ml \cdot kg^{-1} \cdot h^{-1}$; Fig. 2-5B). When expressed as a clearance ratio (analogue/urea) for all collection periods where simultaneous measurements were made, the ratios were 1.97 ± 0.24 (5) for thiourea, and only 0.19 ± 0.09 (5) for acetamide, a highly significant difference.

Plasma Na^+ concentration in both the acetamide and thiourea series was on average 7-fold greater than urine concentrations (Table 2-3). In contrast, plasma and urine Cl^- concentrations were not significantly different from one another. When expressed as secretion clearance rates, Cl^- and urea exhibited a linear proportional relationship ($r = 0.800$, $P < 0.001$) with a slope of 2.70 and an intercept not significantly different than zero (Fig. 2-6A). An almost identical relationship was observed plotting

water movement and urea secretion rates (r = 0.820, $P < 0.001$; slope 2.94). Thus, on a relative basis, 2.7-2.9x more urea was secreted by the kidney than Cl^- or water movement. Predictably, there was no quantitative relationship between the secretion of Na^+ and urea (r = 0.344, n.s.; slope not significantly different than zero (Fig. 2-6)), which is consistent with the finding that Na^+ was more concentrated in the plasma whereas urea was more concentrated in the urine. Overall, there was no net secretion of Na^+.

The secretion clearance of thiourea exhibited a linear proportional relationship with that of Cl^- (r = 0.683, $P < 0.001$) with a slope of 2.08 and an intercept not significantly different from zero (Fig. 2-6B). A similar relationship was observed plotting thiourea secretion and water movement rates (r = 0.797, $P < 0.001$; slope 2.58). Thus, like urea, the renal secretion of thiourea was 2-2.6x more than either Cl^- secretion or water movement. Unlike the patterns of secretion observed for both urea and thiourea, the secretion clearance of acetamide exhibited a 1:1 relationship with both Cl^- and water (Cl^-: r = 0.963, $P < 0.001$; slope 0.88; and water: r = 0.962, $P < 0.001$; slope 0.94) (Fig. 2-6C). Therefore, on a relative basis, acetamide moved into the kidney tubule at the same rate as Cl^- and water.

Discussion

The basis of this study was to exploit the differential handling of urea and its analogues by urea transport mechanisms in order to determine whether a urea transporter was present in the kidney as well as to further characterize the previously documented facilitated diffusion transport mechanism in the gills (Walsh, 1997; Wood et al. 1998; Smith et al. 1999). Our results suggested that there are two distinct transport mechanisms

involved in the excretion of urea in the gulf toadfish, one in the gill and one in the kidney, both of which differentially handle urea, acetamide and thiourea, but with differing capabilities. Notably, acetamide and urea were handled similarly by the gills, with acetamide clearance exhibiting a linear relationship to urea clearance but 50-65% less than urea clearance. In contrast, the relationship of thiourea excretion to urea excretion was much weaker and relative thiourea clearance was at most only 16% of that of urea. The opposite trend was observed in the kidney, where it appeared that thiourea and urea were handled similarly, accumulating in the urine against a concentration gradient. Conversely, acetamide appeared to equilibrate between the plasma and urine. The secretion of thiourea was equal to or greater than that of urea, whereas that of acetamide was less than 25% of urea.

Differences between the handling of urea and analogues amongst organs of the same species is a phenomenon that is not unprecedented. A distinction has also been noted in the handling of urea and analogues by the skin and urinary bladder of the toad, *Bufo viridis* (Garcia-Romeu et al. 1981; Shpun and Katz, 1990). Using 4x more analogue than urea, thiourea was found to competitively interact, thereby inhibiting urea transport by the facilitated diffusion transport mechanism in the urinary bladder, while acetamide did not have an effect (Shpun and Katz, 1990). In contrast, in the toad skin, acetamide, in 5x greater concentrations than urea, inhibited the active transport of urea while thiourea did not interact with the transporter (Garcia-Romeu et al. 1981).

In *Rana esculenta*, the absorption of urea by the skin is believed to be *via* an ion-independent active transporter and although it has the same analogue transport qualities,

it is quite unlike the facilitated diffusion mechanism in the toadfish gill which is suspected to be under AVT control (Gilmour et al. 1998; Perry et al. 1998). However, the urinary bladder of the toad, *Bufo viridis*, in which there is believed to be an AVT-sensitive facilitated diffusion mechanism, exhibited an acetamide permeability that was 40% that of urea, and a thiourea permeability that was only 1% of urea (Levine et al. 1973). This is similar to the present observation on toadfish gills. Thus, although it appears that analogue permeability is a good tool for discriminating between transport mechanisms within one species, one cannot be certain of the type of urea transport mechanism based entirely on the handling of analogues amongst species.

Increases in urea, acetamide and thiourea permeability of the gills were observed during pulsing events, although analogue permeability was significantly less than that of urea. The present findings on branchial thiourea handling during pulses were quantitatively comparable to those of Wood et al. (1998), who demonstrated a significant, although much reduced increase in thiourea permeability compared to urea permeability. Despite small differences in calculated oil/water partition coefficients and lipid permeabilities (Goldstien and Solomon, 1960; Lippe, 1969), all three substances had similar permeabilities during non-pulsing periods, suggesting that the gill handled urea, acetamide and thiourea identically when the facilitated diffusion transporter was not activated.

In the present study, thiourea appeared to inhibit the branchial excretion of urea by reducing the pulse frequency rather than the size of the individual events. Studies done on toad bladder and rat inner medullary collecting ducts (IMCD) have demonstrated the

inhibitory effects of both acetamide and thiourea to urea transport, with the latter being more effective in this regard (Levine et al. 1973; Eggena, 1973; Chou and Knepper, 1989). Chou and Knepper (1989) demonstrated a 74% inhibition by thiourea and a 35% inhibition by acetamide at analogue concentrations of 200 mM. The concentrations of urea and analogue used for that study were greater than concentrations used in the present study (about 4 mM, approximately equal to plasma urea concentrations). Correspondingly, the extent of inhibition in the present study was much less, thiourea inhibiting urea excretion by approximately 50% and acetamide showing no inhibition of urea transport. Using a high level of thiourea (60 mM) in the external seawater, Wood et al. (1998) demonstrated a 73% inhibition of urea influx permeability during pulse events.

The appearance of [^{3}H]-PEG 4000 in the urine of the toadfish supports findings by Lahlou et al. (1969) showing the appearance of [^{14}C]-inulin in the urine of the oyster toadfish, *Opsanus tau*. In examining whether in fact the radioactivity appearing in the urine was incorporated in inulin, they ascertained that inulin was being degraded into a substance of lower molecular weight, resulting in only one-third of the [^{14}C]-label in the urine actually being inulin. With everything taken into account, Lahlou et al. (1969) estimated that the true urine to plasma (U/P) ratio for inulin was probably 0.1-0.2. PEG 4000 is thought to be a more accurate indicator of GFR than inulin derivatives since it undergoes minimal metabolic breakdown (Beyenbach and Kirschner, 1976; Erickson and Gingrich, 1986; Curtis and Wood, 1991). The U/P ratio found for PEG 4000 was approximately 0.19, a value at the high end of the range proposed by Lahlou et al. (1969) for the oyster toadfish. Consequently, the appearance of [^{3}H]-PEG 4000 in the urine

confirms that there are a small number of functional glomeruli present in the kidney of the gulf toadfish, *Opsanus beta*, and probably in the oyster toadfish, *Opsanus tau* as well.

Our results suggest the presence of a urea transport mechanism in the toadfish kidney that is unlike that facilitated diffusion transporter in the gill (Smith et al. 1998) for two reasons. Firstly, as mentioned above, the analogues are handled differently by the kidney than by the gill, suggesting a unique transporter in the kidney. Secondly, the transport of thiourea and urea could not occur exclusively *via* a facilitated diffusion mechanism. The renal secretion rate of urea was greater than the secretion of acetamide, although significantly less than the secretion of thiourea. Both thiourea and urea appeared to be secreted at least 2-3x more effectively than Cl^- and water. Acetamide, on the other hand, was well correlated with Cl^- secretion, being secreted in a 1:1 ratio. Both substances were found in equal concentrations in the urine and plasma and thus appeared to passively equilibrate between the two fluids. Since the relative secretion of thiourea and urea was greater than the relative movement of Cl^- or water, their passage into the kidney tubule cannot be accounted for by simple diffusion. The transport of urea and thiourea also occurred against a concentration gradient, supporting the theory of an active transport mechanism.

What transport mechanism could be involved with the secretion of urea into the kidney tubule of the gulf toadfish? While a facilitated diffusion transporter has been cloned for shark kidney (Smith and Wright, 1999), the most studied active transport mechanism is the Na^+-dependent urea transporter originally identified in the elasmobranch kidney (Schmidt-Nielsen et al. 1972) and more recently in the mammalian

kidney of protein depleted animals (Isozaki et al.1994a,b). One hypothesis for the toadfish kidney is that urea secretion is *via* a Na^+-urea co-transporter, urea moving with Na^+ down its concentration gradient. Further down the tubule, Na^+ would then be actively reabsorbed against its concentration gradient. A second and more simple hypothesis is a Na^+-urea antiporter, for which evidence has recently been provided in the deep portions of the inner medullary collecting duct ($IMCD_3$) of the mammalian kidney; urea appears to move into the kidney tubule in direct counter-transport with active Na^+ reabsorption (Kato and Sands, 1998a,b). This Na^+-urea antiport mechanism appears to have characteristics of two different transporters. It is similar to both the facilitated diffusion mechanism (UT-A1) in that it is stimulated by arginine vasopressin (AVP) and inhibited by phloretin, but also similar to the Na^+-coupled transporter in that it is inhibited by ouabain and a lack of Na^+ on the mucosal side (Kato and Sands, 1998a).

Further investigation is needed in order to establish whether urea secretion in the kidney of the toadfish is an active transport mechanism that relies on the movement of Na^+, or conceivably a unique mechanism not yet observed in other systems.

Table 2-1: The pattern of branchial urea excretion in gulf toadfish prior to and following treatment with the urea analogues, acetamide or thiourea. Values are means ± 1 S.E.M.

	Control	Analogue Treatment
Acetamide Series (N = 5)		
Urea excretion rate ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	65.7 ± 16.3	52.4 ± 7.3
Pulse frequency ($pulses \cdot 12h^{-1}$)	1.20 ± 0.20	0.95 ± 0.35
Pulse size ($\mu mol\ urea \cdot kg^{-1} \cdot pulse^{-1}$)	664 ± 172	606 ± 79
Thiourea Series (N = 5)		
Urea excretion rate ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	47.7 ± 11.4	20.2 ± 2.75*†
Pulse frequency ($pulses \cdot 12\ h^{-1}$)	1.20 ± 0.37	0.50 ± 0.11*
Pulse size ($\mu mol\ urea \cdot kg^{-1} \cdot pulse^{-1}$)	510 ± 124	512 ± 71

* significantly different ($P < 0.05$) from pre-analogue control

† significantly different ($P < 0.05$) from acetamide series

Table 2-2: Gill permeability to urea, acetamide and thiourea during non-pulsing and pulsing periods. Values are means ± 1 S.E.M.

	Urea Permeability ($cm \cdot sec^{-1} \times 10^{-6}$)	Analogue Permeability ($cm \cdot sec^{-1} \times 10^{-6}$)
Acetamide Series (N = 5)		
Non-pulsing	0.41 ± 0.17	0.27 ± 0.10
Pulsing	10.48 ± 2.66*†	4.71 ± 1.01*
Thiourea Series (N = 5)		
Non-pulsing	0.11 ± 0.04	0.10 ± 0.02
Pulsing	5.10 ± 1.45*†	0.56 ± 0.07*

* significantly different (P <0.05) from non-pulsing permeability

† significantly different (P <0.05) from analogue permeability during the same period

Table 2-3: Na^+ and Cl^- concentrations in the plasma and urine of both acetamide and thiourea treated fish. Values are means +/- 1 S.E.M

	$[Na^+]_{plasma}$ ($mmol \cdot l^{-1}$)	$[Na^+]_{urine}$ ($mmol \cdot l^{-1}$)	$[Cl^-]_{plasma}$ ($mmol \cdot l^{-1}$)	$[Cl^-]_{urine}$ ($mmol \cdot l^{-1}$)
Acetamide treated fish (N = 5)	158.1 ± 3.2*	18.7 ± 5.7	127.4 ± 4.7	117.3 ± 14.9
Thiourea treated fish (N = 5)	150.1 ± 4.4*	27.1 ± 5.4	125.9 ± 2.2	93.7 ± 15.1

* significantly different ($P < 0.05$) from urine concentrations. There were no significant differences between the two series.

Figure 2-1: Data from two individual fish showing the appearance of (A) acetamide and urea and (B) thiourea and urea in the surrounding water over time. Branchial pulsatile excretion of both urea and acetamide is evident. In contrast, thiourea pulses are poorly defined. Values are corrected for fish body weight.

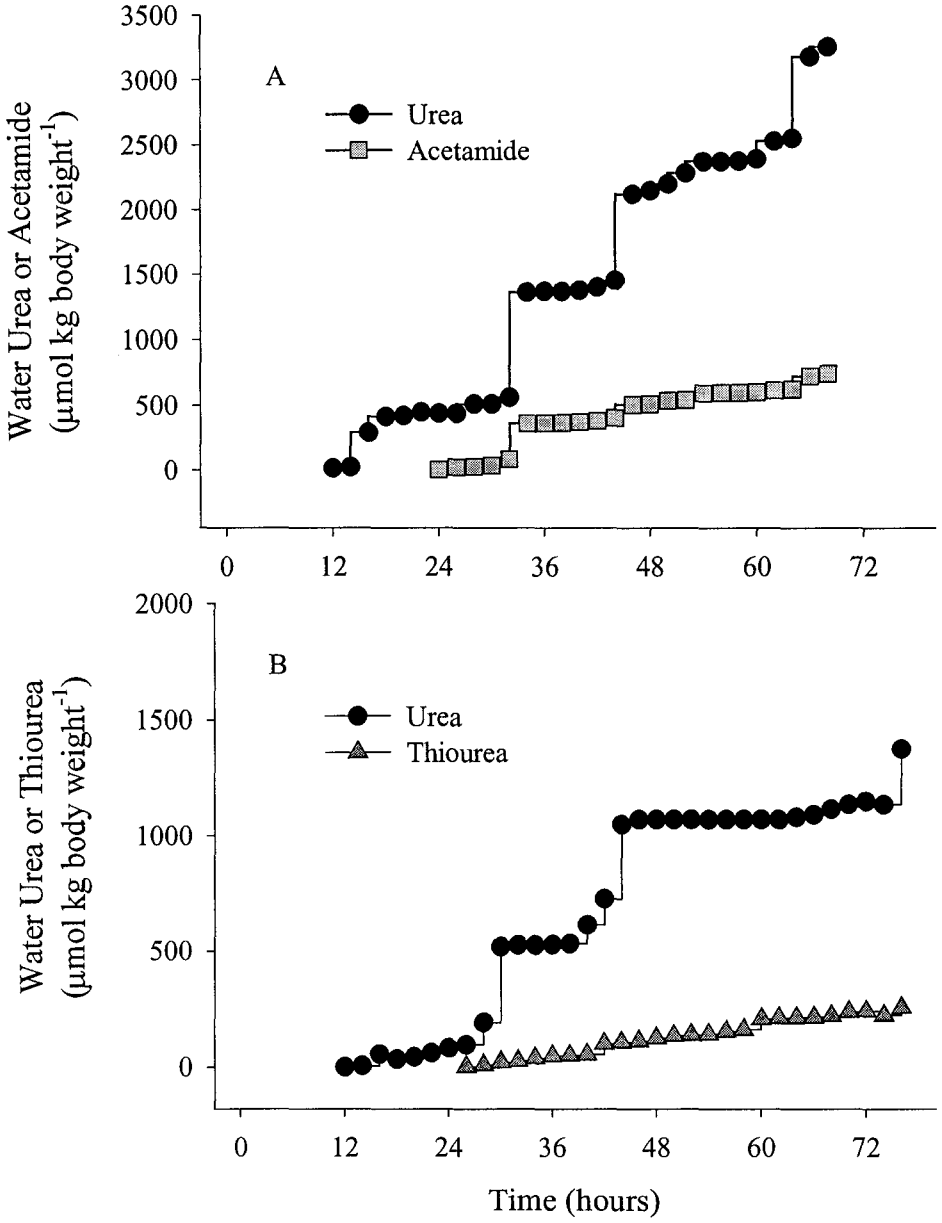

A
Urea
Acetamide
Water Urea or Acetamide
(µmol kg body weight-1)
3500
3000
2500
2000
1500
1000
500
0
0
12
24
36
48
60
72
B
Urea
Thiourea
Water Urea or Thiourea
(µmol kg body weight-1)
2000
1500
1000
500
0
0
12
24
36
48
60
72
Time (hours)

Figure 2-2: Linear regressions of the branchial clearance of analogue (y-axis) *versus* the branchial clearance of urea (x-axis) denoting an analogue clearance that is less than that of urea. The equation of the acetamide line and the significance of the correlation is $y = 0.352x + 12.089$ $r = 0.851$; $P < 0.05$ ($N = 18$) showing that an acetamide pulse is 35% the size of a urea pulse. The equation of the thiourea line and the significance of the correlation is $y = -0.030x + 15.359$ $r = 0.241$; $P < 0.05$ ($N = 9$) demonstrating that thiourea clearance is not strongly correlated with urea clearance and is poorly cleared by the gills.

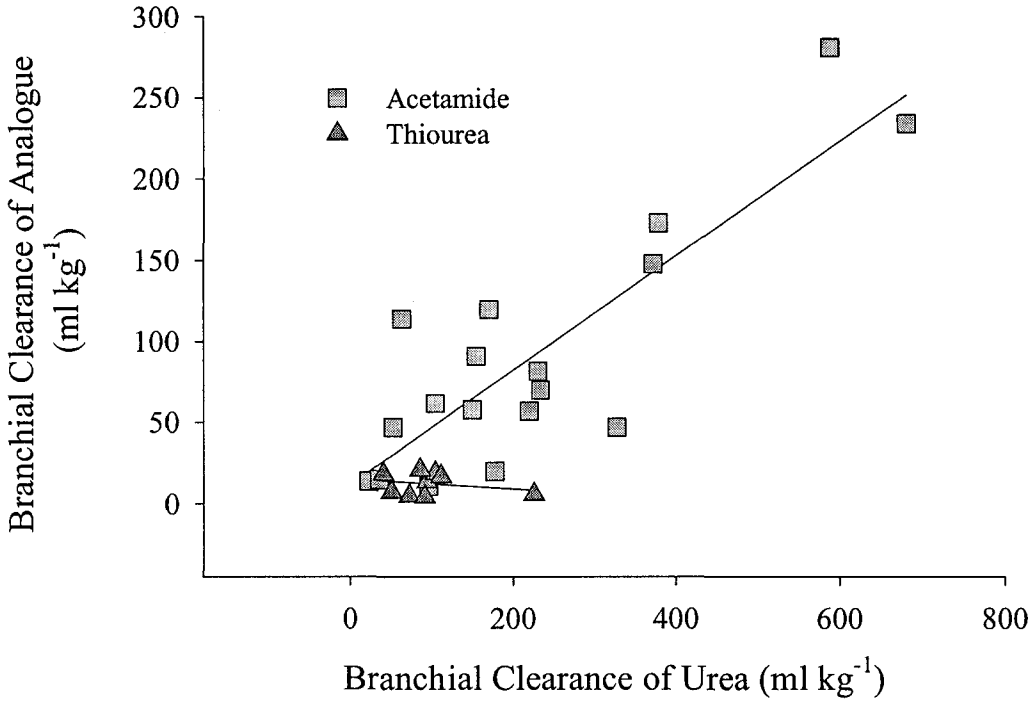

Branchial Clearance of Analogue (ml kg-1)
300
250
200
150
100
50
0
Acetamide
Thiourea
0
200
400
600
800
Branchial Clearance of Urea (ml kg-1)

Figure 2-3: Concentrations of (A) urea and (B) acetamide in the urine and plasma demonstrating differences in the renal handling of the two substances. Dotted line indicates time of acetamide injection. Values are means ± 1 S.E.M. (N = 5); * $P < 0.05$ compared to plasma concentrations.

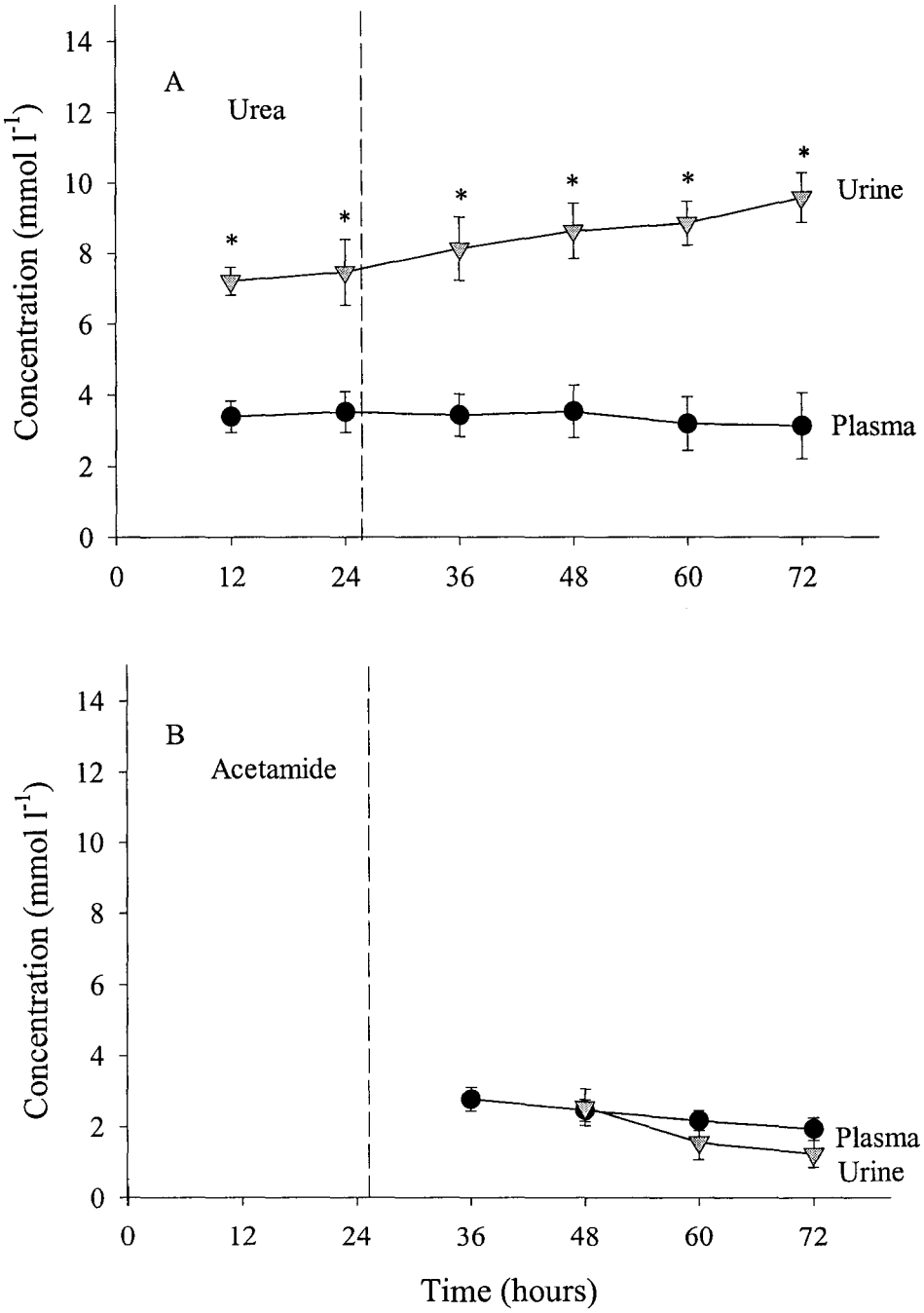

A
Urea
Urine
Plasma
Concentration (mmol l-1)
0
2
4
6
8
10
12
14
0
12
24
36
48
60
72
B
Acetamide
Plasma
Urine
Concentration (mmol l-1)
Time (hours)

Figure 2-4: Concentrations of (A) urea and (B) thiourea in the urine and plasma demonstrating similar renal handling of the two substances. Dotted line indicates time of thiourea injection. Values are means ± 1 S.E.M. (N = 5); * $P < 0.05$ compared to plasma concentrations.

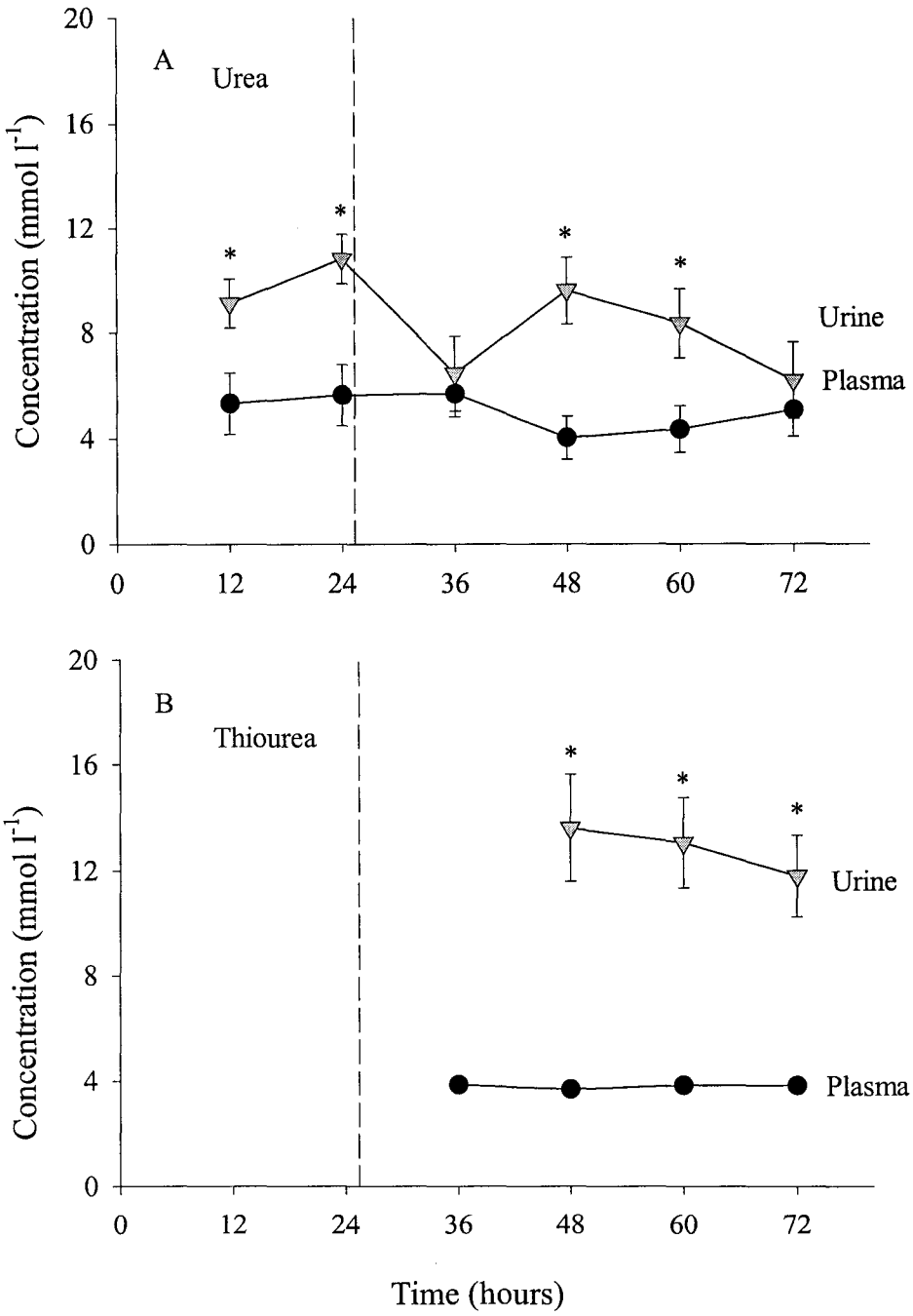
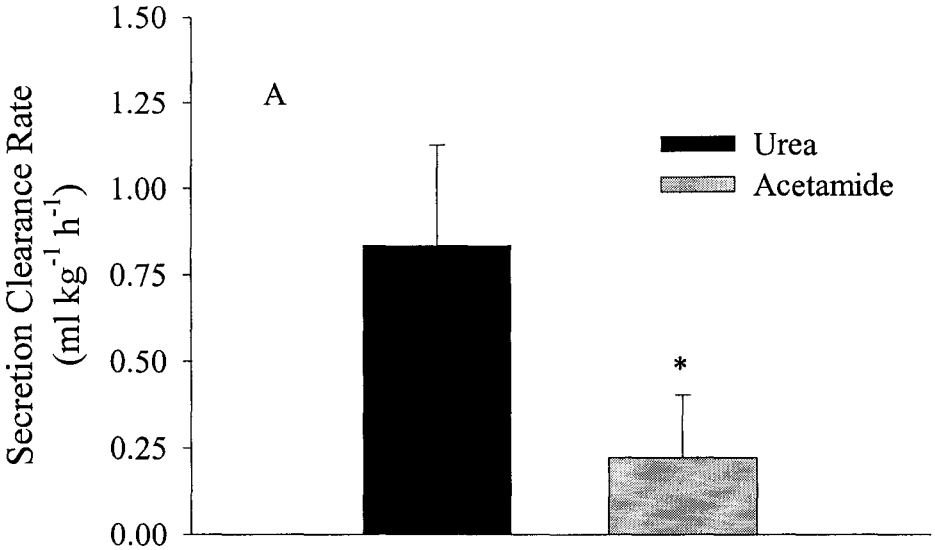

A
Urea
Concentration (mmol l-1)
20
16
12
8
4
0
0
12
24
36
48
60
72
Urine
Plasma
B
Thiourea
Concentration (mmol l-1)
Urine
Plasma
Time (hours)

Figure 2-5: The secretion clearance rate (ml kg^{-1} h^{-1}) of fish in the (A) acetamide series showing a urea clearance rate that is significantly greater than the clearance rate of acetamide. In contrast, (B) the clearance rate of urea is significantly less than the clearance rate of thiourea. Values are means ± 1 S.E.M. (N = 5 for each treatment); * $P < 0.05$ compared to urea.


A
Urea
Acetamide
*
Secretion Clearance Rate
(ml kg-1 h-1)
1.50
1.25
1.00
0.75
0.50
0.25
0.00

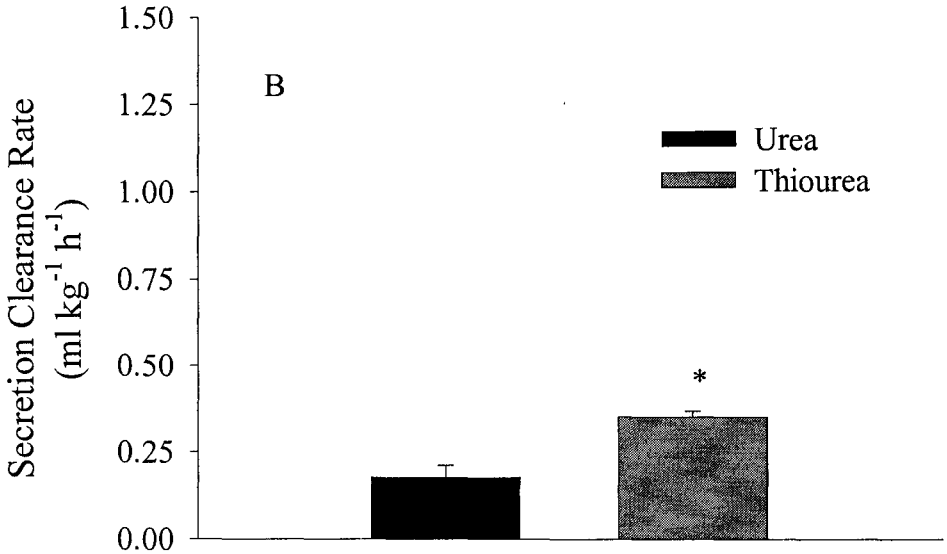
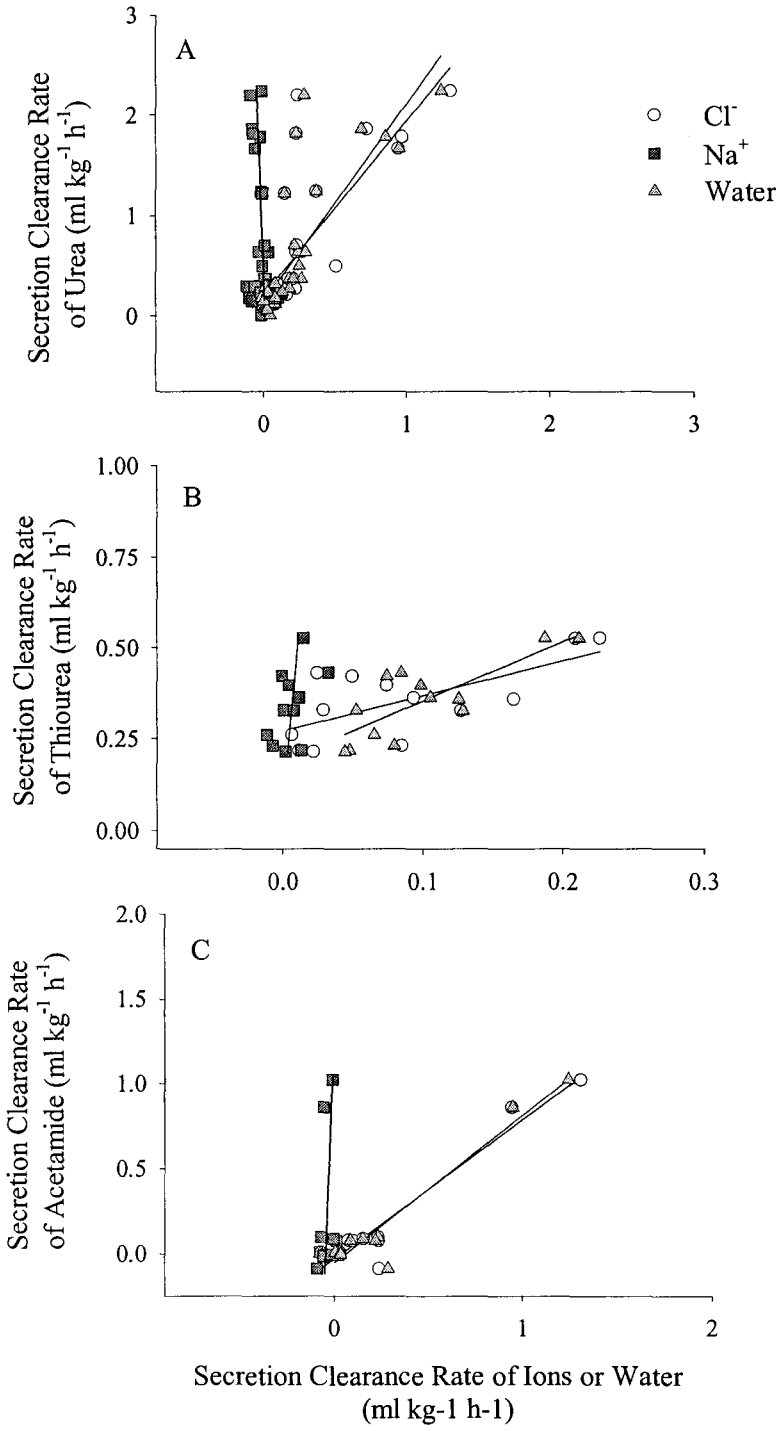

B
Urea
Thiourea
*
Secretion Clearance Rate
(ml kg-1 h-1)
1.50
1.25
1.00
0.75
0.50
0.25
0.00

Figure 2-6: Linear regressions of the secretion clearance rates of (A) urea (B) thiourea and (C) acetamide (y-axis) *versus* water, Na^+ and Cl^- (x-axis) demonstrating a secretion of urea and thiourea at a greater relative rate than the movement of water and Cl^- and a secretion of acetamide comparable to both Cl^- and water. In all cases there was no net secretion of Na^+.


A
Secretion Clearance Rate of Urea (ml kg-1 h-1)
3
2
1
0
0
1
2
3
Cl-
Na+
Water
B
Secretion Clearance Rate of Thiourea (ml kg-1 h-1)
1.00
0.75
0.50
0.25
0.00
0.0
0.1
0.2
0.3
C
Secretion Clearance Rate of Acetamide (ml kg-1 h-1)
2.0
1.5
1.0
0.5
0.0
0
1
2
Secretion Clearance Rate of Ions or Water
(ml kg-1 h-1)

Chapter 3

The branchial and renal handling of urea in the gulf toadfish, *Opsanus beta*: the effect of exogenous urea loading

Abstract

The objective of this study was to determine whether the pulsatile facilitated diffusion transport mechanism (tUT) found in the gills of the gulf toadfish (*Opsanus beta*) and the active secretion transporter thought to be present in its kidney could be saturated when faced with elevated plasma urea concentrations. Toadfish were fitted with indwelling caudal artery and ureteral catheters and infused with four consecutive exogenous urea loads at a rate of 0, 150, 300 and 600 μmol kg^{-1} h^{-1}. Initial plasma and urine urea concentrations were 8.1 +/- 0.9 (6) mmol·l^{-1} and 12.4 +/- 1.5 (6) mmol·l^{-1}, respectively, and steadily increased with increasing infused loads of urea to a maximum of 36.8 +/- 2.8 (6) mmol·l^{-1} in the plasma and 39.8 +/- 6.5 (4) mmol·l^{-1} in the urine. There was no change in pulse frequency and only a very weak relationship (r = 0.17) between pulse size (measured as branchial excretion during pulsatile excretion of urea) and plasma urea concentration (slope = 9.79 μmol-N kg^{-1}/ mmol-N l^{-1}; $P < 0.05$) suggesting that the branchial excretion mechanism was already saturated at normal plasma urea concentrations. Urine flow rate (0.15 ± 0.03 (6) ml kg^{-1} h^{-1}) and glomerular filtration rate

(0.025 ± 0.004 (6) ml kg^{-1} h^{-1}) remained constant throughout the experiment despite the increased volume load. Initially, urine urea concentrations were significantly greater than those in the plasma but by the end of the 300 μmol kg^{-1} h^{-1} infusion and for the remainder of the experiment, urine and plasma urea concentrations were not significantly different. However, renal urea secretion rate maintained a strong linear relationship ($r = 0.84$) to plasma urea levels (slope = 0.391 μmol-N kg^{-1} h^{-1}/ mmol-N l^{-1}; $P < 0.001$) with no observable transport maximum, suggesting that the renal secretory transport mechanism could not be saturated even at plasma levels well above normal, in contrast to the branchial excretion mechanism.

Introduction

The gulf toadfish (*Opsanus beta*) is unique with respect to urea metabolism and excretion. Unlike most teleost fish, the toadfish has a fully functional ornithine-urea cycle (OUC), giving it the ability to excrete its nitrogenous wastes as urea, a less toxic but more energetically-expensive alternative to ammonia (Mommsen and Walsh, 1989). However, toadfish are not obligately ureotelic, like the equally unusual Lake Magadi tilapia (Randall et al. 1989; Wood et al. 1989). Rather, toadfish are facultatively ureotelic, excreting predominantly ammonia under normal conditions but will switch to excreting predominantly urea when stressed by exposure to air, ammonia, crowding or confinement (Walsh et al. 1990; Walsh et al. 1994a; Walsh and Milligan, 1995). Most of the urea (> 90%) leaves the fish through its gills where the cDNA for a urea transport protein (tUT) has been cloned that shows higher than 60% homology at the amino acid level to mammalian urea transporters of the hormonally controlled facilitated diffusion (UT) type (Smith et al. 1998; Walsh et al. 2000). This facilitated diffusion mechanism in the toadfish appears to be periodically activated or inserted into the gill membrane; the permeability of the gill to urea intermittently increases, allowing urea to be excreted in distinct 0.5–3 hour pulses, on average once or twice every 24 hours (Wood et al. 1995; Wood et al. 1997; Wood et al. 1998). During pulsing periods, tUT allows the passage of the urea analogues, acetamide and thiourea. However, the increased permeability of the gill for these two substances is notably less (17-fold and 6-fold, respectively) than the 36-fold increase observed for urea (Chapter 2). During non-pulsing periods, all three of these substances are handled similarly by the gill, passing *via* simple diffusion.

In addition to tUT in the gill, recent evidence suggests a transport mechanism in the kidney of the toadfish (Chapter 2). Although very low glomerular filtration has been reported, the kidney of the gulf toadfish is primarily secretory and is the secondary route of nitrogenous waste excretion, contributing to <10% of total urea excretion. However, toadfish urine urea concentrations generally exceed plasma levels by at least 30%, suggesting the presence of an active urea transport mechanism. The toadfish kidney also demonstrates differential handling of urea and analogues, though with a different pattern from that of the gill. Urea and thiourea are more concentrated in the urine than in the plasma while acetamide appears to passively equilibrate between the two. In Chapter 2, it was speculated that a Na^+-dependent active urea transporter similar to that originally described in the elasmobranch kidney (Schmidt-Nielsen et al. 1972) or more recently the mammalian kidney (Isozaki et al. 1994a,b; Kato and Sands, 1998a,b), could be involved in the uphill movement of urea into the toadfish kidney tubule.

In mammals, characterizing urea transport kinetics has proven difficult as the urea concentrations required to saturate UT-type transporters are detrimentally high, making saturation difficult to distinguish from membrane degradation (Chou et al. 1990; reviewed by Sands et al. 1997). To date, the kinetic variables K_m and V_{max} have not been determined for cloned mammalian UT-type transporters and recent evidence suggests that these mechanisms behave more like channels than typical carriers due to their high turnover number (Kishore et al. 1997; reviewed by Smith and Rousselet, 2001). However, saturation kinetics have been observed for both facilitated and active urea transport mechanisms in cases of lower vertebrates including amphibians, elasmobranchs

and teleosts (Levine et al. 1973a; Shpun and Katz, 1989, 1990; Pilley and Wright, 2000; Fines et al. 2001). In amphibian skin, where an active transport mechanism for urea exists, the K_m is low (< 2 mmol·l^{-1}) compared to the high K_m (88-107 mmol·l^{-1}) of the UT-type mechanism found in the urinary bladder (Levine et al. 1973a, Katz et al. 1981; Shpun and Katz, 1989; Dytko et al. 1993).

As of yet, the saturation kinetics for the branchial tUT transporter and the suggested urea secretory mechanism in the kidney of the toadfish have not been investigated. The objective of the present study was to determine the pattern of renal and branchial handling of urea when faced with elevated plasma urea levels and determine whether the movement of urea through either of these two organs can be saturated. The results indicate very different response patterns by branchial *versus* renal transport mechanisms.

Materials and Methods

Experimental Animals

Gulf toadfish (*Opsanus beta*) were caught by commercial shrimpers in Biscayne Bay, Florida between November and December 1999. The toadfish were held in an outdoor tank at the shrimpers' holding facility with running sea water (ambient seasonal conditions) for no longer than 24 h following capture, then transferred to the laboratory. Fish were treated with a dose of malachite green (final concentration 0.05 mg·l^{-1}) in formalin (15 mg·l^{-1}) (AquaVet, Hayward, CA, USA) on the day of transfer to the laboratory in order to prevent infection by the cilate, *Cryptocaryon irritans* (Stoskopf,

1993). Initially the fish were kept in 50 L polypropylene tubs with flowing, aerated seawater. Fish were maintained in this relatively crowded environment (> 10 fish per tank) in order to initiate a switch to ureotelism (Walsh et al. 1994a). In order to ensure full expression of ureotelic behaviour, the fish were then transferred in groups of four to plastic tubs (6 L) served with flowing seawater at least 48 h prior to surgery. The water temperature was 26°C. Fish were fed weekly with shrimp and squid throughout the experimental period.

Experimental Protocol

The specific goal was to examine the pattern of branchial and renal handling of urea in the face of elevated plasma urea levels. In addition to branchial and renal handling of urea, urine flow rate (UFR), glomerular filtration rate (GFR) and the handling of Na^+, Cl^-, Mg^{++} by the kidney were also monitored.

As outlined in Chapter 2, caudal artery and ureteral catheterization were performed simultaneously on fish anaesthetized with MS-222 (0.5 $g \cdot l^{-1}$; Sigma-Aldrich Canada) and wrapped with wet towels. Caudal artery catheters were inserted as described by Wood et al. (1997). The caudal vertebrae were exposed by a 1.5-2.0 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with Clay-Adams PE50 tubing filled with Hank's saline (Walsh, 1987) containing 50 $i.u \cdot ml^{-1}$ sodium heparin (Sigma-Aldrich Canada). A heat-flared PE160 sleeve was glued in place with cyanoacrylate tissue cement (Vetbond; 3M Corporation) and sutured at the site of exit in order to secure the catheter. The wound was treated with oxytetracycline powder,

in order to prevent infection, and sutured securely with 2-0 silk.

The technique for inserting indwelling ureteral catheters is described in detail in Chapter 2 and is a modification of the original method described by Howe and Gutknecht (1978) and Lahlou et al. (1969). Our goal was to examine the function of the kidney alone by bypassing the urinary bladder, thereby eliminating any reabsorptive/secretory roles of the bladder. Briefly, the end of a catheter, made of Clay Adams PE10 tubing, was filled with distilled water and advanced dorsally through the urogenital papilla into the urinary sinus. The catheter was held in place by three 3-0 silk ligatures around the papilla. A heat-flared PE60 sleeve was threaded onto the catheter and moved as close to the papilla as possible. The sleeve was glued in place with Vetbond and sutured to the body at the site of exit in order to secure the catheter. A ventral incision was then made just anterior to the urogenital papilla. Both urinary bladders were isolated and ligated close to the ureter end with 2-0 silk, thereby negating the storage function of the bladders. Oxytetracycline powder was dusted into the body cavity and the ventral musculature and skin were then sutured securely. The urine drained directly from the ureters into the urinary sinus where the catheter tip was located. During recovery and experimentation, urine was collected continuously with the catheter emptying into a vial approximately 3.0 cm below the water level of the box. The urine flow rate (UFR) was determined gravimetrically. Following surgery, fish were kept in darkened individual containers (minimum volume = 1.5 L) that were continually aerated and supplied with flowing water (200 $ml \cdot min^{-1}$).

Following the procedure outlined by McDonald and Wood (1998) and in Chapter 2,

[^{3}H]-polyethylene glycol (PEG, Mr4000) was used as a marker for glomerular filtration rate (GFR). PEG 4000 is considered a more accurate and conservative indicator of GFR in fish than other commonly used markers (*e.g.* inulin derivatives), as it undergoes minimal radioautolysis, metabolic breakdown, or post-filtration reabsorption across tubules and bladder (Beyenbach and Kirschner, 1976; Erickson and Gingerich, 1986; Curtis and Wood, 1991).

Prior to injection of PEG 4000, the fish (0.119 ± 0.012 (6) kg ranging from 0.085 to 0.158 kg) were allowed to recover from surgery for at least 12 h, a period during which the patency of the arterial and urinary bladder catheters was confirmed. A dose of 10 $\mu Ci \cdot 100g^{-1}$ of [^{3}H]-PEG 4000 (New England Nuclear) in 0.25 $\mu l \cdot 100g^{-1}$ saline was injected *via* the caudal arterial catheter followed by an additional 0.3 ml of saline in fish where both catheters were deemed successful. Immediately following the [^{3}H]-PEG 4000 injection, the arterial catheters of the fish were connected to a peristaltic pump and fish were infused with four consecutive isosmotic (300 mOsm kg^{-1}) loads of urea (0, 50, 100, 200 $mmol \cdot l^{-1}$) balanced with NaCl. In each case, one channel of a Gilson-8 channel peristaltic pump was used at an infusion rate of 3 ml kg^{-1} h^{-1}; the rate was checked by periodic measurements of the weight of the infusion reservoir. Branchial urea excretion was measured by closing the flux box and recording the appearance of urea in the external water. Twelve hours after the start of the first infusion, a blood sample was taken (200 μl; with saline plus red blood cell replacement), a fresh urine collection was started, water flow to the fish box was stopped and the water level was set to an exact volume mark of 1.5 L. An initial water sample was taken for measurement of urea and

ammonia concentration. Thereafter, water samples (5 ml) were taken every two hours for the remainder of the experiment and the box was rapidly flushed with fresh seawater over a 15-minute period at 24 h intervals. Vigorous aeration maintained PO_2 close to air saturation during times when water flow to the box was stopped. Blood and urine samples were taken every 12 hours, making a total of approximately 60 water samples (at 2 h intervals), 10 blood samples (at 12 h intervals) and 10 urine samples (each spanning a 12 h collection period). After the initial 24 h infusion with 0 μmol kg^{-1} h^{-1} urea, the rate of urea infusion was increased to 150 μmol kg^{-1} h^{-1} urea and the same protocol of box closure, water, urine and blood sampling repeated over the next 36 h. At the 60 h and 96 h mark the rate of urea infusion was again increased to 300 μmol kg^{-1} h^{-1} and 600 μmol kg^{-1} h^{-1} urea respectively. The final infusion period was terminated at 132h.

Thus, for the first infusion (isosmotic NaCl only), 1 blood sample was taken and 1 measurement of renal and branchial urea excretion was recorded. For the second, third and fourth infusions, 3 blood samples were taken and 3 measurements of renal and branchial urea excretion were recorded. Blood samples were centrifuged at 10 000g for 1 minute and the plasma decanted. The red blood cells were then resuspended in saline and re-infused. Plasma and urine were frozen and stored at -20°C for later analysis of Na^+, Cl^-, Mg^{++}, urea and [^{3}H] PEG 4000. Water samples were analyzed immediately for urea and ammonia.

Analytical Techniques and Calculations

Urea concentrations in blood, urine and water were measured using the diacetyl

monoxime method of Rahmatullah and Boyde, (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in the three media. Ammonia concentrations in the water were measured by the method of Ivancic and Degobbis (1984). Na^+ and Cl^- concentrations in plasma and urine were measured using a Varian 1275 Atomic Absorption Spectrophotometer and a Radiometer CMT10 Chloridometer respectively. For measurements of [^{3}H]-PEG 4000, plasma and urine samples (20 μl) were added to 4 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217.

The branchial excretion (B; μmol kg^{-1}) of any substance (*X*) was calculated from the increase in concentration of the substance in the water $[\Delta X]_w$ during a pulse corrected for fish body weight (wt) and calculated as:

$$B_X = \frac{[\Delta X]_w \times V_f}{wt}, \qquad (1)$$

where V_f is the volume of water surrounding the fish. A pulse was identified as a sudden increase in urea, acetamide or thiourea appearance in the surrounding water of at least 20 μmol kg^{-1}. The branchial clearance (C_B; ml kg^{-1}) of any substance (*X*) was calculated from the increase in concentration of the substance in the water $[\Delta X]_w$ factored by fish body weight (wt) and plasma concentration $[X]_p$ and calculated as:

$$C_{BX} = \frac{[\Delta X]_w \times V_f}{wt \times [X]_p}, \qquad (2)$$

where V_f is the volume of water surrounding the fish. The branchial permeability (P) of any substance (*X*) was determined as:

$$P_X = \frac{C_{BX}}{T \times SA_B}, \quad (3)$$

using the calculated values of branchial clearance (C_B) and branchial surface area for toadfish (SA_B) (1920 cm^2 kg^{-1}; Hughes and Grey (1972)), where T is the period duration.

All the following rates were related to fish body weight by expressing urinary flow rate (UFR) in $ml \cdot kg^{-1} \cdot h^{-1}$. Urinary excretion rates (U; $\mu mol\ kg^{-1}\ h^{-1}$) of any substance (*X*) were calculated as:

$$U_X = [X]_u \times UFR, \quad (4)$$

using measured values of urine flow rates (UFR) and urine concentrations $[X]_u$. Glomerular filtration rate (GFR; $ml\ kg^{-1}\ h^{-1}$) was calculated as the clearance of [^{3}H]-PEG 4000 - *i.e.* the excretion of radioactivity in the urine (cpm_u) relative to its concentration in the blood plasma (cpm_p):

$$GFR = \frac{cpm_u \times UFR}{cpm_p}. \quad (5)$$

The filtration rate (FR; $\mu mol\ kg^{-1}\ h^{-1}$) of a substance *X* at the glomeruli was calculated as:

$$FR_X = [X]_p \times GFR, \quad (6)$$

and consequently the net tubular secretion rate (TS; $\mu mol\ kg^{-1}\ h^{-1}$) of *X* was calculated as:

$$TS_X = U_X - FR_X. \quad (7)$$

The renal clearance rate by secretion (CR; ml kg^{-1} h^{-1}) of *X* was calculated as:

$$CR_X = \frac{TS_X}{[X]_p} \quad . \tag{8}$$

Statistics

Data were reported as means ± 1 S.E.M (N = number of fish). Regression lines were fitted by the method of least squares, and the significance ($P < 0.05$) of the Pearson's correlation coefficient r assessed. The significance of differences between means was evaluated using Student's paired or unpaired, two-tailed or one-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977). An ANOVA with time as the main factor was followed by a comparison of individual means using the Bonferroni correction for multiple sample comparisons.

Results

Exogenous urea loading in the gulf toadfish was performed to elevate plasma urea levels well beyond concentrations measured during resting conditions so as to determine whether the pulsatile branchial UT mechanism or renal secretion of urea could be saturated. Mean data for plasma and urine composition, UFR and GFR are reported in Table 3-1. There was no significant change in most of these variables over the 132-h infusion period; however, urine Cl^- increased significantly by the end of the experimental period. As a consequence of exogenous urea loading, plasma and urine urea concentrations increased by 4.6-fold and 3.2-fold, respectively, reaching a maximum of 36.8 +/- 2.8 (6) $mmol \cdot l^{-1}$ in the plasma and 39.8 +/- 6.5 (4) $mmol \cdot l^{-1}$ in the urine (Fig. 3-1).

The toadfish used in this study were predominantly ureotelic, excreting 63.8 ± 3.1 (7) % urea-N (198.2 ± 8.4 (6) μmol-N·kg^{-1}·h^{-1}) and 36.2 ± 3.1 (7) % ammonia-N (100.6 ±19.2 (6) μmol-N·kg^{-1}·h^{-1}) when averaged over the experimental period of 132 hours. Increasing plasma urea concentrations appeared to have a slight but significant negative effect on branchial ammonia excretion, the linear relationship (r = -0.40) having a negative slope of –1.10 μmol-N·kg^{-1}·h^{-1} per mmol-N·l^{-1} ($P < 0.01$; Fig. 3-2A). The % ureotelism increased (r = 0.42) with increasing plasma urea concentration (slope = 0.434 % per mmol·l^{-1} ($P < 0.005$; Fig. 3-2B) and in the last infusion period fish excreted an average of 76.1 ± 3.3 (7) % urea. During the 12 h control period, when fish were infused with urea at a rate of 0 μmol·kg^{-1}·h^{-1}, the gills were the major route of urea excretion, averaging 63.5 ± 20.6 (6) μmol·kg^{-1}·h^{-1} relative to a urinary excretion rate of only 1.85 ± 0.79 (5) μmol·kg^{-1}·h^{-1}, which did not change over the course of the experiment.

On average, more than 99% (99.6 ± 0.1 (6) %) of the branchial excretion of urea occurred in discrete pulses and the occurrence of pulsatile *versus* non-pulsatile excretion did not vary significantly over the experimental period (Fig. 3-3). The permeability of the gills to urea was significantly greater during pulse events (2.4 ± 0.9 x 10^{-6} (7) cm·sec^{-1}) than during non-pulsing periods (0.12 ± 0.02 x 10^{-6} (6) cm·sec^{-1}) and gill permeability to urea did not change over the course of the experiment. Pulses occurred with a mean frequency of 3.0 ± 0.4 (6) pulses every 12 h, frequency staying relatively constant throughout the experiment and not increasing with the increasing plasma urea concentrations. The mean pulse size was 429 ± 274 (6) μmol·kg^{-1}·pulse^{-1} during the

initial 24h control infusion period and 429 ± 214 (6) $\mu mol \cdot kg^{-1} \cdot pulse^{-1}$, 433 ± 93 (6) $\mu mol \cdot kg^{-1} \cdot pulse^{-1}$ and 426 ± 104 (6) $\mu mol \cdot kg^{-1} \cdot pulse^{-1}$ during the respective subsequent infusions. Overall there was only a very weak positive relationship (r = 0.17) between pulse size (measured as branchial excretion during pulsatile excretion of urea) and plasma urea concentration (slope = 9.797 $\mu mol\text{-}N \cdot kg^{-1}$ per $mmol\text{-}N \cdot l^{-1}$; $P < 0.05$; Fig. 3-4). Branchial excretion rates were only 35.9%, 29.6% and 20.1% of the respective infusion rates (150, 300 and 600 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). It would appear that the excretion mechanism was saturated at normal plasma urea concentrations as there was a negligible increase in excretion, despite plasma urea concentrations being well above normal physiological levels.

With respect to the kidney, urine flow rate (0.15 ± 0.03 (6) $ml \cdot kg^{-1} \cdot h^{-1}$) and glomerular filtration rate (0.025 ± 0.003 (6) $ml \cdot kg^{-1} \cdot h^{-1}$) did not change significantly despite the increased volume load (Table 3-1). Initial urine urea concentrations (12.5 ± 1.6 (6) $mmol \cdot l^{-1}$) were significantly greater than those in the plasma (8.1 ± 0.9 (6) $mmol \cdot l^{-1}$), however, by the end of the 300 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ infusion, and for the remainder of the experiment, urine and plasma urea concentrations were not significantly different (Fig. 3-1). Renal excretion rates were only 2.3%, 1.4% and 1.3% of the respective infusion rates (150, 300 and 600 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). Despite these observations, renal urea secretion rate maintained a strong linear relationship (r = 0.84) to plasma urea levels (slope = 0.391 $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$ per $mmol\text{-}N \cdot l^{-1}$, $P < 0.001$) with no observable transport maximum (Fig. 3-5).

On average, Na^+ concentrations in the plasma (111.9 ± 3.4 (7) mmol·l^{-1}) were 9-fold greater than those in the urine (12.7 ± 5.5 (6) mmol·l^{-1}; Table 3-1) while Cl^- concentrations in the plasma (116.8 ± 2.4 (7) mmol·l^{-1}) and urine (71.4 ± 17.6 (6) mmol·l^{-1}) were not significantly different. In contrast, Mg^{++} concentrations were 50-fold greater in the urine (49.9 ± 7.3 (7) mmol·l^{-1}) than in the plasma (0.97 ± 0.26 (7) mmol·l^{-1}). When expressed as secretion clearance rates, Cl^- (mean: 0.072 ± 0.026 (6) ml·kg^{-1}·h^{-1}) and urea (mean: 0.220 ± 0.038 (6) ml·kg^{-1}·h^{-1}) exhibited a linear relationship with each other (r = 0.67) with a slope of 0.45 ml·kg^{-1}· h^{-1} per ml·kg^{-1}·h^{-1} (note: units cancel) ($P < 0.001$; Fig. 3-6A) indicating that the secretion clearance of Cl^- was only 45% of that of urea. Similarly, the relationship between the secretion clearance rate of water (mean: 0.139 ± 0.028 (6) ml kg^{-1} h^{-1}) and urea was also linear, (r = 0.97) with a slope of 0.66 ($P < 0.001$; Fig. 3-6B). Thus, on a relative basis, the movement of water and Cl^- into the kidney tubule is only 45-66% the secretion of urea. The relationship between Mg^{++} (mean: 12.61 ± 3.43 (6) ml·kg^{-1}·h^{-1}) and urea was linear, (r = 0.72) with a slope of 59.6 ($P < 0.001$), indicating that only a small proportion of urea enters the kidney tubule compared to Mg^{++}, which is thought to be actively secreted (Fig. 3-6C). There was no quantitative relationship between the secretion clearance of Na^+ (mean: -0.011 ± 0.004 (6) ml·kg^{-1}·h^{-1}; a negative value for secretion clearance indicating reaborption) and urea (r = 0.25, slope not significant from zero) and a low rate of net reabsorption rather than secretion was measured for Na^+ in most cases (Fig. 3-6D). The relationships between the secretion clearance rate of urea, Na^+, Cl^-, water and Mg^{++} were linear despite non-physiologically high plasma urea concentrations in contact with the kidney tubule.

Discussion

The purpose of this study was to increase circulating urea concentrations by exogenous urea loading in an attempt to saturate the branchial tUT and the renal urea secretory mechanism *in vivo*. In addition, the renal handling of urea in the face of urea loading was compared to the handling of Na^+, Cl^-, water and Mg^{++}. Our results indicate that excretion occurred at a limited capacity in the face of progressive urea loading, resulting in a 4.6-fold increase in plasma urea concentrations. The principal limitation appeared to occur with the gill excretion mechanism, which accounts for about 97% of whole body urea excretion under normal conditions. Branchial urea excretion exhibited a negligible increase in the face of a large elevation in plasma urea concentration (Fig. 3-3). Although pulse size exhibited a very slight rise with plasma urea concentration, it did not rise in proportion to plasma urea concentration (Fig. 3-4). Furthermore, pulse frequency did not change with the increased infusion load, supporting previous observations that pulse occurrence was not correlated with a threshold plasma urea concentration (Wood et al. 1997). Taken together, these results suggest the pulsatile tUT mechanism in the toadfish gill normally operates close to saturation.

The situation was very different for the renal secretion rate, which increased linearly with plasma concentration, and showed no evidence of reaching a transport maximum. The renal handling of Mg^{++}, Cl^- and water but not Na^+ were proportional to the renal handling of urea and the relationships between the renal handling of these

substances under urea loading were not substantially different than those observed under resting conditions (Chapter 2).

In mammalian systems, facilitated diffusion urea transport mechanisms are characteristically difficult to saturate and technical difficulties often arise in trying, due to the high urea concentrations that are often required (Chou et al. 1990). Data from kinetic studies up until this point indicate that mammalian UT-type transporters have a very high turnover number (V_{max}), uncharacteristic of most carrier-like transporters that must alternate the specific binding site to either membrane face. The high V_{max} of UT-type transporters suggests a channel-based function that could handle millions of molecules per second (Kishore et al. 1997; reviewed by Smith and Rousselet, 2001). Recently however, saturation of urea transporters has been described in fish, namely *Squalus acanthias* gill (K_m = 10.1 mmol·l^{-1} and V_{max} = 0.34 μmol·mg protein^{-1}·h^{-1}; Fines et al. 2001) and *Oncorhynchus mykiss* embryos (K_m = 2.0 mmol·l^{-1} and V_{max} = 10.5 nmol·g embryo^{-1}·h^{-1}; Pilley and Wright, 2000). In fact, inward urea transport by the dogfish gill appears to be *via* a secondary active mechanism (Fines et al. 2001), while outward/inward urea movement in trout embryo may be mediated by UT-facilitated diffusion (Pilley and Wright, 2000). Saturable urea transport appears to carry over in adult trout as well, as phloretin-sensitive uptake of urea by trout gill basolateral membrane vesicles has a K_m = 1.17 mmol·l^{-1} and a V_{max} = 0.42 μmol·mg protein^{-1}·h^{-1} (Chapter 6). This observation supports preliminary Northern blot and sequence analysis that revealed a mRNA from rainbow trout gill with approximately 2000 bases

homologous to the mRNA of the gulf toadfish tUT (C.M. Pilley, P.W. Wright and P.J. Walsh, unpublished data).

The branchial excretion rates during the consecutive 150, 300 and 600 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ urea infusions were only 35.9%, 29.6% and 20.1% of the infusion rates and the renal excretion rates were only 2.3%, 1.4% and 1.3%, respectively; the kidney contributing less than 5% to total urea excretion. Thus, mainly as a consequence of the limitation in branchial urea excretion, plasma urea concentrations increased dramatically, and 80% of the infused load that was not excreted can be accounted for, assuming that this rise in plasma urea levels is distributed throughout the body water compartment. It is not surprising that internal urea concentrations can increase to this extent without any apparent negative effects in the toadfish as similar observations have been made with respect to the plainfin midshipman (*Porichthys notatus*), a close relative of the toadfish (Chapter 4). In the toadfish, there appears to be no homeostatic regulation of plasma urea loads. When midshipmen were infused at 15 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ urea (lower than rates used in the present study), they demonstrated an initial ability to maintain resting plasma urea concentrations (Chapter 4). However, when infused at 240 and 480 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, plasma urea concentrations in the midshipman began to rise and the branchial excretion of urea was only 11-14% of the respective infusion rates, lower than the clearance efficiency of toadfish under similar loading conditions (36-20%).

In contrast to batrachoidid fish (toadfish and midshipmen), the freshwater rainbow trout appears to have a tighter regulation of plasma urea concentrations in the face of exogenous loading (McDonald and Wood, 1998). When infused with increasing

exogenous loads of urea, the branchial excretion rate of the trout initially kept pace with the infusion rate and internal urea levels remained stable. When the infusion rate reached 240 μmol kg^{-1} h^{-1}, branchial excretion accounted for 75% of infusion rate and 55% when the rate of infusion was increased to 420 μmol·kg^{-1}·h^{-1}, resulting in a rise in internal urea concentrations that eventually proved toxic. However, these clearance efficiencies were much greater than in the toadfish (36-20%) under similar loading conditions. The physiological significance of these findings is uncertain. For the toadfish, a lower branchial clearance efficiency compared to the trout could be a limitation of the activation of its pulsatile excretion mechanism (*i.e.* the pulse frequency was not increased) since pulse events are thought to be under hormonal control (Wood et al. 2001).

The very weak correlation between branchial excretion and plasma urea concentration in the gulf toadfish suggests that tUT, when activated, is running very close to saturation under normal conditions. In support of this finding, the toadfish tUT demonstrates a branchial excretion rate that is 10-35 times the rates achieved in trout and midshipman (~ 400 μmol-N·kg^{-1}·h^{-1} during pulse events in toadfish *cf.* 40 μmol-N·kg^{-1}·h^{-1} and 12 μmol-N·kg^{-1}·h^{-1} in trout; McDonald and Wood (1998) and midshipmen; Chapter 4, respectively). Considering the above, tUT operating at or near saturation agrees with the entire premise behind the cryptic pulsing behaviour in toadfish: to eliminate the daily nitrogen waste production in as short a period of time as possible (Wood et al. 1995). An excretion mechanism that ran half maximally in this case would be disadvantageous. It is

curious, however, why pulse frequency or duration were not increased during urea loading; possibly, the hormonal stimuli were not elicited by urea loading.

The kidney of the gulf toadfish is primarily secretory in function and the amount of glomerular filtration that does occur is minor in comparison. The filtration of urea accounts for only approximately 10% of the total amount excreted in the urine. Therefore, the majority of urea must enter the kidney tubule by secretion, but *via* what mechanism? With respect to tubular secretion, urea and the urea analogues, thiourea and acetamide are handled differently, as urea and thiourea are both secreted against a concentration gradient while acetamide is found in equal concentrations in plasma and urine (Chapter 2). With acetamide found equally in the two media, the possibility that thiourea and urea are concentrated in the tubule as a consequence of water reabsorption can be eliminated. In addition, the tubular secretion of urea is 1.6-2.2 times greater than the movement of Cl^- and water, disqualifying solvent drag as the only mechanism of entry.

In the present study, urea secretion initially occurred against a concentration gradient, however, this gradient was not maintained at plasma urea concentrations greater than 18 $mmol \cdot l^{-1}$ (Fig. 3-1). Nonetheless, the renal secretory mechanism did not demonstrate saturation under the conditions of the present experiment (Fig. 3-5). Possibly, if plasma urea concentrations had been further increased, saturation of the renal transport mechanism might have been observed. Alternatively, the fluid loading caused by 84 hours of infusion may be influencing urea movement into the kidney tubule, making saturation difficult to observe. In mammalian kidney tubules, water loading

upregulates active urea secretion by 200% in the $IMCD_3$ of rats that are not given food and 500% in the $IMCD_3$ of rats that are given food (Kato and Sands, 1998b). Under infusion conditions similar to the present experiment, individual toadfish experienced a 20% increase in weight, attributed to an increase in body water content as a consequence of an aglomerular kidney not designed to rid the body of excess fluid (MD McDonald, CM Wood, M Grosell and PJ Walsh, unpublished observations). This water loading may have upregulated active urea secretion as in mammals (Kato and Sands, 1998b). Furthermore, since urine is formed predominantly by secretion in the toadfish, there might have been further increased urea movement into the kidney tubule as a consequence of increased water secretion (evident in the slight increase in UFR; Table 3-1) making saturation of urea transport difficult to observe.

If the renal secretion of urea is carrier-mediated, there are several mechanisms that could potentially be involved. Presently, there are three Na^+-dependent, secondary active urea transport mechanisms functionally characterized in mammalian IMCD subsegments (reviewed by Sands, 1999). One allows for urea-Na^+ co-reabsorption across the apical membrane of $IMCD_1$ (Isozaki et al. 1993; Isozaki et al. 1994a,b), a second allows for active urea secretion by counter exchange with Na^+ across the apical membrane of $IMCD_3$ (Kato and Sands, 1998b) and a third allows for active urea reabsorption with counter-transport of Na^+ across the basolateral membrane of the $IMCD_1$ (Kato and Sands, 1998a). Of the three, urea secretion in conjunction with Na^+ reabsorption seems to be the most physiologically feasible when considering the direction of movement in the toadfish kidney. If that were the case, a negative correlation between

urea secretion clearance and Na^+ secretion clearance (negative values indicating reabsorption) should have been evident. Instead, no clear relationship between urea and Na^+ secretion clearance rates was measured. Perhaps Mg^{++}, being such an important component of secretory kidney urine formation, plays a role in urea secretion in a unique co-secretory mechanism in toadfish and other aglomerular teleosts. Alternatively, urea secretion could involve a H^+-urea cotransporter or perhaps a primary active transporter, as debated in the case of the amphibian skin (Rappoport et al. 1988, 1989; Lacoste et al. 1991).

Additional research using *in vitro* methods, such as isolated kidney membrane vesicles (Wolf et al. 1987), is needed now in order to further our understanding of the tubular urea secretion in the toadfish and to determine whether in fact, urea enters the kidney tubule by a conventional method described in other organisms or by a mechanism as equally unique as the toadfish itself.

Table 3-1: Average values for plasma and urinary ion composition, urine flow rate and glomerular filtration rate.

Time (h)	UFR (ml kg^{-1} h^{-1})	GFR (ml kg^{-1} h^{-1})	$[Na^+]_p$ (mmol·l^{-1})	$[Na^+]_u$ (mmol·l^{-1})	$[Cl^-]_p$ (mmol·l^{-1})	$[Cl^-]_u$ (mmol·l^{-1})	$[Mg^{++}]_p$ (mmol·l^{-1})	$[Mg^{++}]_u$ (mmol·l^{-1})
12	0.13 ± 0.04 (5)	0.020 ± 0.004 (5)	119.0 ± 4.1 (6)	8.0 ± 1.0 (5)	120.4 ± 2.7 (6)	40.0 ± 11.1 (5)	0.64 ± 0.08 (6)	55.3 ± 8.5 (6)
24	0.17 ± 0.03 (5)	0.028 ± 0.002 (5)	113.0 ± 4.5 (6)	7.3 ± 0.9 (5)	116.9 ± 2.0 (6)	39.0 ± 8.7 (5)	0.65 ± 0.05 (6)	62.8 ± 9.1 (5)
36	0.18 ± 0.03 (5)	0.031 ± 0.007 (5)	115.0 ± 2.9 (6)	8.0 ± 1.6 (5)	116.1 ± 2.8 (6)	42.8 ± 16.4 (5)	0.80 ± 0.09 (6)	62.5 ± 12.5 (5)
48	0.18 ± 0.04 (6)	0.028 ± 0.007 (6)	113.7 ± 2.8 (7)	7.5 ± 1.1 (6)	117.8 ± 1.5 (7)	58.2 ± 17.0 (6)	0.75 ± 0.18 (7)	53.5 ± 9.0 (6)
60	0.17 ± 0.04 (6)	0.021± 0.006 (6)	114.0 ± 4.0 (7)	7.5 ± 1.4 (5)	118.1 ± 3.1 (7)	56.9 ± 12.7 (6)	1.31 ± 0.38 (7)	49.7 ± 12.2 (6)
72	0.13 ± 0.04 (6)	0.025 ± 0.007 (5)	108.6 ± 3.5 (6)	9.6 ± 3.4 (5)	114.9 ± 3.1 (6)	60.2 ± 15.9 (5)	0.98 ± 0.26 (6)	54.1 ± 13.6 (5)
84	0.14 ± 0.05 (5)	0.019 ± 0.007 (5)	106.7 ± 3.5 (5)	7.0 ± 1.1 (5)	113.2 ± 3.2 (5)	69.2 ± 17.4 (5)	0.84 ± 0.40 (6)	43.0 ± 11.5 (5)
96	0.15 ± 0.06 (5)	0.023 ± 0.008 (4)	110.9 ± 5.4 (6)	7.7 ± 1.7 (5)	119.7 ± 6.5 (5)	100.8 ± 36.5 (5)	0.92 ± 0.41 (6)	49.5 ± 11.6 (4)
108	0.18 ± 0.08 (5)	0.017 ± 0.009 (4)	107.9 ± 5.5 (6)	7.8± 1.2 (4)	114.0 ± 5.3 (5)	105.7 ± 58.8 (4)	1.06 ± 0.36 (2)	45.9 ± 5.8 (2)
120	0.18 ± 0.08 (3)	0.031 ± 0.001 (2)	101.8 ± 7.9 (6)	5.4 ± 0.8 (3)	106.2 ± 8.4 (5)	106.4 ± 23.5 (3)	1.09 ± 0.42 (6)	42.6 ± 11.1 (2)

Data are shown as mean ± S.E.M. (N); UFR = urine flow rate, GFR = glomerular filtration rate, p = plasma and u = urine.

Figure 3-1: Steadily increasing urea concentrations in plasma and urine over the 132 h experimental period. Values are means ± 1 S.E.M. (N = 6 for plasma samples, (N) for urine samples); * $P < 0.05$ compared to initial concentrations; † $P < 0.05$ compared to plasma concentrations.

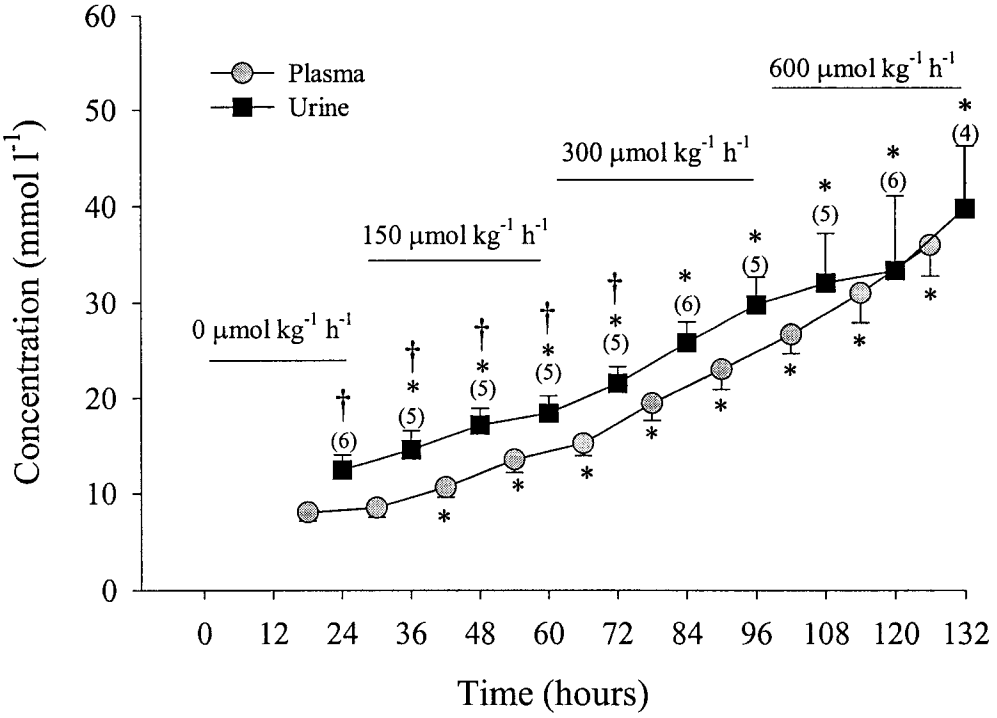

Plasma
Urine
0 μmol kg-1 h-1
150 μmol kg-1 h-1
300 μmol kg-1 h-1
600 μmol kg-1 h-1
Concentration (mmol l-1)
Time (hours)
0
10
20
30
40
50
60
0
12
24
36
48
60
72
84
96
108
120
132

Figure 3-2: (A) Branchial ammonia excretion rate (y-axis) is negatively correlated with plasma urea concentration (x-axis). The equation of the regression line and the significance of the correlation is $y = -2.21x + 137.2$ $r = 0.40$, $P < 0.01$ (N = 42 points from 7 fish). (B) The % ureotelism (y-axis) *versus* plasma urea concentrations (x-axis) demonstrating a weak but significant positive correlation. The equation of the regression line and the significance of the correlation is $y = 0.868x + 46.5$ $r = 0.42$, $P < 0.001$ (N = 42 points from 7 fish).

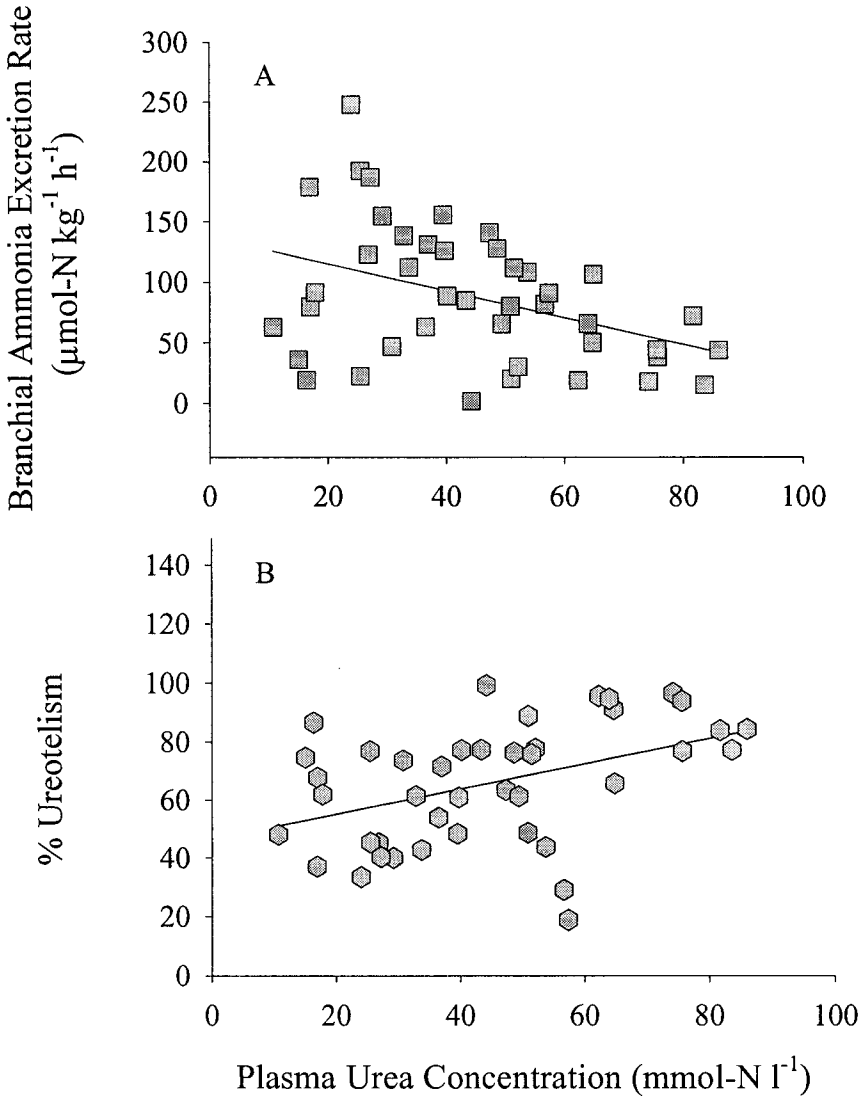

Branchial Ammonia Excretion Rate
(µmol-N kg-1 h-1)
300
250
200
150
100
50
0
A
0
20
40
60
80
100
% Ureotelism
140
120
100
80
60
40
20
0
B
0
20
40
60
80
100
Plasma Urea Concentration (mmol-N l-1)

Figure 3-3: Total urea excretion, pulsatile urea excretion and non-pulsatile urea excretion *via* the gills during the four different series of infusions demonstrating little change in these parameters despite large increases in infused rates of urea. Values are means ± 1 S.E.M. (N=7).

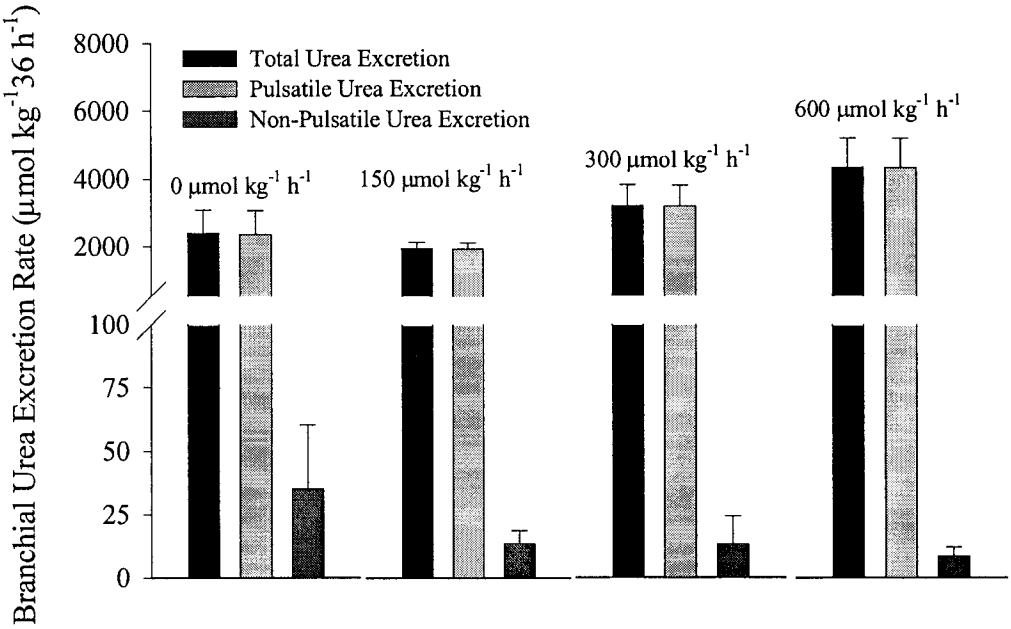

Branchial Urea Excretion Rate (µmol kg-1 36 h-1)
8000
6000
4000
2000
100
75
50
25
0
Total Urea Excretion
Pulsatile Urea Excretion
Non-Pulsatile Urea Excretion
0 µmol kg-1 h-1
150 µmol kg-1 h-1
300 µmol kg-1 h-1
600 µmol kg-1 h-1

Figure 3-4: Branchial urea excretion during pulses > 20 μmol kg^{-1} (y-axis) *versus* plasma urea concentration (x-axis) demonstrating only a very weak correlation between the two variables. The equation of the regression line and the significance of the correlation is $y = 9.80x + 431.3$ $r = 0.17$, $P < 0.05$ (N = 214 points from 7 fish).

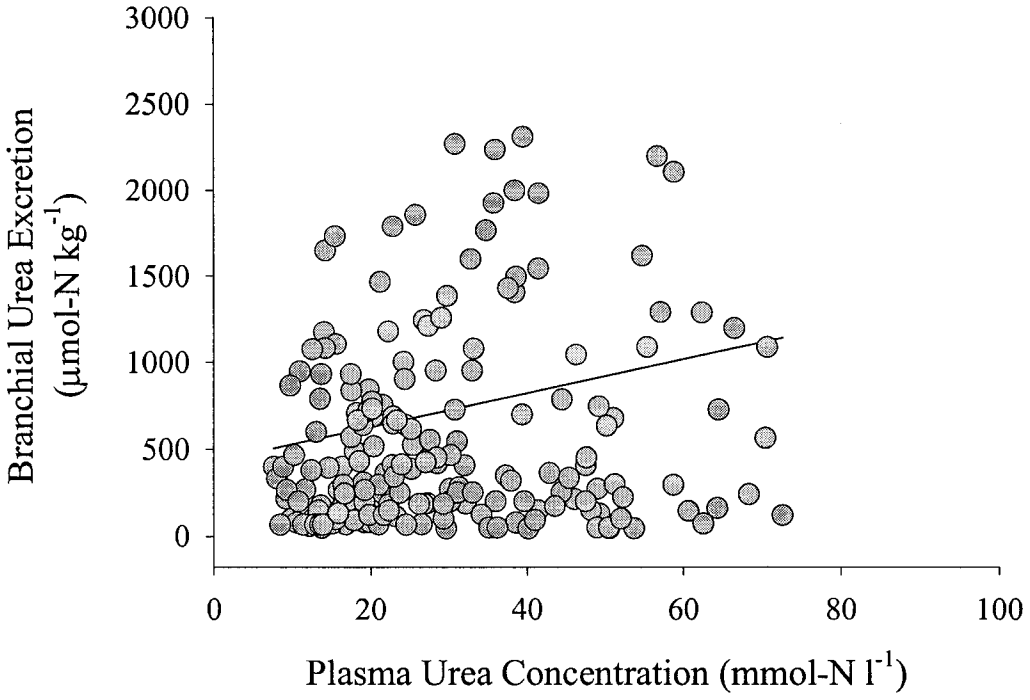

Branchial Urea Excretion
(µmol-N kg-1)
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
Plasma Urea Concentration (mmol-N l-1)

Figure 3-5: Linear regression of the rate of renal urea secretion (y-axis) *versus* the plasma urea concentration (x-axis) demonstrating a strong correlation between the two variables and a lack of a transport maximum (N = 36 data points from 6 fish) despite plasma urea concentrations that were greatly elevated. The equation of the regression line and the significance of the correlation are $y = 0.391x - 2.60$ $r = 0.84$, $P < 0.001$.

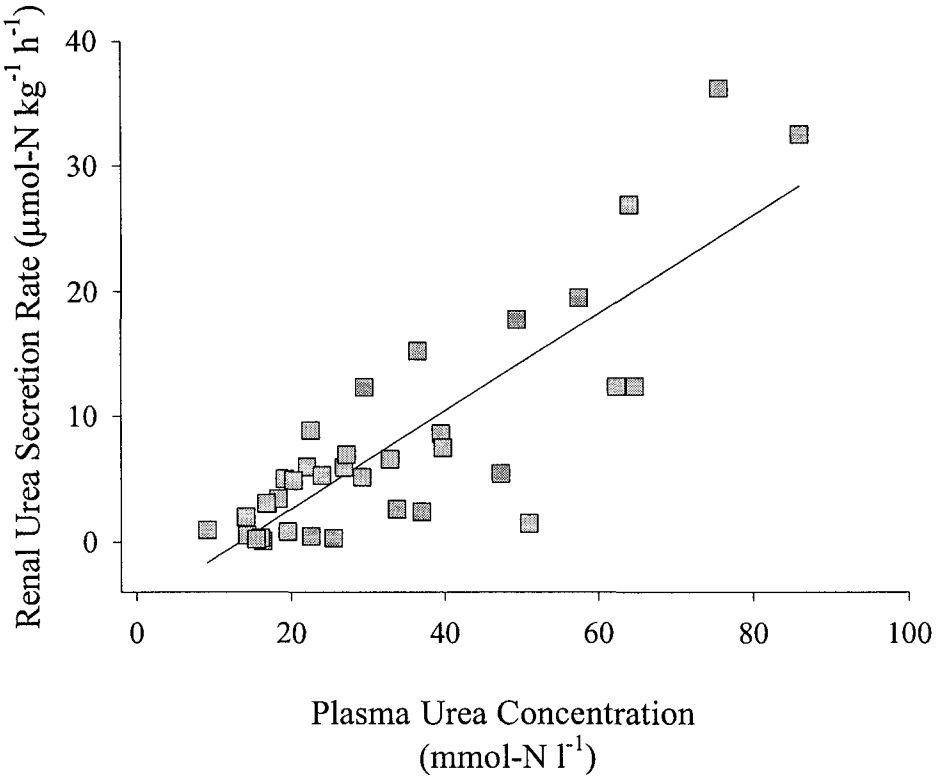
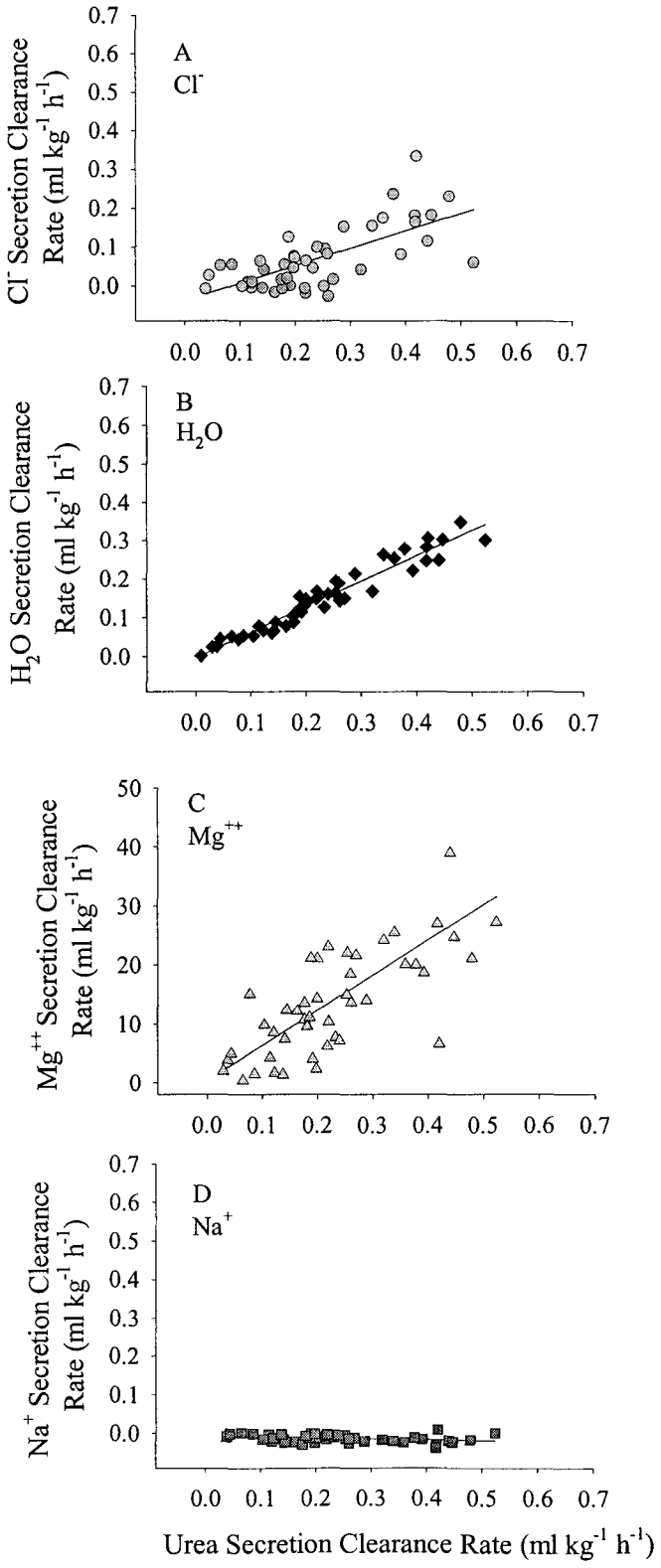

Renal Urea Secretion Rate (μmol-N kg-1 h-1)
40
30
20
10
0
0
20
40
60
80
100
Plasma Urea Concentration
(mmol-N l-1)

Figure 3-6: Secretion clearance rates of (A) Cl^-, (B) water, (C) Mg^{++} and (D) Na^+ (y-axis) *versus* the secretion clearance rate of urea (x-axis) indicating a secretion of urea that was much less than the secretion of Mg^{++} but greater than Cl^- and water. There was no net secretion of Na^+. The equation of the Cl^- regression line is $y = 0.450x - 0.038$) $r = 0.69$, $P < 0.0001$; the equation of the water regression line is $y = 0.659x - 0.003$ $r = 0.97$, $P < 0.001$; the equation of the Mg^{++} regression line is $y = 59.6x - 0.373$ $r = 0.72$, $P < 0.001$; and the equation of the Na^+ regression line is $y = -0.021x - 0.008$ $r = 0.26$, n.s.


A
Cl⁻
Cl⁻ Secretion Clearance Rate (ml kg⁻¹ h⁻¹)
B
H2O
H2O Secretion Clearance Rate (ml kg⁻¹ h⁻¹)
C
Mg++
Mg++ Secretion Clearance Rate (ml kg⁻¹ h⁻¹)
D
Na+
Na+ Secretion Clearance Rate (ml kg⁻¹ h⁻¹)
Urea Secretion Clearance Rate (ml kg⁻¹ h⁻¹)

Chapter 4

Branchial and renal excretion of urea and urea analogues in the plainfin midshipman, *Porichthys notatus*

Abstract

This study investigated whether urea transport mechanisms were present in the gills of the ammoniotelic plainfin midshipman (*Porichthys notatus*), similar to those recently documented in its ureotelic relative, the gulf toadfish (*Opsanus beta*), both being members of the family Batrachoididae. Midshipmen were fitted with internal urinary and caudal artery catheters for repetitive sampling of urine and blood in experiments and radiolabelled urea analogues ([^{14}C]-thiourea and [^{14}C]-acetamide) were used to evaluate the handling of these substances. Isosmotically balanced infusions of urea were employed to raise plasma and urine urea concentrations to levels surpassing physiological levels by 8.5-fold and 6.4-fold, respectively. Despite urea levels greatly exceeding normal resting urea concentrations, there was no observable transport maximum in either renal or branchial urea excretion rate, a result that was mirrored in the total uptake of fish exposed to a range of environmental urea concentrations. Permeability to urea appeared to be symmetrical in the two directions. At comparable plasma concentrations the branchial clearance rate of acetamide was 74% that of urea while branchial clearance rate of thiourea was 55% that of urea. For influx, the comparable values were 60% and 36%,

indicating the same pattern. In contrast, the secretion clearance rate of acetamide by the kidney was 56% that of urea while the rate of thiourea secretion clearance was 137% greater than that of urea, with both urea and thiourea being more concentrated in the urine than in the plasma. In addition, the secretion clearance rates of thiourea and urea were significantly greater than those of water and Cl^-, whereas acetamide, water and Cl^- were found equally in the plasma and urine, appearing to passively equilibrate between the two fluids. Based on the findings of this study there appear to be two distinct transport mechanisms involved in urea excretion in the plainfin midshipmen, one in the gill (a facilitated diffusion type transporter) and one in the kidney (an active transport mechanism), each of which does not saturate even at plasma urea concentrations that greatly exceed physiological levels. These transporters appear to be similar to those in the midshipman's ureotelic relative, the gulf toadfish.

Introduction

In an aqueous environment fish are able to eliminate their nitrogenous wastes directly as ammonia as it diffuses readily through the gills and is subsequently diluted by the surrounding water. In this way, fish bypass the energy-consuming detoxification steps that are necessary for land-dwelling animals. However, there are a handful of adult teleost (bony) fish species that have a fully functional ornithine-urea cycle giving them the ability to detoxify ammonia into urea when environmental conditions are unfavorable for ammonia excretion (Saha and Ratha, 1987; Randall et al. 1989; Mommsen and Walsh, 1989; Wood et al. 1989). One of the best studied teleosts in this regard is the gulf toadfish, *Opsanus beta*, which has the unprecedented ability to switch from ammoniotelism to ureotelism when in a stressful environment (crowding, confinement, air or ammonia exposure; Walsh et al. 1990; Walsh et al. 1994a; Walsh and Milligan, 1995).

In the gulf toadfish the primary route of urea excretion is *via* the gills (> 90 % total urea excretion), where the cDNA for a urea transport protein (tUT) has been cloned that shows 62 % homology at the amino acid level to mammalian hormonally-controlled facilitated diffusion (UT) urea transporters (Smith et al. 1998; Walsh et al. 2000). This facilitated transport mechanism in toadfish allows for urea excretion that is not continuous but occurs in distinct 0.5-3 hour pulses, on average once or twice every 24 hours (Wood et al. 1995, 1997). During pulsing periods, the activation of tUT gives rise to an average 36-fold increase in urea permeability and at the same time a corresponding increase in the permeability of the urea analogues acetamide and thiourea of only 17-fold

and 6-fold respectively (Wood et al. 1998; Chapter 2). During non-pulsing periods the permeability of all three of these substances are not significantly different (Chapter 2). The aglomerular kidney of the toadfish, which forms urine primarily by secretion, is the secondary route of nitrogenous waste excretion (< 10% total urea excretion). Toadfish urine urea concentrations generally exceed plasma levels by at least 30%, suggesting the presence of an active urea transport mechanism (Chapter 2). Similar to the gill, the toadfish kidney demonstrates differential handling of urea and analogues; urea and thiourea are more concentrated in the urine than in the plasma while acetamide appears to passively equilibrate between the two (Chapter 2).

The plainfin midshipman, *Porichthys notatus*, found in the northern Pacific, is a close relative of the warm-water gulf toadfish, both being members of the family Batrachoididae. To date, much of the research on the midshipman has focussed on its vocal-acoustic signaling ability and the several hundred dermal luminescent photophores present on its ventral surface (for reviews see Bass et al. 1994; Baguet, 1975). With respect to nitrogen metabolism and excretion, the midshipman shows a remarkable insensitivity to ammonia, being 2.5x more tolerant than most teleosts, although not as tolerant as the gulf toadfish (Wang and Walsh, 2000). Unlike the toadfish where ammonia tolerance is for the most part due to the conversion of ammonia into urea, the midshipman does not express the ornithine urea cycle and does not have the ability to switch to ureotelism when under stressful conditions (Wang and Walsh, 2000; Walsh et al. 2001a). Nonetheless, the midshipman does excrete some (< 10%) of its nitrogenous waste as urea but unlike the toadfish, branchial urea excretion is continuous (reviewed by

Walsh, 1997). The distinct vesicular trafficking evident in the gill epithelium of pulsing toadfish, thought to play a role in pulsatile urea excretion, is not present in the gills of midshipman (Wang and Walsh, 2000; Laurent et al. 2001; Walsh et al. 2001a). Despite these differences, when a ^{32}P-labelled cDNA probe based on the toadfish branchial urea transporter (tUT) was used to examine gill mRNA by Northern blot analysis, the relative signal strength was strong for the plainfin midshipman (Walsh et al. 2001a). Although mRNA expression does not necessarily reflect protein expression, this result suggests the possibility of a branchial urea transport mechanism in the midshipman.

The objectives of this study were twofold. Firstly, we set out to characterize the branchial excretion of urea in the plainfin midshipman by examining the handling of urea in the face of urea loading, the handling of urea compared to the urea analogues acetamide and thiourea, and the uptake of urea from the water. At the same time, the aglomerular kidney of the midshipman was examined for potential urea transport mechanisms. Our results suggest the presence of two distinct transport mechanisms involved in urea excretion in the plainfin midshipman, found in the gill and the kidney, each of which does not saturate, even at plasma urea concentrations that greatly exceed physiological levels. In addition, both the gill and the kidney differentially handle urea, acetamide and thiourea, the gill moving urea preferentially over both acetamide and thiourea and the kidney moving urea over solely acetamide. Even though the midshipman is ammoniotelic, the characteristics of its transporters appear to be similar to those found in its ureotelic relative, the toadfish.

Materials and Methods

Experimental Animals

Plainfin midshipman (*Porichthys notatus*) were obtained by chartered trawl in Berkley Sound, British Columbia, Canada, in July and August 1999 and 2000. At Bamfield Marine Station, the fish were held for 1 week prior to experimentation in large outdoor tanks served with running seawater at the experimental temperature (12 ± 1°C), salinity (30 ± 2‰) and pH (7.90 ± 0.15). Fish were not fed throughout the experimental period.

Experimental Protocol

Caudal artery and ureteral catheterization were performed simultaneously on male fish anaesthetized with MS-222 (0.07 $g \cdot l^{-1}$; Sigma-Aldrich Canada) and artificially ventilated on an operating table. Caudal artery catheters were inserted as described by Wood et al. (1997). The caudal vertebrae were exposed by a 1.5-2.0 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with Clay-Adams PE50 tubing filled with Hank's saline (Walsh, 1987) containing 50 $i.u \cdot ml^{-1}$ sodium heparin (Sigma-Aldrich Canada). A heat-flared PE160 sleeve was glued in place with cyanoacrylate tissue cement (Vetbond; 3M Corporation) and sutured at the site of exit in order to secure the catheter. The wound was treated with oxytetracycline powder, in order to prevent infection, and sutured securely with 2-0 silk.

The technique for inserting indwelling ureteral catheters is described in detail in Chapter 2. This technique was modified from the protocol described by Howe and

Gutknecht (1978) and Lahlou et al. (1969). Our goal was to examine the function of the kidney alone by bypassing the urinary bladder and eliminating any reabsorptive/secretory roles of the bladder. Briefly, the end of a catheter, made of Clay Adams PE10 tubing, was filled with distilled water and advanced dorsally through the urogenital papilla into the urinary sinus of male fish. Females were not used because their anatomy made it difficult to avoid the oviducts when entering the urinary sinus. The catheter was held in place by three 2-0 silk ligatures around the papilla. A heat-flared PE60 sleeve was threaded onto the catheter and moved as close to the papilla as possible. The sleeve was glued in place with Vetbond and sutured to the body at the site of exit in order to secure the catheter. A ventral incision was then made just anterior to the urogenital papilla. Both urinary bladders were isolated and ligated close to the ureter end with 2-0 silk, thereby negating the storage function of the bladders. The urine therefore drained directly from the ureters into the urinary sinus where the catheter tip was located. Oxytetracycline powder was dusted into the body cavity and the ventral musculature and skin were then sutured securely. During recovery and experimentation, urine was collected continuously with the catheter emptying into a vial approximately 3.0 cm below the water level of the box. The urine flow rate (UFR) was determined gravimetrically. Following surgery, fish were kept in darkened individual containers (minimum volume = 1.5 L) that were continually aerated and supplied with flowing water (200 $ml \cdot min^{-1}$).

Following the procedure outlined by McDonald and Wood (1998), [^{3}H]-polyethylene glycol (PEG, Mr4000) was used as a marker for glomerular filtration rate (GFR) in order to detect whether there were any functional glomeruli. PEG 4000 was chosen because it

is considered a more accurate and conservative indicator of GFR in fish than other commonly used markers (*e.g.* inulin derivatives), as it undergoes minimal radioautolysis, metabolic breakdown, or post-filtration reabsorption across tubules and bladder (Beyenbach and Kirschner, 1976; Erickson and Gingrich, 1986; Curtis and Wood, 1991). Prior to injection of PEG 4000, the fish were allowed to recover from surgery for at least 12 h, a period during which the patency of the arterial and urinary bladder catheters was confirmed. A dose of 9 $\mu Ci \cdot 100g^{-1}$ of [^{3}H]-PEG 4000 (New England Nuclear) was injected *via* the caudal arterial catheter followed by an additional 0.3 ml of saline in fish where both catheters were deemed successful. The [^{3}H]-PEG 4000 was then allowed to equilibrate throughout the extracellular space for 12h before sampling commenced.

In order to determine the normal composition of bladder urine, separate urine samples were taken directly from the bladder of 6 midshipmen and analysed for Na^+, Cl^-, Mg^{++} and urea concentration.

Experimental Series

Series i: Urea handling during urea loading

This series examined the response of the fish to infusions of urea. The specific goal was to examine the pattern of branchial and renal handling of urea in the face of elevated plasma urea levels. In addition to branchial and renal handling of urea, UFR, GFR, urine composition, plasma composition and the handling of Na^+, Cl^-, Mg^{++} and PEG 4000 by the kidney were also monitored.

The protocol used in this series is described in detail by McDonald and Wood (1998). Following the [^{3}H]-PEG 4000 injection and recovery period, two trials consisting of successive 36-h infusions *via* the caudal artery catheter were performed. In *Trial A,* 8 fish were infused with isosmotic solutions (300 mOsm kg^{-1}) containing 0, 5 and 20 $mmol \cdot l^{-1}$ urea respectively, balanced with NaCl. Mean weights of fish in *Trial A* were 0.123 ± 0.006 kg ranging from 0.105 to 0.146 kg. In *Trial B,* 6 fish were infused with isosmotic solutions containing 0, 80 and 140 $mmol \cdot l^{-1}$ urea respectively balanced with NaCl. Mean weights of fish in *Trial B* were 0.100 ± 0.004 kg ranging from 0.082 to 0.109 kg. The 0 $mmol \cdot l^{-1}$ urea infusion served as a control for the effect of volume loading alone. In each case, one channel of a Gilson-8 channel peristaltic pump was used at an infusion rate of 3 ml kg^{-1} h^{-1}; the rate was checked by periodic measurements of the weight of the infusion reservoir. Branchial urea excretion was measured by closing the flux box and recording the appearance of urea in the external water. At the start of each infusion period, a blood sample was taken (200 μl, with saline plus red blood cell replacement), a fresh urine collection was started, water flow to the fish box was stopped and the water level was set to an exact volume mark of 1L. An initial water sample was taken for measurement of urea concentration. Vigorous aeration maintained PO_2 close to air saturation. At 12 h, urine was sampled and a fresh urine collection was started, a blood sample was taken together with a second (final) water sample and the box was then rapidly flushed over a 15-minute period. The box was then closed again for another 12 h, with water sampling at the beginning and water, blood and urine sampling at the end. After 36 h, the infusion solution was then changed to a higher urea concentration and the

same protocol of box closure, water, urine and blood sampling repeated over the next 36 h. The final infusion for each trial was 24 h.

Thus, for the first and second infusions of each trial 3 blood samples were taken and 3 measurements of renal and branchial urea excretion were recorded. For the third infusion of each trial, only 2 blood samples were taken and 2 measurements of renal and branchial urea excretion were recorded. Blood samples were centrifuged at 10 000g for 1 minute and the plasma decanted. The red blood cells were resuspended in saline and re-infused. Plasma and urine were frozen and stored at -20°C for later analysis of Na^+, Cl^-, Mg^{++}, urea and [^{3}H] PEG 4000. Water samples were analyzed immediately for urea and ammonia.

Two fish from each of *Trial A* and *Trial B* of this series were chosen to continue on with the successive 36-h infusions. The two fish from *Trial A* continued to be infused with isosmotic solutions containing 140, 200 and 300 $mmol \cdot l^{-1}$ urea respectively, balanced with NaCl. In *Trial B*, the two fish continued to be infused with 200 and 300 $mmol \cdot l^{-1}$ urea, balanced with NaCl. Thus, for these fish, additional blood samples were taken and measurements of renal and branchial urea excretion were made.

Series ii: Urea handling compared to analogues

In this series, the patterns of branchial and renal urea excretion were compared to the handling of analogues, acetamide and thiourea, similar in size and structure to urea. Two trials were performed: (A) 10 fish treated with acetamide and (B) 10 fish treated

with thiourea. Mean weights of fish in the acetamide trial were 0.089 ± 0.013 kg, ranging from 0.056 to 0.168 kg. Mean weights of fish in the thiourea trial were 0.071 ± 0.008 kg, ranging from 0.061 to 0.163 kg. Following the [^{3}H]-PEG 4000 injection and 12-h equilibration period, urine collection was started and blood and water samples were taken. Water flow to the box was then stopped and the volume was set to at least 1 L. Thereafter, blood, urine and water samples were taken every 12 h, the water being changed at 12-h intervals.

Twenty-four hours after the [^{3}H]-PEG 4000 injection (*i.e.,* after two control blood, water and urine samples) the fish were injected with a dose of 5μCi·100g body weight^{-1} of [^{14}C]-labeled thiourea <u>or</u> acetamide in 160 μmol·100g body weight^{-1} of isosmotic cold analogue (concentration = 300 mmol·l^{-1}) to render internal analogue concentrations approximately equal to internal urea concentrations (2 mmol·l^{-1}). Samples were taken for an additional four 12-hour periods. As described above, blood samples were immediately centrifuged. Plasma and urine were stored at -20°C for later analysis of Na^+, Cl^-, Mg^{++}, urea, [^{14}C]-analogue and [^{3}H]-PEG 4000 concentrations. Water samples were analyzed for urea, ammonia and [^{14}C]-analogue concentrations only.

Series iii: Total uptake of urea from the surrounding water

Four different trials were performed using a total of 77 fish. Similar to the protocol of Wright et al. (1995b) and Chapter 5, smaller midshipmen (average weight = 0.035 ± 0.008 kg) in groups of five to seven were placed into aerated flux chambers with flow-through seawater and were left to acclimate for 24 h. After the acclimation period,

water flow was stopped, the volume of the chamber was set to 2 L and the fish became a part of one of the following trials. *Trial A* investigated the saturability of total urea uptake by placing individual groups of midshipmen in 0.5, 1, 2, 5, or 10 $mmol \cdot l^{-1}$ urea + 100 μCi [^{14}C]-urea in the surrounding seawater. In *Trial B* a direct comparison was made between the uptake rates of urea and two urea analogues, acetamide and thiourea, using concentrations that were comparable to urea concentrations found in blood plasma *in vivo*. In this trial, one group of fish was exposed to 2 $mmol \cdot l^{-1}$ urea + 100 μCi [^{14}C]-urea in the surrounding seawater, a second group was exposed to 2 $mmol \cdot l^{-1}$ acetamide + 100 μCi [^{14}C]-acetamide and a third group was exposed to 2 $mmol \cdot l^{-1}$ thiourea + 100 μCi [^{14}C]-thiourea in the surrounding seawater. *Trial C* investigated analogue competition with urea using urea concentrations slightly greater than internal concentrations and analogue concentrations 3x greater than urea concentrations. The first group of fish was exposed to 5 $mmol \cdot l^{-1}$ urea + 100 μCi [^{14}C]-urea, the second set to 5 $mmol \cdot l^{-1}$ urea, 100 μCi [^{14}C]-urea plus 15 $mmol \cdot l^{-1}$ acetamide and the third to 5 $mmol \cdot l^{-1}$ urea, 100 μCi [^{14}C]-urea plus 15 $mmol \cdot l^{-1}$ thiourea in the external water. *Trial D* examined the effect of phloretin, a urea transport blocker, on urea uptake. In this trial the first group of fish was exposed to 2 $mmol \cdot l^{-1}$ urea + 100 μCi [^{14}C]-urea + 0.250 $mmol \cdot l^{-1}$ phloretin in 0.04% ethanol. A second group was exposed to 2 $mmol \cdot l^{-1}$ urea + 100 μCi [^{14}C]-urea + 0.04% ethanol in order to test the effect of the vehicle alone.

In all four trials, after a 12-h flux period, the fish were sacrificed by a blow to the head, weighed, placed in 50 $mmol \cdot l^{-1}$ of "cold" urea or analogue solution (to remove any surface binding of [^{14}C]-label), blotted dry, and then homogenized in 8% perchloric acid

(2 parts acid: 1 part fish) using a Proctor-Silex Blend Master blender. A sample of this solution was centrifuged and the supernatant was analyzed for [^{14}C] counts.

Analytical Techniques and Calculations

Urea concentrations in blood, urine and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in the three media and correction for the presence of thiourea and acetamide, both of which tended to depress color development in a linear fashion. This correction was done by adding the calculated concentration of analogue present in the urine, plasma or water in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. Ammonia concentrations in the water were measured by the method of Ivancic and Degobbis (1984). Na^+ and Mg^{++} concentrations in plasma and urine were measured using a Varian 1275 Atomic Absorption Spectrophotometer. Cl^- was measured using a Radiometer CMT10 Chloridometer. For measurements of [^{3}H]-PEG 4000, [^{14}C]-urea and/or [^{14}C]-analogue, blood and urine samples (25 μl plus 5 ml of seawater), seawater samples (5 ml) or whole body extracts (200μl plus 5 ml seawater) were added to 10 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 Counter using an onboard quench correction program to separate [^{3}H] and [^{14}C] counts when necessary.

The concentration of analogue [*A*] in the plasma, urine, water or whole body was determined from the original specific activity (*S*; cpm·μmol^{-1}) of the injected solution by converting the radioactivity found in the samples into concentration (μmol·ml^{-1}):

$$S = \frac{cpm_{in}}{[A]_{in}}, \quad (1)$$

$$[A]_s = \frac{cpm_s}{S}, \quad (2)$$

where cpm_{in} (cpm·ml^{-1}) indicates the radioactivity of the injected solution, $[A]_{in}$ indicates the total analogue concentration of the injected solution, cpm_s is the radioactivity in the sample and $[A]_s$ is the analogue concentration in the sample. These analogues are not known to occur endogenously in fish.

The branchial clearance rate (CB; ml·kg^{-1}·h^{-1}) of any substance (*X*) was calculated by dividing the concentration of the substance appearing in the water $[X]_w$ by fish body weight (wt), plasma concentration $[X]_p$ and time (*t*):

$$CB_X = \frac{[X]_w \times V_f}{wt \times [X]_p \times t}, \quad (3)$$

where V_f is the volume of water surrounding the fish.

The amount of any substance *X* taken up from the water was determined from the specific activity of the surrounding water (S_w; cpm·μmol^{-1}) by converting the radioactivity measured in fish (cpm_f) into concentration (μmol·kg^{-1} body weight). The relative uptake (RU) of a substance *X* was then determined by dividing the amount of *X* taken up from the water by $[X]_w$ and by time (*t*).

$$X = \frac{cpm_f}{S_w}, \quad (4)$$

$$RU_X = \frac{X}{[X]_w \times t}. \quad (5)$$

All the following renal rates were related to fish body weight by expressing urinary flow rate (UFR) in $ml \cdot kg^{-1} \cdot h^{-1}$. Urinary excretion rates (U) of any substance (X) were calculated as:

$$U_X = [X]_u \times UFR, \quad (6)$$

using measured values of urine flow rates (UFR) and urine concentrations $[X]_u$. Glomerular filtration rates (GFR) were calculated as the clearance of [^{3}H]-PEG 4000 - *i.e.,* the excretion of radioactivity in the urine (cpm_u) relative to its concentration in the blood plasma (cpm_p):

$$GFR = \frac{cpm_u \times UFR}{cpm_p}. \quad (7)$$

The filtration rate (FR; $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of a substance X at the glomeruli was calculated as:

$$FR_X = [X]_p \times GFR, \quad (8)$$

and consequently the tubular secretion rate (TS; $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of X was calculated as:

$$TS_X = U_X - FR_X. \quad (9)$$

The renal clearance rate by tubular secretion (C_R; $ml \cdot kg^{-1} \cdot h^{-1}$) of X was calculated as:

$$C_{RX} = \frac{TS_X}{[X]_p}. \quad (10)$$

Statistics

Data are reported as means ± 1 S.E.M. (N = number of fish). Regression lines were fitted by the method of least squares, and the significance ($P < 0.05$) of the Pearson's correlation coefficient, r, was assessed. The significance of differences between means was evaluated using Student's paired, unpaired or one-sample two-tailed or one-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977). An ANOVA with time as the main factor was followed by a comparison of individual means using the Bonferroni correction for multiple sample comparisons.

Results

Resting values

Under resting conditions, concentrations of urea in the ureteral urine (3.83 ± 0.25 (20) $mmol \cdot l^{-1}$) were significantly greater than those in the plasma (2.68 ± 0.19 (20) $mmol \cdot l^{-1}$) but were not significantly changed further by the urinary bladder (4.77 ± 0.58 (6) $mmol \cdot l^{-1}$; Fig. 4-1A). Na^+ concentrations were approximately 8-fold greater in the plasma (159.3 ± 5.9 (20) $mmol \cdot l^{-1}$) than in the ureteral urine (20.3 ± 3.4 (20) $mmol \cdot l^{-1}$) and were unmodified by the urinary bladder (16.5 ± 6.1 (6) $mmol \cdot l^{-1}$; Fig. 4-1B). There was no significant difference between Cl^- concentrations in the ureteral urine (159.8 ± 7.7 (20) $mmol \cdot l^{-1}$), plasma (162.1 ± 4.9 (20) $mmol \cdot l^{-1}$) or bladder urine (142.2 ± 8.2 (6) $mmol \cdot l^{-1}$; Fig. 4-1C). Mg^{++} concentrations in the ureteral urine (125.7 ± 8.56 (13) $mmol \cdot l^{-1}$) were approximately 70-fold greater than concentrations in the plasma (1.8 ± 0.3 (14)

mmol·l^{-1}) with no further modification by the urinary bladder (117.2 ± 5.1 (6) mmol·l^{-1}; Fig. 4-1D).

The midshipmen used in this study were ammoniotelic, excreting 80% ammonia-N (111.7 ± 13.4 (19) μmol-N kg^{-1} h^{-1}) and 20% urea-N (24.1 ± 5.2 (20) μmol-N kg^{-1} h^{-1}) when averaged over a resting period. The gills were the major route of urea excretion relative to a urinary excretion rate of only 2.6 ± 0.3 (20) μmol-N kg^{-1} h^{-1}.

Based on the renal clearance of the glomerular filtration rate marker [^{3}H]-PEG 4000, the midshipmen had a glomerular filtration rate (0.040 ± 0.003 (20) ml kg^{-1} h^{-1}) that was significantly greater than zero, suggesting the presence of a small number of functional glomeruli. However, the urine flow rate (0.63 ± 0.12 (20) ml kg^{-1} h^{-1}) was substantially greater (16-fold) than the glomerular filtration rate indicating that the urine was largely formed by secretion.

Urea handling during urea loading

Urea infusions were performed in *Series i* with the goal of raising the plasma urea concentration and therefore both the excretion rate of urea at the gill and the secretion rate of urea at the kidney tubule, to determine whether either of these processes could be saturated. Mean data for plasma and urine composition, UFR and GFR during *Trial A* (low dose infusion) and *Trial B* (high dose infusion) of this series are reported in Tables 4-1 and 4-2. Although there was no significant change in most of these variables over the 96-h infusion protocol, GFR decreased significantly during the low dose infusion only (Table 4-1).

On average in both trials, initial urine urea concentrations (3.04 ± 0.17 (14) $mmol \cdot l^{-1}$) were significantly greater than plasma urea concentrations (2.14 ± 0.14 (14) $mmol \cdot l^{-1}$). Plasma urea concentrations during *Trial A* remained fairly stable through the infusion with 0 $mmol \cdot l^{-1}$ urea and up until the end of the 5 $mmol \cdot l^{-1}$ urea infusion (loading rate = 15 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) after which they rose significantly (Fig. 4-2A). Plasma urea concentrations continued to significantly rise when the infusate concentration was raised to 20 $mmol \cdot l^{-1}$ (loading rate = 60 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) reaching a maximum concentration of 3.78 ± 0.19 (8) $mmol \cdot l^{-1}$. Urine urea concentrations stayed relatively constant throughout the experiment, rising only in the final 24 h reaching a maximum concentration of 5.53 ± 0.29 (8) $mmol \cdot l^{-1}$ (Fig. 4-2A).

Plasma urea concentrations during *Trial B* were relatively stable during the 0 $mmol \cdot l^{-1}$ urea infusion but concentrations increased significantly during the 80 $mmol \cdot l^{-1}$ (loading rate = 240 $\mu mol\ kg^{-1}\ h^{-1}$) and 140 $mmol \cdot l^{-1}$ (loading rate = 420 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) infusions reaching a maximum concentration of 18.09 ± 1.14 (6) $mmol \cdot l^{-1}$ (Fig. 4-2B). Urine urea concentrations showed the same trend reaching a high of 19.35 ± 0.38 (6) $mmol \cdot l^{-1}$ (Fig. 4-2B). Urine and plasma urea concentrations reached maxima that were 6.5-9.5x higher, respectively, than the resting levels reported above.

The branchial excretion rate of urea ($\mu mol\ kg^{-1}\ h^{-1}$) exhibited a linear relationship ($r = 0.77$, $P < 0.0001$) to plasma urea concentration ($mmol \cdot l^{-1}$) with a slope of 3.00 $\mu mol\ kg^{-1}\ h^{-1}$ per $mmol \cdot l^{-1}$ (Fig. 4-3A). There was no evident limit in transport rate even though many plasma urea concentrations were well beyond physiological levels. In the opposite direction, in the experiment of *Series iii-A*, total uptake rate ($\mu mol\ kg^{-1}\ h^{-1}$) of urea from

the external water exhibited a similar relationship ($r = 0.96$, $P < 0.007$) to water urea concentration ($mmol \cdot l^{-1}$) with a comparable slope of 3.82, indicating that urea is capable of moving effectively in both directions (Fig. 4-3B). Similar to branchial excretion, there was no observable transport maximum in the uptake of urea at the gills. In addition, in *Series iii-D*, the rate of urea uptake was found to be insensitive to phloretin, a blocker of UT-type transport mechanisms, placed on the apical side of the gill (data not shown).

Urinary excretion rate of urea also showed a linear relationship ($r = 0.72$, $P < 0.0001$) to plasma urea concentration with a slope of 0.38 $\mu mol\ kg^{-1}\ h^{-1}$ per $mmol \cdot l^{-1}$ (Fig. 4-4). This slope was approximately one order of magnitude lower than the slope observed between branchial excretion rate and plasma urea concentration, a difference that was also reflected in the mean resting values of branchial and urinary excretion rate. Again, similar to branchial urea handling, there was no observable transport maximum in the urinary excretion rate of urea.

Urea handling compared to analogues

In *Series ii*, branchial and renal handling of urea was compared to the handling of analogues based on the premise that if a carrier is involved it would differentially transport urea *versus* analogues. However, if movement is *via* simple diffusion, urea would not be differentially transported. In the acetamide series (*ii-A*), the branchial clearance rate ($ml\ kg^{-1}\ h^{-1}$) of urea (3.62 ± 0.90 (10) $ml\ kg^{-1}\ h^{-1}$) was not different than that of acetamide (2.40 ± 0.48 (10) $ml\ kg^{-1}\ h^{-1}$; Fig. 4-5A). In contrast, in the thiourea series (*ii-B*), the branchial clearance rate of urea (7.45 ± 1.41 (10) $ml\ kg^{-1}\ h^{-1}$) was approximately 3x greater than the clearance rate of thiourea (2.52 ± 0.34 (10) $ml\ kg^{-1}\ h^{-1}$;

Fig. 4-5B). When expressed as a clearance ratio (analogue/urea) for all collection periods where simultaneous measurements were made, the ratios were 0.74 ± 0.10 (9) for acetamide and 0.55 ± 0.13 (10) for thiourea, both showing a significant difference from unity. The same pattern (acetamide/urea ratio = 0.60; thiourea/urea ratio = 0.36) was observed in *Series iii-B* when measuring the total uptake rate of these substances (urea =8.15 ± 1.94 (12) ml kg^{-1} h^{-1}, acetamide = 4.93 ± 0.67 (6) ml kg^{-1} h^{-1}, thiourea = 2.95 ± 0.67 (6) ml kg^{-1} h^{-1}; Fig. 4-5C, D), thereby supporting bidirectional transport in independent experiments.

Interestingly, the total uptake rate of urea in *Series iii-B* (measured in the absence of analogue) and the branchial clearance rate of urea in the thiourea series (*ii-B*) (measured in the presence of thiourea) were quite similar. However, both were significantly greater than the branchial clearance rate of urea measured in the presence of acetamide (*ii-A*), suggesting a possible inhibitory effect of acetamide on urea clearance. In investigating this possibility further using the uptake protocol in *Series iii-C*, urea movement was found to be unaffected by the presence of analogues, even at analogue concentrations in the external water that were 3-fold greater than urea concentrations (data not shown). Thus, there appears to be no inhibitory effect of urea analogues on branchial urea movement.

Fish treated with internal acetamide loading (*Series ii-A*) demonstrated urine urea concentrations (4.04 ± 0.40 (9) mmol·l^{-1}) that were significantly greater than plasma concentrations (2.92 ± 0.33 (9) mmol·l^{-1}) throughout most of the experimental period, *i.e.,* before and after the acetamide injection (Fig. 4-6A). Both plasma and urine urea

concentrations showed a tendency to increase during the experimental period, although this increase was not significant. Plasma and urine acetamide concentrations were not significantly different from one another (1.65 ± 0.12 (9) mmol·l^{-1} and 1.98 ± 0.14 (9) mmol·l^{-1} respectively; Fig. 4-6B) and by the end of the experiment, acetamide levels were actually slightly greater in the plasma than in the urine.

As in the acetamide series, urea concentrations in the urine of fish treated with internal thiourea loading (*Series ii-B*; 4.44 ± 0.38 (10) mmol·l^{-1}) were significantly greater than plasma levels (2.85 ± 0.23 (10) mmol·l^{-1}) both before and after injection of the analogue (Fig. 4-6C). Thiourea concentrations in the urine and plasma showed the same trend as urea concentrations, in that the thiourea was much greater in the urine (3.37 ± 0.45 (10) mmol·l^{-1}) than in the plasma (1.29 ± 0.17 (10) mmol l^{-1}). These data suggest that the kidney handles thiourea in the same way that it handles urea (Fig. 4-6D) and very differently from the way it handles acetamide (Fig. 4-6B).

In simultaneous secretion clearance measurements, urea secretion clearance (0.54 ± 0.13 (9) ml kg^{-1} h^{-1}) was 1.8x greater than acetamide secretion clearance (0.30 ± 0.05 (9) ml kg^{-1} h^{-1}; Fig. 4-7A). In contrast, in the presence of thiourea, the secretion clearance of urea (0.45 ± 0.07 (10) ml kg^{-1} h^{-1}) was less than that of thiourea (0.71 ± 0.12 (10) ml kg^{-1} h^{-1}; Fig. 4-7B). When expressed as clearance ratios (analogue/urea) for all collection periods where simultaneous measurements were made, the ratios were 2.43 ± 0.66 (10) for thiourea and only 0.59 ± 0.08 (9) for acetamide, a highly significant difference showing that thiourea was cleared more effectively than urea whereas acetamide was cleared less effectively.

As mentioned earlier, urine Na^+ concentrations were on average 8-fold lower than plasma concentrations (Fig. 4-1B). Conversely, in accordance with active secretion is the observation that urine Mg^{++} concentrations were 70-fold greater than plasma concentrations (Fig. 4-1D). However, plasma and urine Cl^- concentrations were not significantly different from one another, suggesting that Cl^- was passively distributed between the plasma and the urine (Fig. 4-1C). When expressed as secretion clearance rates, the rate of Mg^{++} secretion clearance (38.22 ± 7.05 (14) ml kg^{-1} h^{-1}) was approximately 100x greater than that of urea secretion clearance rate (0.48 ± 0.07 (20) ml kg^{-1} h^{-1}; Fig. 4-8A). However, urea secretion clearance rate was significantly greater than the secretion of both Cl^- (0.27 ± 0.04 (20) ml kg^{-1} h^{-1}) and water (0.26 ± 0.03 (20) ml kg^{-1} h^{-1}), which were not significantly different from each other. Thus, on a relative basis, 1.8x more urea was secreted by the kidney than Cl^- or water movement. Not surprisingly, urea secretion clearance rate was significantly greater than the secretion clearance rate of Na^+. Overall there was no net secretion of Na^+.

As observed for urea, the secretion clearance rate of thiourea (0.71 ± 0.12 (10) ml kg^{-1} h^{-1}) was significantly less than Mg^{++} (28.5 ± 9.2 (10) ml kg^{-1} h^{-1}) but significantly greater than Cl^- (0.26 ± 0.11 (10) ml kg^{-1} h^{-1}) and water (0.25 ± 0.09 (10) ml kg^{-1} h^{-1}; Fig. 4-8B). Thus, the renal secretion of thiourea was 2.7-2.8x more than either Cl^- or water movement.

The secretion clearance rate of acetamide (0.31 ± 0.05 (9) ml kg^{-1} h^{-1}), like thiourea and urea, was significantly less than Mg^{++} (59.8 ± 17.6 (3) ml kg^{-1} h^{-1}; Fig. 4-8C). However, the clearance of acetamide was not significantly different than Cl^- (0.23 ±

0.07 (9) ml kg^{-1} h^{-1}) or water (0.22 ± 0.05 (9) ml kg^{-1} h^{-1}). Therefore, on a relative basis, acetamide moved into the kidney tubule at the same rate as Cl^- and water.

Discussion

A cDNA probe based on the toadfish branchial urea transporter (tUT) produced a strong signal when tested against gill mRNA from the plainfin midshipman, suggesting that there is message for a UT-type urea transporter in the gills (Walsh et al. 2001a). However, this does not necessarily mean that the protein is expressed and/or functional. Hence, the objectives of this study were to examine and characterize the movement of urea through the gills of the plainfin midshipman and to determine whether the branchial excretion of urea is in fact carrier-mediated. In addition, the kidney was examined for urea transport mechanisms. Our results suggested that despite a lack of saturation kinetics there is evidence for two distinct transport mechanisms involved in the excretion of urea, one in the gill and one in the kidney, both of which demonstrate differential handling of urea and analogues. However, the pattern of urea *versus* analogue handling in the two organs is not the same.

When plasma urea concentrations of midshipman were elevated by infusion of exogenous urea loads, there was no observable transport maximum in the branchial excretion of urea, even at plasma urea concentrations well exceeding physiological levels. Although the lack of a transport maximum is usually indicative of simple diffusion through cell membranes, UT-type urea transport mechanisms are notably difficult to saturate (Chou et al. 1990). However, saturation kinetics have been described

in urea transport mechanisms in lower vertebrates (Levine et al.1973a, Shpun and Katz, 1989, 1990; Pilley and Wright, 2000; Fines et al. 2001). Similar to observations in the present study, a linear relationship between branchial excretion rate and plasma urea concentration was observed in the freshwater rainbow trout, *Oncorhynchus mykiss,* upon exogenous urea loading at the same rates (McDonald and Wood, 1998). However, using the *in vitro* membrane vesicle preparation of Perry and Flik (1998), saturation kinetics for urea transport across the basolateral membrane of the trout gill (K_m = 1.17 mmol·l^{-1} and V_{max} = 0.42 μmol·mg protein^{-1}·h^{-1}) has recently been demonstrated (Chapter 6). Thus, perhaps in using an *in vitro* approach to study the midshipman gill, it would be possible to determine saturation kinetics for urea movement.

In addition to the linear relationship between branchial excretion rate and concentration there was a very similar relationship between total uptake rate and concentration; the two functions exhibited similar slopes, indicating that urea passes symmetrically in both directions. This same symmetry of transport in two directions has been found in the gill of the gulf toadfish (Wood et al. 1998) as well as the tidepool sculpin (*Oligocottus maculosus*; Wright et al. 1995) and is characteristic of facilitated diffusion transporters for urea (Chou et al. 1990).

The similarity between branchial excretion and total uptake of urea argues that uptake is through the gill and the contribution of the skin and gut is likely minor. The permeability of midshipman skin to urea may be similar to that of toadfish; the measured permeability of toadfish skin to urea is low (5.07 ± 0.56 (8) x 10^{-7} cm sec^{-1}) compared to the permeability of tritiated water (72.0 ± 15.0 (8) x 10^{-7} cm sec^{-1}; Part et al. 1999). With

respect to the contribution of the gut, toadfish drink only 0.75 ± 0.06 (24) ml kg^{-1} h^{-1} seawater (Grosell, Wood, McDonald and Walsh, unpublished), hence the gut could account for only a small component urea uptake from the water if drinking rate in midshipmen is similar. Conceivably, the slightly greater slope observed in uptake *versus* concentration could be due to the small contribution of the gut.

Further evidence for the presence of a branchial urea transport mechanism in the midshipman is the preferential clearance of urea over both acetamide and thiourea. The ratio of analogue/urea clearance revealed a clearance of acetamide that was 74% of urea clearance while thiourea clearance was only 55% that of urea. In the influx direction, these ratios were 60% and 36%, respectively. This pattern is similar to that in the gills of the gulf toadfish where there were increases in permeability in the order urea > acetamide > thiourea of toadfish gills observed during pulses when the facilitated diffusion transporter (tUT) was activated (Chapter 2). At this time, the branchial clearance of acetamide was 35-50% that of urea clearance and the relative thiourea clearance was at most only 16% of urea clearance during pulsing events. However, all three substances had similar permeabilities during non-pulsing periods, suggesting that the gill handled urea, acetamide and thiourea identically when tUT was not activated despite small differences in calculated oil/water partition coefficients and lipid permeabilities of the three substances (Goldstein and Solomon, 1960; Lippe, 1969; Galluci et al. 1971). Similar to the toadfish during a pulse event, the UT-type transporter in the Lake Magadi tilapia (mtUT with 75% identity to tUT) demonstrated a permeability to thiourea that was 19% that of urea permeability (Walsh et al. 2001b). Therefore, it seems probable that a

facilitated diffusion transporter for urea, similar to those in the ureotelic species, is continually expressed at low levels in the gills of the plainfin midshipman, in accord with recent molecular evidence (Walsh et al. 2001a). Indeed this type of transporter may be present in the gills of many other ammoniotelic teleosts (Walsh et al. 2001a) as well as in teleost embryos (Pilley and Wright, 2000). It would be interesting to know whether the transporter is subject to physiological regulation in ammoniotelic fish.

Differentiation between urea and analogues by urea transport mechanisms is not an uncommon observation, as studies characterizing urea transport mechanisms in other organisms have shown that urea transporters customarily move analogues less effectively than urea and can often distinguish between analogues. Classic work on the kidney of the spiny dogfish (*Squalus acanthias*) showed that acetamide and methylurea were reabsorbed nearly as well as urea but thiourea was not (Schmidt-Nielsen and Rabinowitz, 1964). Since then, Smith and Wright (1999) have cloned a renal urea transporter (ShUT) of the dogfish that appears related to the mammalian UT-A family of facilitated diffusion transporters. A study by Schmidt-Nielsen and Shrauger (1963) on the kidney of the frog *Rana catesbeiana* demonstrated the apparent active secretion of thiourea but the lack of acetamide transport. In rat inner medullary collecting ducts (IMCD), the permeability of thiourea was much lower than the permeability of urea through the vasopressin-sensitive urea transport mechanism (UT-A1; Chou et al. 1990). In addition, Naccache and Sha'afi (1973) reported the permeability of urea in human erythrocytes (through UT-B transporters) to be 30-fold greater than the permeability of thiourea.

Competitive interactions between urea and analogues are often observed in cases where UT-type transport mechanisms are involved including the gills of both the gulf toadfish (tUT; Wood et al. 1998; Walsh et al. 2000) and the Lake Magadi tilapia (mtUT; Walsh et al. 2001b) and even in the rainbow trout (*Oncorhynchus mykiss*) embryo (Pilley and Wright, 2000), where a facilitated diffusion transport mechanism for urea is believed to be present. Interestingly, there was no inhibition of analogues on urea transport when analogues were injected into the fish or when looking at the uptake of [^{14}C]-urea in the presence of an excess of cold thiourea or acetamide. It could be that the high capacity of this transporter could allow for both acetamide and thiourea to pass through freely with no effect on urea. Alternatively, acetamide and thiourea may not be moving through the transport mechanism at all, but rather diffusing through the membrane by simple diffusion and thus not interfering with urea movement. Phloretin, a potent inhibitor of UT-type transport mechanisms (Chou and Knepper, 1989), had no effect when placed in the water (having access to the apical membrane), suggesting that a UT-type transport mechanism is not present at least on the apical membrane of the gill. However, phloretin-insensitivity on the apical side of the cell does not rule out the possibility that a phloretin-sensitive mechanism could be present on the basolateral membrane. In the gulf toadfish, phloretin introduced systematically *via* a caudal artery catheter (having access to the basolateral membrane) significantly inhibited pulse frequency and urea output as pulses, causing a significant internal accumulation of urea-N, though interpretation was confounded because of the toxicity of the drug when administered internally (Wood et al. 1998).

To maintain osmotic equilibrium marine teleosts drink seawater, consequently ingesting monovalent and divalent ions that, if not excreted, would increase in the extracellular fluid to physiologically intolerable concentrations. Reflecting this, the dominant osmolytes in the urine of seawater-acclimated toadfish are Mg^{++}, Cl^- and SO_4^{--}; Mg^{++} and SO_4^{--} is found in significantly higher concentrations in the urine than in the plasma while Cl^- is found equally in both media (Howe and Gutknecht, 1978; Baustain et al. 1997; Chapter 2). It was therefore not surprising that Mg^{++} concentrations were low in the plasma and high in the urine of the midshipman, while Cl^- concentrations were in equal concentrations in plasma and urine. Although SO_4^{--} was not measured in this study, it can be postulated that it would make up the anion deficit as high levels of SO_4^{--} are found in the urine of several marine teleosts (Hickman and Trump, 1969; Renfro and Dickman, 1980).

In addition to drinking seawater to maintain osmotic equilibrium, marine teleosts generally exhibit a degenerated glomerulus that is smaller than that found in freshwater teleosts, as there is no need for excreting high amounts of water in marine animals (Marshall, 1929; Hickman and Trump, 1969). The extreme case of glomerular degeneration is the aglomerular kidney believed to be present in goosefish (*Lophius americanus*), toadfish and midshipmen (Marshall, 1929; Lahlou et al. 1969; Baustain et al. 1997). The appearance of [^{3}H]-PEG 4000 in the urine of the midshipman supports findings in Chapter 2 showing the presence of [^{3}H]-PEG 4000 in the urine of the gulf toadfish, *Opsanus beta.* In addition, an earlier study by Lahlou et al. (1969) showed the appearance of [^{14}C]-inulin in the urine of the oyster toadfish, *Opsanus tau.* PEG 4000 is

thought to be a more accurate indicator of GFR than inulin since it undergoes minimal metabolic breakdown (Beyenbach and Kirschner, 1976; Erickson and Gingrich, 1986; Curtis and Wood, 1991). Therefore, there appear to be a small number of functional glomeruli present in the kidney in the family Batrachoididae. However, in the case of the midshipman (as in the toadfish) GFR is less than 15% of UFR so despite measurable glomerular filtration, urine in the midshipman is formed mainly by secretion.

Our results suggest the presence of a urea transport mechanism in the midshipman kidney that is unlike the facilitated diffusion transporter that may be present in the gill for two reasons. Firstly, the analogues are handled differently by the kidney than by the gill, suggesting a different transporter in the kidney. In contrast to the gill where acetamide and urea were handled similarly (and different from thiourea), the opposite trend was observed in the kidney where it appeared that thiourea and urea were handled similarly, accumulating in the urine against a concentration gradient. Conversely, acetamide appeared to equilibrate between the plasma and urine. The secretion of thiourea was 37% greater than that of urea, whereas that of acetamide was 45% less than that of urea.

Secondly, the secretion of thiourea and urea could not occur entirely *via* a facilitated diffusion mechanism since they appear to move against a concentration gradient, suggesting the involvement of an active transport mechanism. In addition, both thiourea and urea appeared to be secreted at least 1.8-2.7x more effectively than Cl^-, which was found equally in the urine and plasma, appearing to passively equilibrate between the two fluids. Acetamide secretion clearance, on the other hand, was not significantly different than Cl^- secretion clearance. Since the relative secretions of

thiourea and urea were greater than the relative movements of Cl^- or water, their passage into the kidney tubule cannot be accounted for by simple diffusion. In addition, with acetamide found equally in both urine and plasma, the possibility that thiourea and urea were concentrated in the tubule as a consequence of water reabsorption can be eliminated.

To date, little research has been done investigating the renal mechanisms for urea handling in fish. Similar to the findings of this study, the aglomerular kidney of the gulf toadfish demonstrated the same pattern in analogue differentiation, with the secretion clearance rates of urea and thiourea being relatively greater than the secretion of acetamide and Cl^- (Chapter 2). In that study, the authors speculated what transport mechanisms could be responsible for the secretion of urea against a concentration gradient into the kidney tubule. The most likely candidate was the Na^+-dependent urea transporter originally identified in the elasmobranch kidney (Schmidt-Nielsen et al. 1972) and more recently in the mammalian kidney of protein depleted animals (Isozaki et al. 1994a,b). Apparent active urea reabsorption has also been seen in the kidney of the freshwater rainbow trout (McDonald and Wood, 1998). One hypothesis for the midshipman and toadfish kidney is that urea secretion occurs *via* a Na^+-urea co-transporter, urea moving with Na^+ down the concentration gradient of the latter (Isozaki et al. 1994a,b; Sands et al. 1996). Further down the tubule, Na^+ would then be actively reabsorbed against its concentration gradient. A less complicated hypothesis would be a Na^+-urea antiporter, as has been suggested to be present in the deep portions of the inner medullary collecting duct ($IMCD_3$) of the mammalian kidney where urea appears to

move into the kidney tubule in direct counter-transport with active Na^+ reabsorption (Kato and Sands, 1998a,b). Unfortunately, this transporter has yet to be cloned.

In summary, it appears that the midshipman has urea transporters in both the gill and the kidney, similar to its close relative, the gulf toadfish. The patterns of analogue *versus* urea handling by the gill and the kidney are similar between the two species, suggesting the involvement of functionally similar transporters. The gill and kidney transporters exhibit different patterns of urea *versus* analogue handling; the gill transporter appears to be of the facilitated diffusion type, while the kidney transporter appears to be of the active type. Further investigation is required to fully understand the mechanisms involved.

Table 4-1: Average values for plasma and urinary ion composition, urine flow rate and glomerular filtration rate during *Trial A* from fish of *Series i*.

Time (h)	UFR ($ml\ kg^{-1}\ h^{-1}$)	GFR ($ml\ kg^{-1}\ h^{-1}$)	$[Na^+]_p$ ($mmol \cdot l^{-1}$)	$[Na^+]_u$ ($mmol \cdot l^{-1}$)	$[Cl^-]_p$ ($mmol \cdot l^{-1}$)	$[Cl^-]_u$ ($mmol \cdot l^{-1}$)	$[Mg^{++}]_p$ ($mmol \cdot l^{-1}$)	$[Mg^{++}]_u$ ($mmol \cdot l^{-1}$)
12	0.24 ± 0.05 (7)	0.044 ± 0.006 (7)	164.4 ± 4.5 (8)	18.3 ± 7.7 (8)	173.7 ± 4.3 (8)	184.0 ± 12.1 (8)	1.2 ± 0.1 (7)	129.6 ± 7.1 (7)
24	0.27 ± 0.04 (8)	0.047 ± 0.004 (8)	159.0 ± 7.0 (8)	6.9 ± 1.3 (8)	177.4 ± 5.4 (8)	164.0 ± 11.6 (8)	1.2 ± 0.2 (8)	128.9 ± 6.5 (8)
36	0.22 ± 0.04 (8)	0.031 ± 0.005 (8)	153.7 ± 5.5 (8)	8.1 ± 1.8 (8)	170.7 ± 5.2 (8)	162.8 ± 11.0 (8)	1.1 ± 0.2 (8)	121.6 ± 5.4 (7)
48	0.30 ± 0.06 (8)	0.027 ± 0.004 (8)	151.7 ± 4.3 (8)	14.3 ± 6.9 (8)	165.9 ± 5.9 (8)	171.8 ± 11.7 (8)	1.1 ± 0.1 (7)	123.3 ± 6.1 (8)
60	0.33 ± 0.10 (8)	0.026± 0.005 (8)	153.4 ± 5.7 (8)	13.6 ± 4.1 (8)	172.9 ± 5.1 (8)	150.3 ± 17.3 (8)	1.2 ± 0.2 (8)	116.6 ± 7.3 (7)
72	0.34 ± 0.13 (8)	0.022 ± 0.004* (8)	151.7 ± 4.5 (8)	17.6 ± 6.4 (8)	165.9 ± 5.7 (8)	145.6 ± 18.5 (8)	1.3 ± 0.2 (8)	122.9 ± 11.8 (7)
84	0.45 ± 0.15 (8)	0.022 ± 0.004* (8)	152.5 ± 6.0 (8)	22.9 ± 8.0 (7)	170.0 ± 4.5 (7)	135.9 ± 21.6 (7)	1.4 ± 0.3 (8)	103.0 ± 12.8 (7)
96	0.37 ± 0.10 (7)	0.018 ± 0.004* (8)	154.9 ± 5.6 (8)	19.2 ± 7.7 (6)	174.4 ± 6.6 (8)	154.1 ± 20.5 (6)	1.9 ± 0.5 (8)	113.0 ± 14.7 (6)

Data are shown as mean ± S.E.M. (N); UFR = urine flow rate, GFR = glomerular filtration rate, p = plasma and u = urine.

*significantly different from first value; $P < 0.05$.

Table 4-2: Average values for plasma and urinary ion composition, urine flow rate and glomerular filtration rate during *Trial B* from fish of *Series i*.

Time (h)	UFR (ml kg^{-1} h^{-1})	GFR (ml kg^{-1} h^{-1})	$[Na^+]_p$ (mmol·l^{-1})	$[Na^+]_u$ (mmol·l^{-1})	$[Cl^-]_p$ (mmol·l^{-1})	$[Cl^-]_u$ (mmol·l^{-1})	$[Mg^{++}]_p$ (mmol·l^{-1})	$[Mg^{++}]_u$ (mmol·l^{-1})
12	0.20 ± 0.06 (6)	0.046 ± 0.005 (5)	157.2 ± 7.9 (6)	13.2 ± 3.2 (6)	193.3 ± 6.3 (6)	134.3 ± 8.5 (5)	1.0 ± 0.2 (6)	113.5 ± 4.2 (5)
24	0.21 ± 0.06 (6)	0.039 ± 0.009 (6)	151.4 ± 4.5 (6)	10.6 ± 1.3 (6)	192.3 ± 2.9 (6)	124.8 ± 15.7 (6)	1.0 ± 0.2 (6)	102.5 ± 6.4 (6)
36	0.28 ± 0.04 (6)	0.045 ± 0.010 (6)	150.0 ± 4.8 (6)	15.3 ± 4.2 (6)	186.7 ± 2.8 (6)	116.4 ± 22.7 (6)	0.9 ± 0.1 (6)	90.1 ± 12.0 (6)
48	0.25 ± 0.04 (6)	0.027 ± 0.006 (6)	148.0 ± 3.2 (6)	16.2 ± 7.0 (6)	191.1 ± 23.0 (6)	102.7 ± 21.4 (6)	1.0 ± 0.1 (6)	88.1 ± 16.0 (6)
60	0.20 ± 0.06 (6)	0.018 ± 0.007 (6)	141.4 ± 2.1 (6)	9.6 ± 2.7 (5)	186.5 ± 2.5 (6)	123.8 ± 26.6 (5)	1.0 ± 0.1 (6)	102.2 ± 14.4 (5)
72	0.22 ± 0.05 (5)	0.022 ± 0.005 (5)	140.9 ± 3.5 (6)	9.8 ± 2.9 (5)	186.0 ± 2.7 (6)	109.3 ± 22.2 (5)	1.1 ± 0.1 (6)	96.6 ± 13.8 (5)
84	0.28 ± 0.04 (4)	0.031 ± 0.011 (3)	143.9 ± 4.4 (6)	21.5 ± 9.9 (4)	185.2 ± 2.5 (6)	127.3 ± 44.6 (4)	1.2 ± 0.2 (6)	117.2 ± 33.7 (4)
96	0.25 ± 0.03 (4)	0.022 ± 0.004 (4)	141.6 + 4.3 (6)	6.1 ± 2.1 (3)	184.6 ± 3.7 (6)	92.9 ± 28.5 (4)	1.2 ± 0.3 (6)	98.1 ± 20.6 (4)

Data are shown as mean ± S.E.M. (N); UFR = urine flow rate, GFR = glomerular filtration rate, p = plasma and u = urine.

Figure 4-1: Concentrations of (A) urea, (B) Na^+, (C) Cl^- and (D) Mg^{++} in the plasma, ureteral urine and bladder urine of the plainfin midshipman under resting conditions. Values are means ± 1 S.E.M. (N); $*P < 0.05$ significantly different from plasma.

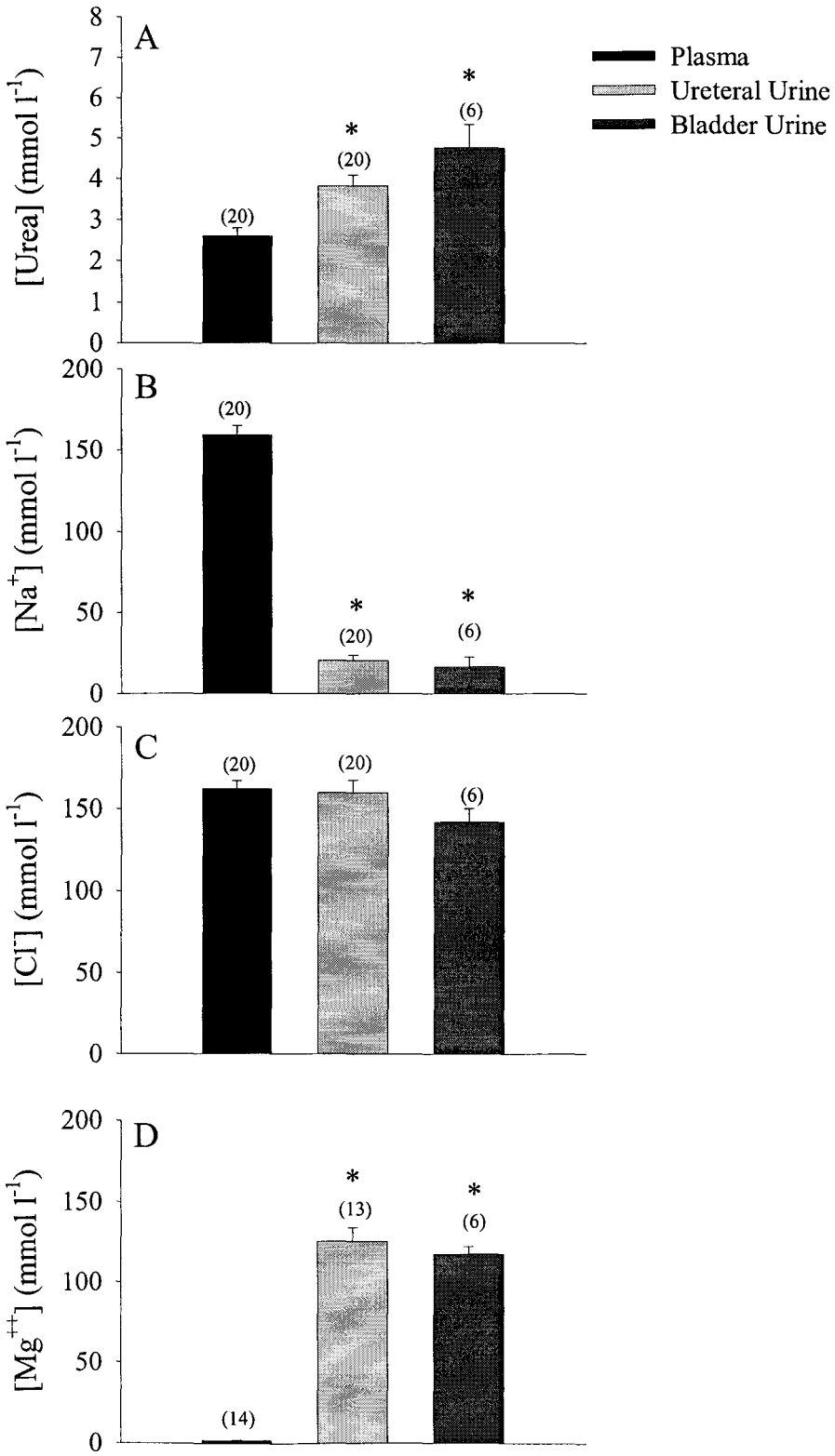

A
B
C
D
Plasma
Ureteral Urine
Bladder Urine
[Urea] (mmol l-1)
[Na+] (mmol l-1)
[Cl-] (mmol l-1)
[Mg++] (mmol l-1)
(20)
(6)
(13)
(14)
*

Figure 4-2: Urea concentrations in the urine and plasma in (A) fish of *Series i Trial A* infused with urea at consecutive rates of 0, 15 and 60 μmol kg^{-1} h^{-1} and (B) fish of *Series i Trial B* infused with urea at consecutive rates of 0, 240 and 420 μmol kg^{-1} h^{-1}. Values are means ± 1 S.E.M. (N = 8 for Trial A, N = 6 for Trial B). *$P < 0.05$ significantly different from the first value.

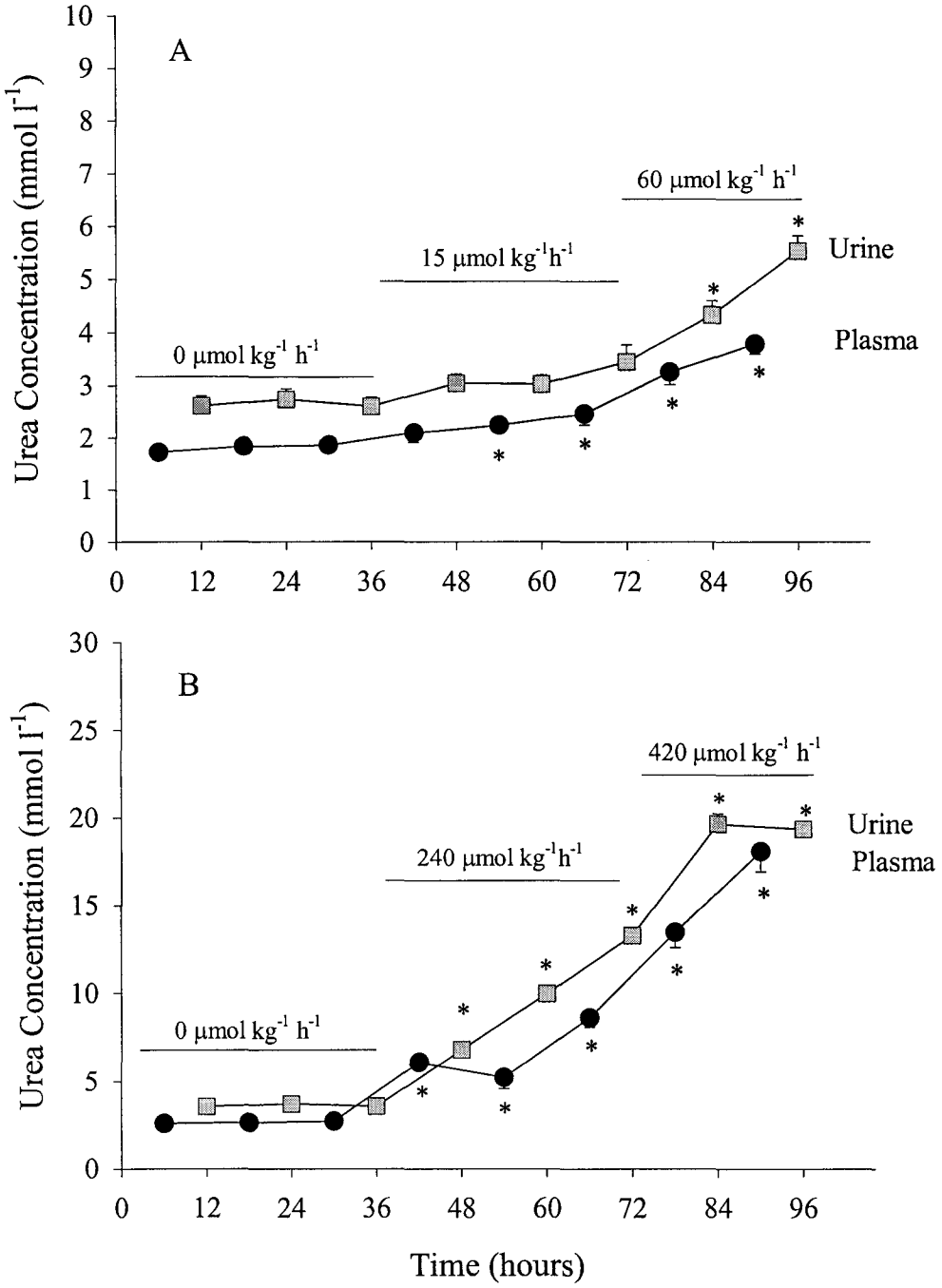
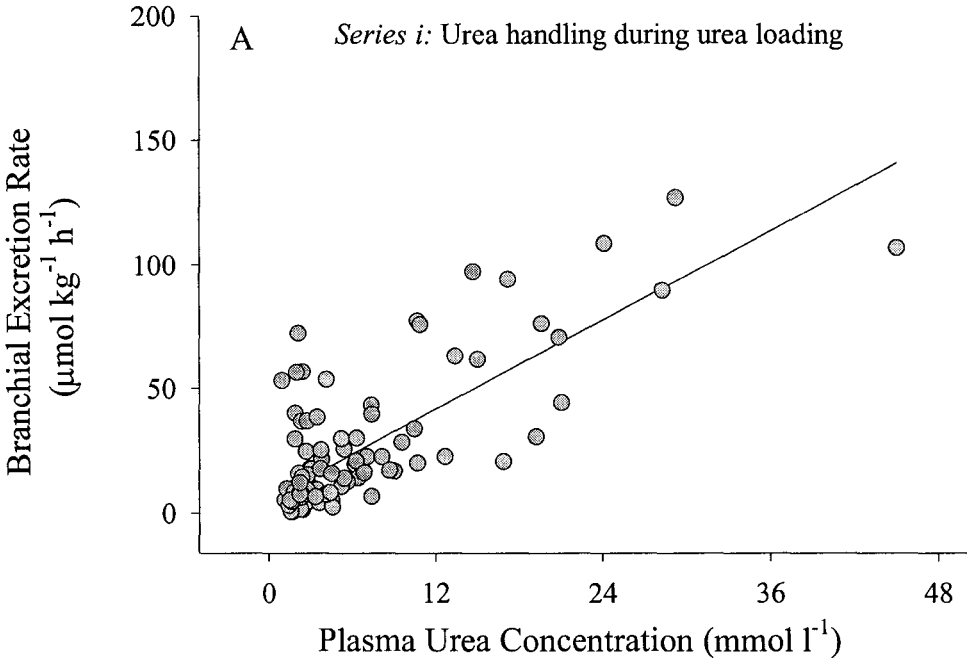

A
Urea Concentration (mmol l-1)
0 µmol kg-1 h-1
15 µmol kg-1h-1
60 µmol kg-1 h-1
Urine
Plasma
B
Urea Concentration (mmol l-1)
0 µmol kg-1 h-1
240 µmol kg-1h-1
420 µmol kg-1 h-1
Urine
Plasma
Time (hours)

Figure 4-3: (A) Linear regression of the branchial excretion rate of urea (y-axis) *versus* the plasma urea concentration (x-axis) indicating a linear, proportional relationship with no apparent transport maximum. The equation of the line and the significance of the correlation are $y = 3.00x + 5.83$ $r = 0.77$, $P < 0.0001$ (N =118 points from 14 fish). (B) A linear regression of the branchial uptake rate (y-axis) *versus* water urea concentration (x-axis) showing the same trend in the reverse direction. The equation of the line and the significance of the correlation are $y = 3.82x + 0.181$ $r = 0.96$, $P < 0.007$. Values are means ± 1 S.E.M. (N).


A
Series i: Urea handling during urea loading
Branchial Excretion Rate
(µmol kg-1 h-1)
200
150
100
50
0
0
12
24
36
48
Plasma Urea Concentration (mmol l-1)

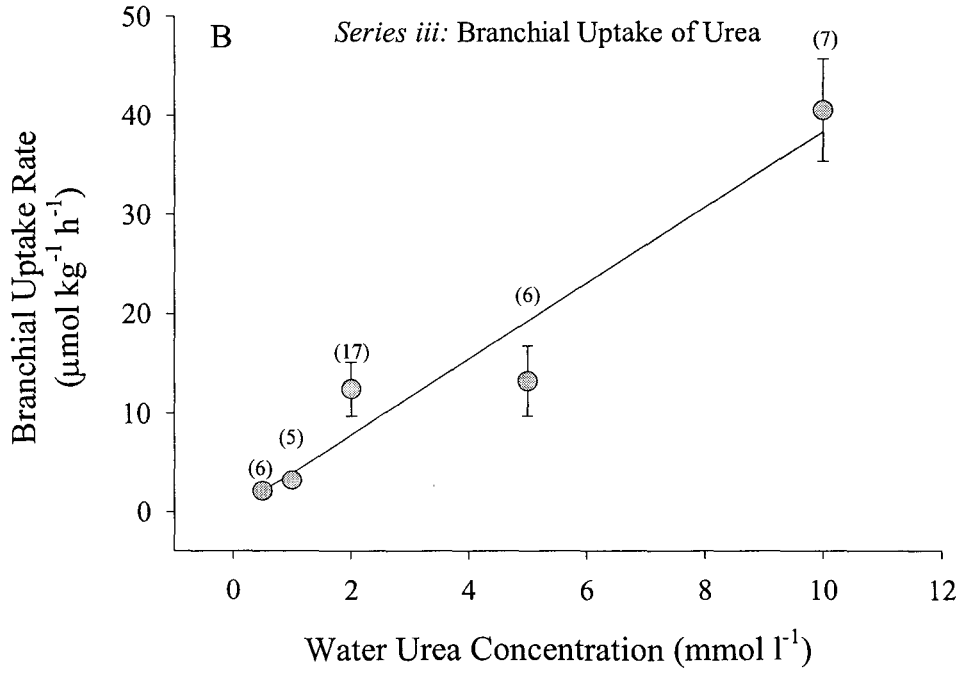

B
Series iii: Branchial Uptake of Urea
Branchial Uptake Rate
(µmol kg-1 h-1)
50
40
30
20
10
0
(7)
(6)
(17)
(5)
(6)
0
2
4
6
8
10
12
Water Urea Concentration (mmol l-1)

Figure 4-4: Linear regression of the urinary excretion rate of urea (y-axis) *versus* the plasma urea concentration (x-axis) demonstrating a linear, proportional relationship with no observable transport maximum. The equation of the line and the significance of the correlation are $y = 0.37x + 0.41$ $r = 0.72$, $P < 0.0001$ ($n = 130$ points from 14 fish).

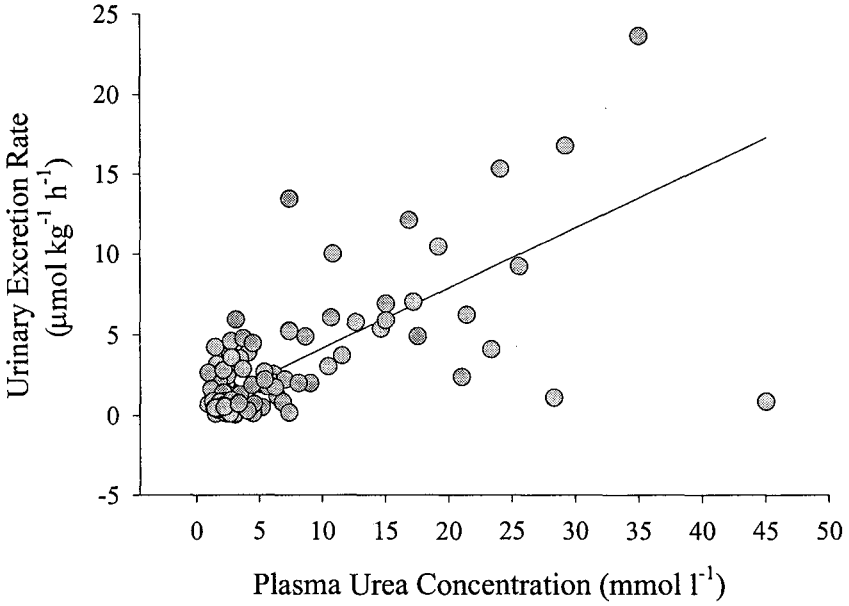

Urinary Excretion Rate
(µmol kg-1 h-1)
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
40
45
50
Plasma Urea Concentration (mmol l-1)

Figure 4-5: The branchial clearance rates (ml kg^{-1} h^{-1}) of fish in the (A) acetamide series demonstrating similar handling of urea and acetamide. Fish in the (B) thiourea series have a significantly lower branchial clearance of thiourea compared to urea. A similar trend is illustrated by measured branchial uptake rates of (C) urea and acetamide and (D) urea and thiourea. Values are means ± 1 S.E.M. (N); * $P < 0.05$ significantly different from analogue.

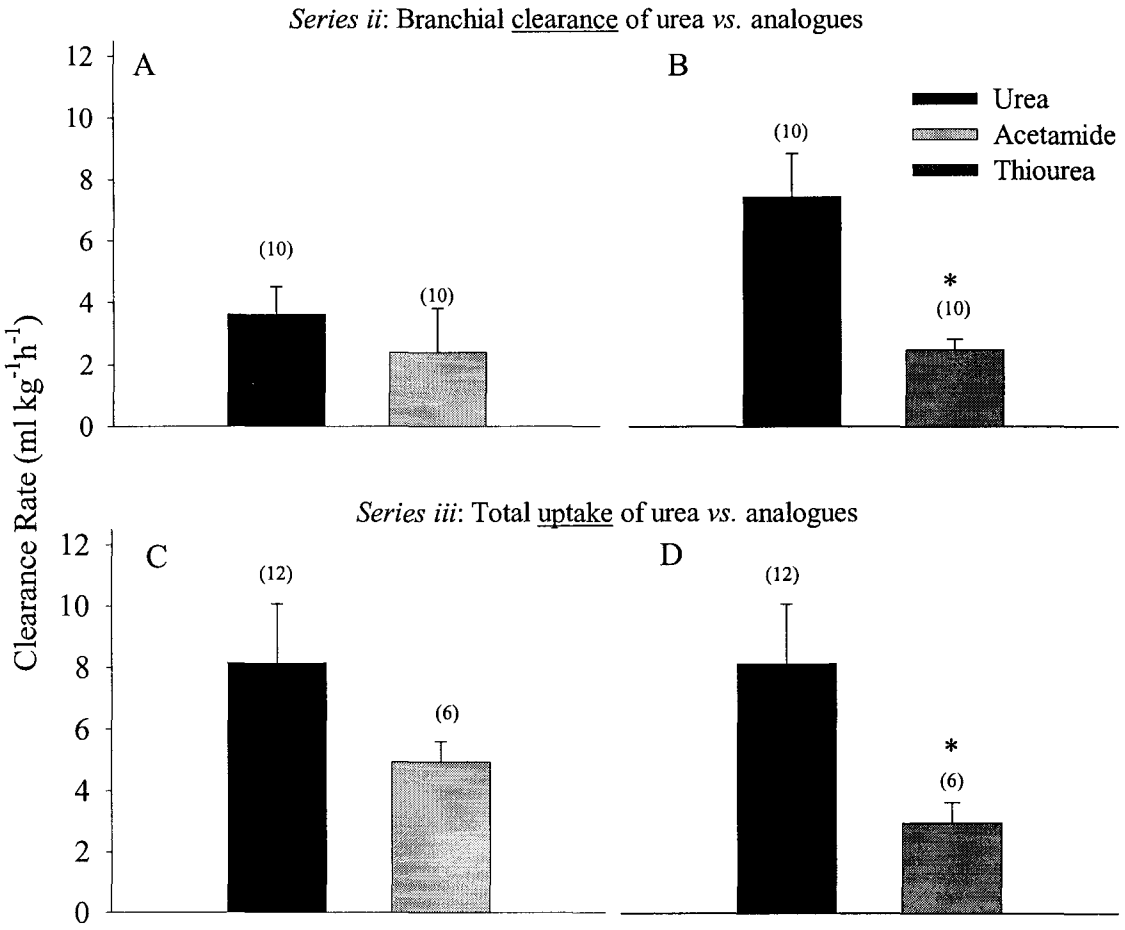

Series ii: Branchial clearance of urea *vs.* analogues
A
B
Urea
Acetamide
Thiourea
(10)
(10)
(10)
(10)
*
Series iii: Total uptake of urea *vs.* analogues
C
D
(12)
(6)
(12)
(6)
*
Clearance Rate (ml kg^{-1}h^{-1})
0
2
4
6
8
10
12

Figure 4-6: Concentrations of (A) urea and (B) acetamide, (C) urea and (D) thiourea in the urine and plasma demonstrating differences in the renal handling of urea and acetamide but similar handling between urea and thiourea. Values are means ± 1 S.E.M. (N = 9 for acetamide series, N=10 for thiourea series); $*P < 0.05$ significantly different from plasma concentrations.

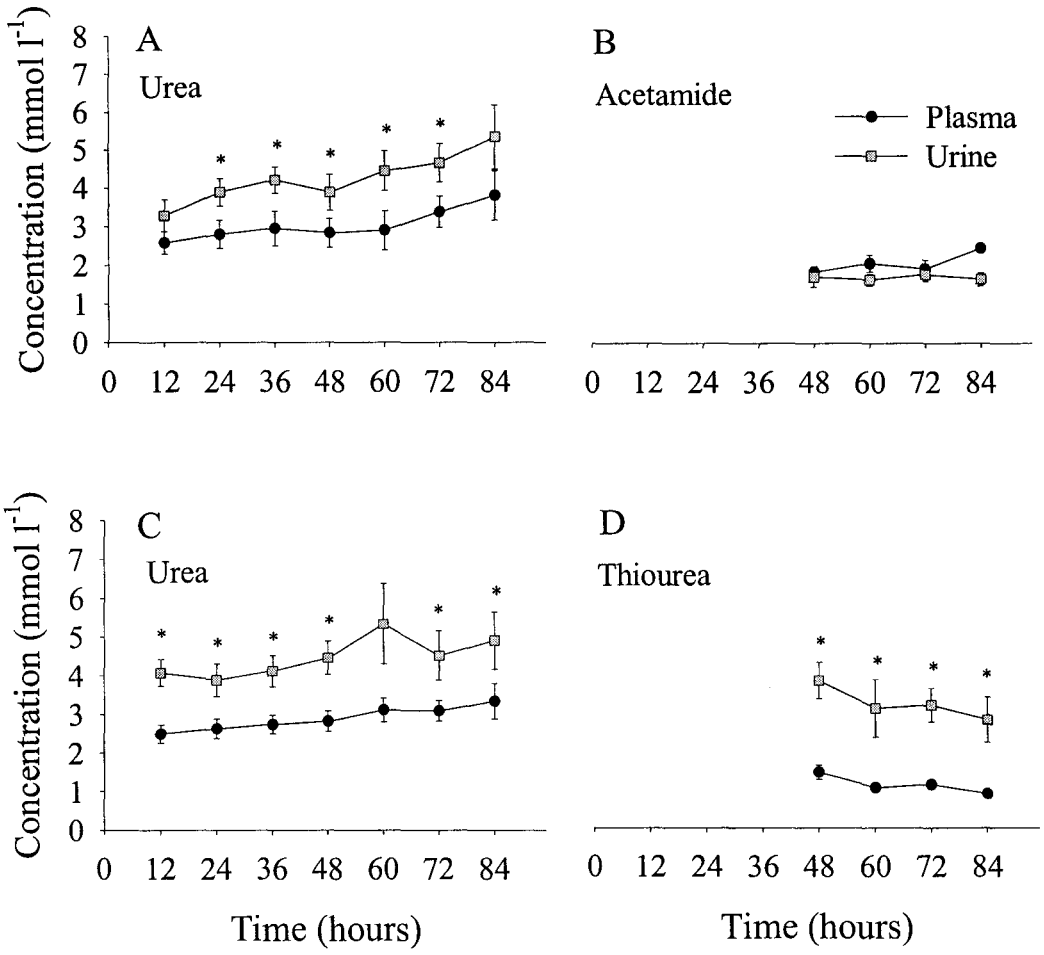
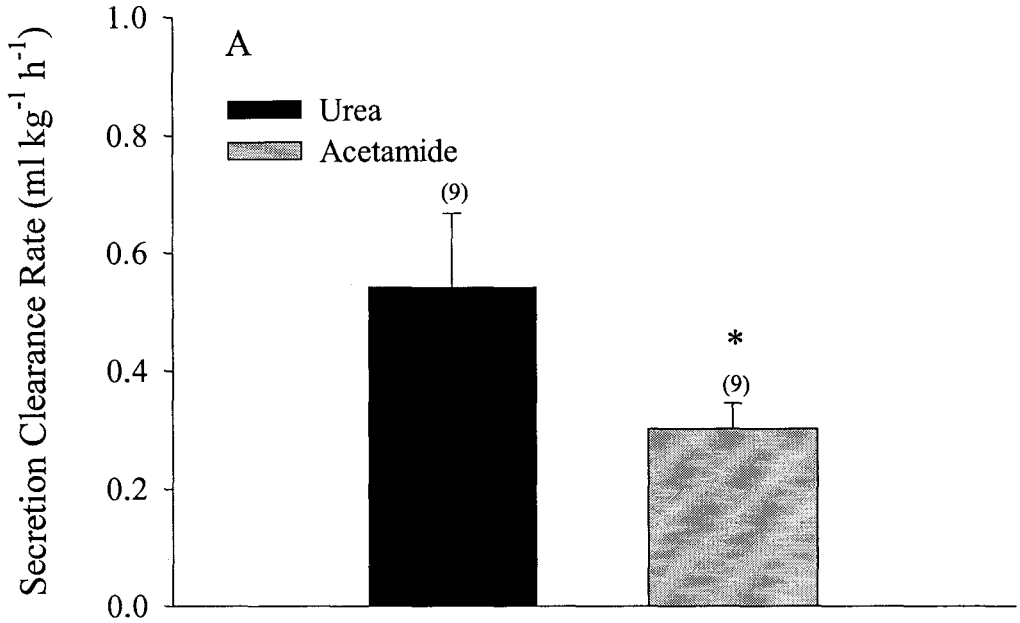

A
Urea
B
Acetamide
Plasma
Urine
C
Urea
D
Thiourea
Concentration (mmol l-1)
Time (hours)
0
1
2
3
4
5
6
7
8
0 12 24 36 48 60 72 84

Figure 4-7: The secretion clearance rate (ml kg^{-1} h^{-1}) of fish in the (A) acetamide series showing a urea clearance rate that is significantly greater than the clearance rate of acetamide. In contrast, fish in the (B) thiourea series demonstrated a clearance rate of urea that was significantly less than the clearance rate of thiourea. Values are means ± 1 S.E.M. (N = 9 for acetamide series, N=10 for thiourea series); * $P < 0.05$ significantly different from analogue.


A
Urea
Acetamide
Secretion Clearance Rate (ml kg-1 h-1)
1.0
0.8
0.6
0.4
0.2
0.0
(9)
*
(9)

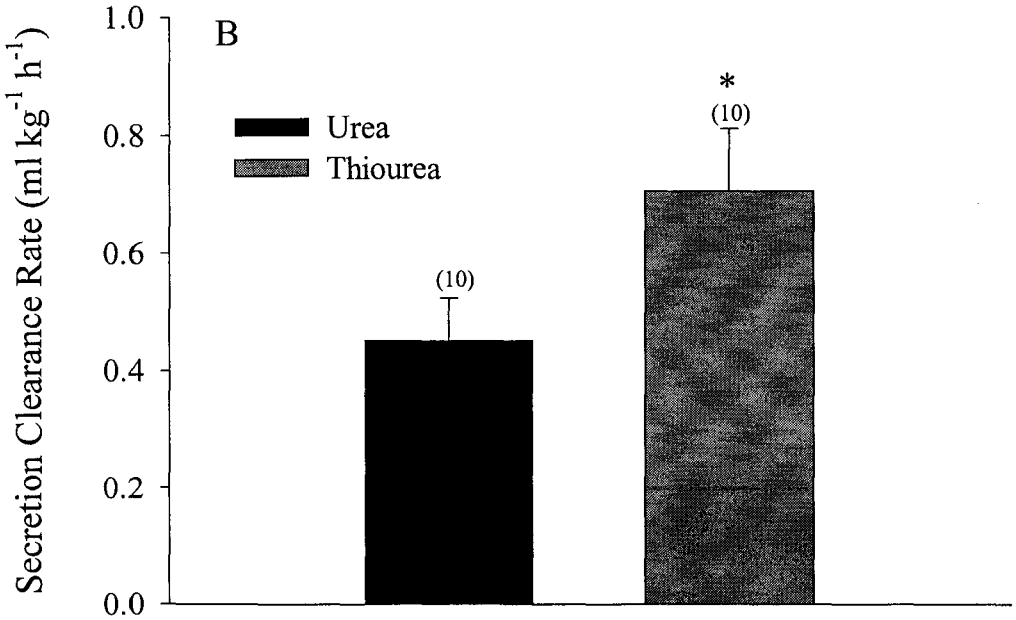
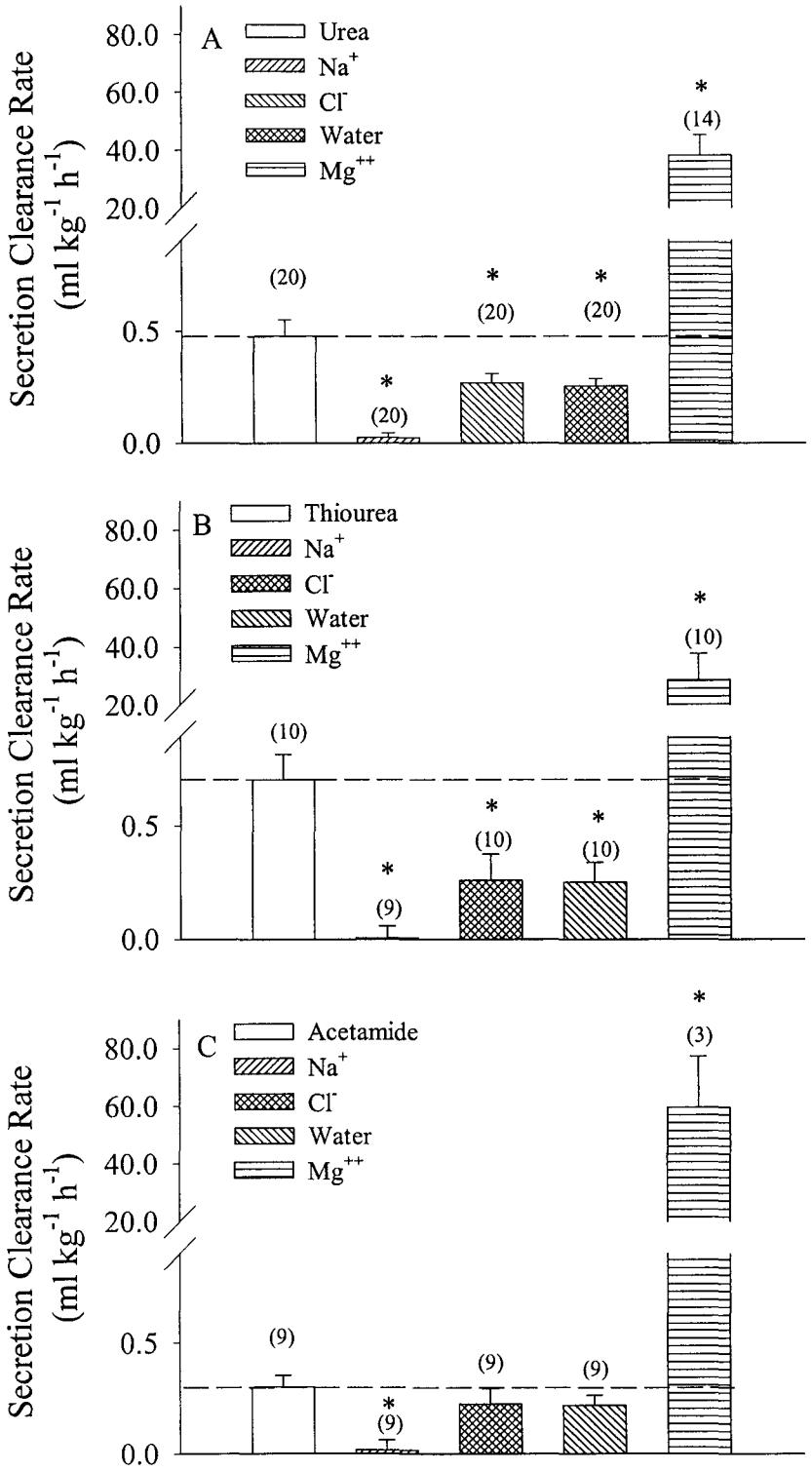

B
Urea
Thiourea
Secretion Clearance Rate (ml kg-1 h-1)
1.0
0.8
0.6
0.4
0.2
0.0
(10)
*
(10)

Figure 4-8: The secretion clearance rates (ml kg^{-1} h^{-1}) of (A) urea, (B) acetamide and (C) thiourea *versus* Na^+, Cl^-, water and Mg^{++} demonstrating a secretion of urea and thiourea at a greater relative rate than the movements of water and Cl^-. The rate of secretion of acetamide was comparable to those of both water and Cl^-. In all cases, there was no net secretion of Na^+. Values are means ± 1 S.E.M. $*P < 0.05$.


A
Urea
Na+
Cl-
Water
Mg++
Secretion Clearance Rate
(ml kg-1 h-1)
80.0
60.0
40.0
20.0
0.5
0.0
(20)
*
(20)
*
(20)
*
(20)
*
(14)
B
Thiourea
Na+
Cl-
Water
Mg++
Secretion Clearance Rate
(ml kg-1 h-1)
80.0
60.0
40.0
20.0
0.5
0.0
(10)
*
(9)
*
(10)
*
(10)
*
(10)
C
Acetamide
Na+
Cl-
Water
Mg++
Secretion Clearance Rate
(ml kg-1 h-1)
80.0
60.0
40.0
20.0
0.5
0.0
(9)
*
(9)
(9)
(9)
*
(3)

Chapter 5

The branchial and renal handling of urea and its analogues in the freshwater rainbow trout (*Oncorhynchus mykiss*)

Abstract

The possible presence of urea transport mechanism(s) in the gill and kidney of the freshwater rainbow trout (*Oncorhynchus mykiss*) was investigated *in vivo* by comparing the branchial and renal handling of the analogues, acetamide and thiourea, with the handling of urea. Trout were fitted with indwelling dorsal aortic catheters and urinary catheters and injected with an isosmotic dose of [^{14}C]-labeled urea analogue (acetamide or thiourea) calculated to bring plasma analogue concentrations close to plasma urea concentrations. Urea and analogue concentrations were significantly greater in the urine that in the plasma. Branchial clearance rate of acetamide was only 48% of urea clearance while the clearance of thiourea was only 22%, a pattern that was also observed in branchial uptake of these substances, and similar to our previous observations in toadfish and midshipmen. The renal secretion clearance rates of urea and acetamide were similar and on average both substances were secreted on a net basis, although reabsorption did occur in some cases. In contrast, thiourea was not handled effectively by the kidney. The secretion clearance rates of both acetamide and urea were well correlated with the secretion clearance rates of Na^+, Cl^- and water while there was no relationship between thiourea and these substances. The pattern of acetamide, thiourea

and urea handling by the gill of the trout is similar to that found in the gills of the midshipman and the gulf toadfish, and strongly suggests the presence of a UT-type facilitated diffusion urea transport mechanism. The pattern of differential handling in the kidney is unlike that of the gill, and also unlike that in the kidney of the midshipman and the gulf toadfish, suggesting a different mechanism. In addition, renal urea secretion is strongly correlated with the reabsorption of Na^+ and occurs against a concentration gradient, suggesting the involvement of an active transport mechanism.

Introduction

During the last few decades, the classic textbook definition of urea as a highly diffusable molecule that could pass freely through lipoprotein membranes unaided has been refuted by the discovery of carrier-mediated urea transport systems. To date, both facilitated diffusion (UT-type transporters) and active transporters for urea have been described. Urea analogues (*i.e.* thiourea, acetamide, N-methylurea) are often used to identify urea transport mechanisms. Their structural similarity to urea enables them to interact competitively at urea transport sites, thereby inhibiting urea transport (Chou and Knepper, 1989; Gillan and Sands, 1993). Although they are often found to interfere with the movement of urea, analogues are not always transported as effectively as urea. In mammals, UT-A1, a hormonally-controlled facilitated diffusion carrier (You et al. 1993; Smith et al. 1995; Shayakul et al. 1996; Karakashian et al. 1999) is difficult to saturate even at urea concentrations well beyond the physiological range (Chou et al. 1990). However, this mechanism can be saturated at relatively low thiourea concentrations, demonstrating that the transport capacity is much lower for thiourea than for urea (Brahm, 1983; Mayrand and Levitt, 1983; Chou et al. 1990).

Studies characterizing urea transport mechanisms in other organisms have shown that urea transporters customarily move analogues less effectively than urea and can often distinguish between analogues. In rat inner medullary collecting ducts (IMCD), the permeability of thiourea was much lower than the permeability of urea through UT-A1 (Chou et al. 1990). In human erythrocytes, the permeability of thiourea (through UT-B transporters) has been reported to be only one thirtieth the permeability of urea

(Naccache and Sha'afi, 1973). In the kidney of the frog *Rana catesbeiana*, there appeared to be active secretion of thiourea but the lack of acetamide transport (Schmidt-Nielsen and Shrauger, 1963). Classic work on the kidney of the spiny dogfish (*Squalus acanthias*) showed that acetamide and methylurea were reabsorbed nearly as well as urea but thiourea was not (Schmidt-Nielsen and Rabinowitz, 1964). It has not yet been determined whether this discrimination was by the recently cloned facilitated diffusion urea transporter (ShUT; Smith and Wright, 1999), or by the hypothesized Na^+-dependent secondary active transporter (Schmidt-Nielsen et al. 1972).

Recently, urea transport mechanisms [tUT from gulf toadfish (*Opsanus beta*), Walsh et al. 2000 and mtUT from Lake Magadi tilapia (*Alcolapia grahami*), Walsh et al. 2001b] have been cloned and physiologically characterized in two teleost fish that, unlike most other teleosts, can excrete urea as their primary nitrogenous waste (Randall et al. 1989; Wood et al. 1989; Walsh et al. 1990; Walsh and Milligan, 1995). When the hormonally-controlled facilitated diffusion urea transporter in the gill of the gulf toadfish (tUT) was activated or inserted into the gill, the permeability for urea increased by 36-fold whereas the permeability for acetamide and thiourea increased by only 17-fold and 6-fold respectively (Wood et al. 1998; Chapter 2). Furthermore, in hepatocytes of the gulf toadfish, phloretin-sensitive urea transport demonstrated a high specificity for urea compared with acetamide, thiourea and N-methylurea (Walsh et al. 1994b). In the Lake Magadi tilapia, the mtUT transporter is continually activated and exhibits a high specificity for urea over thiourea (Walsh et al. 2001b).

While for the most part, freshwater teleosts excrete the majority of their nitrogenous wastes as ammonia, many do retain circulating concentrations of urea many-fold higher than those of ammonia (Wood, 1993), suggesting a role for urea transport mechanisms even in ammoniotelic species. In the ammoniotelic plainfin midshipman (*Porichthys notatus*), a close relative of the facultatively ureotelic gulf toadfish, there is evidence for urea transporters in both the gill and the kidney (Walsh et al. 2001a; Chapter 4). McDonald and Wood (1998) presented circumstantial evidence suggesting that rainbow trout (*Oncorhynchus mykiss*) may regulate endogenous urea concentrations by urea transport mechanisms in the gill and kidney (McDonald and Wood, 1998).

In the trout, branchial excretion of urea is continuous and accounts for > 90% of total urea excretion. Specifically, during the first stages of exogenous urea loading, the gill effectively cleared the plasma of excess urea, maintaining plasma urea concentrations constant and suggesting a role for a facilitated diffusion UT-type transport mechanism for urea in the branchial epithelium (McDonald and Wood, 1998). As infused rates continued to increase, branchial excretion rate did not keep up, indicating possible saturation of the transport mechanism. The secondary route of excretion (< 10 %) was the kidney, which forms primary urine by glomerular filtration and appears to reabsorb urea against a concentration gradient, suggesting the presence of an active transport mechanism (McDonald and Wood, 1998). When infused with exogenous urea loads, net renal urea reabsorption in trout increased in direct proportion to the filtered urea load, and saturation of the reabsorptive mechanism was not apparent, even at urea filtration rates well beyond physiological.

The two main objectives of the present study were to investigate the possibility of urea transport mechanisms in the gill and kidney by quantitatively comparing the handling of urea and two urea analogues, acetamide and thiourea. In addition, the renal handling of urea and analogues was compared to that of other substance such as Na^+, Cl^- and water. Our results indicate that there exist two distinct transport mechanisms involved in urea excretion in the rainbow trout, found in the gill and the kidney, each of which differentially handles urea, acetamide and thiourea.

Materials and Methods

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Farm in Mono Mills, Ontario. The fish were acclimated to seasonal water temperatures (11°C - 14°C) and were fed with commercial trout pellets every second day until the time of surgery. Acclimation was carried out in dechlorinated Hamilton tapwater [in $mmol \cdot l^{-1}$: Ca^{++} = 1.8; Cl^- = 0.8; Na^+ = 0.6; Mg^{++} = 0.5; K^+ = 0.04; titration alkalinity (to pH 4.0) = 1.9; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0].

Experimental Protocol

Dorsal aortic and urinary catheterizations were performed simultaneously on fish anaesthetized with MS-222 (0.07 $g \cdot l^{-1}$; Sigma-Aldrich Canada) and artificially ventilated on an operating table. Dorsal aortic catheters (Clay-Adams PE50 tubing) were implanted as described by Soivio et al. (1972) and were filled with Cortland saline (Wolf, 1963) containing 50 i.u. per ml lithium heparin (Sigma-Aldrich Canada). Internal urinary catheters were inserted using a technique described by Curtis and Wood (1991) and

Wood and Patrick (1994), in which the catheter (heat-molded Clay Adams PE60 tubing) is placed in the urinary bladder so as to drain urine continuously as it is produced from the ureters. Any reabsorptive/secretory role of the bladder is negated so as to examine the function of the kidney alone. During recovery and experimentation, urine was collected continuously with the catheter emptying into a vial approximately 3 cm below the water level of the box. Following surgery, fish were kept in darkened individual containers (minimum volume = 4 L) that were continually aerated and supplied with flowing freshwater (200 $ml \cdot min^{-1}$).

Following the procedure outlined by McDonald and Wood (1998), [^{3}H]-polyethylene glycol (PEG, Mr4000) was used as a marker for glomerular filtration rate (GFR). PEG 4000 was chosen because it is considered a more accurate and conservative indicator of GFR in fish than other commonly used markers (*e.g.* inulin derivatives), as it undergoes minimal radioautolysis, metabolic breakdown, or post-filtration reabsorption across tubules and bladder (Beyenbach and Kirschner, 1976; Erickson and Gingerich, 1986; Curtis and Wood, 1991). Prior to injection of PEG, the fish were allowed to recover from surgery for at least 12 hours, a period during which the patency of the arterial and urinary bladder catheters was confirmed. A dose of 5 $\mu Ci \cdot 100g$ body $weight^{-1}$ of [^{3}H]-PEG 4000 (New England Nuclear) in 0.25 $ml \cdot 100$ g body $weight^{-1}$ was injected *via* the dorsal aortic catheter followed by an additional 0.3 ml of saline in fish where both catheters were deemed successful. The [^{3}H]-PEG 4000 was then allowed to equilibrate throughout the extracellular space for 24h before sampling commenced.

Series i: Urea handling compared to analogues

In this series, the patterns of branchial and renal urea excretion were compared to the handling of acetamide and thiourea, which are similar in size and structure to urea. Two trials were performed: 6 fish treated with acetamide and 5 fish treated with thiourea. Mean weights of fish in the acetamide trial were 0.259 ± 0.017 (6) kg ranging from 0.187 to 0.296 kg. Mean weights of fish in the thiourea trial were 0.268 ± 0.004 (5) kg ranging from 0.256 to 0.276 kg. Following the [^{3}H]-PEG 4000 injection and 24 h equilibration period, a blood sample was taken, (200 μl with saline plus red blood cell replacement), a fresh urine collection was started, water flow to the fish box was stopped and the water level was set to an exact volume mark of 4L. An initial water sample was taken for measurement of urea concentration. Thereafter, water, urine and blood samples were taken every 12 hours and the fish box was flushed thoroughly over a 15 minute period at 12 hour intervals. Vigorous aeration maintained PO_2 close to air saturation during times when water flow to the box was stopped. Twenty four hours after sampling commenced, the fish were injected with a dose of 5μCi·100g body weight^{-1} of [^{14}C]-labeled thiourea <u>or</u> acetamide in 160 μmol·100g body weight^{-1} of isosmotic cold urea analogue (concentration = 300 mmol·l^{-1}) in order to render internal analogue concentrations approximately equal to internal urea concentrations (1.8 mmol·l^{-1}). Sampling continued for the remainder of the experiment (72 hours) to make a total of 6 water samples, 6 blood samples and 6 urine samples. The 2 samples of the 6 that were taken before the analogue injection provided control values while the 4 that were taken after the analogue

injection provided experimental values. Blood samples were immediately centrifuged (10, 000g for 2 min.). Plasma and urine were stored at -20°C for later analysis of Na^+, Cl^-, urea, [^{14}C]-analogue and [^{3}H]-PEG 4000 concentrations. Water samples were analyzed for urea and [^{14}C]- analogue only.

Series ii: Total uptake of urea from the surrounding water

Three different trials were performed using a total of 42 fish. Similar to the protocol of Wright et al. (1995b) and Chapter 4, small rainbow trout (average weight = 0.025 ± 0.004 (42) kg) in groups of six were placed into aerated flux chambers with flow-through freshwater and were left to acclimate for 12 h. After the acclimation period, water flow was stopped, the volume of the chamber was set to 2 L and the fish became a part of one of the following trials. In *Trial A,* a direct comparison was made between the uptake rates of urea and two urea analogues, acetamide and thiourea, using concentrations that were comparable to urea concentrations found in blood plasma *in vivo*. In this trial, one group of fish was exposed to 2 mmol·l^{-1} urea + 25 μCi·l^{-1} [^{14}C]-urea in the surrounding water, a second group was exposed to 2 mmol·l^{-1} acetamide + 25 μCi·l^{-1} [^{14}C]-acetamide and a third group was exposed to 2 mmol·l^{-1} thiourea + 25 μCi·l^{-1} [^{14}C]-thiourea in the surrounding water. *Trial B* investigated analogue competition with urea using urea concentrations slightly greater than internal concentrations and analogue concentrations 3x greater than urea concentrations. The first group of fish was exposed to 2 mmol·l^{-1} urea + 25μCi·l^{-1} [^{14}C]-urea, the second set to 2 mmol·l^{-1} urea, 25 μCi·l^{-1} [^{14}C]-urea plus 10 mmol·l^{-1} acetamide and the third to 2 mmol·l^{-1} urea, 25 μCi·l^{-1} [^{14}C]-

urea plus 10 $mmol \cdot l^{-1}$ thiourea in the external water. *Trial C* examined the effect of phloretin, a urea transport blocker, on urea uptake. In this trial the first group of fish was exposed to 2 $mmol \cdot l^{-1}$ urea + 25 $\mu Ci \cdot l^{-1}$ [^{14}C]-urea + 0.250 $mmol \cdot l^{-1}$ phloretin in 0.04% ethanol. A second group was exposed to 2 $mmol \cdot l^{-1}$ urea + 25 $\mu Ci \cdot l^{-1}$ [^{14}C]-urea + 0.04% ethanol in order to test the effect of the vehicle alone.

In all three trials, after a 12-h flux period, the fish were sacrificed by a blow to the head, weighed, placed in 50 $mmol \cdot l^{-1}$ of "cold" urea or analogue solution (to displace any surface binding of [^{14}C]-label), blotted dry, and then homogenized in 8% perchloric acid (2 parts acid: 1 part fish) using a Proctor-Silex Blend Master blender. A sample of this solution was centrifuged and the supernatant was analyzed for [^{14}C] counts.

Analytical Techniques and Calculations

Urea concentrations in blood, urine, and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in the three media and correction for the presence of thiourea and acetamide. This correction was done by adding the calculated concentration of analogue present in the urine, plasma or water (see Equation 1 and 2) in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. Na^+ and Cl^- concentrations in plasma and urine were measured using a Varian 1275 Atomic Absorption

Spectrophotometer and a Radiometer CMT10 Chloridometer respectively. For measurements of [^{3}H]-PEG 4000 and [^{14}C]-analogue, blood and urine samples (25 μl plus 5 ml of freshwater) or water samples (5 ml) were added to 10 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 Counter using an onboard quench correction program to separate [^{3}H] and [^{14}C] counts. Samples of [^{14}C]-urea or [^{14}C]-analogue in supernatant (5 ml) were added to 10 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 Counter. Quench curves using various amounts of supernatant plus 1 μCi [^{14}C]-urea or [^{14}C]-analogue were made in order to account for the quench associated with supernatant color.

The concentration of analogue [*A*] in the plasma, urine, water or whole body was determined from the original specific activity (*S*; cpm·μmol^{-1}) of the injected solution by converting the radioactivity found in the samples into concentration (μmol·ml^{-1}):

$$S = \frac{\mathrm{cpm_{in}}}{[A]_{\mathrm{in}}}, \qquad (1)$$

$$[A]_{\mathrm{s}} = \frac{\mathrm{cpm_s}}{\mathrm{S}}, \qquad (2)$$

where $\mathrm{cpm_{in}}$ (cpm·ml^{-1}) indicates the radioactivity of the injected solution, $[A]_{\mathrm{in}}$ indicates the total analogue concentration of the injected solution, $\mathrm{cpm_s}$ is the radioactivity in the sample and $[A]_{\mathrm{s}}$ is the analogue concentration in the sample. These analogues are not known to occur endogenously in fish.

The branchial clearance rate (C_B; ml kg^{-1} h^{-1}) of any substance (*X*) was calculated by dividing the concentration of the substance appearing in the water $[X]_{\mathrm{w}}$ by fish body

weight (wt), plasma concentration $[X]_p$ and time (t):

$$C_{BX} = \frac{[X]_w \times V_f}{wt \times [X]_p \times t}, \quad (3)$$

where V_f is the volume of water surrounding the fish. The amount of any substance X taken up from the water was determined from the specific activity of the surrounding water (S_w; cpm·μmol^{-1}) by converting the radioactivity measured in fish (cpm_f) into concentration (μmol·kg^{-1} body weight). The relative uptake (R_U; ml kg^{-1} h^{-1}) of a substance X was then determined by dividing the amount of X taken up from the water by $[X]_w$ and by time (t).

$$X = \frac{cpm_f}{S_w}, \quad (4)$$

$$R_{UX} = \frac{X}{[X]_w \times t}. \quad (5)$$

All the following renal rates were related to fish body weight by expressing urinary flow rate (UFR) in ml·kg^{-1}·h^{-1}. Urinary excretion rates (U) of any substance (X) were calculated as:

$$U_X = [X]_u \times UFR, \quad (6)$$

using measured values of urine flow rates (UFR) and urine concentrations $[X]_u$. Glomerular filtration rates (GFR) were calculated as the clearance of [^{3}H]-PEG 4000 - *i.e.,* the excretion of radioactivity in the urine (cpm_u) relative to its concentration in the blood plasma (cpm_p):

$$GFR = \frac{cpm_u \times UFR}{cpm_p} . \quad (7)$$

The filtration rate (FR; $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of a substance *X* at the glomeruli was calculated as:

$$FR_X = [X]_p \times GFR, \quad (8)$$

and consequently the tubular secretion rate (TS; $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of *X* was calculated as:

$$TS_X = U_X - FR_X. \quad (9)$$

A $FR_X > U_X$ results in a negative TS_X indicating net tubular reabsorption. The renal clearance rate by tubular secretion (SCR; $ml \cdot kg^{-1} \cdot h^{-1}$) of *X* was calculated as:

$$SCR_X = \frac{TS_X}{[X]_p} . \quad (10)$$

A negative SCR value signifies a negative renal "clearance rate" by tubular reabsorption.

The concept of clearance ratio (CRx ; see Wood, 1995) relates the clearance of a substance X to the GFR (*i.e.* to the clearance of [^{3}H] PEG 4000):

$$CR_X = \frac{[X]_u \times UFR}{[X]_p \times GFR} = \frac{U_X}{FR_X} . \quad (11)$$

Assuming that a substance X is filtered at the glomeruli with the same efficiency as [^{3}H]-PEG 4000, a relatively safe assumption with the small neutral molecules (urea, acetamide, thiourea, water) and monovalent ions (Na^+, Cl^-) of the present study, then the clearance ratio provides quantitative information on the tubular handling of X. If the clearance ratio is greater than 1, then there is net secretion of X, if less than 1 then net reabsorption has occurred. For example $CR_X = 0.05$ would indicate 95% net reabsorption of the filtered load of X.

Statistics

Data have been reported as means ± 1 S.E.M (N = number of fish). Regression lines were fitted by the method of least squares, and the significance ($P < 0.05$) of the slope assessed. The significance of differences between means was evaluated using Student's paired or unpaired, two-tailed or one-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977).

Results

The theory behind this study is that if branchial and renal handling of urea are carrier-mediated, then the transporter involved would differentially transport urea *versus* analogues. However, if urea moves through the gill and kidney by simple diffusion, urea would not be differentially transported. In fish treated with acetamide, the branchial clearance rate of urea (11.39 ± 1.50 (6) ml kg^{-1} h^{-1}) was 2-fold greater than the clearance of acetamide (4.97 ± 0.60 (6) ml kg^{-1} h^{-1}; Fig. 5-1A). In thiourea-treated fish, the branchial clearance rate of urea (8.59 ± 1.77 (5) ml kg^{-1} h^{-1}) was approximately 4x greater than the clearance rate of thiourea (2.10 ± 0.81 (5) ml kg^{-1} h^{-1}; Fig. 5-1B). The branchial clearance rates of urea in both trials were not significantly different and the branchial clearance in the presence of analogue was not significantly different than that measured during control periods (control in acetamide trial: 10.89 ± 3.41 (6) ml kg^{-1} h^{-1}; control in thiourea trial: 6.84 ± 1.25 (5) ml kg^{-1} h^{-1}). When expressed as a clearance ratio (analogue/urea) for all collection periods where simultaneous measurements were made,

the ratios were 0.48 ± 0.08 (6) for acetamide and 0.22 ± 0.05 (5) for thiourea, both showing a significant difference from unity.

Although a similar pattern (acetamide/urea ratio = 0.71; thiourea/urea ratio = 0.32) was observed when measuring the total uptake rate of these substances, the relative uptake ratio was substantially greater in the case of acetamide/urea. The total uptake rates of urea (4.44 ± 0.22 (6) ml kg^{-1} h^{-1}) and acetamide (3.16 ± 0.44 (6) ml kg^{-1} h^{-1} Fig. 5-1C) were significantly lower than their clearance rates (10.12 ± 1.17 (11) ml kg^{-1} $h^{-1}$and 4.97 ± 0.60 (6) ml kg^{-1} h^{-1}, respectively) while the total uptake rate of thiourea (1.41 ± 0.41 (6) ml kg^{-1} h^{-1}; Fig. 5-1D) was not significantly different from its clearance rate (2.10 ± 0.81 (5) ml kg^{-1} h^{-1}).

The uptake protocol was also used to investigate the possibility of competitive interactions between urea and urea analogues. Urea movement was found to be unaffected by the presence of analogues, even at analogue concentrations in the external water that were 5-fold greater than urea concentrations (data not shown). Thus, there appears to be no inhibitory effect, in accord with the clearance data. No conclusions could be drawn about the effect of phloretin, a urea-transport blocker, on the rate of urea uptake because all fish treated with phloretin died during the experiment. Fish treated with the vehicle (0.04% ethanol) alone had urea uptake rates that were not significantly different than control (data not shown).

In both analogue-injection trials, the mean plasma urea concentration (1.20 ± 0.13 (11) mmol·l^{-1}) was only about 48% of that in the urine (2.51 ± 0.30 (11) mmol·l^{-1}). Urine flow rate (UFR) was about two thirds of the measured glomerular filtration rate

(GFR; Table 5-1). The concentrations of Na^+ and Cl^- in the ureteral urine were less than 10% of plasma levels. Both ions on average had very low clearance ratios (CR) indicating close to 95 % reabsorption from the kidney tubules (Table 5-2). In contrast, CR_{water} was 0.71 indicating only 30% reabsorption by the kidney tubules. On average CR_{urea} was 1.46, whereas $CR_{acetamide}$ was 1.33 indicating 46% and 33% net secretion by the kidney tubules, respectively. $CR_{thiourea}$ was not significantly different from 1.0 indicating that it was neither net secreted nor net reabsorbed by the kidney tubule (Table 5-2).

In the acetamide series, urea levels remained relatively stable in the urine (2.01 ± 0.33 (6) $mmol \cdot l^{-1}$) and in the plasma (1.10 ± 0.17 (6) $mmol \cdot l^{-1}$); urine levels were significantly greater than plasma levels throughout most of the experiment (Fig. 5-2A). Acetamide showed a similar trend, being significantly more concentrated in the urine (1.79 ± 0.06 (6) $mmol \cdot l^{-1}$) than in the plasma (0.98 ± 0.05 (6) $mmol \cdot l^{-1}$; Fig. 5-2B). However, the concentration of acetamide in the plasma and the urine appeared to steadily decline as it was cleared by the kidney and gills.

As in the acetamide series, fish treated with thiourea had relatively stable urine (3.08 ± 0.28 (5) $mmol \cdot l^{-1}$) and plasma (1.32 ± 0.15 (5) $mmol \cdot l^{-1}$) urea concentrations, with urine levels being significantly greater than plasma levels at all times (Fig. 5-2C). Thiourea was also found in greater concentrations in the urine (1.55 ± 0.17 (5) $mmol \cdot l^{-1}$) than in the plasma (1.00 ± 0.07 (5) $mmol \cdot l^{-1}$) although early in the experiment, urine and plasma thiourea concentrations were the same (Fig. 5-2D). Similar to acetamide, thiourea concentrations tended to fall with time.

The handling of urea and acetamide by the kidney was complicated by the fact that the two substances were both reabsorbed and secreted. When comparing the relative renal secretion clearance rates (SCR_X, Equation 10), the $SCR_{acetamide}$ exhibited a strong linear, proportional relationship (r= 0.98) to the SCR_{urea} with a slope of 1.08 ($P < 0.001$), indicating that the secretion/reabsorption clearance rate of acetamide was approximately 100% of the rate for urea (Fig. 5-3). In contrast, there was no relationship between the $SCR_{thiourea}$ and that of urea (r =0.17; slope not significantly different than zero).

Plasma Na^+ and Cl^- concentrations in both the acetamide and thiourea series were greater than urine concentrations and on average had very similar SCR values (Na^+: -3.58 ± 0.48 (11) ml kg^{-1} h^{-1}; Cl^-: -3.57 ± 0.48 (11) ml kg^{-1} h^{-1}), the negative values indicating net reabsorption and not secretion. The SCR values of both Na^+ and Cl^- exhibited linear proportional relationships (r = 0.75 and r = 0.76, respectively) to SCR_{urea} with slopes of 0.692 ($P < 0.0001$; Fig. 5-4A) and 0.693 ($P < 0.0001$; Fig. 5-4B), respectively. The relationship of SCR_{water} (-1.74 ± 0.32 (11) ml kg^{-1} h^{-1}) to SCR_{urea} was linear (r = 0.89) with a slope of 0.965 ($P < 0.0001$; Fig. 5-4C). Thus, on a relative basis, the SCR_{urea} was approximately 70% the SCR values of Na^+ and Cl^- and equal to the movement of water.

$SCR_{acetamide}$ exhibited an almost 1:1 relationship with both Na^+ (r = 0.93; slope 0.950, $P < 0.0001$) and Cl^- (r = 0.93; slope 0.946, $P < 0.0001$; Fig. 5-5A, B). $SCR_{acetamide}$ and SCR_{water} exhibited a linear proportional relationship (r = 0.96) with a slope of 1.03 ($P < 0.0001$; Fig. 5-5C). Thus, $SCR_{acetamide}$ was approximately 95% of the SCR values of Na^+ and Cl^- and equal to the movement of water. In contrast, there was no relationship

between the negligible $SCR_{thiourea}$ and the SCR values of Na^+, Cl^- or water (data not shown).

Discussion

The objectives of this study were to characterize the branchial and renal handling of urea in the rainbow trout in order to determine whether the excretion of urea through the gills or kidney is in fact carrier-mediated. Our results suggest that there is evidence for a distinct transport mechanism involved in the excretion of urea through the gill that has characteristics similar to those of the UT-family of urea transporters and therefore similar to those found in the gills of the gulf toadfish (Chapter 2) and the plainfin midshipman (Chapter 4). There is also evidence for carrier-mediated renal secretion of urea *via* a transport mechanism that is distinct from that in the gill and also distinct from that in the kidneys of the other two species examined in this thesis.

The trout gill exhibited the same pattern of differential handling as observed in the facultatively ureotelic gulf toadfish and the ammoniotelic plainfin midshipman, where the branchial clearance rate of urea > acetamide > thiourea. Through the trout gill, the ratio of analogue/urea clearance was 48% for acetamide and only 22% for thiourea. In the influx direction, these ratios were 71% and 32%, respectively. In the gulf toadfish (Chapter 2), a similar pattern is observed through tUT, a hormonally-controlled, pulsatile, facilitated diffusion mechanism in the gill that shows 62% homology at the amino acid level to mammalian (UT) urea transporters (Smith et al. 1998; Walsh et al. 2000). In the toadfish, the branchial clearance of acetamide when tUT is activated is 35-50% that of

urea clearance and the relative thiourea clearance is at most only 16% of urea clearance during pulsing events. However, all three substances exhibit similar permeabilities during non-pulsing periods, suggesting that the gill handles urea, acetamide and thiourea identically when tUT is not activated, despite small differences in calculated oil/water partition coefficients and lipid permeabilities of the three substances (Goldstein and Solomon, 1960; Lippe, 1969; Galluci et al. 1971).

In the ammoniotelic midshipman, a cDNA probe based on tUT produced a strong signal when tested against gill mRNA, suggesting that there is a message for a UT-type transporter in the gills (Walsh et al. 2001a). The clearance of acetamide through midshipman gill was 74% of urea clearance while thiourea clearance was only 55%, with similar patterns in the influx direction (Chapter 4). In the obligately ureotelic Lake Magadi tilapia, mtUT (with 75% identity to tUT) demonstrated a permeability to thiourea that was 19% that of urea permeability; acetamide was not tested (Walsh et al. 2001b). Thus, this pattern of analogue *versus* urea handling appears to be characteristic of branchial UT-type transport mechanisms in both ureotelic and ammoniotelic organisms. That this pattern is also evident in rainbow trout strongly suggests the presence of a UT-type facilitated diffusion transporter in the gill, and supports preliminary Northern blot and sequence analysis that revealed a mRNA from trout gill with approximately 2000 bases homologous to the mRNA of the toadfish tUT (C.M. Pilley, P.W. Wright and P.J. Walsh, unpublished data).

Recent molecular evidence (mRNA hybridization with a cDNA based on tUT) indicates that facilitated diffusion transporters may be present in the gills of many other

ammoniotelic teleosts (Walsh et al. 2001a). In addition, trout embryos appear to have a UT-type transport mechanism, facilitating the diffusion of urea through the chorion, as a significant proportion (26%) of their nitrogenous wastes is excreted as urea (Wright and Land, 1998; Pilley and Wright, 2000). Trout embryos also appear to have relatively high levels of at least four OUC enzymes at the time of hatching, which suggests the possibility that ammoniotelic teleosts may have retained the genes for OUC enzymes and urea transporters because they are important during embryogenesis (Wright et al. 1995a).

A common tool for characterizing UT-type transport mechanisms is to determine whether analogues will competitively inhibit urea movement. Competition between thiourea and urea has been observed through the toadfish tUT (Wood et al. 1998; Walsh et al. 2000). In addition, urea transport through the Lake Magadi tilapia mtUT was inhibited by the presence of acetamide, N-methylurea and thiourea (Walsh et al. 2001b). However, there was no inhibition of analogues on urea transport in the trout either when analogues were present internally (injected) or when present externally (uptake of [^{14}C]-urea in the presence of an excess of cold thiourea or acetamide). These results are similar to those determined *in vivo* for the plainfin midshipman (Chapter 4).

Interestingly, these *in vivo* patterns with analogue (differential handling but no inhibitory effect) differ from those seen *in vitro* in a study of transport by gill basolateral membrane vesicles of trout (Chapter 6). In that study, thiourea was transported at the same rate as urea, but urea transport was inhibited by a 5-fold excess of thiourea. In contrast, acetamide was transported at a lower rate than urea but urea transport was not inhibited by a 5-fold excess of acetamide. The potential reasons for these differences

versus the *in vivo* situation are explored in Chapter 6. Alternatively, it could be that the analogue concentrations in the present study were simply not high enough to cause a substantial effect on urea transport *in vivo*, especially when dealing with both apical and basolateral membranes. In both the toadfish and the tilapia competition uptake studies, the urea and analogue concentrations in the external water were much higher than those used in the present study (Wood et al. 1998; Walsh et al. 2001b). In the toadfish study, 30 $mmol \cdot l^{-1}$ urea was placed in the external water with double the amount of thiourea and in the tilapia study, 20 $mmol \cdot l^{-1}$ urea was placed with triple the amount of analogue (Wood et al. 1998; Walsh et al. 2001b).

The trout kidney appeared to handle urea and acetamide similarly. On average, both were added to the kidney ultrafiltrate by net secretion by the kidney tubule, although reabsorption occurred in some circumstances (see below). In contrast, thiourea was not handled by the kidney tubule, but entered the urine entirely by filtration. These results are similar to those of the elasmobranch kidney in which acetamide and urea were handled similarly by what was believed to be a Na^+-coupled secondary active transport mechanism while thiourea was not (Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen et al. 1972). It is possible that the pattern observed in both the trout and shark kidney is characteristic of the same type of mechanism. It is unlikely that the urea transport mechanisms in the gill and kidney are the same, based on the different pattern observed in the kidney, plus the fact that urea is moving against a concentration gradient. In addition, the pattern of urea and analogue handling by the trout kidney is different from that observed in the toadfish and midshipman where $SCR_{thiourea} > SCR_{urea} >$

$SCR_{acetamide}$ (Chapter 2; Chapter 4). This latter difference is most likely a reflection of different urea transport mechanisms and morphology; the handling of urea in batrachoidides is not correlated with Na^+ as observed in trout, and the kidney of toadfish and midshipmen is aglomerular while the kidney of the trout is glomerular.

Interestingly, previous evidence indicated that rainbow trout reabsorbed urea against a concentration gradient and urea concentrations in the plasma were almost double the concentrations in the urine (reviewed by Wood, 1993; McDonald and Wood, 1998). In McDonald and Wood (1998), the mean clearance ratio of urea (0.27) was significantly less than 1.0 indicating that 73% of the urea filtered at the glomerulus was then reabsorbed by the kidney tubule. In contrast, in the present study, plasma urea concentrations are significantly less than urine urea concentrations and the mean clearance ratio for urea is 1.46. This indicates that in addition to the filtered urea load, 46% more urea is secreted into the urine against a concentration gradient. However, consistent in both studies is the fact that the movement of urea is highly correlated with the reabsorption of Na^+ (Fig. 5-6). In the present study, the secretion of urea was highly correlated with Na^+ and Cl^- reabsorption (and passive reabsorption of water) and net urea secretion (positive SCR_{urea}) only occurred when Na^+ reabsorption was reduced (low negative values of SCR_{Na+}). If Na^+ reabsorption was elevated, then urea reabsorption was observed. Compared to McDonald and Wood (1998), mean SCR_{Na+} is not significantly different and the linear relationship between SCR_{Na+} and SCR_{urea} is consistent (slope = 0.73, $P < 0.001$; y-intercept not significantly different than zero; previously unreported data from McDonald and Wood, 1998; Fig. 5-6). A major difference between the two

relationships, aside from urea being predominantly secreted in the present study, is that the linear relationship appears to have shifted so that the y-intercept is significantly different than zero. Possibly, two different types of transport mechanisms, both involving Na^+ but with different affinities for urea, are at work in the kidney of the trout and depend on the physiological status of the fish.

Similar dichotomies have been seen in mammals. For example, a Na^+-dependent, secondary active, urea reabsorptive mechanism is only present in rats fed a low-protein diet for at least 3 weeks and is not expressed in rats fed a normal (18%) protein diet (Isozaki et al. 1994a,b). At the same time, urea secretion through Na^+-urea counter transport mechanisms has been observed in rats that are given free access to food and water (Kato and Sands, 1998b). While trout of the present study were fed up until the time of surgery, trout used in McDonald and Wood (1998) were starved for one week prior to this point. It would be interesting to investigate the long-term effect of a low protein diet on trout and its implications for urea metabolism and excretion. Another possible factor, stress, through the mobilization of cortisol, may effect urea transport and metabolism (Mommsen et al. 1999; Naruse et al. 1997; Peng et al. 2002) and has been investigated in Chapter 7.

In summary, it appears that the freshwater rainbow trout has urea transporters in both the gill and the kidney. The patterns of analogue *versus* urea handling by the gill is similar to observations in three other teleost species, suggesting not only the involvement of a UT-type transporter in the gill of the trout but a characteristic pattern of analogue handling by this type of branchial urea transporter, across a broad range of teleost

species. The gill transporter appears to be of the facilitated diffusion type. Urea and analogue handling by the trout kidney exhibits a different pattern from the gill, suggesting different transport mechanisms in the two organs. Indeed, the kidney transporter of the trout appears to be of the active type, and may be Na^+-linked. Further investigation is required to fully understand the implications for urea handling at the level of the kidney with respect to the physiological state of the whole animal.

Table 5-1: Average values for plasma and urinary ion composition, urine flow rate and glomerular filtration rate.

UFR ($ml\ kg^{-1}\ h^{-1}$)	GFR ($ml\ kg^{-1}\ h^{-1}$)	$[Urea]_p$ ($mmol \cdot l^{-1}$)	$[Urea]_u$ ($mmol \cdot l^{-1}$)	$[Na^+]_p$ ($mmol \cdot l^{-1}$)	$[Na^+]_u$ ($mmol \cdot l^{-1}$)	$[Cl^-]_p$ ($mmol \cdot l^{-1}$)	$[Cl^-]_u$ ($mmol \cdot l^{-1}$)
2.1 ± 0.1	3.7 ± 0.5	1.20 ± 0.13	2.51 ± 0.30	140.8 ± 2.8	8.3 ± 1.7	133.0 ± 2.8	7.2 ± 0.3

Data are shown as mean ± 1 S.E.M. (N = 11); UFR = urine flow rate, GFR= glomerular filtration rate, p = plasma, u = urine.

Table 5-2: The calculated clearance ratios for water, Na^+, Cl^-, urea, acetamide and thiourea for each fish in *Series i*.

CR_{water}	CR_{Na+}	CR_{Cl-}	CR_{urea}	$CR_{acetamide}$	$CR_{thiourea}$
0.71 ± 0.07 (11)	0.05 ± 0.01 (11)	0.04 ± 0.01 (11)	1.46 ± 0.12 (11)	1.33 ± 0.12 (6)	1.25 ± 0.29 (5)

Data are shown as mean ± 1 S.E.M. (N).

Figure 5-1: The branchial clearance rates (ml kg^{-1} h^{-1}) of fish in the (A) acetamide series and fish in the (B) thiourea series showing a significantly lower branchial clearance of acetamide and thiourea compared to urea. A similar trend is illustrated by measured branchial uptake clearance rates of (C) urea *versus* acetamide and (D) urea *versus* thiourea. Values are means ± 1 S.E.M. (N); * $P < 0.05$ significantly different from urea.

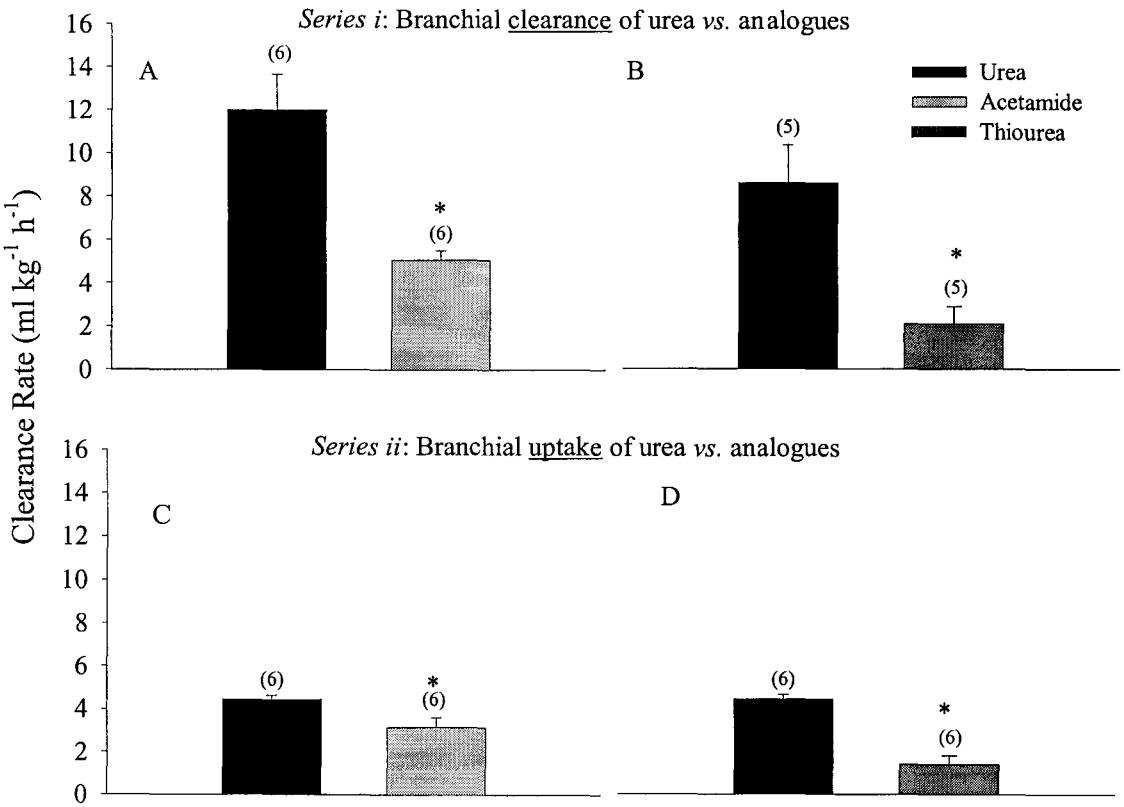

Series i: Branchial clearance of urea vs. analogues
Series ii: Branchial uptake of urea vs. analogues
Clearance Rate (ml kg-1 h-1)
A
B
C
D
Urea
Acetamide
Thiourea
(6)
(5)
*
0
2
4
6
8
10
12
14
16

Figure 5-2: Urine and plasma concentrations of (A) urea and (B) acetamide in the same fish, and (C) urea and (D) thiourea in the same fish demonstrating that all three substances are more concentrated in the urine than in the plasma. Values are means ± 1 S.E.M. (N = 6 for acetamide series, N=5 for thiourea series); $*P < 0.05$ significantly different from plasma concentrations.

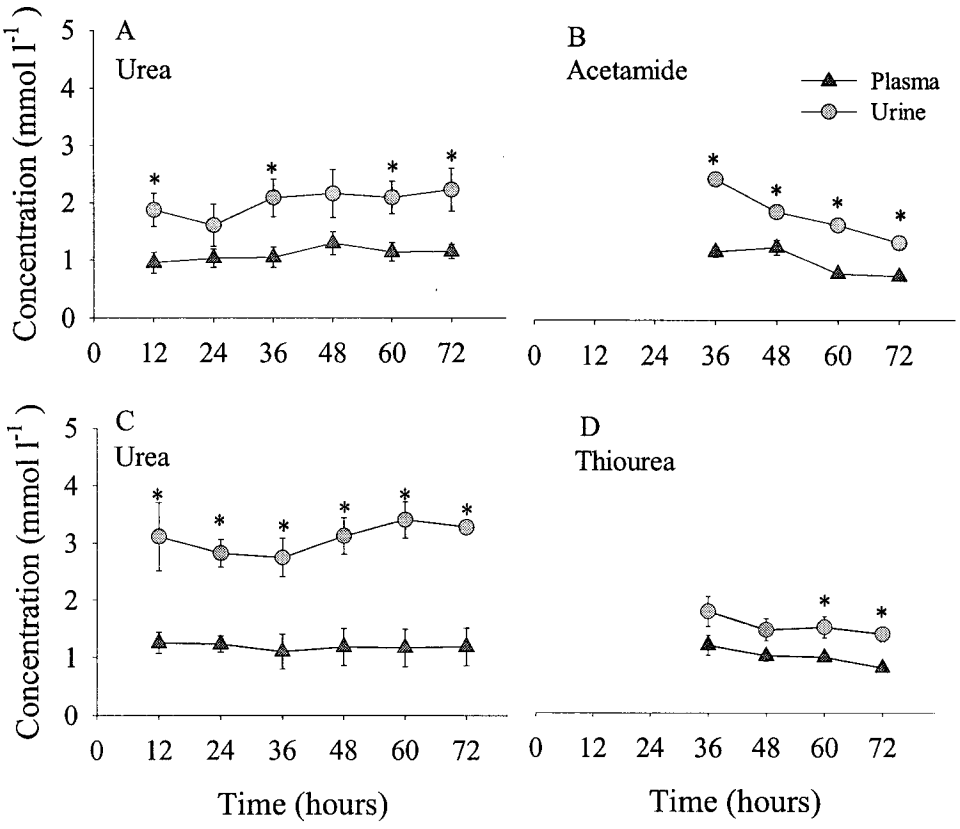

A
Urea
B
Acetamide
Plasma
Urine
C
Urea
D
Thiourea
Concentration (mmol l-1)
Time (hours)
0
1
2
3
4
5
0
12
24
36
48
60
72

Figure 5-3: Linear regressions of the secretion clearance rates (SCR_X; Equation 10) of analogue (y-axis) and urea (x-axis) demonstrating a $SCR_{acetamide}$ that is equal to that of urea. The equation of the acetamide line and the significance of the correlation is $y = 1.08x + 0.088$ $r = 0.98$, $P < 0.001$ (N = 20 simultaneous measurements from 6 individual fish). In contrast, thiourea does not appear to be transported by the kidney tubule. The equation of the thiourea line is $y = 0.070x - 0.200$ $r = 0.17$, slope n.s (N = 13 simultaneous measurements from 5 individual fish).

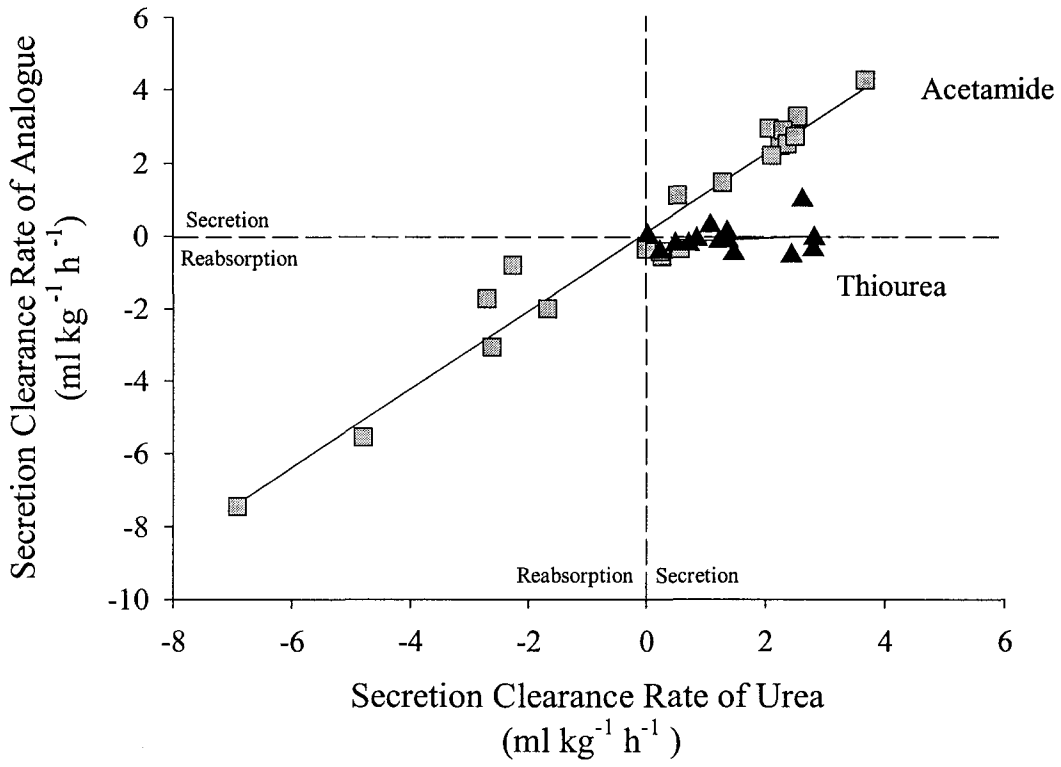

Secretion Clearance Rate of Analogue
(ml kg-1 h-1)
6
4
2
0
-2
-4
-6
-8
-10
Secretion
Reabsorption
Acetamide
Thiourea
Reabsorption
Secretion
-8
-6
-4
-2
0
2
4
6
Secretion Clearance Rate of Urea
(ml kg-1 h-1)

Figure 5-4: Linear regressions of the secretion clearance rate (SCR_X; Equation 10) of urea (y-axis) *versus* (A) Na^+ (B) Cl^- and (C) water (x-axis). The SCR_{urea} is highly correlated with the SCR values of all three substances (negative SCR values represent reabsorption). Urea is both reabsorbed and secreted by the kidney whereas Na^+, Cl^- and water are only reabsorbed. The equation of the Na^+ line and the significance of the correlation is $y = 0.692x + 2.92$ $r = 0.75$, $P < 0.0001$. The equation of the Cl^- line and the significance of the correlation is $y = 0.693x + 2.927$ $r = 0.76$, $P < 0.0001$. The equation of the water line and the significance of the correlation is $y = 0.965x + 2.13$ $r = 0.89$, $P < 0.0001$ (N = 56 simultaneous measurements from 11 individual fish).

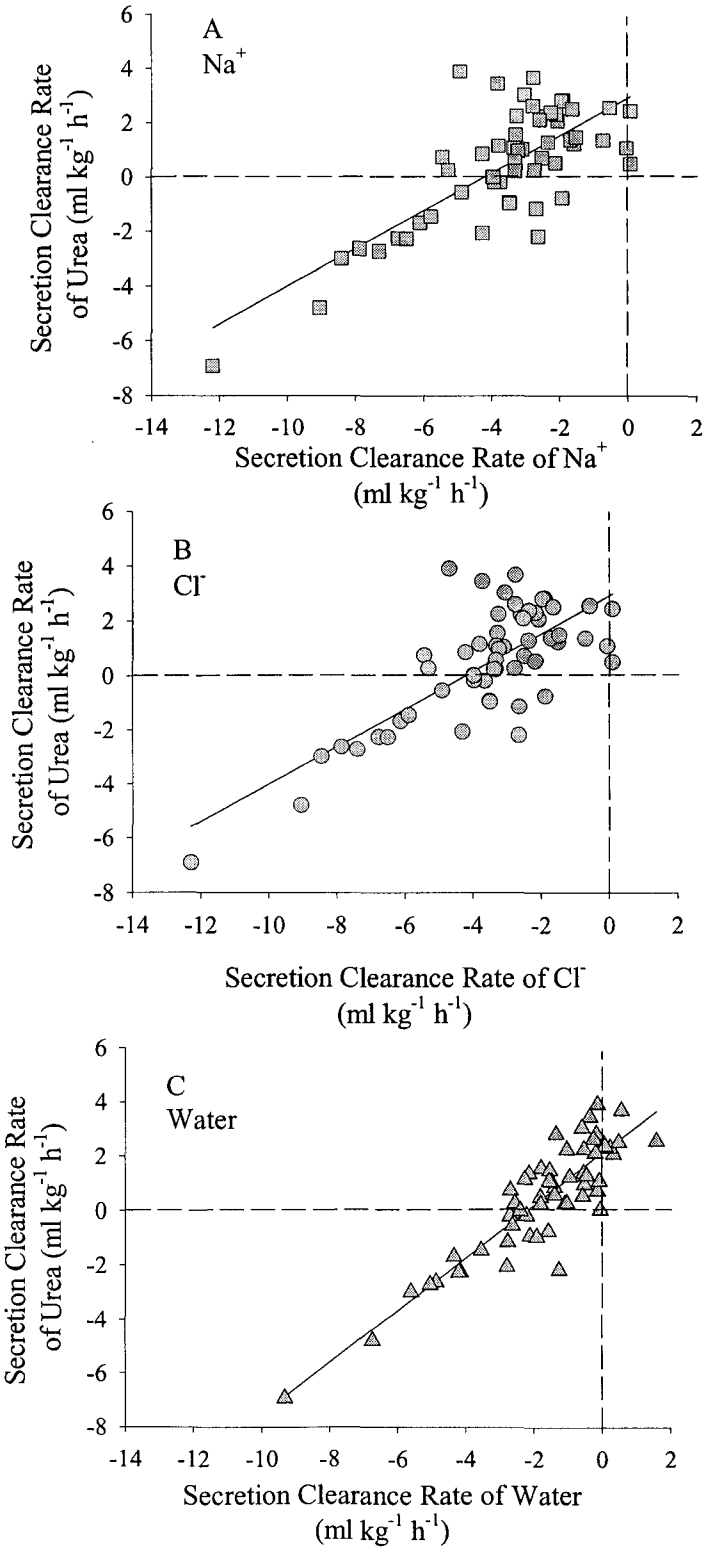

A
Na+
Secretion Clearance Rate of Urea (ml kg-1 h-1)
Secretion Clearance Rate of Na+ (ml kg-1 h-1)
B
Cl-
Secretion Clearance Rate of Urea (ml kg-1 h-1)
Secretion Clearance Rate of Cl- (ml kg-1 h-1)
C
Water
Secretion Clearance Rate of Urea (ml kg-1 h-1)
Secretion Clearance Rate of Water (ml kg-1 h-1)

Figure 5-5: Linear regressions of the secretion clearance rate (SCR_X; Equation 10) of acetamide (y-axis) *versus* (A) Na^+ (B) Cl^- and (C) water (x-axis). The $SCR_{acetamide}$ is highly correlated to the SCR values of all three substances (negative SCR values represent reabsorption). Acetamide, like urea, is both reabsorbed and secreted by the kidney whereas Na^+, Cl^- and water are only reabsorbed. The equation of the Na^+ line and the significance of the correlation is $y = 0.950x + 4.17$ $r = 0.93$, $P < 0.0001$. The equation of the Cl^- line and the significance of the correlation is $y = 0.946x + 4.18$ $r = 0.93$, $P < 0.0001$. The equation of the water line and the significance of the correlation is $y = 1.03x + 2.22$ $r = 0.96$, $P < 0.0001$ (N = 20 simultaneous measurements from 6 individual fish).

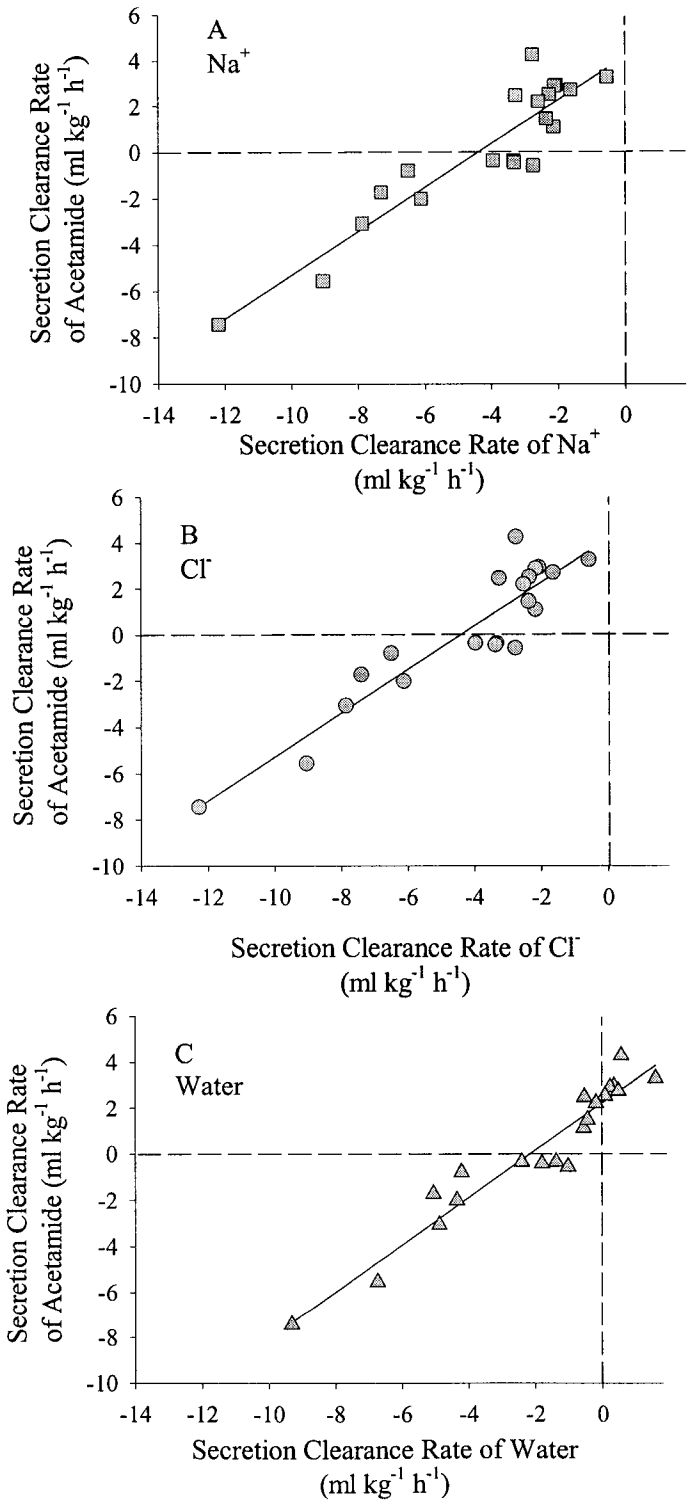
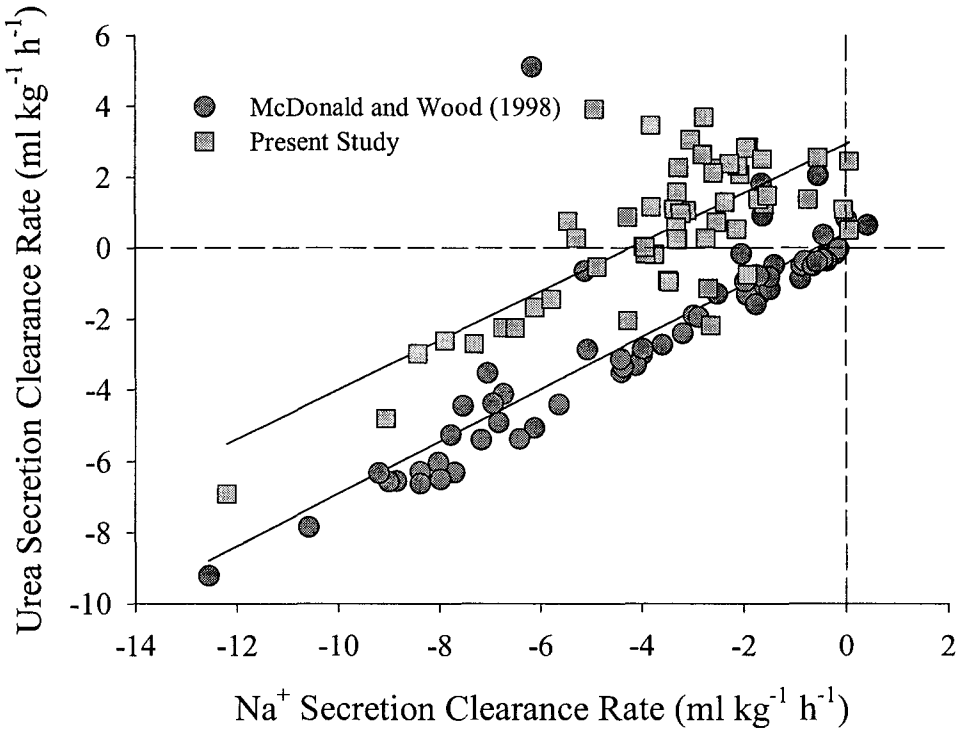

A
Na+
Secretion Clearance Rate of Acetamide (ml kg-1 h-1)
Secretion Clearance Rate of Na+ (ml kg-1 h-1)
B
Cl-
Secretion Clearance Rate of Acetamide (ml kg-1 h-1)
Secretion Clearance Rate of Cl- (ml kg-1 h-1)
C
Water
Secretion Clearance Rate of Acetamide (ml kg-1 h-1)
Secretion Clearance Rate of Water (ml kg-1 h-1)

Figure 5-6: Linear regressions of the secretion clearance rates (SCR_X; Equation 10) of Na^+ (x-axis) and urea (y-axis) from the present study *cf.* the relationship found in McDonald and Wood (1998). The two relationships have similar slopes but different y-intercepts. The equation of the line from the present study and the significance of the correlation is $y = 0.692x + 2.92$ $r = 0.75$, $P < 0.0001$ (N = 56 simultaneous measurements from 11 individual fish). The equation of the line from McDonald and Wood (1998) is $y = 0.733x + 0.421$ $r = 0.87$, $P < 0.0001$ (N = 66 simultaneous measurements from 15 individual fish).


McDonald and Wood (1998)
Present Study
Urea Secretion Clearance Rate (ml kg-1 h-1)
Na+ Secretion Clearance Rate (ml kg-1 h-1)
6
4
2
0
-2
-4
-6
-8
-10
-14
-12
-10
-8
-6
-4
-2
0
2

Chapter 6

Evidence for facilitated diffusion of urea by the basolateral membrane of the gill of the rainbow trout (*Oncorhynchus mykiss*)

Abstract

Recent *in vivo* evidence suggests that the mechanism of branchial excretion of urea in the ammoniotelic rainbow trout (*Oncorhynchus mykiss*) is carrier-mediated. Further characterization of this mechanism was achieved by using an *in vitro* membrane vesicle preparation, isolating the basolateral membrane of the gill. Basolateral membrane vesicles (BLMV) demonstrated urea uptake with a saturable component (K_m = 1.17 ± 0.43 mmol·l^{-1}; V_{max} = 0.42 ± 0.08 μmol mg protein^{-1} h^{-1}) at low, physiologically relevant urea concentrations and a non-saturable component at urea concentrations of up to 17.5 mmol·l^{-1}. Uptake of urea at 1 mmol·l^{-1} by BLMV was reduced by 62.5% when incubated with 0.25 mmol·l^{-1} phloretin, a potent blocker of UT-type transport mechanisms. BLMV demonstrated differential handling of urea *versus* urea analogues at 1 mmol·l^{-1} concentrations: analogue/urea uptake ratios were 32% for acetamide and 84% for thiourea. In support of these findings, urea uptake at 1 mmol·l^{-1} was significantly reduced by 73% in the presence of 5 mmol·l^{-1} thiourea but was not affected by 5 mmol·l^{-1} acetamide or 5 mmol·l^{-1} N-methylurea. Urea uptake at 1 mmol·l^{-1} by BLMV was

sensitive to temperatures above and below the temperature of acclimation with a $Q_{10} > 2$ suggesting a protein carrier-mediated process.

Introduction

Like most other freshwater teleosts, the rainbow trout (*Oncorhynchus mykiss*) excretes the majority of its nitrogenous wastes across the gills as ammonia. However, a handful of teleosts expend energy to detoxify ammonia and excrete urea as their primary waste product (Saha and Ratha, 1987; Randall et al. 1989; Wood et al. 1989; Walsh et al. 1990), made possible by a fully functional ornithine-urea cycle (OUC) in internal tissues (especially the liver) and specialized facilitated diffusion urea transport mechanisms in the gills (UT-type transporters; Walsh et al. 2000; Walsh et al. 2001b). While adult rainbow trout do not have a full complement of OUC enzymes, they do maintain a surprisingly high level of circulating urea compared to endogenous ammonia concentrations (5–50x more urea) and urea makes up about 10% of total nitrogen waste excretion (reviewed by Wood, 1993). In addition, recent pharmacological evidence suggests that, as in ureotelic fish, urea excretion is carrier-mediated in ammoniotelic organisms such as the rainbow trout (McDonald and Wood, 1998; Chapter 5) and the plainfin midshipman (*Porichthys notatus*; Chapter 4).

To date, evidence suggesting the involvement of protein carriers in trout urea excretion has been obtained through *in vivo* studies (McDonald and Wood, 1998; Chapter 5). In Chapter 5, acetamide and thiourea, two urea analogues, were used as tools to characterize urea transport, as the specificity of a transporter leads it to preferentially transport urea over substances that are similar to it. Amongst teleosts where branchial UT mechanisms have been cloned (tUT; gulf toadfish; Walsh et al. 2000 and mtUT; Lake Magadi tilapia: Walsh et al. 2001b) or are suspected (plainfin midshipman; Walsh et al.

2001a), a consistent pattern of urea and analogue handling is observed where acetamide clearance at the gills is 35-60% of urea clearance and thiourea clearance is only 16-19% (Chapter 2; Chapter 4; Walsh et al. 2001b). This highly conserved pattern of differential urea and analogue handling was also observed in the rainbow trout where the ratio of analogue/urea branchial clearance was 48% for acetamide and only 22% for thiourea, strongly suggesting the presence of a UT-type diffusion mechanism (Chapter 5).

Upon infusion with exogenous urea loads, the gill of the trout effectively cleared the plasma of excess urea, suggesting a role for a facilitated diffusion UT-type transport mechanism for urea (McDonald and Wood, 1998). Furthermore, saturation of this mechanism was suggested when branchial excretion rate could not keep up with infusion rate, but observations were complicated by the lethality of the elevated internal urea levels (McDonald and Wood, 1998). Recently, saturation of several urea transporters has been described in fish (Pilley and Wright, 2000; Fines et al. 2001), in contrast to their mammalian counterparts that prove difficult to saturate (Chou et al. 1990).

Investigation of the trout gill using an *in vitro* protocol is therefore desirable to further understand the mechanism(s) by which urea is excreted through this epithelium (see Appendix for an investigation of urea transport mechanisms using the *in vitro* urinary bladder of the trout and toadfish as potential models for the kidney). The purpose of the present study was to characterize any urea transport mechanism that might be present in the basolateral membrane of the trout gill through the use of the branchial basolateral membrane vesicle (BLMV) preparation developed by Perry and Flik (1988). Our results indicate the presence of a phloretin-sensitive, facilitated urea transport

mechanism in the basolateral membrane of the gill that is saturated at low, physiologically relevant urea concentrations (K_m = 1.17 mmol·l^{-1} and V_{max} = 0.42 μmol mg protein^{-1} h^{-1}).

Materials and Methods

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Farm in Mono Mills, Ontario. The fish were acclimated to 14°C and were fed with commercial trout pellets every second day. Acclimation was carried out in dechlorinated Hamilton tapwater [in mmol·l^{-1}: Ca^{++} = 1.8; Cl^- = 0.8; Na^+ = 0.6; Mg^{++} = 0.5; K^+ = 0.04; titration alkalinity (to pH 4.0) = 1.9; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0].

Preparation of Gill Basolateral Membrane Vesicles (BLMV)

BLMV were prepared using the method of Perry and Flik (1988) as modified by Bury et al. (1999). For each preparation, five to seven adult rainbow trout (250-400 g) were stunned by a blow to the head and killed by severance of the spine. The gills were then perfused with approximately 60 ml ice-cold perfusion saline (0.9% NaCl, 0.5mM ethylenediaminetetraacetic acid-disodium salt (Na_2-EDTA), 20 i.u.·ml^{-1} heparin; adjusted to pH 7.8 with Tris), quickly removed from the fish and placed in ice-cold perfusion saline without heparin. All subsequent procedures were performed at 1-4°C.

The gill epithelium without red blood cells was scraped from the cartilage of the filaments with a glass slide and a small aliquot from each fish was frozen at -80°C for

later analysis of enzyme activity. The remaining tissue (5-10 g wet weight) was combined and homogenized with a Dounce homogenizer (Kontes) with 30 strokes of a loose fitting pestle in 30 ml of a hypotonic solution (25 mM NaCl, 1 mM Hepes, 1mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF); adjusted to pH 8.0 with Tris). After homogenization, the volume was adjusted to a final value of 80 ml with the hypotonic solution, and then centrifuged at 550 g for 15 minutes to remove nuclei and cellular debris. Floating debris on the supernatant were removed and the supernatant was then decanted into a clean centrifuge tube and centrifuged at 50000 g for 30 minutes. The resulting pellet consisted of a top fluffy white layer (plasma membranes) and a firm brownish bottom (mitochondria). The white portion of the pellet was carefully resuspended in 5-10 ml of isotonic buffer (250 mM sucrose, 5 mM $MgSO_4$, 5 mM Hepes; adjusted to pH 7.4 with Tris) by gentle agitation. The resuspended pellet was adjusted to a volume of 20 ml and homogenized for 100 strokes using the tight fitting pestle of a Dounce homogenizer (Kontes). This second homogenate was centrifuged at 1000 g for 10 minutes and then at 10000 g for 10 minutes, producing a pellet containing the remainder of contaminating membranes. The supernatant was decanted into a clean centrifuge tube and centrifuged at 50000 g for 20 minutes to produce a final pellet of enriched basolateral membranes. A small sample of this final pellet was taken and stored at -80°C for subsequent enzyme analysis. The remainder of the pellet was resuspended in 1-3 ml of resuspension buffer (250 mM sucrose, 10 mM KNO_3, 0.8 mM $MgSO_4$, 0.5 mM Na_2-EDTA, 20 mM Hepes; adjusted to pH 7.4 with Tris) and was used immediately for protein concentration determination and urea transport assays. Typically, such a

procedure yields the BLMV preparation as 20% inside-out vesicles, 30% right side out vesicles and the remaining 50% of the vesicle membranes as unsealed (Perry and Flik, 1988).

Transport Measurements

Transport of [^{14}C]-urea was measured at trout acclimation temperature (14°C) by a rapid filtration technique described by van Heeswijk et al. (1984). Freshly prepared BLMV pellets were resuspended at a protein concentration of approximately 1 mg membrane protein·ml^{-1} in resuspension buffer and allowed to equilibrate for 30 minutes on ice. Thorough mixing and formation of vesicles was achieved by 10 passages through a 23 gauge needle both before and after the equilibration period. BLMV urea uptake was assessed by prewarming the 135 μl of assay media (250 mM sucrose, 10 mM KNO_3, 11 mM $MgSO_4$, 6 mM Na_2-EDTA, 20 mM Hepes, 1mM urea plus 5 μCi [^{14}C]-urea; adjusted to pH 7.4 with Tris) to 14°C, after which 35 μl of vesicle solution was added, the solution mixed on a vortex mixer and routinely incubated for 5 secs. Different incubation duration and temperatures were used in some tests, as outlined below. After incubation, 150 μl of the vesicle/assay mixture was immediately filtered by rapid filtration and the vesicles collected on nitrocellulose filters (Schleicher and Schüell Ltd.; 25 mm; 0.45 μm). The filters had been incubated in ice-cold stop solution with a high "cold" urea content (200 mM sucrose, 10 mM KNO_3, 10 mM $MgSO_4$, 20 mM Hepes, 50 mM urea; adjusted to pH 7.4 with Tris) to reduce the non-specific binding of the [^{14}C]-urea to the filter. The filtered membranes were washed twice with 1 ml aliquots of this ice-cold stop solution to

displace surface-bound [^{14}C]-urea then placed in individual glass scintillation vials. While 1 mmol·l^{-1} urea, typical of blood and tissue levels *in vivo* (McDonald and Wood, 1998) was used routinely in transport assays, other concentrations were used in some trials, as outlined below.

Urea Transport Assays

Time dependence of [^{14}C]-urea uptake was determined by incubating BLMVs in assay solution for 5, 8, 10 or 20 seconds. In order to verify that urea had been transported into the vesicular space, BLMVs were loaded with urea using the standard resuspension and assay conditions over a 30 second period, after which either 10 μl of 10% Triton-X in assay solution (to lyse the vesicles) or 10 μl of assay solution alone (control) was added to the vesicles. The concentration of radiolabeled urea present in the vesicles was measured at 3 and then 10 minutes after this point.

In order to establish concentration-dependence, urea uptake was measured over a range of urea concentrations (in mmol·l^{-1}: 0.09, 0.18, 0.51, 1.0, 2.0, 5.9, 11.7, 17.5). Part of the sucrose (BLMV) in the assay solution was reciprocally substituted with urea, keeping the osmolality of every solution the same and eliminating the effects of osmotic differences. Inhibitory effects of analogues on urea transport was examined by adding 5 mmol·l^{-1} of either thiourea, acetamide or N-methylurea to the assay solution in reciprocal substitution for sucrose (BLMV). To measure acetamide or thiourea uptake, 1 mmol·l^{-1} of respective analogue plus [^{14}C]-analogue replaced urea in the assay solution. Analogue uptake was then measured as described above. Non-competitive inhibition in BLMV was

investigated by resuspending the isolated membranes in a resuspension media with 0.25 $mmol \cdot l^{-1}$ of phloretin dissolved in ethanol so that the total ethanol concentration in the media did not exceed 0.04%. To measure urea uptake, phloretin-exposed vesicles were then incubated in assay media with the same concentration of phloretin. Control vesicles were resuspended and then incubated in 0.04% ethanol alone. In order to determine temperature sensitivity, urea uptake was measured in BLMVs incubated in assay solutions at temperatures of 1°C, 7°C, 14°C and 23°C.

Analytical Techniques and Calculations

The activity of Na^+K^+-ATPase, found only on the basolateral membrane of the gill, was used as an indicator of the relative enrichment of the final BLMV preparation. Na^+K^+-ATPase activity was measured as described by McCormick et al. (1993) in the initial gill homogenate and the final pellet of BLMV.

Urea concentration in the assay solution was measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges and correction for the presence of thiourea and acetamide in the competition and analogue uptake studies. This correction was done by adding the experimental concentration of analogue in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. Protein concentration of the BLMV was

determined by a commercial kit (Bradford's reagent; Sigma-Aldrich Canada) using bovine serum albumin standards. Non-specific binding of [^{14}C]-urea to the nitrocellulose filters was measured by filtering through 150 µl of assay solution only. For measurements of [^{14}C]-urea or [^{14}C]-analogue uptake by BLMV, the nitrocellulose filters in glass scintillation vials received 10 ml of ACS fluor (Amersham), and were analyzed by scintillation counting on an LKB Rackbeta 1217 Counter. Tests confirmed that quench was uniform, therefore no correction was necessary.

Uptake of [^{14}C]-labeled urea or analogue (U_X; µmol mg protein^{-1} h^{-1}) by BLMV's was determined by the equation:

$$U_X = \frac{cpm_f}{SA \times [P] \times t}, \qquad (1)$$

where cpm_f is the counts present on the nitrocellulose filters, SA is the specific activity of the media (cpm µmol $^{-1}$), [P] represents the concentration of protein in the BLMV filtered (mg ml^{-1}) and t is the time of incubation (hours).

The Q_{10} factor is defined as the ratio of two rates for a 10°C difference in temperature and was calculated as follows:

$$Q_{10} = \frac{J_{K2}^{(10/(K2-K1))}}{J_{K1}}, \qquad (2)$$

where J_{K1} and J_{K2} are the mean flux rates at a low and high temperature respectively in µmol mg protein^{-1} h^{-1} and K1 and K2 are the low and high temperatures respectively in °C. The Q_{10} factor for a physical process such as diffusion is about 1. Q_{10} factors for

biochemical reactions including carrier-mediated transport are typically 2 to 3 (Withers, 1992).

Statistical Analysis

Values are expressed as mean ± 1 S.E.M. where N = replications of basolateral membrane preparations combined from 5-7 fish. Statistical comparisons were made by Student's two-tailed, unpaired t-test as appropriate, with a significance levels of $P < 0.05$. Michaelis-Menten kinetic constants were derived from non-linear regression analysis and best fit curves were generated using the SigmaPlot 2000 computer package.

Results

Trout gill basolateral membrane vesicle preparations were enriched in Na^+K^+-ATPase activity by approximately 6-fold compared to the initial crude homogenate (Table 6-1). The standard incubation period for BLMV for use in most assays was determined by first investigating the time dependence of urea uptake (Fig.6-1). The uptake of urea (at 1 $mmol \cdot l^{-1}$ in the incubation media) into the vesicles at 14°C was fast and readily saturated by about 20 seconds (½ time to saturation = 3.7 sec). An incubation time of 5 sec was therefore selected for further assays as it was before the vesicles reached their maximum filling capacity. Urea appeared to be accumulating in the vesicular space of BLMV, rather than adsorbing to the outside, as exposure to the detergent Triton-X for either 3 minutes or 10 minutes essentially eliminated total urea uptake when compared to control vesicles kept for the same period of time (Fig.6-2).

Urea uptake by BLMV was measured over a range of urea concentrations in the incubation medium, revealing two components of urea uptake (Fig. 6-3). At high concentrations (up to 17.5 $mmol \cdot l^{-1}$) urea uptake was linearly dependent on the urea concentration (r^2 =0.99) with a slope of 0.073 $\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}/mmol \cdot l^{-1}$ ($P < 0.005$; Fig. 6-3A). However, at urea concentrations in the physiological range (0.1-2 $mmol \cdot l^{-1}$), urea uptake exhibited saturation kinetics (Fig.6-3B). The non-linear regression revealed that transport was significantly correlated with urea concentration (r = 0.99; $P < 0.001$) and yielded a Michaelis constant (K_m) of 1.17 ± 0.43 $mmol \cdot l^{-1}$ and a maximal velocity (V_{max}) of 0.42 ± 0.08 $\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$ consistent with values determined with an Eadie-Hofstee regression (K_m = 1.30 $mmol \cdot l^{-1}$ and V_{max} = 0.39 $\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$).

Urea uptake by BLMV at 1 $mmol \cdot l^{-1}$ urea was sensitive to the non-competitive urea transport blocker, phloretin: urea uptake in the ethanol controls (0.277 ± 0.043 (7) $\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$) was 2.7 times greater than the uptake by the vesicles in the presence of 0.25 mM phloretin plus ethanol (0.104 ± 0.022 (8) $\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$; Fig. 6-4).

The basolateral membrane of the gill differentiated between urea and acetamide but not between urea and thiourea. At a concentration of 1 $mmol \cdot l^{-1}$, the uptake of urea into the vesicles was approximately 3 times greater than the uptake of acetamide, while urea and thiourea uptake were not significantly different (Fig.6-5A). When expressed as an uptake ratio (analogue/urea), the ratios were 0.84 for thiourea and 0.32 for acetamide. Reflecting these results, thiourea inhibited urea uptake by BLMV: urea uptake under control conditions (1 $mmol \cdot l^{-1}$) was 3.7 times greater than uptake in the presence of 5

$mmol \cdot l^{-1}$ thiourea (Fig. 6-5B). However, urea uptake was not significantly reduced by the presence of 5 $mmol \cdot l^{-1}$ acetamide or 5 $mmol \cdot l^{-1}$ N-methylurea (Fig.6-5B) in the assay solution.

Urea uptake in the physiological range of concentration was substantially altered by changes in temperature. A decrease in temperature from 14°C (temperature of acclimation) down to 7°C caused a decrease in uptake with a Q_{10} of 4.81 (Fig.6-6). However, urea uptake was not inhibited further when temperature was reduced to 1°C. When temperature was increased to 23°C, uptake was significantly stimulated with a Q_{10} of 2.04 (Fig.6-6).

Discussion

In using the *in vitro* approach of the present study, it was possible to pursue the investigation of urea transport mechanisms in an individual membrane without the confounding effects of the whole animal. Rainbow trout BLMV preparations showed a significant 5.9-fold enrichment in Na^+K^+-ATPase activity compared to the initial crude homogenate, a value similar to that reported by both Perry and Flik (1988) and Bury et al. (1999) for the same trout gill preparation. As this preparation has 20% inside-out vesicles, 30% right-side out vesicles and 50% unsealed membranes (Hogstrand et al.1996; Perry and Flik, 1988; Bury et al. 1999), the facilitated diffusion urea transporter characterized in this study would be assayed in about 50% of the total protein, since facilitated diffusion transporters are typically bidirectional. In the present study, five

lines of evidence give support to the hypothesis of a facilitated diffusion transport mechanism for urea in the basolateral membrane of the trout gill.

The first indication of carrier-mediated urea transport was that urea uptake by isolated basolateral membranes of the trout gill demonstrated saturation kinetics. Urea transport by BLMV exhibited saturation kinetics at low urea concentrations (< 2 $mmol \cdot l^{-1}$) with a K_m (1.17 $mmol \cdot l^{-1}$) similar to physiological urea concentrations (1-2 $mmol \cdot l^{-1}$; McDonald and Wood, 1998) and a V_{max} of 0.39-0.42 $\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$. Since approximately 50% of the vesicles are unformed in the preparation (Hogstrand et al.1996; Perry and Flik, 1988; Bury et al. 1999) the V_{max} is underestimated by approximately the same amount. Up until now, only *in vivo* approaches have been used to investigate urea handling in the freshwater rainbow trout and it has not been possible to definitively show whether saturation kinetics are present or not (McDonald and Wood, 1998, Chapter 5). In retrospect however, both the saturable and non-saturable components of urea transport evident in BLMVs may be apparent in the data from trout infused with exogenous urea loads *in vivo* (McDonald and Wood, 1998). Upon exogenous urea loading at low rates, branchial urea excretion rate in trout matched infusion rate; the excess urea was effectively eliminated across the gill and plasma urea concentrations remained close to physiological levels (< 2 $mmol \cdot l^{-1}$; McDonald and Wood, 1998). Most likely, the effective clearance of urea across the gills at these low urea concentrations was achieved by the facilitated diffusion mechanism described by the present study. However, as the rates of exogenous urea loading increased, plasma urea concentrations rose to levels greater than physiological and the branchial excretion of urea fell to 75% and then 55%

of infusion rate, possibly due to the saturation of the urea transport mechanism, as observed in BLMVs. However, saturation of urea excretion *in vivo* was not observed, probably because the linear component was recruited by the high plasma urea levels. Furthermore, transport kinetics were perhaps complicated by the lethality of the elevated internal urea levels as 4 of 7 trout died when internal urea levels reached concentrations > 9 $mmol \cdot l^{-1}$ (McDonald and Wood, 1998). *In vivo*, the relationship between excretion rate and plasma urea concentration remained linear over the concentration range of 0.77 to 11.7 $mmol \cdot l^{-1}$ (slope = 19.59 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ per $mmol \cdot l^{-1}$, $P < 0.0001$, r = 0.83; previously unreported data, McDonald and Wood, 1998), corresponding to the linear component above 2 $mmol \cdot l^{-1}$ identified in the BLMV preparation (Fig. 6-3A).

A facilitated diffusion UT-type mechanism is also suspected in rainbow trout embryos in which the production and effective excretion of urea is believed to be an essential mechanism for ammonia detoxification during early life stages (Pilley and Wright, 2000). Like the trout gill, there appears to be two components of urea transport in the trout embryo, a saturable component at low concentrations, believed to be facilitated diffusion of urea, and a second, non-saturable component at concentrations of up to 20 $mmol \cdot l^{-1}$, which could be simple diffusion or movement through non-specific pathways (Pilley and Wright, 2000). Urea uptake by trout embryos displayed saturation kinetics with a K_m = 2.0 $mmol \cdot l^{-1}$ that was similar to internal urea concentrations (1.8 $mmol \cdot l^{-1}$) and a V_{max} = 10.5 $nmol \cdot g\ embryo^{-1} \cdot h^{-1}$. The V_{max} of embryonic trout is much lower than that measured in adult BLMV (4000-fold lower capacity assuming 60 mg $protein \cdot g\ embryo^{-1}$; Brauner and Wood, 2002 and correcting for the underestimated V_{max}

in BLMV), despite similar circulating levels of urea. However, both embryonic and adult mechanisms have similar affinities (K_m) which are close to circulating urea concentrations, indicating that the transporter has a relatively high affinity for urea.

The second line of evidence for facilitated diffusion of urea across the trout gill basolateral membrane was phloretin sensitivity. Phloretin, a potent inhibitor of UT-type facilitated diffusion transport mechanisms (Chou and Knepper, 1989), caused a significant inhibition of urea uptake by BLMV in the saturable, physiologically relevant range of urea concentration, suggesting the presence of this type of mechanism on the basolateral membrane of the trout gill. Phloretin sensitivity has also been observed in other preparations where UT-type mechanisms from teleost fish have been studied. When tUT of the gulf toadfish was injected into oocytes and exposed to 0.5 $mmol \cdot l^{-1}$ of phloretin, there was a 75% inhibition in urea transport (Walsh et al. 2000). Additionally, phloretin sensitivity was apparent in oocytes injected with mtUT of Lake Magadi tilapia, 1.0 $mmol \cdot l^{-1}$ of phloretin causing an approximate 70% reduction in urea uptake (Walsh et al. 2001b), and in the trout embryo, where 0.05 $mmol \cdot l^{-1}$ caused a 100% reduction in urea uptake (Pilley and Wright, 2000).

The third finding in support of carrier-mediated transport of urea was the capacity of BLMV to differentiate between urea and urea analogues with urea transport ≥ thiourea transport > acetamide transport. However *in vivo*, the branchial clearance of urea and analogues in the rainbow trout exhibits a pattern where urea transport > acetamide transport > thiourea transport, in both the excretion and the uptake direction (Chapter 5). This *in vivo* pattern of urea *versus* analogue handling is observed not only in the rainbow

trout but also by the gill of the plainfin midshipman (Chapter 4), by tUT of the gulf toadfish (Chapter 2) and by mtUT of the lake Magadi tilapia (Walsh et al. 2001b). While the BLMV pattern of handling is representative of the basolateral membrane alone, the *in vivo* pattern of analogue handling is a combination of both apical and basolateral membranes. Hypothetically, the apical membrane could then be responsible for the differential handling of thiourea and urea observed *in vivo*, while handling urea and acetamide identically, suggesting a second urea transport mechanism on the apical membrane of the gill.

Supporting the trend observed in the uptake of urea, acetamide and thiourea is the fourth piece of evidence for facilitated urea diffusion: inhibition of urea transport by urea analogues, a well-described characteristic of UT-type mechanisms (Chou and Knepper, 1989; Gillan and Sands, 1993; Wood et al. 1998; Walsh et al. 2000). The presence of thiourea in concentrations 5-times greater than urea significantly inhibited urea uptake by BLMV. Together with the finding that thiourea and urea uptake rate by BLMV are similar, the inhibitory effect of thiourea on urea uptake suggests that thiourea and urea are moving *via* the same mechanism and are interacting with each other. In contrast, neither acetamide nor N-methylurea inhibited urea uptake when present in 5-fold greater concentrations. The differential handling between urea and acetamide (only 30% of urea transport at the same 1 $mmol \cdot l^{-1}$ concentration) could therefore be a consequence of urea moving through a specific transport mechanism while acetamide is moving by simple diffusion, thus not interfering with urea movement. Thiourea has also proven to be a potent inhibitor of urea transport *via* the toadfish gill (which contains tUT), causing a

73% reduction in the urea uptake *in vivo* when present in 2-fold excess in the external media of the gulf toadfish (Wood et al. 1998). Similarly, urea influx in the Lake Magadi tilapia gill (which contains mtUT) was inhibited by approximately 40% in the presence of thiourea, by only 30% in the presence of N-methylurea and by 15% in the presence of acetamide, all in 3-fold excess of urea (Walsh et al. 2001b).

The fifth and final line of evidence for carrier-mediated transport of urea across the trout gill basolateral membrane was temperature sensitivity. A change in incubation temperature, either above or below the temperature of acclimation, caused an alteration in urea uptake rate by BLMV with a Q_{10} factor > 2, indicative of the involvement of a carrier protein. While the increased uptake rate measured at 23°C could possibly be due to a lipid phase transition, the rate change measured at more physiological temperatures (from 14°C to 7°C: Q_{10} factor > 4) supports the presence of a urea transporter in the basolateral membrane. Urea transport by BLMV was insensitive to a further decrease in temperature from 7°C to 1°C but conceivably, at these low temperatures, carrier-mediated transport of urea may have slowed to a point where urea movement across the membrane occurs solely by simple diffusion. Temperature sensitivity of urea efflux has also been measured in toadfish hepatocytes, shown to have phloretin-sensitive urea efflux (Walsh and Wood, 1996).

Thus, the present study has demonstrated that urea uptake by BLMV (1) displays saturation kinetics at physiological urea concentrations, (2) is phloretin sensitive, (3) is greater than acetamide uptake, (4) is inhibited by thiourea and (5) demonstrates temperature sensitivity typical of carrier-mediated transport. Taken together, these

findings strongly suggest the involvement of a facilitated diffusion transport mechanism for urea across the basolateral membrane of the trout gill, similar to those identified in the toadfish (tUT) and the tilapia (mtUT) and those suspected in the trout embryo and midshipman.

Table 6-1: Na^+K^+-ATPase activity in the crude homogenate, in the isolated basolateral membrane (BLM) and the calculated magnitude of enrichment. Values are means ± 1 S.E.M. (6); * $P < 0.05$ significantly different than crude homogenate.

	Na^+K^+-ATPase Activity ($\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$)
Crude Homogenate	16.9 ± 1.5
Isolated BLM	99.7 ± 18.5*
Enrichment Factor	5.9

Figure 6-1: Time course of [^{14}C]-urea uptake by basolateral membrane vesicles (BLMV) of rainbow trout gill. Each data point is a mean ± 1 S.E.M. (N = 3 replications) of basolateral membrane preparations combined from 5-7 fish.

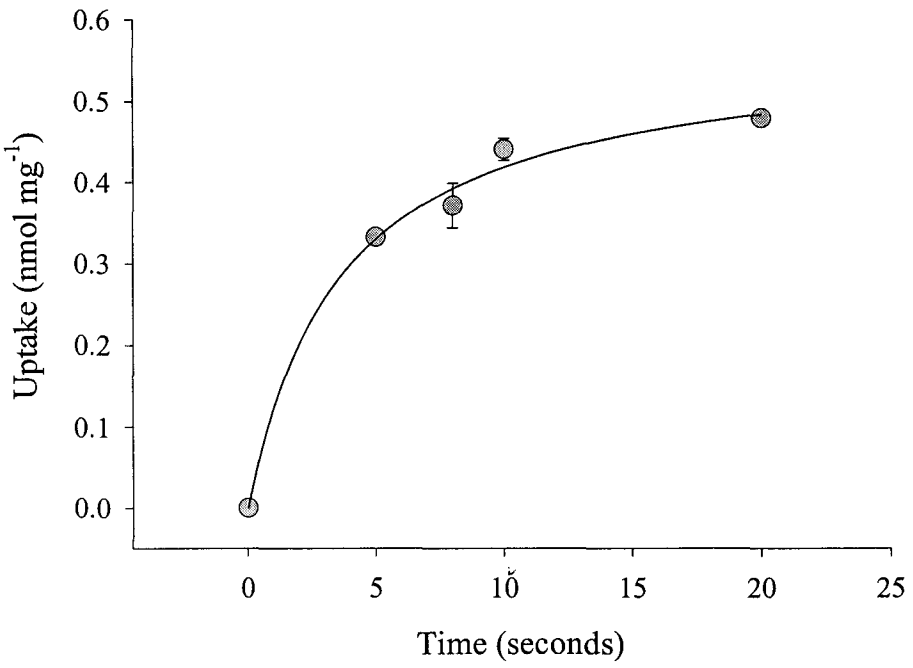

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Uptake (nmol mg-1)
0
5
10
15
20
25
Time (seconds)

Figure 6-2: [^{14}C]-urea uptake by trout gill BLMV after 30 seconds, 3 minutes and 10 minutes under control conditions and when incubated in Triton-X after the initial 30 second sample. Each data point is a mean ± 1 S.E.M. (N = 3 replications) of basolateral membrane preparations combined from 5-7 fish; * $P < 0.05$ significantly different from the corresponding control value.

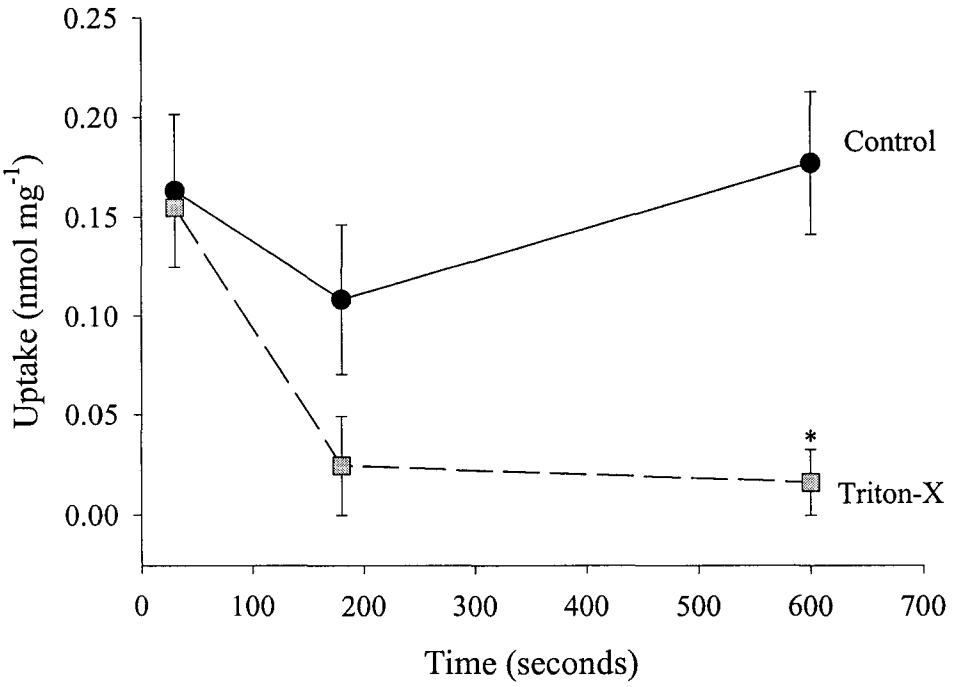

0.25
0.20
0.15
0.10
0.05
0.00
Uptake (nmol mg-1)
Control
Triton-X
*
0
100
200
300
400
500
600
700
Time (seconds)

Figure 6-3: (A) The rate of [^{14}C]-urea uptake by trout gill BLMV demonstrating two components of urea uptake. A saturable component is evident at concentrations of up to 2 mmol·l^{-1}, whereas beyond this concentration [^{14}C]-urea uptake becomes linear. (B) The Michealis-Menton fit of the saturable component gives a K_m of 1.17 mmol·l^{-1}and a V_{max} of 0.42 μmol·mg protein^{-1}·h^{-1}; r = 0.99, $P < 0.01$. The equation for the linear component of uptake corrected for the saturable component, is y = 0.073x – 0.042 r = 0.996, $P <$ 0.005. Each data point is a mean ± 1 S.E.M. (N = 6 replications) of basolateral membrane preparations combined from 5-7 fish.

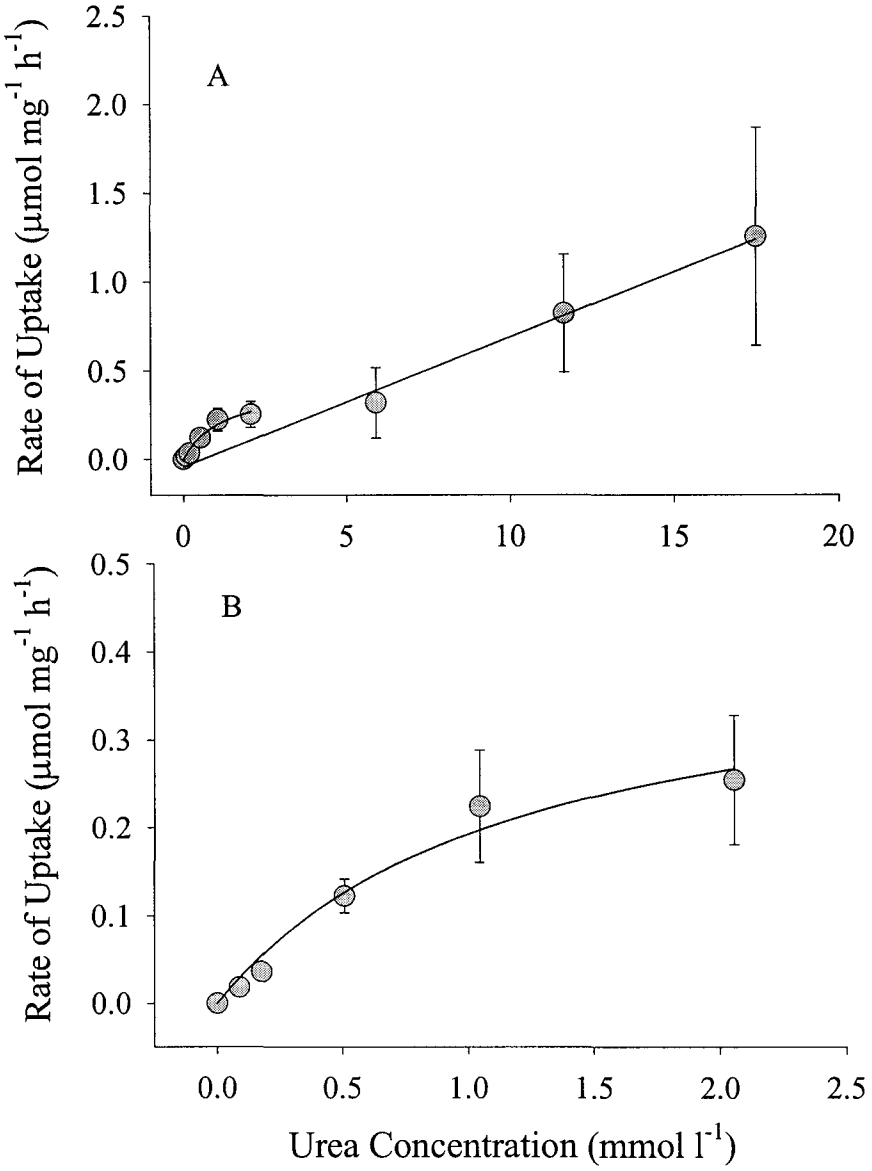

A
B
Rate of Uptake (µmol mg-1 h-1)
Rate of Uptake (µmol mg-1 h-1)
Urea Concentration (mmol l-1)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.5
1.0
1.5
2.0
2.5

Figure 6-4: The rate of [^{14}C]-urea uptake by BLMV incubated at 1 mmol·l^{-1} in 0.25 mmol·l^{-1} phloretin, a urea transport blocker, was significantly less than vehicle controls. Vesicles were pre-incubated for one hour in standard incubation media with 0.04% ethanol ± 0.250 mmol·l^{-1}phloretin and added to a standard assay media with 0.04% ethanol ± 0.250 mmol·l^{-1}phloretin. Each data point is a mean ± 1 S.E.M. (N = replications) of basolateral membrane preparations combined from 5-7 fish; * $P < 0.05$ significantly different from controls.

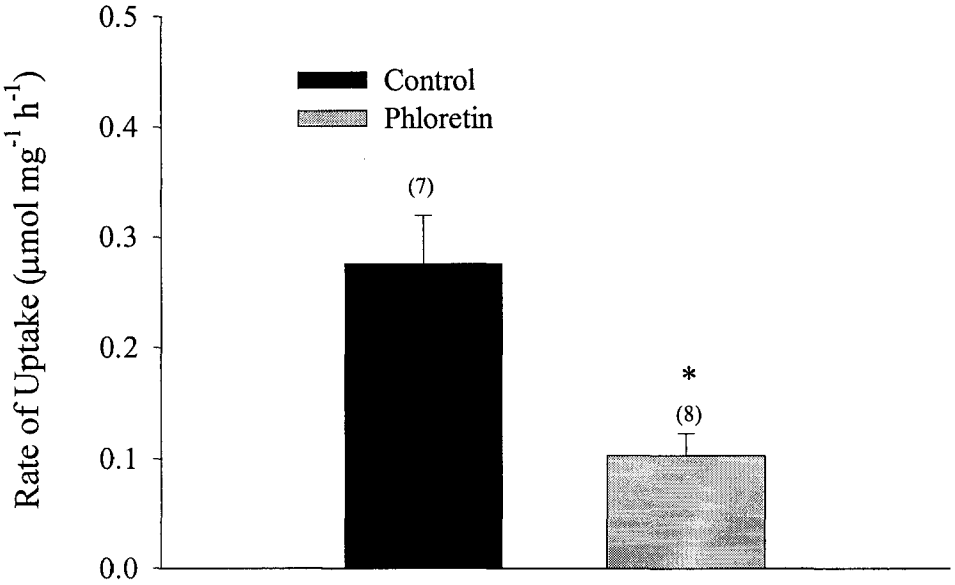
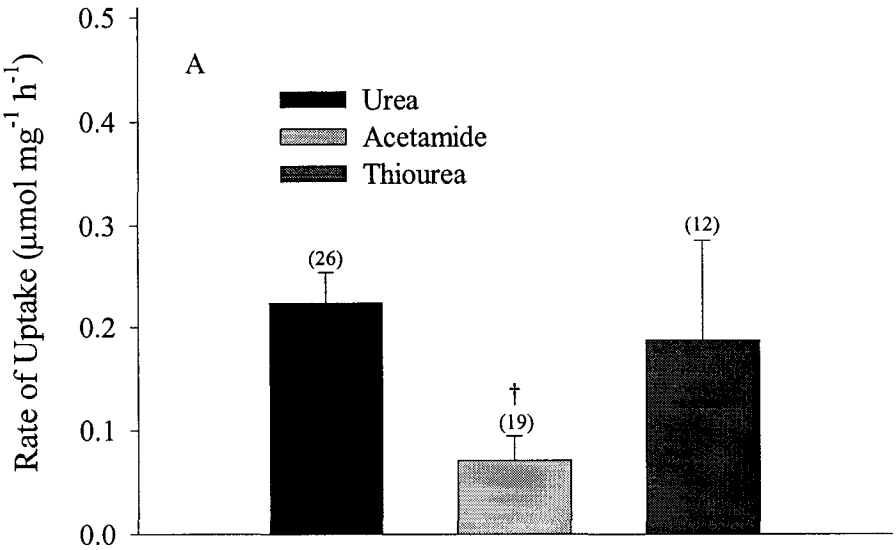

Rate of Uptake (µmol mg-1 h-1)
0.5
0.4
0.3
0.2
0.1
0.0
Control
Phloretin
(7)
*
(8)

Figure 6-5: (A) Mean uptake rates of [^{14}C]-urea, [^{14}C]-acetamide and [^{14}C]-thiourea at 1 mmol·l^{-1} concentration in the incubation media demonstrating an uptake of [^{14}C]-urea that was significantly greater than [^{14}C]-acetamide uptake. However, the uptake rates of [^{14}C]-thiourea and [^{14}C]-urea were not significantly different. The similar handling of thiourea and urea is reflected in (B) the inhibition by 5 mmol·l^{-1} thiourea on [^{14}C]-urea uptake at 1 mmol·l^{-1}. The presence of acetamide and N-methylurea in the assay solution had no significant effect. Each data point is a mean ± 1 S.E.M. (N = replications) of basolateral membrane preparations combined from 5-7 fish; † $P < 0.05$ significantly different from urea uptake rate; * $P < 0.05$ significantly different from the corresponding control value.


A
Urea
Acetamide
Thiourea
Rate of Uptake (µmol mg-1 h-1)
0.5
0.4
0.3
0.2
0.1
0.0
(26)
†
(19)
(12)

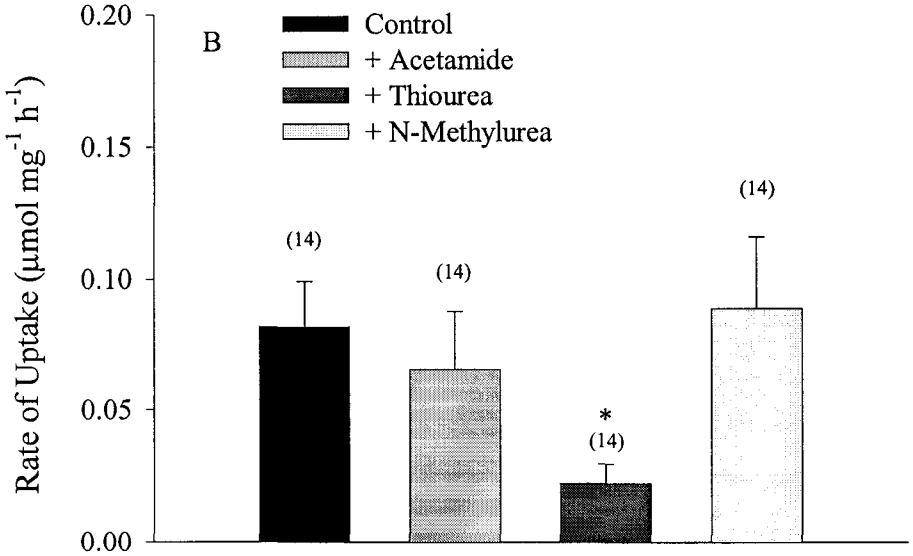
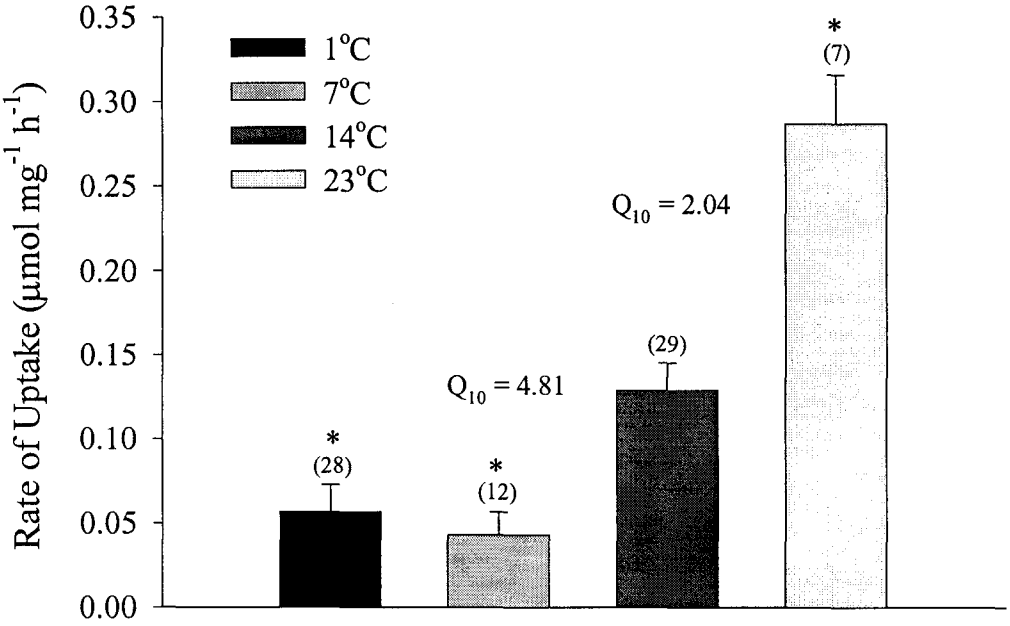

B
Control
+ Acetamide
+ Thiourea
+ N-Methylurea
Rate of Uptake (µmol mg-1 h-1)
0.20
0.15
0.10
0.05
0.00
(14)
(14)
*
(14)
(14)

Figure 6-6: Mean uptake rates of [^{14}C]-urea by BLMV incubated at temperatures above or below the temperature of acclimation (14°C). An increase in temperature causes a significant increase in uptake by a Q_{10} of 2.04. A decrease in temperature to 7°C causes a significant decrease in urea uptake by a Q_{10} of 4.81 with no further decrease observed at 1°C. Each data point is a mean ± 1 S.E.M. (N = replications) of basolateral membrane preparations combined from 5-7 fish; * $P < 0.05$ significantly different from the temperature of acclimation.


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Rate of Uptake (µmol mg-1 h-1)
1°C
7°C
14°C
23°C
*
(28)
*
(12)
Q10 = 4.81
(29)
Q10 = 2.04
*
(7)

Chapter 7

The effect of chronic cortisol elevation on urea metabolism and excretion in the rainbow trout (*Oncorhynchus mykiss*)

Abstract

The objective of this study was to investigate the possible involvement of cortisol in urea metabolism and excretion in the ammoniotelic rainbow trout (*Oncorhynchus mykiss*). Trout fitted with dorsal aortic and internal urinary catheters received either no implant (Control), or were implanted with coconut oil (Sham), RU486 in coconut oil, cortisol in coconut oil or cortisol + RU486 in coconut oil, and monitored over 72 hours. Rainbow trout implanted with cortisol alone or cortisol + RU486 had similarly elevated plasma cortisol concentrations (663.2 ± 98.9 (7) $ng \cdot ml^{-1}$ and 646.2 ± 63.6 (5) $ng \cdot ml^{-1}$, respectively) when compared to control (132.5 ± 36.8 (8) $ng \cdot ml^{-1}$) and sham (107.0 ± 26.3 (8) $ng \cdot ml^{-1}$) fish. Fish implanted with RU486 alone had cortisol levels (217.9 ± 33.6 (8) $ng \cdot ml^{-1}$) that were significantly elevated relative to sham fish. Fish implanted with cortisol alone had 3-fold greater plasma (2.09 ± 0.33 (8) $mmol \cdot l^{-1}$) and urine (4.21 ± 0.39 (8) $mmol \cdot l^{-1}$) urea concentrations relative to fish of all other groups (0.72 ± 0.04 (29) $mmol \cdot l^{-1}$ and 1.40 ± 0.10 (29) $mmol \cdot l^{-1}$, respectively), including fish with similarly high cortisol levels but implanted with RU486. In cortisol-implanted fish, a positive correlation (r = 0.72) was observed between plasma cortisol and plasma urea

concentration (slope = 0.002 $mmol \cdot l^{-1}$ per $ng \cdot ml^{-1}$; $P < 0.001$) that was abolished in fish implanted with cortisol + RU486. Cortisol-treated fish had significantly elevated branchial (two-fold higher) and urinary (three-fold higher) excretion rates of urea but not ammonia compared to sham-implanted fish. However, as branchial and renal urea clearance were unaffected, there appears to be no stimulation or inhibition of urea excretion mechanisms in the gill or kidney separate from effects due to changes in plasma urea concentrations. Thus in trout, cortisol and glucocorticoid receptors appear to be involved in the regulation of endogenous urea production and therefore plasma concentrations, but not in the control of urea excretory mechanisms.

Introduction

Cortisol is the principal corticosteroid in teleost fish and circulating cortisol levels rise dramatically upon exposure to a stressor. However, the physiological consequences of cortisol elevation are subject to extensive debate (reviewed by Mommsen et al. 1999). While metabolic reactions to highly elevated cortisol levels are variable amongst teleosts, several studies postulate that endogenous cortisol elevation in fish enhances hepatic gluconeogenesis from amino acids (Freeman and Idler, 1973; Whiting and Wiggs, 1977; Chan and Woo, 1978; Leach and Taylor, 1982; Davis et al. 1985; Vijayan et al. 1996, 1997; Milligan, 1997). Consequently, with a rise in endogenous cortisol comes an elevation in circulating glucose and an increase in ammonia production and excretion (Chan and Woo, 1978).

Recent evidence indicates that cortisol may also contribute to the regulation of urea production in fish (Vijayan et al. 1996; Hopkins et al. 1995, reviewed by Mommsen et al. 1999). Cortisol has been shown to stimulate hepatic glutamine synthetase in the facultatively ureotelic gulf toadfish, *Opsanus beta* (Hopkins et al. 1995). Glutamine synthetase is the feeder enzyme for ammonia nitrogen entry into the ornithine-urea cycle in toadfish hepatocytes *in vitro* and is believed to be a contributing factor in the switch from ammoniotelism to ureotelism in the marine toadfish (Hopkins et al. 1995). Hepatic glutamine synthetase (GS) induction by corticosteroids has also been observed in the predominantly ammoniotelic sea raven (*Hemitripterus americanus*) when exposed to cortisol *in vivo* (Vijayan et al. 1996), although the sea raven does not have all the other OUC enzymes. In addition to GS, hepatic arginase and allantoicase, two enzymes that

contribute to urea production in most teleost fish *via* the direct breakdown of dietary arginine or through uricolysis respectively, are also stimulated by cortisol in the sea raven. The stimulation of these mechanisms of urea production in cortisol-implanted sea raven led to a 3-fold increase in plasma urea concentrations compared to sham fish (Vijayan et al. 1996). There was no effect of cortisol on plasma ammonia concentrations.

While elevated cortisol levels appear to have a stimulatory effect on amino acid metabolism, which consequently elevates plasma nitrogen concentrations and nitrogen excretion, glucocorticoids also have a paradoxical inhibitory effect on vasopressin-sensitive UT-type facilitated diffusion urea transport mechanisms (Knepper et al. 1975; Naruse et al. 1997; Klein et al. 1997; Wood et al. 1997, 2001; Peng et al. 2002). In the mammalian kidney, glucocorticoids cause a decline in facilitated urea reabsorption in rat terminal IMCD (Knepper et al. 1975; Naruse et al. 1997; Peng et al. 2002). New evidence suggests that glucocorticoids regulate urea transport by significantly inhibiting the activity of a promoter region, resulting in a decrease mRNA expression (Peng et al. 2002). In teleost fish, cortisol appears to inhibit the activation or insertion of tUT, the facilitated diffusion urea transporter found in the gills of the gulf toadfish (Wood et al. 1997, 2001). Sharp, periodic declines in plasma cortisol concentrations lead to the pulsatile activation of this excretory mechanism (Wood et al. 1997, 2001) and the pulsatile activation of tUT can be greatly reduced if the periodic reduction in plasma cortisol concentrations is prohibited by exogenous cortisol loading, a response that appears to be mediated through glucocorticoid-type receptors (MD McDonald, CM Wood, M Grosell and PJ Walsh, unpublished results).

With respect to urea transport mechanisms in freshwater teleosts, work by McDonald and Wood (1998) and Chapter 5, 6 has suggested the presence of two different urea transport mechanisms in the ammoniotelic rainbow trout (*Oncorhynchus mykiss*): a branchial facilitated diffusion mechanism and a renal active transport mechanism, the latter occurring against a concentration gradient in either the reabsorptive or secretory direction. These observations suggest that endogenous urea in the trout may be under homeostatic regulation and that trout may elect to retain or eliminate urea depending on the circumstances. In this respect, cortisol could potentially play an important role.

The hypothesis of this study was that stress-induced elevation of cortisol may have a regulatory effect on either urea metabolism, urea excretory mechanisms, or both in the freshwater rainbow trout. Thus, the objectives of this study were to characterize urea production as well as branchial and renal handling of urea in the presence and absence of cortisol. The role of glucocorticoid receptors was investigated using the glucocorticoid receptor blocker, RU486. Possible effects on ammonia metabolism and excretion were examined simultaneously. Our results indicate that cortisol does have a specific effect on urea metabolism in the trout, but does not appear to be involved in the regulation of urea transport mechanisms.

Materials and Methods

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Farm in Mono Mills, Ontario. The fish were acclimated to seasonal water temperatures

(11°C - 14°C) and were fed with commercial trout pellets every second day until the time of surgery. Acclimation was carried out in dechlorinated Hamilton tapwater [in $mmol \cdot l^{-1}$: Ca^{++} = 1.8; Cl^{-} = 0.8; Na^{+} = 0.6; Mg^{++} = 0.5; K^{+} = 0.04; titration alkalinity (to pH 4.0) = 1.9; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0].

Experimental Protocol

Dorsal aortic, urinary catheterizations and coconut oil implantations were performed simultaneously on fish anaesthetized with MS-222 (0.07 $g \cdot l^{-1}$; Sigma-Aldrich Canada) and artificially ventilated on an operating table. Dorsal aortic catheters (Clay-Adams PE50 tubing) were implanted as described by Soivio et al. (1972) and were filled with Cortland saline (Wolf, 1963) containing 50 i.u. per ml lithium heparin (Sigma-Aldrich Canada). The internal urinary catheterization technique described by Curtis and Wood (1991) and Wood and Patrick (1994) was used, in which the catheter (heat-molded Clay Adams PE60 tubing) is placed in the urinary bladder so as to drain urine continuously as it is produced from the ureters. Any reabsorptive/secretory role of the bladder is negated so as to allow examination of the function of the kidney alone.

After dorsal aortic and urinary catheterizations, trout were either left to recover (Control fish; 0.336 ± 0.020 (8) kg ranging from 0.250 to 0.414 kg) or were then implanted with the following as described by Vijayan et al. (1994) and Gregory and Wood (1998): Sham fish (0.335 ± 0.035 (8) kg ranging from 0.225 to 0.494 kg) were implanted with 10 $ml \cdot kg$ body $weight^{-1}$ coconut oil, RU486-implanted fish (0.325 ± 0.015 (8) kg ranging from 0.267 to 0.375 kg) were implanted with RU486 (mifepristone; 11β -

[4-Dimethylamino] phenyl-17β-hydroxy-17 [1-propynyl] estra-4,9-dien-3-one) in coconut oil at a dose of 10 $mg \cdot ml^{-1} \cdot 100\ g^{-1}$, cortisol-implanted fish (0.275 ± 0.007 (8) kg ranging from 0.240 to 0.298 kg) were implanted with cortisol (11β, 17α, 21-Trihydroxy-pregn-4-ene-3,20-dione; Sigma-Aldrich Canada) in coconut oil at a dose of 15 $mg \cdot ml^{-1} \cdot 100\ g^{-1}$) and cortisol + RU486-implanted fish (0.203 ± 0.032 (5) kg ranging from 0.153 to 0.329 kg) were implanted with both cortisol and RU486 in coconut oil at a dose of 15 mg cortisol and 150 mg RU486 $ml^{-1} \cdot 100\ g^{-1}$. Implantation of cortisol using coconut oil as a vehicle has been shown to produce a slow release of cortisol into the circulation (Vijayan and Leatherland, 1989). After injection with the warmed coconut oil, an ice pack was placed against the site of injection to facilitate the solidification of the implant.

During recovery and experimentation, urine was collected continuously with the catheter emptying into a vial approximately 3 cm below the water level of the box. Following surgery, fish were kept in darkened individual containers (minimum volume of 4 L) that were continually aerated and supplied with flowing freshwater (200 $ml \cdot min^{-1}$).

Following the procedure outlined by McDonald and Wood (1998), [^{3}H]-polyethylene glycol (PEG, Mr4000) was used as a marker for glomerular filtration rate (GFR). PEG 4000 was chosen because it is considered a more accurate and conservative indicator of GFR in fish than other commonly used markers (Beyenbach and Kirschner, 1976; Erickson and Gingrich, 1986; Curtis and Wood, 1991). Prior to injection of PEG, the fish were allowed to recover from surgery for at least 12 hours, a period during which the patency of the arterial and urinary bladder catheters was confirmed. In fish where both catheters were deemed successful, a dose of 5 $\mu Ci \cdot 100g$ body $weight^{-1}$ of [^{3}H]-PEG 4000

(New England Nuclear) in 0.25 ml·100 g body weight^{-1} was injected *via* the dorsal aortic catheter followed by an additional 0.3 ml of saline. The [^{3}H]-PEG 4000 was then allowed to equilibrate throughout the extracellular space for 24h before sampling commenced. Following the [^{3}H]-PEG 4000 injection and 24 h equilibration period, a blood sample (200 μl with saline plus red blood cell replacement) was taken, a fresh urine collection was started, water flow to the fish box was stopped and the water level was set to an exact volume mark of 4L. An initial water sample was taken for measurement of urea and ammonia concentration. Thereafter, water, urine and blood samples were taken every 12 hours and the fish box was flushed thoroughly over a 15 minute period at 12 hour intervals. Vigorous aeration maintained PO_2 close to air saturation during times when water flow to the box was stopped. Sampling continued for the remainder of the experiment (72 hours) to make a total of 12 water samples, 6 blood samples and 6 urine samples. Blood samples were immediately centrifuged (10, 000g for 2 min.). Plasma and urine were stored at -20°C for later analysis of urea and [^{3}H]-PEG 4000 concentrations in both fluids and ammonia, cortisol, and glucose in plasma only. Water samples were analyzed for urea and ammonia.

Analytical Techniques and Calculations

Urea concentrations in blood plasma, urine, and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in the three media. Ammonia concentrations in the water were measured by the method of Ivancic

and Degobbis (1984) and in the plasma by a Sigma Diagnostics ammonia (L-glutamate dehydrogenase) kit. Plasma glucose concentrations were measured with a Sigma Diagnostics Infinity glucose (hexokinase) kit. For measurements of [^{3}H]-PEG 4000, blood and urine samples (25 µl) were added to 4 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 Counter. Tests demonstrated that quench was constant, thus no correction was necessary.

The branchial excretion rate (B; µmol-N·kg^{-1}·h^{-1}) of any substance (*X*) was calculated by dividing the concentration of the substance appearing in the water $[X]_w$ by fish body weight (wt) and time (*t*):

$$B_X = \frac{[X]_w \times V_f}{wt \times t}, \qquad (1)$$

where V_f is the volume of water surrounding the fish.

The branchial clearance rate (C_B; ml·kg^{-1}·h^{-1}) of any substance (*X*) was then calculated by dividing its branchial excretion rate by plasma concentration $[X]_p$:

$$C_{BX} = \frac{B_X}{[X]_p}. \qquad (2)$$

All the following renal rates were related to fish body weight by expressing urinary flow rate (UFR) in ml·kg^{-1}·h^{-1}. Urinary excretion rates (U; µmol-N·kg^{-1}·h^{-1}) of urea was calculated as:

$$U_{urea} = [urea]_u \times UFR, \qquad (3)$$

using measured values of urine flow rates (UFR) and urine concentrations $[urea]_u$.

Glomerular filtration rates (GFR) were calculated as the clearance of [^{3}H]-PEG 4000 - *i.e.,* the excretion of radioactivity in the urine (cpm_u) relative to its concentration in the blood plasma (cpm_p):

$$GFR = \frac{cpm_u \times UFR}{cpm_p} . \quad (4)$$

The urinary clearance (Cu; $ml \cdot kg^{-1} \cdot h^{-1}$) of urea was calculated by dividing the urinary excretion rate of urea (Equation 3) by the concentration of urea in the plasma:

$$Cu = \frac{U_{urea}}{[urea]_p} . \quad (5)$$

The concept of clearance ratio (CR; see Wood, 1995) relates the clearance of urea to the GFR (*i.e.* to the clearance of [^{3}H] PEG 4000) and to the concentration of urea in the plasma:

$$CR = \frac{[urea]_u \times UFR}{[urea]_p \times GFR} . \quad (6)$$

Assuming that a substance X is filtered at the glomeruli with the same efficiency as [^{3}H]-PEG 4000, then the CR_X provides quantitative information on the tubular handling of X. If the clearance ratio is greater than 1, then there is net secretion of X, if less than 1 then net reabsorption has occurred. For example CRx = 0.05 would indicate 95% net reabsorption of the filtered load of X.

Statistics

Data were reported as means ± 1 S.E.M (N = number of fish). Regression lines were fitted by the method of least squares, and the significance ($P < 0.05$) of the slope assessed. The significance of differences between means was evaluated using Student's paired or unpaired, two-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977). An ANOVA with time as the main factor was followed by a comparison of individual means using the Bonferroni correction for multiple sample comparisons.

Results

When averaged over the entire period, rainbow trout with sham implants had plasma cortisol concentrations (107.0 ± 26.3 (8) $ng \cdot ml^{-1}$) that were not significantly different than control fish (132.5 ± 36.8 (8) $ng \cdot ml^{-1}$) and relatively constant over the 72 h experimental period (Fig.7-1). Rainbow trout implanted with either cortisol or cortisol + RU486 had similarly elevated plasma cortisol concentrations (663.2 ± 98.9 (7) $ng \cdot ml^{-1}$ and 646.2 ± 63.6 (5) $ng \cdot ml^{-1}$ respectively) when compared to control and sham groups and were not significantly different from eachother except at 12 h (Fig.7-1). On average, fish implanted with RU486 alone had cortisol levels (217.9 ± 33.6 (8) $ng \cdot ml^{-1}$) that were significantly greater than in sham fish (Fig.7-1). However, when looking at individual time points it is evident that endogenous cortisol levels in RU486 implanted fish exhibited a transient elevation and were significantly greater than shams only for the first 36 h of the experiment, and then returned to control levels by 48 h.

There were no significant differences in plasma glucose concentration among the different treatments, though cortisol-treated fish tended to have higher values (Table 7-1). Fish implanted with cortisol alone had plasma (2.09 ± 0.33 (8) $mmol \cdot l^{-1}$) and urine (4.21 ± 0.39 (8) $mmol \cdot l^{-1}$) urea concentrations that were approximately 3-fold greater than urea concentrations in fish of other groups (plasma = 0.72 ± 0.04 (29) $mmol \cdot l^{-1}$ and urine = 1.40 ± 0.10 (29) $mmol \cdot l^{-1}$; Fig.7-2). The response was blocked by RU486. Thus, fish implanted with cortisol + RU486 had plasma (0.71 ± 0.05 (5) $mmol \cdot l^{-1}$) and urine (1.30 ± 0.14 (5) $mmol \cdot l^{-1}$) urea concentrations that were significantly lower than fish implanted with cortisol alone and not significantly different from sham or control fish (Fig.7-2).

While there was no significant relationship between plasma urea and plasma cortisol concentration in control (Fig.7-3A), sham (Fig.7-3B) and RU486-implanted fish (Fig.7-3C), a positive correlation (r = 0.72) was observed in fish implanted with cortisol alone (slope = 0.0019 $mmol \cdot l^{-1}$ per $ng \cdot ml^{-1}$; $P < 0.001$; Fig.7-3D). This relationship, however, was abolished when fish were implanted with RU486 in addition to cortisol (r = 0.42, slope not significantly different than zero; Fig.7-3E).

The branchial excretion of urea from fish of each group was relatively constant throughout the 72-h experimental period (Fig. 7-4A). When averaged over the entire period, sham-implanted fish had branchial excretion rates (47.3 ± 6.4 (8) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$) that were not significantly different from control fish (39.2 ± 6.3 (8) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$; Fig.7-4A). Fish implanted with cortisol alone had an approximately two-fold elevation in branchial urea excretion rate (81.8 ± 7.0 (8) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$) when compared to shams, controls and fish implanted with cortisol + RU486 (38.9 ± 5.0 (5) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$); fish

implanted with cortisol + RU486 were not significantly different than sham implanted fish. In contrast, fish implanted with RU486 alone had significantly lower branchial urea excretion rates (28.6 ± 3.5 (8) μmol-N·kg^{-1}·h^{-1}) when compared to sham fish (47.3 ± 6.4 (8) μmol-N·kg^{-1}·h^{-1}) over the last 24 hours of the experiment.

Notably, the calculated branchial clearance rates of urea were the same across all groups (Fig.7-4B). Therefore the differences observed in branchial urea excretion rates were accounted for by differences in plasma urea concentration.

Similar to branchial urea excretion, the branchial excretion of ammonia from fish of most groups was fairly constant throughout the 72-h experimental period (Fig. 7-5A). However, in contrast to urea excretion, branchial ammonia excretion rates in fish from all five groups were not significantly different from each other, except for fish implanted with cortisol alone that were significantly different from sham fish at 12 h. However, the overall means in these two treatments were not significantly different (374.2 ± 40.3 (8) μmol-N·kg^{-1}·h^{-1} *versus* 340.3 ± 60.9 (8) μmol-N·kg^{-1}·h^{-1} in cortisol and sham fish, respectively). Fish implanted with cortisol + RU486 had branchial ammonia excretion rates (237.8 ± 18.0 (5) μmol-N·kg^{-1}·h^{-1}) that were similar to cortisol-implanted, sham fish, control fish (244.6 ± 36.6 (8) μmol-N·kg^{-1}·h^{-1}) and fish treated with RU486 alone (245.4 ± 15.4 (8) μmol-N·kg^{-1}·h^{-1}; Fig.7-5A). Due to a technical mishap, plasma ammonia data was not recorded from control or fish treated with RU486 alone but cortisol-implanted fish had plasma ammonia concentrations (179.7 ± 23.9 (7) μmol·l^{-1}) that were not significantly different from those of sham-implanted fish (134.9 ± 19.1 (6) μmol·l^{-1}; Fig.7-5B) over the 72 h experimental period. However, fish implanted with

cortisol + RU486 had circulating ammonia levels (79.4 ± 6.7 (5) $\mu mol \cdot l^{-1}$) that were significantly less than those in fish implanted with cortisol alone. Despite this, the branchial clearance rates of ammonia in sham (2425 ± 653 (7) $ml \cdot kg^{-1} \cdot h^{-1}$), cortisol-implanted (2434 ± 410 (6) $ml \cdot kg^{-1} \cdot h^{-1}$) and cortisol + RU486 implanted fish (2998 ± 230 (5) $ml \cdot kg^{-1} \cdot h^{-1}$) were not significantly different from one another (Fig.7-5C), though almost two orders of magnitude greater than the branchial clearance rates of urea (*cf.* Fig. 7-4B).

With respect to the kidney, glomerular filtration rate (GFR) and urine flow rate (UFR) were relatively constant over the 72 h experimental period. Sham-implanted fish had a GFR (4.72 ± 0.71 (8) $ml \cdot kg^{-1} \cdot h^{-1}$) and UFR (2.70 ± 0.23 (8) $ml \cdot kg^{-1} \cdot h^{-1}$) that were not significantly different from the GFR (3.84 ± 0.55 (8) $ml \cdot kg^{-1} \cdot h^{-1}$) and UFR (2.57 ± 0.40 (8) $ml \cdot kg^{-1} \cdot h^{-1}$) measured in control fish. GFR was significantly lower in fish implanted with RU486 alone (2.15 ± 0.30 (8) $ml \cdot kg^{-1} \cdot h^{-1}$) than fish in all other groups (4.37 ± 0.33 (29) $ml \cdot kg^{-1} \cdot h^{-1}$), including fish implanted with RU486 + cortisol (4.43 ± 0.20 (5) $ml \cdot kg^{-1} \cdot h^{-1}$; Fig.7-6A). In reflection of this, UFR was significantly reduced in RU486-implanted fish (1.27 ± 0.23 (8) ml kg^{-1} h^{-1}) compared to fish of all other groups (2.77 ± 0.19 (29) $ml \cdot kg^{-1} \cdot h^{-1}$; Fig.7-6B).

Fish implanted with cortisol alone had a urinary excretion rate of urea (24.2 ± 4.9 (8) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$) that was significantly greater by about three-fold than those of shams (7.6 ± 0.6 (8) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$), controls (7.2 ± 1.3 (8) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$) and fish implanted with cortisol + RU486 (7.3 ± 0.7 (5) $\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$; Fig.7-7A). In contrast,

fish implanted with RU486 alone had a urinary excretion rate of urea (3.2 ± 0.7 (8) μmol-N·kg^{-1}·h^{-1}) that was significantly less than fish of all other groups for most of the experiment (Fig.7-7A). Taking the plasma urea concentration into account, fish implanted with cortisol had a urinary clearance rate of urea (5.31 ± 0.74 (8) ml·kg^{-1}·h^{-1}) that was not significantly different than those of sham fish (5.41 ± 0.56 (8) ml·kg^{-1}·h^{-1}), control fish (4.97 ± 0.72 (8) ml·kg^{-1}·h^{-1}) and fish implanted with cortisol + RU486 (4.52 ± 0.32 (8) ml·kg^{-1}·h^{-1}; Fig. 7-7B). In contrast, fish implanted with RU486 alone has a urinary clearance rate of urea (2.29 ± 0.42 (5) ml·kg^{-1}·h^{-1}) that was significantly less than fish of all other groups (Fig. 7-7B). However, when plasma urea concentration and GFR are taken into account, the clearance ratios (CR) of fish from all groups were not significantly different from eachother or from unity. However, $CR_{cortisol}$ (1.36 ± 0.21 (8)), CR_{sham} (1.39 ± 0.26 (8)) and $CR_{control}$ (1.21 ± 0.12 (8)) showed a slight trend towards secretion compared to fish treated with RU486 (CR_{RU486} = 0.93 ± 0.07 (8) and $CR_{cortisol+RU486}$ = 1.03 ± 0.08 (8); Fig.7-7C. While these CR data suggest that urea was neither secreted nor reabsorbed in these fish, it is more likely a reflection of averaging individual fish that were either reabsorbing urea (CR < 1) or secreting urea (CR > 1).

Discussion

The objective of this study was to determine whether elevated cortisol levels have a regulatory effect on urea metabolism, urea excretion or both in the freshwater rainbow trout. Our results indicate that cortisol does have specific effect on urea metabolism, causing a significant rise in circulating urea concentrations and a significant elevation in

branchial and renal urea excretion, demonstrating that urea production rate is approximately doubled. Furthermore, this elevation was not observed in fish treated with the glucocorticoid receptor blocker, RU486. While branchial and renal urea excretion were elevated in cortisol-treated fish, this elevation was due to the rise in circulating urea concentrations, because the clearance rates in these fish were not significantly different than in sham-treated fish. The regulation of branchial and renal urea transport mechanisms in the trout does not appear to be mediated by cortisol and glucocorticoid receptors.

The impact of cortisol on fish intermediary metabolism tends to be controversial because the outcome of an individual experiment is highly dependent on the species of fish and the experimental design used (Gamperl et al. 1994; reviewed by Mommsen et al. 1999). In the present study, trout were fed up until the time of surgery, thus caution should be taken in comparing these findings to results on starved trout as nutritional status has an important influence in nitrogen metabolism and excretion (reviewed by Wood, 2001). In addition, the intraperitoneal implantation of cortisol and/or RU486 with coconut oil allowed for the slow release of these two substances over the experimental period, a factor that could also be influential to the outcome (Vijayan and Leatherland, 1989). The plasma cortisol concentrations measured in control fish (approximately 100 ng ml^{-1}) were typical of cannulated and confined fish, where chronic stress appears to elevate plasma cortisol from true resting values (< 10 ng ml^{-1}; reviewed by Barton and Iwama, 1991). Fish implanted with cortisol ± RU486 experienced cortisol concentrations that were 5-6 fold greater than control fish and were comparable to levels measured in

rainbow trout after 4 h of swimming (643 ng ml^{-1}; Hill and Fromm, 1968) or 2 h of mild agitation (900 ng ml^{-1}; Wedemeyer, 1969).

Similar to mammals, mean plasma cortisol concentrations in the rainbow trout were elevated following treatment with RU486 alone, likely due to altered negative feedback control of cortisol release from the interrenal cells of the head kidney, which is analogous to the mammalian adrenal cortex (Healy et al. 1983; Bertagna et al. 1984; Gaillard et al. 1985). The stimulation of cortisol production observed with RU486 treatment is in contrast to previous observations of an absence of RU486-mediated cortisol elevation in rainbow trout (Vijayan et al. 1994). However, the cortisol elevation in RU486-treated fish in the present study was transient; cortisol levels in the plasma greatly exceeded those in sham fish initially but then gradually decreased and were not significantly different after 48 hours. Both the present study and Vijayan et al. (1994) used the same dose of RU486 but the latter study would have probably missed this temporary effect of RU486 since sampling began 7 days post-implantation (*versus* 1.5 days post-implantation in the present study).

Mammalian studies have demonstrated that glucocorticoids modulate hepatic glucose metabolism, generally stimulating the production of glucose *de novo* by gluconeogenesis (reviewed by Kraus-Friedman, 1984). In the present study plasma glucose concentrations were elevated in cortisol-treated trout compared to trout of other groups, though this trend was not significant. However, a significant hyperglycemia is often associated with cortisol treatment in fish, and is usually attributed to an enhanced rate of hepatic gluconeogenesis (Butler, 1968; Inui and Yokote, 1975; Lidman et al.

1979; Leach and Taylor, 1982). Indeed, cortisol has been reported to significantly increase the activities of all the key gluconeogenic enzymes, namely glucose-6-phosphatase (Inui and Yokote, 1975; Chan and Woo, 1978), fructose-1, 6-bisphosphatase (Inui and Yokote, 1975; Chan and Woo, 1978) and phosphoenolpyruvate carboxykinase (PEPCK; Foster and Moon, 1986; Vijayan et al. 1997; reviewed by Mommsen et al. 1999).

Many studies have also suggested that corticosteriods are important in regulating the catabolism of amino acids as a substrate for gluconeogenesis, and transaminase activities are often stimulated by cortisol elevation (Freeman and Idler, 1973; Chan and Woo, 1978; Vijayan et al. 1997). Specifically, in the hepatocytes of the marine sea raven (*Hemitripterus americanus*), cortisol enhanced glycogen breakdown and stimulated the oxidation of alanine (Vijayan et al. 1993). Furthermore, Vijayan and coworkers (1994) showed that cortisol implantation significantly increased gluconeogenesis and oxidation from [^{14}C]-alanine in rainbow trout hepatocytes, a stimulation that was abolished in the presence of RU486.

A consequence of amino acid catabolism during gluconeogenesis is an increased formation of nitrogenous wastes, however, few studies have directly measured nitrogen metabolism under these circumstances in fish. In mammalian studies, an increased glucose production has for decades been associated with an increased urea production, the primary nitrogenous waste in mammals, and elevated urea excretion has been used as an indicator of protein breakdown in response to glucocorticoids (Ingle, 1952; Christowitz et al. 1981; reviewed by Kraus-Friedmann, 1984). Rather than excreting

urea, most teleost fish predominantly excrete their nitrogenous wastes as the more toxic ammonia, which is the direct result of amino acid breakdown, as it diffuses easily through the gills and is readily diluted by the surrounding environment. Yet in the present study, fish with a 6-fold elevation in cortisol did not a show a significant rise in circulating ammonia levels or in branchial ammonia excretion rate compared to sham-implanted fish. These findings are similar to those by DeBoeck et al. (2001) who found no change in ammonia excretion in juvenile rainbow trout implanted with cortisol over a similar time period. However, they are in contrast to observations by Chan and Woo (1978) who measured a 48% increase in branchial ammonia excretion in the eel, *Anguilla japonica*, injected with cortisol.

Despite being ammoniotelic, most teleosts also produce and excrete a small amount of urea and circulating urea concentrations in trout are 5-50 times greater than plasma ammonia concentrations (reviewed by Wood, 1993). Trout treated with cortisol demonstrated a 3-fold elevation in plasma and urine urea concentrations compared to sham fish, an elevation that was abolished in fish implanted with cortisol + RU486 suggesting that cortisol, mediated by glucocorticoid receptors, stimulates urea production. Likewise, the ammoniotelic marine sea raven, with an elevation in plasma cortisol concentration comparable to the trout of the present study, demonstrated plasma urea concentrations that were 3-fold greater than in fish with sham coconut oil implants (Vijayan et al. 1996). Similar to rainbow trout, a rise in circulating ammonia levels in response to cortisol-implantation was not observed in the sea raven (Vijayan et al. 1996). In the present study, RU486 treatment lowered plasma ammonia in trout, suggesting a

chronic elevation in plasma ammonia, driven by endogenous cortisol mobilization. Upregulation of urea synthesis in response to corticosteriods has been observed in mammals; rats treated with cortisol demonstrated a stimulation of 3 out of 5 urea cycle enzymes, namely carbamoyl phosphate synthetase (CPS), arginosuccinate synthetase (ASS) and arginase (Christowitz et al. 1981). An upregulation of ureogenic pathways in response to cortisol has also been observed in fish with reports of increased GS, arginase and allantoicase (See Introduction).

Paradoxical to the stimulatory effect of cortisol on endogenous urea production is evidence that glucocorticoids have an inhibitory effect on urea transport mechanisms, namely facilitated diffusion UT-type transporters in both mammals (Naruse et al. 1997; Peng et al. 2002) and in the ureotelic gulf toadfish (Wood et al. 1997, 2001). In the toadfish, the response appears to operate through glucocorticoid receptors as it is blocked by RU486 (MD McDonald, CM Wood, M Grosell and PJ Walsh, unpublished data). Recent evidence indicates that the branchial excretion of urea in the ammoniotelic trout is mediated at least in part by a phloretin-sensitive, UT-type facilitated diffusion mechanism (Chapter 6). However in the present study, the branchial clearance rate of urea, which corrects for differences in excretion due to differences in plasma concentration, was not significantly altered by cortisol or RU486, or indeed by any of the experimental treatments. Thus, branchial urea excretion does not appear to be controlled by cortisol in this ammoniotelic teleost.

Evidence suggests the presence of an active urea transport mechanism in the kidney of the rainbow trout that can move urea in either the reabsorptive or secretory

direction (McDonald and Wood, 1998; Chapter 5). However, in the present study, a role for cortisol in the homeostatic control of renal urea retention or elimination was not apparent. The clearance ratio (CR) for urea, which corrects for variations in plasma urea concentration and glomerular filtration rate, was not significantly different among the five treatment groups. Thus, as with branchial clearance of urea, cortisol and glucocorticoid receptors do not appear to play a role in the regulation of renal urea handling.

However, an important modifying factor in the urinary excretion of any substance by a freshwater fish is the glomerular filtration rate (GFR), which directly affects the urine flow rate (UFR). In at least one teleost species, the ureotelic freshwater catfish, *Heteropneustes fossilis*, cortisol has been shown to have a diuretic effect *via* a stimulation of GFR leading to elevated UFR (Parwez et al. 1984; Parwez and Goswami, 1985). In the present study, GFR and UFR were significantly decreased in trout treated with RU486 alone compared to fish of all other groups, suggesting the involvement of glucocorticoid receptors in the control of GFR. On the other hand, cortisol treatment alone did not elevated GFR or UFR, so perhaps the response operates only over a low range of cortisol concentrations -*i.e.* diuresis may be much more sensitive to cortisol elevation than gluconeogenesis.

To conclude, glucocorticoid receptors appear to play a role in urea production but not in the regulation of urea excretory mechanisms in the ammoniotelic rainbow trout. Further research is necessary to fully understand the mechanisms involved in the

regulation of urea excretory mechanisms in the gill and the kidney and elucidate the functional role, if any, of circulating urea in ammoniotelic teleost fish.

Table 7-1: Mean plasma glucose concentrations in the fish of each group. Values are means ± 1 S.E.M. (N). There were no significant differences.

	$[Glucose]_{plasma}$ ($mmol \cdot l^{-1}$)
Control	3.53 ± 0.21 (8)
Sham Implanted	4.91 ± 1.02 (8)
RU486 Implanted	4.55 ± 0.72 (8)
Cortisol Implanted	6.31 ± 1.48 (8)
Cortisol + RU486 Implanted	4.82 ± 0.57 (5)

Figure 7-1: Plasma cortisol concentrations ($ng \cdot ml^{-1}$) of fish from all five groups over the 72 h experimental period. Cortisol concentrations in cortisol-implanted and cortisol + RU486 implanted fish were significantly higher than in sham-implanted fish for most of the 72 h. Cortisol concentrations in the two groups implanted with cortisol were not significantly different except at 12 h. RU486-implanted fish had significantly elevated cortisol levels up until 48 h. Values are means ± 1 S.E.M. (N= 5 for cortisol + RU486, N = 8 for other groups); * $P < 0.05$ relative to sham-implanted fish; † $P < 0.05$ relative to cortisol-implanted fish.

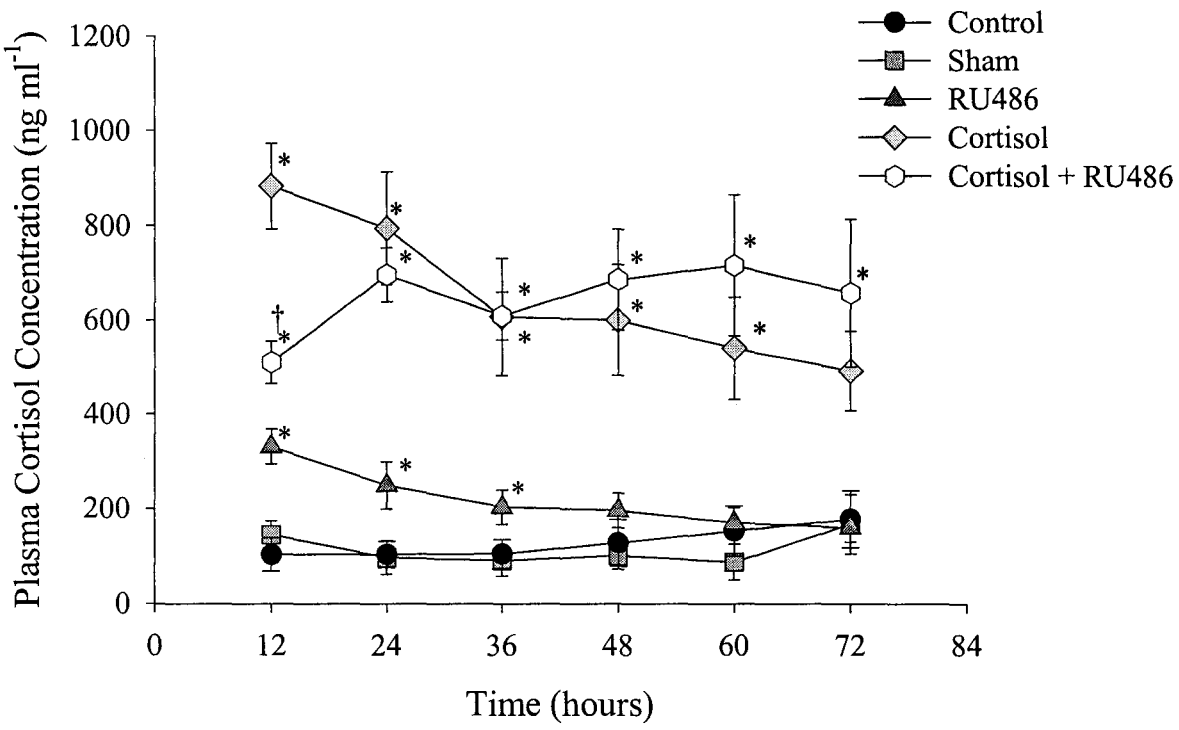

Control
Sham
RU486
Cortisol
Cortisol + RU486
Plasma Cortisol Concentration (ng ml-1)
1200
1000
800
600
400
200
0
0
12
24
36
48
60
72
84
Time (hours)

Figure 7-2: Mean plasma and urine urea concentrations in fish from all five groups demonstrating a significant elevation in urea levels in cortisol-implanted fish that was abolished in fish implanted with cortisol + RU486. Values are means ± 1 S.E.M. (N= 5 for cortisol + RU486, N = 8 for other groups); $P < 0.05$, a different letter denotes a significant difference.

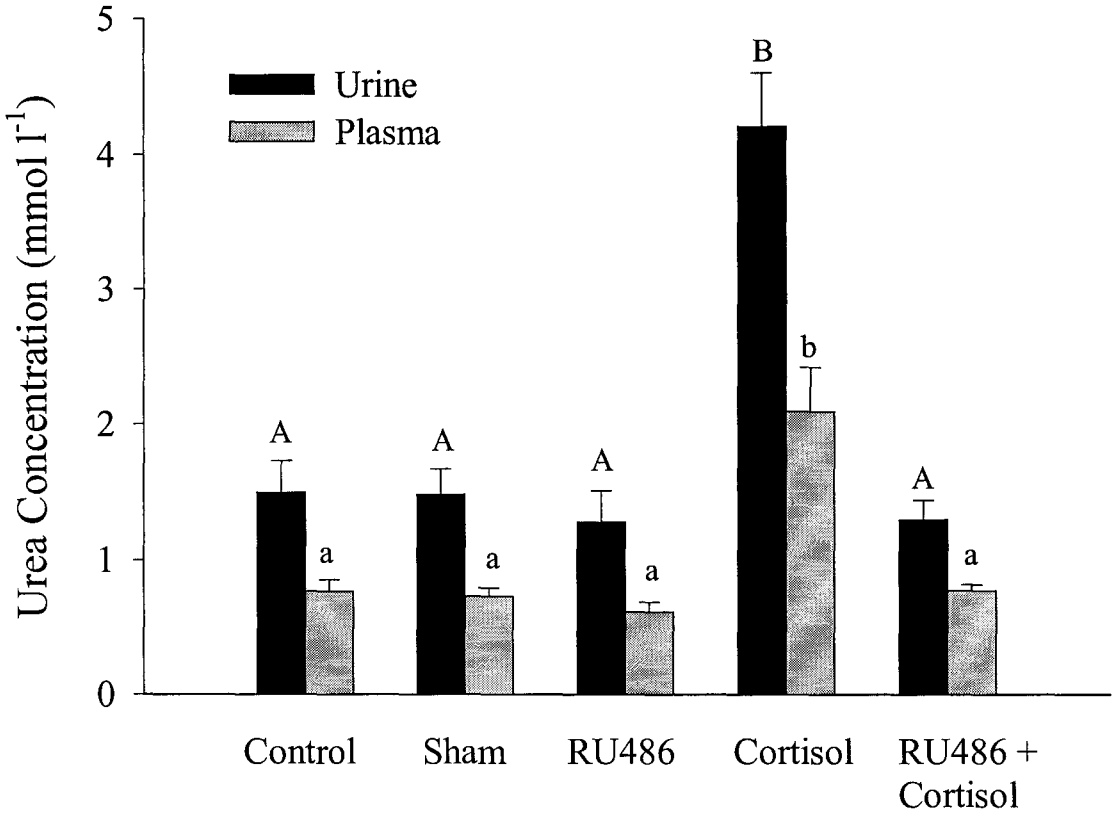

Urea Concentration (mmol l-1)
5
4
3
2
1
0
Urine
Plasma
A
a
A
a
A
a
B
b
A
a
Control
Sham
RU486
Cortisol
RU486 +
Cortisol

Figure 7-3: Linear regressions of plasma urea concentration (y-axis) *versus* plasma cortisol concentration (x-axis) demonstrating a significant linear relationship in fish implanted with cortisol that was abolished in fish implanted with cortisol + RU486. In cortisol-implanted fish the equation of the line is $y = 0.002x + 1.17$ $r = 0.72$, $P < 0.001$. No significant correlation was found in fish of any other group.

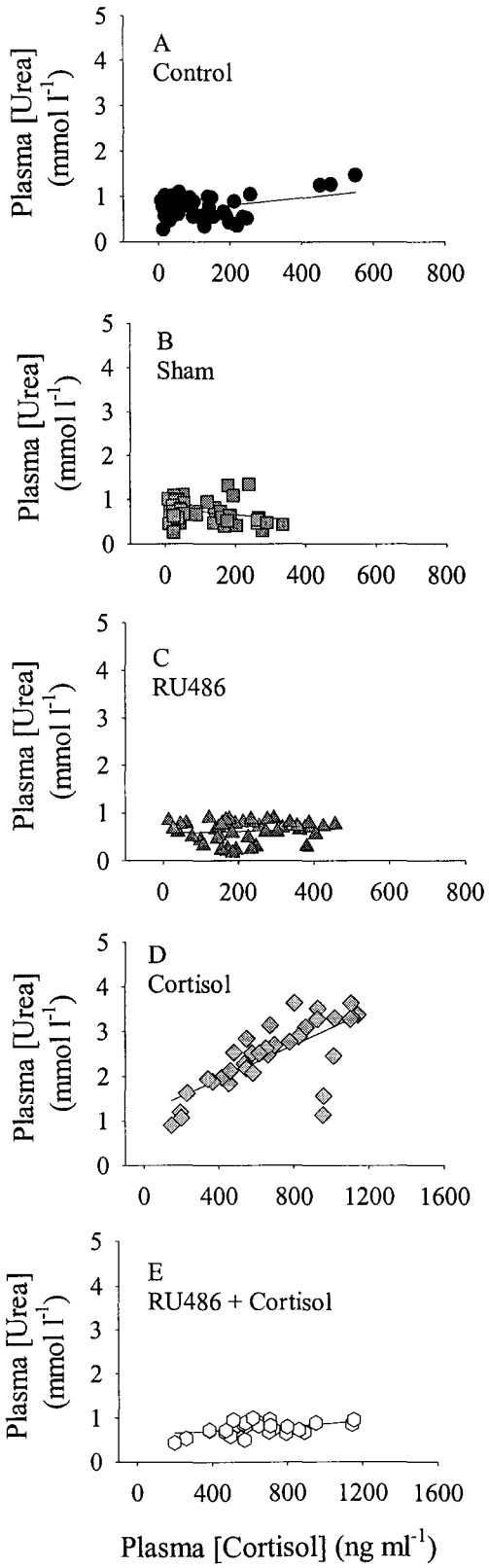
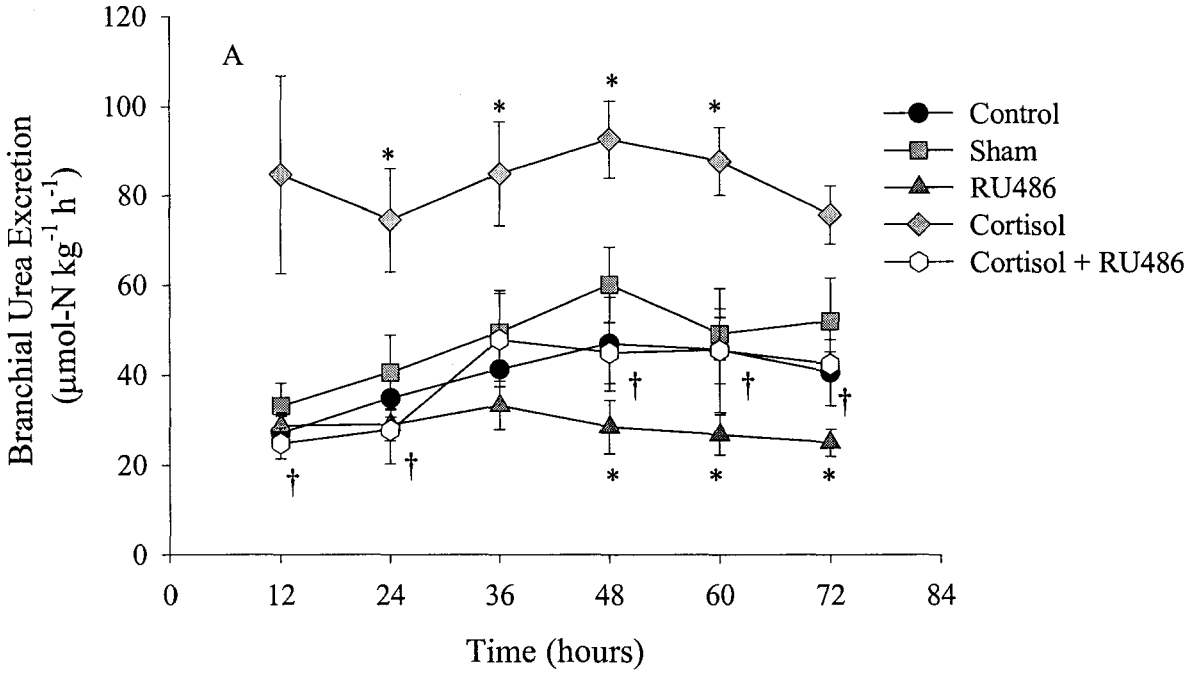

A
Control
B
Sham
C
RU486
D
Cortisol
E
RU486 + Cortisol
Plasma [Urea] (mmol l-1)
Plasma [Cortisol] (ng ml-1)
0
1
2
3
4
5
0
200
400
600
800
1200
1600

Figure 7-4: (A) The rates of branchial urea excretion in fish of all five groups over the 72 hour experimental period demonstrating the stimulatory effect of cortisol on excretion rate when compared to sham fish. Cortisol + RU486-implanted fish had excretion rates that were not different than sham fish. Fish implanted with RU486 alone had significantly lower rates by 48 h compared to shams. (B) Mean branchial urea clearance rates are similar in fish from all five groups. Values are means ± 1 S.E.M. (N= 5 for cortisol + RU486, N = 8 for other groups) * $P < 0.05$ relative to sham-implanted fish; † $P < 0.05$ relative to cortisol-implanted fish.


A
Branchial Urea Excretion
(µmol-N kg-1 h-1)
120
100
80
60
40
20
0
0
12
24
36
48
60
72
84
Time (hours)
Control
Sham
RU486
Cortisol
Cortisol + RU486

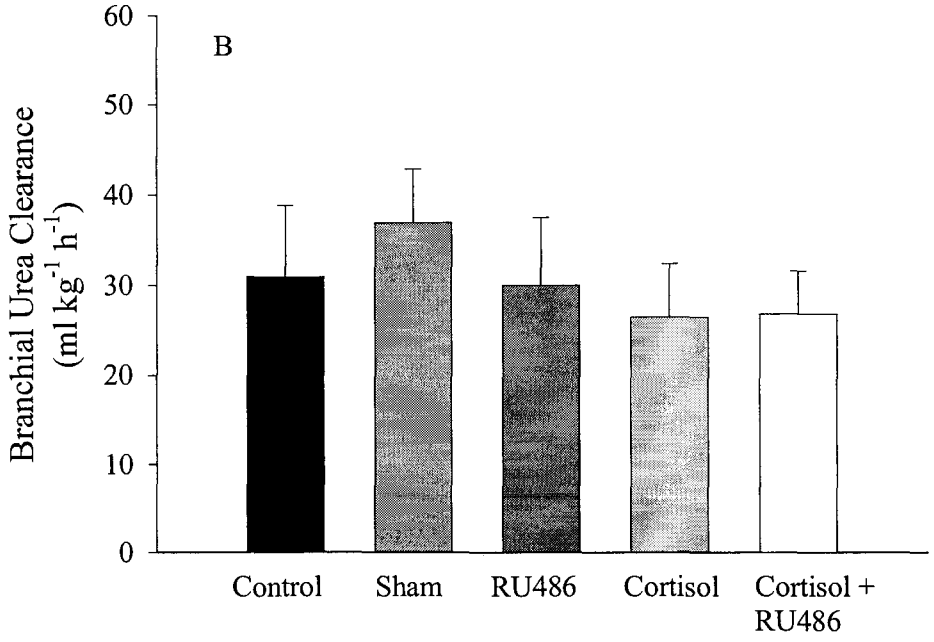

B
Branchial Urea Clearance
(ml kg-1 h-1)
60
50
40
30
20
10
0
Control
Sham
RU486
Cortisol
Cortisol +
RU486

Figure 7-5: (A) The rates of branchial ammonia excretion in fish of all five groups over the 72 hour experimental period were not significantly different from eachother. (B) Plasma ammonia concentrations showed no significant difference between cortisol-implanted fish and sham fish, but the former were significantly elevated relative to those treated with RU486. (C) Mean rates of branchial ammonia clearance in fish of the three groups were not significantly different. Values are means ± 1 S.E.M. (N= 5 for cortisol + RU486, N = 8 for other groups); * $P < 0.05$ relative to sham-implanted fish; † $P < 0.05$ relative to cortisol-implanted fish.

.

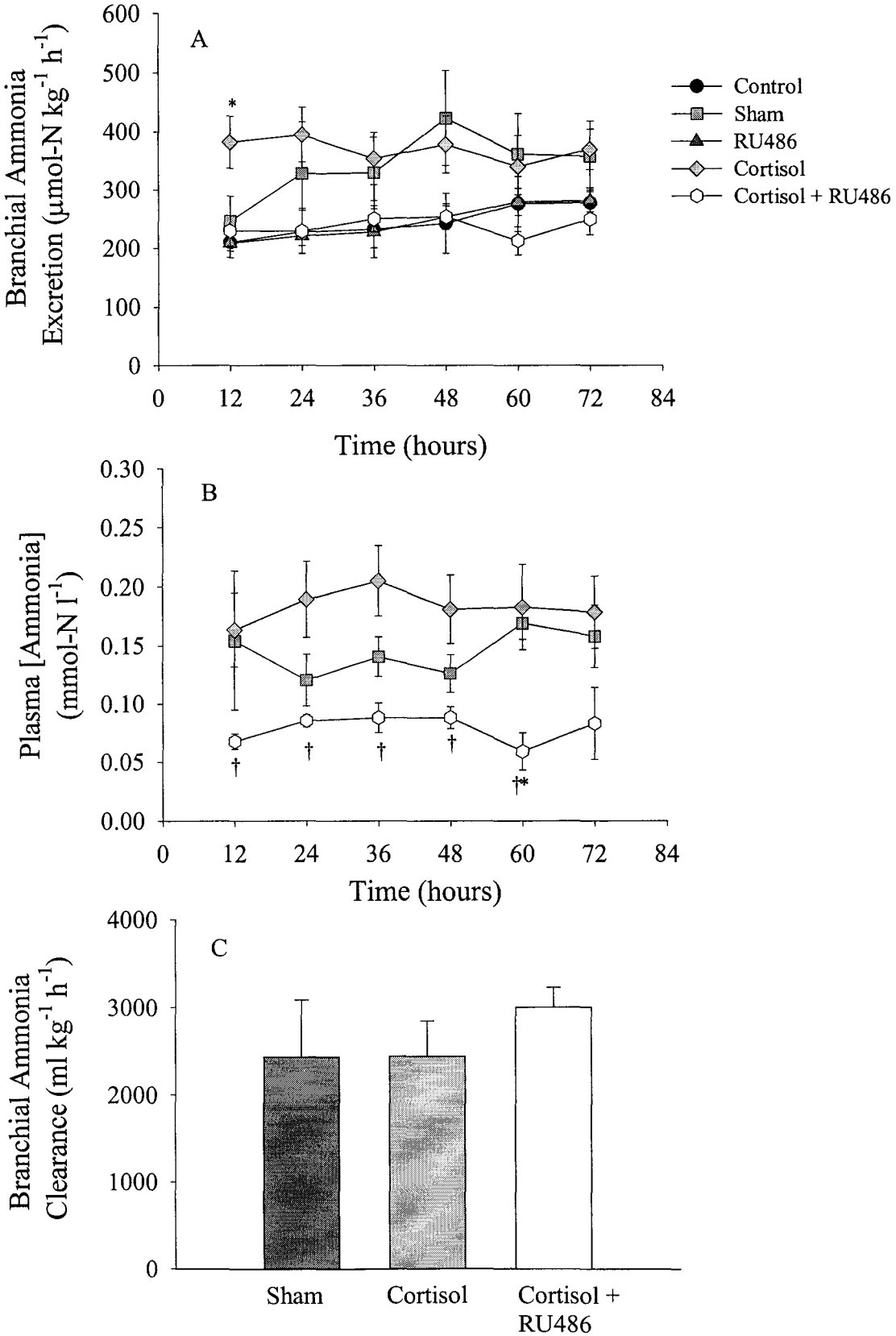
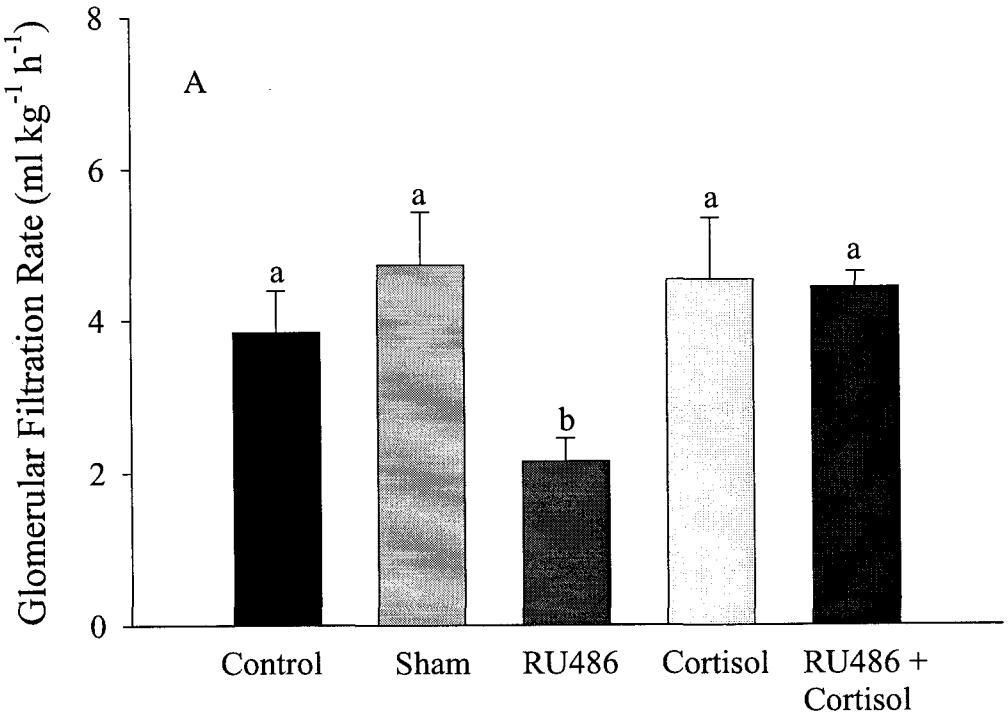

A
Branchial Ammonia Excretion (µmol-N kg-1 h-1)
600
500
400
300
200
100
0
*
0
12
24
36
48
60
72
84
Time (hours)
Control
Sham
RU486
Cortisol
Cortisol + RU486
B
Plasma [Ammonia] (mmol-N l-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
†
†
†
†
†*
0
12
24
36
48
60
72
84
Time (hours)
C
Branchial Ammonia Clearance (ml kg-1 h-1)
4000
3000
2000
1000
0
Sham
Cortisol
Cortisol + RU486

Figure 7-6: (A) Glomerular filtration rates and (B) urine flow rates demonstrating rates in RU486-implanted fish that are significantly lower than measured rates of fish in all other groups. Values are means ± 1 S.E.M. (N= 5 for cortisol + RU486, N = 8 for other groups). $P < 0.05$, a different letter denotes a significant difference.


A
Glomerular Filtration Rate (ml kg-1 h-1)
8
6
4
2
0
a
a
b
a
a
Control
Sham
RU486
Cortisol
RU486 + Cortisol

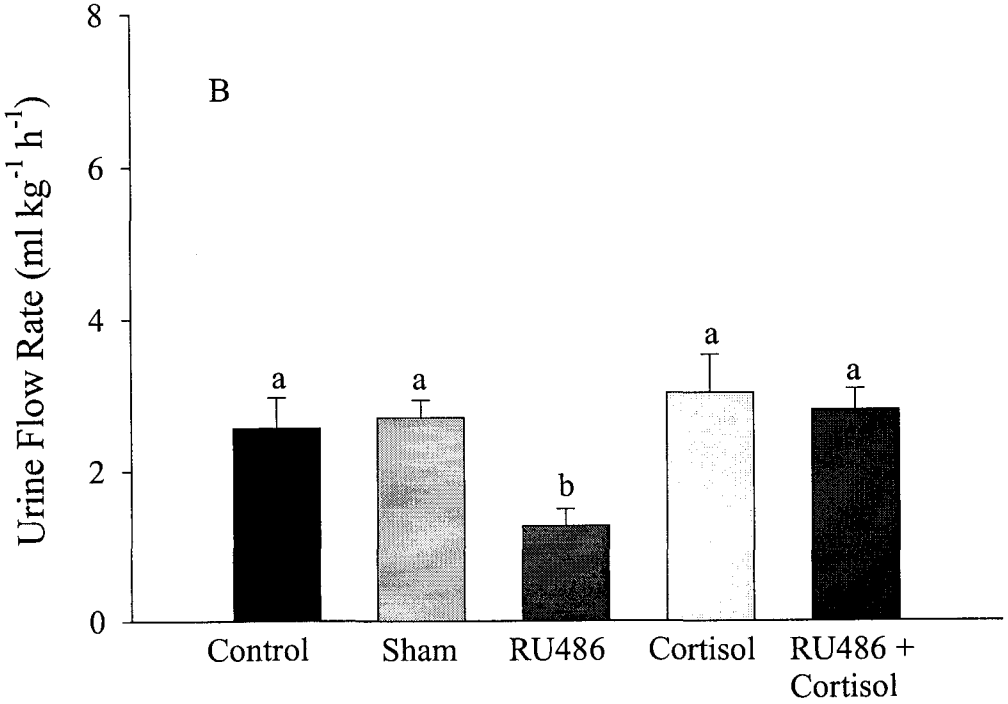
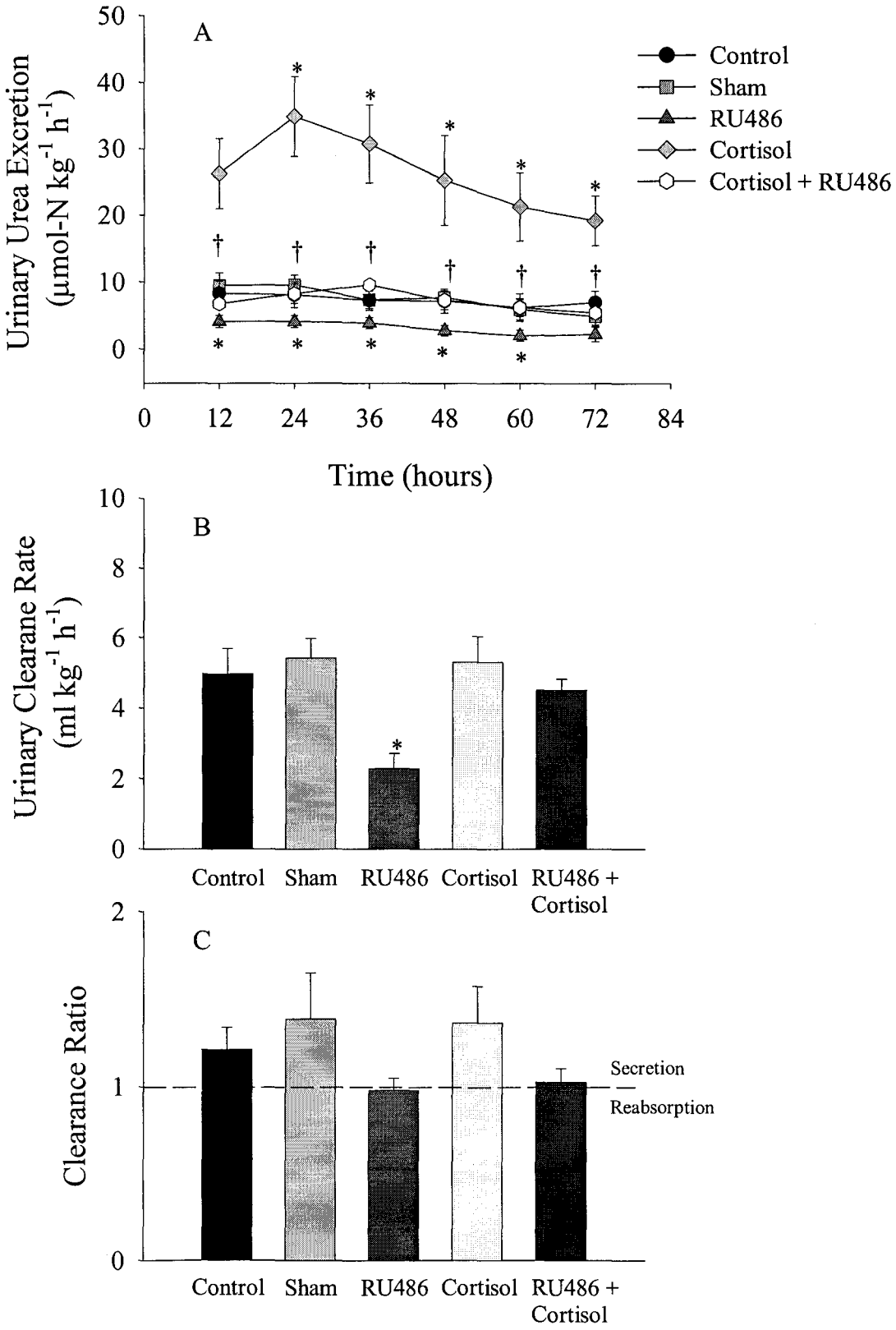

B
Urine Flow Rate (ml kg-1 h-1)
8
6
4
2
0
a
a
b
a
a
Control
Sham
RU486
Cortisol
RU486 + Cortisol

Figure 7-7: (A) The urinary excretion rates of urea in fish of all five groups during the 72 hour experimental period showing an elevation of excretion rate in cortisol-implanted fish compared to sham fish that was abolished when fish were implanted with cortisol + RU486. Fish implanted with RU486 alone had significantly lower urinary excretion rates of urea. (B) Mean urinary clearance rates of fish in all five groups demonstrating a significant lower clearance rate in fish implanted with RU486 alone. The rates in fish of all other groups were not significantly different. (C) Mean clearance ratios of urea were not significantly different in fish from all groups. Values are means ± 1 S.E.M. (N= 5 for cortisol + RU486, N = 8 for other groups); * $P < 0.05$ relative to sham-implanted fish; † $P < 0.05$ relative to cortisol-implanted fish.

.


A
Urinary Urea Excretion
(µmol-N kg-1 h-1)
50
40
30
20
10
0
0
12
24
36
48
60
72
84
Time (hours)
Control
Sham
RU486
Cortisol
Cortisol + RU486
B
Urinary Clearane Rate
(ml kg-1 h-1)
10
8
6
4
2
0
Control
Sham
RU486
Cortisol
RU486 +
Cortisol
C
Clearance Ratio
2
1
0
Secretion
Reabsorption
Control
Sham
RU486
Cortisol
RU486 +
Cortisol

Chapter 8

General Discussion

From the present work, it is apparent that the branchial and renal excretion mechanisms for urea in the freshwater, ammoniotelic rainbow trout (*Oncorhynchus mykiss*) and in two marine species of the family Batrachoididae, the facultatively ureotelic gulf toadfish (*Opsanus beta*) and the ammoniotelic plainfin midshipman (*Porichthys notatus*) are carrier-mediated. This conclusion is based on the physiological and pharmacological characteristics of urea movement uncovered in the present work and on the distinguishing features of urea transport mechanisms previously defined in mammalian, amphibian and teleostean models.

The marine gulf toadfish, *Opsanus beta*, is one of the best-studied teleosts with respect to urea metabolism and excretion. It has the unique ability to switch from ammoniotelism to ureotelism when in a stressful environment (crowding, confinement, air or ammonia exposure; Walsh et al. 1990; Walsh et al. 1994a; Walsh and Milligan, 1995). To facilitate urea excretion, a urea transport protein (tUT) is present in the gills of the toadfish that shows 62 % homology at the amino acid level to mammalian hormonally-controlled facilitated diffusion (UT) urea transporters (Smith et al. 1998; Walsh et al. 2000). The activation of tUT is periodic, which results in the excretion of urea that is not continuous but occurs in distinct 0.5-3 hour pulses on average once or

twice a day every 24 hours (Wood et al. 1995, 1997, 2001). In the periods between pulses there is little excretion of urea.

When tUT was characterized on the basis of urea and analogue handling, it differentially handled urea, acetamide and thiourea (Chapter 2). Since tUT is periodically activated, it was possible to quantify the handling of urea and analogues during periods of activation, when the three substances would be moving through the transporter, and during periods of inactivation, when the three substances would be moving through the lipid bilayer unaided. The observed movement of urea through the toadfish gill was in accord with the premise that carrier-mediated transport would result in the differential handling of the three substances, and simple diffusion would result in all three substances moving similarly. The branchial clearance of acetamide when tUT was activated was 35-50% that of urea clearance and the relative thiourea clearance was at most only 16% of urea clearance during pulsing events (Chapter 2; Fig. 8-1). Thus, during pulsing periods the activation of tUT gave rise to an average 36-fold increase in urea permeability and at the same time a corresponding increase in the permeability of acetamide and thiourea of only 17-fold and 6-fold, respectively (Chapter 2; Fig. 8-2). However, all three substances exhibited similar permeabilities during non-pulsing periods. Thus, the lipid membrane of the gill handled urea, acetamide and thiourea identically when tUT was not activated, despite small differences in calculated oil/water partition coefficients and lipid permeabilities of the three substances (Goldstein and Solomon, 1960; Lippe, 1969; Galluci et al. 1971; Fig. 8-2).

In determining the transport kinetics of tUT *in vivo* by infusing toadfish with consecutive isosmotic loads of urea, urea movement through tUT appeared to be saturated, as pulse size did not increase in proportion to infused urea loads (Chapter 3). In addition, the branchial urea excretion rates measured in toadfish were 10-35 times higher than the rates measured in teleosts such as the midshipman and trout. From these findings, it was hypothesized that tUT is running fairly close to its transport maximum under resting conditions, most likely as a strategy to eliminate as much urea in as short a period of time as possible (Chapter 3).

A much less studied route of nitrogen excretion in the toadfish is its "aglomerular" kidney, which is secondary to the gill as it accounts for < 10% total urea excretion. The toadfish kidney forms urine primarily by secretion but does have a measurable glomerular filtration rate (< 10 % of urine flow rate; Chapter 2). Preliminary evidence suggested the possibility of an active urea secretory mechanism in the kidney of the toadfish, since toadfish urine urea concentrations generally exceed plasma levels by at least 30% (Wood et al. 1995). When toadfish were infused with exogenous urea loads, the secretion clearance rate of urea (SCR_{urea}) into the kidney tubule showed a strong, linear relationship to plasma urea concentrations, with no evidence of a transport maximum even at plasma urea levels that were well beyond resting concentrations (Chapter 3). As in the gill, the toadfish kidney demonstrated differential handling of urea and analogues, however the pattern was different. Urea and thiourea were more concentrated in the urine than in the plasma and acetamide appeared to passively

equilibrate between the two, suggesting a urea secretory mechanism that is unlike the branchial excretory mechanism (Chapter 2; Fig. 8-3).

Although closely related to the gulf toadfish, the plainfin midshipman does not express the ornithine urea cycle. It is obligately ammoniotelic and unable to switch between ammoniotelism and ureotelism (Wang and Walsh, 2000; Walsh et al. 2001a). The midshipman does excrete some (< 10%) of its nitrogenous waste as urea but the branchial excretion of urea is continuous and not pulsatile as in the toadfish (reviewed by Walsh, 1997). Despite being ammoniotelic, a strong signal was observed when a ^{32}P-labelled cDNA probe based on the toadfish branchial urea transporter (tUT) was used to examine midshipman gill mRNA by Northern blot analysis, suggesting the presence of a facilitated diffusion transporter in the midshipman gill (Walsh et al. 2001a). Supporting this initial finding, the present study characterized two distinct transport mechanisms, which appeared to be involved in urea excretion in the plainfin midshipman. Found in the gill and the kidney, neither of these mechanisms saturated even at plasma urea concentrations that greatly exceeded physiological levels (Chapter 4). The branchial clearance of acetamide through the midshipman gill was 74% of urea clearance while thiourea clearance was only 55%, with similar patterns in the influx direction, a pattern that was similar to that observed for the toadfish tUT (Chapter 4; Fig. 8-1). In addition, the aglomerular secretory kidney of the midshipman, similar to the toadfish in that glomerular filtration is measurable, demonstrated a comparable pattern of renal handling as the toadfish, where $SCR_{thiourea} > SCR_{urea} > SCR_{acetamide}$ (Fig. 8-3). Though the

midshipman is ammoniotelic, the characteristics of its urea transporters appear to be similar to those found in its ureotelic relative, the toadfish.

The midshipman, while related to the toadfish, has many characteristics of more typical teleosts, such as the freshwater rainbow trout (*Oncorhynchus mykiss*). Midshipmen and trout continuously excrete their nitrogenous waste as ammonia but retain a constant level of urea in the blood (McDonald and Wood, 1998; Chapter 4, 5, 7). Early evidence suggested homeostatic regulation of endogenous urea concentrations in the rainbow trout that involved urea transport mechanisms in the gill and the kidney (McDonald and Wood, 1998). The trout gill exhibited the same pattern of differential handling as observed in the facultatively ureotelic gulf toadfish and the ammoniotelic plainfin midshipman, where the branchial clearance rate of urea > acetamide > thiourea. Similar to both the toadfish and midshipman, the ratio of analogue/urea branchial clearance in the trout was 48% for acetamide and only 22% for thiourea (Chapter 5; Fig. 8-1). A similar difference in urea and thiourea handling is also observed in the obligately ureotelic Lake Magadi tilapia, in which mtUT (with 75% identity to tUT) demonstrated a permeability to thiourea that was 19% that of urea permeability (Walsh et al. 2001b). Thus, this pattern of analogue *versus* urea handling appears to be characteristic of branchial UT-type transport mechanisms in both ureotelic and ammoniotelic organisms. The occurrence of this pattern in rainbow trout strongly suggests the presence of a UT-type facilitated diffusion transporter in the gill, and supports preliminary Northern blot and sequence analysis that revealed a mRNA from trout gill with approximately 2000

bases homologous to the mRNA of the toadfish tUT (CM Pilley, PW Wright and PJ Walsh, unpublished data).

The isolated basolateral membrane vesicle (BLMV) preparation gave additional support for a facilitated diffusion transport mechanism in the trout gill; urea uptake by vesicles was sensitive to phloretin, a well-described blocker of facilitated type transport (Chapter 6). Urea uptake by BLMV displayed saturation kinetics at physiological concentrations with a $K_m = 1.17$ mmol·l^{-1} and a $V_{max} = 0.42$ μmol mg protein^{-1} h^{-1}. The basolateral membrane of the gill displayed differential handling of urea and acetamide, similar to the pattern observed in whole gill *in vivo* in trout, midshipmen and toadfish (Chapter 6). However, in contrast to the whole gill, the uptake of urea and thiourea were not significantly different. In addition, urea uptake in the presence of thiourea was significantly lower than in control conditions, suggesting competitive interaction between these two substances. Urea uptake in the presence of acetamide and N-methylurea was not affected. Taken together, these results suggest that the differential handling of urea and some analogues could actually be a consequence of urea moving through the transporter while some analogues are moving by simple diffusion and thus not interfering with urea movement. Thiourea, which inhibited urea uptake by BLMV, was also taken up at the same rate as urea, suggesting that they were moving *via* the same mechanism.

The renal handling of urea by the trout proved to be complicated. While an earlier study by McDonald and Wood (1998) suggested reabsorption of urea against a concentration gradient in the trout, in the present studies renal secretion (and not reabsorption) of urea against a concentration gradient was observed in most fish, though

there were some instances of reabsorption (Chapter 5, Chapter 7). The pattern of urea and analogue handling by the trout kidney ($SCR_{urea} = SCR_{acetamide} > SCR_{thiourea}$) was different than that observed in the toadfish and midshipman where $SCR_{thiourea} > SCR_{urea} > SCR_{acetamide}$ (Chapter 2, 4, 5; Fig. 8-3). This difference is most likely due to different urea transport mechanisms and morphology; the handling of urea in batrachoidid fish is not correlated with Na^+ as observed in trout, and the kidneys of toadfish and midshipmen are aglomerular while the kidney of the trout is glomerular. Based on the different pattern observed in the kidney, plus the fact that urea is moving against a concentration gradient, it is unlikely that the urea transport mechanisms in the gill and kidney are the same.

While it is not difficult to comprehend the purpose of urea transport proteins in the facultatively ureotelic gulf toadfish, it is harder to explain why an ammoniotelic teleost fish would possess specific urea excretory mechanisms when urea makes up only 10% of their total nitrogenous waste. Growing evidence suggests a role for urea in early life stage development of ammoniotelic teleosts. Wright and coworkers (1995a) have measured an elevation in three of the five OUC enzymes and GSase in larval rainbow trout post-hatch, suggesting the presence of a functional OUC. It is possible that all teleosts may have retained OUC genes but for most species these are expressed only during early stages of development and are silenced in the adult phase (Wright et al. 1995a; Chadwick and Wright, 1999; Terjesen et al., 2000, 2001). Wright and coworkers (1995a) argued that in a few teleosts, OUC expression may maintained throughout the life cycle as a mechanism to cope with unusual environmental conditions, as in the case

of the gulf toadfish (Walsh et al. 1990; Walsh et al. 1994a; Walsh and Milligan, 1995) and the Lake Magadi tilapia (Randall et al. 1989; Walsh et al. 1993).

Urea synthesis in larval trout is believed to be a strategy to avoid ammonia toxicity and the expression of OUC enzymes are thought to be a mechanism for ammonia detoxification (Wright et al. 1995a; Pilley and Wright, 2000; Steele et al., 2001q). In adult rainbow trout, an elevation in internal cortisol caused a rise in endogenous urea concentrations and in urea excretion (Chapter 7). Since cortisol has been documented to stimulate gluconeogenesis and proteolysis in fish, the glucocorticoid-mediated increase of endogenous urea synthesis could be a potential mechanism of ammonia detoxification. It is possible that the ability to upregulate urea synthesis during periods of elevated ammonia has been extended from early life stages into adults and can be called upon when the situation presents itself. Furthermore, a bidirectional, facilitated diffusion urea transport mechanism in trout embryos (Pilley and Wright, 2000) has been described that is characteristically similar to that found in the basolateral membrane of the adult trout gill (Chapter 6). Thus, urea transport mechanisms could have followed the same evolutionary path as urea synthesis pathways but expression of these mechanisms continued in adult teleosts long after they were no longer obligatory.

The consequences and implications of this research span not only the general topic of nitrogen metabolism and excretion but specifically touch on ureotelism *versus* ammoniotelism, the evolution of urea transport mechanisms and the potential role of urea in ammonia detoxification. That many fish species synthesize urea, either through a fully functional OUC, or *via* uricolysis or arginolysis, suggests that the ability to make urea

was likely present well before vertebrate emergence onto land (reviewed by Walsh, 1997). The research described in this thesis indicates that specific urea transport mechanisms are well-developed in fish and thus were also most likely present before terrestrial invasion. From an evolutionary perspective, the ability to excrete urea effectively may have facilitated the movement onto land. The existence of urea transport mechanisms in ammoniotelic aquatic species provides insight regarding the evolutionary link between ammoniotelic and ureotelic organisms.

Figure 8-1: The branchial clearance rates (ml kg^{-1} h^{-1}) of toadfish, midshipmen and trout in the (left panels) acetamide series and (right panels) in the thiourea series. Note the significantly lower branchial clearance of acetamide and thiourea compared to urea and the similar trends among the three different species. Values are means ± 1 S.E.M. (N); * $P < 0.05$ significantly different than urea.

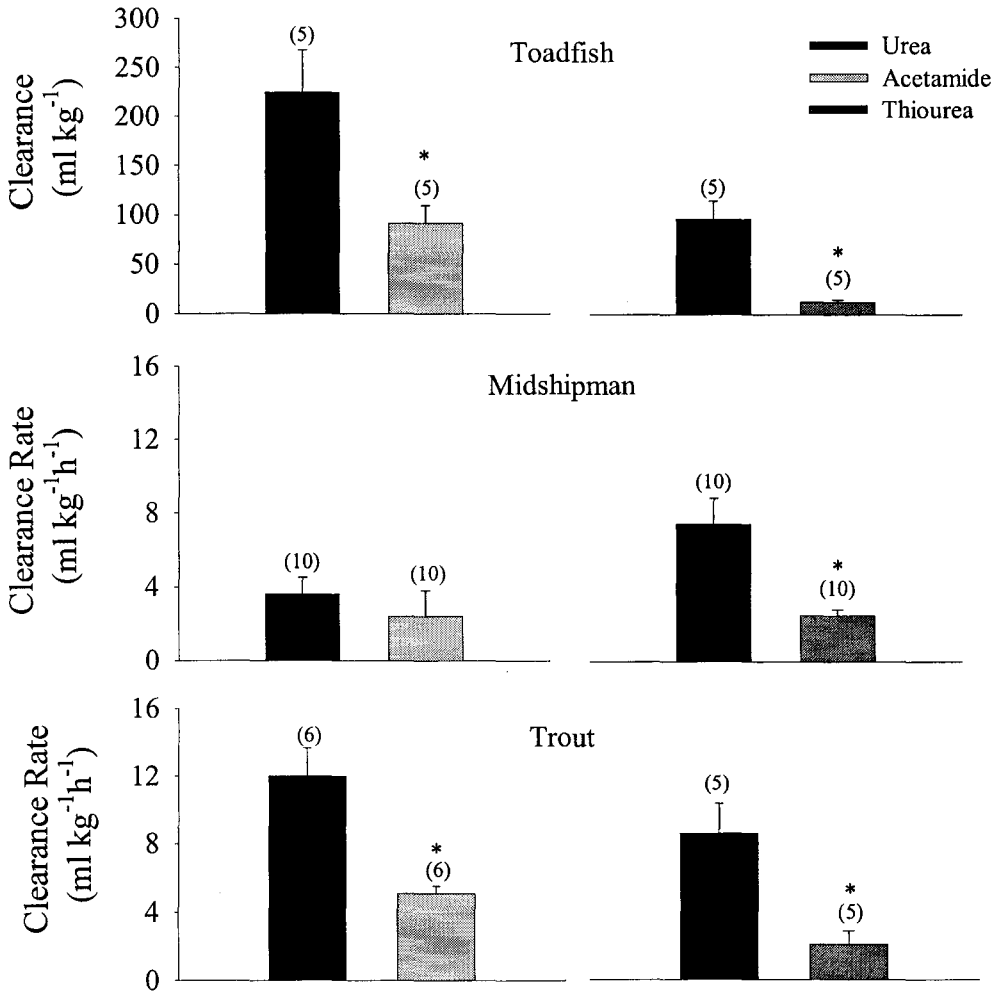

Toadfish
Urea
Acetamide
Thiourea
Clearance (ml kg-1)
300
250
200
150
100
50
0
(5)
*
(5)
(5)
*
(5)
Midshipman
Clearance Rate (ml kg-1h-1)
16
12
8
4
0
(10)
(10)
(10)
*
(10)
Trout
Clearance Rate (ml kg-1h-1)
16
12
8
4
0
(6)
*
(6)
(5)
*
(5)

Figure 8-2: Gill permeability of toadfish to (A) urea and (B) acetamide and (C) urea and (D) thiourea during pulsing and non-pulsing periods indicating a significant increase in the gill permeability to all three substances during pulsing periods. The branchial permeability to urea was significantly greater than its permeability to analogues. During non-pulsing periods, all three analogues demonstrated similar low permeabilities. Values are means ± 1 S.E.M. (N) * $P < 0.05$ significantly different from non-pulsing periods, † $P < 0.05$ significantly different from analogue permeability.

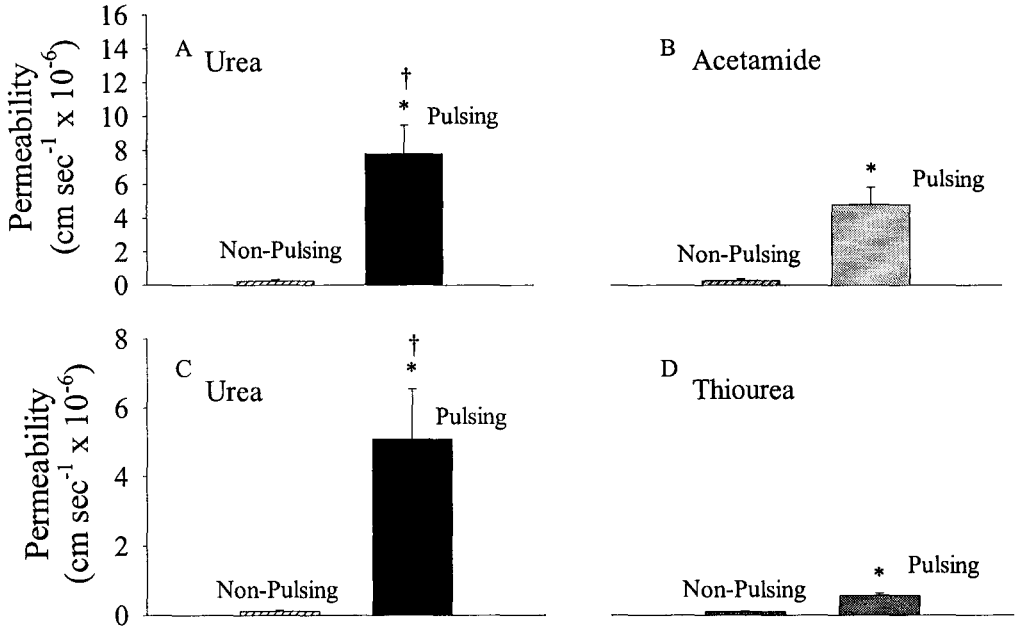
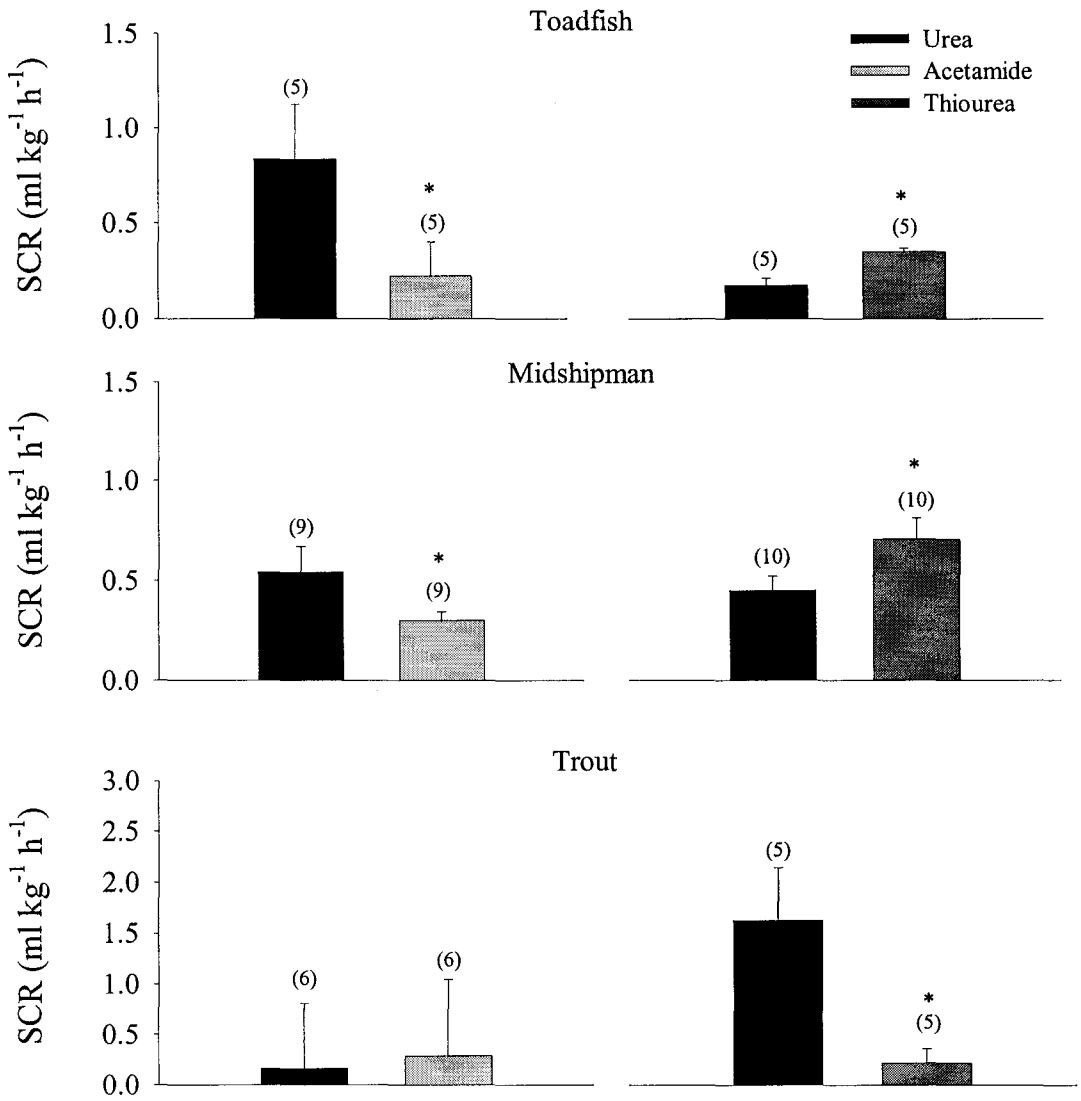

A Urea
B Acetamide
C Urea
D Thiourea
Permeability (cm sec-1 x 10-6)
Non-Pulsing
Pulsing
*
†
0
2
4
6
8
10
12
14
16

Figure 8-3: The secretion clearance rates (SCR; ml kg^{-1} h^{-1}) of the kidney in toadfish, midshipmen and trout in the (left panels) acetamide series and the (right panels) thiourea series. Note that for aglomerular toadfish and midshipmen acetamide clearance was less than urea clearance, while thiourea clearance was greater than urea clearance. However, a different pattern of renal handling was observed in the glomerular trout in which urea clearance and acetamide clearance were not significantly different but thiourea clearance was significantly less than urea clearance. Values are means ± 1 S.E.M. (N); * $P < 0.05$ significantly different than urea.


Toadfish
Urea
Acetamide
Thiourea
SCR (ml kg-1 h-1)
1.5
1.0
0.5
0.0
(5)
*
(5)
(5)
*
(5)
Midshipman
SCR (ml kg-1 h-1)
1.5
1.0
0.5
0.0
(9)
*
(9)
(10)
*
(10)
Trout
SCR (ml kg-1 h-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(6)
(6)
(5)
*
(5)

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Appendix

Transport physiology of the urinary bladder in teleosts: a suitable model for renal urea handling?

Abstract

The transport physiology of the urinary bladder of both the freshwater rainbow trout (*Oncorhychus mykiss*) and the marine gulf toadfish (*Opsanus beta*) was characterized with respect to urea and the suitability of the urinary bladder as a model for renal urea handling was investigated. Through the use of the *in vitro* urinary bladder sac preparation, urea handling was characterized under control conditions and in the presence of pharmacological agents traditionally used to characterize urea transport such as urea analogues (thiourea, acetamide), urea transport blockers (phloretin, amiloride) and hormonal stimulation (arginine vasotocin; AVT). Na^+-dependence and temperature sensitivity were also investigated. Under control conditions the *in vitro* trout bladder behaved as *in vivo* demonstrating significant net reabsorption of Na^+, Cl^-, water, glucose and urea. Bladder urea reabsorption was not affected by pharmacological agents and, in contrast to renal urea reabsorption, was not correlated to Na^+. However, the trout bladder showed a 3-fold greater urea permeability compared to artificial lipid bilayers, a prolonged phase transition with a lowered E_a between 5°C and 14°C, and differential handling of urea and analogues, all suggesting the presence of a urea transport mechanism. The *in vitro* toadfish bladder did not behave as *in vivo,* showing significant net reabsorption of Na^+ but not of Cl^-, urea or water. As in the trout bladder,

pharmacological agents were ineffective. The toadfish bladder showed no differential transport of urea and analogues, consistent with a low permeability storage organ and intermittent urination. Our results therefore suggest the possibility of a urea transport mechanism in the urinary bladder of the rainbow trout but not the gulf toadfish. While the bladders may not be suitable models for renal urea handling, the habit of intermittent urination by ureotelic tetrapods and toadfish seems to have selected for a low permeability storage function in the urinary bladder.

Introduction

The teleost urinary bladder is a morphological extension of the kidney, originating from the mesonephric duct (Nishimura and Imai, 1982). The urinary bladder, like the kidney, selectively reabsorbs salt and water on a net basis (Fossat and Lahlou, 1977, 1979; Renfro, 1977; Marshall, 1986). For this reason, the urinary bladder has been used extensively as a model for renal tubular function in fish, though some difference in mechanisms between the two organs appear to exist. For example, the urinary bladders of both the freshwater rainbow trout (*Oncorhynchus mykiss*) and the marine gulf toadfish (*Opsanus beta*) have been documented to actively reabsorb Na^+ and Cl^- *in vivo* (Howe and Gutknecht, 1978; Curtis and Wood, 1991), complementing kidney function in both fish, aiding in salt conservation in the case of the trout, while contributing to water recovery in the case of the toadfish. However, this trend does not necessarily hold true for other solutes: in both trout and toadfish, for example, Mg^{++} is actively secreted into the kidney tubule but not into the bladder (Beyenbach and Kirschner, 1975; Howe and Gutknecht, 1978).

In amphibians, the urinary bladder has a different embryological origin than in teleosts, being of endodermal origin (Phillips, 1975). Urea is an important osmolyte in these animals, and in many species of frogs and toads, facilitated diffusion urea transporters (UT), similar to those found in mammalian models (Brahm, 1983; Chou and Knepper, 1989; Chou et al. 1990) have been discovered in the urinary bladder and characterized both on a physiological level and a molecular level (Eggena, 1973; Levine et al. 1973a,b; Ardizzone and Lippe, 1982; Shpun and Katz, 1990; Martial et al. 1991;

Zhang and Verkman, 1991; Couriaud et al. 1999). In the toads *Bufo marinus*, *Bufo viridis* and *Bufo bufo*, urea transport by the urinary bladder is stimulated by the hormone arginine vasotocin (AVT; analogous to the mammalian arginine vasopressin), which is thought to induce or activate more urea transporters or channels of the facilitated diffusion type (*ie.* increasing V_{max}) without changing the affinity (K_m) to urea (Levine et al. 1973a; Shpun and Katz, 1989). Such UT-type transporters are typically difficult to saturate, although saturation kinetics have been observed in some cases (Levine et al. 1973a; Imai et al. 1988; Shpun and Katz, 1989; Shpun and Katz, 1990; Chou et al. 1990). Facilitated diffusion of urea in the amphibian urinary bladder does not depend on the presence of Na^+ and is inhibited both by the urea transport blocker, phloretin and by urea analogues, which are thought to compete for a common site on the membrane carrier molecule (Eggena, 1973; Levine et al. 1973a,b; Shpun and Katz, 1990).

In contrast, urea transport by the mesonephros-derived urinary bladder of teleost fish has yet to be studied. However, recent evidence suggests that carrier-mediated transport of urea occurs in the kidneys of both rainbow trout, an ammoniotelic species (McDonald and Wood, 1998), and gulf toadfish, a species which is facultatively ureotelic (Chapter 2). In the kidney of the trout, which forms primary urine by glomerular filtration, urea is reabsorbed against a concentration gradient, suggesting an active transport mechanism (McDonald and Wood, 1998). When infused with exogenous urea loads, net renal urea reabsorption in trout increases in direct proportion to the filtered urea load, and saturation of the reabsorptive mechanism is not apparent, even at urea filtration rates well beyond physiological (McDonald and Wood, 1998). Furthermore,

analysis of *in vivo* data (M.D. McDonald and C.M.Wood, unpublished results) from the same study suggests that urea reabsorption and sodium reabsorption are closely correlated in the kidney of the trout. In the kidney of the toadfish, which forms primary urine largely by secretion, a similar net uphill movement of urea is observed, but in the direction of secretion (Chapter 2). Moreover, in both fish, the kidneys have been shown to preferentially transport urea rather than analogues, namely acetamide and thiourea (Chapter 2, 5). However, the patterns of renal handling of these substances show distinct interspecific differences: the trout kidney transports urea and acetamide similarly, but not thiourea, while the toadfish kidney transports urea and thiourea similarly but not acetamide. As a consequence of the similarities in embryonic development and function between the urinary bladder and kidney in teleosts, the presence of a urea transport mechanism(s) in the urinary bladder, similar to that found in the kidney, is a distinct possibility.

The objectives of this study were twofold. Our first goal was to characterize the transport physiology of the urinary bladder of both the rainbow trout and the gulf toadfish with respect to urea. Secondly, we set out to determine whether the urinary bladder is a suitable model for renal handling of urea. Our results provide evidence for a urea transport mechanism in the urinary bladder of the rainbow trout but not the gulf toadfish, but indicate that the urinary bladders of neither fish are suitable models for renal urea handling.

Materials and Methods

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Farm in Mono Mills, Ontario. The fish were acclimated to seasonal water temperatures (11°C - 14°C) and were fed with commercial trout pellets every second day until the time of surgery. Acclimation was carried out in dechlorinated Hamilton tapwater [in $mmol \cdot l^{-1}$: Ca^{++} = 1.8; Cl^- = 0.8; Na^+ = 0.6; Mg^{++} = 0.5; K^+ = 0.04; titration alkalinity (to pH 4.0) = 1.9; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0]. Gulf toadfish (*Opsanus beta*) were caught by commercial shrimpers in Biscayne Bay, Florida in November and December. The toadfish were held in outdoor tanks at the shrimpers' holding facility with running seawater (ambient seasonal conditions; 30-32 ‰, 22-26°C) for no longer than 24 hours following capture, then transferred to the laboratory. Fish were treated with a dose of malachite green (final concentration 0.5 mg l^{-1}) in formalin (15 mg l^{-1}) (Aquavet, Hayward, CA, USA) on the day of transfer to the laboratory in order to prevent infection by the cilate, *Cryptocaryon irratans* (Stoskopf, 1993). Initially the fish were kept in 50 L glass aquaria with flowing, aerated seawater. Fish were maintained in this relatively crowded environment (> 15 fish per tank) in order to initiate a switch to ureotelism (Walsh et al. 1994). Fish were fed squid weekly up until surgery.

Bladder Sac Experiments

Rainbow trout and gulf toadfish were terminally anaesthetized using MS-222 (1 g l^{-1}). The peritoneal cavity was cut open, the ureter end of the bladder was tied with one

3.0 silk ligature and the urinary bladder was removed carefully from the body cavity. In the case of the gulf toadfish, which has a bi-lobed bladder, the larger of the two lobes was used for all experiments. A heat-flared catheter, made out of Clay-Adams PE 50 tubing, was advanced through the small opening at the papilla-end of the bladder and held in place by two or three 3.0 silk ligatures secured tightly around the bladder. The ligatures were tested for leaks by injecting a small amount of saline. The urinary bladder was then filled with saline (Cortland saline for trout; Wolf, 1963 (in $mmol \cdot l^{-1}$) : 124.0 NaCl, 5.1 KCl, 1.6 $CaCl_2$, 0.9 $MgSO_4$, 11.9 $NaHCO_3$, 2.9 NaH_2PO_4 and 5.6 Glucose; Hank's saline for toadfish; Walsh, 1987 (in $mmol \cdot l^{-1}$) : 151.0 NaCl, 5.9 KCl, 0.9 $MgSO_4$, 0.46 Na_2HPO_4, 0.48 KH_2PO_4, 5.0 $NaHCO_3$, 11.0 Hepes, 1.0 $CaCl_2$ and 3.0 Glucose), containing typical plasma urea concentrations for each respective fish (1.8 $mmol \cdot l^{-1}$ urea for trout under resting conditions; McDonald and Wood, 1998; 7.0 $mmol \cdot l^{-1}$ urea for toadfish held in crowded conditions; Wood et al. 1995) plus 1 μCi of [^{14}C]-urea (57.0 mCi $mmol^{-1}$; Amersham). The radio-labeled urea on the mucosal side allows unidirectional urea movement to be monitored. Every bladder was suspended individually in a 20 ml glass vial containing 10 ml of an identical non-radioactive saline and left to incubate 3-4 hours. The solution bathing the serosal surface of the bladder contained the same concentration of urea and was bubbled with a 99.7 % O_2: 0.3% CO_2 mix gas (P_{CO2} = 1.7 torr) and left at ambient holding water temperature for each respective fish. Typical saline pH was 7.6 for both trout and toadfish.

Control series were performed simultaneously with all experimental treatments. Experimental treatments involved filling the bladder with the aforementioned saline plus

a given amount of urea analogue (thiourea = 10 $mmol \cdot l^{-1}$ for trout and 20 $mmol \cdot l^{-1}$ for toadfish; acetamide = 10 $mmol \cdot l^{-1}$ for trout), hormone (arginine vasotocin (AVT; the fish homologue of arginine vasopressin = 10^{-9} $mol \cdot l^{-1}$) or inhibitor (phloretin = 0.1 $mmol \cdot l^{-1}$ in 0.04% ethanol for trout and 0.250 $mmol \cdot l^{-1}$ in 0.5% DMSO for toadfish; amiloride = 0.1 $mmol \cdot l^{-1}$ in 0.02% DMSO for trout). All reported concentrations of analogue, hormone, inhibitor and vehicle were final concentrations in saline. Analogue concentrations were much higher than urea concentrations to ensure a competitive interaction between them and urea. In the inhibitor experiments, the vehicle alone (ethanol, DMSO) was tested at the same concentration in the respective control series. The effect of AVT (10^{-9} $mol \cdot l^{-1}$) on thiourea-exposed (10 $mmol \cdot l^{-1}$) bladders of trout was investigated by combining these two treatments. In the above-mentioned experimental groups, both mucosal and serosal salines were identical, aside from [^{14}C]-urea on the mucosal side.

The possibility of a Na^+-dependent urea transport mechanism was investigated by removing Na^+ from the saline on the mucosal side (trout Na^+-free saline (in $mmol \cdot l^{-1}$): 124.0 N-methyl-D-glucamine, 2.1 KCl, 1.6 $CaCl_2$, 0.9 $MgSO_4$, 11.9 $C_5H_{14}NO \cdot HCO_3$, 2.9 K_2HPO_4, 5.6 Glucose, 1.8 Urea, pH balanced to 7.6 with HCl; toadfish Na^+-free saline (in $mmol \cdot l^{-1}$): 156.9 N-methyl-D-glucamine, 0.9 $MgSO_4$, 0.46 K_2HPO_4, 0.48 KH_2PO_4, 5.0 $KHCO_3$, 11.0 Hepes, 1.0 $CaCl_2$, 3.0 Glucose, 7.0 Urea, pH balanced to 7.6 with HCl), ensuring that the Na^+-free solution on the mucosal side had the same concentration of urea and the same osmolality as the serosal bath. The effect of temperature was examined by altering the incubation temperature of separate groups of bladders. The effect of a

concentration gradient was investigated by placing the bladder in a serosal bath with the urea replaced by NaCl, ensuring the same osmolality on both sides of the bladder.

The handling of thiourea and acetamide by the urinary bladder of trout and toadfish was evaluated by replacing the cold and radio-labeled urea (1.8 $mmol \cdot l^{-1}$ in trout, 7.0 $mmol \cdot l^{-1}$ in toadfish plus 1 μCi [^{14}C]-urea) in the saline with analogue (thiourea or acetamide) at the same concentration plus 1μCi of [^{14}C]-thiourea (19.8 mCi $mmol^{-1}$; Sigma-Aldrich Canada) or [^{14}C]-acetamide (50 mCi $mmol^{-1}$; American Radioactive Chemicals).

Net urea flux ($J_{net\ urea}$) was measured following a procedure similar to that outlined by Hirano et al. (1973) where $J_{net\ urea}$ was calculated from the difference in initial and final mucosal urea concentrations and mucosal saline volume. Unidirectional urea flux ($J_{in\ urea}$) in the direction of reabsorption was measured following a protocol by Eggena (1973) and Levine et al. (1973a,b). Briefly, serosal samples were taken at 0.5 h intervals throughout the incubation period and monitored for the appearance of [^{14}C]-urea. Unidirectional analogue flux rate was monitored in a similar fashion in experiments using [^{14}C]-acetamide or [^{14}C]-thiourea. Net water movement was measured gravimetrically by weighing the bladders (after blotting away excess fluid) at the beginning and end of the 3-4h experiments.

For measurements of unidirectional urea flux ($J_{out\ urea}$) in the direction of secretion, 2 μCi of [^{14}C]-urea was placed in the serosal bath and initial and final mucosal samples were taken to monitor the appearance of [^{14}C]-urea at the inner surface.

Analytical Techniques and Calculations

Urea concentrations in serosal and mucosal solutions were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges and correction for the presence of thiourea and acetamide. This correction was done by adding the concentration of analogue present in the saline in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. For measurements of [^{14}C]-urea, [^{14}C]-thiourea or [^{14}C]-acetamide, samples (25 μl) were added to 4 ml of Ecolume fluor and analyzed by β-scintillation counting (LKB Rackbeta 1217 counter). Tests demonstrated that quench was constant because the samples were counted in the same matrix, so cpms were used directly in all calculations. Bladder surface area was determined by making a ventral cut along the bladder, spreading it on graph paper and tracing it carefully. Osmolality was determined using a Wescor Inc. 5100C Vapor Pressure Osmometer. Na^+ and Cl^- concentrations were measured using a Varian 1275 atomic absorption spectrophotometer and a Radiometer CMT10 chloridometer respectively. Glucose was measured enzymatically (hexokinase, glucose-6-phosphate dehydrogenase) using a commercial kit (Sigma-Aldrich Canada).

The concentration ratio of X (CR_X) was calculated from experiments where [^{14}C]-urea was placed initially on both sides of the bladder in equal concentrations:

$$CR_X = \frac{[X]_m}{[X]_s}, \qquad (1)$$

where $[X]_m$ and $[X]_s$ are the final mucosal and serosal concentrations of X, respectively, after 3-4 hours of incubation. A final ratio that was significantly less than 1.0 indicated net reabsorption independent of water movement, a ratio greater than 1.0 indicated net secretion independent of water movement and a ratio equal to 1.0 indicated no net transport independent of water movement. Only in calculating CR_{urea} was [^{14}C]-urea used on both sides of the bladder for increased sensitivity.

Net flux rates of water, urea and other substances were determined by an approach similar to that of Hirano et al. (1973). The net flux rate of water ($J_{net\ water}$) in ml $cm^{-2}\ h^{-1}$ for bladder sacs was calculated using:

$$J_{net\ water} = \frac{\Delta V}{A \times t}, \qquad (2)$$

where ΔV is the change in volume estimated by the change in weight between the beginning and end of the experiment in ml, A is the surface area of the urinary bladder in cm^2 and t is the time in hours.

The net flux rate of X ($J_{net\ X}$) in μmol $cm^{-2}\ h^{-1}$ in isolated bladder sacs was found by calculating the change in concentration of substance X before and after incubation with respect to mucosal volume as follows:

$$J_{net\ X} = \frac{([X]_i \times V_i) - ([X]_f \times V_f)}{A \times t}, \qquad (3)$$

where $[X]_i$ and $[X]_f$ are the initial and final concentrations in μmol ml^{-1}, V_i and V_f are the initial and final mucosal volumes in ml, A is the bladder surface area in cm^2 and t is the time in hours.

Preliminary tests showed that there were small but detectable changes in the internal specific activity (SA) which followed a simple exponential decline over the course of the experiment. Unidirectional flux rate of X ($J_{in\,X}$) in the direction of reabsorption in μmol cm^{-2} h^{-1} in isolated bladder sacs was therefore determined by first calculating the specific activity (SA) on the mucosal side of the bladder at different times during the experiment. The initial and final SA could be calculated directly by dividing the measured amount of radioactivity (cpm) on the mucosal side at the beginning and end of the incubation period by the respective unlabelled X concentration at those two time periods.

$$SA = \frac{cpm_m}{[X]}, \qquad (4)$$

where cpm_m is the radioactivity in cpm ml^{-1} measured on the mucosal side and $[X]$ is the concentration of unlabelled substance X on the mucosal side in μmol ml^{-1}. The mucosal SA's at intermediate times during the experiment (between the initial and final mucosal sampling periods when mucosal samples were not taken) were approximated by fitting an exponential regression against time to the initial and final mucosal SA's (Shaw, 1959).

Unidirectional flux rate of X ($J_{in\,X}$) in the direction of reabsorption in isolated bladder sacs for any period was then calculated as follows:

$$J_{in\,X} = \frac{(cpm_s^{\,b} \times V^b) - (cpm_s^{\,a} \times V^a)}{t \times SA \times A}, \qquad (5)$$

where $cpm_s^{\,a}$ and $cpm_s^{\,b}$ are the radioactivities per ml measured at time 1 and time 2 respectively in serosal samples, V^a and V^b are the volumes of the serosal bath at times 1 and 2 (taking removed sample volumes into account) in ml, t is the time between samples in hours, SA is the specific activity in cpm μmol^{-1} at that given sample period, and A is the bladder surface area in cm^2.

The permeability (P) of the bladder to a substance X in cm s^{-1} was calculated using the following equation:

$$P_X = \frac{\Delta cpm_s \times V_s}{cpm_m \times T \times A}, \qquad (6)$$

where Δcpm_s is the change of radioactivity between two successive samples measured on the serosal side (cold side) in cpm ml^{-1}, cpm_m is the mean radioactivity measured on the mucosal side (hot side) in cpm ml^{-1}, V_s is the volume in ml (or cm^3) on the serosal side, T is the time between the two samples in seconds and A is the bladder surface area in cm^2.

The transient urea concentration (T_{urea}) in mmol·l^{-1} was calculated by dividing $J_{net\ urea}$ in $\mu mol\ cm^{-2}\ h^{-1}$ by $J_{net\ water}$ in ml $cm^{-2}\ h^{-1}$ at a given sample period as follows:

$$T_{urea} = \frac{J_{net\ urea}}{J_{net\ water}}. \qquad (7)$$

The Q_{10} factor is defined as the ratio of two rates for a 10°C difference in temperature and was calculated as follows:

$$Q_{10} = \frac{J_{K2}^{(10/(K2-K1))}}{J_{K1}}, \quad (8)$$

where J_{K1} and J_{K2} are flux rates at a low and high temperature respectively in μmol cm^{-2} h^{-1} and K1 and K2 are the low and high temperatures respectively in °C. The Q_{10} factor for a physical process such as diffusion is about 1. Q_{10} factors for biochemical reactions including carrier-mediated transport are typically 2 to 3 (Withers, 1992).

An Arrhenius plot displays the natural logarithm of transport rate *versus* the inverse of temperature in degrees Kelvin (K^{-1}), the slope of which is equal to:

$$m = \frac{-E_a}{R}, \quad (9)$$

where m is the slope of the line, E_a is the activation energy in J mol^{-1} and R is the gas constant (8.314 J K^{-1} mol^{-1}). Using this equation, the activation energy of a biological system can be calculated.

Statistical Analysis

Data have been reported as means ± S.E.M. (N = number of bladders). Regression lines have been fitted by the method of least squares, and the significance (P <0.05) of the Pearson's correlation coefficient r assessed. The significance of difference between means was evaluated using Student's paired, unpaired or one-sample two-tailed t-test (P <0.05) as appropriate, with the Bonferroni correction (Nemenyi et al. 1977) for multiple comparisons.

Results

Rainbow Trout

Under control conditions, the isolated urinary bladder of the rainbow trout incubated *in vitro* demonstrated net flux (J_{net}; positive values signifying reabsorption) of urea (0.019 ± 0.003 (39) μmol cm^{-2} h^{-1}) and water (0.012 ± 0.002 (39) ml cm^{-2} h^{-1}); $J_{net\ urea}$ and $J_{net\ water}$ were both significantly different from zero (Fig. A-1A). Unidirectional urea flux ($J_{in\ urea}$; 0.098 ± 0.029 (39) μmol cm^{-2} h^{-1})) from the bladder was 5-fold greater than simultaneous $J_{net\ urea}$ (0.019 ± 0.003 (39) μmol cm^{-2} h^{-1}) indicating a substantial backflux component to urea movement. Similar to observations *in vivo*, the isolated bladder demonstrated substantial net reabsorption of Na^+ (2.17 ± 0.17 (20) μmol cm^{-2} h^{-1}), Cl^- (1.92 ± 0.28 (11) μmol cm^{-2} h^{-1}) and glucose (0.71 ± 0.26 (8) μmol cm^{-2} h^{-1}; Fig. A-1B). Calculated concentration ratios for urea (0.98 ± 0.01 (6)) and glucose (0.91 ± 0.07 (6)) at the end of the 3-4 h flux periods were not significantly different than 1.0 indicating that there was no net movement of these two substances independent of water movement under symmetrical conditions (Fig. A-1C). In contrast, the final concentration ratios for both Na^+ (0.59 ± 0.04 (6)) and Cl^- (0.57 ± 0.05 (6)) were significantly less than 1.0 ($P < 0.05$), indicating a significant reabsorption that is independent of water movement.

The average urea permeability of the sample population of control bladders was 10.8 ± 0.8 (40) x 10^{-6}cm s^{-1}. This permeability is 3-fold higher than the theoretical

permeability of urea through artificial lipid bilayers (3.7 ± 0.6 (6) x 10^{-6} cm s^{-1}; Galluci et al. 1971) and suggests the presence of a urea transport mechanism in the bladder of the rainbow trout.

Despite this higher permeability, none of the pharmacological agents traditionally used to characterize urea transport mechanisms had any significant effect on $J_{in\ urea}$, $J_{net\ urea}$ or $J_{net\ water}$ (Table A-1). The pharmacological agents used in this study were placed on both sides of the bladder and can be grouped into three categories. The first category consisted of urea analogues (thiourea; 10 mmol·l^{-1} and acetamide; 10 mmol·l^{-1}) in 5-6 fold excess of urea concentrations. The second category contained potential urea transport blockers (amiloride; 0.1 mmol·l^{-1} and phloretin; 0.1 mmol·l^{-1}) and the third category consisted of a hormone (AVT; 10^{-9} mol·l^{-1}) that could potentially stimulate facilitated diffusion of urea. The effect of AVT when urea movement was being "inhibited" by the presence of thiourea was also tested. None of the treatments had significant effects.

In agreement with the lack of amiloride influence, there was also no change in $J_{in\ urea}$, $J_{net\ urea}$ or $J_{net\ water}$ when Na^+ was removed from the mucosal side of the isolated bladder indicating that urea movement is not dependent on Na^+ (Table A-1).

Isolated bladders from fish acclimated to 11°C-14°C appeared to have a range of homeostasis for the transport of urea and water with respect to acute changes in temperature, with $J_{in\ urea}$, $J_{net\ urea}$ and $J_{net\ water}$ remaining relatively unchanged from 5-20°C (Fig. A-2). However, the bladders were sensitive to extreme temperatures, showing a significant decrease in $J_{in\ urea}$ when exposed to 0°C (0.049 ± 0.005 (6) μmol cm^{-2} h^{-1}) and

a significant increase in $J_{in\ urea}$ when exposed to 30°C (0.150 ± 0.015 (6) μmol cm^{-2} h^{-1}) when compared to $J_{in\ urea}$ at control temperatures (0.080 ± 0.011 (6) μmol cm^{-2} h^{-1}; Fig A-2A). The Q_{10} from 0°C to 5°C was 2.4 and the Q_{10} from 20°C to 30°C was only 1.5. An Arrhenius plot of these data showed a prolonged phase transition with a lowered E_a of 0.4 KJ mol^{-1} between 5°C and 14°C (Fig. A-2B). A similar trend in temperature sensitivity was also observed in measured $J_{net\ urea}$ and $J_{net\ water}$ values but was only significant for fluxes at 30°C (Fig. A-2C, 2D).

Isolated bladders exposed to a 1.8 mmol·l^{-1} urea concentration gradient achieved by removing urea on the serosal side showed a significantly greater $J_{net\ urea}$ (0.054 ± 0.009 (6) μmol cm^{-2} h^{-1}) compared to controls (0.011 ± 0.003 (6) μmol cm^{-2} h^{-1}; Fig. A-3A). There was no significant change in $J_{in\ urea}$. The gradient conditions essentially removed the backflux component of urea movement (urea was unavailable on the serosal side to move back into the bladder), consequently $J_{in\ urea}$ (0.052 ± 0.011 (6) μmol cm^{-2} h^{-1}) and $J_{net\ urea}$ (0.054 ± 0.009 (6) μmol cm^{-2} h^{-1}), although measured independently, became equal. There was no effect on the movement of water.

The "transient urea concentration" (6.47 ± 1.04 (6) mmol·l^{-1}) of these gradient-exposed bladders was 3-fold greater than the concentration of urea measured initially in the mucosal solution (2.11 ± 0.02 (6) mmol·l^{-1}; Fig. 3B). This difference in urea concentration between the "transient solution" and the mucosal solution was not evident in isolated bladders from any other treatment group (Fig. A-3B).

In support of carrier-mediated transport, when radiolabelled analogues were all tested at the same concentration (1.8 mmol·l^{-1}) in separate experiments, the urinary

bladder preferentially transported urea over both acetamide and thiourea. $J_{in\ urea}$ (0.098 ± 0.029 (39) μmol cm^{-2} h^{-1}) was significantly greater than $J_{in\ acetamide}$ (0.030 ± 0.007 (6) μmol cm^{-2} h^{-1}) and to an even greater extent $J_{in\ thiourea}$ (0.014± 0.003 (6) μmol cm^{-2} h^{-1}; Fig. A-4). Correspondingly, the permeability of the bladder to urea (P_{urea}; 10.8 ± 0.8 (40) x 10^{-6} cm s^{-1}) was 2.5-fold greater than $P_{acetamide}$ (4.3 ± 0.9 (6) x 10^{-6} cm s^{-1}) and 3.4-fold greater than $P_{thiourea}$ (2.3 ± 0.5 (6) x 10^{-6} cm s^{-1}).

Gulf Toadfish

Under control conditions, the isolated urinary bladder of the gulf toadfish demonstrated unidirectional urea flux rates ($J_{in\ urea}$; positive values signifying reabsorption) of 0.125 ± 0.020 (33) μmol cm^{-2} h^{-1} which were much greater than net urea flux rates ($J_{net\ urea}$; -0.039 ± 0.020 (32) μmol cm^{-2} h^{-1}; Fig. A-5A). In most cases, $J_{net\ urea}$ was not significantly different than zero indicating that the backflux component to urea movement was equal to $J_{in\ urea}$. $J_{net\ water}$ (-0.003 ± 0.002 (32) ml cm^{-2} h^{-1}) was not significantly different from zero. Similar to observations *in vivo*, the isolated bladder demonstrated net reabsorption of Na^+ (0.75 ± 0.33 (20) μmol cm^{-2} h^{-1}) and Cl^- (0.43 ± 0.21 (19) μmol cm^{-2} h^{-1}), although the latter was not significantly different than zero (Fig. A-5B). Final concentration ratios of urea (0.98 ± 0.01 (8)), Na^+ (1.04 ± 0.07 (8)) and Cl^- (1.08 ± 0.04 (8)) were not significantly different than 1.0 indicating that there was no net movement of these three substances under symmetrical conditions independent of the movement of water (Fig. A-5C).

The average urea permeability of a sample population of control toadfish bladders was 8.0 ± 1.2 (34) cm s^{-1} x 10^{-6}, which was not significantly different from the value for the trout bladder. This measured permeability is 2.2-fold greater than the theoretical permeability of urea through artificial lipid bilayers (3.7 ± 0.6 (6) x 10^{-6} cm s^{-1}; Galluci et al. 1971), which again may suggest carrier-mediated transport of urea by the urinary bladder.

However, similar to the trout bladder, pharmacological agents traditionally used to characterize urea transport mechanisms did not affect $J_{in\ urea}$, $J_{net\ urea}$ or $J_{net\ water}$ in the bladder of the gulf toadfish (Table A-2). Again, three main treatments were used. A urea analogue (thiourea; 20 mmol·l^{-1}) in 3-fold excess of urea concentrations, a urea transport blocker (phloretin 0.250 mmol·l^{-1}), and the hormone AVT (10^{-9} mol·l^{-1}) were investigated. All pharmacological agents were placed on both sides of the bladder. Furthermore, removing Na^+ from the mucosal side of the bladder had no effect on $J_{in\ urea}$ (0.122 ± 0.038 (13) μmol cm^{-2} h^{-1}) when compared to controls (0.101 ± 0.037 (13) μmol cm^{-2} h^{-1}). Na^+-free conditions did not affect either $J_{net\ urea}$ or $J_{net\ water}$.

When urea was removed from the serosal side of six bladders, creating a urea concentration gradient of approximately 7 mmol·l^{-1}, there was no effect on $J_{in\ urea}$ (0.244 ± 0.051 (6) μmol cm^{-2} h^{-1}) when compared to simultaneous controls (0.143 ± 0.066 (7) μmol cm^{-2} h^{-1}). However, there was a significant increase in $J_{net\ urea}$ (0.252 ± 0.102 (6) μmol cm^{-2} h^{-1}), compared to controls (0.004 ± 0.009 (7) μmol cm^{-2} h^{-1}). Note that as in trout, $J_{net\ urea}$ in gradient exposed bladders became essentially equal to $J_{in\ urea}$. There was no effect on the movement of water. Consistent with the variability of $J_{net\ urea}$ and $J_{net\ water}$

measured in both experimental and control toadfish bladders, the calculated concentrations of urea in the transient solution were also exceedingly variable. For that reason, no significant differences were observed between urea concentrations in the transient fluid (overall control means: 10.19 ± 6.12 (26) $mmol \cdot l^{-1}$) *versus* those in the initial mucosal solution (control: 7.07 ± 0.13 (27) $mmol \cdot l^{-1}$) in any of the experimental treatments, including the gradient experiment (data not shown).

Isolated bladders from toadfish acclimated to 26°C were sensitive to temperatures both above and below the temperature of acclimation (Fig. A-6A). Bladders exposed to 15°C and 20°C showed a increase in $J_{in\ urea}$ (0.458 ± 0.083 (6) $\mu mol\ cm^{-2}\ h^{-1}$ and 0.254 ± 0.004 (6) $\mu mol\ cm^{-2}\ h^{-1}$ respectively) compared to control bladders incubated at 26°C (0.126 ± 0.021 (6) $\mu mol\ cm^{-2}\ h^{-1}$). Bladders incubated at 30°C demonstrated an elevated $J_{in\ urea}$ (0.239 ± 0.035 (6) $\mu mol\ cm^{-2}\ h^{-1}$) compared to controls by a Q_{10} factor of 4.6. In addition, bladders incubated at 35°C had a greatly elevated $J_{in\ urea}$ (1.690 ± 0.533 (6) $\mu mol\ cm^{-2}\ h^{-1}$), greater than control values by a Q_{10} factor of 17.3. An Arrhenius plot of these data showed a sharp phase transition at 26°C (Fig. A-6B). The same trend in temperature sensitivity is evident in $J_{net\ urea}$ however, these observations were not significant (Fig. A-6C, A-6D).

When serosal to mucosal urea flux ($J_{out\ urea}$) was examined in a separate series, measured $J_{out\ urea}$ (-0.041 ± 0.008 (6) $\mu mol\ cm^{-2}\ h^{-1}$) was not significantly different than $J_{in\ urea}$ (0.146 ± 0.063 (6) $\mu mol\ cm^{-2}\ h^{-1}$), emphasizing zero net urea movement by the urinary bladder of the toadfish.

In contrast to the trout urinary bladder, the toadfish urinary bladder sac did not differentiate between urea, thiourea and acetamide. $J_{in\ urea}$ (0.125 ± 0.020 (33) μmol cm^{-2} h^{-1}) was not significantly different than either $J_{in\ thiourea}$ (0.131± 0.036 (6) μmol cm^{-2} h^{-1}) or $J_{in\ acetamide}$ (0.210 ± 0.075 (7) μmol cm^{-2} h^{-1}; Fig. A-7). Likewise, P_{urea} (8.0 ± 1.2 (34) x 10^{-6} cm s^{-1}) was not significantly different than $P_{thiourea}$ (5.8 ± 1.3 (6) x 10^{-6} cm s^{-1}) nor $P_{acetamide}$ (6.8 ± 2.1 (7) x 10^{-6} cm s^{-1}).

Discussion

The objectives of this study were to investigate the transport physiology of the urinary bladder in the gulf toadfish and rainbow trout and to determine whether the urinary bladder would make a suitable model for renal urea handling in these two fish. Our results suggest the presence of a urea transport mechanism in the urinary bladder of the rainbow trout but in not the gulf toadfish and indicate that the urinary bladders of neither fish are suitable models for the handling of urea by the kidney. The bladder of the trout shows a net reabsorption of urea when exposed to symmetrical conditions, *i.e.*, in the absence of osmotic or electrochemical gradients. In addition, when the backflux component was eliminated, it was apparent that urea flux was not associated with water flux; the urea concentration in fluid leaving the bladder ("the transient concentration") was 3-fold more concentrated than the initial mucosal solution. Thus, urea reabsorption is not a consequence of solvent drag. Furthermore, the trout urinary bladder demonstrates an ability to differentiate between urea and the urea analogues, thiourea and

acetamide, showing a specificity towards urea transport, P_{urea} being greater than $P_{thiourea}$ and $P_{acetamide}$.

The urinary bladder of the rainbow trout plays a significant role in salt and urea reabsorption *in vivo*; urinary excretion rates of Na^+, Cl^- and urea are significantly lower for resting fish compared those whose bladder function is by-passed by internal urinary catheterization (Curtis and Wood, 1991). The net rates of reabsorption for Na^+ and Cl^- determined *in vivo* by Curtis and Wood (1991) were 0.7-2.5 μmol cm^{-2} h^{-1}, thus comparable to the rates determined using the *in vitro* bladder sac preparation (1.9-2.2 μmol cm^{-2} h^{-1}) in the present study. However, the net rate of reabsorption of urea *in vivo* (approximately 0.2 μmol cm^{-2} h^{-1}) was approximately double the rate obtained *in vitro* (0.098 μmol cm^{-2} h^{-1}). Despite this small inconsistency, it appears in the case of the trout that the *in vitro* bladder sac preparation functions similarly to the *in vivo* bladder.

Using this *in vitro* preparation, we determined that the urinary bladder probably does not function similarly to the kidney *in vivo* with respect to urea handling. Renal urea reabsorption measured in resting trout showed a linear proportional relationship to the reabsorption of Na^+ (slope = 0.011 μmol-urea kg^{-1} h^{-1} / μmol-Na^+ kg^{-1} h^{-1}) that is well correlated (r^2 = 0.76; unpublished data from the study of McDonald and Wood, 1998). These data suggest that a Na^+-coupled transport mechanism may exist in the kidney of the rainbow trout, similar to that observed in mammalian inner medullary collecting ducts and elasmobranch kidney tubules (Schmidt-Nielsen et al. 1972; Isozaki et al. 1994a,b). In contrast, reabsorption of urea by the *in vitro* bladder sac was not affected when Na^+

was removed from the mucosal bath (Table 1). Thus, unlike the kidney, there is no correlation between urea and Na^+ movement through the urinary bladder, thereby eliminating the possibility that urea transport is Na^+-coupled in this organ.

The measured permeability of the trout bladder for urea (10.8 ± 0.8 cm s^{-1} x 10^{-6}) was 3-fold higher than that observed in artificial lipid bilayers that lack transport proteins to facilitate urea movement (Galluci et al. 1971). The permeability of urea through human red blood cell membranes (1.2 x 10^{-3} cm s^{-1}; RBC), in which a UT-B transporter is present, is approximately 1000-fold higher than that observed in artificial membranes (Mayrand and Levitt, 1983). However, the permeability of urea through vasopressin-stimulated, UT-A1 transport mechanisms is generally much lower than that measured in RBC's. In fact, the urinary bladders of amphibians, under control conditions, have measured permeabilities for urea ranging from 7.7 to 12.7 x 10^{-6} cm s^{-1}, in the same range as the current measurements for the trout bladder and at most 3-fold greater than membranes without transport proteins (Levine et al. 1973a,b; Eggena et al. 1973; Levine and Worthington, 1976; Sphun and Katz; 1990). In addition, only a 2.5-fold rise in permeability has been noted in *Xenopus* oocytes injected with the branchial facilitated diffusion urea transporter (a UT-A type transporter) of the Lake Magadi tilapia (mtUT; Walsh et al. 2001). Therefore, the measured permeability of the trout urinary bladder for urea is comparable with permeabilities observed in other systems when UT-A type transporters are present.

In contrast to the transporter of the amphibian bladder, the transporter believed to be present in the trout bladder is not AVT-sensitive. Then again, not all facilitated

diffusion mechanisms for urea demonstrate AVT sensitivity. Although UT-A1 transporters (4.0 kDa mRNA) are stimulated by ADH (vasopressin in mammals, vasotocin in amphibians) and cAMP, UT-A2 transporters (2.9 kDa mRNA) are not (You et al. 1993; Smith et al. 1995; Shayakul et al. 1996). In addition, UT-A2 transport mechanisms are less sensitive to inhibition by urea analogues (Chou et al. 1990; You et al. 1993). The urinary bladder of the trout appeared not to be sensitive to urea analogues, even at analogue concentrations that were 5x greater than urea concentrations. To date, urea transport mechanisms in teleost fish are not well defined. Although UT transporters in teleosts demonstrate a high amino acid sequence homology to mammalian and amphibian UT transporters (> 60%; Walsh et al. 2000; 2001), they show unconventional sensitivity and regulation patterns and may be controlled by entirely different mechanisms than UT transporters in other systems (Wood et al. 2001).

The trout urinary bladder shows a differential permeability towards urea, acetamide and thiourea, P_{urea} being 2.5-fold greater than $P_{acetamide}$ and 3.4-fold greater than $P_{thiourea}$. Thiourea and urea show similar permeabilities through artificial lipid bilayers while the permeability of acetamide through artificial bilayers is greater than both of these substances (Galluci et al. 1971). Differential handling of urea and thiourea is an observation consistent with the handling of these substances by the trout kidney *in vivo* (Chapter 5). In the same *in vivo* experiment, similar handling of acetamide and urea by the trout kidney was observed, contrary to the handling of these substances by the urinary bladder, and giving further evidence that the bladder is not an appropriate model for the kidney. Differential handling of urea *versus* both thiourea and acetamide has been

observed by the branchial facilitated diffusion transporter (tUT) of the gulf toadfish, which has a branchial clearance of urea that is greater than acetamide and much greater than thiourea (Chapter 2). The ability of the urinary bladder to distinguish between urea and both these substances that are similar to urea in size and molecular weight suggests the presence of a carrier protein that is specific for urea.

The trout bladder shows a discontinuity in temperature dependence with unidirectional urea flux rate not significantly changing between 5°C and 14°C. Below 5°C and above 14°C, Q_{10} values were consistent with carrier-mediated transport. Pure phospholipids are known to exhibit sharp phase transitions due to temperature, whereas more complex phospholipid membranes exhibit prolonged thermal transitions (Linden et al. 1973). A prolonged thermal transition such as that observed in the trout bladder from 5°C to 14°C is defined by a distinct beginning and end, marking the beginning (t_l) and end (t_h) of the process of lateral phase separation. Often a lower activation energy for transport is observed during phase separation, as the energy required for the insertion of a transport-associated protein into a membrane in this phase is small (Linden et al. 1973). At temperatures below t_l, the activation energy for transport is again large.

In contrast to the trout bladder, there is little evidence for a urea transporter in the urinary bladder of the gulf toadfish. The toadfish bladder does not show net transport of urea under symmetrical conditions. Although the permeability of the toadfish bladder (8.0 ± 1.2 cm s^{-1} x 10^{-6}) to urea is higher than in artificial lipid bilayers, this could be due to the higher temperature at which toadfish live. In addition, the urinary bladder of the toadfish does not differentially handle urea, acetamide or thiourea, (similar

permeabilities), suggesting that a urea-specific transport mechanism that preferentially moves urea is not present in the urinary bladder of the toadfish. The toadfish bladder does exhibit temperature sensitivity, namely a sharp phase transition at 26°C, however, this is indicative of a membrane consisting of pure phospholipids (Linton et al. 1973). At low temperatures there is a "paradoxical" increase in urea flux rate, which is similar to observations of passive potassium flux in mammalian erythrocytes (Hall and Willis, 1986).

In vivo, the urinary bladder of *Opsanus tau*, a close relative of the gulf toadfish *Opsanus beta*, recovers 60% of the fluid excreted from the kidneys by reabsorbing an isosmotic absorbate containing primarily NaCl (Lahlou et al. 1969; Howe and Gutknecht, 1978; Baustain et al. 1997). In contrast, the *in vitro* urinary bladder of *Opsanus beta* does not show significant net reabsorption of Cl^- or water (though Na^+ reabsorption is significant), nor does it significantly transport urea in either the reabsorptive or secretory direction. Ionic measurements suggest that the bladder of *Opsanus beta in vivo* reabsorbs Na^+ and Cl^-, but without knowledge of water movement it is impossible to determine whether urea is also transported (Chapter 2).

The key conclusion is that in the gulf toadfish the kidney itself regulates the transport and excretion of urea while the urinary bladder is used as a low permeability storage organ in which urea will not be secreted or absorbed. A secondary conclusion is that the toadfish urinary bladder *in vitro* is not a good model for renal urea handling *in vivo*, where active secretion of urea by the kidney occurs (Chapter 2). Active secretion of urea does not occur in the bladder *in vitro*. Furthermore, unlike the bladder, the toadfish

kidney has the ability to differentiate between urea and the urea analogue acetamide, preferentially transporting urea but not acetamide (Chapter 2). Because the *in vitro* urinary bladder has identical low permeability to urea and acetamide, it represents an epithelium that does not selectively transport urea.

In conclusion, the data of the present study indicate that the *in vitro* urinary bladder preparations of both species are not suitable models for understanding the handling of urea by the kidney *in vivo*. However, these data do suggest the possibility of a urea transport mechanism in the urinary bladder of the rainbow trout, which holds urine for approximately 30 minutes *in vivo* (Curtis and Wood, 1991), however further investigation is needed to understand this mechanism. This study also demonstrated urinary bladder function in toadfish as being primarily a storage organ of low permeability consistent with long urine holding periods and the intermittent urine release in this species (Wood et al. 1995). It is interesting to speculate that the habit of intermittent urine release in tetrapods and toadfish should in both cases be associated with bladder epithelium of low (basal) permeability, a possible example of convergent evolution.

Table 1: The influence of various pharmacological treatments on urea flux rates in *in vitro* urinary bladder sac experiments from the rainbow trout (J_{in}: unidirectional flux in the direction of reabsorption; J_{net}: net flux; positive values signify reabsorption from the bladder; values are means ± 1 S.E.M. (N)).

Experiment	$J_{in\ urea}$ ($\mu mol\ cm^{-2}\ h^{-1}$)	$J_{net\ urea}$ ($\mu mol\ cm^{-2}\ h^{-1}$)	$J_{net\ water}$ ($ml\ cm^{-2}\ h^{-1}$)
Control (6)	0.087 ± 0.010	0.048 ± 0.009	0.030 ± 0.006
Thiourea (6)	0.072 ± 0.014	0.027 ± 0.004	0.014 ± 0.002
Control (9)	0.127 ± 0.023	0.009 ± 0.003	0.007 ± 0.001
Acetamide (6)	0.098 ± 0.016	0.011 ± 0.005	0.006 ± 0.003
Control (6)	0.077 ± 0.020	0.011 ± 0.003	0.007 ± 0.001
Amiloride† (6)	0.099 ± 0.017	0.020 ± 0.008	0.011 ± 0.004
Control (6)	0.193 ± 0.031	0.026 ± 0.014	0.014 ± 0.006
Phloretin‡ (6)	0.142 ± 0.018	0.015 ± 0.004	0.009 ± 0.001
Control (6)	0.056 ± 0.016	0.015 ± 0.005	0.008 ± 0.002
AVT (6)	0.058 ± 0.011	0.014 ± 0.004	0.007 ± 0.002
Control (6)	0.056 ± 0.016	0.015 ± 0.005	0.008 ± 0.002
AVT + Thiourea (6)	0.053 ± 0.007	0.009 ± 0.002	0.006 ± 0.001
Control (6)	0.031 ± 0.010	0.008 ± 0.005	0.120 ± 0.002
Na^+-free (6)	0.041 ± 0.010	0.011 ± 0.002	0.007 ± 0.002

†DMSO alone had no effect on $J_{in\ urea}$, $J_{net\ urea}$ or $J_{net\ water}$

‡Ethanol alone had no effect on $J_{in\ urea}$, $J_{net\ urea}$ or $J_{net\ water}$

Urea concentration in all experiments was 1.8 $mmol \cdot l^{-1}$.

There was no significant difference ($P > 0.05$) between experimental and simultaneous control treatments.

Table 2: The influence of various pharmacological treatments on urea flux rates in *in vitro* urinary bladder sac experiments from the gulf toadfish (J_{in}: unidirectional flux in the direction of reabsorption; J_{net}: net flux; positive values signify reabsorption from the bladder; negative values signify secretion into the bladder; values are means ± 1 S.E.M. (N)).

Experiment	$J_{in\ urea}$ ($\mu mol\ cm^{-2}\ h^{-1}$)	$J_{net\ urea}$ ($\mu mol\ cm^{-2}\ h^{-1}$)	$J_{net\ water}$ ($ml\ cm^{-2}\ h^{-1}$)
Control (6)	0.192 ± 0.028	0.127 ± 0.085	0.011 ± 0.006
Thiourea (5)	0.203 ± 0.072	-0.048 ± 0.061	-0.008 ± 0.009
Control (6)	0.192 ± 0.028	0.127 ± 0.085	0.011 ± 0.006
Phloretin‡ (6)	0.159 ± 0.056	0.009 ± 0.020	0.002 ± 0.001
Control (8)	0.097 ± 0.023	-0.015 ± 0.011	-0.001 ± 0.001
AVT (12)	0.186 ± 0.058	0.032 ± 0.039	0.002 ± 0.001
Control (7)	0.143 ± 0.066	0.004 ± 0.009	0.005 ± 0.001
Gradient (6)	0.244 ± 0.051	0.252 ± 0.102	0.006 ± 0.002
Control (13)	0.101 ± 0.037	-0.001 ± 0.010	0.002 ± 0.001
Na^+-free (13)	0.122 ± 0.038	0.021 ± 0.024	0.004 ± 0.004

‡DMSO alone had no effect on $J_{in\ urea}$, $J_{net\ urea}$ or $J_{net\ water}$

Urea concentration in all experiments was 7 $mmol \cdot l^{-1}$.

There were no significant differences ($P > 0.05$) between experimental and simultaneous control treatments.

Figure A-1: Control rates for (A) unidirectional ($J_{in\ urea}$) and net fluxes of urea ($J_{net\ urea}$) and water ($J_{net\ water}$) and (B) Na^+, Cl^- and glucose in isolated bladders of the rainbow trout showing unidirectional flux of urea in the direction of reabsorption plus significant net reabsorption of urea, water, Na^+, Cl^- and glucose. (C) Final concentration ratios at the end of 3-4h flux experiments indicate a significant reabsorption of Na^+ and Cl^- independent of water movement, but not of urea or glucose. Values are means ± 1 S.E.M. (N); * P <0.05 significantly different than 1.0.

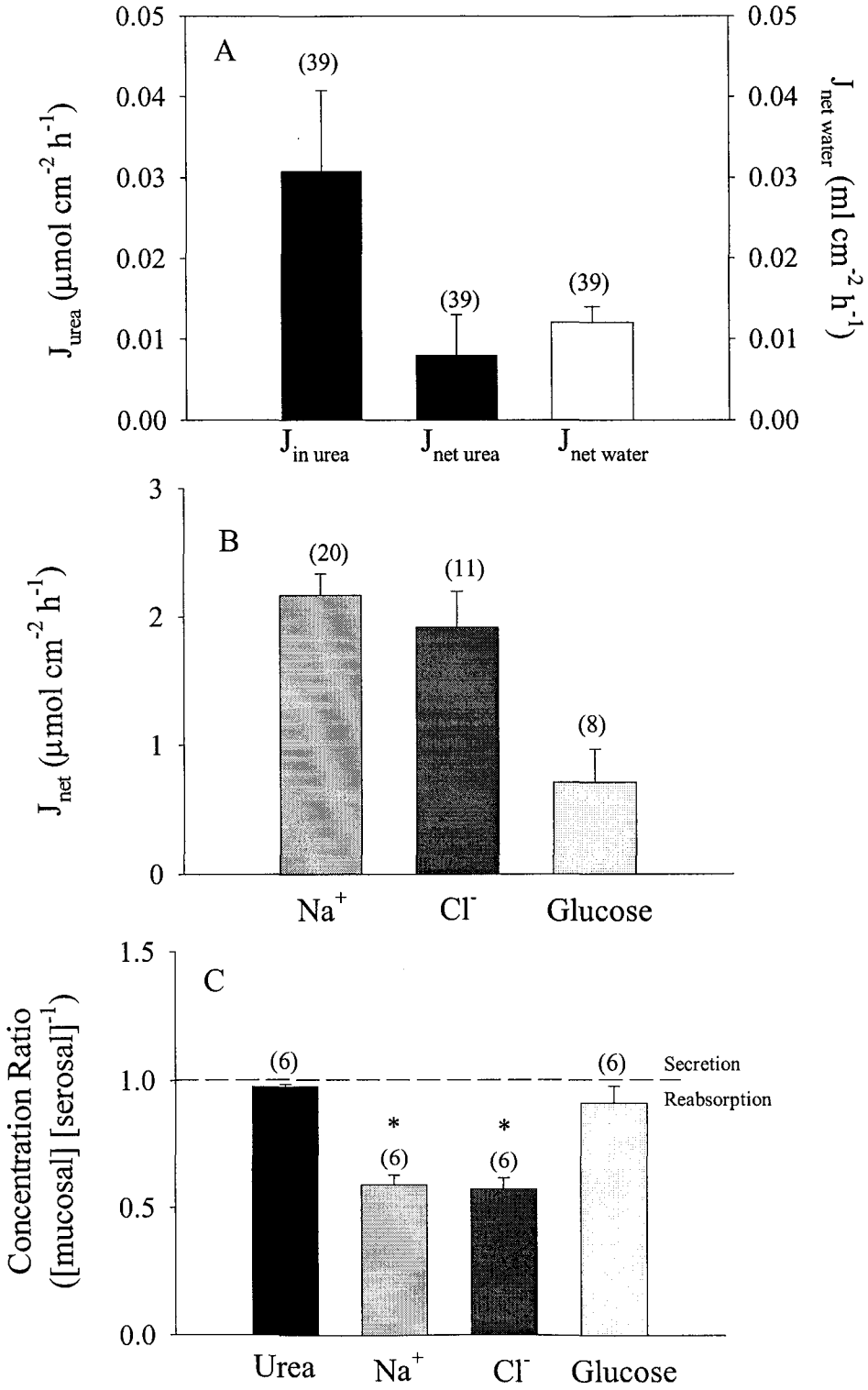

A
B
C
0.05
0.04
0.03
0.02
0.01
0.00
J_{urea} (µmol cm^{-2} h^{-1})
$J_{net\ water}$ (ml cm^{-2} h^{-1})
(39)
(39)
(39)
$J_{in\ urea}$
$J_{net\ urea}$
$J_{net\ water}$
3
2
1
0
J_{net} (µmol cm^{-2} h^{-1})
(20)
(11)
(8)
Na^+
Cl^-
Glucose
1.5
1.0
0.5
0.0
Concentration Ratio
([mucosal] [serosal]$^{-1}$)
(6)
(6)
(6)
(6)
*
*
Secretion
Reabsorption
Urea
Na^+
Cl^-
Glucose

Figure A-2: Flux rates of isolated bladders of the rainbow trout acclimated to 11-14°C and then acutely exposed to different temperatures. A significant decrease in (A) $J_{in\ urea}$ (C) $J_{net\ urea}$ and (D) $J_{net\ water}$ at 0°C and an increase at 30°C is evident. A lateral phase transition is evident in the Arrhenius plot (B). Values are means ± 1 S.E.M. (N); * $P <$ 0.05 significantly different from rates at temperature of acclimation.

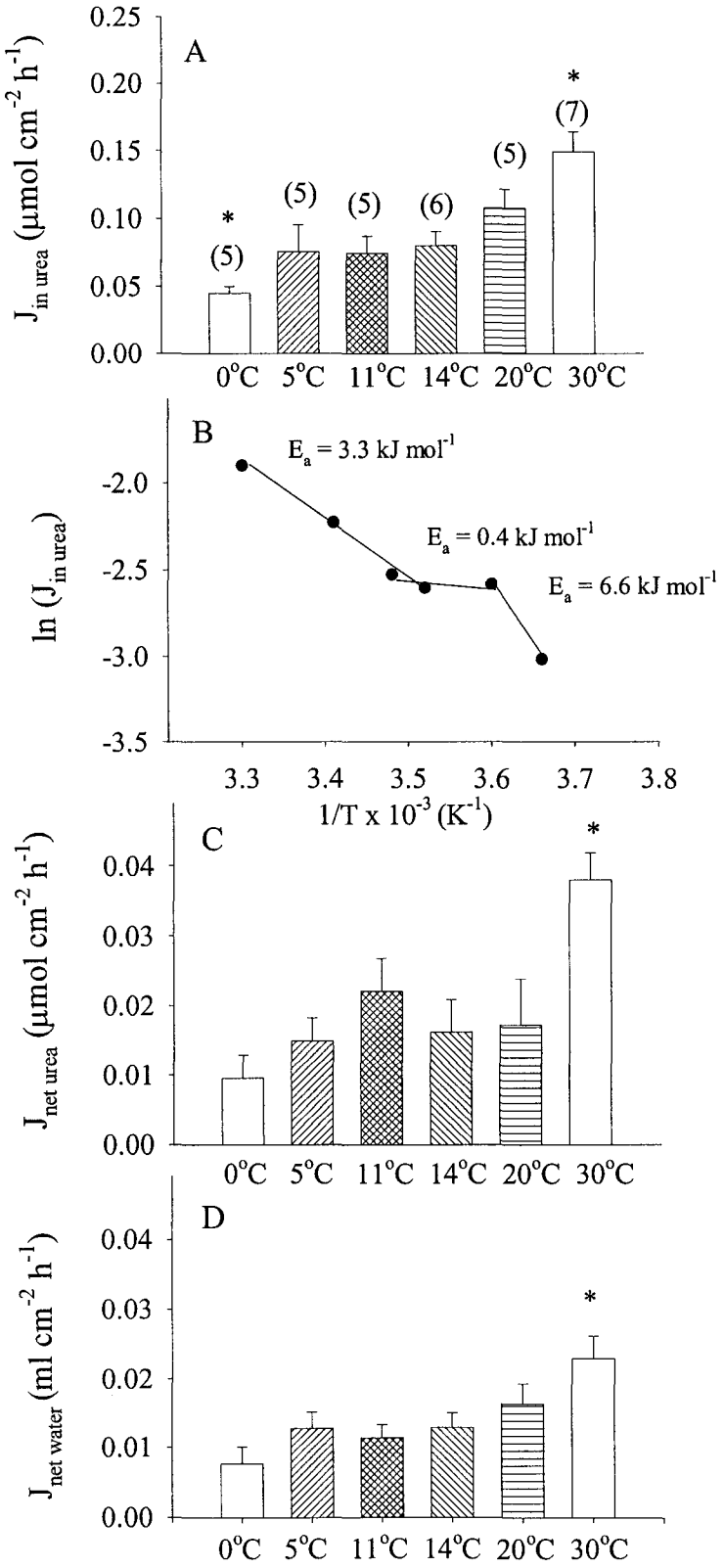
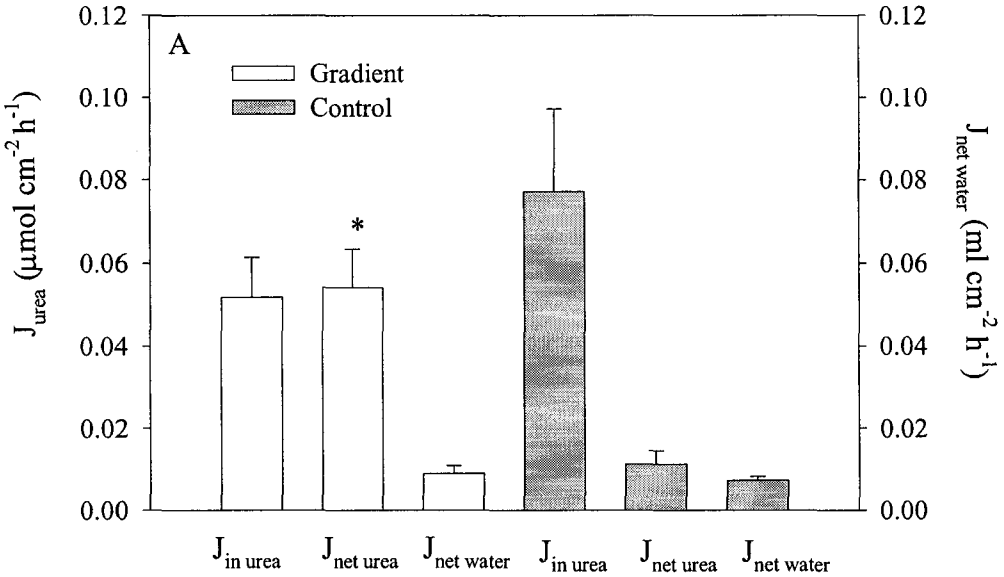

A
$J_{in\ urea}$ (µmol cm^{-2} h^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
*
(5)
(5)
(5)
(6)
(5)
*
(7)
0°C 5°C 11°C 14°C 20°C 30°C
B
ln ($J_{in\ urea}$)
E_a = 3.3 kJ mol^{-1}
E_a = 0.4 kJ mol^{-1}
E_a = 6.6 kJ mol^{-1}
-2.0
-2.5
-3.0
-3.5
3.3 3.4 3.5 3.6 3.7 3.8
1/T x 10^{-3} (K^{-1})
C
$J_{net\ urea}$ (µmol cm^{-2} h^{-1})
0.04
0.03
0.02
0.01
0.00
*
0°C 5°C 11°C 14°C 20°C 30°C
D
$J_{net\ water}$ (ml cm^{-2} h^{-1})
0.04
0.03
0.02
0.01
0.00
*
0°C 5°C 11°C 14°C 20°C 30°C

Figure A-3: (A) Flux rates of urinary bladders of rainbow trout exposed to a 1.8 $mmol \cdot l^{-1}$ urea concentration gradient, from mucosal to serosal surface (urea-free) showing a significant increase in $J_{net\ urea}$ relative to controls. No increase in $J_{net\ water}$ is apparent. (B) There is a significant difference between the urea concentration in the initial mucosal solution and that in the transient solution in bladders exposed to a gradient compared to those in all other treatment groups. Values are means ± 1 S.E.M. (N = 6); * $P < 0.05$ significantly different from control flux rates: † $P < 0.05$ significantly different from mucosal urea concentrations.


A
Gradient
Control
*
0.12
0.10
0.08
0.06
0.04
0.02
0.00
J_{urea} (µmol cm^{-2} h^{-1})
$J_{net\ water}$ (ml cm^{-2} h^{-1})
$J_{in\ urea}$
$J_{net\ urea}$
$J_{net\ water}$
$J_{in\ urea}$
$J_{net\ urea}$
$J_{net\ water}$

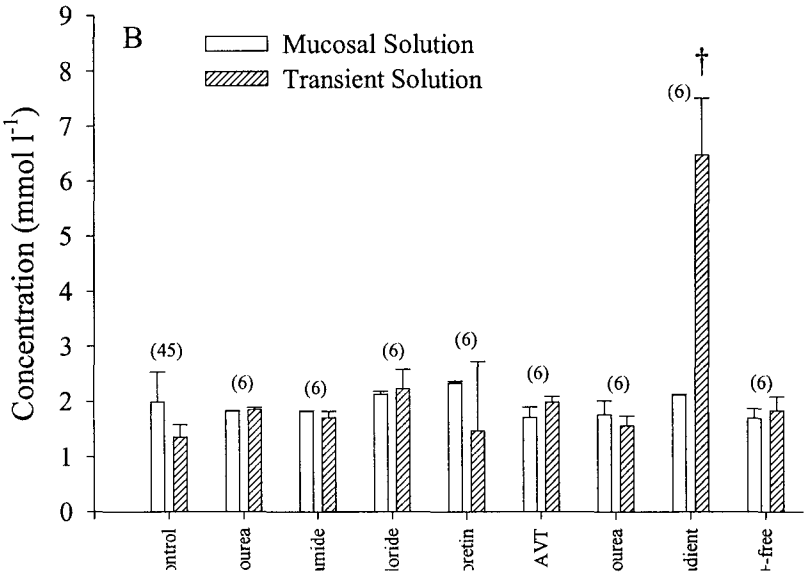

B
Mucosal Solution
Transient Solution
Concentration (mmol l^{-1})
9
8
7
6
5
4
3
2
1
0
(45)
(6)
(6)
(6)
(6)
(6)
(6)
(6)
(6)
†
ontrol
ourea
umide
loride
oretin
AVT
ourea
adient
-free

Figure A-4: Measurements of urea (1.8 $mmol \cdot l^{-1}$), thiourea (1.8 $mmol \cdot l^{-1}$) and acetamide (1.8 $mmol \cdot l^{-1}$) unidirectional flux rates and accompanying net water flux rates by the isolated urinary bladder of the rainbow trout. The urinary bladder appears to differentiate between urea and both thiourea and acetamide. Values are means ± 1 S.E.M. (N = 6 for thiourea and acetamide groups, N = 39 for urea); * $P < 0.05$ significantly different from urea flux rate.

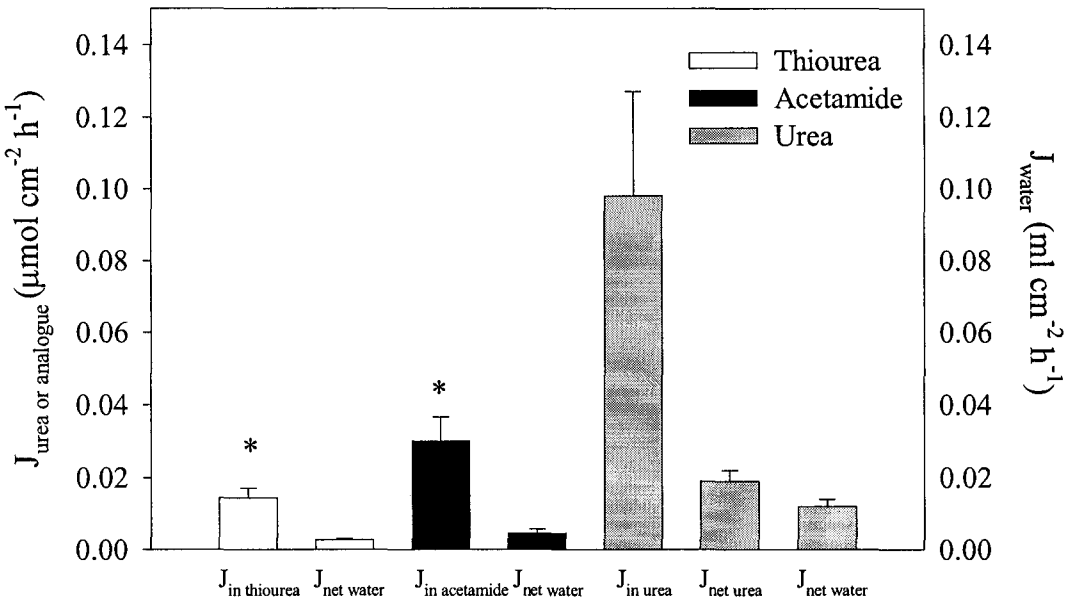

Thiourea
Acetamide
Urea
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
*
$J_{urea\ or\ analogue}$ (μmol cm^{-2} h^{-1})
J_{water} (ml cm^{-2} h^{-1})
$J_{in\ thiourea}$
$J_{net\ water}$
$J_{in\ acetamide}$
$J_{net\ water}$
$J_{in\ urea}$
$J_{net\ urea}$
$J_{net\ water}$

Figure A-5: Control rates for (A) unidirectional ($J_{in\ urea}$) and net fluxes of urea ($J_{net\ urea}$) and water ($J_{net\ water}$) and (B) Na^+ and Cl^- ($J_{net\ ions}$) in isolated bladders of the gulf toadfish showing significant unidirectional flux of urea and net flux of Na^+ in the direction of reabsorption but values of $J_{net\ urea}$, $J_{net\ water}$ and $J_{net\ Cl-}$ that are not significantly different from zero. (C) Final concentration ratios at the end of 3-4 h flux experiments indicate that there is no significant movement of urea, Cl^- or Na^+ independent of the movement of water. Values are means ± 1 S.E.M. (N).

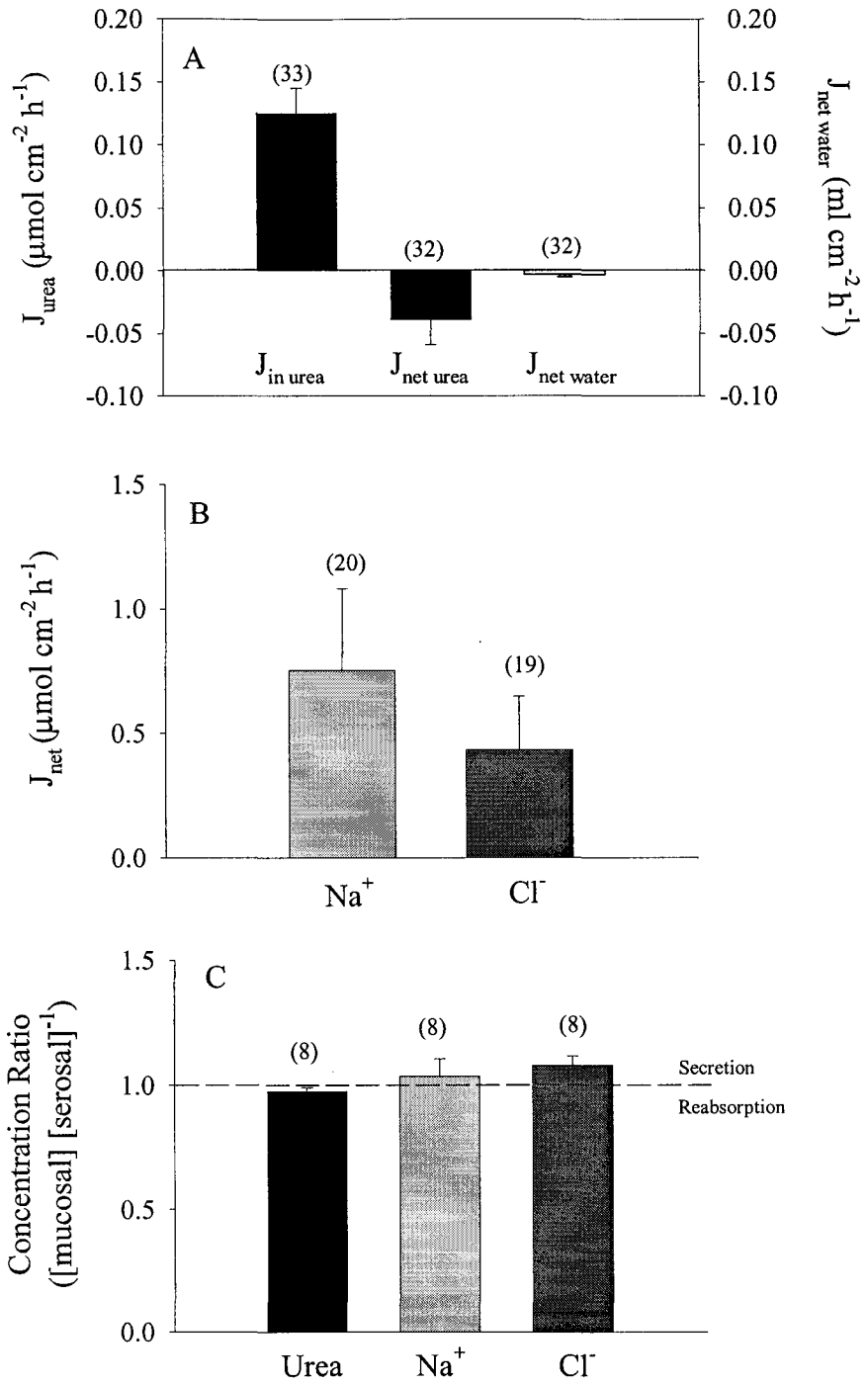

A
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
(33)
(32)
(32)
J_{urea} (µmol cm^{-2} h^{-1})
$J_{net\ water}$ (ml cm^{-2} h^{-1})
$J_{in\ urea}$
$J_{net\ urea}$
$J_{net\ water}$
B
1.5
1.0
0.5
0.0
(20)
(19)
J_{net} (µmol cm^{-2} h^{-1})
Na^+
Cl^-
C
1.5
1.0
0.5
0.0
(8)
(8)
(8)
Secretion
Reabsorption
Concentration Ratio
([mucosal] [serosal]$^{-1}$)
Urea
Na^+
Cl^-

Figure A-6: Flux rates of isolated urinary bladders of the gulf toadfish acclimated to 26°C and then acutely exposed to different temperatures. A significant increase in (A) $J_{in\ urea}$ is evident with temperatures above and below the temperature of acclimation. A similar trend, though not significant, is also apparent in (C) $J_{net\ urea}$ and (D) $J_{net\ water}$ values. A sharp phase transition is evident in the Arrhenius plot (B). Values are means ± 1 S.E.M. (N = 6); $*P < 0.05$ significantly different from rates at temperature of acclimation.

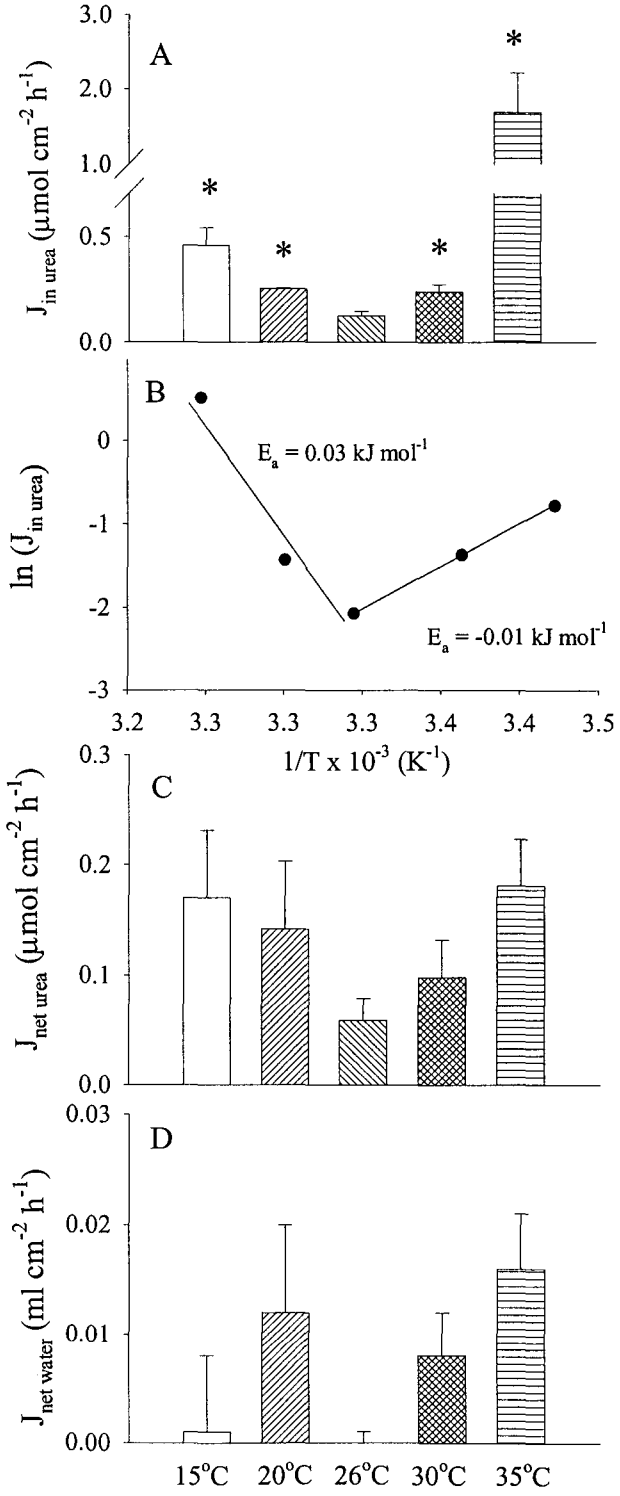
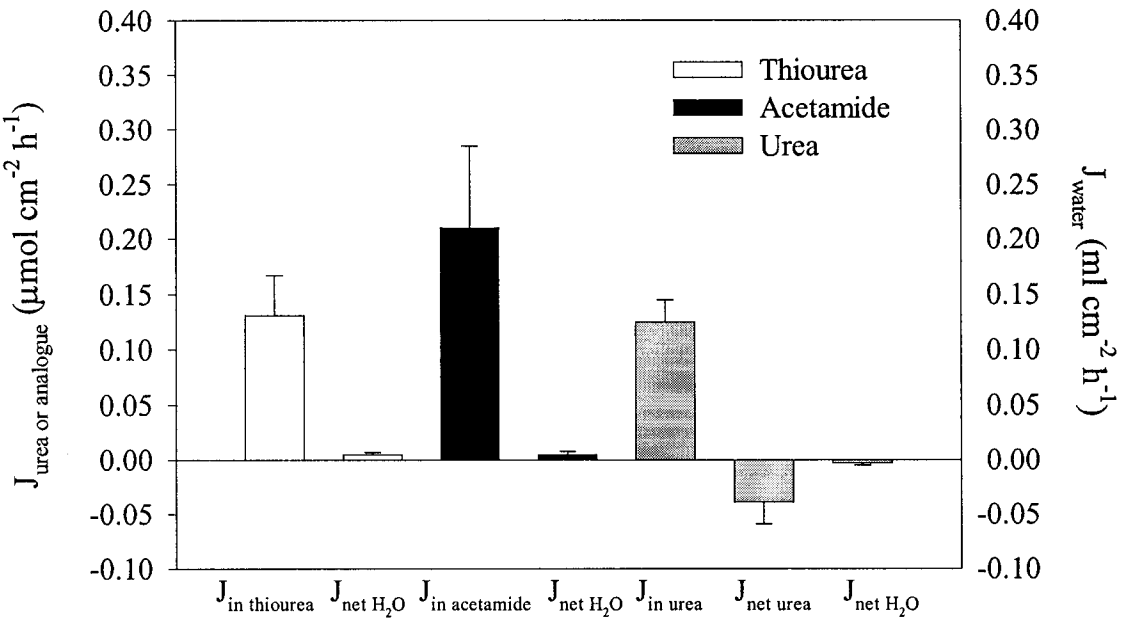

A
B
C
D
$J_{in\ urea}$ (µmol cm^{-2} h^{-1})
ln ($J_{in\ urea}$)
E_a = 0.03 kJ mol^{-1}
E_a = -0.01 kJ mol^{-1}
1/T x 10^{-3} (K^{-1})
$J_{net\ urea}$ (µmol cm^{-2} h^{-1})
$J_{net\ water}$ (ml cm^{-2} h^{-1})
15°C
20°C
26°C
30°C
35°C

Figure A-7: Measurements of urea (7 $mmol \cdot l^{-1}$), thiourea (7 $mmol \cdot l^{-1}$) and acetamide (7 $mmol \cdot l^{-1}$) unidirectional flux rates, and accompanying net water flux rates by the isolated urinary bladder of the gulf toadfish. The urinary bladder does not appear to differentiate between the three substances. Values are means ± 1 S.E.M. (N = 6 for thiourea and acetamide groups, N = 33 for urea), no significant differences ($P > 0.05$).


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
Thiourea
Acetamide
Urea
$J_{urea\ or\ analogue}$ (μmol cm^{-2} h^{-1})
J_{water} (ml cm^{-2} h^{-1})
$J_{in\ thiourea}$
$J_{net\ H_2O}$
$J_{in\ acetamide}$
$J_{net\ H_2O}$
$J_{in\ urea}$
$J_{net\ urea}$
$J_{net\ H_2O}$

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