FUNCTIONAL CHARACTERIZATION OF RENAL AMMONIA TRANSPORT AND ACID-BASE REGULATION IN TELEOST AND ELASMOBRANCH FISHES

FUNCTIONAL CHARACTERIZATION OF RENAL AMMONIA TRANSPORT AND ACID-BASE REGULATION IN TELEOST AND ELASMOBRANCH FISHES

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ABSTRACT

Teleost fishes incorporate renal ammonia excretion as part of a greater acid-base regulatory system. However, the transport mechanisms employed by the renal epithelium to excrete ammonia are relatively unknown. I hypothesized that, under metabolic acidosis, increased renal ammonia excretion would be the product of tubular secretion and involve a Na^+/NH_4^+ exchange metabolon mediated through Rhesus (Rh) glycoproteins. To induce metabolic acidosis, goldfish (*Carassius auratus*) were exposed to a low pH environment (pH 4.0; 48-h). There was a clear signal of metabolic acidosis: a reduction in both plasma [HCO_3^-] and blood pH with no influence on plasma P_{CO2}. Goldfish demonstrated an elevation in total plasma [ammonia] with a reduction in P_{NH3} under acidosis. Metabolic acidosis induced higher rates of urinary excretion of acidic equivalents in the form of both NH_4^+ and titratable acidity-HCO_3^- (TA-HCO_3^-) excretion. Urinary Na^+ excretion was not affected by acidosis and urine [Na^+] did not correlate with urinary [ammonia]. Alanine aminotransferase activity in the kidney was higher in acidotic goldfish. Glomerular filtration rate and urine flow rate were not affected by acidosis. Increased renal NH_4^+ excretion was due to increased secretion, and not increased filtration, of ammonia. There was a corresponding elevation in Rhcg1b mRNA expression but no change in renal Na^+ reabsorption. My data support a secretion-based mechanism of teleost renal ammonia transport. This system is Na^+ independent and is likely mediated by Rh glycoproteins and H^+ ATPase, involving a parallel H^+/NH_3 secretion mechanism. To investigate effects of metabolic acidosis on elasmobranch fish, Pacific spiny dogfish (*Squalus acanthias suckleyi*) were infused with an acidic saline (125 mM HCl/375 mM

NaCl; 3 ml/kg/h; 24-h). The results are preliminary, with no marked effects of HCl infusion on plasma acid-base or N-status, but increased branchial NHE2 and lower renal NHE3 protein expressions. These data are summarized in an Appendix.

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THESIS ORGANIZATION AND FORMAT

The following thesis is arranged in the "sandwich" format in accordance with the recommendations and approval of my supervisory committee. This thesis consists of three chapters. Chapter 1 outlines a general introduction and background of information relevant to my thesis work including thesis objectives and hypotheses. Chapter 2 contains my manuscript that is being prepared for submission to a peer-reviewed scientific journal. My final chapter summarizes the general findings and relevance of my thesis and provides future directions for subsequent research. There is an additional appendix containing all data pertaining to elasmobranch infusion material that is relevant to my thesis work.

Chapter 1: **General introduction, thesis objectives and hypotheses**

Chapter 2: **Physiological responses of the goldfish (*C. auratus*) to metabolic acidosis and potential mechanisms of renal ammonia transport.**

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Chapter 3: **General conclusions and future directions**

Appendix: **Data pertaining to elasmobranch acid-infusion experiments**

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ABBREVIATIONS

P_{CO2} – Partial pressure of CO_2

TA-HCO_3^- – Titratable acid minus bicarbonate

HAT – H^+ ATPase

NHE – Na^+/H^+ exchanger

P_{NH3} – Partial pressure of ammonia gas

Rh – Rhesus

CA – Carbonic anhydrase

NKCC – $Na^+/K^+/2Cl^-$ cotransporter

T_{amm} – Total ammonia

P_i – Inorganic phosphate

GFR – Glomerular filtration rate

[^{3}H]PEG-4000 – Polyethylene glycol MW 4000

CD – Collecting duct

UFR – Urine flow rate

NADH – Nicotinamide adenine dinucleotide

mRNA – Messenger ribonucleic acid

RT-qPCR – Real-time quantitative polymerase chain reaction

cDNA – Complementary deoxyribonucleic acid

EF1α – Elongation factor-1α

ND – Not detected

EIPA – 5-(N-ethyl-N-isopropyl)amiloride

HPI – Hypothalamic-pituitary-interrenal

ACTH – Adrenocorticotropic hormone

CRF – Corticotropin-releasing factor

CHAPTER 1: GENERAL INTRODUCTION

Ammonia and its Physiological Roles:

Ammonia is a nitrogenous compound common to the metabolism of almost all vertebrate clades. Ammonia exists in two forms, the gas, NH_3, and the ion, NH_4^+,which are interconvertible with a pK of about 9.5; thus at physiological pH (6.8-8.2), the vast majority exists in the form of NH_4^+. In this thesis, the term "ammonia" encompasses both forms, and the chemical symbols are used to refer to the particular species.

Ammonia is the primary metabolic waste product of most fishes encompassing teleosts (Smith 1929; Wright 1995) and a small group of freshwater elasmobranchs, the *Potamotrygon* stingrays of the Amazon River basin (Goldstein and Forster 1971; Wood et al. 2002; Ip et al. 2003). Ammonia is considered to be quite toxic to life and, in fish, minute concentrations in the water are known to have serious pathological ramifications (Burrows 1964; Thurston et al. 1981; Colt and Armstrong 1981; Meade 1985) including gill damage (Smart 1976; Benli et al. 2008), energy metabolism dysfunction (Arillo et al. 1981) and membrane depolarization (Tsui et al. 2004; Walsh et al. 2007). The latter effect is believed to be the most significant pathological mode of action of ammonia in fish causing significant damage to the central nervous system (Randall and Tsui 2002; Tsui et al. 2004; Walsh et al. 2007). Therefore, to avoid toxicosis, ammonotelic fish need to continuously excrete ammonia and, in teleosts, this occurs at two main exchange sites, the branchial (Smith 1929; McDonald and Wood 1981; Zimmer et al. 2014) and renal epithelia (Smith 1929; McDonald and Wood 1981; King and Goldstein 1983b; Wood et al. 1999; Zimmer et al. 2014).

While ammonia is quite toxic to fish, it can also be beneficial in the physiological maintenance of acid-base homeostasis. In vertebrates, metabolic acidosis is characterized by a concurrent reduction in blood pH and plasma [HCO_3^-] in the absence of an accumulation of PCO_2 (Hills 1973). In order to re-establish systemic acid-base balance and restore the buffering capacity of the blood, H^+ must be excreted, thereby returning new HCO_3^- to the body fluids (Hills 1973; Weiner and Verlander 2014). One mechanism by which this can occur is through the metabolism of amino acids, liberating equimolar amounts of NH_4^+ and HCO_3^- on a net basis. The subsequent excretion of ammonia as NH_4^+ results in an equimolar retention of a HCO_3^- (Krebs 1973; Hills 1973; Marren 1988; Knepper et al. 1989; Taylor and Curthoys 2004; reviewed in Weiner and Verlander 2014).

In specific detail, renal amino acid catabolism liberates NH_3 in two deamination stages, collectively referred to as transdeamination, which includes the catalysis of glutamine to glutamate via glutaminase and glutamate to α-ketoglutarate via glutamate dehydrogenase (Curthoys and Watford 1995; Wright 1995). The α-ketoglutarate is then further metabolised by α-ketoglutarate dehydrogenase producing succinate, which can be used in ATP synthesis via Kreb's cycle, and CO_2. Finally, carbonic anhydrase quickly catalyzes the reaction of CO_2 with water to form HCO_3^- and H^+. The resulting H^+ reacts, in equal proportions, with NH_3 forming an acid equivalent, NH_4^+ (NH_3+H^+), which can be subsequently excreted. NH_4^+ excretion thereby permits the net formation of HCO_3^- in the kidney which is subsequently shuttled to the extracellular fluid to restore acid-base balance (reviewed in Knepper et al. 1989 and Weiner 2010). While this specific mechanism is based on a metabolic pathway, in any circumstance where ammonia excretion facilitates the release/elimination of metabolic H^+, either directly as NH_4^+, or indirectly by diffusion trapping of an excreted H^+ (i.e. $NH_3 + H^+$), a metabolic HCO_3^-

(a basic equivalent) is left behind and net acidic equivalent excretion is achieved. Thus, ammonia excretion represents a significant mechanism by which acid-base homeostasis can be achieved in vertebrates.

In practical terms, every molecule of ammonia excreted in the urine is counted as an acidic equivalent in acid-base balance theory (Hills 1973). The ammonia has either directly entered the urine as NH_4^+, or has diffused into the urine as NH_3, trapping a metabolic H^+ to become an NH_4^+. A negligible amount of total ammonia exists as NH_3 at typical urine pH. In light of the high pK (9.5), NH_4^+ is not measured in standard procedures (titration with base back to blood pH) used to quantify other metabolic H^+ ions excreted in the urine (e.g. those bound to phosphate), and so net acidic equivalent excretion is calculated as total ammonia + titratable acid minus HCO_3^- ($TA\text{-}HCO_3^-$) excretion (Hills 1973). In common parlance, ammonia is often referred to as a major urinary "buffer", as it prevents the urinary pH from becoming very acidic, by removing metabolic H^+ from solution.

The use of ammonia excretion in acid-base regulatory processes is quite widespread among vertebrate taxonomic groups. Metabolic acidosis results in a significant elevation in ammonia excretion in mammals (Sajo et al. 1981; Cheval et al. 2006), birds (Wolbach 1955), amphibians (Yoshimura et al. 1961; Vanatta and Frazier 1981), teleosts (Wood and Caldwell 1978; Evans 1982; McDonald and Wood 1981; King and Goldstein 1983b; Wood et al. 1999) and elasmobranch fishes (King and Goldstein 1983a; Wood et al. 1995). This effect is also believed to be an important response in regulating crocodilian acid-base homeostasis (Lemieux et al. 1984). In fish, metabolic acidosis stimulates an elevation in ammonia excretion at both the renal (McDonald and Wood 1981; Evans 1982; King and Goldstein 1983a; King and Goldstein 1983b; Wood et al. 1999) and branchial (McDonald and Wood 1981; Evans 1982) epithelia,

presumably as a means of facilitating acid-base regulation. However, it should be noted that under most circumstances the gill (not the kidney) contributes by far the largest proportion to both ammonia (Smith 1929; McDonald and Wood 1981; Evans 1982; Wood et al. 1995) and acidic equivalent excretion (Cross et al. 1969; McDonald and Wood 1981; Evans 1982; Wood et al. 1995) in elasmobranch and teleost fishes.

The excretion of ammonia in teleost fish is also a significant process with regard to Na^+ uptake from the environment. Teleost freshwater fishes occupy an environment that is hypotonic relative to their tissues and, as a result, experience passive ion loss mainly across the gills (Krogh 1939; Evans 2008). As the loss of ionic homeostasis can lead to severe metabolic impairment (Bowlus and Somero 1972; Somero 1986; Yancy 2001), teleost fishes employ mechanisms to actively take up ions, namely Na^+ and Cl^-, from the external environment. This ion uptake primarily occurs in the teleost gill and is believed to be maintained through two distinct transport proteins; H^+ ATPase (HAT; paired with an as yet unidentified Na^+ channel) and Na^+/H^+ exchanger (NHE). In both instances, H^+ is translocated from the gill cell cytoplasm thereby generating favourable electrochemical gradients to facilitate an influx of Na^+ across the branchial epithelium (reviewed in Evans 2008). However, the influx of Na^+ has often been observed to be coupled with branchial ammonia excretion (Krogh 1939; Maetz and Romeau 1964; Wright and Wood 1985; McDonald and Prior 1988). Previous work has found that fish exposed to amiloride, a blocker of both epithelial Na^+ channels (Alvarez de la Rosa 2000) and NHE (Demaruex et al. 1995), experienced impaired branchial ammonia efflux (Wright and Wood 1985; Wilson et al. 1994) with similar effects occurring under low environmental Na^+ as well (Evans 1982). Thus, a branchial Na^+/NH_4^+ exchange mechanism was proposed whereby the excretion of NH_4^+ generates thermodynamically favourable conditions for the operation of an NHE and/or HAT-

Na^+ channel system to facilitate the uptake of Na^+ across the gill (reviewed in Wright and Wood 2009, 2012). However, Na^+-independent ammonia excretion has also been demonstrated in the gill causing debate into specific ammonia transport mechanism(s) within the scientific community (Kerstetter et al. 1970; Maetz 1972; MacDonald and Prior 1988). Recently though, the discovery of Rhesus (Rh) glycoproteins in the gills of teleost fish that transport ammonia and advances in molecular transport physiology have lent considerable evidence to the existence of Na^+/NH_4^+ exchange through the proposal of a Na^+/NH_4^+ exchange metabolon (reviewed in Weihrauch et al. 2009; Wright and Wood 2009, 2012. Ito et al. 2013).

Teleost Branchial Ammonia Excretion Mechanisms:

Historically, branchial ammonia excretion was hypothesized to be the product of a number of different physiological processes including simple ammonia gas diffusion (NH_3), NH_4^+/Na^+ exchange and NH_4^+ diffusion (reviewed in Wilkie 1997). In the last decade though, there has been a considerable paradigm shift with regard to the mechanism(s) of branchial ammonia transport, resulting from the discovery of Rhesus (Rh) glycoproteins (Nakada et al. 2007a; Nakada et al. 2007b; Nawata et al. 2007). These proteins are believed to be involved in the facilitated diffusion of NH_3, between cellular compartments, along a favourable partial pressure gradient (P_{NH3}) (Knepper and Agre 2004; Javelle et al. 2007; Nawata et al. 2010b). The expression of these proteins has been found to localize heavily in the gills of a number of freshwater teleost fish species (Hung et al. 2007; Nakada et al. 2007a; Nakada et al. 2007b; Nawata et al. 2007; Nawata and Wood 2008; Tsui et al. 2009; Nawata et al. 2010a; Zimmer et al. 2010; Cooper et al. 2013) existing in two main isoforms; a basolateral Rhbg (Nakada et al. 2007a; Nakada et al. 2007b; Cooper et al. 2013) and an apical Rhcg (Nakada et al. 2007a; Nakada et al. 2007b; Cooper et al. 2013). Generally, Rhcg and Rhbg are often found to co-

localize in the pavement cells (Nakada et al. 2007b; Claiborne et al. 2008b; Braun et al. 2009; Cooper et al. 2013) of the gill whereas Rhcg is solely expressed in the branchial mitochondria rich cells (Nakada et al. 2007a; Nakada et al. 2007b; Claiborne et. 2008; Cooper et al. 2013).

The arrangement of these Rh proteins works to facilitate a pathway by which NH_3 can freely diffuse along a blood-to-water P_{NH3} gradient. However, working in isolation, branchial ammonia efflux would cease as NH_3 begins to accumulate in the boundary layer of the gill thereby reducing the blood-to-water P_{NH3} gradient (Wright and Wood 2009). In order to promote continued ammonia efflux across the gills, a variety of H^+ excretion processes acidify the boundary layer of the branchial epithelium. This is beneficial to sustaining P_{NH3} gradients as the relatively high pK of ammonia (~9.5) dictates its existence in the ionized state (NH_4^+) in low pH environments. Boundary layer acidification acts to reduce the local concentration of NH_3 in this microenvironment thereby sustaining continued NH_3 diffusion (Wright et al. 1986; Randall and Wright 1989; Wright et al. 1989; Randall et al. 1991). Indeed, buffering of the gill boundary layer (Wright et al. 1989) or exposure to a highly alkaline environment (Wright et al. 1993) can result in the reduction of the capacity of the gill to effectively excrete ammonia. Currently, it is believed that boundary layer acidification is maintained by three main mechanisms in the gill: an apical, extracellular carbonic anhydrase (CA) (Wright et al. 1986; Randall and Wright 1989; Wright et al. 1989), an HAT (Nawata et al. 2007; Perry et al. 2000; Wilson et al. 2000; Wright and Wood 2009) and/or through an NHE (Perry et al. 2000; Hirata et al. 2003; Ivanis et al. 2008b; Tsui et al. 2009; Nawata and Wood 2009; Wright and Wood 2009; Zimmer et al. 2010). In support of the relationship with ammonia transport, immunohistological analysis has determined that both the NHE (Hirata et al. 2003; Ivanis et al. 2008b; Cooper et al. 2013) and HAT (Lin et al. 1994; Sullivan et al. 1995; Wilson et al. 2000) have an apical expression in the

gill often co-localizing with apical Rhcg proteins (Nakada et al. 2007a; Cooper et al. 2013). Similarly, in circumstances where branchial Rh protein expression is upregulated, there is a parallel up-regulation of NHE (Nawata et al. 2010a; Zimmer et al. 2010) and/or HAT (Nawata et al. 2007; Nawata and Wood 2008; Nawata et al. 2010a; Zimmer et al. 2010) mRNA transcripts further supporting the importance of these two proteins in regulating branchial ammonia excretion. This system is also believed to work in the opposite manner in that increasing the rate of ammonia excretion can result in favourable electrochemical gradients for H^+ excretion (McDonald and Wood 1981; Evans et al. 2005; Wright and Wood 2009).

This arrangement also facilitates the uptake of Na^+ across the gills of the fish. In the case of the NHE, there is believed to be a direct exchange of Na^+ and H^+ (Avella and Bornancin 1989; Boisen et al. 2003; Yan et al. 2007). As alluded to earlier, ammonia excretion is believed to generate favourable chemical gradients for the operation of branchial NHE and, consequently, this leads to a loose coupling between the transport rates of the two substances (Krogh 1939; Maetz and Romeau 1964; Wright and Wood 1985; McDonald and Prior 1988; Wright and Wood 2009, 2012; Zimmer et al. 2010). In much the same manner as the NHE, the diffusion of NH_3 across the gills via apical Rhcg, is believed to permit an enhanced operation of HAT thereby facilitating continued Na^+ uptake across the epithelial Na^+ channel (Reid et al. 2003; Parks et al. 2008). While the ENaC has yet to be discovered in teleost fishes (Perry et al. 2003; Hiroi et al. 2008), there is recent evidence to suggest that HAT works in tandem with an acid-sensitive ion channel (ASIC) to facilitate branchial Na^+ uptake (Dymowska et al. 2014). Regardless, this system aids the fish in not only excreting ammonia but in ionoregulating as well (reviewed in Wilkie 1997 and Evans et al. 2005). It should be reiterated though that the coupling is loose and indirect, and branchial ammonia excretion is not necessarily coupled to branchial Na^+ uptake

under all circumstances (Kerstetter et al. 1970; Maetz 1972; McDonald and Prior 1988; Zimmer et al. 2010).

The interplay between Rh glycoproteins, H^+ and Na^+ transport mechanisms in the gill is collectively referred to as the Na^+/ NH_4^+ exchange metabolon and is believed to be the primary mechanism of branchial ammonia excretion (Fig. 1.1) (reviewed Wright and Wood 2009, Wright and Wood 2012, and Ito et al. 2013). However, the possible existence of this exchange metabolon has yet to be assessed in the renal epithelium of the kidney, an important site of acid-base regulation and, to a lesser degree, ammonia excretion in teleost fishes (McDonald and Wood 1981; McDonald 1983; King and Goldstein 1983b; Wood et al. 1999). While teleost renal ammonia transport information is limited, insights may be garnered through observations into the mechanisms employed by the mammalian kidney.

Mammalian Acid-Base Regulation:

In mammals, the kidney provides the primary site of acid-base regulation (reviewed in Knepper et al. 1989). Under metabolic acidosis, the mammalian kidney is capable of increasing the rate of urinary net acid excretion through two main components; TA-HCO_3^- (Sartorius et al. 1949; Hamm and Simon 1987) and ammonia (Sartorius et al. 1949; Leonard and Orloff 1955; Pitts et al. 1963; Sajo et al. 1981; Hamm and Simon 1987; Cheval et al. 2006) with the latter effectively representing the major portion of the acid excreted by the kidney during metabolic acidosis (Sartoruis et al. 1949; Hamm and Simon 1987). Alterations to the rate of renal ammonia excretion arise mostly as a result of secretion by the renal tubule cells of the nephron (Glabman et al. 1963; Sajo et al. 1981; Simon et al. 1985; Reviewed in Weiner and Verlander 2011).

The synthesis and excretion of ammonia in mammals, as outlined earlier, is crucial to the maintenance of acid-base homeostasis through the production of HCO_3^- in this process. The synthesis of ammonia is believed to occur primarily in the cells lining the proximal tubule (Curthoys and Lowry 1973; Good and Burg 1984) through a metabolic pathway involving the catabolism of amino acids, namely glutamine (see *Ammonia and its Physiological Roles*). Under metabolic acidosis, the overall ammoniogenic capacity of the kidney is greatly increased whereby the activities/expression of enzymes mediating glutamine catabolism are significantly upregulated (Wright and Knepper 1990; Wright et al. 1992; Curthoys 2001; Curthoys et al. 2007). Ultimately, this results in an elevation in the synthesis of both renal ammonia (Vinay et al. 1980) and HCO_3^-(Wright et al. 1992) whereby the HCO_3^- is transported to the extracellular fluid to restore acid-base homeostasis. The ammonia is then translocated into the lumen of the proximal tubule through the use of an apically expressed NHE (Amemiya et al. 1995). Here, ammonia is secreted through electroneutral exchange wherein H^+ is replaced by an intracellularly derived NH_4^+ and is exchanged against a luminal Na^+ by the NHE (Kinsella and Aronson 1981; Aronson et al. 1983; Nagami 1988; Nagami et al. 1989; Simon et al. 1992). This is supported quite strongly by the work of Nagami (2004) whereby proximal tubular secretion of ammonia in acidotic mice was greatly reduced when the cells were exposed to a saline containing low Na^+ and amiloride, a blocker of NHE. The results of this experiment were later confirmed by Nagami (2008) who also demonstrated a significant upregulation of NHE3 protein expression, relative to controls, in the proximal tubule of acidotic mice. In addition to this mechanism of transport, a proportion of the ammonia synthesized in the proximal tubule is believed to diffuse as NH_3 across the apical membrane through simple diffusion (Nagami 1988) or an unidentified transporter (Weiner and Verlander 2011) and/or facilitated diffusion of NH_4^+ via a K^+ channel

(Simon et al. 1992), thereby trapping a metabolic H^+. Rh glycoproteins do not appear to be involved with proximal tubule ammonia secretion (reviewed in Weiner and Verlander 2011). The net HCO_3^- produced in the proximal tubule from NH_4^+ secretion is subsequently shuttled into the extracellular fluid, via a basolateral Na^+/HCO_3^- cotransporter (NBC), to restore extracellular fluid buffering capacity and help restore systemic acid-base homeostasis (reviewed in Weiner and Verlander 2011)

The vast majority of the ammonia that is produced and secreted in the proximal tubule does not stay in the urine but is reabsorbed by the thick ascending limb of the loop of Henle specifically in the form of NH_4^+ (reviewed in Good 1994). Here, ammonia is translocated across the apical membrane by an apical $Na^+/K^+/2Cl^-$ cotransporter (NKCC) whereby NH_4^+ is substituted for K^+ (Good et al. 1984; Kinne et al. 1986; Watts and Good 1994; Kaplan et al. 1996). Indeed, the addition of bumetanide (Kinne et al. 1986) and furosemide to the nephron lumen (Good et al. 1984; Watts and Good 1994), both potent blockers of NKCC, results in a significant reduction of the loop of Henle's ability to reabsorb ammonia. This ammonia is subsequently transported from the tubule cell to the interstitium of the kidney via a basolateral NHE (Chambrey et al. 2001; Bourgeois et al. 2010; Weiner and Verlander 2011). Here, the ammonia is returned to the filtrate, through diffusive processes, at the thick descending limb establishing a counter-current exchange system whereby ammonia can accumulate in the medulla of the kidney (Sullivan 1965; Stern et al. 1985; Packer et al. 1991; reviewed in Weiner and Verlander 2011, 2014). This results in a concentration gradient of ammonia existing over the transition between the renal cortex (lowest [ammonia]) and the renal medulla (highest [ammonia]). The high medullary [ammonia] generated by the counter current exchange is critical in maintaining favourable gradients for continued collecting duct ammonia secretion (Buerkert

et al. 1982; reviewed in Weiner and Verlander 2011)(Fig. 1.2). As such, under metabolic acidosis where collecting duct ammonia secretion is elevated (Sajo et al. 1981), there is an increase in ammonia delivery to the loop of Henle (Buerkert et al. 1982) as well as simultaneous elevations in ascending limb ammonia reabsorption (Buerkert et al. 1983; Good 1990), NHE-3 mRNA (Lahgmani et al. 1997; Kim et al. 1999) and the expression of both NKCC protein and mRNA (Attmane-Elakeb et al. 1998). Overall, this results in a stronger ammonia concentration gradient between the medulla and the cortex of the kidney thereby facilitating a greater degree of collecting duct ammonia secretion (Stern et al. 1985; Packer et al. 1991; reviewed in Weiner and Verlander 2011).

The collecting duct cells are the primary site of ammonia secretion in the mammalian kidney (Pitts et al. 1958; Glabman et al. 1963; Sajo et al. 1981). The collecting duct is also the primary site of Rh glycoprotein expression (Quentin et al. 2003; Verlander et al. 2003) reflecting its key role in renal ammonia secretion and acid-base regulation. Here, similar to the teleost gill, ammonia secretion is mediated entirely by Rh glycoproteins which consist of two major isoforms; a basolateral Rhbg (Eladari et al. 2002; Quentin et al. 2003; Kim et al. 2007; Verlander et al. 2002) and, unlike the case in teleost gills, an Rhcg which demonstrates both apical (Eladari et al. 2002; Quentin et al. 2003; Han et al. 2006; Seshadri et al. 2006b; Kim et al. 2007; Verlander et al. 2002) and basolateral expression (Han et al. 2006; Seshadri et al. 2006b; Kim et al. 2007). Under metabolic acidosis, when ammonia excretion is elevated, the mammalian kidney exhibits considerable increases in Rh glycoprotein expression at both the transcriptional (Cheval et al. 2006; Bishop et al. 2010) and protein level (Seshadri et al. 2006a; Seshadri et al. 2006b). The knockout of Rhcg from the collecting ducts of acidotic mice not only impairs renal ammonia excretion under acidosis but also simultaneously reduces the ability of mice to acid-

base regulate (Lee et al. 2009; Lee et al. 2010; Lee et al. 2014). Similar effects have been demonstrated with Rhbg knockout in acidotic mice (Bishop et al. 2010) however, the relevance of Rhbg in moderating ammonia secretion is a topic of debate (Chambrey et al. 2005; Weiner and Verlander 2014). Apical Rhcg proteins have also been observed to co-localize with an apical HAT in the collecting duct (Eladari et al. 2002; Han et al. 2006; Lee et al. 2009) whereby acidosis results in concurrent increases in the mRNA transcripts of both of these proteins (Cheval et al. 2006). This has provided strong evidence to support a model of ammonia transport based on apical parallel transport of $H^+ + NH_3$ in the collecting duct of the mammalian kidney (Fig. 1.3). Here, ammonia (as NH_3), is permitted to diffuse across the basolateral surface of the collecting duct tubule cell via Rhbg and/or Rhcg. It then diffuses from the intracellular space to the lumen of the nephron through an apical Rhcg with cell-to-lumen P_{NH3} gradients being maintained through H^+ secretion into the boundary layer microenvironment of the tubule cell (reviewed in Weiner and Verlander 2014). Unlike the gills in teleosts though (Wright and Wood 2009), this transport is not coupled with Na^+ uptake in any appreciable way (Weiner and Verlander 2011; Weiner and Verlander 2014). In this manner, mammals are able to excrete ammonia and produce HCO_3^-, thereby working to maintain acid-base homeostasis.

Teleost Renal Ammonia Transport:

While the mechanisms of branchial ammonia excretion and acid-base regulation are fairly well understood, little is known about these processes in the teleostean kidney. Under resting physiological conditions, the teleost kidney accounts for a relatively small proportion of the total ammonia excreted by the animal (Smith 1929; McDonald and Wood 1981; Zimmer et al. 2014). However, metabolic acidosis results in a marked increase in renal ammonia excretion well above baseline levels in rainbow trout (Wood and Caldwell 1978; McDonald and Wood

1981; Wood et al. 1999) and goldfish (King and Goldstein 1983b). Increases in renal ammoniogenic enzymes, including glutamate dehydrogenase and alanine aminotransferase, have also been documented under metabolic acidosis suggesting an endogenous ammonia synthesis system via renal amino acid catabolism similar to that of the mammalian kidney (Wood et al. 1999). However, this system is believed to differ in one respect in that rather than metabolising glutamine, the teleost kidney instead uses alanine and/or aspartate as its preferred substrate(s) (Wood et al. 1999; King and Goldstein 1983b). Under this model, alanine/aspartate would undergo transamination via alanine/aspartate aminotransferases to form glutamate. This glutamate is metabolised in much the same as in the mammalian kidney ultimately resulting in the formation of NH_4^+ and HCO_3^- (Wood et al. 1999). However, this has yet to be thoroughly evaluated.

Information pertaining to the specific mechanisms of renal ammonia transport is lacking. However, the same transporter proteins as in the branchial epithelium appear to be present in the teleost kidney, suggesting the possible presence of a Na^+/NH_4^+ exchange metabolon. Rh glycoproteins have been documented in the kidneys of teleost fish with Nakada et al. (2007a) first demonstrating the expression of an apical Rhcg1 in the zebrafish nephron. Since then, Rh glycoproteins as either transcripts or proteins, including Rhcg and Rhbg isoforms, have been identified in the kidneys of a number of teleost species including the rainbow trout (Nawata et al. 2007), the common carp (Wright et al. 2014) and the mangrove killifish (Hung et al. 2007; Cooper et al. 2013). In much the same manner as the teleost gill (reviewed in Wright and Wood 2009) and mammalian collecting duct (reviewed in Weiner and Verlander 2014), Rhcg1 appears to be apically expressed in the distal tubule (Nakada et al. 2007a; Cooper et al. 2013; Wright et al. 2014), and collecting duct (Nakada et al. 2007a) of the teleost nephron. Additionally, these

proteins have been found to co-localize with many of the classical Na^+ transport proteins. Here, Rhcg1 has been found to co-localize with a basolateral NKA in distal tubule cells of the zebrafish (Nakada et al. 2007a), common carp (Wright et al. 2014) and mangrove killifish (Cooper et al. 2013). Additionally, NHE3 is also found to be expressed on the apical distal tubule membrane alongside Rhcg1 (Cooper et al. 2013). Further support for a Na^+ dependent mode of renal ammonia transport is found in Ivanis et al. (2008a) whereby the proximal tubule cells of rainbow trout exhibit a basolaterally expressed NKA alongside apical expressions of both NHE3 and HAT.

To date, few studies have attempted to consolidate the molecular and physiological findings into an overall model of renal ammonia transport. Wright et al. (2014) provided some evidence to suggest a Na^+/NH_4^+ exchange metabolon because metabolic acidosis elevated the expression, at the transcriptional level, of Rhcg1a, Rhcg1b, NHE3, HAT and NKA in the common carp. However, despite an elevation in urinary [ammonia], acidosis did not result in the expected increase in renal ammonia excretion. This was believed to be a result of a reduction in urine flow rate (UFR) which served to reduce ion loss in a low pH environment, representing an "acid/base-ion balance compromise". This compromise represents the animal's "decision' to favour either urinary ion conservation or acid excretion under acidosis through a decrease in UFR or an increase in urinary ammonia excretion, respectively (Wright et al. 2014).

Methods Used for Teleost Analysis:

In the present study, to address the mechanism(s), role, and contribution to acid-base balance of renal ammonia excretion, goldfish (*Carassius auratus*) were fitted with urinary bladder catheters (Wood and Patrick 1994) and exposed to either a control (pH=8.2) or a low pH

(pH=4.0) water environment for a duration of 48-h. Urine was collected every 12-h and was analysed for a variety of parameters. These included urinary volume, pH, total ammonia concentration (T_{amm}), [TA-HCO_3^-], inorganic phosphate (P_i), urea, Na^+ and Cl^-. At 48-h, fish were quickly euthanized, and plasma and renal tissues were taken for further analysis of the fish's acid-base status. Additionally, renal tissues were investigated at the enzymatic and transcriptional level to address responses to metabolic acidosis in renal nitrogen metabolism and transporter physiology.

A subset of fish were pre-injected with radio labelled polyethylene glycol MW 4000 ([^{3}H]PEG-4000) to address changes in glomerular filtration rate (GFR) in response to acidosis (Curtis and Wood 1991; Robertson and Wood 2014). [^{3}H]PEG-4000 was employed here as it has been shown to be an ideal glomerular marker as it has a high renal clearance ratio as well as a low bladder reabsorption rate, relative to other glomerular markers (e.g. inulin, glofil) (Bayenbach and Kirschner 1976), thus providing a reasonable estimate of glomerular filtration rate. The filtration of the blood at the glomerulus is the first stage of urine production wherein only small substances (>40-115 Å; >68,000 MW), including my glomerular marker (4000 MW), are permitted to pass through the filtration barrier (Bott and Richards 1941; Caulfield and Farquhar 1974; Ohlson et al. 2000; Ohlson et al. 2001). This forms the primary urine which is reflective of the ionic/molecular composition of the plasma (Smith 1952). As such, changes in the filtration rate can greatly influence the excretion rate of a particular metabolite. In mice, increases in the GFR can correspond with an elevated renal ammonia excretion (Ditella et al. 1978). This effect has been suggested to occur in teleosts as well (King and Goldstein 1983b), reflecting the need to measure GFR to fully understand the specific contributions of the filtration and secretion to renal ammonia excretion.

By measuring GFR and the concentration of a particular metabolite in the blood plasma, we can calculate the net amount of that metabolite appearing in the urine that originated from glomerular filtration rather than from renal secretory processes (Smith 1952). This calculation assumes that the primary filtrate has the same composition as the blood plasma, so the filtered input is calculated as the product of the plasma composition of a moiety (e.g. ammonia) times its GFR. By subtracting the filtered input of a particular metabolite from the total urinary excretion value, we can directly determine the role of the kidney in the secretion and/or reabsorption of said metabolite. If the net excretion rate of a substance exceeds its filtration rate, then the difference represents the net secretion rate. If the net excretion rate is less than the filtration rate, then net reabsorption (i.e "negative secretion") must have occurred. This is an important consideration for implicating Rh glycoproteins in renal ammonia secretion (Weiner and Verlander 2014). Thus, by measuring the GFR and plasma composition we can determine the specific tubular processes mediating changes in the urinary excretion of a variety of metabolites relevant to acid-base regulation.

The experimental treatment (exposure to low water pH) was chosen as it has previously been shown to induce a metabolic acidosis in which renal ammonia excretion is significantly upregulated (McDonald and Wood 1981; King and Goldstein 1983b; Wood et al. 1999). Low pH water is advantageous in renal ammonia transport assessment as it results in a favourable gradient for branchial H^+ uptake and an unfavourable gradient for branchial H^+ excretion. In this situation, acid-base regulation is primarily mediated by the kidney (McDonald and Wood 1981; King and Goldstein 1983b; McDonald 1983; Wood et al. 1999; Wright et al. 2014). In essence, the fish loads with acidic equivalents at the gills which must be excreted via the kidney.

The selection of goldfish for use in this study was dependent on a number of factors. First, goldfish had been previously shown to survive at pH=4.0, exhibiting clear signs of metabolic acidosis as well as an upregulation of renal acid excretion in the form of ammonia (King and Goldstein 1983b). As well, because of previous work (Bradshaw et al. 2012; Sinha et al. 2013; Sinha et al. unpubl.), there is a full suite of molecular probes available to assess changes in renal transport mechanisms at the molecular level. Lastly, these fish are readily available, cost effective and need only basic housing requirements. Indeed, the goldfish is the ideal model fish in assessing renal ammonia transport.

Urinary bladder catheterization (reviewed in Wood and Patrick 1994) has proven to be a reliable and an effective method for collecting urine in variety of teleost fish species (Wood and Caldwell 1978; McDonald and Wood 1981; Evans 1982; King and Goldstein 1983b; Wood et al. 1999; Wright et al. 2014; Zimmer et al. 2014). However, this technique has one potential flaw in that it does not entirely represent the final urine product, because it continually drains the urine from the bladder, thereby preventing normal bladder function. It has been established that the teleost urinary bladder is an important site of ion regulation wherein the bladder epithelium is capable of reabsorbing a variety of ions/solutes from the primary urine (Curtis and Wood 1991; Curtis and Wood 1992; Wood and Patrick 1994). In comparisons between urinary bladder cannulae and externally fitted cannulae (representing the final urine product) in rainbow trout, urinary bladder cannulae can artificially increase the overall excretion rate of some of these substances, including Na^+ and Cl^-, by as much as 50% (Curtis and Wood 1991; Curtis and Wood 1992). However, under a low pH environment (pH= 4.4), the excretion rates of Na^+, Cl^- and acidic equivalents were found to be consistent between the bladder urine and the final urine in trout (Patrick and Wood unpubl. *In* Wood and Patrick 1994). Thus, in the context of my study,

this may not be a problem. Regardless, the technique allows assessment of renal function alone, without complications of final urine re-processing in the bladder

Elasmobranchs; the Interaction of Acidosis and Ammonia:

Marine elasmobranchs are considered to be both ureotelic and ureosmotic in that they produce and retain in their body fluids high quantities of urea as a metabolic by-product and as an essential osmolyte (Smith 1931). Urea is synthesized in the mitochondria of liver and white muscle tissue through the ornithine-urea cycle (OUC). This process requires the use of glutamine as its primary substrate wherein this component is either directly imported or synthesized within the mitochondria through the addition of an ammonia (i.e. amination) to glutamate via glutamine synthetase (reviewed by Ballantyne 1997). Rather than expelling urea, elasmobranchs tend to accumulate it within their tissues reaching concentrations as high as 600 mM (Smith 1931; Yancy and Somero 1980) thus allowing the animal to reach the osmolarity of the external environment and achieve osmoconformity (Smith 1931; Yancy 2001; reviewed in Ballantyne and Robinson 2010). Given the importance of urea in marine elasmobranch survival, the substrates for urea synthesis, including glutamine and ammonia, are maintained at relatively low concentrations in the blood (King and Goldstein 1983a; Bedford 1983; Wood et al. 1995; Grosell et al. 2003). Indeed, relative to freshwater teleost fish, whole body ammonia excretion rates are very low, reflecting the high demand for ammonia in urea synthesis (Evans 1982; Claiborne and Evans 1992; Wood et al. 1995). However, the development of both metabolic (King and Goldstein 1983a; Wood et al. 1995; Nawata, Walsh, and Wood, unpubl.) and respiratory acidosis (Evans 1982; Cross et al. 1969; Claiborne and Evans 1992) has been shown to elevate the rate of whole body and renal ammonia excretion in elasmobranch fishes. Additionally, elevations in TA-HCO_3^- excretion at both exchange sites have been observed under acidosis and, unlike the

responses in teleosts, these constitute a greater proportion of the net acid excretion, likely reflecting an ammonia conservation mechanism (Evans 1982; King and Goldstein 1983a; Wood et al. 1995; Claiborne and Evans 1992).

However, in both the branchial and renal epithelia, the mechanism(s) by which ammonia is translocated to the external environment under these conditions remain relatively unknown. The current evidence suggests that this system may involve Rh glycoproteins. Nakada et al. (2010) demonstrated the expression of a primitive Rh glycoprotein, Rhp2, which was restricted to the basolateral membranes of the sinus zone of the nephron in the Japanese hound shark (*Triakis scyllium*) and which co-localized with an apical HAT in this region. Given that Rhp2 was responsive to environmental salinity changes, the authors proposed that this system was likely being used to recover ammonia from the urine for use in urea synthesis (Fig. 1.4). However, this notion has yet to be confirmed. Additionally, transcripts of Rhp2, Rhbg, and NHE2 have been reported to occur in the kidneys and gills of the Pacific spiny dogfish (*Squalus acanthias suckleyi*) (Nawata, Walsh, and Wood unpubl.) and of Rhbg in the little skate (*Leucoraja erinacea*) (Anderson et al. 2010). In the Atlantic spiny dogfish (*Squalus acanthias acanthias*), it has been determined that Rhcg protein is localized to the apical membrane in both the gills and kidneys and that there is a significant presence of the mRNA transcripts of NHE2 and Rhcg in both of these regions (Wright, Lawrence, Currie, MacLellan, Wood and Edwards unpubl.). However, the functional aspects of these transporter proteins remain unknown.

In my thesis, I attempted to resolve the mechanisms of ammonia transport in elasmobranch fish at both the level of the gill and the kidney. In addressing this question, the Pacific spiny dogfish (*Squalus acanthias suckleyi*) was employed as we have a number of molecular and immunohistochemical probes to address the composition and changes of the

transporter physiology of these animals (Wright, Lawrence, Currie, MacLellan, Wood and Edwards unpubl.; Nawata, Walsh, and Wood unpubl.). In previous work, it was demonstrated that urinary ammonia excretion increases under metabolic acidosis (King and Goldstein 1983a; Wood et al. 1995). In assessing ammonia transport physiology, animals were fitted with arterial (De Boeck et al. 2001b) and urinary bladder (Wood and Patrick 1994) cannulae. A no-infusion flux period was first conducted to assess baseline levels of whole body ammonia, urea and TA-HCO_3^- effluxes through water analysis. Blood samples, via the arterial cannula, were also taken during this time to address basic blood acid-base parameters in these animals. Fish were then infused with either a 500 mM NaCl saline (control fish) or a 375 mM NaCl/125 mM HCl saline (acid-loaded fish) at a rate of 3 ml/kg/h over a 24-h period with the same parameters being assessed as above. Following infusion, fish were quickly euthanized with terminal gill, kidney, rectal gland, white muscle and liver tissues being sampled. These are currently being used in molecular analyses of transport physiology (Wright, Lawrence, Currie, MacLellan, Wood and Edwards unpubl). Unfortunately, due to seminal fluid contamination of the urine, urinary responses (e.g. ammonia and TA- HCO_3^- excretion rates) could not be determined during this exposure series. There was also a lack of a clear sign of metabolic acidosis in the acid-infused fish. Therefore, the data from the elasmobranch studies do not parallel those collected on the goldfish. Under the recommendation of my committee, they have been summarized in the Appendix of this thesis.

Thesis Objectives:

The aim of my thesis was to characterize the response of the fish kidney on a physiological, biochemical and molecular level to metabolic acidosis. This information would then be integrated to determine a working model of renal ammonia transport. This model is

specifically for freshwater teleost fishes. Due to the above-mentioned problems with the elasmobranch experiments, comparisons will not be made between the two taxonomic groups investigated here. Nevertheless, the preliminary data obtained on the dogfish shark remain valuable, and so are summarized in the Appendix. My thesis investigates the specific mechanism of renal ammonia transport, a largely ignored aspect of teleost physiology. Additionally, it contributes a working model of the physiological responses to low environmental pH, a significant issue with respect to industrial waste effluents and anthropogenic environmental acidification. In this way we can better predict how these animals might be able to cope with increases in environmental acid stress and provide tools in mitigating harmful effects of this stress at the population level. To address these concerns, my thesis had the following specific objectives:

1. To expose goldfish to a low environmental pH (pH=4.0) to address the effects of metabolic acidosis on urinary acid and metabolite excretion, as well as whole kidney tissue acid-base status and enzymatic activity.
2. To use the radio-labelled glomerular marker [^{3}H]PEG-4000 to determine changes in glomerular filtration rate (GFR), and thereby allow assessment of the relative roles of filtration *versus* renal secretion and reabsorption processes in alterations in renal metabolite excretion in the goldfish. The focus here was particularly on renal ammonia handling.
3. To investigate the potential coupling between Na^+ and ammonia excretion/secretion in order to assess the possible presence a renal Na^+/NH_4^+ exchange metabolon in teleost fish.

4. To make use of the available molecular probes to determine the effects of metabolic acidosis on the transcriptional expression of genes potentially involved in mediating renal Na^+/NH_4^+ exchange. The particular focus here was the role of Rh glycoproteins in acid-base regulation and renal ammonia secretion.
5. To integrate all of the above to develop a working model of renal ammonia transport in the goldfish.
6. To assess the physiological and molecular changes of the elasmobranch kidney and gills to the infusion of an acidic saline (HCl-loading), with specific reference to nitrogen metabolism and ammonia transport.

In my thesis, I assessed the following hypotheses:

H1: In the goldfish, elevations in urinary ammonia excretion during metabolic acidosis are predominately mediated through secretion by the renal tubule cells of the nephron as opposed to filtration by the glomerulus.

H2: Tubular ammonia secretion is mediated through an increase in renal Rh glycoprotein expression coupled to a Na^+ dependent mechanism.

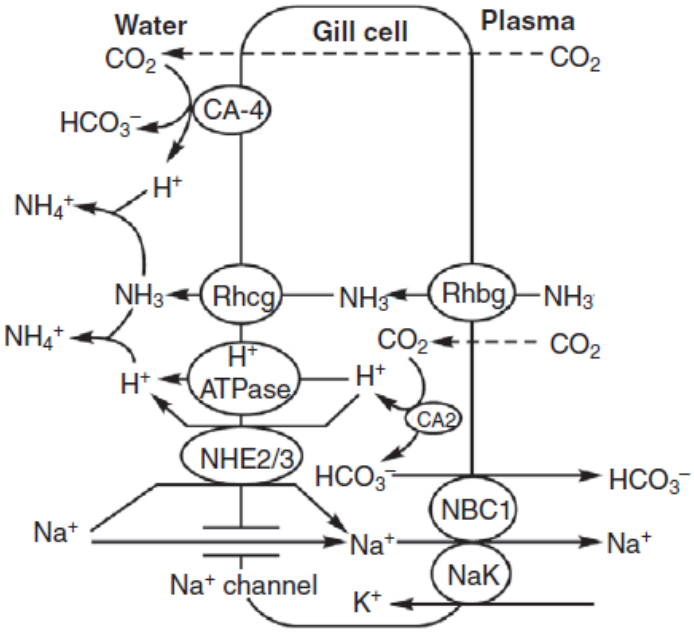

Figure 1.1: Proposed mechanisms of branchial ammonia transport in a freshwater teleost fish. Here, ammonia excretion is dependent upon a Na^+/NH_4^+ exchange complex that can function in acid-base regulation as well as in ion regulatory processes. CA=carbonic anhydrase, NaK= Na^+/K^+ ATPase, NBC=Na^+/HCO_3^- cotransporter. Adapted from Wright and Wood (2009).

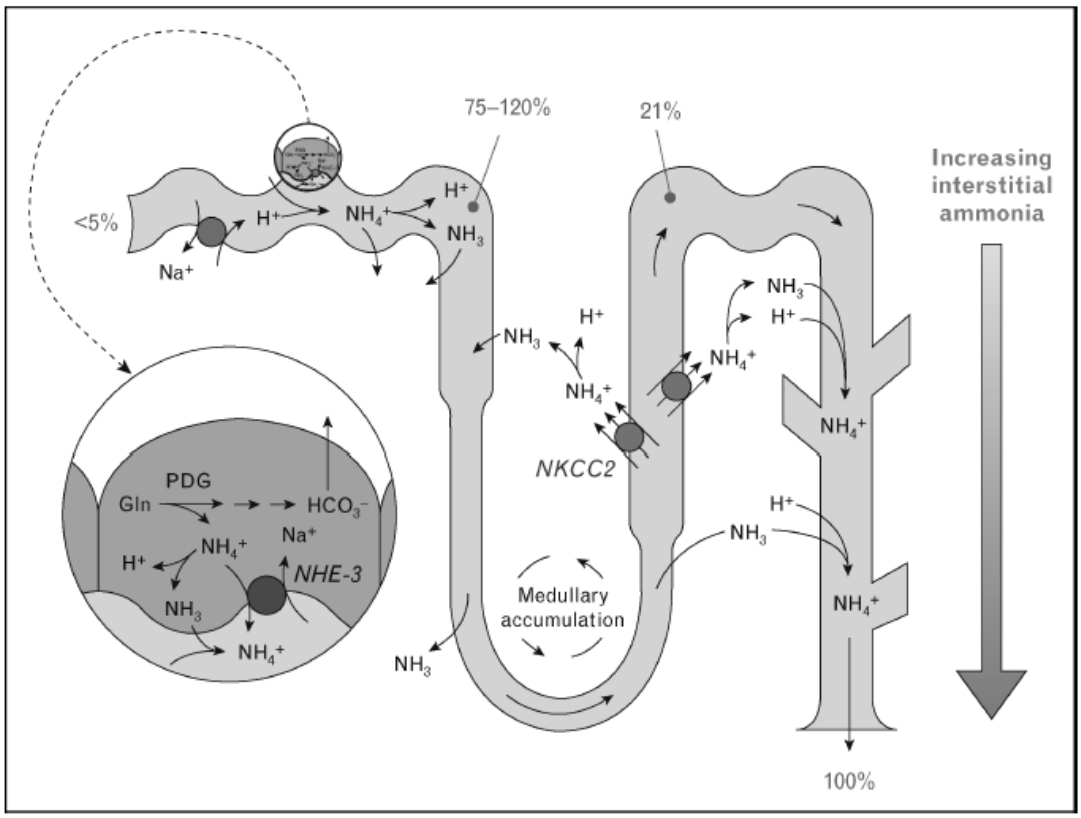

Figure 1.2: Proposed model of mammalian proximal tubule secretion and renal medulla ammonia accumulation. Proximal tubule secretion is mediated through Na^+/NH_4^+ via NHE-3. Accumulation of medullary ammonia occurs through a counter-current exchange system in the loop of Henle whereby ammonia is cycled via $Na^+/K^+/2Cl^-$ cotransporter (NKCC) (Adapted from Weiner and Verlander 2010).

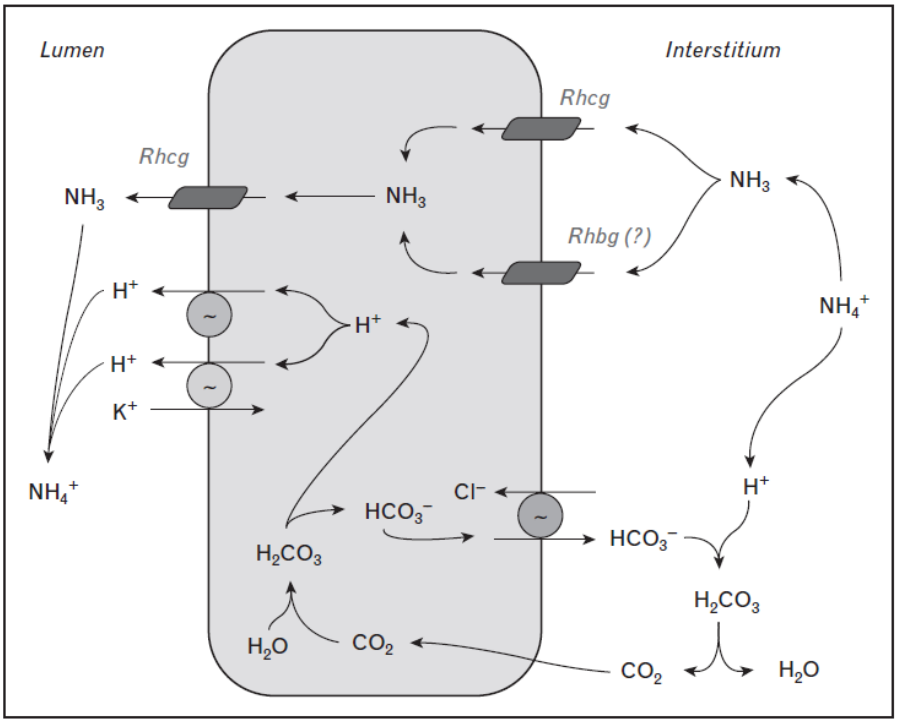

Figure 1.3: Proposed model of ammonia transport in the mammalian collecting duct. This system involves a parallel translocation of H^+ and NH_3 through the actions of an apical Rhcg and an H^+ ATPase and to a lesser extent, an H^+/K^+ ATPase. Adapted from Weiner and Verlander (2010).

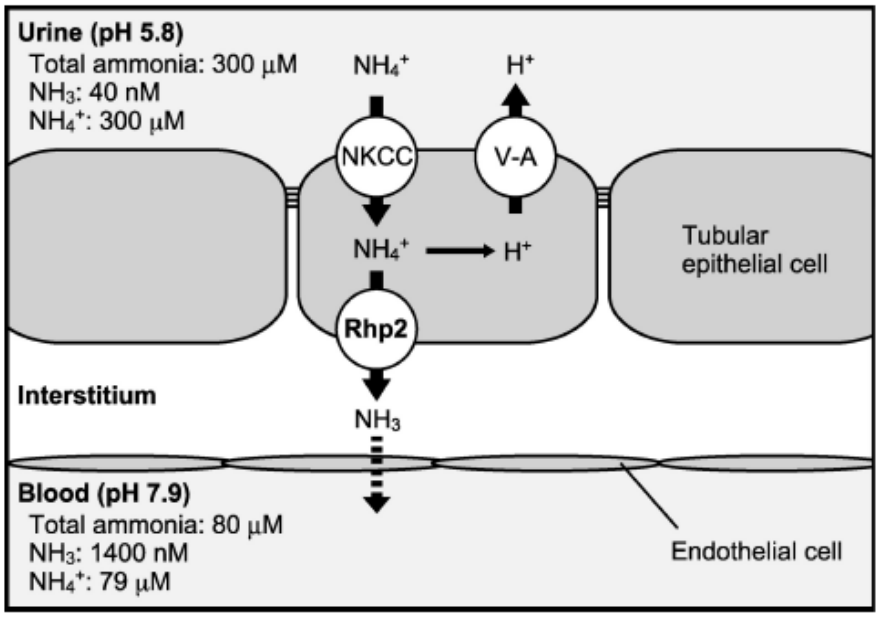

Figure 1.4: Proposed model of renal ammonia reabsorption in the sinus zone of the nephron of the Japanese hound shark (*Triakis scyllium)*. Here, an H^+ ATPase (V-A) generates a chemical gradient by removing H^+ to liberate NH_3 allowing the uptake of an NH_4^+ across the apical surface via NH_4^+/K^+ substitution on an apical NKCC ($Na^+/K^+/2Cl^-$ cotransporter). The H^+ on the NH_4^+ is stripped from it forming NH_3 which is allowed to diffuse across the basolateral surface along a partial pressure gradient via Rhp2. Adapted from Nakada et al. (2010).

CHAPTER 2: PHYSIOLOGICAL RESPONSES OF THE GOLDFSIH (*C. AURATUS*) TO METABOLIC ACIDOSIS AND POTENTIAL MECHANISMS OF RENAL AMMONIA TRANSPORT

Abstract:

Teleost acid-base regulatory processes can be mediated through renal ammonia excretion although little is known about the specific transport mechanisms. We hypothesized that an elevation in renal ammonia excretion during metabolic acidosis in the goldfish (*Carassius auratus*) is the product of tubular secretion through a Na^+/NH_4^+ exchange metabolon involving Rhesus (Rh) glycoproteins. Fish were exposed to a low pH environment (pH 4.0; 48-h) and demonstrated a characteristic metabolic acidosis: reductions in blood pH and $[HCO_3^-]$ with no change in plasma P_{CO2}. Acidosis induced an increase in total plasma [ammonia] but reduced plasma P_{NH3}. The intracellular environment of renal cells remained unchanged with regard to intracellular ammonia, lactate and pH. Urinary excretion of net acid, ammonia, titratable acidity (TA)-HCO_3^-, P_i and urea were elevated during metabolic acidosis. However, ion excretion (Na^+ and Cl^-) was unaffected by acidosis. Urine $[Na^+]$ was not correlated with urine [ammonia]. There was an elevation in renal alanine aminotransferase activity under acidosis. Acidosis had no influence on the glomerular filtration rate, urinary flow rate and Na^+ reabsorption but did correspond with an elevation in renal ammonia secretion. This was mirrored by an elevation in the expression of Rhcg1b mRNA. These data directly indicate a secretion based model of renal ammonia transport that is likely mediated through Rh glycoproteins and occurs independent of Na^+ transport. Rather, we propose a model of parallel H^+/NH_3 transport as the primary mechanism of renal ammonia secretion that is likely dependent on renal amino acid catabolism.

Introduction:

Renal ammonia excretion plays an important role in maintaining acid-base homeostasis in freshwater fishes. Under normal physiological conditions, the contribution of the kidney to whole body ammonia excretion is minimal, typically representing < 20% of total N-excretion, with > 80% being excreted at the gills (Smith 1929; McDonald and Wood 1981; Zimmer et al. 2014). However, metabolic acidosis dramatically elevates the rate of renal ammonia excretion in some species (McDonald and Wood 1981; McDonald, 1983; King and Goldstein 1983b; Wood et al. 1999). Specifically, renal ammonia excretion represents the shuttling of acid equivalents, in the form of NH_4^+ (or $NH_3 + H^+$) from the body of the fish to the urine for subsequent excretion, with the return of HCO_3^- to the extracellular fluid. This mechanism may become quantitatively more important than titratable acid (TA) excretion (e.g. P_i, organic acids) in its contribution to total urinary acid excretion during metabolic acidosis in fish (McDonald and Wood 1981; McDonald 1983; King and Goldstein 1983b; Wood et al. 1999). In mammals, the majority of NH_4^+ excreted in the urine results not from filtration, but rather from elevated amino acid catabolism in the renal tubular cells, a process which produces equimolar amounts of NH_4^+ for secretion and HCO^-_3 for restoration of extracellular pH on a net basis (Knepper et al. 1989; Atkinson 1992; Wright et al. 1992; Curthoys 2001).

In teleost fishes, the limited studies to date suggest that similar mechanism(s) may be at play during metabolic acidosis. In response to acidosis, ammoniogenic enzymes in the kidney increase in activity in trout (*Oncorhynchus mykiss*) (Wood et al. 1999), while plasma ammonia concentrations, and therefore by inference glomerular filtration of ammonia, rise only marginally in both trout and goldfish (*Carassius auratus*) (King and Goldstein 1983b; Wood et al. 1999).

However, elevated renal secretion of ammonia, and the cellular and molecular mechanisms by which it may occur, have not yet been directly demonstrated in teleosts.

In mammals, epithelial ammonia transport is facilitated by a specialized group of membrane proteins, the Rhesus (Rh) glycoproteins (Weiner 2004), and in fish, recent evidence suggests that the same is true, at least in the gills (Nakada et al. 2007b; Hung et al. 2007; Nawata et al. 2007; see Wright and Wood 2009 for review). These proteins appear to function as channels for the translocation of ammonia gas (NH_3) along favourable partial pressure gradients (P_{NH3}) (Knepper and Agre 2004; Javelle et al. 2007; Nawata et al. 2010). In the mammalian kidney, Rh proteins typically exist in two isoforms: Rhbg has a strict basolateral localization (Eladari et al. 2002; Quentin et al. 2003; Kim et al. 2007; Verlander et al. 2003) and Rhcg which can have dual localizations to both the apical (Eladari et al. 2002; Quentin et al. 2003; Seshadri et al. 2006b; Kim et al. 2007; Verlander et al. 2010) and basolateral surfaces (Seshadri et al. 2006b; Kim et al. 2007) of the renal tubule cell. Rh proteins are abundant in the collecting duct (CD) of the kidney (Quentin et al. 2003; Verlander et al. 2003) where the majority of renal tubular ammonia secretion occurs (Sajo et al. 1981). As Rh proteins facilitate NH_3 diffusion, blood-to-lumen P_{NH3} gradients are maintained through parallel transport of H^+ (Knepper et al. 1984; Wagner et al. 2009) via H^+-ATPase (HAT), an active transporter found to co-localize with apical Rh proteins in the CD (Eladari et al. 2002; Han et al. 2006; Lee et al. 2009). Ammonia excretion is coupled to Na^+ uptake in the proximal tubule, mediated through a Na^+/H^+ exchanger (NHE), whereby intracellular NH_4^+ substitutes for H^+ and is directly exchanged against luminal Na^+ (Aronson et al. 1983; Nagami 1988; Nagami et al. 1989; Simon et al. 1992). There is no evidence for Rh proteins in the mammalian proximal tubule though (reviewed in Weiner and Verlander 2011). During metabolic acidosis, there is also an upregulation of HAT, Rhcg and

Rhbg glycoprotein mRNA (Cheval et al. 2006) as well as an elevation in Rhcg protein expression (Seshadri et al. 2006a) in the CD. Indeed, the silencing of Rh glycoprotein genes in the CD reduces renal ammonia excretion as well as hindering systemic acid-base regulation in mice (Lee et al. 2009). As such, Rh glycoproteins are critical in maintaining mammalian acid-base balance.

In the teleost gill, ammonia excretion is mediated by a Na^+/NH_4^+ exchange complex or metabolon consisting of several key transporters (Rh proteins, HAT, NHE, carbonic anhydrase, Na^+/K^+ ATPase) which transport $NH_3 + H^+$ into the external water while simultaneously facilitating active Na^+ uptake from the water to the blood, thereby contributing to systemic ionic and acid-base homeostasis (reviewed by Wright and Wood 2009). However, information on the mechanisms of renal ammonia transport and acid-base regulation in fish is relatively sparse. To date, the mRNA transcripts of Rhbg and multiple isoforms of Rhcg have been found in the kidney of the common carp (Wright et al. 2014) and mangrove killifish (Hung et al. 2007), while Rhbg expression has been reported in the rainbow trout kidney (Nawata et al. 2007). Immunohistochemical techniques have localized Rhcg1 to the CD and distal tubule of the zebrafish nephron (Nakada et al. 2007a). There also exists a co-localization of a basolateral Na^+/K^+ ATPase (NKA) and apical Rhcg1 in the distal tubule of the zebrafish (Nakada et al. 2007a). Cooper et al. (2013) demonstrated a similar profile in the distal tubule of the mangrove killifish where Rhcg1 and NHE3 are on the apical membrane with a basolateral NKA. Apical NHE3 also co-localizes with an apical HAT and basolateral NKA in the proximal tubules of rainbow trout (Ivanis et al. 2008a). These observations suggest the presence of a Na^+ -coupled mechanism of renal ammonia secretion similar to that of the gill (Wright and Wood 2009). However, the only study which has examined this issue found an elevation in urine [ammonia]

and increased expression of Rhcg1a and Rhcg1b mRNA in the kidney of the common carp (*Cyprinus carpio*) during metabolic acidosis, yet there was no relationship between urinary ammonia excretion and Na^+ excretion (Wright et al. 2014). However, interpretation of the data was confounded by the simultaneous decrease in urine flow rate in this species under acid exposure such that there was no increase in urinary ammonia excretion. Thus, the purpose of the present study was to characterize the physiological, biochemical and molecular responses of the kidney to metabolic acidosis and develop a working model of renal ammonia transport in teleost fish.

We hypothesized that elevated urinary excretion of ammonia during metabolic acidosis in the goldfish would be predominately mediated through renal tubular secretion, rather than by increased glomerular filtration. Secondly, in light of the possible linkage of ammonia secretion via Rh proteins to Na^+ reabsorption discussed earlier, we predicted that increased expression of apical Rh glycoprotein (Rhcg1a and 1b) mRNA would occur, and that there would be a decrease in renal Na^+ excretion and an increase in renal tubular Na^+ reabsorption during acidosis. To test these hypotheses, we used the goldfish, *C. auratus*, one of the fish species in which renal ammonia excretion was first studied directly (King and Goldstein 1983b), and for which a suite of molecular probes are now available to investigate relevant transporter responses (Bradshaw et al. 2012; Sinha et al. 2013; Sinah unpubl.). Low environmental pH exposure (water pH = 4.0) was employed as an effective tool for inducing metabolic acidosis, as the gills take up rather than excrete acidic equivalents, and the kidney becomes the sole route of net acid excretion in this circumstance (McDonald and Wood 1981; McDonald 1983; King and Goldstein 1983b; Wood et al. 1999; Wright et al. 2014). Under this exposure, a sustained metabolic acidosis is developed in teleost fish (McDonald and Wood 1981; King and Goldstein 1983b; Wood et al. 1999; Wright et

al. 2014). These hypotheses were tested in the context of a detailed description of the response of the goldfish kidney to metabolic acidosis *in vivo*, with measurements of excretion, filtration, secretion, and reabsorption rates of ammonia, TA-HCO_3^-, inorganic phosphate (P_i), Na^+, Cl^-, and urea, together with comparable plasma measurements, and mRNA expression levels of potential transporters, and enzymatic activities in the kidney. Animals were fitted with urinary bladder catheters and exposed to control water at pH 8.2 or acid water at pH 4.0. Urine was collected over successive 12-h intervals for 48-h, then blood and renal tissue was terminally sampled.

Materials and Methods:

All procedures were approved by the McMaster University Animal Research Ethics Board (AUP 12-12-45) and were in accordance with the Guidelines of the Canadian Council on Animal Care. Unless otherwise noted, all chemicals were purchased from the Sigma-Aldrich Corporation (Oakville, ON, Canada)

Animal Care and Cannulation:

Goldfish [*Carasssius auratus* (Linnaeus 1758); 33.9± 0.8 g] were obtained from Aquality Inc. (Mississauga, ON, Canada) and held at McMaster University at 18-20 °C under a 12-h L:12-h D photoperiod in 200-L tanks served with recirculating filters and dechlorinated Hamilton tapwater. This moderately hard water had the following composition: [Na^+] = 0.6 mequiv/l, [Cl^-] = 0.8 mequiv/l, [Ca^{2+}] = 1.8 mequiv/l, [Mg^{2+}] = 0.3 mequiv/l, [K^+] = 0.05 mequiv/l; titration alkalinity 2.1 mequiv/l; pH ~8.2; hardness ~140 mg/l as $CaCO_3$ equivalents. The fish were fed to satiation 3 times per week with flaked food (Big Al's Canada, Woodbridge, ON, Canada), but fasted for 7 days prior to experimentation to avoid confounding effects of feeding status on ammonia excretion (Zimmer et al. 2010).

For urinary cannulation, fish were anaesthetized in a 200 mg/L tricaine methane sulfonate solution (Syndel Laboratories, Qualicum Beach, B.C., Canada) which had been neutralized with 1 M KOH. Fish were weighed before being placed on an operating table. The gills were artificially ventilated with a dilute MS-222 solution (100 mg/L). The urinary bladder was fitted with an indwelling catheter (Clay-Adams PE-50 tubing; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) as described in Wood and Patrick (1994) with the following exception. The last 2.5 cm of the distal end of the tubing was bent at a 90° angle so that it remained perpendicular to the midline of the fish while conforming to the fish's urinary tract to accommodate the unique anatomy of the goldfish urogenital system. In a subset of fish used for glomerular filtration rate measurements, 1 µCi of [^{3}H]polyethylene glycol-4000 (PEG-4000; Sigma-Aldrich, St. Louis, MO, USA) in 140 mM NaCl was injected into the caudal hemal arch at 0.003 ml/g-fish.

After cannulation, the animal was ventilated with anaesthetic-free water until strong breathing movements resumed, and then transferred to the experimental chamber for recovery. Each chamber comprised a small plastic box (~1-L) fitted with a perforated piece of 3.8 cm diameter PVC pipe (to prevent the animal from turning around), an aeration device, and an independent water source (1000 ml/min) from a pH-controlled (8.2) 250-L reservoir. Urine was collected continuously by gravity (head ~3 cm) into a glass vial.

Experimental Protocol:

There were two experimental series: control fish exposed to water pH=8.2 ±0.1 for 48-h, and experimental fish exposed to acidic water (pH 4.0 ±0.1) for 48-h. This water was continually pumped through each of the flux chambers from the pH-controlled 250-L reservoir. Water pH was maintained within the desired range through the use of a pH-stat system consisting of a

PHM82 pH meter (Radiometer-Copenhagen, Brønshøj, Denmark) coupled with a Radiometer GK24O1C combination glass pH electrode. This meter worked in conjunction with an auto-titration controller (Radiometer TTT-80) which regulated the water pH at the desired level through the metering of a 0.1 N HCl solution via a solenoid valve into the continuously mixed reservoir.

After at least 18-h of recovery at pH 8.2, exposure to pH 4.0 or 8.2 was initiated (time 0-h). Urine was collected over successive 12-h intervals. Urine flow rate (UFR), pH, and TA-HCO_3^- were measured immediately, and the remainder of the urine was frozen at -20°C for subsequent analysis of the concentrations of urinary total ammonia (T_{amm}), urea, Na^+, Cl^-, P_i, and [^{3}H]PEG-4000 radioactivity (if applicable).

In the subset of fish used for GFR measurements, it was necessary to quantify the loss of [^{3}H]PEG-4000 radioactivity through the gills. This was achieved by stopping the water flow to each chamber from 44 h to 48 h. Mixing was maintained by aeration. As the chambers were isolated from the pH-stat system, the appropriate pH was maintained over this period through manual titration (0.1 N HCl) in combination with a handheld pH meter (SympHony SP70C, VWR Inc, Edmonton, AB, Canada). At the start and end of this closed period, a 4-ml water sample was collected and frozen at -20°C for later analysis of [^{3}H]PEG-4000 radioactivity.

At 48-h, fish were quickly euthanized with a lethal dose of MS-222 (750 mg/L) that was pH balanced (1 M KOH) to match the experimental pH. A terminal blood sample was drawn by caudal puncture into a 1-ml syringe rinsed with lithium heparinized (300 mg/L) Cortland saline (Wolf 1963). The sample was quickly transferred to a 0.5-ml centrifuge tube and blood pH was measured, taking care to minimize air exposure. The blood was immediately spun at 1,500 g in a

microfuge (Mandel, Guelph, ON, Canada) for 1 min, and the plasma was decanted and flash-frozen in liquid N_2 for later analysis of plasma T_{amm}, urea, Na^+, Cl^-,P_i, HCO_3^-, lactate, glucose and cortisol concentrations and [^{3}H]PEG-4000 radioactivity. Lastly, the kidneys were removed, and flash-frozen in liquid N_2, for later measurements of enzymatic activity and mRNA expression. Samples were stored at -80°C.

Analytical techniques

Urinary analyses:

UFR was measured gravimetrically and urine pH was measured at the experimental temperature (18±1°C) with a pH microelectrode (Orion PerpHecT ROSS , Thermo Fischer Scientific, Toronto, ON, Canada) coupled to a pH meter (SympHony SP70C, VWR Inc, Edmonton, AB, Canada). Urinary [TA-HCO_3^-] was determined within 24-h using double endpoint titration (Hills 1973) and standardized procedures for fish urine (McDonald and Wood 1981; Wood 1988). A glass pH electrode (Radiometer-Copenhagen GK2401C, Brønshøj, Denmark) coupled to a H160 pH meter (Hach, Mississauga, ON, Canada) and 2-ml microburettes (Gilson, Middleton, WI, USA) filled with standardized solutions, 0.02 N HCl or 0.02 N NaOH, were employed. All samples were titrated first to below pH 4.0 and aerated with CO_2-free air to eliminate HCO_3^-, and then back to a control blood pH of 8.00 (interpolated from Tzaneva et al. 2011).

Total urinary ammonia and urea concentrations were determined through the colorimetric salicylate (Verdouw et al. 1978) and monoxime (Rahmatullah and Boyde 1980) methods, respectively, on a microplate reader (SpectraMax 340PC, Molecular Devices Sunnyvale, CA, USA). Urinary Na^+ concentrations were determined by flame spectrophotometry (Spectra AA

220FS, Varian, Palo Alto, CA, USA), whereas urine Cl^- was determined through a mercury thiocyanate based colorimetric assay (Zall et al. 1956). [^{3}H]PEG-4000 radioactivity (beta emissions) was determined by scintillation counting (Tri-Carb 2900TR Liquid Scintillation Analyzer, PerkinElmer Inc., Waltham, MA, USA). Samples (100 µl) were incubated in a 1:4 ratio (sample:fluor) with Optiphase HiSafe fluor (PerkinElmer Inc., Waltham, MA, USA). Total P_i in the urine was measured with a commercial kit (Pointe Scientific, Canton, MI, USA).

Plasma analyses:

Whole blood pH was measured at experimental temperature (18±1°C) using a pH microelectrode (Thermo Fisher Scientific, Toronto, ON, Canada) and a pH meter (SympHony SP70C, VWR Inc, Edmonton, AB, Canada). Plasma T_{amm} was assayed using an enzymatically based commercial kit (Raichem, Cliniqa, San Marcos, CA, USA). Plasma ions and urea were measured as for urinary parameters. Plasma cortisol was determined with a commercial radioimmunoassay kit (Gammacoat, DiaSorin, Missauga, ON, Canada) and read on a gamma counter (Wizard 1480 300 Auto Gamma Counter, PerkinElmer Inc., Waltham, MA, USA). Lactate and glucose were measured with a handheld lactate meter (Lactate Pro, Arkray Inc., Kyoto, Japan) and a commercially available reagent kit (Infinity Glucose Hexokinase Liquid Stable Reagent, Thermo Fisher Scientific, Toronto, ON, Canada), respectively. Total plasma bicarbonate was measured by the double endpoint titration method described earlier. Plasma [^{3}H]PEG-4000 radioactivity was determined by scintillation counting as described above, but using Optima Gold scintillation fluor (PerkinElmer Inc., Waltham, MA, USA; 1:2; sample:fluor ratio). The total volume of each sample was made up to 1-ml using nanopure water (0.9-ml). Water [^{3}H]PEG-4000 radioactivity was analyzed using a method similar to that used for urinary analysis.

Enzymatic analyses:

The activities of glutamate dehydrogenase (*EC 1.4.1.2*), alanine aminotransferase (*EC 2.6.1.2*), aspartate aminotransferase (*EC 2.6.1.1*), arginase (*EC 3.5.3.1*), glutamine synthetase (*EC 6.3.1.2*) and glutaminase (*EC 3.5.1.2*) were assayed using a common homogenization buffer. Frozen kidney tissue from each fish was weighed to an appropriate mass (~40 mg) and immediately sonicated on ice in 200 µl of glycerol buffer (50% glycerol, 20 mM K_2HPO_4, 10 mM HEPES, 0.5 mM EDTA, 1 mM DTT; pH=7.5). The homogenate was centrifuged at 4°C (µSpeedFuge SFR13K, Savant, Toronto, ON, Canada) at 11,500 g for 3 minutes, and the supernatant was assayed. Specific assay conditions for glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase and arginase followed the methods reported by Mommsen et al. (1980) and those for glutamine synthetase and glutaminase followed those of Webb and Brown (1976) and Walsh et al. (1990), respectively. Kinetic assays measured the disappearance of NADH using zero order reaction kinetics in a linear fashion.

NKA (*EC 3.6.3.9*) and HAT (*EC 3.6.3.6*) activities were determined using the methods of McCormick (1993) and Nawata et al. (2007), respectively. Samples were homogenized (Power Gen 125 homogenization unit, Thermo Fisher Scientific, Toronto, ON, Canada) in an imidazole buffer (50 mM imidazole, 125 mM sucrose, 5 mM EGTA; pH=7.5) at 4°C. Activity was normalized to total protein content as measured with Bradford's reagent. Enzymatic activities were measured on a Molecular Devices microplate reader (SpectraMax 340PC, Sunnyvale, CA, USA) at room temperature.

Carbonic anhydrase (CA; *EC 4.2.1.1.*) activity was assayed using the method of Henry (1991). Frozen tissues were homogenized in a buffer solution (10 mM NaH_2PO_4, 225 mM

mannitol, 10 mM Tris, 75 mM sucrose; pH 7.4 using H_3PO_4), and the supernatant obtained by centrifugation (4°C) at 11,500 g for 1 minute. Changes in pH during the reaction were recorded using the Radiometer GK2401C glass pH electrode and Hach H160 pH meter described earlier. Activity was normalized to total protein content.

Whole Tissue Analyses:

Frozen kidney tissue was ground under liquid nitrogen with a chilled mortar and pestle. Whole tissue ammonia was assayed on tissues that had been deproteinized (8% PCA, 1 mM EDTA) and returned to physiological pH (1 M KOH; ~7.5). Samples were centrifuged (4°C; µSpeedFuge SFR13K, Savant, Toronto, ON) at 11,500 g for 3 minutes. The resulting supernatant was assayed for total ammonia content using the same Raichem kit as for plasma. Whole tissue lactate was measured on the same supernatant using the lactate:hydrazine sink method as outlined in Walsh (1987) but with a slightly modified reaction buffer solution (0.4 M hydrazine, 2 mM EDTA, 1 M glycine; pH=9.5).

Intracellular pH was measured by the method of Portner et al. (1990). Tissue powdered under liquid nitrogen (0.1 g) was incubated in buffer (1 ml) (6 mM sodium nitrilotriacetate, 150 mM KF), then centrifuged at 11,500 g for 3 minutes (4°C) using the µSpeedFuge centrifuge (see above). The same pH microelectrode and pH meter combination, at experimental temperature, was used as for blood pH.

mRNA Expression:

Total RNA was extracted from previously frozen renal tissue, stored at -80°C, using a TRIzol (Invitrogen, Burlington, ON) based extraction method. Samples were then assessed optically for total RNA purity (Nanodrop ND-1000, Nanodrop Technologies,Wilmington, DE,

USA) in which the 260/280 and 260/230 ratios were determined. The RNA quality was further evaluated by gel electrophoresis (1% agarose gel). Samples were of acceptable quality if both ratios were 2.00±0.1. cDNA was generated from total RNA (1 µg) by incubating with SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada), oligo (dT17) primers (Invitrogen) and excess deoxyribonucleotide triphosphate (DNTP). Genomic DNA was removed from each sample through the addition of DNase I (Invitrogen). cDNA was stored at -20°C.

mRNA transcript expression of candidate genes was ascertained using quantitative real-time PCR (RT-qPCR). Primer sequences were derived from Sinha et al. (2013), Sinha et al. (unpubl.) and Bradshaw et al. (2012) (Table 2.1). qPCR reactions consisted of a total volume of 10 µl; 4 µl of cDNA sample, 5 µl of 2x SSoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 0.4 µl (10 µM) of both the reverse and forward primers (Mobix, Hamilton, ON, Canada) and 0.2 µl of RNase free water. Reactions were conducted in a real time-PCR unit (CFX Connect Real-Time PCR detection system, Bio-Rad Laboratories Inc., Hercules, CA, USA). The following protocol was utilized: polymerase activation (98°C, 2 min), a two-stage amplification (1^{st}: 98°C, 2 s. 2^{nd}: 60°C, 5 s) x39 cycles. A melt curve was generated by heating samples from 75°C to 95°C by 0.2°C every 10 s to ensure the production of a single gene product. A no-template control (RNase free water) and no-reverse transcriptase sample was run on every plate. The efficiency of each primer set reaction was determined through a standard curve derived from a pool of all cDNA samples (Table 1). mRNA expression data were normalized against two housekeeping genes, EF1α and β-actin, using the GeNorm algorithm (Primer Design Ltd., Southampton University, Highfield Campus, Southampton Haunts, UK; Vandesompele et al. 2002).

Calculations:

Urine flow rate (UFR) was calculated as the total urine produced (V) divided by the mass of the fish (m) and the duration of the collection time (t):

(1) $UFR=V/(m*t)$

The excretion rate of a metabolite was calculated as the product of UFR and the concentration of a metabolite in the urine ($[M]_u$):

(2) $\text{Excretion Rate} = UFR*[M]_u$

Negative values indicate a net efflux of a metabolite via the urine.

Glomerular filtration rate (GFR) was calculated as the product of the urinary [^{3}H]PEG-4000 excretion rate for a given period divided by the estimated mean plasma [^{3}H]PEG-4000 ($[PEG\text{-}4000]_p$]) for the same period :

(3) $GFR = UFR *[PEG\text{-}4000]_u/[PEG\text{-}4000]_p$

Mean plasma [^{3}H]PEG-4000 radioactivity ($[PEG\text{-}4000]_p$) represents an average of the two values bracketing the urine collection interval. Other than terminal measurements (48-h), radioactivity was estimated at each time as follows. First, branchial [^{3}H]PEG-4000 losses ($J_{PEG\text{-}4000gill}$) were tabulated as being the product of [[^{3}H]PEG-4000] in the water ($[PEG\text{-}4000_{H2O}]$) and the effective volume of the flux chamber (V_E) while also accounting for the mass of the fish (m) and the flux time (t) (equation 4). In each fish, $J_{PEG\text{-}4000gill}$ was no more than a few percent of the simultaneous urinary efflux and this percentage was assumed to be constant over the duration of the experimental series and was used to calculate the particular value of $J_{PEG\text{-}4000gill}$ used in equation 5, which estimated the absolute loss ($PEG\text{-}4000_{gill}$) for each timeframe:

(4) $J_{PEG\text{-}4000gill} = ([PEG\text{-}4000_{H2O}]^* V_E)/m/t$

(5) $PEG\text{-}4000_{gill} = J_{PEG\text{-}4000gill}^*t^*m$

The [^{3}H]PEG-4000 radioactivity, in absolute counts, at any given time point in the plasma ($PEG\text{-}4000_t$) was calculated as the sum of the plasma radioactivity in next time step ($PEG\text{-}4000_{t+1}$), the branchial loss ($PEG\text{-}4000_{gill}$) and urinary loss ($PEG\text{-}4000_{urine}$) (equation 6). The two plasma values were then averaged and expressed as a concentration using the extracellular fluid volume (193 ml/kg-fish) given in Munger et al. (1991) (equation 7)

(6) $PEG\text{-}4000_t = (PEG\text{-}4000_{t+1} + PEG\text{-}4000_{gill} + PEG\text{-}4000_{urine})$

(7) $[PEG\text{-}4000]_p = ((PEG\text{-}4000_f + PEG\text{-}4000_i)^* 1/2)/(193^*m)$

While GFR values were calculated for individual periods for each fish, values were subsequently averaged across time for each fish for use in the subsequent filtration, secretion, and reabsorption calculations.

The filtration rate of a metabolite was calculated as the product of the GFR and concentration of the metabolite in the plasma ($[M]_p$):

(8). Filtration Rate $= GFR^*[M]_p$

The secretion rate was calculated as the difference between the excretion rate and the filtration rate:

(9). Secretion Rate = $(UFR^*M_u) - GFR^*[M]_p$

Here a positive value represents a net secretion rate, and a negative value a net reabsorption rate.

Net acid excretion is the sum of two components, titratable acids (e.g. P_i^-, organic acids) and non-titratable acid where the latter effectively represents the total ammonia excretion (Hill 1973). As such, total urinary acid excretion rate was calculated as the sum of urinary total ammonia (T_{amm}) excretion rate and the TA-HCO_3^- excretion rate.

Constants derived from Cameron and Heisler (1983) at the experimental temperature were used in ammonia partitioning calculations. Plasma ammonium ion (NH_4^+) and ammonia gas (NH_3) were calculated using a re-arranged Henderson-Hasselbalch equation with the dissociation constant (pK), blood pH (pH) and the measured total plasma ammonia concentration ($[T_{amm}]_p$):

(10). $[NH_4^+]_p = [T_{amm}]_p/(1+(\text{antilog}(pH-pK))$

(11). $[NH_3]_p=[T_{amm}]_p-[NH_4^+]_p$

The partial pressure of ammonia gas (P_{NH3}) was calculated from $[NH_3]_p$ and the solubility of ammonia (αNH_3) :

(12). $P_{NH3}=[NH_3]_p/\alpha NH_3$

Equations 10-12 were also used in an analogous manner in model calculations to partition ammonia in renal cells (using tissue total ammonia concentration and intracellular pH).

The partial pressure of CO_2 in the plasma (P_{CO2}) was determined at the experimental temperature using measured blood plasma pH and $[HCO_3^-]_p$ values and constants taken from Boutilier et al. (1984) for the apparent dissociation constant (pK) and the solubility of CO_2 in plasma (αCO2). The Henderson-Hasselbalch equation was re-arranged as described by Severinghaus (1966) (equation 13).

(13). P_{CO2}=10^ (-1*(pH-pK-log ([HCO_3^-]$_p$)+log (αCO2)))

Statistical Analyses:

Data have been reported as means ± 1 SEM (N) throughout, and significance of differences was accepted at 5%. All statistical analyses were performed using SigmaPlot v10.0 (Systat Software Inc., San Jose, CA, USA). A two-way ANOVA model (factors = time, treatment) combined with a Tukey's post-hoc test was employed for urinary excretion parameters. A one-way ANOVA model with a Tukey post-hoc test was used in the analysis of filtration and secretion parameters. Differences in plasma parameters, enzymatic activities and relative mRNA expression patterns between control and acid-exposed groups were evaluated by Student's unpaired two-tailed t-tests.

Results:

Blood and Tissue Acid-Base Status and Plasma Parameters:

Fish exposed to pH 4.0 for 48-h had a lower blood pH by almost 0.4 units, a 40% lower plasma [HCO_3^-] and no significant changes in plasma P_{CO2} relative to control values (Table 2.2), indicative of pure metabolic acidosis (Hills 1973). Plasma [T_{amm}] was higher by about 60%, while plasma P_{NH3} was lower by 35% relative to control values. Plasma lactate and glucose were unresponsive to acid exposure, but plasma cortisol and urea were 85% and 59% higher, respectively, compared to control values (Table 2.2). Acid exposure also resulted in a significantly lower plasma Na^+ concentration by approximately 25%, relative to control values, while both plasma P_i and Cl^- were unchanged.

In the kidney, intracellular pH was maintained at a constant value of about 6.9 (Table 2.3) despite the marked acidosis in the blood plasma. Similarly, the renal tissue lactate, total ammonia and NH_4^+ concentrations, as well as tissue P_{NH3} were not significantly different (Table 2.3).

Urinary Responses, Ammonia and Acid-Base Excretion Rates

In both control and experimental groups, UFR increased significantly over the 48-h exposure period, but UFR was largely unaffected by exposure to a low pH environment (Fig. 2.1A). However, between 12 and 24-h, acid-exposed fish exhibited a 4.6-fold higher UFR relative to the control group but this was a transient difference (Fig. 2.1A). Changes in UFR were significantly influenced by both time ($P<0.001$) and exposure treatment ($P<0.001$) but no interaction between the two was detected.

Urine pH was significantly lower, within the first 12-h of acid exposure, relative to the control group, and this difference was maintained throughout the 48-h exposure (Fig. 2.1B). Urine pH significantly decreased over time in both control and acid-exposed fish, but more so in the latter (Fig. 2.1B). Exposure treatment ($P<0.001$) and time ($P<0.001$) were both shown to have a significant influence on urinary pH but no interaction was demonstrated.

There were large changes in the magnitude and direction of net urinary acid excretion in fish exposed to acid (Fig. 2.2A). Note that by convention (see Methods) negative values represent net acid excretion by the kidney, and positive values net base excretion. In the control group, the kidney excreted base at a constant rate, whereas acid-exposed fish excreted acid within the first 12-h and throughout the acid exposure period (Fig. 2.2A). Exposure treatment was the only significant factor ($P<0.001$) contributing to changes in net acid excretion. Urinary ammonia excretion was higher under acid-exposure, 5-10 fold greater than in control fish by 12-

h of exposure, a difference which was maintained throughout (Fig. 2.2B). Both time ($P<0.001$) and exposure treatment ($P<0.001$) were found to have significant influences on urinary ammonia excretion but there was no interaction demonstrated between the two.

TA-HCO_3^- excretion was reversed in acid-exposed fish relative to control animals (Fig. 2.2C). Urine P_i excretion was significantly elevated throughout the acid exposure reaching values substantially higher than seen in the control series (Fig. 2.2D). Exposure treatment ($P<0.001$) was the only contributing factor in the observed differences in both these parameters and no interaction was detected. TA-HCO_3^- and P_i concentrations in the urine were significantly positively correlated in both a 2^{nd} ($P<0.05$; $r^2=0.193$) and 3^{rd} ($P<0.05$; $r^2=0.289$) order polynomial regression model in that increases in urine [P_i] were associated with higher urine [titratable acid] (Fig. 2.3).

Urine urea excretion was elevated several fold over control rates immediately following and throughout the 48-h acid exposure period (Fig. 2.4A), an effect which was found to be influenced by both treatment ($P<0.001$) and time ($P=0.002$). Urinary Na^+ excretion was variable over time in both control and acid groups, but Na^+ excretion rates were significantly higher at 12-24-h of acid treatment relative to control values (Fig. 2.4B). Urinary Na^+ excretion was influenced by time ($P=0.013$) and exposure group ($P<0.001$) but the two showed no significant interaction. Urine [Na^+] did not significantly correlate with urinary [ammonia] ($P>0.05$; $r^2=0.00$) (Fig. 2.5). Cl^- excretion was unaffected by acid exposure (Fig. 2.4C).

Renal Enzyme Activity:

In fish exposed to acid, there was a significantly higher activity of alanine aminotransferase (32%) relative to control fish. Despite a strong trend towards higher activities

of both glutamate dehydrogenase (P=0.26) and aspartate aminotransferase (P=0.09) during acid exposure, no other renal enzyme was significantly altered by the acid treatment (Table 2.4).

Filtration, Secretion and Reabsorption:

Exposure to pH 4.0 did not influence the mean UFR or GFR relative to mean values for the control group (Fig. 2.6). These data were used to determine mean rates of filtration, secretion, and reabsorption (= negative secretion) of metabolites entering the urine. Water filtration and reabsorption were not appreciably affected by acid exposure (Fig. 2.7A). Under control conditions, ammonia was both filtered and secreted at relatively low rates and was largely a product of filtration (Fig. 2.7B). In acid-exposed fish, there was a significant elevation (~15-fold) in the rate of ammonia secretion but no significant increase in ammonia filtration rate (Fig. 2.7B).

The filtration rates of P_i, Na^+, Cl^-, and urea were not significantly different between control and acid-exposed fish (2.7C-F). Although, the rate of P_i reabsorption demonstrated a trend towards decrease (~68%) under pH 4.0 relative to control fish, however this was non-significant (Fig. 2.7C). Overall, the rest of these substances were reabsorbed to the same degree in fish exposed to neutral or acid water. Renal Na^+ reabsorption did not correlate with renal ammonia secretion in any appreciable manner (P=0.88. r^2=0.00).

mRNA expression:

Of the three Rh glycoproteins assessed in this study, only the mRNA expression of Rhcg1b demonstrated any statistically significant changes under the acidic environment. The expression of renal Rhcg1b mRNA was elevated by a factor of 3.5 relative to control fish (Fig.

2.8). While the expression of NKA and UT were unaltered, HAT expression declined by ~50%. The mRNA transcripts of NHE3 and NHE2 were below the level of detection.

Discussion:

Overview

Goldfish exposed to water pH = 4.0 exhibited a classical metabolic acidosis with renal compensation by increased urinary excretion of acidic equivalents in the form of both TA-HCO_3^- and NH_4^+. In contrast to previous studies in which interpretation was confounded by a decrease in UFR (Wright et al. 2014) and/or an absence of ammonia filtration and secretion measurements (McDonald and Wood 1981; Wood et al. 1999; King and Goldstein 1983b), the present study clearly demonstrated that the increased NH_4^+ excretion occurs via increased secretion, not by increased filtration, thereby supporting our first hypothesis. However our second hypothesis was only partially supported. As predicted, this renal response was associated with an upregulation of Rhcg1b mRNA expression and increased activities of ammoniogenic enzymes in the kidney, but there was no evidence linking increased NH_4^+ secretion to increased Na^+ reabsorption. Thus, as in mammals (see Introduction), elevated tubular ammonia secretion is likely mediated through Rh glycoproteins, but as in the fish gill under certain circumstances (e.g. Kerstetter et al. 1970; Maetz 1972; MacDonald and Prior 1988; Salama et al. 1999; Zimmer et al. 2010), there may be no obligatory coupling to Na^+ counter-transport. Fig. 2.9 provides an overview diagram.

Secretion of Ammonia:

We have direct evidence to indicate renal ammonia secretion in that GFR and UFR did not increase significantly while tubular secretion of ammonia was markedly elevated under metabolic acidosis. Increased GFR (Ditella et al. 1978) and UFR (MacKnight et al. 1962; King

and Goldstein 1983b) correlate strongly with increases in renal ammonia excretion in other studies. Thus, alterations in these parameters here would have been indicative of glomerular filtration input. In contrast to our findings, King and Goldstein (1983b) found that acidosis increased goldfish GFR, UFR, and renal ammonia excretion implying a role for filtration in ammonia output. However, King and Goldstein (1983b) argued that the elevation in ammonia filtration was not enough to explain the overall response, and suggested that the observed increase in urine [ammonia] and ammoniogenic capacity of the kidney tissue *in vitro* reflected an increase in ammonia secretion as well. In rainbow trout experiencing metabolic acidosis, UFR was stable while both urinary [ammonia] and excretion rates increased (McDonald and Wood 1981; Wood et al. 1999) suggesting, a secretion-based mechanism of ammonia transport. However, caution must be exercised in this assumption as plasma [T_{amm}] can influence the primary filtrate composition, thereby affecting the total ammonia excretion rate (Smith 1952). In our study, unlike King and Goldstein (1983b), plasma [T_{amm}] did increase significantly, but not enough to significantly increase the filtration rate of ammonia, given the variability in the data. As in mammals (see Introduction), our results clearly show that increased tubular secretion is the primary urinary ammonia output mechanism during metabolic acidosis. In mammals, this secretion is localized to the CD and, to a lesser extent, the proximal tubules (Glabman et al. 1963; Sajo et al. 1981; Simon et al. 1985; reviewed in Weiner and Verlander 2014). Given that Rh proteins localize to the distal tubule and CD in teleosts (see Introduction; Nakada et al. 2007a; Cooper et al. 2013; Wright et al. 2014), we can suggest that secretion is likely to occur here as well in freshwater fish. However, isolated nephron perfusion experiments would be required to confirm this conclusion.

Renal Ammonia Transport Mechanisms:

Rh protein involvement in increased renal ammonia secretion in the teleost kidney seems very probable. As predicted, metabolic acidosis resulted in a concurrent elevation in renal Rhcg1b mRNA and in both urinary ammonia secretion and excretion. Wright et al. (2014) reported that acidotic carp similarly experience concurrent increases in renal Rhcg1 expression and urinary [ammonia], but they did not detect increased urinary ammonia excretion, probably because UFR declined greatly, in contrast to the present study. In both mammals (Lee et al. 2009; Lee et al. 2010) and fish (Braun et al. 2009; Shih et al. 2009), the knockdown/out of Rh glycoproteins severely impaired the organism's ability to excrete ammonia. However, in fish, it is not clear whether this effect occurs in the kidney, or in the gills alone. Furthermore, in acidotic mammals, the knockout of Rhcg impaired the animal's acid-base regulatory capacity (Lee et al. 2009; Lee et al. 2010) in circumstances where these proteins are normally upregulated to facilitate increased renal ammonia excretion (Cheval et al. 2006; Seshadri et al. 2006a; Seshadri et al. 2006b). Overall, we can conclude that Rhcg is probably involved in regulating renal ammonia transport and, therefore, critical to renal acid-base balance in the goldfish.

In addition to Rh glycoprotein involvement, we predicted that increased ammonia secretion would be coupled with increased Na^+ reabsorption. However, in opposition to this hypothesis, metabolic acidosis had no influence on urinary Na^+ reabsorption or excretion and these parameters did not correlate with their respective ammonia equivalents. In other teleosts, similar effects have been noted in that urinary Na^+ excretion rates were not influenced by metabolic acidosis (King and Goldstein 1983b; McDonald and Wood 1981). Overall, these data suggest that NH_4^+ secretion is not directly coupled to Na^+ reabsorption, similar to the uncoupling seen in the gill under certain circumstances (Kerstetter et al. 1970; Maetz 1972; MacDonald and Prior 1988; Salama et al. 1999; Zimmer et al. 2010). Furthermore, the fact that the mRNA

expression of NHE2 and NHE3, the two transport proteins believed to cause Na^+/NH_4^+ coupling (Shih et al. 2009; Zimmer et al. 2010), were below detectable levels in the kidney (note: they were easily detectable in gill tissue), as well as a lack of NKA response, provides compelling evidence against a renal Na^+/NH_4^+ exchange complex in the goldfish kidney similar to that thought to be present in the gills (Wright and Wood 2009).

We suggest a model of renal ammonia transport involving parallel H^+/NH_3 transport independent of Na^+ uptake, similar to the protein arrangement observed in the mammalian collecting duct (reviewed by Weiner and Verlander 2014). This is supported by the expression of Rhcg1a, Rhcg1b and HAT mRNA coupled with relatively high activity of renal HAT. In support of this model, previous work has demonstrated the localization of both Rhcg1 (Wright et al. 2014) and HAT (Perry and Fryer 1997; Ivanis et al. 2008a) to the apical membranes of the proximal tubules in teleost fish. HAT activity in the kidney was not affected by acidosis, but its activity was greater than reported in the goldfish gill (Sinha et al. 2013) where ammonia excretion rates were ~20-50 fold greater (Maetz 1972; Sinha et al. 2013) than observed here, suggesting sufficient operational capacity to facilitate renal ammonia secretion. Furthermore, the lower urinary pH during acidosis would tend to inhibit operation of NHE in kidney tubules (Parks et al. 2007). HAT would be more likely to secrete H^+ for NH_3 trapping under these conditions. Under this model, it was expected that HAT mRNA would increase under acidosis, but the opposite effect was observed. The cause is unknown; perhaps this represents an energy conservation strategy to cope with the long term energetic costs of chronic acidosis (Ferguson and Boutlier 1988; Kalinin and Gesser 2002; Deigweiher et al. 2010).

Ammonia Production:

The increased ammonia excreted in the urine during metabolic acidosis was probably produced in the kidney. Under acidosis, there was an elevation in plasma [T_{amm}] however, due to a reduction in blood pH, plasma P_{NH3} was decreased thereby reducing available NH_3 for plasma-to-tubule cell ammonia translocation. Additionally, similar to the carp (Wright et al. 2014), there was a trend towards a reduction in Rhbg mRNA expression under acidosis. Together, this suggests an endogenous production of ammonia in the kidney itself, because the diffusive conductance of the basolateral surface of the tubular epithelial cells to ammonia is potentially limited. This implies that endogenous renal ammonia production is probably the primary source of urinary ammonia, mediated through renal amino acid catabolism as seen in other teleosts whereby aspartate and alanine act as the primary substrates (King and Goldstein 1983b; Wood et al. 1999). Here, there was a significant increase in alanine aminotransferase activity (plus non-significant elevations in glutamate dehydrogenase and aspartate aminotransferase) under acidosis demonstrating comparable responses to both other teleosts (Wood et al. 1999) and mammals (Schoolwerth et al. 1978; Wright et al. 1992; Schroeder et al. 2003; Nowik et al. 2008). In concert with the current teleost model, we propose that ammonia in the goldfish kidney forms from glutamate dehydrogenase catabolism and that alanine and/or aspartate, but not glutamine, act as the primary substrates in NH_4^+/HCO_3^- synthesis. This conclusion reflects the lack of detectable glutaminase activity in the kidney of goldfish (present study), the enzyme which is required to catalyze the deamination of glutamine to glutamate (Hirata et al. 2003). This enzyme has been demonstrated to occur in the kidneys in other teleost studies, but it is generally restricted to a low level of activity (King and Goldstein 1983b; Wood et al. 1999; Wright et al. 2014). Additionally, King and Goldstein (1983b) demonstrated high activities of both alanine aminotransferase and aspartate aminotransferase in the goldfish kidney whereby the incubation

of renal homogenates with their corresponding amino acid substrate (alanine or aspartate) resulted in a 3-4x greater ammonia production than comparative incubations with glutamine, further supporting an alanine/aspartate based system. In our study, as well as others (King and Goldstein 1983b; Wood et al. 1999; Wright et al. 2014), glutamine synthetase activities were found to be much lower than the activities of glutamate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase, underscoring the importance of alanine and aspartate in teleost renal ammonia synthesis. As well, the synthesis of ammonia here is likely to be occurring at a rate equal to that of secretion as indicated by the constancy of the renal intracellular conditions. In producing ammonia through transdeamination, α-ketoglutarate is the final metabolite formed in this series of reactions. The subsequent catalysis of this metabolite via α-ketoglutarate dehydrogenase yields CO_2 which is quickly converted to H^+ and HCO_3^- through CA (Wood et al. 1999; Wright 1995).

General Acid-Base and Ion Responses:

Goldfish urinary responses were qualitatively similar to those seen in previous teleost (McDonald and Wood 1981; King and Goldstein 1983b; Wood et al. 1999) and mammalian studies (Sartorius et al. 1949; Hills 1973; Hamm and Simon 1987) on metabolic acidosis, with elevation in acid excretion resulting from an increase in both ammonia excretion and TA-HCO_3^- excretion, the latter consisting of mostly P_i. Unlike ammonia, the urinary P_i and, by proxy TA-HCO_3^- excretion (Wheatly et al. 1984), appeared to result from decreased tubular reabsorption rather than direct secretion of P_i. In mammals (Strickler et al. 1964; Agus et al. 1971), changes in P_i reabsorption similarly regulate the degree of urinary P_i excretion.

Under acidosis, plasma ion loss was also evident. This effect has been characterized previously in acid-exposed teleosts (McDonald et al. 1980; McDonald and Wood 1981; Ultsch et al. 1981; Fugelli and Vislie 1982; Wright et al. 2014) and is believed to be a product of unfavourable electrochemical gradients impeding active branchial ion uptake and promoting increased diffusive ion losses (McDonald and Wood 1981; Ultsch et al. 1981). There also appeared to be no renal compensation for reducing ion loss as renal Na^+ and Cl^- reabsorption rates were unaffected by acidosis. This suggests that goldfish elect to acid-base regulate rather than ionoregulate under acidosis, in contrast to carp, as part of the "acid/base-ion balance compromise" (Wright et al. 2014)

Transport and Ammonia Synthesis Regulation:

Plasma cortisol nearly doubled under acidosis implying a regulatory role in nitrogen metabolism. Here, cortisol likely aids in improving the supply of amino acids through a stimulation of proteolysis (Milligan 1996; Wiseman et al. 2003; reviewed in Mommsen et al. 1999) as well as elevating the activities/expression of ammoniogenic enzymes so as to promote ammonia synthesis in the kidney (Chan and Woo 1978; Wood et al. 1999; Ortega et al. 2005). However, increased cortisol appears to be restricted to metabolic acidosis (Brown et al. 1986; Wood et al. 1999) and was not observed during respiratory acidosis in fish (Wood and LeMoigne 1991; Wood et al. 1999). Metabolic acidosis, relative to respiratory acidosis, stimulates a much larger change in renal ammonia excretion suggesting an important role of cortisol in mediating the ammonia secretory response (Wood et al. 1999). As well, previous work in fish has demonstrated that increased circulating cortisol aids in stimulating the ammoniogenic capacity (Chan and Woo 1978; Mommsen et al. 1992; Vijayan et al. 1996), with plasma [cortisol] correlated with plasma [ammonia] (Ortega et al. 2005) and ammonia excretion rates (Chan and

Woo 1978). Additionally, the HPI axis is believed to play a pivotal role in mediating the expression of Rh glycoproteins in fish (Nawata and Wood 2009; Tsui et al. 2009) and therefore may be regulating the increased levels of Rhcg1b mRNA observed here.

Urea metabolism was greatly affected by acidosis, with increased renal urea excretion rates and higher plasma [urea]. The elevation in plasma urea likely represents a detoxification mechanism to prevent ammonia toxicity in the fish, thereby maintaining circulating [ammonia] within an ideal homeostatic range (Fromm and Gillette 1968; Olson and Fromm 1971; Arillo et al. 1981; Mommsen and Walsh 1992; Ip et al. 2004). This action has been demonstrated previously in goldfish exposed to high environmental ammonia (Olson and Fromm 1971; Sinha et al. 2013). Renal arginase activity was not affected by acidosis in the present study and thus, urea synthesis probably occurs outside the goldfish kidney. In accordance with this idea, the elevation in urinary urea excretion was the product of glomerular filtration, with the kidney performing urea reabsorption similar to that seen in trout (McDonald and Wood 1998), a very different situation from the renal handling of ammonia. This conclusion is in accord with the unchanged expression of UT mRNA (Mistry et al. 2005; reviewed in McDonald et al. 2006), as UT would be involved in urea reabsorption, not urea filtration.

Conclusions:

Increased renal ammonia excretion during metabolic acidosis in goldfish is the result of tubular secretion, and the mechanism does not seem to involve a Na^+/NH_4^+ exchange system. Ammonia transport is likely mediated through a parallel H^+/NH_3 transport facilitated by Rhcg1 and HAT in concert with enhanced endogenous renal cell ammonia synthesis via amino acid metabolism (Fig. 2.9). Overall, this system exemplifies a classic teleost renal acid-base

regulatory response that is probably regulated through the HPI axis. While this study lays the foundation for understanding teleost renal ammonia transport, further work is needed to address specific sites of renal ammonia secretion as well as the localization of renal transporter proteins.

Table 2.1: Primer sequences and reaction efficiency values in the goldfish kidney

Gene	Sequence	Efficiency (%)	Source
Rhcg1a	F: gctggttccattctctggac R: atcttcggcatggaggacag	99.9	Sinha et al. (2013)
Rhcg1b	F: attgtgggcttcttctgtgg R: ggcacacgtttctcaaaagc	110.8	Sinha et al. (2013)
Rhbg	F: atgatgaaacggatgccaag R: tcctggaaactgggataacg	107.9	Sinha et al. (2013)
NKA	F: gtcatgggtcgtattgcatc R: gttacagtggcagggagacc	111	Sinha et al. (2013)
HAT	F: ctatgggggtcaacatggag R: ccaacacgtgcttctcacac	102.6	Sinha et al. (2013)
UT	F: tgttaaagggcagggtgaag R: cggatataacggcatcttgg	99.5	Sinha et al. (2013)
β-actin	F: ggcctccctgtctatcttcc R: ttgagaggtttgggttggtc	101.6	Sinha et al. (2013)
EF-1α	F: tttcaccctgggagtcaaac R: tcttccatcccttgaaccag	111.7	Sinha et al. (Unpubl.)
NHE3	F: gtgtcatttggaggctcgtt R: atccatgttggcggtaatgt	N/A	Bradshaw et al. (2012)
NHE2	F: gagcgctgggatacattctc R: attttcggcgtactgtttgg	N/A	Bradshaw et al. (2012)

Table 2.2: Blood and plasma parameters for the goldfish exposed to control (pH 8.2) and acidic (pH 4.0) water conditions for 48 hours.

Parameter	**Exposure Group**	
	Control water (pH 8.2)	**Acid water (pH 4.0)**
Whole Blood pH	7.83±0.02	7.47±0.02***
Plasma [HCO_3^-]	9.02±0.98	5.46±0.99*
P_{CO2} (mmHg)	3.06±0.33	4.62±0.84
T_{amm}	0.136±0.02	0.212±0.03*
P_{NH3} (μTorr)	54.37±6.12	35.34±4.82*
[glucose]	2.18±0.30	2.69±0.33
[lactate]	0.93±0.19	1.20±0.31
[P_i]	1.83±0.14	1.94±0.16
[Na^+]	125.51±4.30	94.48±4.94***
[Cl^-]	82.68±5.10	71.45±3.96
[urea] (mmol-N/l)	1.05±0.21	2.53±0.42***
[cortisol] (ng/ml)	103.97±19.99	193.54±38.52*

Means ± 1 SEM (N $\geq$7), * (P<0.05) and *** (P<0.001) denote significant differences vs. control fish. Unless otherwise noted, all values are expressed as mmol/L.

Table 2.3: Acid-base status and nitrogen metabolism parameters in the kidney of goldfish exposed to control (pH 8.2) and acidic (pH 4.0) water conditions for 48 hours.

Renal parameter	Control Water (pH=8.2)	Acid Water (pH=4.0)
Intracellular pH	6.89±0.03	6.95±0.06
Whole Tissue Ammonia (mmolkg)	1.38±0.14	1.17±0.22
P_{NH3} *(µTorr)*	72.39±9.65	85.16±24.02
$[NH_4^+]$ *(mmol/kg)*	1.37±0.14	1.16±0.21
Whole Tissue Lactate (mmol/kg)	0.69±0.13	0.85±0.08

Means ± 1 SEM (N ≥6). No significant differences.

Table 2.4: Renal enzymes activities of goldfish exposed to control (pH 8.2) and acidic (pH 4.0) water conditions for 48 hours.

Renal Enzyme	Control Water (pH=8.2)	Acid Water (pH=4.0)
Alanine aminotransferase	5.44±0.56	8.04±1.23*****
Glutamate Dehydrogenase	1.87±0.26	2.90±0.65
Glutamine Synthetase	0.49±0.11	0.27±0.06
Aspartate aminotransferase	32.41±8.59	55.55±8.77
Arginase	5.15±0.72	5.19±1.66
Glutaminase	ND	ND
Na^+/K^+ ATPase (µmol ADP/mg protein/h)	3.70±0.73	4.38±1.02
H^+ ATPase (µmol ADP/mg protein/h)	1.21±0.18	1.12±0.14
Carbonic Anhydrase (1/mg protein)	0.14±0.015	0.12±0.014

Means ± 1 SEM ($N \geq 6$); all activities expressed in µmol/g/min unless otherwise noted. *denotes significant differences ($P<0.05$) vs. control fish. ND=Not Detected

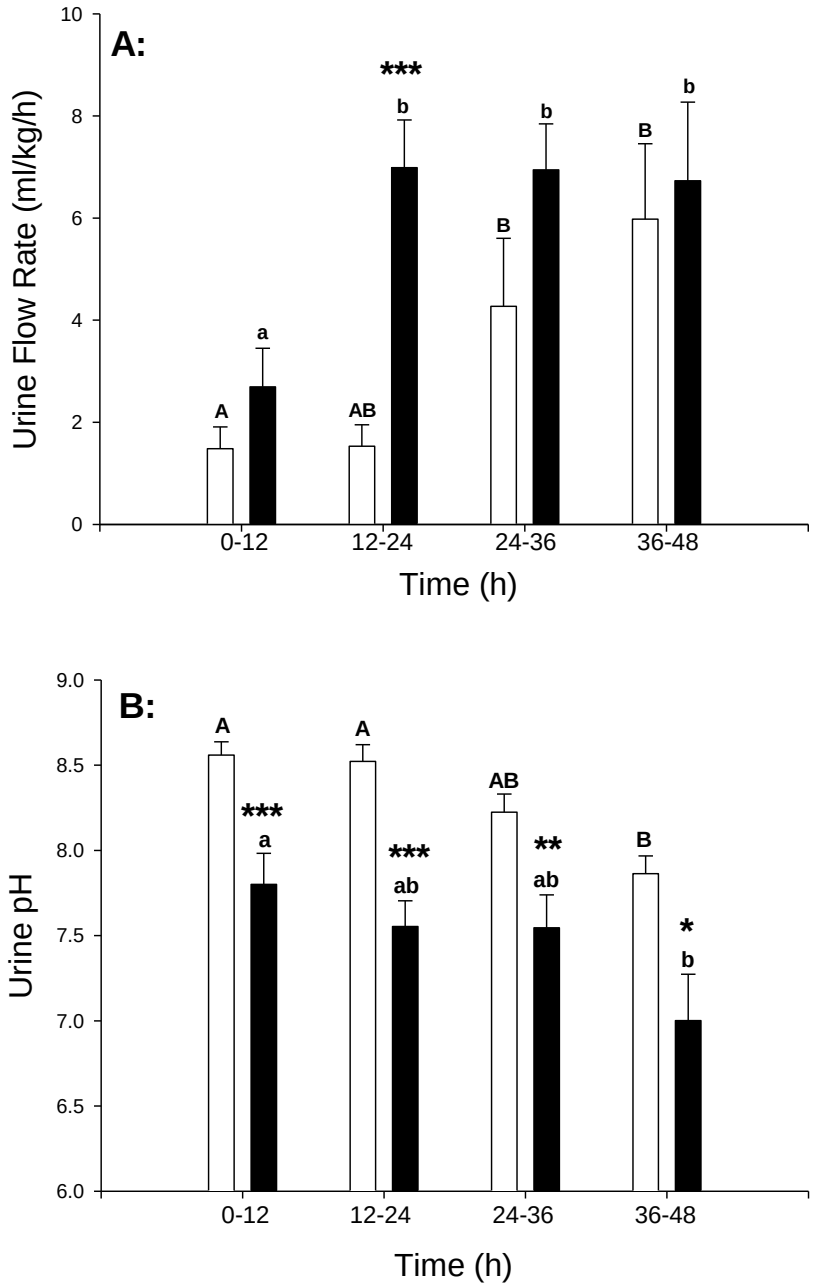

Figure 2.1: **(A)** Urine flow rate (N ≥ 7) and (**B**) urine pH (N ≥ 6) of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. Means ± 1 SEM. Asterisks denote significant differences (*** P<0.001, ** P<0.01, * P<0.05) between control and acid exposed fish at a specific time interval whereas unique letters denote significant differences (P<0.05) within a treatment group.

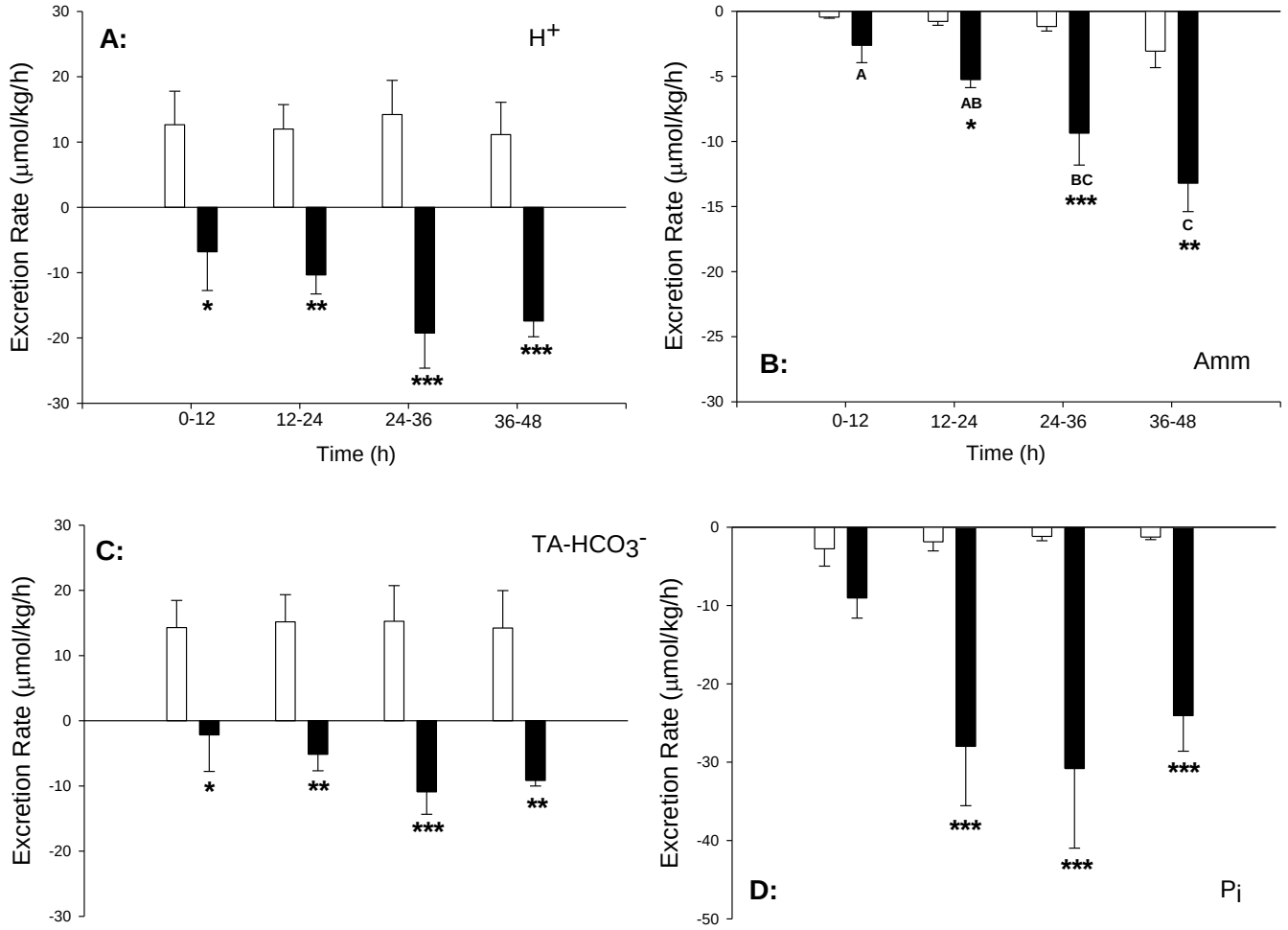

Figure 2.2 : Components of urinary acid excretion in goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. (**A**) net H^+ excretion ($N \geq 4$), (**B**) total ammonia (Amm) excretion ($N \geq 6$), (**C**) titratable acid-HCO_3^-($TA\text{-}HCO_3^-$) excretion ($N \geq 5$), and **(D)** inorganic phosphate (P_i) excretion ($N \geq 6$). Means ± 1 SEM. Asterisks denote significant differences (*** $P<0.001$, ** $P<0.01$, * $P<0.05$) between control and acid exposed fish at a specific time interval whereas unique letters denote significant differences ($P<0.05$) within a treatment group.

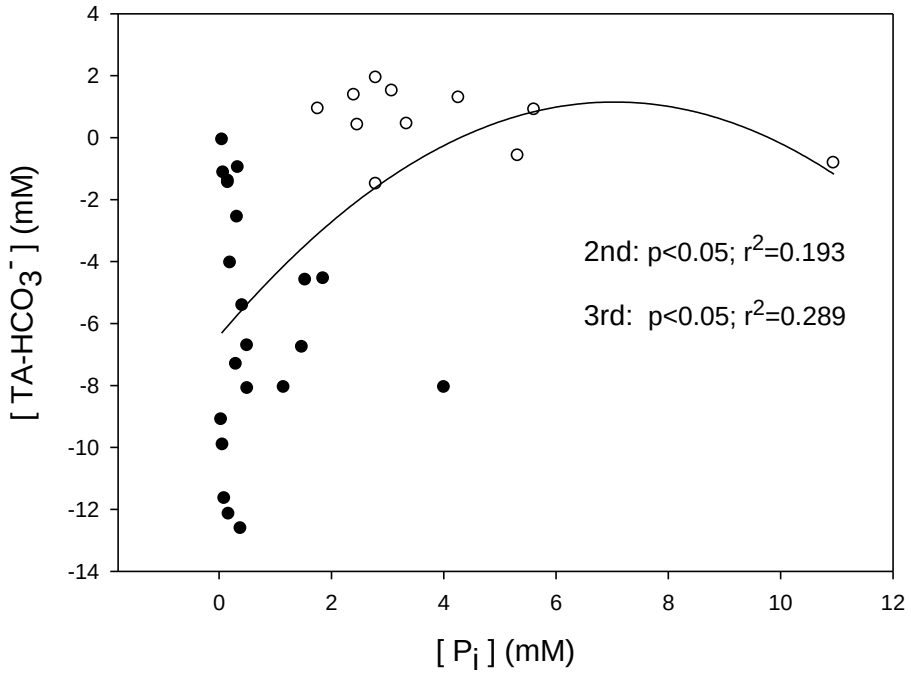

Figure 2.3: Simultaneous measurements of urinary titratable acid-bicarbonate ($TA\text{-}HCO_3^-$) and urinary inorganic phosphate (P_i) concentrations from acid (open circles) and control (filled circles) goldfish populations fit to a polynomial regression model. Positive values indicate a net acid concentration (in the case of $TA\text{-}HCO_3^-$). Increases in urinary phosphate concentrations correlate with an elevation in $TA\text{-}HCO_3^-$ (acid) concentrations for both a 2nd ($p<0.05$; $r^2=0.193$) and 3rd ($p<0.05$; $r^2=0.289$) order polynomial function.

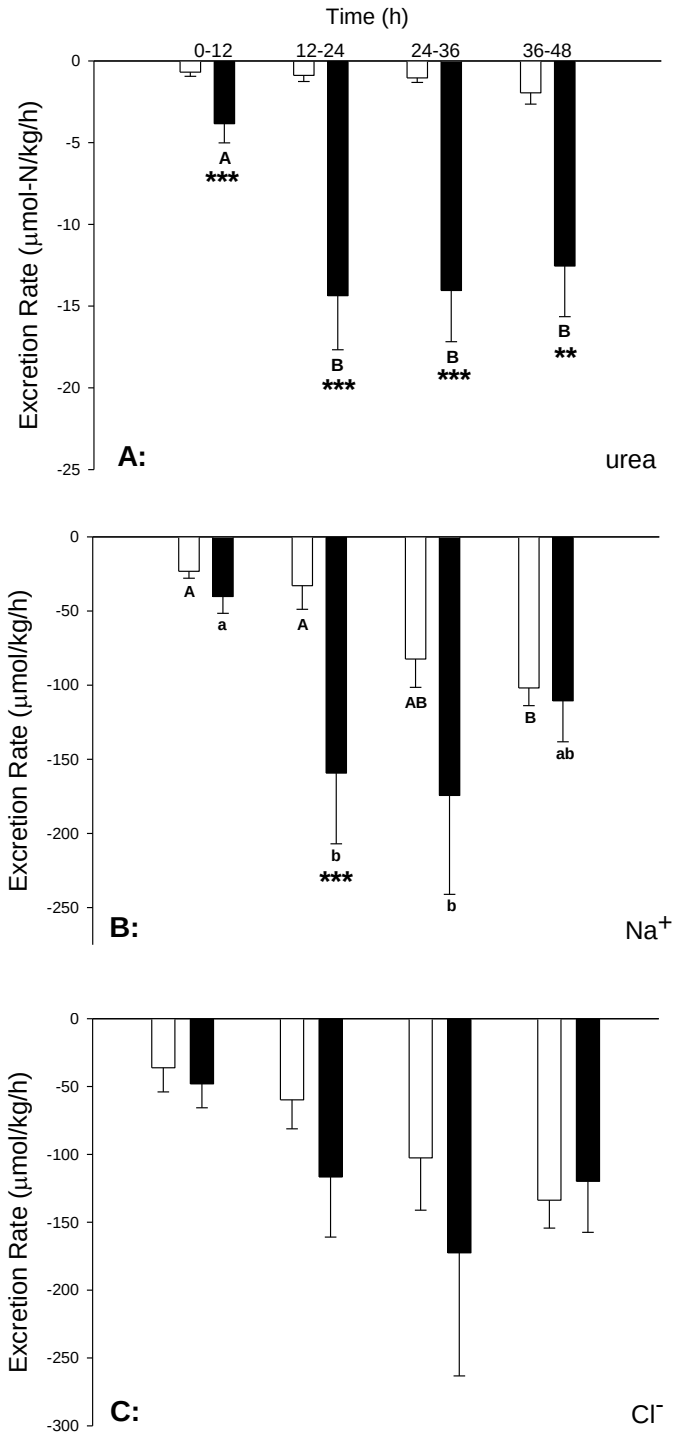

Figure 2.4: Urinary (**A**) urea excretion (N ≥ 6), (**B**) Na^+ excretion (N ≥ 6) and (**C**) chloride excretion (N ≥ 3) of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. Means ± 1 SEM. Asterisks denote significant differences (*** P<0.001, ** P<0.01) between control and acid exposed fish at a specific time interval whereas unique letters denote significant differences (P<0.05) within a treatment group.

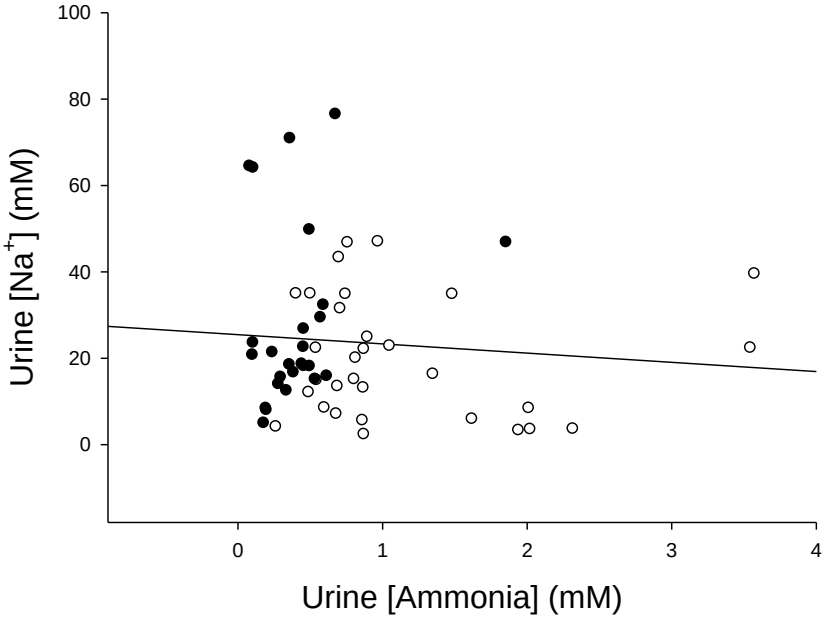

Figure 2.5: Simultaneous measurements of urinary ammonia and Na^+ concentrations fit to a simple linear regression model representing acid (open circles) and control goldfish populations (filled circles). There was no significant relationship between the two parameters ($P>0.05$; $r^2=0.00$).

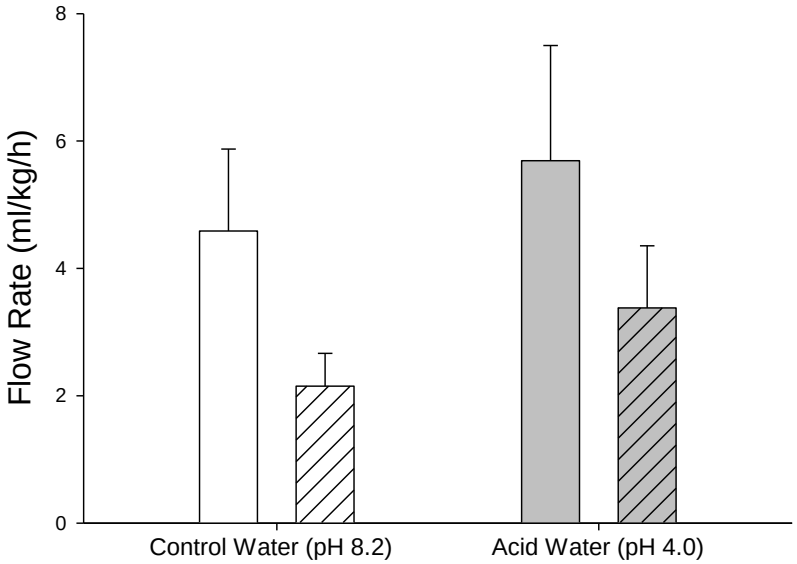

Figure 2.6: Comparison of the mean glomerular filtration rate (solid bars; N=8) and mean urinary flow rate (hatched bars; N=8) of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; grey bars) over 48-h. Means ± 1 SEM. There were no significant treatment effects.

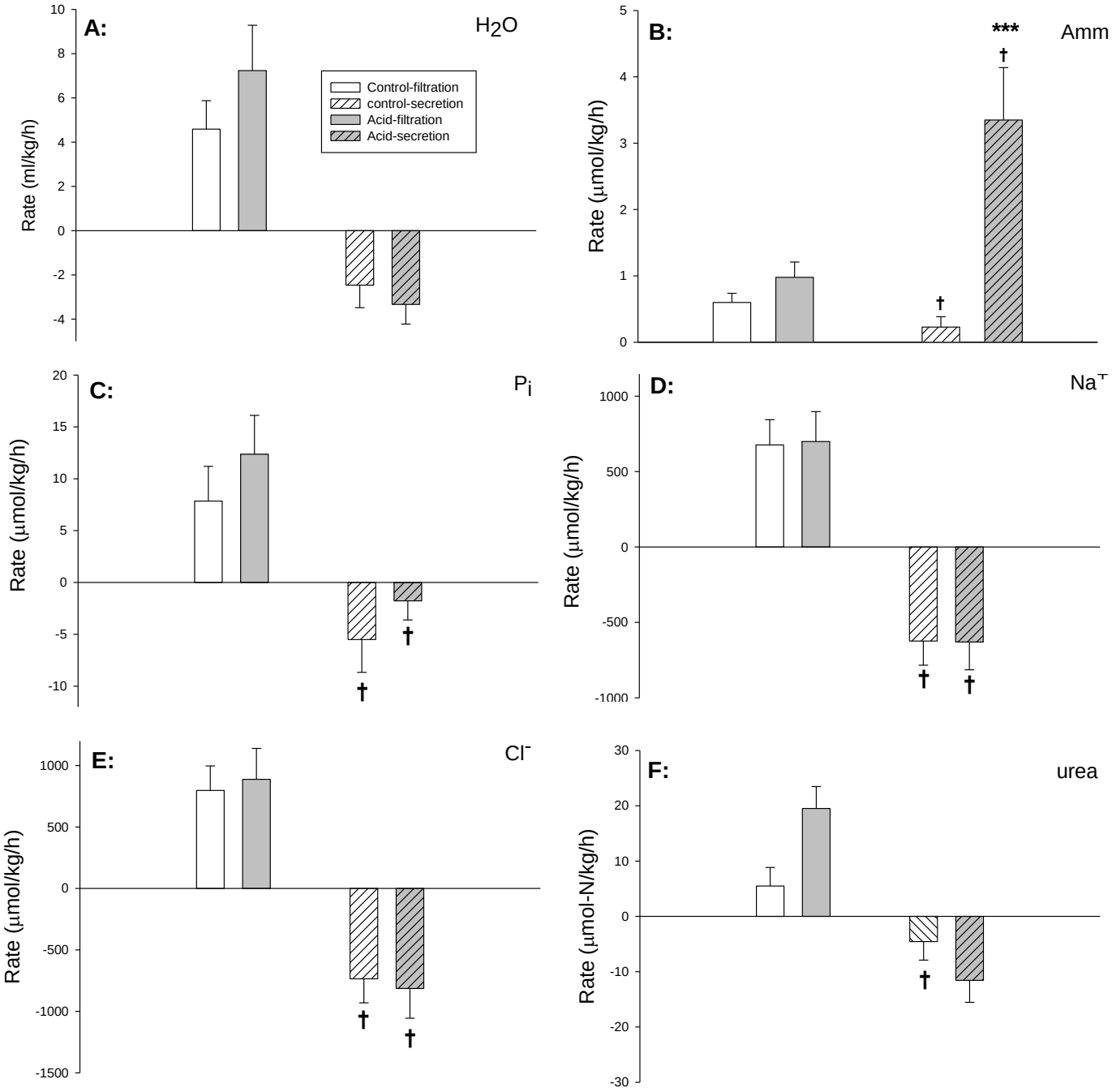

Figure 2.7: Mean glomerular filtration rates (solid bars) and mean tubular secretion rates (hatched bars) of (**A**) water (N>6), (**B**) ammonia (N ≥ 5), (**C**) P_i (N ≥ 5), (**D**) Na^+ (N ≥ 5), (**E**) Cl^- (N ≥ 5) and (**F**) urea (N ≥ 5) in the kidney of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; grey bars) over 48-h. Note that negative net secretion rates represent net reabsorption rates Means ± 1 SEM. Asterisks (***P<0.001) denote differences between control and acid exposed fish for a particular parameter. Daggers (†) denote a difference (P<0.01) between filtered load and secreted load within a treatment group.

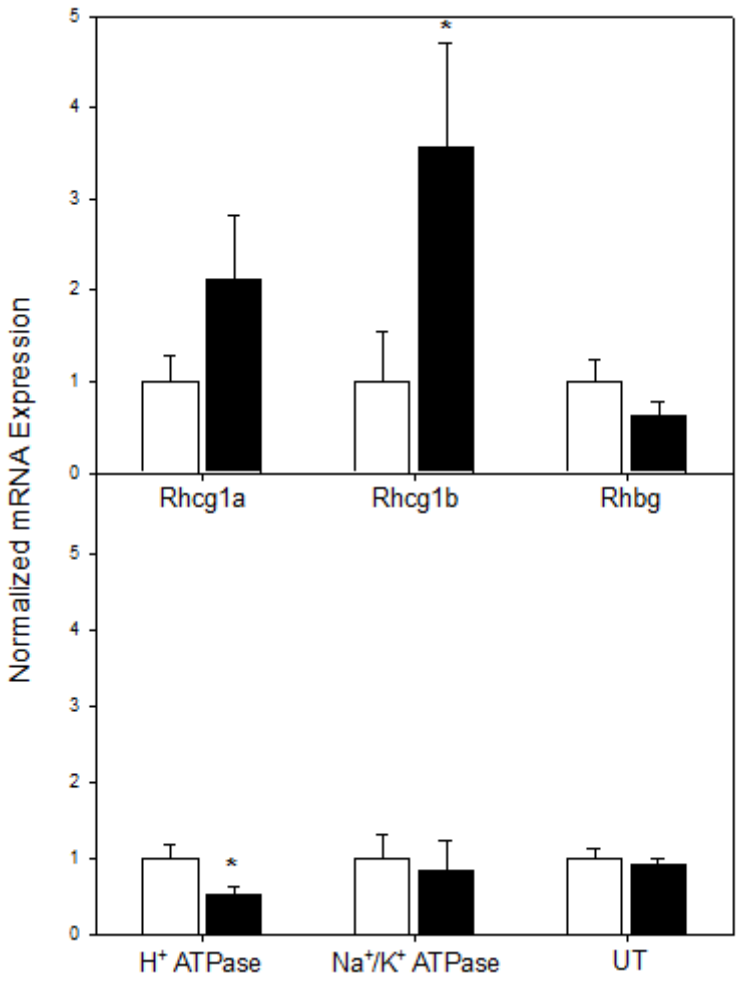

Figure 2.8: Normalized mRNA expression of various transport proteins found in the kidney in the kidney of goldfish exposed to control (pH=8.2; white bars) and low environmental pH (pH=4.0; black bars) over 48-h. Means ± 1 SEM ($N \geq 5$). Asterisks denotes a significant difference (*$P<0.05$) between control and acid exposed fish.

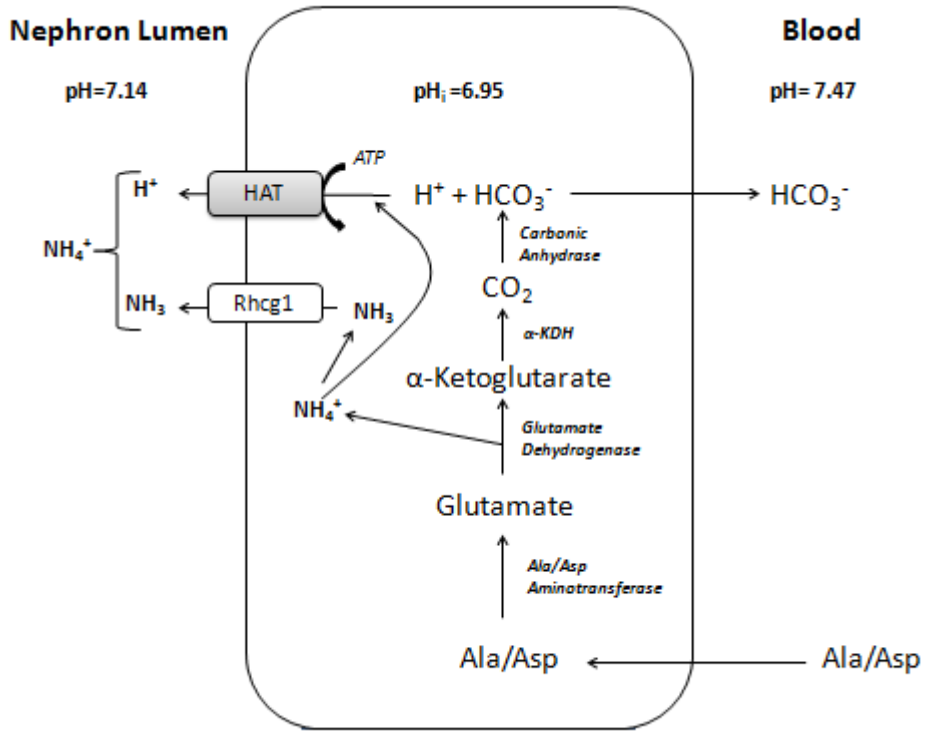

Figure 2.9: A proposed model for ammonia transport in the goldfish (*C. auratus*) kidney. Alanine (Ala) and/or aspartate (Asp) enter the tubule cell and are catalyzed via aminotransferases to form glutamate. Glutamate is subsequently catabolised by glutamate dehydrogenase (GDH) to form α-ketoglutarate simultaneously liberating ammonia. α-ketoglutarate is further metabolized to succinate and CO_2 via α-ketoglutarate dehydrogenase (α-KDH). Carbonic anhydrase (CA) mediates the reaction of the CO_2 and H_2O to form H^++HCO_3^-. Newly synthesized HCO_3^- is transported to the extracellular fluid while H^+ is translocated to the urine along with the ammonia.

CHAPTER 3: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Conclusions:

In this study, I evaluated the mechanisms by which renal ammonia transport occurs and physiologically characterized the acid-base response to metabolic acidosis, with particular reference to nitrogen metabolism, of a model teleost fish. Goldfish (*Carassius auratus*) were exposed to a low pH environment (pH 4.0) and exhibited a clear sign of metabolic acidosis: a concurrent reduction in whole blood pH and plasma [HCO_3^-] with no change in plasma P_{CO2} (Table 2.2, Chapter 2). As in previous works (Caldwell and Wood 1978; McDonald and Wood 1981; King and Goldstein 1983b; Wood et al. 1999), metabolic acidosis stimulated an elevation in the urinary excretion of acidic equivalents in the form of ammonia and TA-HCO_3^-. I demonstrated that renal ammonia transport in the goldfish is a direct product of tubular secretion as metabolic acidosis had no effect on GFR and UFR while renal ammonia secretion increased by 15-fold (Fig. 2.7B, Chapter 2) thus confirming what has been suggested by others (King and Goldstein 1983b; Wood et al. 1999; Wright et al. 2014). Similarly, ammonia excretion in the mammalian kidney is largely a product of tubular secretion occurring at the collecting ducts via Rh glycoproteins (Glabman et al. 1963; Sajo et al. 1981; Simon et al. 1985; reviewed in Weiner and Verlander 2014).

This thesis did support the involvement of Rh glycoproteins in mediating renal ammonia transport. The development of metabolic acidosis in goldfish resulted in a 3.5-fold increase in Rhcg1b mRNA expression (Fig. 2.8, Chapter 2) that occurred in concert with elevations in renal ammonia excretion and secretion. This provides evidence to support the role of these proteins in mediating renal ammonia transport here. This mRNA response has been described previously in carp where renal Rhcg1 expression and urinary [ammonia] were both elevated (Wright et al.

2014) but interpretation was confounded in that study by an ion conservation mechanism that worked to reduce UFR and thus ammonia excretion rates. In mammals, the transcripts (Cheval et al. 2006) and protein expression (Seshadri et al. 2006a; Seshadri et al. 2006b) of Rh glycoproteins are also elevated in response to metabolic acidosis. However, the knockout of Rhcg in the collecting ducts results in a reduction in urinary ammonia excretion as well as a hindered/muted acid-base regulatory response in these animals (Lee et al. 2009; Lee et al. 2010). Thus, it is very probable that the elevation in Rh glycoprotein expression in the goldfish was supporting increased renal ammonia excretion/secretion and, consequently, acid-base regulation. However, inferences made from mRNA expression alone are not conclusive evidence for Rh glycoprotein involvement; thus, further study is required to assess the expression of Rh proteins in the kidney via Western blot analysis and immunohistochemistry.

I had originally hypothesized that renal ammonia secretion would be mediated through a Na^+ -dependent mechanism as seen in the teleost gill (Wright and Wood 2009). However, both urinary Na^+ excretion (Fig 2.4B, Chapter 2) and renal Na^+ reabsorption (Fig 2.7D, Chapter 2) were unaffected by acidosis. Urine [Na^+] and renal Na^+ reabsorption did not correlate with their respective ammonia equivalents (Fig. 2.5, Chapter 2). Finally, the lack of detectable NHE2 and NHE3 mRNA in the kidney, the proteins believed to mediate Na^+/NH_4^+ exchanges (Avella and Bornancin 1989; Wright and Wood 2009; Zimmer et al. 2010), provides additional support for the conclusion that renal ammonia secretion is likely a Na^+-independent mechanism. However, one must be cautious in assuming no renal NHE involvement based on an absence of NHE mRNA. For example, there may exist a different NHE isoform that was not addressed in this study. Given the high activity of HAT (Table 2.4, Chapter 2) and the elevations in Rhcg1b mRNA, I proposed a parallel H^+/NH_3 transport mechanism similar to that found in the

mammalian collecting duct (Fig. 2.9, Chapter 2) (reviewed in Weiner and Verlander 2014). Due to the elevation in renal alanine aminotransferase activity (Table 2.4, Chapter 2), ammonia secretion likely originates from endogenous production via amino acid catabolism by the renal tubule cells themselves (Wright 1995). However, unlike mammals (Schoolwerth et al. 1978; Wright et al. 1992; Wright 1995), this system likely uses aspartate and/or alanine as its primary substrates as reported previously in teleost fish (King and Goldstein 1983b; Wood et al. 1999). A near doubling in plasma cortisol (Table 2.2, Chapter 2) during metabolic acidosis suggests a possible regulatory role of the HPI axis in moderating increases in ammonia production (Chan and Woo 1978; Mommsen et al. 1992; Vijayan et al. 1996; Ortega et al. 2005), excretion (Chan and Woo 1978) and transporter expression (Nawata and Wood 2009; Tsui et al. 2009; Wright et al. 2014) seen here.

Future Directions:

There remain a number of questions that were not addressed during this study. While a general increase in renal ammonia secretion was observed, my investigation did not address the specific region of the nephron by which secretion occurs. In mammals, Rh proteins are heavily localized to the collecting duct of the nephron where the majority of secretion occurs (reviewed in Weiner and Verlander 2014). In teleosts, Rh proteins are expressed throughout the nephron localizing specifically to the distal tubule (Nakada et al. 2007a; Cooper et al. 2013; Wright et al. 2014) and the collecting duct (Nakada et al. 2007a). This would suggest that renal ammonia secretion is likely to be localized in these regions; however, the current study did not address this issue. It is recommended then that an isolated tubule perfusion study be conducted to address the specific region(s) of renal ammonia secretion in the goldfish. This could be carried out in a similar manner to those performed in mouse nephrons (Sajo et al. 1981; Nagami 1988; Nagami

1989) and the malpighian tubules of insects (Maddrell and O'Donnell 1992; O'Donnell and Maddrell 1984). Additionally, to further discern the presence/absence of particular transporters, these perfusions could involve the use of pharmacological blockers. Specifically, EIPA and bafilomycin would ideally be the preferred blockers to test as they have quite specific inhibitory actions on the NHE (Kleyman and Cragoe 1988) and HAT (Bowman et al. 1988) respectively. As well, immunohistological work should be conducted to evaluate the specific localization of the proteins involved in mediating renal ammonia secretion as, in the case of Rh glycoproteins, expression patterns can be quite variable (reviewed in Wright and Wood 2009). Additionally, a comprehensive analysis of the localization of the proteins believed to be involved in goldfish renal ammonia transport has not been conducted.

To date, the literature has only investigated the renal responses to metabolic acidosis of three species of teleost fish: the rainbow trout (*Oncorhynchus mykiss*) (Wood and Caldwell 1978; McDonald and Wood 1981; Wood et al. 1999), the goldfish (*C. auratus*) (King and Goldstein 1983b; this study) and the common carp (*Cyprinius carpio*) (Wright et al. 2014). While there does seem to be a common pattern of an elevated renal ammonia excretion under metabolic acidosis among the species studied thus far (Wood and Caldwell 1978; McDonald and Wood 1981; Wood et al. 1999; King and Goldstein 1983b), this response can be relatively variable. Wright et al. (2014) observed that common carp experiencing metabolic acidosis reduce urine output to prevent ion loss as part of an acid/base-ion balance compromise. Unlike the rainbow trout (Wood and Caldwell 1978; McDonald and Wood 1981; Wood et al. 1999) and the goldfish (King and Goldstein 1983b; this study), renal ammonia excretion was unaffected by acidosis and elevated TA-HCO_3^- excretion was the primary form of urinary acid excretion in the carp. Additionally, this response seems to be variable within species whereby rainbow trout

(*Oncorhynchus myksiss*) injected with lactic acid, thereby inducing metabolic acidosis, did not experience altered renal ammonia excretion rates (Kobayashi and Wood 1980). This also begs the question that in scenarios where the gill is able to participate in acid-base regulation (unlike the situation with exposure to water pH = 4.0), will the kidney still be a relevant site of acid-base homeostasis? The gills do provide the bulk of the acid-base regulatory capacity in teleosts fish (McDonald and Wood 1981) and, despite the lack of change in renal ammonia excretion in Kobayashi and Wood (1980), the kidney may indeed still contribute to acid-base regulation via ammonia excretion under metabolic acidosis in normal pH environments (Wood 1988). However, this issue has not been investigated in teleosts to any great degree. Returning to the taxonomic restrictions of my thesis, it should be noted that, besides my thesis work, Wright et al. (2014) is the only study to attempt to resolve the role of Rh glycoproteins in teleost renal ammonia transport and acid-base regulation. Given that the renal response to metabolic acidosis can be variable among taxonomic groups and the general lack of data concerning renal ammonia transport mechanisms in teleost fishes, it is recommend that future studies should investigate these topics in a wider range of teleost species.

This study was also limited as to the type of acidosis experienced by the goldfish. Respiratory acidosis, which was not investigated here, results from a rise in plasma P_{CO2} (hypercapnia) alongside a reduction in blood pH (Hill 1973). It has been established that this too can result in an increase in renal ammonia excretion (Perry et al. 1987; Wood et al. 1999) with this effect being less pronounced than in metabolic acidosis (Wood et al. 1999). However, this effect is quite variable among teleost fishes. In catfish (*Ictalurus punctatus*), respiratory acidosis resulted in a variable contribution of ammonia to net renal acidic equivalent excretion (Cameron 1980). Additionally, in two species of Amazonian air-breathing fish, hypercapnia had no

influence on renal ammonia excretion (Cameron and Wood 1978). Given the variable responses among teleosts, it would be interesting to assess the role of the kidney in moderating respiratory acidosis in goldfish while comparing, at the physiological and mechanistic level, how this response differs to what I observed under metabolic acidosis. The lack of a glucocorticoid response in the trout to respiratory acidosis (Wood et al. 1999) seems to suggest that the regulatory mechanism may differ been the two types of acidosis, resulting in a differing physiological/biochemical response. On this note, do Rh glycoproteins still play an important role in mediating renal ammonia exchanges under respiratory acidosis? All of these questions have yet to be addressed but may provide valuable insight into the inner workings of the acid-base regulatory machinery of the teleostean kidney.

The role of the HPI axis in moderating teleost renal ammonia excretion and transporter regulation was not investigated in great detail here. I found that plasma [cortisol], the principal corticosteroid in teleosts (reviewed in Mommsen et al. 1999), nearly doubled under acidosis (Table 2, Chapter 2) implying a role in the regulation of nitrogen metabolism and acid-base regulation. Indeed, cortisol has been implicated before in the regulation of ammonia excretion (Chan and Woo 1978), ammonia production rate (Chan and Woo 1978; Wood et al. 1999; Ortega et al. 2005) and ammonia transporter (i.e. Rh glycoprotein) regulation (Nawata and Wood 2009; Tsui et al. 2009; Wright et al. 2014) in teleost fishes. However, cortisol implants in rainbow trout (*Oncorhynchus mykiss*) failed to induce alterations to the animal's nitrogen metabolism and excretion (De Boeck et al. 2001a), and a preliminary study of the responses of the goldfish to cortisol implants showed no clear influence on the animal's nitrogen metabolism/excretion as well (Ng and Lawrence, Unpubl.). To date, the interactions of the HPI axis, metabolic acidosis and nitrogen metabolism have not been thoroughly investigated in teleost fish. This could be

evaluated by specifically inhibiting the various components of the HPI axis in acidotic goldfish. The injection/implantation of RU486, a known antagonist of the glucocorticoid receptor in fish (Bury et al. 1998; Weyts et al. 1998; Kelly and Wood 2002), metyrapone, a chemical that impairs cortisol biosynthesis (Bernier et al. 1999; Bernier and Peter 2001; Doyon et al. 2006; Liu et al. 2013), or spironolactone, an antagonist of mineralcorticoid receptors (Sloman et al. 2001; Scott et al. 2005), could be employed as a means to delineate the effects of cortisol on acid-base regulation and nitrogen metabolism under acidosis. If cortisol is responsible for the observed elevations in ammonia excretion, Rhcg1b mRNA and ammoniogenic enzyme activity, we would expect to see these parameters reduced in acidotic goldfish under cortisol inhibition. The use of metyrapone in addressing this question could be beneficial as it can provide insight into the involvement of the HPI axis in regulating cortisol production. With metyrapone treatment, the secretions of both ACTH and CRF are alleviated from cortisol negative feedback actions (Bernier et al. 1999; Bernier and Peter 2001; Doyon et al. 2006; Liu et al. 2013). This approach could detect whether increases in ACTH and CRF play a regulatory role in moderating the acid-base regulatory responses exhibited by the kidney. Furthermore, the injection of α-helical $CRH_{(9-41)}$, a potent antagonist of the CRH receptor in teleosts (Bernier and Peter 2001; Rotllant et al. 2001; Clements et al. 2002), could be used to further discern the upstream regulation of cortisol and the renal acid-base regulatory responses by the HPI axis.

With reference to the elasmobranch study (see Appendix), this part of the thesis needs to be re-evaluated. I found no clear evidence to support the development of a metabolic acidosis despite the fact that previous work, using comparable HCl infusion rates, have produced this effect in spiny dogfish (King and Goldstein 1983a; Tresguerres et al. 2005; Nawata, Walsh, and Wood unpubl.). The sharks that I worked on had very little time to acclimate to captivity post-

capture and perhaps this high level of stress confounded the results. It may be that the control fish were perhaps experiencing a slight acidosis from stress associated arising from capture/handling which has been reported to occur in a number of elasmobranch species caught in a similar manner to the sharks used in my study (Mandelman and Skomal 2009). This is supported to a degree in that I observed a relatively low arterial blood pH, in control sharks, in comparison to what has been reported before in dogfish under resting conditions (Wood et al. 1995). The data presented here demonstrated a non-significant tendency for greater net acidic equivalent excretion in response to HCl infusion, and provide some indication of an increased acid-base regulatory capacity of the gills at the molecular level. Therefore, it is recommended that this experiment be repeated with fully acclimated sharks to produce a less variable data set to address the true effects of acid-infusion on these animals. As well, due to issues with seminal fluid contamination of the urine, it is recommended that this experiment be repeated with female dogfish instead. While more difficult to cannulate (reviewed in Wood and Patrick 1994), the results would be representative of the actual composition of the urine.

Overall, this study found that the development of metabolic acidosis in a teleost fish results in an increase in renal ammonia excretion and secretion. Here, I have shown that renal ammonia transport is a product of renal tubular cell secretion that is likely mediated through Rh glycoproteins. However, rather than a Na^+-dependent mechanism of action, ammonia transport here likely involves a parallel H^+/NH_3 secretion mediated by Rhcg and HAT. The results of this thesis also suggest an endogenous renal ammonia synthesis via aspartate/alanine catabolism. The physiological responses to metabolic acidosis are likely mediated through the actions of the HPI axis regulating transporter gene expression and nitrogen metabolism to promote acid-base

regulation. Lastly, elasmobranch results are inconclusive due to the failure of the experiment to induce metabolic acidosis and yield uncontaminated urine, and, as such, should be repeated.

APPENDIX

Introduction:

Elasmobranchs osmotically conform to their environment through the production and accumulation of high quantities of urea in their tissues (Smith 1931; Yancy and Somero 1980). Urea synthesis occurs through the ornithine-urea cycle (OUC) localized to the mitochondria of liver and skeletal muscle. Here, glutamine provides the basic N-substrate for urea synthesis. Glutamine can either be directly imported from the cytosol or formed through the addition of an NH_3 to glutamate via glutamine synthetase (reviewed by Ballantyne 1997). Given the importance of ammonia in urea synthesis, elasmobranchs typically having low circulating levels of ammonia (Wood et al. 1995; Grosell et al. 2003) and whole body and renal ammonia excretion rates are also kept quite low (Evans 1982; Claiborne and Evans 1992; Wood et al. 1995). However, metabolic acidosis prompts a marked increase in both renal (King and Goldstein 1983b; Wood et al. 1995) and branchial ammonia excretion rates (Wood et al. 1995; Nawata, Walsh, and Wood, unpubl.), presumably as a method of acidic equivalent excretion. However, the mechanism(s) by which ammonia is translocated to the external environment in these fish is currently unknown. Interestingly, Rh glycoproteins, which are thought to be ammonia gas (NH_3) transporters in teleost fishes (see Ch1: General Introduction), have been identified at the mRNA transcript or protein level in a number of elasmobranch fish including the Pacific spiny dogfish (*Squalus acanthias suckleyi*) (Nawata, Walsh, and Wood, unpubl.), the Atlantic spiny dogfish (*Squalus acanthias acanthias)* (Wright, Lawrence, Currie, MacLellan, Wood and Edwards unpubl.), the little skate (*Leucoraja erinacea*) (Anderson et al. 2010) and the Japanese hound shark (*Triakis scyllium*) (Nakada et al. 2010). Nakada et al. (2010) proposed that renal Rh proteins function in recovery of ammonia from the urine, but their functional purpose in these fish has yet to be fully

characterized. Thus, the objective of this study was to characterize the physiological and molecular responses of a model elasmobranch, the Pacific spiny dogfish (S. *acanthias suckleyi*), to metabolic acidosis specifically with reference to changes in nitrogen metabolism. In order to induce metabolic acidosis, dogfish were fitted with arterial and urinary bladder catheters and then infused intravascularly with an acidic saline solution (125 mM HCl/375 mM NaCl; 3 ml/kg/hr) over a 24h period. Repeated water and urine sampling during this infusion period was performed to assess changes in total ammonia, titratable acid minus bicarbonate (TA-HCO_3^-) and urea excretion at the gills and kidney. Arterial blood samples were taken throughout to assess blood acid-base status. Terminal tissue samples of gill and kidney were taken to quantify changes in the protein expression of genes relevant (Rhcg, NHE2, NHE3) to ammonia transport via Western blot analysis.

Materials and Methods:

Animals Care and Cannulation:

Sexually mature, male Pacific spiny dogfish [*Squalus acanthias suckleyi* (Ebert et al. 2010); 1.88± 0.06 kg] were obtained through longline fishing in Barkley Sound (British Columbia, Canada). Sharks were held in a 151,000-L holding facility, maintained on a flow-through system, at the Bamfield Marine Sciences Centre (Bamfield, BC, Canada) at 12±1 °C under a 12-h D: 12-h L photoperiod. Fish were fed to satiation, three times a week on a diet of dead hake (Kajimura et al. 2006). Five days prior to experimentation, sharks were moved to a smaller, outdoor tank (~1500 L) and fasted over this time to avoid acid-base disturbances associated with feeding (Wood et al. 2007).

Each shark was fitted with two cannulae, an arterial catheter and a urinary bladder catheter. The animal was first anesthetized using a 200 mg/L tricaine methane sulphonate (Sigma-Aldrich, Oakville, ON, Canada) solution, weighed, and placed on a surgical table where the gills were artificially ventilated with the same MS-222 solution. The arterial cannula (PE-50 tubing; BD Intramedic, Mississauga, ON, Canada), containing lithium heparin (150,000 I.U./L) in an isosmotic saline (500 mM NaCl), was inserted into the artery of the haemal canal as outlined De Boeck et al. (2001b). During recovery and subsequent experimentation, the heparinized saline was retained within the catheter to prevent clotting. The urinary bladder cannula (PE-50 tubing; BD Intramedic , Mississauga, ON, Canada), filled with an isomotic saline (500 mM NaCl), was inserted into the urinary papilla and stitched to the lateral side of the animal. To prevent the cannula from sliding out of the papilla, a knot was tied around the distal end of the urinary papilla using surgical thread. The animal was revived in anesthetic–free seawater and allowed to fully recover for 8-h in the experimental chamber (~40-L; Wood et al. 1995). Each chamber was maintained on a flow-through arrangement with ample aeration in an external water bath that was served with flowing sea water at the same temperature.

Experimental Series:

Pre-Infusion Period:

Sharks were first subjected to a 12-h, overnight, no-infusion flux period to act as an in-group control series. During this time, water flow was stopped to each chamber and the water level in the fish boxes was set to a level which equalled external hydrostatic pressure, yielding a volume of ~19-L. Chambers were maintained at 12±1°C through continuous external water flow. Water samples (30-ml) were taken at the onset and termination of this 12-h flux period, of which

20-ml was frozen and stored at -20°C for later analysis of total ammonia (T_{amm}) and urea concentrations. A small portion (~10-ml) was not frozen and was used to determine water TA-HCO_3^- concentrations. Urine was collected over this period and was immediately analyzed for volume, pH and [TA-HCO_3^-] with a small portion frozen and stored at -20°C for later analysis of urinary [ammonia] and [urea]. A single blood sample was taken 10-h into this period at which time the cannula was cleared of all heparinized saline, and a 0.5-ml blood sample was drawn into a chilled, heparinized 1-ml syringe. It was immediately measured for arterial blood pH. Blood was then quickly centrifuged at 1,500 g using a microcentrifuge (Mandel, Guelph, ON, Canada). Plasma was decanted and immediately assessed for total CO_2 content. Remaining plasma was flash-frozen, in liquid nitrogen, for subsequent analysis of plasma [T_{amm}] and [urea]. At the end of this flux period, the chamber was flushed out thoroughly over the course of an hour with fresh sea water.

Infusion Period:

Sharks were infused with one of two saline solutions: acid-loaded fish received a 125 mM HCl/375 mM NaCl saline, while controls received a 500 mM NaCl saline. Saline was infused (time=0) through the arterial cannula at a rate of 3 ml/kg/hr (Nawata, Walsh, and Wood, unpubl.) via a peristaltic pump (Minipuls 3 peristaltic pump; Gilson Inc., Middleton, WI, USA). Sharks were then subjected to a series of flux periods at the onset of infusion as follows; t=0-6 h, t=7-13 h, t=14-24 h. In between each flux period, experimental chambers were flushed out as described by Wood et al. (1995). Water samples (30-ml) were taken at the onset and termination of each flux period for analysis of water [TA-HCO_3^-], [T_{amm}] and [urea] in the same way as for

the pre-infusion period. An additional water sample (10-ml) was taken at t=3 h and t=10 h and frozen (-20°C) for later analysis of water [ammonia] and [urea].

A 0.5-ml blood sample was taken at each of the following time points during infusion: t=3, 10, 13, and 24 h. However, before a sample was drawn, remaining infusion saline was removed from the cannula and replaced with a isotonic (500 mM) heparinized saline (150,000 I.U./L), capped off and allowed to sit for 3 minutes. This saline was then removed and a blood sample then taken. This was done to avoid confounding effects of the infusion saline on the blood sample's acid-base status. The sample was then processed in the same manner as the blood in the pre-infusion series.

At 24-h infusion, sharks were euthanized using a lethal dose of pH balanced (1 M KOH) MS-222 (750 mg/L). The kidney, rectal gland, liver, gills and white muscle tissue of the animal were quickly removed, flash frozen in liquid nitrogen and stored at -80°C.These tissues were then used to conduct protein quantification via Western blot analysis. A subset of the gill, rectal gland and renal tissues were fixed overnight in a 4% paraformaldehyde solution (Bucking et al. 2013). These samples were then stored in refrigerated (4°C) 75% ethanol for later use in immunohistochemical analysis. These procedures are being performed by a collaborator, Dr. S. Edwards at Appalachian State University (Boone, NC, USA).

Analytical Techniques and Procedures

Water Analyses:

[TA-HCO_3^-] was determined within 24-h of sample collection using a double endpoint method of titration (Hills 1973) on a 10-ml water sample. A pH meter (Radiometer PHM82;

Brønshøj, Denmark) with a glass pH electrode (Radiometer-Copenhagen GK2401C, Brønshøj, Denmark) was employed to track changes in sample pH. A 2-ml microburette (Gilson, Middleton, WI, USA) was used to dispense either a standardized 0.02 N HCl or 0.02 N NaOH solution. Samples were titrated below pH 4.0, while being aerated with CO_2-stripped air (bubbled through 1 M KOH), to liberate all HCO_3^- from solution (Claiborne and Evans 1992). Samples were then titrated back to a control blood pH of 7.88 (Wood et al. 1995).

The monoxime (Rahmatullah and Boyde, 1980) and salicylate (Verdouw et al. 1978) assays were employed to determine total [urea] and [ammonia] in the water respectively.

Blood and Plasma Analyses:

Measurements of whole blood pH were conducted at the physiological temperature of the shark (12±1°C) using a pH microelectrode (Orion PerpHecT ROSS , Thermo Fischer Scientific, Toronto, ON, Canada) and a pH meter (H160 pH meter; Hach, Mississauga, ON, Canada). Plasma [T_{amm}] was assessed using a commercially available kit (Raichem, Cliniqa, San Marcos, CA, USA) while plasma [urea] was measured using the monoxime method (Rahmatullah and Boyde 1980). Total plasma [CO_2] was determined using a Corning 965 CO_2 analysis unit (Corning Life Sciences, Tewksbury, MA, USA).

Urine analyses:

Urinary volume was determined gravimetrically. Total urine [ammonia], [urea] and [TA-HCO_3^-] were assessed in much the same way as the water parameters. The only exception was that samples for urinary TA-HCO_3^- were made up to a volume of 10-ml using a 20 mM NaCl solution (MacDonald and Wood 1981; Wood 1988). Urinary pH was determined in the same

manner as whole blood pH. However, due to seminal fluid contamination, urinary values have not been reported.

Tissue Analyses:

Quantification of the protein concentration of the transporters relevant to acid-base regulation in the gill and kidney where conducted using standardized Western blot analysis (Edwards et al. 2005). Antibodies employed for Rhcg, NHE2 and NHE3 were derived from Edwards et al. (2014), Claiborne et al. (2008a) and Choe et al. (2007), respectively.

Calculations:

Whole body fluxes of specific metabolites (J_M) were expressed as a function of the difference between the initial ($[M_i]$) and final concentrations ($[M_f]$) of the metabolite in the water, time (t), body mass (m) and effective volume of the fish box (V_E) (1). These included ammonia, urea, and TA-HCO_3^-.

1. $J_M = ([M_f - M_i] * V_E)/m/t$

Net gill acid excretion (J_H) was calculated as the sum of the branchial ammonia excretion (J_{amm}) and the branchial TA-HCO_3^- excretion ($J_{TA\text{-}HCO3\text{-}}$) (2).

2. $J_H = J_{amm} + J_{TA\text{-}HCO3\text{-}}$

The partial pressure of CO_2 in arterial plasma (P_{CO2}) was calculated from measurements of plasma total CO_2 (T_{CO2}) and pHa using the dissociation constants of CO_2 (pK_{CO2}) and the solubility coefficient of CO_2 (αCO_2) from Boutilier et al. (1984) for shark plasma at 12°C and a re-arrangement of the Henderson-Hasselbalch equation (3).

3. $P_{CO2}=(T_{CO2})/(\text{antilog}((pH-pK)*\alpha CO_2))$

Plasma $[HCO_3^-]$ was then determined as follows:

4. $[HCO_3^-]=T_{CO2}-(\alpha CO_2*P_{CO2})$

Ammonia partitioning in the plasma was calculated using the constants derived from Cameron and Heisler (1983). The plasma ammonium concentration ($[NH_4^+]$), was determined using a re-arrangement of the Henderson-Hasselbalch equation which includes the total plasma ammonia ($[T_{amm}]_p$), the blood plasma pH (pH_a) and the dissociation constant (pK) (5).

5. $[NH_4^+]_p= [T_{amm}]_p/(1+(\text{antilog}(pH_a-pK))$

The concentration of ammonia gas ($[NH_3]_p$) in the plasma as follows:

6. $[NH_3]_p=[T_{amm}]-[NH_4^+]_p$

Using the solubility of ammonia gas (αNH_3) and $[NH_3]_p$, the partial pressure of ammonia gas in arterial plasma (P_{NH3}) could be determined:

7. $P_{NH3}=[NH_3]_P/\alpha NH_3$

Statistical Analyses:

Data have been reported as means ± 1 SEM (N) with statistical significance being accepted at 5%. Statistical analyses were performed using the SigmaPlot v10.0 (Systat Software Inc., San Jose, CA, USA) software package. All data were analysed through a one-way repeated measures ANOVA model with a Tukey's post-hoc test to determine within

treatment group differences. Individual two-tailed, Student's two-tailed unpaired t-test's were used to determine differences between treatment groups at a specific time point as well as differences in protein quantification.

Results:

Plasma:

Acid-infused sharks did not demonstrate a strong sign of metabolic acidosis. Blood pH was consistent with controls throughout the infusion of acid except there was a lower blood pH by 0.2 units, relative to controls, at 13-h of infusion with this effect being transient (Fig. A.1A). Similarly, plasma [T_{CO2}], P_{CO2} and [HCO_3^-] were largely unaffected by acid-infusion (Fig. A.1B-D). However, both plasma P_{CO2} (54%; Fig. A.1C) and [HCO_3^-] (18.5%; Fig. A.1D) were higher than in control fish at 13-h of infusion although this effect was transient as well. Acid-infusion had no influence on plasma total [ammonia] (Fig. A.2A), P_{NH3} (Fig. A.2B), [NH_4^+] (Fig.2C) and [urea] (Fig. A.2D). However, plasma ammonia parameters were variable within this infusion group (Fig. A.2A-C).

Whole Body Effluxes:

Net acid excretion, while demonstrating a trend towards a higher degree of excretion relative to controls, was unaffected by acid-infusion (Fig. A.3A). Similar occurrences were observed in whole body TA-HCO_3^- excretion as well (Fig. A.3B). Whole body ammonia excretion was not influenced by acid-infusion but did demonstrate significant variability within both treatment groups (Fig. A.3C). Whole body urea excretion was also unchanged but did demonstrate a degree of variability within the acid-infused fish (Fig. A.3D).

Protein Expression:

Rhcg proteins were found to occur in the gills (Fig. A.4A) and kidneys (Fig. A.5A) of Pacific spiny dogfish although their expression was unaffected by acid-infusion. Branchial NHE2 protein expression was found to be significantly upregulated during acid-infusion (23%) relative to control fish (Fig. A.4B). Branchial NHE3 protein expression was unresponsive to acid-infusion (Fig. A.4C). Renal NHE2 protein expression was unaffected by acid-infusion (Fig. A.5B) while renal NHE3 protein expression was lower (45%) in acid-infused fish relative to controls (Fig. A.5C).

Discussion:

In contrast to the teleostean study (see Chapter 2), elasmobranch responses to acidosis were less conclusive. These fish demonstrated no clear signs of metabolic acidosis with blood pH being lower than controls only at 13-h of infusion; blood pH recovered to control levels by 24h. At this same time point, there was a higher arterial plasma [HCO_3^-] and P_{CO2} (Fig. A.1B, Fig. A.1C) relative to controls. This accumulation of CO_2 in the arterial plasma causing respiratory acidosis is quite unusual. The high ventilatory rate of fish and high diffusive capacity of CO_2 in water generally prevent fish from developing hypercapnia naturally (Rahn 1966). Perhaps the rate of conversion of endogenous HCO_3^- to CO_2 by the infused HCl was too great for the fish to excrete all of this extra CO_2. Plasma urea levels remained quite stable and were comparable to those reported in previous elasmobranch studies (Goldstein and Forster 1971; Withers et al. 1994; Wood et al. 1995). However, plasma T_{amm} was relatively variable across time and was much lower than reported previously (Wood et al. 1995; Grosell et al. 2003).

Whole body effluxes of net acidic equivalent, Tamm, TA-HCO_3^-, and urea were unresponsive to acid-infusion as well. However, net acidic equivalent and titratable acid excretion (Fig. A.3B) showed a trend towards a greater rate of efflux relative to controls. TA-HCO_3^- effluxes constituted the majority of the total net acidic equivalent efflux which is similar to what has been observed in previous studies (King and Goldstein 1983a; Wood et al. 1995). Urinary responses were not addressed due to sample contamination with seminal fluids resulting from the common urogenital tract found in male elasmobranch fish (Wood and Patrick 1994).

Using Western blot analysis, it was determined that branchial NHE2 protein expression was upregulated during acid-infusion which occurred in concert with a decrease in renal NHE3 protein expression. Rhcg expression was not affected in both of these tissue types. Without a clear development of acidosis in the shark, it is impossible to determine why the expression of NHE proteins was altered. However, it should be noted that the gill comprises a large majority of the acid-base regulatory capacity in elasmobranch fish (Evans 1982; Wood et al. 1995) and the changes in NHE protein expression may represent a shift towards branchial acid-base regulation. However, as there was no clear sign of acidosis, this is impossible to determine at this time.

Conclusions:

In summary, Pacific spiny dogfish were generally unresponsive to acid-infusion although they did show tendencies towards a greater acid-base regulatory capacity at the level of the gill However, this study does illustrate the presence of Rhcg protein in the gill and kidney. Results were generally inconclusive and the study must be re-done. Future work should use female dogfish as a means of ensuring uncontaminated urine samples.

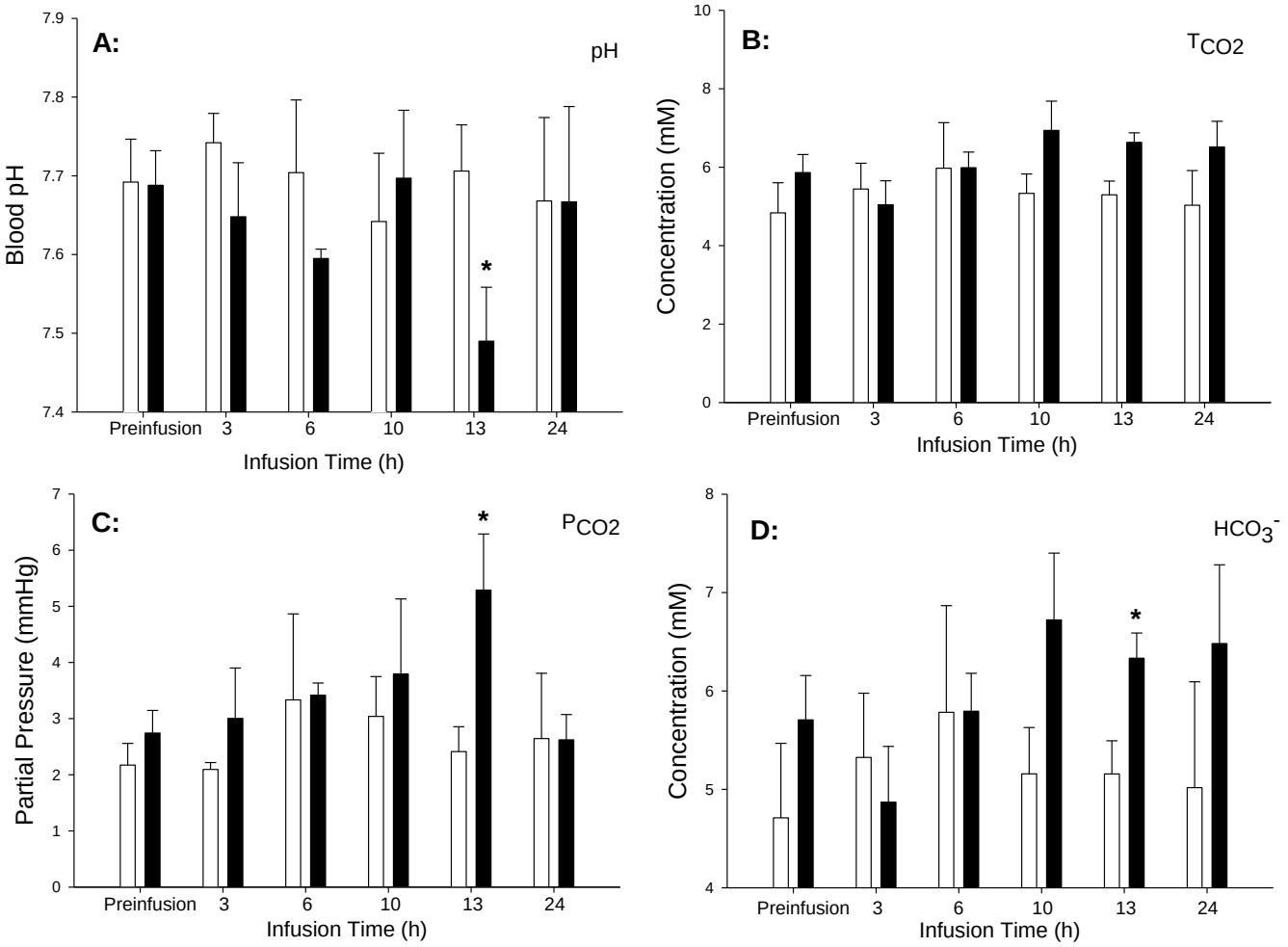

Figure A.1: The effects of the infusion of an acid (125mM HCl, 375 NaCl; Black bars) and neutral (500mM NaCl; White bars) saline on (**A**) whole blood pH (N≥5), (**B**) total plasma [CO_2] (T_{CO2}; N≥5), (**C**) partial pressure of plasma CO_2 gas (P_{CO2}; N≥5) and (**D**) plasma [HCO_3^-] (N≥5) in the spiny dogfish shark (*S. acanthias suckleyi*) over a 24-h infusion period. Values are mean ± 1 SEM. Asterisks (* $P<0.05$) denote differences between control and acid infused fish.

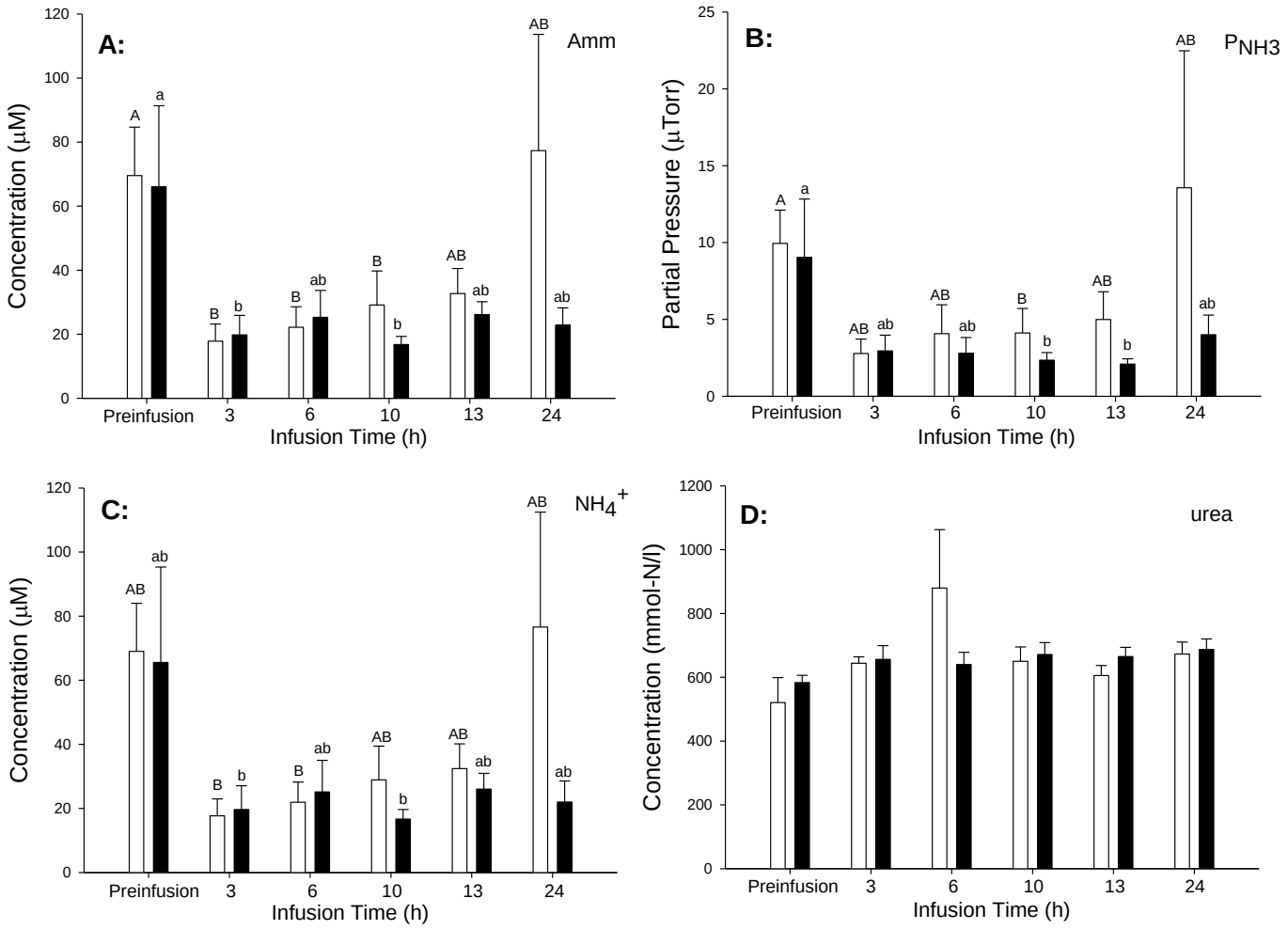

Figure A.2: The effects of the infusion of an acid (125mM HCl, 375 NaCl; Black bars) and neutral (500mM NaCl; White bars) saline on (**A**) total plasma ammonia (Amm; N≥4), (**B**) partial pressure of ammonia gas (P_{NH3}; N≥4), (**C**) [NH_4^+] (N≥4) and (**D**) urea (N≥4) in the spiny dogfish shark (*S. acanthias suckleyi*) over a 24-h infusion period. Values are mean ± 1 SEM. Asterisks (* P<0.05) denote differences between control and acid infused fish whereas unique letters denote significant differences (P<0.05) within a treatment group.

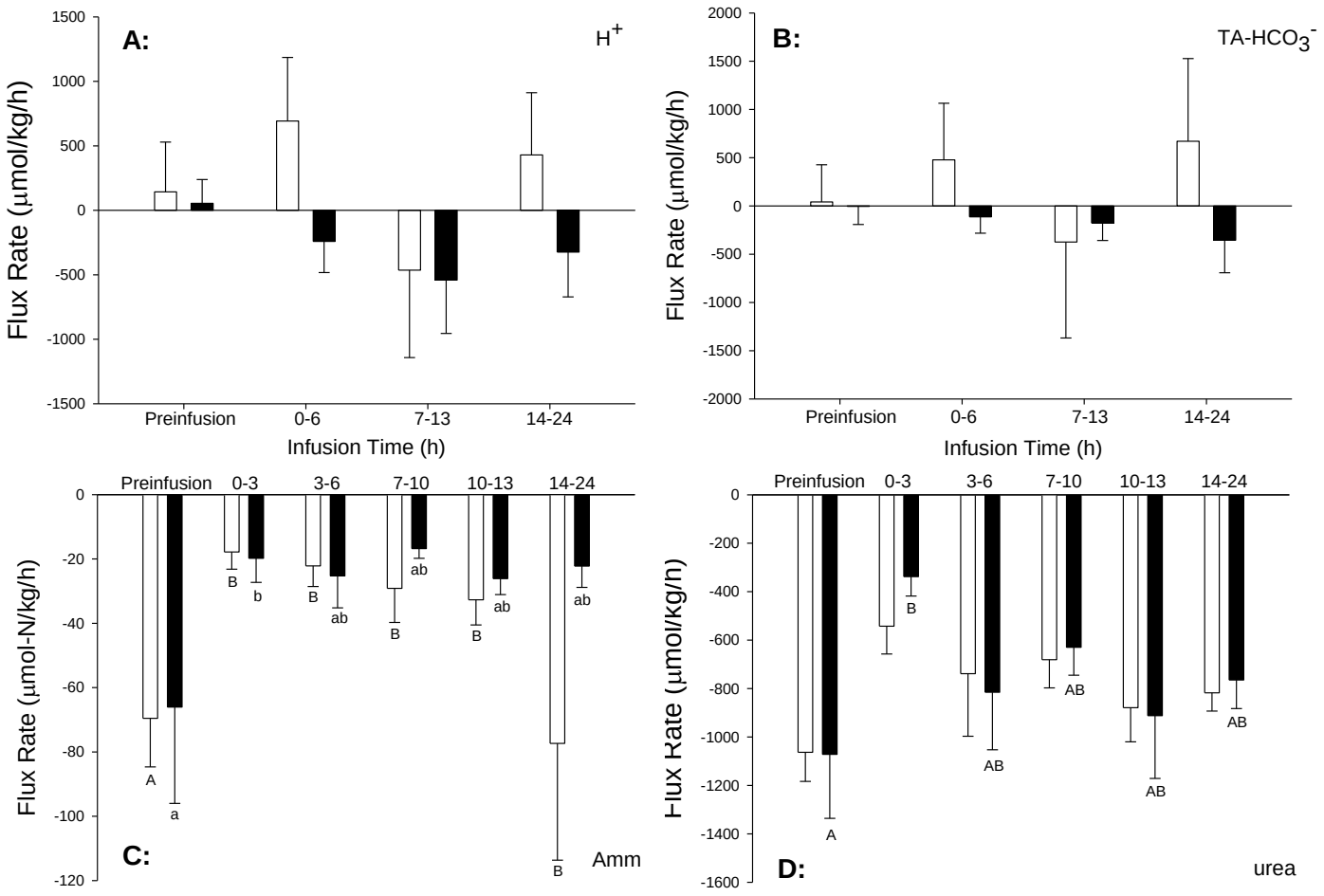

Figure A.3: The effects of the infusion of an acid (125mM HCl, 375 NaCl; Black bars) and neutral (500mM NaCl; White bars) saline on (**A**) whole body net H^+ (N≥3), (**B**) titratable acid-HCO_3^- (TA-HCO_3^-; N≥3), (**C**) total ammonia (Amm; N≥4) and (**D**) urea (N≥4) effluxes in the spiny dogfish shark (*S. acanthias suckleyi*) over a 24-h infusion period. Values are mean ± 1 SEM. Asterisks (* P<0.05) denote differences between control and acid infused fish whereas unique letters denote significant differences (P<0.05) within a treatment group.

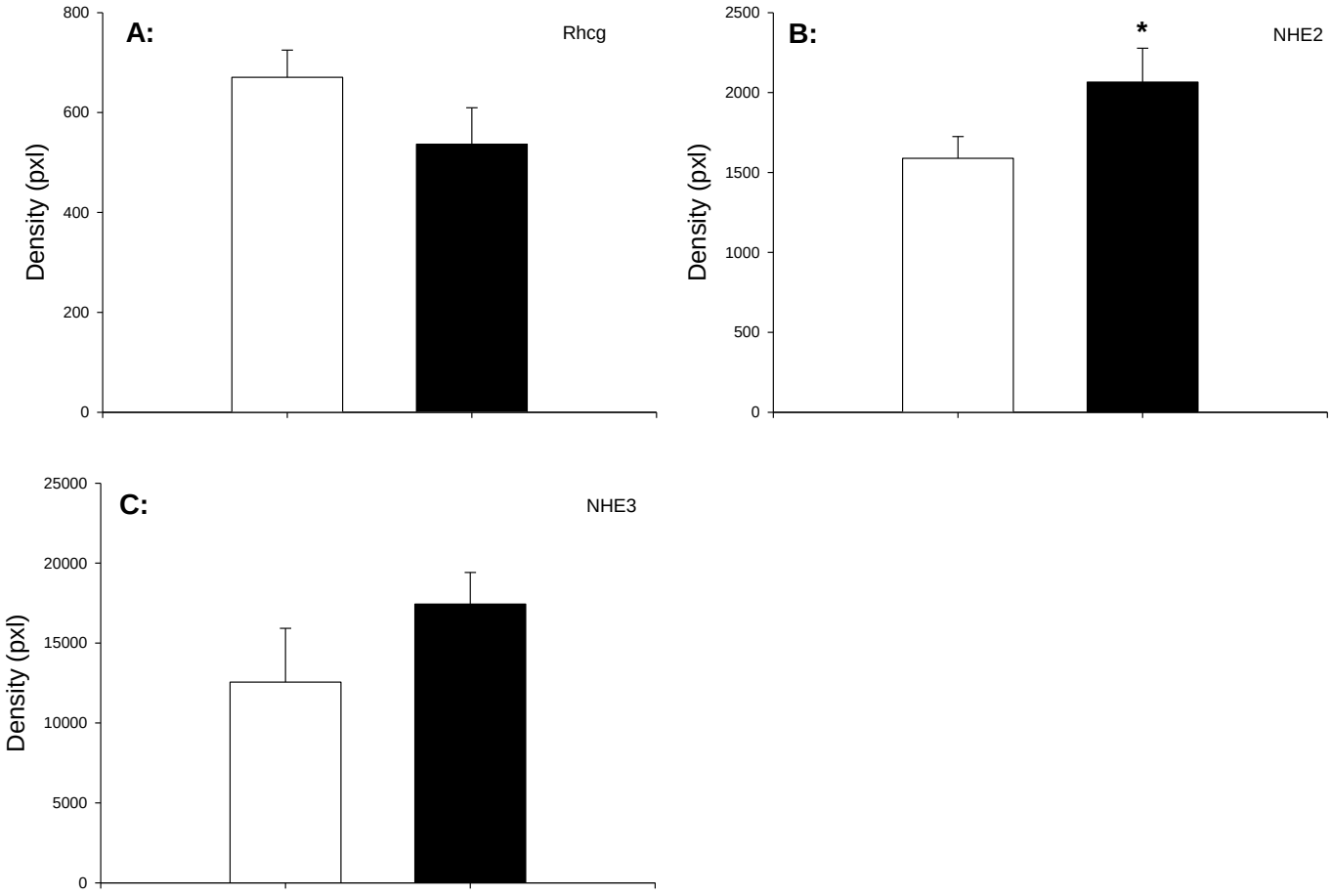

Figure A.4: The effects of the infusion of an acid (125mM HCl, 375 NaCl; Black bars) and neutral (500mM NaCl; White bars) saline on the protein expression of branchial (**A**) Rhcg (N=3), (**B**) NHE2 (N=3) and (**C**) NHE3 (N=3) in the spiny dogfish shark (*S. acanthias suckleyi*) over a 24-h infusion period. Values are mean ± 1 SEM. Asterisks (* $P<0.05$) denote differences between control and acid infused fish.

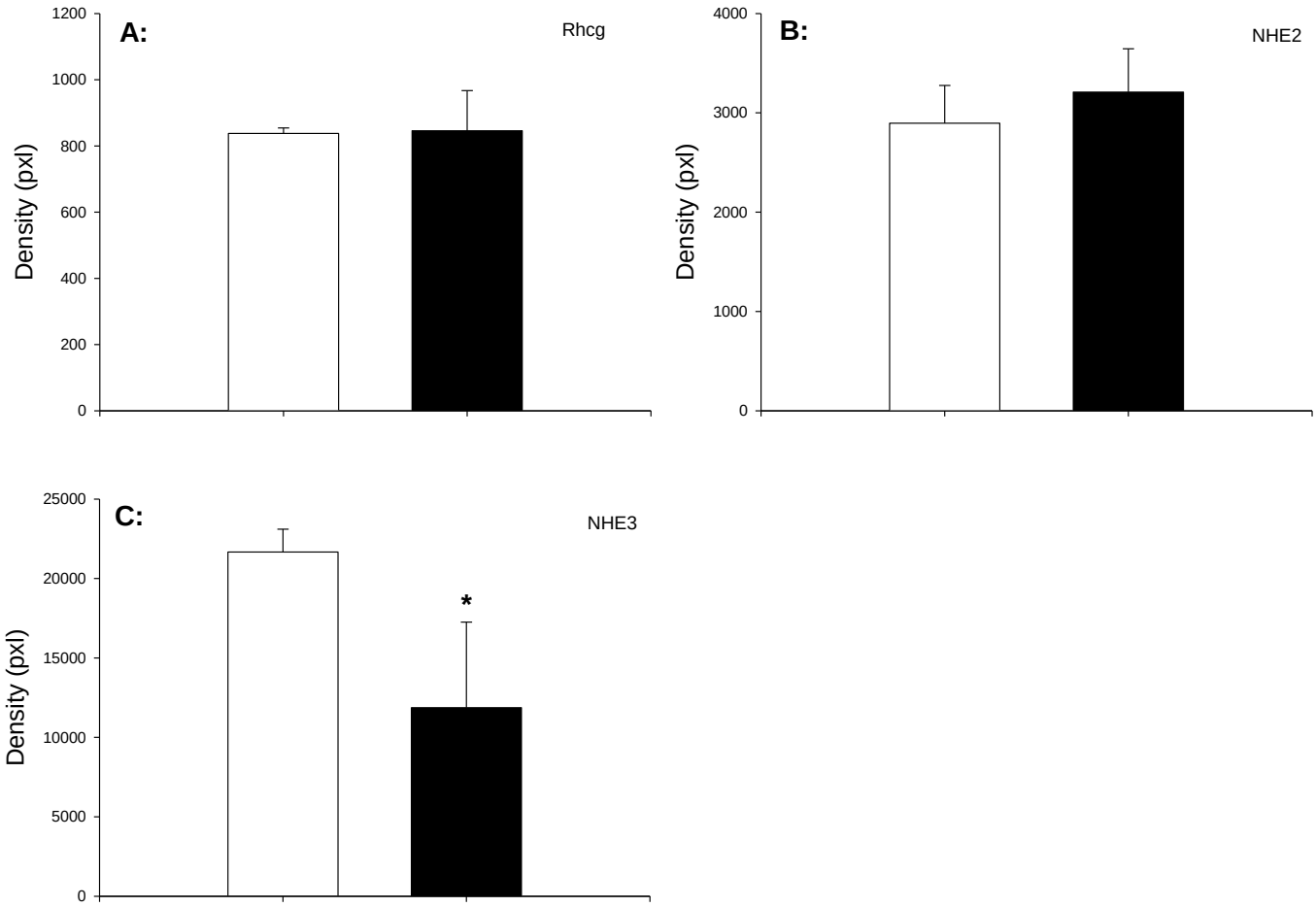

Figure A.5: The effects of the infusion of an acid (125mM HCl, 375 NaCl; Black bars) and neutral (500mM NaCl; White bars) saline on the protein expression of renal (**A**) Rhcg (N=3), (**B**) NHE2 (N=3) and (**C**) NHE3 (N=3) in the spiny dogfish shark (*S. acanthias suckleyi*) over a 24-h infusion period. Values are mean ± 1 SEM. Asterisks (* $P<0.05$) denote differences between control and acid infused fish.

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