DEVELOPMENT OF A NOVEL NH_4^+-SELECTIVE MICROELECTRODE

MEASUREMENT OF AMMONIUM IN HAEMOLYMPH AND MALPIGHIAN TUBULE SECRETION IN *DROSOPHILA MELANOGASTER*: APPLICATION OF A NOVEL AMMONIUM-SELECTIVE MICROELECTRODE

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TITLE: Measurement of ammonium in haemolymph and Malpighian tubule secretion in *Drosophila melanogaster*: Application of a novel ammonium-selective microelectrode

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Abstract

The transport of ammonia by various tissues throughout the body is of fundamental importance for nitrogen excretion in invertebrates, yet sites and mechanisms of ammonia transport are not presently well understood. In this thesis a novel ammonium-selective microelectrode was developed using the crown ether ionophore TD19C6, which is approximately 3800-fold more selective for NH_4^+ than Na^+ compared with the 100-fold difference of the neutral antibiotic nonactin used in previous microelectrodes. We investigated the accuracy of the ammonium microelectrode in solutions simulating haemolymph (25 mM K^+) and secreted fluid (120 mM K^+). In haemolymph-like solutions, ammonium could be measured down to about 1 mM, with an error of 0.5 mM, while in secreted fluid-like conditions ammonium could be determined to within 0.3 mM down to a level of 1 mM NH_4^+ in the presence of 100 to 140 mM K^+. These results suggested that the ammonium microelectrode could be used to measure ammonium in the presence of physiological levels of potassium, unlike previous studies. With the use of the novel NH_4^+-selective microelectrode ~ 20 mM NH_4^+ was found in the diet and 1.5 ± 0.3 mM NH_4^+ in the haemolymph, suggesting under control conditions 3rd instar *Drosophila melanogaster* larvae were able to maintain low levels of ammonium in their haemolymph despite high environmental ammonia. We also quantified ammonium secretion by the Malpighian (renal) tubules of larvae. The results from the Ramsay assay revealed ammonium concentrations of secreted fluid were consistently equivalent to or above ammonium concentrations of bathing salines. With a lumen-positive transepithelial potential, these results suggested an active secretory mechanism for ammonia transport. Under conditions of low K^+ concentrations, the ability of the tubules to concentrate ammonium in secreted fluid was significantly enhanced, indicating some level of competition between NH_4^+ and K^+ for common transporters. The new ammonium-selective microelectrode is sufficiently sensitive to detect ammonium at the picomol level.

Acknowledgments

I am going to tell you a story, a rather short one, about the people who have led me to where I am today – writing a Master's thesis. In this way, I acknowledge and sincerely thank all of the individuals mentioned. This story begins with my parents, Michael and Faye Browne, who have given me the freedom to explore and instilled a sense of wonder in me. They are the reason why my favourite question is Why? With my toy tractors in one hand and the question of How? in the other, my mechanistic view undoubtedly led me to science. My first lab experience was with Virginia Walker, my always inspiring undergraduate thesis supervisor at Queen's University. I always felt more confident and determined walking out of her office than when I went in and she is the reason I pursued graduate studies in the first place. From high school to Queen's to McMaster, Mike Delorme is a life-long friend who I seem to be perpetually following, and is the reason I pursued graduate studies in the Biology department at McMaster. Mike lead me to Michael O'Donnell, a professor at McMaster. When I stepped into his office for the first time he was so interested in my interest of his interests that I decided to join his lab. There simply is no better person I would rather work for. Upon my arrival there was Ryan Belowitz, Sara Chahine, Wida Naikkhwah and Jean-Paul Paluzzi. Ryan taught me about microelectrodes, Sara taught me the Ramsay assay, Wida taught me dissections and was the life of our lab, Jean-Paul Paluzzi is a future professor and my buddy in the lab. Shortly thereafter came Sara Seabrooke, who is the definition of a mentor, has helped me grow and is the reason I am pursuing further graduate studies. After a year in, Mark Vanderveken and Evan Pacey joined the lab. Mark is my partner in crime and my future lawyer, while Evan is my favourite person to work alongside in the evenings. Now you know the story of how I got to where I am today and some of the people who have helped me along the way. I will pay it forward by supporting and encouraging others on their way to achieving their goals just as others have done for me. The end.

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Chapter 1
Introduction

The transport of ammonia by various tissues throughout the body is of fundamental importance for nitrogen excretion in invertebrates, yet sites and mechanisms of ammonia transport are not presently well understood. The significance of ammonia derives from its ubiquitous production through the deamination of amino acids. Once produced, the broad toxicity of ammonia (NH_3 and NH_4^+) necessitates its detoxification, tolerance, or removal from the body. Ammonia transport is likely a crucial step in all these strategies for maintaining homeostasis in the presence of this toxic nitrogenous waste product. It has long been understood that insects excrete mostly uric acid rather than urea or ammonia in order to conserve body water. However, in recent years several studies have suggested that some insects may also excrete significant amounts of ammonia. This thesis proposes to develop a novel ammonium-selective microelectrode in order to re-examine the extent and mechanisms of ammonia transport by insect epithelia.

1 Ammonia

1.1 Toxicity and Nitrogen Excretion Strategies

Throughout this thesis, ammonia refers to both NH_3 and NH_4^+. Ammonia (NH_3) is a weak base which exists in two forms: neutral ammonia (NH_3) and ionic ammonium (NH_4^+). With a pKa of about 9 to 10, at physiological pH (7) about 99% of the ammonia is in the form of NH_4^+ (Emerson et al., 1975). Both forms are highly toxic with 0.5 to 5 mM total ammonia (NH_3 + NH_4^+) being lethal to many invertebrates and vertebrates (Withers, 1992). Mechanisms of

ammonia toxicity involve alterations at a molecular level that affect cellular function. Ammonia is known to disrupt extracellular and intracellular pH. Upon NH_4Cl addition to bathing solutions in astrocytes, an initial transient alkalinization followed by a prolonged acidification of the intracellular compartment has been observed (Nagaraja and Brookes, 1998). These observations are consistent with rapid NH_3 entry followed by slower inward transport of NH_4^+. In general, NH_3 is thought to pass through cellular membrane quite readily, while NH_4^+ is relatively impermeable, thus requiring a membrane-spanning transporter. Due to their similar hydrated radii, competition between NH_4^+ and K^+ for K^+ transporters affects intracellular ion concentrations (Moser, 1987, Martinelle and Haggstrom, 1993). Reduction in potassium transport as well as NH_4^+ transport may alter membrane potential. High concentrations of ammonium chloride were found to depolarize both neurons and astrocytes, presumably via the entry of positively charged NH_4^+ into the cytosol (Bosoi and Rose, 2009). Although the increase in membrane potential (depolarization) was below the threshold for evoking action potentials, hyper-excitability may still result.

At the organismal level, pathological states may arise in animals, especially from cells with excitable membranes due to the electrical disturbances of NH_4^+. Ammonia has been shown to lead to the activation of N-methyl-D-aspartate (NMDA) receptors in mammalian systems. The mechanism of activation in not clear but it may involve membrane depolarization via NH_4^+ entry as previously discussed. Inhibitors of the NMDA receptor were able to abolish the ammonia-induced death of animals indicating excessive activation of NMDA receptors was responsible for the observed toxicity (Hermenegildo et al., 1996). Excessive activation of ATP-dependent transporters or enzymes may also alter cellular metabolism. With all the possible cellular

disruption it is likely that ammonia may result in an increased demand for energy to maintain homeostasis (Martinelle and Haggstrom, 1993). Further, ammonia-induced alterations in the mitochondrial respiratory chain may lead to decreased synthesis of ATP and to increased formation of free radicals (Llansola et al., 2007). Clearly, there are multiple mechanisms of ammonia toxicity whose effects are wide spread and many are still speculative. Organisms must therefore have the ability to cope with such a systemic toxicant to survive.

The toxicity of ammonia necessitates its excretion, although it may not necessarily be excreted in an unchanged form. The most common nitrogenous excretory molecules are ammonia, urea and uric acid (Wright, 1995). Insects are predominantly considered uricotelic coinciding with the Baldwin-Needham hypothesis (Baldwin and Needham, 1934) that terrestrial organisms developed less soluble nitrogenous excretion products such as urea, uric acid or allantoin, in order to conserve body water. More recently, it has become clear that some insects do not follow this trend calling into question the generality of this hypothesis. Excretion of predominantly ammonia or ammoniotely has been demonstrated in cockroaches, blowflies and mosquitoes (Prusch, 1972, Mullins and Cochran, 1973, Scaraffia et al., 2005). Desert locusts (*Schistocerca gregaria*) excrete significant amounts of ammonium urate (Phillips et al., 1994). It is important to note that ammoniotely, ureotely and uricotely are not fixed states as many organisms can excrete combinations of ammonia, urea and uric acid simultaneously. Alterations in diet can also shift the relative abundances of all three nitrogenous wastes (Briegel, 1986).

1.2 Ammonia Production

Insects in their natural milieu are unlikely to ingest foodstuffs containing high levels of ammonia, rather it is produced endogenously via the deamination of amino acids derived from dietary protein (Campbell, 1991). The exception would be for insects that feed in environments that have been subject to decay by ammonia-producing microorganisms. The main tissue involved in ammonia production is likely the fat body, which has a functional glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle whereas the midgut does not (Scaraffia et al., 2010). The main pathway for amino acid catabolism involves the deamination of glutamate to α-ketoglutarate and NH_3 by the exclusively intramitochondrial enzyme glutamate dehydrogenase (GDH). Alternatively α-amino groups may be linked to the purine nucleotide cycle (Lowenstein, 1972). The latter involves aspartate, GTP and H_2O supplying the cycle that terminates with the deamination of adenosine monophosphate (AMP) by the predominantly extramitochondrial enzyme adenylate deaminase forming inosine monophosphate (IMP) and NH_3. It is thought that the purine nucleotide cycle may play a larger role in invertebrate ammonia production (D. Weihrauch, personal communication) as high levels of adenylate deaminase have been observed in a few species. Yet, enhanced GDH expression has been observed following a blood meal in the fat body of mosquitoes, suggesting that this tissue facilitates ammonia production/detoxification, as the GDH reaction is reversible (Scaraffia et al., 2005).

Bacterial endosymbionts of insects may aid in catabolic use of amino acids and nitrogen excretion. It has been suggested that endosymbionts may provide their hosts with ‘new’ enzymes that allow their hosts to exploit new dietary regimes and as such have reverted back to ancestral

ammoniotely from uricotely (Lopez-Sanchez et al., 2009). A return to ammonotely may be due to excess ammonia generated by the symbiotic bacteria or perhaps the metabolic costs of catabolism of the host are reduced by the bacteria and the benefit outweighs the cost of additional water loss. It may be that these endosymbionts produce significant amounts of ammonia, but the magnitude of their role is at present unknown.

1.3 Ammonia Metabolism/Detoxification

Increased expression of a possible ammonia detoxification enzymes such as glutamate dehydrogenase (GDH), glutamine synthetase (GS) and pyrroline-5-carboxylate reductase (P5CR) were identified in the mosquito *Aedes aegypti* in response to a blood meal/ammonia challenge (Scaraffia et al., 2005). The GS/GOGAT cycle comprises the following sequence: the enzyme GS is involved in sequestering free ammonia by incorporating it onto the δ-carbon of glutamate, forming glutamine. For every molecule of glutamine, two molecules of glutamate are produced by the enzyme glutamate synthase, also known as GOGAT. Glutamate is then transported into the mitochondria where it is deaminated by GDH forming NH_3 and α-ketoglutarate from each molecule of glutamate. Downstream of the GS/GOGAT cycle, the glutamate may also be converted into a less toxic proline molecule via P5CS and P5CR enzymes. It is likely that proline and glutamine act as temporary nitrogen sinks in order to avoid build-up of ammonia in the haemolymph during digestion of protein-rich meals. Scaraffia also provides evidence that mosquitoes are able to use proline as an energy substrate for flight muscle, indicating that a proline pathway may be more useful than previously thought (Scaraffia and Wells, 2003).

2 Ion Transport by Malpighian Tubules

In most insect species, Malpighian tubules (MTs) are secretory organs that are involved in the production of primary urine. In some insects, downstream segments may also be involved in modification of the primary urine by reabsorption of water and/or ions. *Drosophila* have two pairs of MTs that are joined through a common ureter to the junction between midgut and hindgut segments of the alimentary canal. Each tubule is approximately 2 mm in length with an outer diameter of about 35 μm and the tubular wall consists of a single layer of epithelium. Urine is produced from the active transepithelial movement of ions and osmotically obliged water from blood (haemolymph) to lumen of the tubules (Ianowski and O'Donnell, 2004). Proximal and distal regions of the tubules appear to be distinct in their functions. In general, the main segment secretes K^+-rich fluid at high rates, while proximal segments reabsorb significant amounts of K^+ and water but also secrete Ca^{2+} and acid equivalents (O'Donnell and Maddrell, 1995). Each of the anterior pair of tubules has a non-secretory distal segment which sequesters Ca^{2+} as lumenal concretions (Dube et al., 2000a). There are two distinct cell types in the main segment: principal cells that are mainly involved in active ion transport and stellate cells, which transport anions passively down an electrical gradient and are also richly-endowed with aquaporins implicated in water transport (O'Donnell et al., 1998). Transport proteins underlying the secretion of fluid by the MTs has been investigated. In principal cells, ion transport is driven mainly by an electrogenic apical V-type H^+-ATPase, which acidifies the lumen and creates a lumen-positive apical membrane potential. Na^+ or K^+/H^+ exchange is energized by the apical proton gradient driving movement of K^+ and Na^+ into the lumen. Basolateral Na^+: K^+: $2Cl^-$ transporters are responsible for entry of K^+, Na^+ and Cl^- entry into tubular cells. Sodium is returned to the blood through a basolateral Na^+/K^+-ATPase (Ianowski and O'Donnell, 2004). Specific pharmacological

antagonists of the various tubular transporters are readily available and may be useful in the study of ammonia transport across Malpighian tubules.

Malpighian tubules have alternate functions other than the formation of urine. Cytochrome P450 and glutathione transferases, genes well known to be involved in xenobiotic detoxification are enriched in the MTs (Wang et al., 2004). In addition, tubules constitutively express low levels of several antimicrobial peptides, suggesting MTs may indeed function as an autonomous immune system (Dow and Davies, 2006).

3 Haemolymph Ammonia

Ammonia absorption from gut lumen to haemolymph has been observed in the tobacco hornworm *Manduca sexta* (Weihrauch, 2006) where haemolymph ammonia concentrations may be tightly regulated. *Manduca* may need to absorb ammonia because their diet is nitrogen-poor. In species with nitrogen rich diets, tight regulation of ammonia haemolymph concentrations may be explained by excretory/secretory modulation and/or detoxification mechanisms. Excretion of ammonia present in the haemolymph may be accomplished through secretion by the Malpighian tubules, hindgut, or rectum. Ammonia secretion has been observed in the hindgut of locusts (rectum) and blowflies and suggested in Malpighian tubules (Prusch, 1974, Thomson et al., 1988, Stagg et al., 1991). Measurements by Stagg et al. (1991) showed a 4 fold increase in ammonia concentrations along with a 0.5 pH unit decrease between haemolymph and tubular fluid, respectively. Active ammonia secretion into the hindgut of the blowfly *Sarcophaga bullata* has also been shown (Prusch, 1976). These data suggest the presence of

vectorial transporters involved in ammonia excretion. The effects of pharmacological inhibitors of putative ammonia transporters could be used to further examine the mechanism of transport.

4 Dietary Ammonia

Although omnivorous insects that feed on living plants are unlikely to encounter foodstuffs containing high levels of ammonia, some insects may very well ingest ammonia directly. For example, insects that feed on decaying, protein-rich food, would likely ingest ammonia derived from ammonifying bacteria/fungi through a process known as ammonification (Payne, 1973). In addition, larval stages may also be exposed to and/or mature in a high ammonia environment, which may provide selective pressure for ammonia tolerance. Interestingly, ammonia concentrations in the diet of *Drosophila* cultures increased from approximately 10 mM after 4 days to about 30 mM after 20 days (Borash et al., 1998). The presence of ammonia in the diet suggests dietary ammonia intake by both larval and adult stages, with larval stages possibly having higher exposure due to their proximity to the diet. *Drosophila* larvae may thus provide a useful model system for studies of ammonia transport.

If some insects do ingest ammonia directly, that ammonia could simply pass through the alimentary canal and be excreted without any modification. However, this possible explanation is at odds with observations that the amount of excreted nitrogen, in the form of NH_4^+, was from five-to-17-fold greater than that ingested in the larval cabbage armyworm *Mamestra brassicae* (Kagata and Ohgushi, 2011). These data are consistent with the hypothesis that metabolically derived ammonia may play a significant role in nitrogen excretion. Further, temporary storage

via a nitrogen sink pathway (conversion from an alternate nitrogen source to NH_4^+) may also explain the source of the excess NH_4^+-nitrogen in the frass.

4.1 Tolerance to High Environmental Ammonia

Drosophila raised in laboratory culture under crowded conditions showed increased ammonia concentrations within the dietary media over time (Borash et al., 1998). As a result of high environmental ammonia, ammonia content within the insect may also increase. Whole body ammonia content of larvae reared on dietary media containing 370 mM ammonia was three times above that of larvae fed a control diet (Borash et al., 2000). Continued survival during increasing concentrations of ammonia in the diet and likely the body suggests that both larvae and adults are tolerant of the toxic effects of ammonia. In addition, larvae fed on increasing doses of NH_4^+ (from about 0.25 mol – 0.5 mol) showed the greatest fitness when assayed on ammonia-supplemented food (Borash et al., 2000). This data indicates that one can indeed select for ammonia tolerance, suggesting that such larvae increase the capacity to detoxify or excrete ammonia.

There is also some evidence for ammonia cross-tolerance, as ammonia-selected larval cultures on urea-supplemented media (266 mM urea) developed significantly faster and had higher viability than unselected controls. Additionally, urea-selected larval cultures on ammonia-supplemented media (350 mM NH_4Cl) developed significantly faster and had higher viability than unselected controls. Together these results provide evidence for cross-tolerance between ammonia and urea.

In the presence of cross-tolerance, it is to be expected that a host of broad detoxifications mechanisms are employed in response to ammonia exposure.

Other insect species may also be tolerant of ammonia. The Malpighian tubules of the blood feeding insect *Rhodnius prolixus* secrete fluid at 40% of the maximal rate in 137.6 mM NH_4Cl in the absence of potassium or sodium (Maddrell, 1969). Further, this indicates that MTs may be quite tolerant of high ammonia levels as they will still function at such high ammonium concentrations. Given that low potassium reduces potassium interference on the ammonium-selective microelectrode described below, it may be feasible to study ammonia transport by *Rhodnius* MTs.

5 Ammonia Transport

It is known that hydrated NH_4^+ and K^+ share the same ionic radius leading researchers to hypothesize that membrane K^+ transporters may also transport NH_4^+ ions (Kikeri et al., 1989, Knepper, 1991, Martinelle and Haggstrom, 1993). Possible transporters of ammonia include: Na^+/K^+-ATPase (Garvin et al., 1985, Wall, 2003, Holtug et al., 2009), Na^+ K^+2Cl^--cotransporter (Kikeri et al., 1989, Knepper, 1991, Evans and Turner, 1998), Na^+or K^+/H^+-exchanger (Grinstein and Rothstein, 1986, Knepper, 1991, Simon et al., 1992), acidified vesicles (Weihrauch, 2006), Rhesus proteins (Nakhoul and Hamm, 2004, Wright and Wood, 2009) and aquaporins (Nakhoul et al., 2001). Interestingly, Rh proteins may also transport methylammonium (MA), such that the use of ^{14}C-MA studies may complement studies looking at ammonium transport (Nakada et al., 2007). Functional expression of Rh50-like glycoprotein showed significantly enhanced

expression in midgut, fat body and Malpighian tubules in response to a blood meal in the mosquito *Aedes albopictus* (Wu et al., 2010). The results are consistent with the hypothesis of Rh proteins as ammonia channels. In a perfusion chamber study by Prusch (1974), where small amounts of external NH_4^+ were added to a hindgut preparation bath, K^+ secretion decreased while NH_4^+ secretion greatly increased. The results were interpreted as evidence for a cation pump with higher specificity for NH_4^+ than K^+. In a subsequent study by the same author it was determined that the reduced K^+ secretion resulted from increased K^+ absorption rather than a decreased secretion of NH_4^+ due to competition (Prusch, 1976). Further competition studies could identify the relative contributions of putative transporter(s).

5.1 Acid Trapping Mechanism

In a diffusion trapping (acid trapping) model, active H^+ secretion in parallel with passive transepithelial NH_3 diffusion results in the conversion of the NH_3 to NH_4^+. In the classical view, NH_4^+ is much less membrane permeable than the non-ionic species NH_3, and as such would become 'trapped' in the latter compartment with little NH_4^+ back flux (Wall, 2003). Both premises – very high NH_3 permeability and very low NH_4^+ permeability – may be untrue in insects. For instance, in conditions of high environmental ammonia (HEA) the diffusion of ammonia would be internally directed, and hence the diffusion model would be a deleterious strategy for reducing ammonia toxicity. As for permeability, variation seems to exist. Little to no permeability to NH_3 has been observed in the medullary thick ascending limb of Henle, which is also highly permeable to NH_4^+ (Kikeri et al., 1989). In contrast, the mammalian proximal tube is quite permeable to NH_3 (Knepper et al., 1989). Due to the low NH_3 permeability in some tissues, others have investigated the possibility of NH_3 transporters and the results indicate that Rh

glycoproteins and aquaporins may facilitate NH_3 conductance (Nakhoul and Hamm, 2004, Zidi-Yahiaoui et al., 2005). Thus the absence of these putative transporters may explain low NH_3 permeability of some tissues. Interestingly, when the pH gradient was reversed across the midgut of *Manduca*, active ammonia uptake still occurred, albeit at a reduced rate. In addition, ammonia transport has been observed even in the presence of symmetrical ammonia applications (Weihrauch, 2006). These data indicated that (1) ammonia transport is not passive, (2) an acidifying gradient enhances ammonia transport but is not an absolute requirement and (3) only a small portion of ammonia transport is pH-dependent. In conclusion, the acid trapping model may help to explain the pH- dependent transport of ammonia but it cannot explain the pH-independent portion, which may be the larger of the two (Weihrauch, 2006).

5.2 H^+-ATPase as a Driving Force for Ammonia Secretion

The proposed function of the apical H^+-ATPase found in Malpighian tubules is to maintain a lumen-directed proton gradient and lumen positive apical membrane potential that provides the driving force for K^+ secretion across the apical membrane via an amiloride-sensitive Na^+ or K^+/H^+ exchanger (Linton and O'Donnell, 1999, Wieczorek et al., 2003, Ianowski and O'Donnell, 2004). With the possibility of NH_4^+ substitution for the K^+ site on the exchanger, addition of apical amiloride may reduce ammonium secretion. Inhibition of the apical H^+-ATPase in colonic epithelia of the chicken *Gallus gallus* significantly reduced serosal-to-mucosal ammonium flux, indicating the importance of a lumen-positive proton gradient in ammonium transport (Holtug et al., 2009). In locust Malpighian tubules, (*Schistocerca gregaria*) Stagg (1991) found a decline in pH of 0.5 units from haemolymph to tubular fluid, which was presumably due to an apical H^+-ATPase.

6 Measuring Ammonia in Nanolitre Volumes

The measurement of ammonia in droplets of fluid secreted by *Drosophila* tubules set up in the Rasmay fluid secretion assay (described below) is difficult because of the small size of the droplets. Malpighian tubules secrete about 20 nl of fluid over a period of 45 minutes. Thus in order to study ammonia transport across the Malpighian tubules using a Ramsay assay we must measure ammonia in nanoliter volumes. Unfortunately, conventional techniques for measuring ammonia require sample volume of about 20 µl or larger. The most common methods for ammonia determination make use of enzymatic reactions where the consumption or production of a fluorescent molecule is well correlated to the consumption or production of ammonia (usually NH_3) within the reaction. Thus the fluorescent intensity measured with a fluorescent microscope could be used to determine ammonia concentrations within a sample using a calibration curve. An enzymatic reaction kit was not used due to the requirement for 20 µl samples and very high absorption/emission spectra that required equipment that was not easily accessible. Another technique for ammonia measurement involves the use of traceable [^{14}C] methylammonium (MA) as an analog of ammonia. The shortcoming of this technique is due to the differences between ammonia and MA. The lack of MA transport by NKCC and the Na^+/K^+-ATPase suggest that the transport of the two analogs may be quite different (Worrell et al., 2008). Gas-sensitive ammonia (NH_3) macroelectrodes also have been used, working on the principle of a gas-permeable membrane allowing separation of NH_3 from sample to filling solution, with NH_3 reacting with water forming OH^- ions generating an electrical potential across the membrane that is proportional to the concentration of NH_3 present in the sample. Since the macroelectrode is large, samples of about 2 ml are required and thus could not be used to study ammonia transport across Malpighian tubules. Ion-selective microelectrodes are well suited to sampling in nanolitre volumes with tip diameters at the micrometer scale. Current ammonium-

selective microelectrodes are based on liquid membranes containing the antibiotic nonactin. Potassium was found to be the main interfering ion with a selectivity coefficient (log) of -0.6 (Fresser et al., 1991). This means the electrode is about 4 times more selective for NH_4^+ compared to K^+. The potassium interference is a significant barrier to ammonia detection as fluid secreted by insect Malpighian tubules contains high levels of potassium (about 100 to 140 mM) (Maddrell and Klunsuwan, 1973). With a selectivity coefficient for potassium of about -1.0 observed with macro- and micro-electrodes, uncorrected measurements would indicate apparent ammonium concentrations 10 to 14 mM greater than actual concentrations. Nonactin based electrodes are also affected by significant interference from high concentrations of Na^+. A novel ionophore, 2,5,12,15,22,26-Hexaoxaheptacyclo [25.4.4.46.11416.21.01.27.06.11.016.21]-tritetracontane (TD19C6), showed greatly reduced sodium interference compared to nonactin in macroelectrodes (Suzuki et al., 2000). With the improvement seen at the macro-scale, we were interested in determining how well the ionophore would function at the micro-scale in ISMEs.

7 Thesis Objectives

The above considerations suggest that *Drosophila* larvae excrete ammonia. The contribution of the Malpighian tubules to ammonia excretion in this species is currently unknown. The goals of my thesis, therefore, are: 1) to develop a novel ammonium-selective microelectrode suitable for measurement of ammonium in the presence of physiological levels of potassium and sodium, and 2) to use these microelectrodes to assess ammonia secretion by isolated insect Malpighian tubules.

Chapter 2
Materials and Methods

8 Ramsay Assay

In order to determine the ammonium content in fluid secreted by the Malpighian tubules, a modified Ramsay assay was used. Briefly, a pair of Malpighian tubules connected by a common ureter was removed from 3rd instar *Drosophila melanogaster* larvae. Dissections were performed in standard bathing medium (SBM), consisting of 117.5 mM NaCl, 20 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 8.5 mM $MgCl_2 \cdot 6H_2O$, 10.2 mM $NaHCO_3$, 8.6 mM HEPES, 20 mM Glucose, 10 mM Glutamine titrated to pH 7.0 with 1M NaOH. The original recipe called for 4.3 mM $NaH_2PO_4 \cdot 2H_2O$ but it was removed due to the formation of a precipitate in the presence of ammonium chloride. Tubules were transferred into 20 µl bathing saline droplets held in wells under paraffin oil contained within a Sylgard® -lined petri dish. Distal portions of the tubules were then drawn outside the bath into the paraffin oil and fixed to a pin such that the ureter was bathed in oil with sufficient space for a secretion droplet to form without fusion with the bathing droplet. Ammonia bathing media (ABM) are essentially the same as the SBM, but with equimolar substitution of NH_4Cl with NaCl, in order to maintain ionic strength. Secreted fluid droplets were removed after 45 minutes and the diameter (d) was recorded using an eye-piece micrometer under 80x magnification. Secreted droplet volume was calculated as $4/3\pi r^3$ and secretion rate (nl min^{-1}) was calculated by dividing droplet volume by the time (min) over which it had formed.

9 Ion-Selective Microelectrodes

Ionophore cocktails consist of an ionophore, a lipophilic salt and a solvent. Note that several cocktails were tested with varying proportions of lipophilic salt and solvent. Of the two solvents used, microelectrodes based on a cocktail containing bis-(1-butylpentyl) adipate (BBPA) had slower response times but slightly higher selectivity compared to electrodes made with nitro 2-nitrophenyl octyl ether (NPOE). The cocktail (ionophore, lipophilic salt and solvent) composition of the ammonium-selective microelectrode that had the highest selectivity with a reasonable response time was 1% TD19C6, 0.25% Potassium tetrakis[3,5-bis-(trifluoromethyl) phenyl] borate (KtktFlMePB) and 98.75% BBPA. The potassium-selective and sodium-selective microelectrodes were prepared from cocktails solutions purchased from Fluka (Potassium Ionophore I – Cocktail B) and Sodium Ionophore X. Electrodes were pulled vertically from borosilicate glass capillaries and lightly salinized with 0.2 µl dichlorodimethylsilane placed in a 10 cm glass petri dish and inverted over batches of 14 micropipettes on a hot plate at 200 ºC. Silanized micropipettes were backfilled with 100 mM NH_4Cl in the case of the ammonium microelectrodes, while 150 mM NaCl was used in sodium microelectrodes, and 150 mM KCl was used in both the potassium and reference microelectrodes. Cocktails were drawn into the tip of the electrodes using negative pressure and allowed to condition in solutions containing 15 mM of the ion of interest for typically 30 min prior to their use. Once positioned on mechanical manipulators, microelectrodes were inserted into calibration solutions submersed in Paraffin oil, the tips of the ion-selective electrodes were broken to a tip diameter of ~ 5 um by touching the tip of one electrode against the shank of the other. This procedure reduced the response time to ~ 10 sec for the NH_4^+-selective microelectrode and to ~ 1 sec for the K^+-selective microelectrode. Nernstian slopes ≥ 50 mV were obtained in calibration solutions (10 fold serial dilutions between 150 and 1.5 mM of the principal ion diluted with 150 mM NaCl to ensure constant ionic strength

of 150 mM) prior to and following all experimentation. An ionic strength of 150 mM was chosen based on analysis of fluid secreted by the Malpighian tubules from a previous study (Linton and O'Donnell, 1999). All potentiometric measurements were performed under paraffin oil unless stated otherwise. The experimental setup involved measurement of ammonium and potassium simultaneously. Chlorided silver wires were connected through a common ground to a high impedance (>10^{12} Ω) input amplifier (A-M systems) connected to a data acquisition system (Power Lab, AD Instruments, Sydney, Australia) using Chart 5 software. The following equation was used to calculate concentrations from voltage traces for each ISME:

$$[Ion]_{Sample} = [Ion]_{Cal} \cdot 10^{(\Delta V/S)}$$

where $[Ion]_{Sample}$ represents an ion concentration in a sampling medium, $[Ion]_{Cal}$ represents an ion concentration in a calibration solution, ΔV represents the difference in voltage recorded between the sample and same calibration solution, and S represents the Nernstian slope of a particular electrode in use at the time. Although ion-selective electrodes measure ion activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions. This assumption is appropriate for fluids secreted by Malpighian tubules, which are of similar ionic strength to the calibration solutions used in this study.

10 Haemolymph and Diet Ion Measurements

Third instar *Drosophila* larvae were rinsed with deionized water on an absorbent pad and allowed to dry for at least 5 minutes. Using forceps the cuticle of each larvae was torn under oil

and pooled haemolymph was immediately drawn into a pipette and transferred to another dish containing oil for sampling with ion-selective microelectrodes (ISMEs).

Samples of diet were taken from the center of culturing vials using a scapula and placed under oil for sampling with ISMEs.

11 Selectivity Coefficients

Selectivity coefficients are a measure of the ability of an ISME to distinguish an interfering ion from the ion of interest. Selectivity coefficients of various interfering ions were determined using a separate solutions method (SSM). For example, 100 mM solutions of each interfering ion were tested against a 100 mM NH_4Cl calibration solution. The Nicolsky-Eisenman equation was used to calculate selectivity coefficients:

Equation 1 $$\log_{10} K_{ij}^{Pot} = \frac{(V_j - V_i)}{58.74} + \log a_i - \log a_j^{1/z_j}$$

where K_{ij}^{Pot} is the selectivity coefficient for the primary ion 'i' relative to the interfering ion 'j', V_j and V_i are the voltages measured in the 100 mM solutions of 'j' and 'i' respectively, a_i and a_j are the corresponding activities and z_j is the valence of the interfering ion. Activity coefficients used in selectivity coefficient calculations were as follows (γ ion): 0.765 NH_4^+, 0.766 K^+, 0.776 Na^+, 0.754 Li^+, 0.761 Rb^+, 0.752 Cs^+, 0.517 Ca^{2+}, 0.535 Mg^{2+}, 0.81 $NMDG^+$, 0.755 $CH_3NH_3^+$, respectively (Robinson and Sinclair, 1934, Verrall, 1975, Goldberg and Nuttall, 1978, Macaskill and Bates, 1986, Chen et al., 2009).

Species-specific selectivity coefficients were also determined using a empirical mixed solution method (EMSM) in solutions intended to mimic fluid secreted by Malpighian tubules of *Drosophila* larvae. EMSM involved determining selectivity coefficients in calibration solutions containing increasing concentrations of NH_4^+ in the presence of a fixed amount of interfering ion (120 mM K^+). Ionic strength of calibration solutions was maintained at 170 mM by equimolar substitution of NaCl for NH_4Cl. Since both the uncorrected and corrected ammonium concentration as well as potassium concentrations were known in calibration solutions, the following equation was used to solve for the selectivity coefficients:

Equation 2 $$[\mathrm{NH_4^+}]_{\mathrm{Corrected}} = [\mathrm{NH_4^+}]_{\mathrm{Uncorrected}} - \left([K^+]\times 10^{\mathrm{K}_{\mathrm{NH_4^+K^+}}^{\mathrm{Pot}}}\right)$$

where $\mathrm{K}_{\mathrm{NH_4^+K^+}}^{\mathrm{Pot}}$ is the log of the selectivity coefficient of potassium for the ammonium ionophore TD19C6.

12 Correcting for Potassium Interference

Uncorrected ammonium and potassium concentrations were first determined. A standard curve of potassium selectivity coefficients over a range of uncorrected ammonium concentrations was used to determine selectivity coefficients for any given uncorrected ammonium concentration. With the appropriate selectivity coefficient and known potassium concentrations of the sample, corrected ammonium concentrations were determined using equation 2.

13 Salines used to Bathe Malpighian Tubules

Malpighian tubules were bathed in solutions intended to mimic that of the haemolymph of *Drosophila* larvae. The saline was adapted from a recipe previously used (Rheault and O'Donnell, 2004). In general, *Drosophila* salines were prepared from two solutions: one containing NH_4Cl at the highest concentration required of the experiment, with the second solution containing NaCl in place of NH_4Cl at the highest concentration required of the experiment. The second solution was used to dilute the first solution to obtain the treatment ammonium concentrations desired for the experiment. The sum of [KCl] + [NaCl] + [NH_4Cl] in the first solution or sum of [KCl] + [NaCl] in the second solution was kept constant at 137.5 mM in order to maintain ionic strength of all solutions at 196.8 mM.

In experiments involving haemolymph-like *Drosophila* saline containing 25 mM K^+, ionic strength was maintained by equimolar substitution of NH_4Cl by N-Methyl-D-glucamine (NMDG) titrated with HCl to produce $NMDG^+$. The composition of the two solutions were (in mM): 29.8 NaCl, 25 KCl, 82.7 $NMDG^+$, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 NaHCO3, 8.6 HEPES and 40 NH_4Cl, 29.8 NaCl, 25 KCl, 42.7 $NMDG^+$, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 NaHCO3, 8.6 HEPES, respectively. Again the solution containing NH_4Cl was diluted with the NH_4Cl-free saline to obtain treatment salines containing the desired ammonium concentration. All salines used to bathe tubules were titrated daily to pH 7.0 prior to their use.

14 Culturing Medias

Drosophila melanogaster stocks were maintained at room temperature (21-23°C) on standard medium (SM) containing (in g): 100 sucrose, 18 agar, 1 potassium dihydrogen orthophosphate, 8 sodium potassium tartrate tetrahydrate, 0.5 NaCl, 0.5 $MgCl_2$, 0.5 $CaCl_2$, 0.5 ferric sulphate, 50 dry active yeast and 7.45 ml/L 10 % tegosept in EtOH and 10 ml/L acid mix (11 parts H_2O, 10 parts propionic acid and 1 part O-phosphoric acid (85%)) diluted with 1 liter of deionized water. Media for experiments involving variation in the ammonia concentration of the diet were prepared from SM with the addition of NH_4Cl so that the only difference between diets was NH_4Cl content.

15 Statistics

Values are presented as mean ± standard error of the mean (SEM) for the indicated number (N) of measurements. Statistical significance of differences were evaluated by one-way analysis of variance (ANOVA). Statistical significance of unpaired differences between measured and predicted ammonium concentrations, for each [NH_4Cl], were evaluated by the Student's *t*-test. A *P*-value of < 0.05 was considered to represent a statistically significant difference. Significance of potassium values between ammonium treatments were indicated using the Greek alphabet, while significance of ammonium values between ammonium treatments were indicated using the English alphabet.

Chapter 3
Results

16 Characterization of the NH_4^+-selective Microelectrode

16.1 Technical Limitations

Microelectrode measurement of NH_4^+ in small volumes posed significant technical challenges. The 95 % response time of NH_4^+-selective microelectrodes under oil was slower (7 seconds) than K^+-selective microelectrodes (typically less than 1 second), thus ammonium microelectrodes were limiting when both K^+ and NH_4^+ were used simultaneously. Preliminary experiments indicated that secreted droplets smaller than 6 nl tended to shrink in ~ 1 hr with a consequent increase in ion concentrations in the droplet (Appendices 7 & 8, Figures A6 & A8). In subsequent experiments, droplets were measured within 1 hr of collection. Consequently, when using the Ramsay assay sampling rates were on the order of 0.14 samples min^{-1}, restricting the number of samples to a maximum of approximately 8 within the 1 hr sampling window. Simultaneous sampling took longer to perform due to an additional calibration solution sampled following each test solution, although it doubled data collection during the experiment. In addition, fluid secretion rate (FSR) was low, especially in conditions of low bathing potassium concentrations and high bathing ammonium concentrations. As a result, volumes of fluid secreted by approximately 30 % the Malpighian tubules were below 6 nl and thus were excluded from the data due to the problem of water loss. In conclusion, the longer response time of the ammonium microelectrode limited the pace of data collection compared to other ion-selective microelectrodes.

16.2 Selectivity Coefficients and Slopes of Microelectrodes

Selectivity coefficients (K_{ij}) are measures of the ability of an ion-selective electrode to distinguish a particular ion from others. Selectivity coefficients are typically presented in log form (log K_{ij}) owing to the wide range of values often observed. Ion-selective electrodes contain selectively permeable membranes that create distributions of charge (potentials). Thus a selectivity coefficient of -1 indicates that a potential associated with a given [NH_4^+] will also be produced by a 10-fold higher concentration of K^+. Positive selectivity coefficients mean the ionophore is more selective for the interfering ion than the principle ion. A previous study has determined selectivity coefficients for the ionophore TD19C6 by utilizing macroelectrodes (Suzuki et al., 2000). Coefficients are not always comparable between macro- and micro-electrodes, thus we measured selectivity coefficients for both the ammonium and potassium-selective micro-electrodes used in our experiments (Fig. 1A and 1B). Potential interfering ions of similar charge to those of NH_4^+ and K^+ were chosen along with ions found commonly in biological fluids. Of the interfering ions on the ammonium microelectrode, potassium was the most significant with a selectivity coefficient of – 0.94 (Fig. 1A). Although methylammonium interference was greater (-0.63), levels of this interfering ion are negligible in biological fluids. Shown for comparison, in the right column, are the selectivity coefficients for interfering ions using a cocktail based on the classical NH_4^+-selective ionophore nonactin (Fresser et al., 1991). In particular, the selectivity coefficients for both potassium (-0.6) and sodium (-2.0) were higher than our values. Sodium, highly represented in extracellular fluids, does not interfere (-3.58) with our ammonium microelectrode to any significant degree. In general, physiological ions interfered less with the potassium-selective microelectrode (Fig 1B). Selectivity coefficients of the potassium microelectrode have been determined previously and are presented in the right column of Fig. 1B (Ammann et al., 1987). Ammonium interference was by far the most prevalent of the

Figure 1. Selectivity coefficients, ($\log_{10}$) of the (A) ammonium and (B) potassium-selective microelectrodes. Values were obtained by the separate solutions method using pure 0.1 M chloride salt solutions of the principal and interfering ions. Membrane compositions of each microelectrode are listed below their respective columns. For comparison, selectivity coefficients obtained by other groups are presented in the right column (Ammann et al., 1987, Fresser et al., 1991). KtktFlMePB, potassium tetrakis[3,5-bis-(trifluoromethyl) phenyl] borate; KtkClPB, Potassium tetrakis(4-chlorophenyl)borate; BBPA, bis-(1-butylpentyl) adipate; NPOE, 2-Nitrophenyl octyl ether; DMNB, 1,2-Dimethyl-3-nitrobenzene.

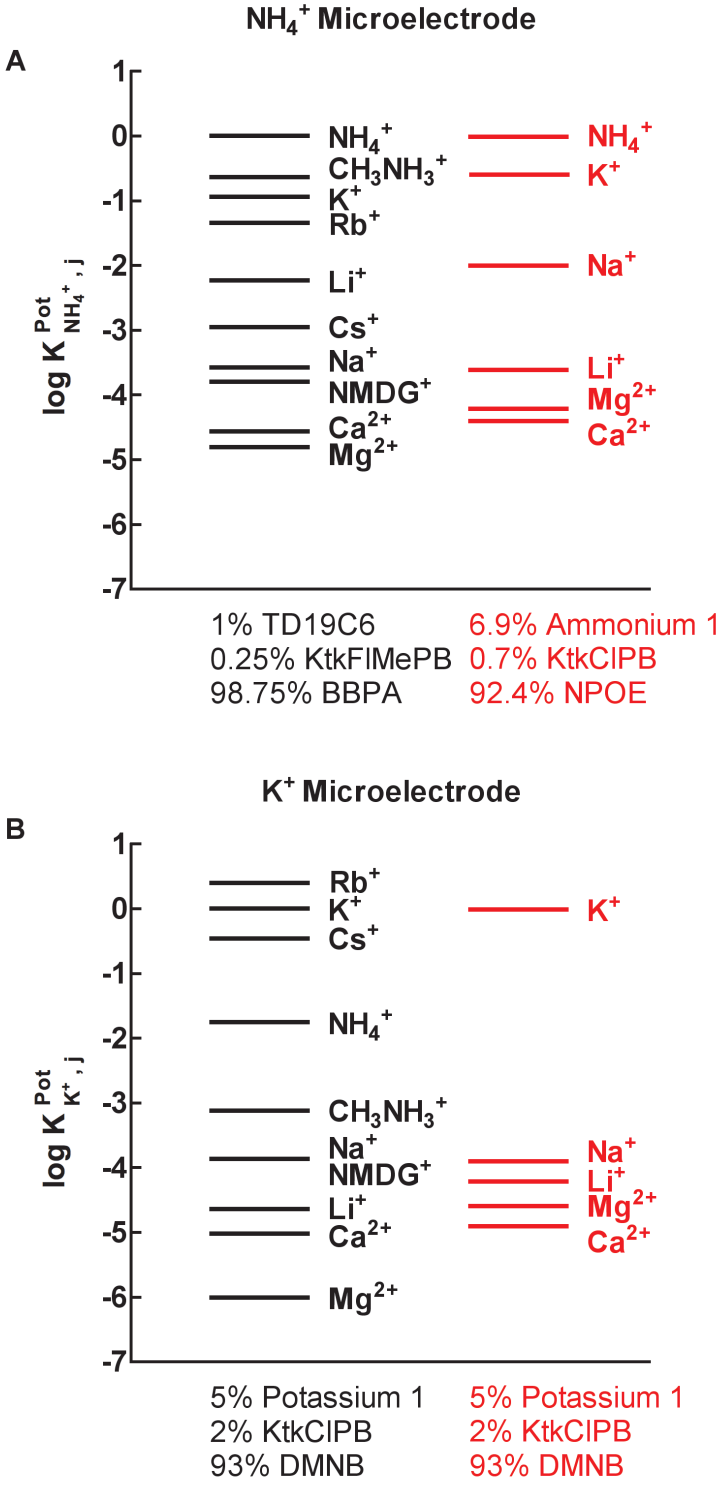
NH4+ Microelectrode
A
log K Pot NH4+, j
1
0
-1
-2
-3
-4
-5
-6
-7
NH4+
CH3NH3+
K+
Rb+
Li+
Cs+
Na+
NMDG+
Ca2+
Mg2+
NH4+
K+
Na+
Li+
Mg2+
Ca2+
1% TD19C6
0.25% KtkFlMePB
98.75% BBPA
6.9% Ammonium 1
0.7% KtkClPB
92.4% NPOE
K+ Microelectrode
B
log K Pot K+, j
1
0
-1
-2
-3
-4
-5
-6
-7
Rb+
K+
Cs+
NH4+
CH3NH3+
Na+
NMDG+
Li+
Ca2+
Mg2+
K+
Na+
Li+
Mg2+
Ca2+
5% Potassium 1
2% KtkClPB
93% DMNB
5% Potassium 1
2% KtkClPB
93% DMNB

Figure 1.

physiological ions in our potassium microelectrode, with a selectivity coefficient of -1.76 shown in the left column (Fig. 1B). As a result, interference due to equimolar ammonium would increase potassium measurements above the true value by 1.74%. In summary, these results suggested that the ammonium microelectrode suffered from significant potassium interference, whereas the potassium electrode is highly selective.

Nernstian slopes of the ammonium-selective microelectrode were evaluated in various solutions in order to determine the conditions in which ammonium could be successfully measured (Table 1). Note that a potential methylammonium-selective microelectrode based on a Cs II ionophore was also evaluated. The microelectrode suffered from significant sodium and potassium interference and was deemed unsuitable for use in physiological solutions (Appendix 1, Figure A1). The theoretical difference in potentials observed between two solutions differing by 10-fold in activity is 2.303 RT/zF, or 59 mV at 23 ^{o}C (Ammann, 1986). Also known as the Nernstian slope, or simply slope, of an electrode is a measure of the sensitivity of an electrode. The sensitivity of an electrode can be reduced in the presence of interfering ions thus the reduction in sensitivity gives empirical estimates of the magnitude of interference. Simply put, the more the slope diminishes from the ideal 59 mV/decade the more interference the electrode has detected. The slope becomes reduced due to additive interference, which disproportionately elevates the signal from the lower activity solution decreasing the slope between the two solutions. In addition, by using several serial dilutions, the lower limit of detection can be estimated. Analysis of the literature indicates that slopes of $\geq$ 50 mV/decade are acceptable for monovalent ions. Using microelectrodes based on 1%

Table 1. Nernstian slopes of microelectrodes based on 3% TD19C6, 1% TD19C6 and 1% Cs II in serial dilutions of 10 mM NH_4^+ containing various background levels of salt solutions. The standard error (SE), number of samples (n) and number of microelectrodes used (Electrode $_n$) are presented in columns 5 through 7. In the description of the background solutions, the term 'Saline' refers to *Drosophila* saline, while all other solutions were made with deionized water.

Table 1.

Ionophore	[Background Soln]	[Range]	Slope (mV)	SE	n	Electrode n
3% TD19C6	150 mM NaCl	10 - 1	-55.79	0.29	48	12
		1 - 0.1	-49.28	0.39	63	12
1% TD19C6	150 mM NaCl	10 - 1	-56.93	0.46	17	11
		1 - 0.1	-51.13	0.83	11	9
		0.1 - 0.01	-29.69	2.90	5	5
1% TD19C6	0 mM KCl Saline	10 - 1	-58.21	0.85	3	3
		1 - 0.1	-52.58	1.37	3	3
		0.1 - 0.01	-25.54	0.75	3	3
1% TD19C6	2 mM KCl Saline	10 - 1	-50.00	1.75	3	3
		1 - 0.1	-30.53	1.23	3	3
		0.1 - 0.01	-7.80	0.71	3	3
1% TD19C6	5 mM KCl Saline	10 - 1	-48.76	2.10	3	3
		1 - 0.1	-22.55	0.72	3	3
		0.1 - 0.01	-4.71	0.28	3	3
1% TD19C6	10 mM KCl Saline	10 - 1	-49.52	4.12	3	3
		1 - 0.1	-25.91	5.96	3	3
		0.1 - 0.01	-7.56	1.52	3	3
1% TD19C6	20 mM KCl Saline	10 - 1	-32.62	1.64	3	3
		1 - 0.1	-7.35	1.15	3	3
		0.1 - 0.01	-1.04	0.48	3	3
1% Cs II	150 mM NaCl	10 - 1	-32.12	12.64	2	1
1% Cs II	15 mM NaCl	10 - 1	-53.14		1	1
		1 - 0.1	-40.10		1	1
		0.1 - 0.01	-15.31		1	1
1% Cs II	100 mM LiCl	10 - 1	-56.28		1	1
		1 - 0.1	-46.20		1	1
		0.1 - 0.01	-16.44		1	1
1% Cs II	0 mM KCl Saline	10 - 1	-45.01		1	1
1% Cs II	2 mM KCl Saline	10 - 1	-38.50		1	1

TD19C6, slopes ≥ 50 mV decade^{-1} were observed during ammonium detection down to 0.1 mM in the presence of 150 mM NaCl (Table 1). In *Drosophila* saline containing up to 2 mM K^+, an acceptable slope was observed down to 1 mM NH_4^+. In K^+-free *Drosophila* saline, concentrations of $NH_4^+ \geq 0.1$ mM could be resolved. In general, increasing concentrations of K^+ in *Drosophila* saline of up to 20 mM progressively reduced the slope of the ammonium microelectrode. In combination, these results indicate that the ammonium microelectrode has significant potassium interference, with detection limited to 1 mM in the presence of *Drosophila* saline containing 2 mM K^+.

16.3 Potassium Interference and Correction

The previous experiments suggested that ammonium could be detected down to 0.1 mM in the absence of potassium, with only slight increases in potassium (by 2 mM) reduced detection to approximately 1 mM ammonium. Our goal was ultimately to measure ammonium in haemolymph and fluid secreted by the Malpighian tubules, in which we would expect to find about 20 and 120 mM potassium, respectively (Naikkhwah and O'Donnell, 2011). In order to address the issue of potassium interference, we opted to attempt to correct for potassium interference rather than perform experiments under potassium-free conditions. Since we expected potassium concentrations to vary in the biological fluids in which we wished to measure ammonium, either among ammonium treatments or between sampled individuals, the magnitude of the interference had to be determined rather than attempting to keep potassium interference at a constant level. A potassium-selective microelectrode, described previously, was employed to precisely determine the concentration of potassium in solutions where ammonium would also be measured. With known potassium concentrations, the amount of potassium interference could be

corrected by simply subtracting the apparent ammonium concentration from the product of the potassium concentration and the antilog of the potassium selectivity coefficient (-0.94) previously determined. For example, an apparent $[NH_4^+]$ of 7 mM in a solution that also contained 17.5 mM K^+ would have an actual $[NH_4^+]$ of: $7 - (17.5)(10^{-0.94}) = 5$ mM. Hereafter, $[NH_4^+]$ free of potassium interference is referred to as corrected $[NH_4^+]$ and $[NH_4^+]$ that includes potassium interference is referred to as uncorrected $[NH_4^+]$. With the method for correcting for potassium interference established we first wanted to test the accuracy of it.

In order to investigate the accuracy of the method for correcting for potassium interference, ammonium was measured in calibration solutions containing increasing concentrations of NH_4^+ with a constant background of 100 mM potassium (Fig 2A and 2B). The mean absolute error ($[NH_4^+]_{Measured} - [NH_4^+]_{Known}$) across all NH_4^+ concentrations prior to correction was 11.94 mM, while following potassium correction it was 0.63 mM. Furthermore, prior to potassium correction all measured values were significantly different from known values, while following potassium correction all values were not significantly different from known values. Thus the potassium correction dramatically improved the accuracy of ammonium detection in the presence of 100 mM K^+. As expected, potassium concentrations were not significantly different across the test solutions but there was a decreasing trend with increasing $[NH_4^+]$. Possible reasons for this phenomenon will be discussed below. In conclusion, these results indicate that the method for correcting for potassium interference dramatically reduces the error in ammonium detection.

Figure 2. The accuracy of ammonium detection (A) prior to and (B) following correction for potassium interference. Ion concentrations measured with microelectrodes are shown on the y-axis, while the NH_4Cl composition of calibration solutions is shown on the x-axis. Dotted lines represent K^+ concentration of the solutions, while solid red lines indicate the NH_4^+ concentration of the particular experimental solution. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. A potassium selectivity coefficient of -0.94 was used to correct for potassium interference. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=2 NH_4^+ N=8-10).

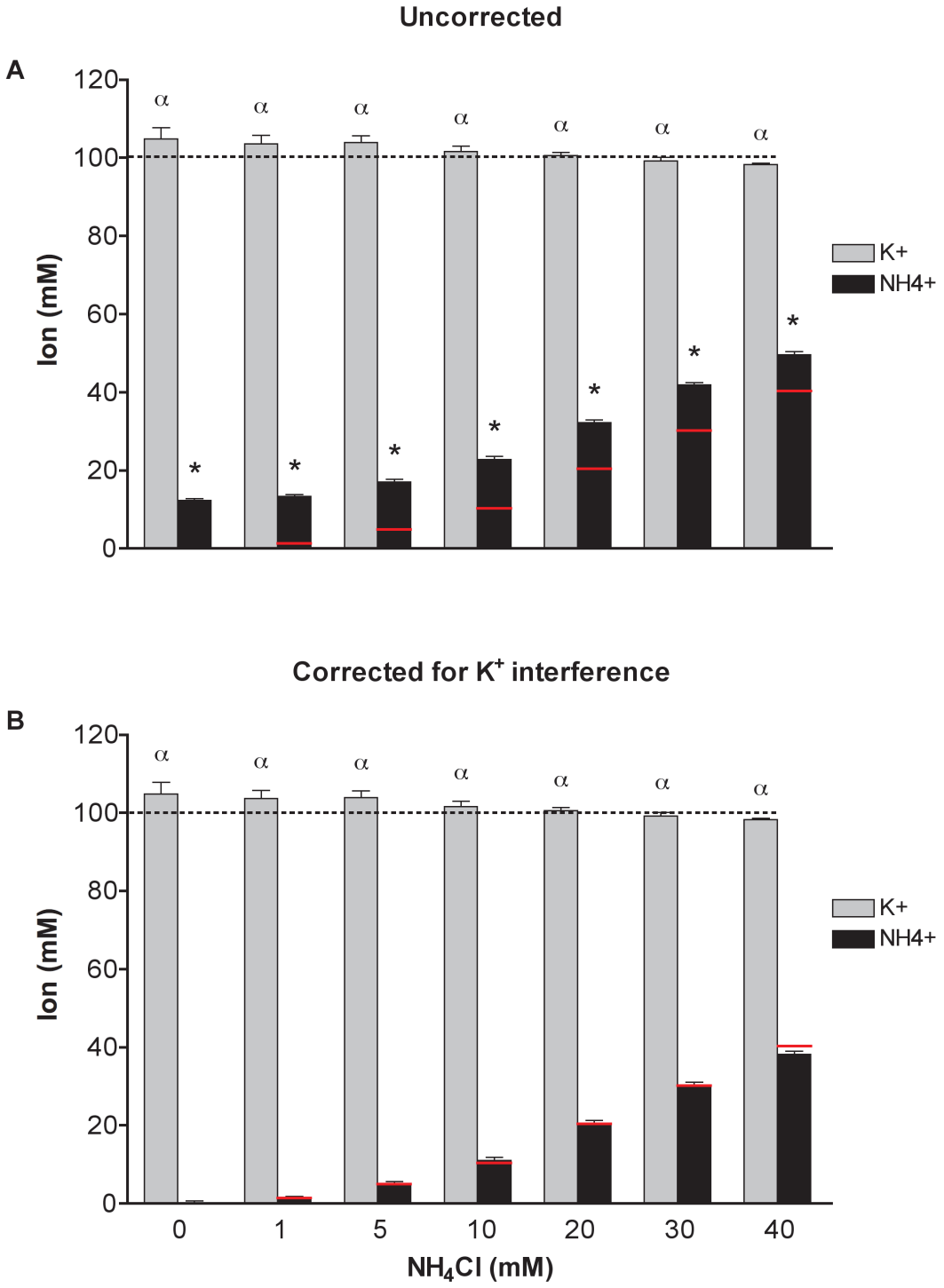
Uncorrected
A
Ion (mM)
120
100
80
60
40
20
0
α
*
K+
NH4+
Corrected for K+ interference
B
Ion (mM)
0
1
5
10
20
30
40
NH4Cl (mM)

Figure 2.

Since the potassium correction resulted in accurate measurements of ammonium in the presence of 100 mM potassium, background potassium levels were increased to 150 mM K^+, a level expected to be the maximum found in fluid secreted by insect Malpighian tubules (Maddrell and Klunsuwan, 1973). The rationale was that if NH_4^+ could be measured under these extreme conditions, then conditions with reduced potassium and therefore reduced interference would only improve the accuracy of ammonium detection. Unfortunately, when the background potassium concentration was elevated to 150 mM K^+, a consistent absolute error of about 3 mM NH_4^+ lower than expected was found across all the ammonia test solutions (Fig. 3A). As a result, corrected ammonium concentrations for the NH_4^+-free and 1 mM test solutions were found have negative values. Conversely, potassium measurements had greater variability as 0 and 10 mM NH_4^+ treatments were significantly higher than all other treatments. Still, all potassium concentrations were within 6 mM K^+ of the 150 mM K^+ known to be in the test solutions, which represents an error of 4%. Negative corrected values for ammonium concentrations indicated that the magnitude of the potassium correction was too large. When the selectivity coefficient for potassium was empirically adjusted to -0.99, rather than the -0.94 observed using the separate solutions method, the mean error was reduced to 0.74 mM from the previous 3 mM NH_4^+ (Fig. 3B). In addition, the empirical adjustment of the potassium selectivity coefficient abolished all significant differences between measured and known values observed prior adjustment. Absolute error on the measurements at 1 mM NH_4Cl improved from 2.15 mM to 0.28 mM NH_4^+ following the adjustment as well, suggesting the detection limit of the ammonium microelectrode was also improved. These findings led us to believe that the accuracy of ammonium detection was indeed perturbed by increased potassium concentrations, contrary to our initial findings in 100 mM K^+ that were very promising. In addition, by empirically adjusting the log $K_{NH4,K}$ the accuracy of ammonium detection could be recovered. Since the empirical adjustment improved the accuracy

Figure 3. Ammonium detection in solutions containing 150 mM K^+ and the effect of empirically adjusting the potassium selectivity coefficient from (A) -0.94 to (B) -0.99. Ion concentrations measured with microelectrodes are shown on the y-axis, while the NH_4^+ composition of calibration solutions is shown on the x-axis. Dotted lines represent K^+ concentration of the solutions, while solid red lines indicate the NH_4^+ concentration of the particular experimental solution. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=5 NH_4^+ N=10).

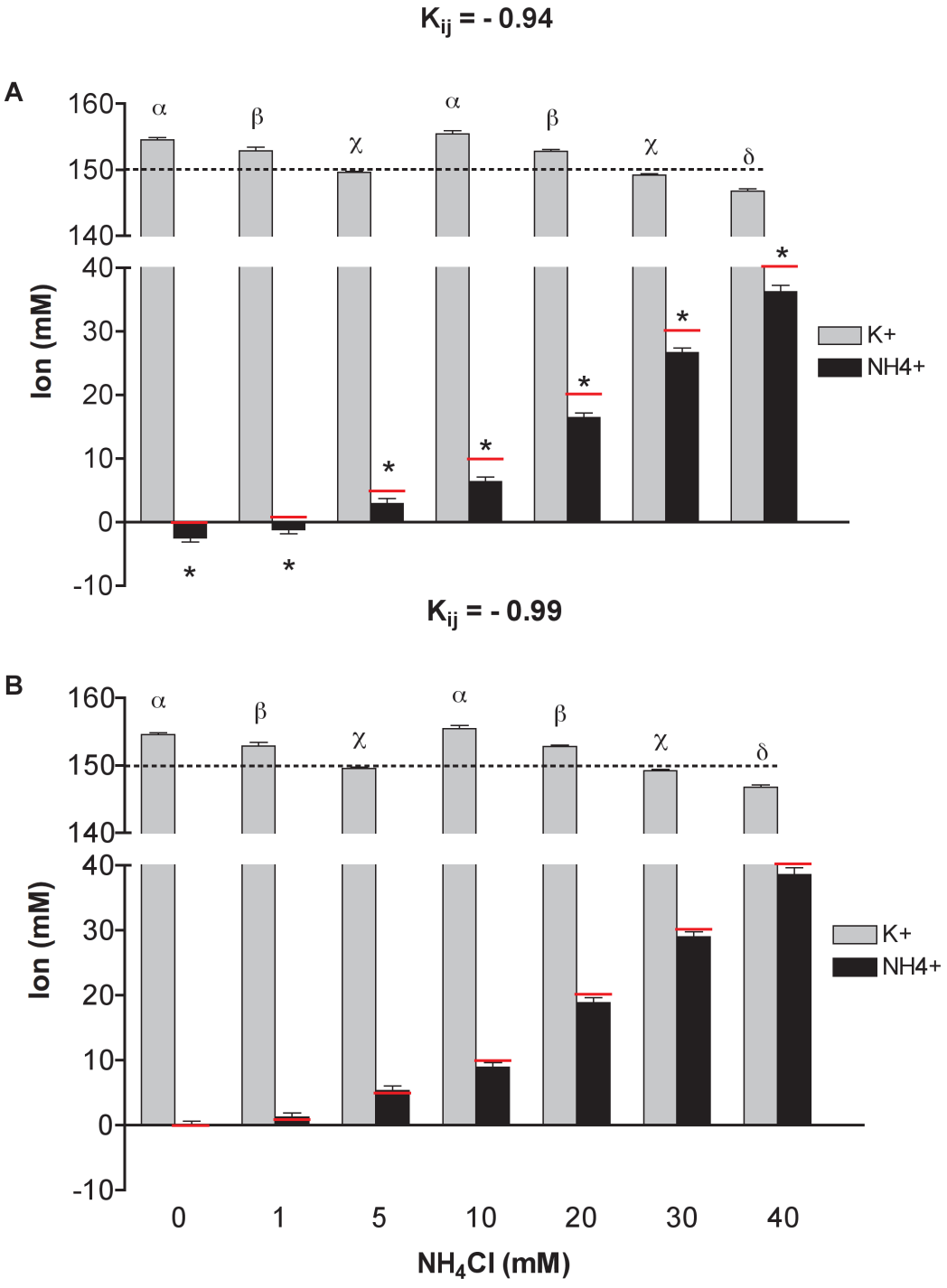
K_{ij} = - 0.94
A
160
150
140
α
β
χ
α
β
χ
δ
40
30
20
10
0
-10
Ion (mM)
*
K+
NH4+
K_{ij} = - 0.99
B
0
1
5
10
20
30
40
NH_4Cl (mM)

Figure 3.

of ammonium detection, this key finding suggested that adjusting/determining the log $K_{NH4,K}$ at each ammonium concentration tested (ie using multiple log $K_{NH4,K}$ values rather than a single value) would improve the accuracy of ammonium detection across all ammonium concentrations on an individual basis. Essentially, we had modified the fixed interference method (FIM), whereby selectivity coefficients are calculated in the presence of a fixed amount of interference rather than in pure solutions. Hereafter, the method used in this paper we will referred to as the modified mixed solution method (MMSM). A more thorough presentation of the calculations involved in the MMSM, including an example, can be found in Appendix 2.

16.4 Calibration in Haemolymph-like and Secreted Fluid-like Solutions

Solutions used in the MMSM experiment were designed to reflect the potassium and sodium content of larval *Drosophila* haemolymph and fluid secreted by Malpighian tubules. The haemolymph of 3rd instar *Drosophila* larvae was sampled and found to contain 26 mM K^+ and 35 mM Na^+ (Fig. 4A). In order to determine physiological levels of potassium and sodium in the secreted fluid, Malpighian tubules were bathed in 'haemolymph-like' *Drosophila* saline based on the results of the previous experiment. Haemolymph-like saline contained 25 mM K^+ and 40 mM Na^+ across all ammonium concentrations (Naikkhwah and O'Donnell, 2011). In order to maintain ionic strength as well as potassium and sodium concentrations, equivalent amounts of NH_4Cl were substituted with N-Methyl-D-glucamine chloride ($NMDG^+$) rather than NaCl. In addition to potassium, sodium was kept constant because it was thought that the high levels of sodium (117.5 mM) found in the standard *Drosophila* saline might have influenced ammonium secretion, considering the physiological level of sodium in the haemolymph determined in our

Figure 4. Na^+ and K^+ concentrations in (A) haemolymph and (B) secreted fluid of 3rd instar *Drosophila* larvae. Secreted fluid was sampled from Malpighian tubules bathed in *Drosophila* saline containing 25 mM KCl and 40 mM NaCl. Both measurements were taken from isolated samples submerged under paraffin oil. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (unpaired, *t*-test, $P< 0.05$, (A) $N=20$ (B) $N=11$-15).

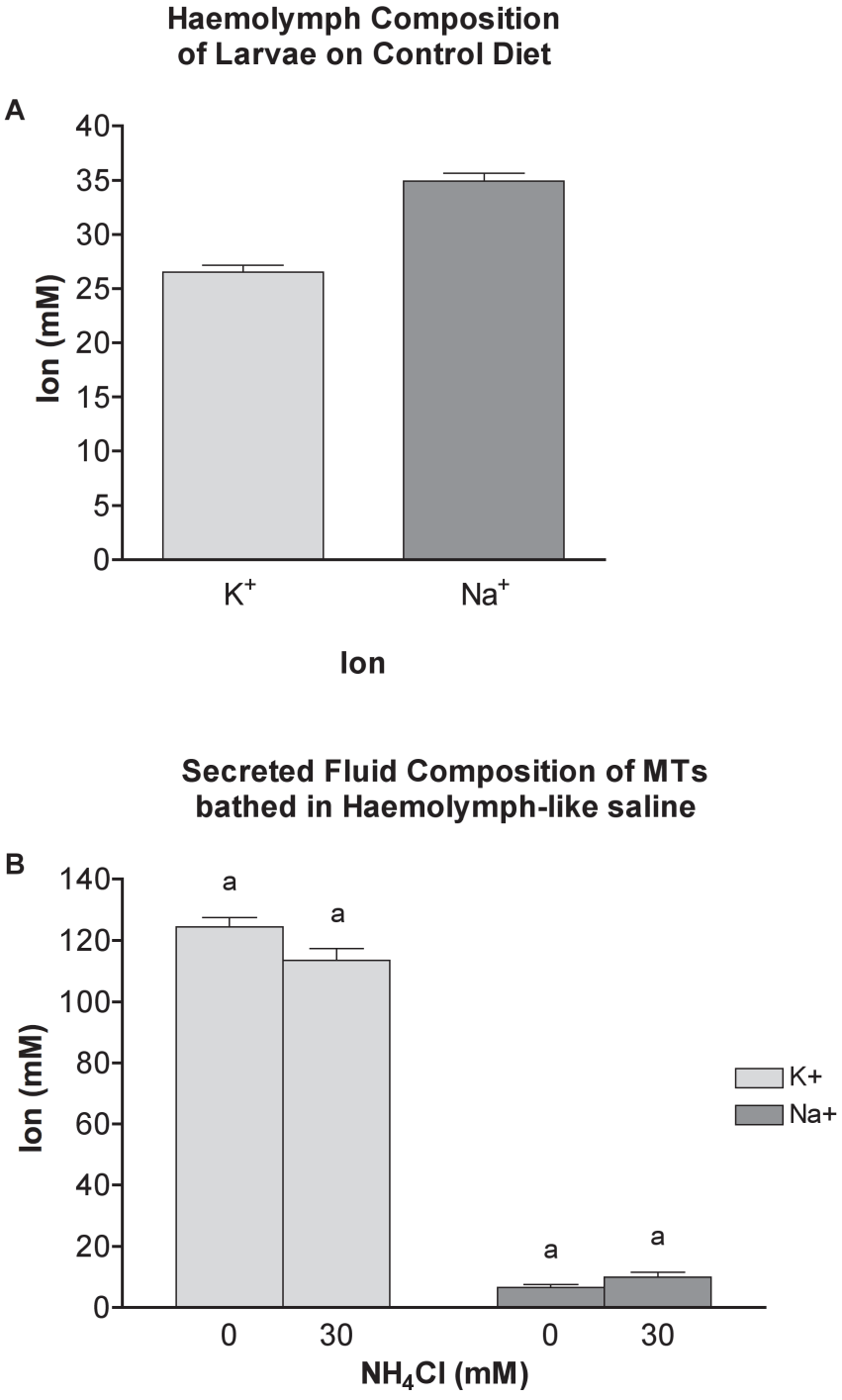
Haemolymph Composition
of Larvae on Control Diet
A
Ion (mM)
40
35
30
25
20
15
10
5
0
K+
Na+
Ion
Secreted Fluid Composition of MTs
bathed in Haemolymph-like saline
B
Ion (mM)
140
120
100
80
60
40
20
0
a
a
a
a
K+
Na+
0
30
0
30
NH4Cl (mM)

Figure 4.

experiments was only 36 mM. A subsequent experiment comparing $NMDG^+$ and NaCl-substituted *Drosophila* salines found no significant difference in secretory rate or composition (Appendices 3A and 3B). In this case, $NMDG^+$ was used and isolated Malpighian tubules bathed in haemolymph-like *Drosophila* saline containing 0 and 30 mM NH_4^+, secreted fluid that contained a mean 119 mM K^+ and 8.3 mM Na^+ (Fig. 4B). In contrast to the saline, both haemolymph-like and secreted fluid-like solutions were substituted with NaCl rather than $NMDG^+$ since no biological tissues were involved and a simpler solution could be used with NaCl-substitution as $NMDG^+$ requires buffering and titration. As a result, haemolymph-like solutions contained 25 mM KCl and 40 to 60 mM NaCl, while secreted fluid-like solutions contained 120 mM KCl and 10-50 mM NaCl.

In the haemolymph-like and secreted fluid-like solutions, log $K_{NH4,K}$ were calculated at each ammonium concentration using the MMSM (Fig. 5A and 5B). From the resulting standard curves, the equation of a linear regression of the means was found to be $y = -0.0053x – 0.956$ for measurements in haemolymph-like solutions and $y = -0.0027x – 0.994$ in secreted fluid-like solutions. In both linear regressions (r^2 values ≥ 0.96) log $K_{NH4,K}$ values significantly declined with increasing ammonium concentrations. An explanation as to why the log $K_{NH4,K}$ values are not constant will be discussed below. The results from the standard curves indicated that potassium selectivity coefficients vary with [NH_4Cl] thus a single selectivity coefficient is not ideally suited to our experiments involving increasing concentration of ammonia.

Figure 5. Empirical adjustment of potassium selectivity coefficients ($\log_{10}$) determined with the ammonium-selective microelectrode in (A) haemolymph-like and (B) secreted fluid-like calibration solutions. Values were obtain using a fixed interference method. Correlation coefficients, p-values and equations of the linear regression of the means are shown on the figure.

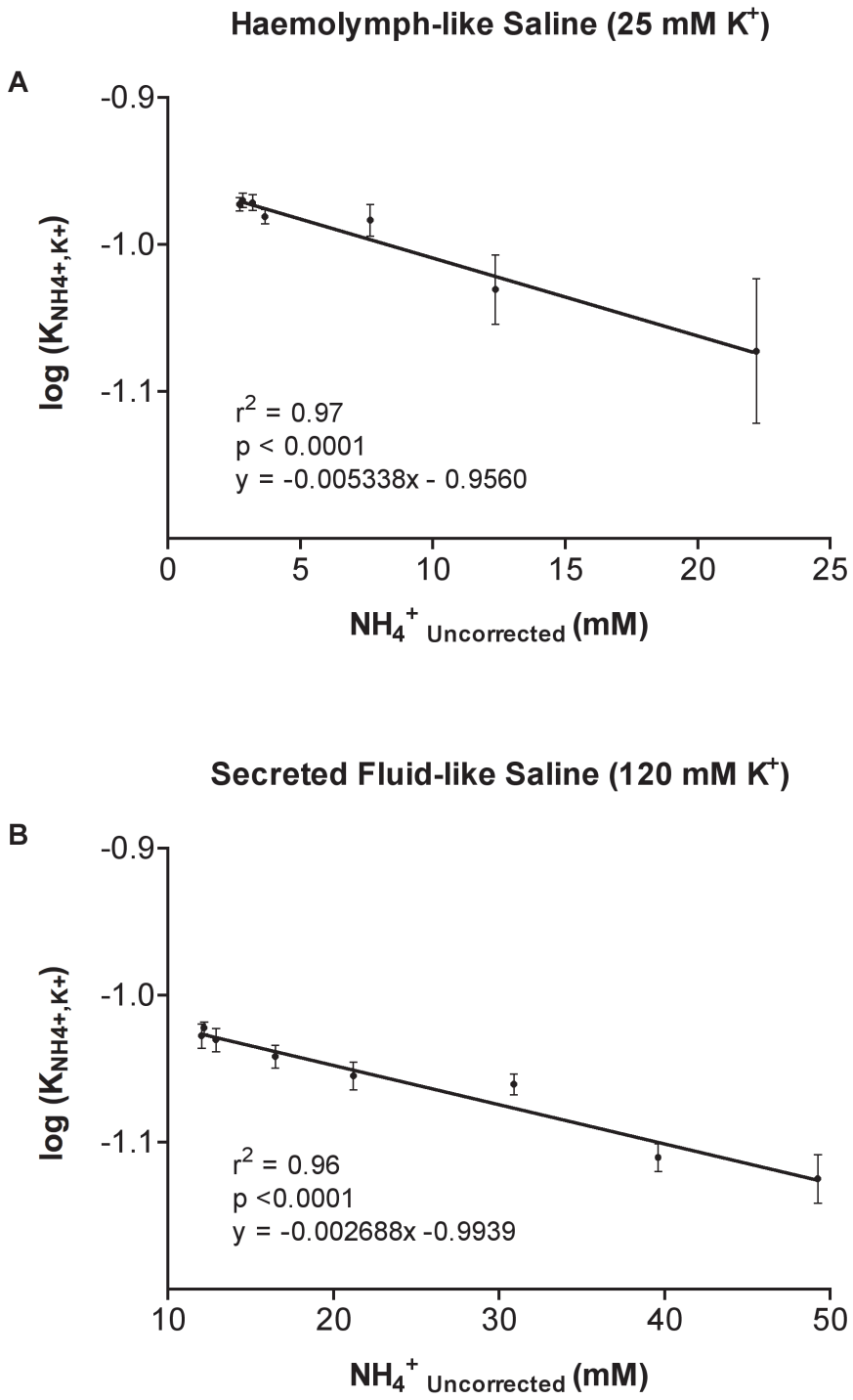
Haemolymph-like Saline (25 mM K+)
A
-0.9
-1.0
-1.1
log (K NH4+,K+)
r2 = 0.97
p < 0.0001
y = -0.005338x - 0.9560
0
5
10
15
20
25
NH4+ Uncorrected (mM)
Secreted Fluid-like Saline (120 mM K+)
B
-0.9
-1.0
-1.1
log (K NH4+,K+)
r2 = 0.96
p <0.0001
y = -0.002688x -0.9939
10
20
30
40
50
NH4+ Uncorrected (mM)

Figure 5.

In another set of calibration solutions, potassium and ammonium concentrations were measured and the log $K_{NH4,K}$ standard curves were used to predict the appropriate log $K_{NH4,K}$ to correct for potassium interference in each solution. In haemolymph-like solutions, the mean error across all ammonium concentrations was 0.6 mM (Fig. 6/Table 2). With the exception of the 10 mM NH_4^+ treatment, all measured ammonium values were significantly different from predicted values.

Although the values diverged from predicted levels the absolute errors were ≤ 1 mM NH_4^+ in all test solutions. On the other hand, potassium concentrations were stable at the desired level across ammonia treatments. In secreted fluid-like solutions, the mean error was 0.2 mM across all ammonium concentrations (Fig. 7/Table 3). All ammonium values were not significantly different from predicted values, suggesting ammonium measurement in secreted fluid-like solutions is accurate, even in the presence of high concentrations of potassium. Unlike haemolymph-like solutions, potassium concentrations were found to significantly decrease in solutions containing 30 and 40 mM NH_4^+ from solutions containing ≤ 10 mM NH_4^+. The errors were not large, with all mean potassium concentrations within 4.9 mM of the desired 120 mM K^+. Note the potassium correction (difference between uncorrected and corrected ammonium concentrations) was smaller in 25 mM K^+ solutions (2.5 mM) than in 120 mM K^+ solutions (11 mM). Together, these results suggest that in haemolymph-like solutions (25 K^+) ammonium measurement is quite accurate, with an error of 0.5 mM at 1 mM NH_4^+, even though the measurements were significantly different than known levels of the test solutions. In secreted fluid (120 K^+) ammonium can be detected down to 1 mM within 0.1 mM even though the potassium interference was much larger.

Figure 6. Measured versus predicted [NH_4^+] in solutions containing 25 mM KCl. Ion concentrations measured with microelectrodes are shown on the y-axis, and the NH_4^+ concentrations of the calibration solutions are shown on the x-axis. Dotted lines represent KCl concentration of the solutions, while solid red lines indicate the NH_4^+ concentration of the particular experimental solution. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0053x – 0.956, where x represents uncorrected ammonium concentrations. Specific key values are presented in **table 2**. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=6 NH_4^+ N=6).

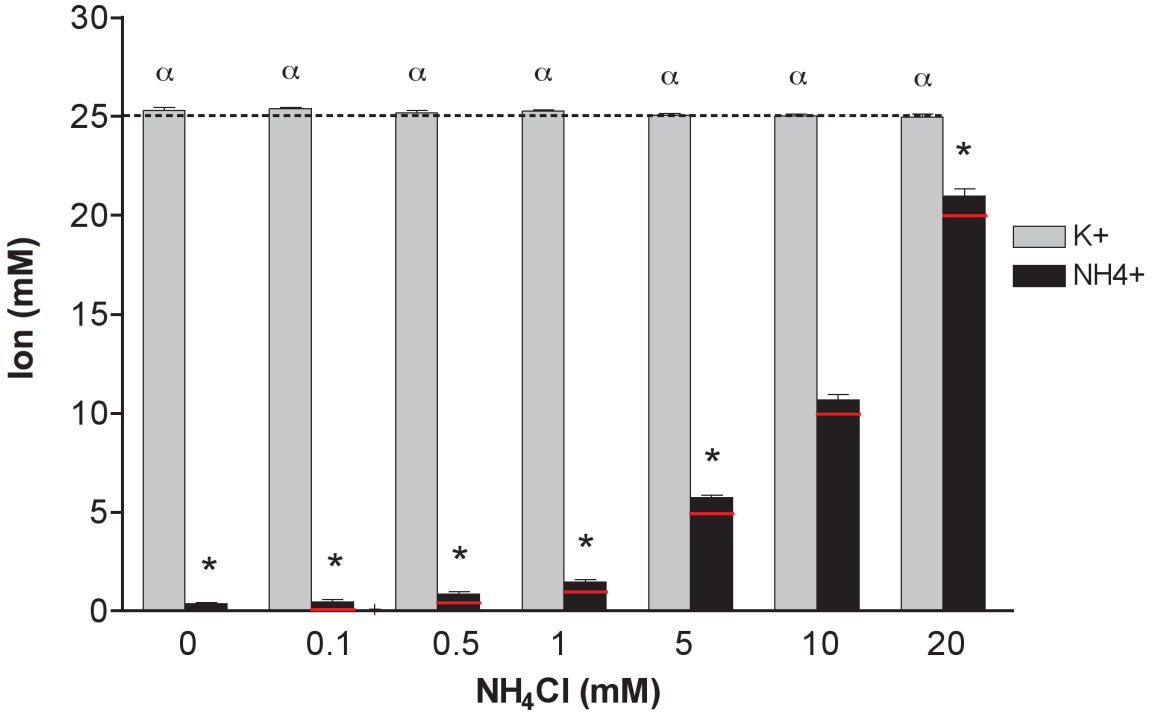

Figure 6.

Table 2.

Haemolymph-like Solutions (25 mM K^+)

	Bathing NH_4Cl (mM)						
	0	0.1	0.5	1	5	10	20
Measured [NH_4^+]							
Mean	0.3	0.5	0.8	1.5	5.7	10.7	21.0
Standard Error	0.1	0.1	0.1	0.1	0.1	0.3	0.4
Absolute Error	0.3	0.4	0.3	0.5	0.7	0.7	1.0
K^+ Correction	2.7	2.7	2.7	2.7	2.5	2.4	2.1

Figure 7. Measured versus predicted [NH_4^+] in solutions containing 120 mM KCl. Ion concentrations measured with microelectrodes are shown on the y-axis, and the NH_4^+ concentrations of the calibration solutions are shown on the x-axis. Dotted lines represent KCl concentration of the solutions, while solid red lines indicate the NH_4^+ concentration of the particular experimental solution. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0027x – 0.994, where x represents uncorrected ammonium concentrations. Specific key values are presented in **table 3**. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=5-12 NH_4^+ N=5-12).

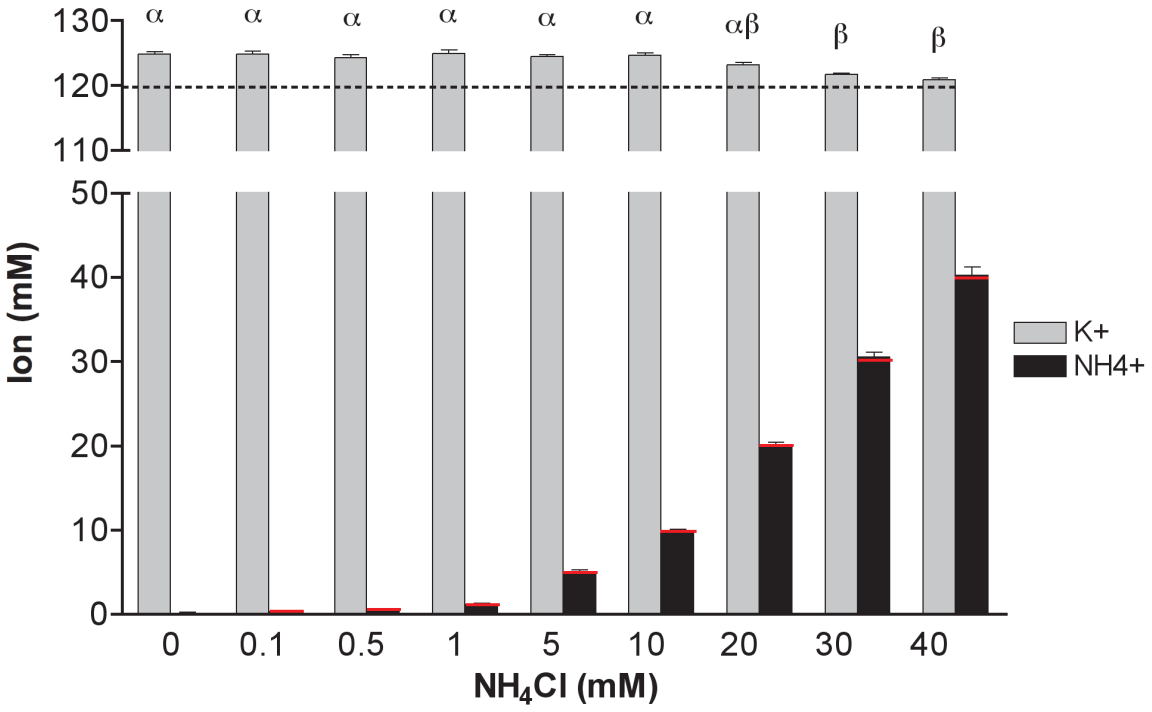

Figure 7.

Table 3.

Secreted Fluid-like Solutions (120 mM K^+)

	Bathing NH_4Cl (mM)								
	0	0.1	0.5	1	5	10	20	30	40
Measured [NH_4^+]									
Mean	0.1	0.3	0.5	1.1	5.1	9.7	20.0	30.5	40.2
Standard Error	0.1	0.1	0.1	0.1	0.2	0.3	0.4	0.6	1.0
Absolute Error	0.1	0.2	0.0	0.1	0.1	0.3	0.0	0.5	0.2
K^+ Correction	11.6	11.8	11.8	11.8	11.5	11.1	10.4	9.6	9.0
n	12	12	12	12	12	5	5	5	5

Fluid secreted by the Malpighian tubules is a much simpler solution compared to insect haemolymph with secreted fluid consisting of mostly sodium, potassium and chloride (Linton and O'Donnell, 1999). Thus the effect of potassium variation on the accuracy of ammonium detection was further examined in solutions resembling secreted fluid. Potassium content of secreted fluid can vary among insects from about 100 to 140 mM (Maddrell and Klunsuwan, 1973). To complement the measurements in solutions containing 120 mM KCl, we measured ammonium in solutions containing 100 and 140 mM KCl. Ammonium detection from 0 to 5 mM NH_4^+ in the presence of 100 mM KCl had a similar mean absolute error of 0.3 mM NH_4^+ compared to the 0.1 mM NH_4^+ error observed in 120 mM KCl (Fig. 8/Table 4). Ammonium concentrations were significantly different from predicted values for all ammonium concentrations tested, although the differences were small (absolute errors of 0.3 to 0.4 mM NH_4^+). On the other hand, potassium measurements were consistent yet elevated by about 5 mM K^+ from the 100 mM KCl known to be in the solutions. An increase in the background potassium concentration to 140 mM resulted in a mean absolute error of 0.1 mM NH_4^+, an improvement from the 0.2 mM NH_4^+ observed in solutions containing 120 mM KCl (Fig. 9/Table 5). No significant differences between measured and predicted ammonium concentrations were observed and detection down to 1 mM NH_4^+ was within 0.2 mM NH_4^+. When comparing 100, 120 and 140 mM KCl experiments, the results indicated that when using the MMSM, the error of ammonium detection varied from 0.1 to 0.3 mM at 1 mM ammonium. In other words, when using the MMSM, variation in potassium from 100 to 140 mM had little effect on the accuracy of ammonium detection. It was concluded that the novel ammonium microelectrode had the ability to detect ammonium within 0.3 mM down to about 1 mM NH_4^+ in the presence of 100 to 140 mM KCl.

Figure 8. Measured versus predicted [NH_4^+] in solutions containing 100 mM KCl. Ion concentrations measured with microelectrodes are shown on the y-axis, and the NH_4^+ concentrations of the calibration solutions are shown on the x-axis. Dotted lines represent KCl concentration of the solutions, while solid red lines indicate the NH_4^+ concentration of the particular experimental solution. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0027x – 0.994, where x represents uncorrected ammonium concentrations. Specific key values are presented in **table 4**. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=7 NH_4^+ N=7).

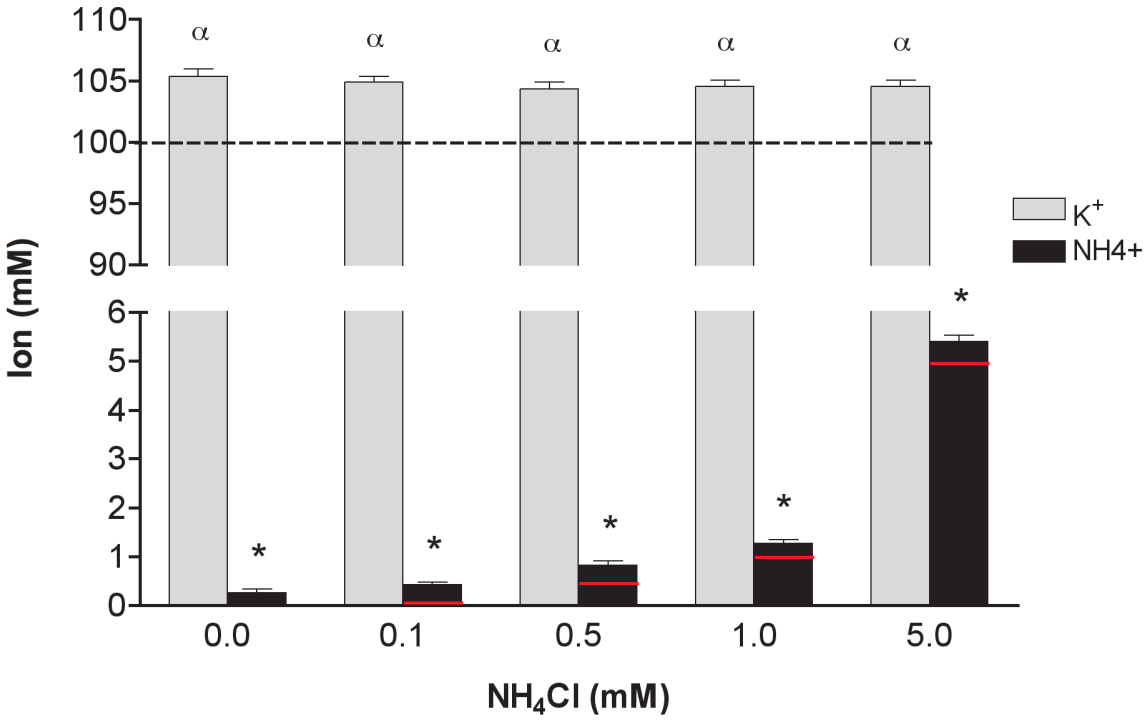

Figure 8.

Table 4.

Secreted Fluid-like Solutions (100 mM K^+)

	Bathing NH_4Cl (mM)				
	0	**0.1**	**0.5**	**1**	**5**
Measured [NH_4^+]					
Mean	0.3	0.4	0.8	1.3	5.4
Standard Error	0.1	0.1	0.1	0.1	0.1
Absolute Error	0.3	0.3	0.3	0.3	0.4

Figure 9. Measured versus predicted [NH_4^+] in solutions containing 140 mM KCl. Ion concentrations measured with microelectrodes are shown on the y-axis, and the NH_4^+ concentrations of the calibration solutions are shown on the x-axis. Dotted lines represent KCl concentration of the solutions, while solid red lines indicate the NH_4^+ concentration of the particular experimental solution. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0027x – 0.994, where x represents uncorrected ammonium concentrations. Specific key values are presented in **table 5**. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=7 NH_4^+ N=7).

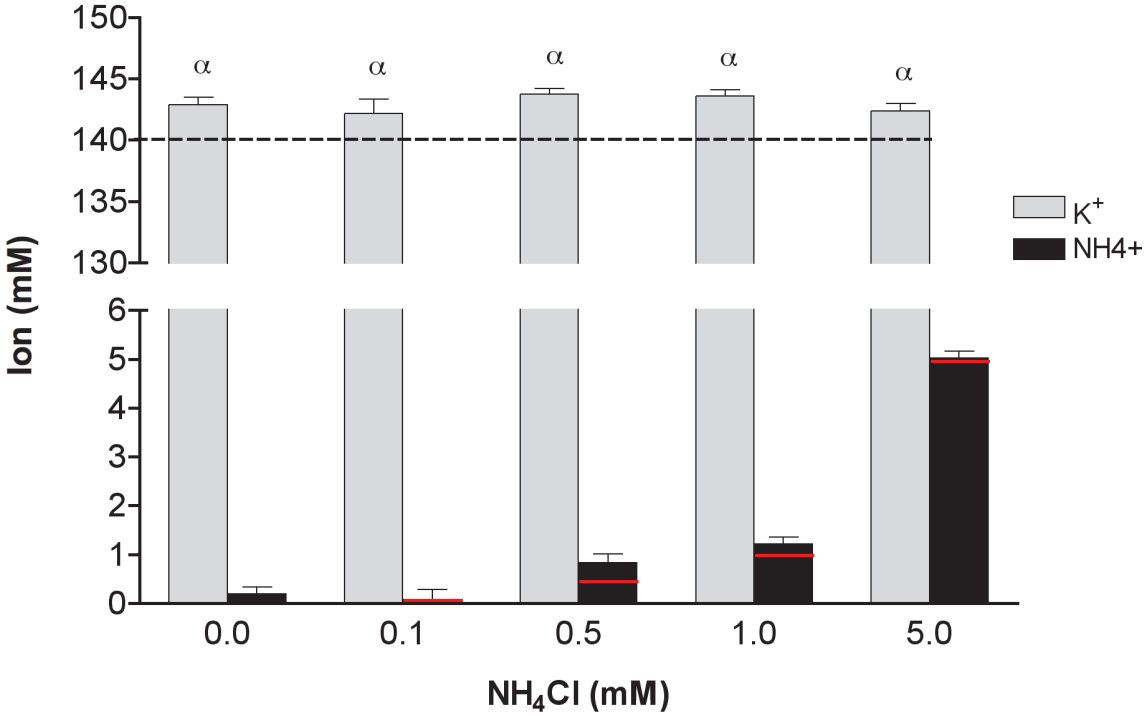

Figure 9.

Table 5.

Secreted Fluid-like Solutions (140 mM K^+)

	Bathing NH_4Cl (mM)				
	0	**0.1**	**0.5**	**1**	**5**
Measured [NH_4^+]					
Mean	0.2	0.1	0.8	1.2	5.0
Standard Error	0.1	0.2	0.2	0.1	0.1
Absolute Error	0.2	0.0	0.3	0.2	0.0

17 Application of the NH_4^+-selective Microelectrode

17.1 Measurement of NH_4^+ in Dietary Media – Effect of Dietary Ammonia

Other groups have observed temporal increases in ammonia concentrations within the diet of cultured *Drosophila* (Borash et al., 1998). Thus we hypothesized that because *Drosophila* experience high environmental ammonia (HEA) they would have the ability to secrete ammonia, via their renal tissues, in order to rid their bodies of ingested ammonia. The ammonium and potassium microelectrodes were used to measure ammonium and potassium in diets spiked with 0, 30 and 100 mM NH_4^+. Upon the addition of a mean 38 flies (uncrowded conditions) to media for a period of 8 days, ammonium levels in the media significantly increased by more than 20 mM in all treatments (Fig. 10A). Potassium measurements showed the opposite trend, as potassium concentrations significantly decreased by about 12 mM irrespective of the diet (Fig. 10B). These results confirmed that our flies experience increases in dietary ammonia exposure and suggest that the larvae had a net uptake of potassium and a net efflux of ammonium or nitrogenous compounds that were degraded into ammonium, from their bodies. The ammonium content of the media had little effect on these trends.

17.2 Measurement of NH_4^+ in Haemolymph – Effect of Dietary Ammonia

Ammonia concentrations in insect haemolymph differs across species, as 0.8 mM has been found in the larvae of the tobacco hornworm *Manduca sexta* and 16 mM in the sheep blowfly *Lucilia cuprina* (Marshall and Wood, 1990, Weihrauch, 2006). Knowledge of the physiological levels would allow us to improve our design of experiments involving Malpighian tubules. Potassium and ammonium concentrations of the haemolymph were measured in 3rd instar larvae under oil

Figure 10. Effect of NH_4Cl-loaded diets on the (A) ammonium and (B) potassium content of *Drosophila* media, with or without flies. A potassium selectivity coefficient of -0.94 was used to correct for potassium interference. Potassium and ammonium measurements were made from the same media (paired). Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (Bonferroni one-way ANOVA, $P< 0.05$, K^+ N=10 NH_4^+ N=10).

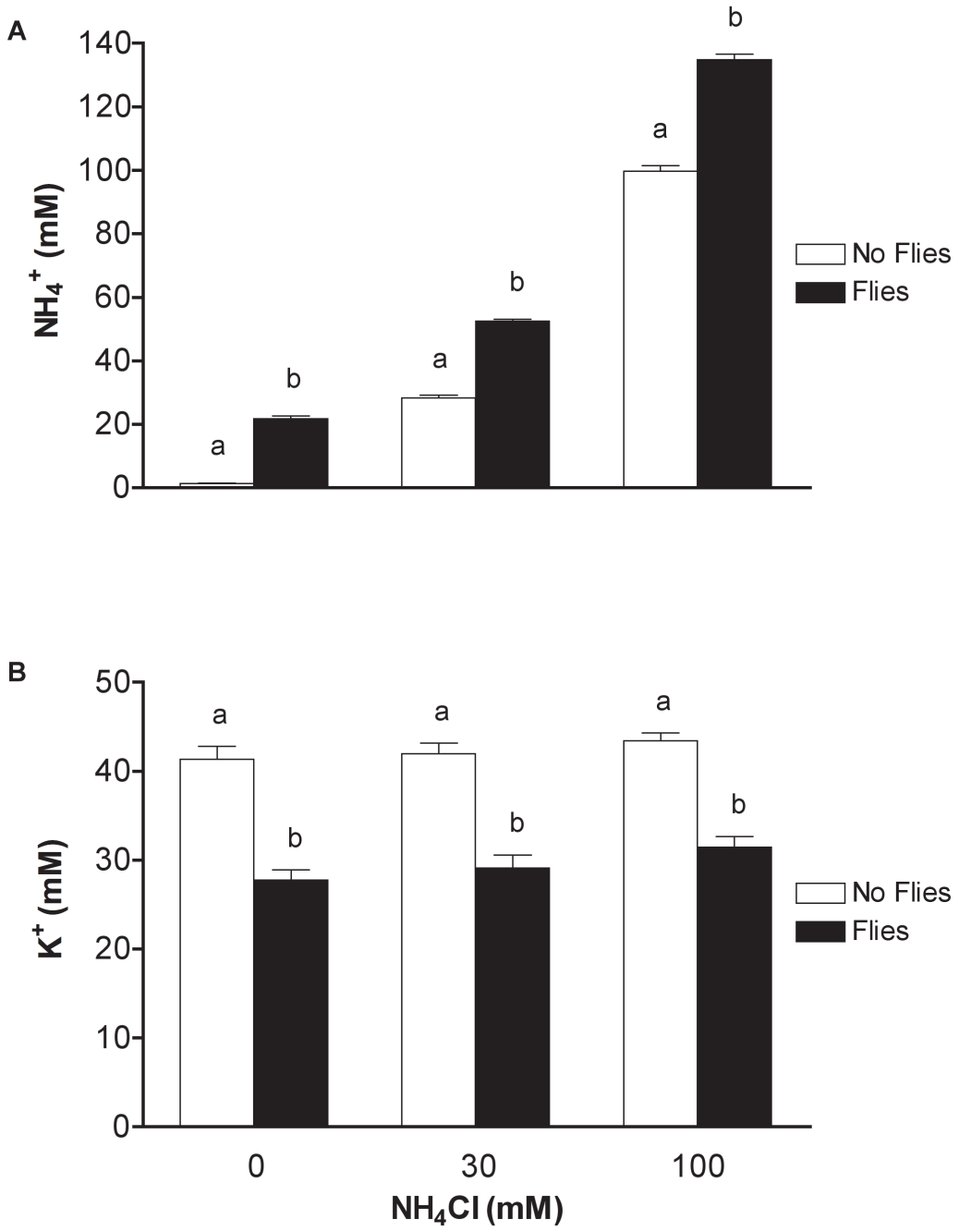
A
140
120
100
80
60
40
20
0
NH_4^+ (mM)
a
b
a
b
a
b
No Flies
Flies
B
50
40
30
20
10
0
K^+ (mM)
a
b
a
b
a
b
No Flies
Flies
0
30
100
NH_4Cl (mM)

Figure 10.

(Fig. 11). Potassium was 26.5 mM K^+ and the 1.5 mM NH_4^+ found in the haemolymph was just within our estimates of detection for haemolymph-like calibration solutions - down to 1 mM with an error of 0.5 mM. Thus, it appeared that *Drosophila* larvae were able to maintain quite low levels of ammonium in their haemolymph while being exposed to quite high levels in their media.

To examine the effect of dietary ammonia on haemolymph ammonium content, we sampled the haemolymph of larvae fed on 0, 30 and 100 mM NH_4Cl diets. 3rd instar *Drosophila* larvae that developed for 8 days in media containing 100 mM NH_4Cl had significantly higher levels of ammonium in their haemolymph than larvae that developed on standard media (Fig. 12). The increase was not large, from 3.7 to 10.0 mM NH_4^+, suggesting haemolymph ammonium levels are well regulated. Potassium concentrations in the haemolymph did not significantly change with dietary ammonia challenge, although all measurements were above the value seen in the previous experiment. These results support the idea that *Drosophila* larvae are quite tolerant of high levels of dietary ammonia.

Ammonium, being a cation with a similar hydrated radius to that of potassium, was hypothesized to compete with potassium for transport (Kikeri et al., 1989). Fluid secretion rate of Malpighian tubules isolated from adult flies was unaffected by NH_4Cl concentration, even though at the highest concentration tested (100 mM NH_4Cl) there appeared to be a reduction (Appendix 4, Figure A3 panel A). 3rd instar larvae were also unaffected by ammonia application in the presence of 20 mM KCl saline (Appendix 4, Figure A3 panel B). The mean fluid secretion rate

Figure 11. Haemolymph concentrations of potassium and ammonium from 3rd instar *Drosophila* larvae reared on control diet. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0053x – 0.956, where x represents uncorrected ammonium concentrations (K^+ *N*=20 NH_4^+ *N*=6).

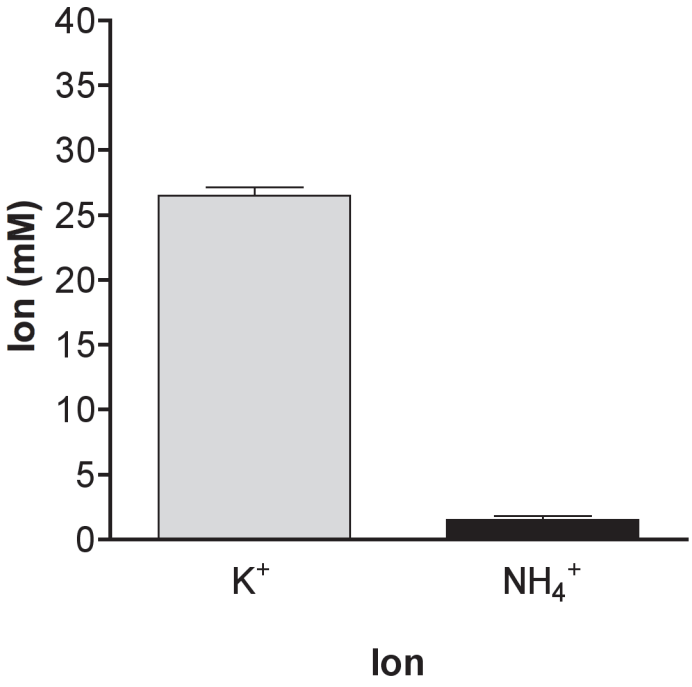

Figure 11.

Figure 12. Effect of dietary NH_4Cl on haemolymph potassium and ammonium concentrations. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0053x – 0.956, where x represents uncorrected ammonium concentrations. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (one-way ANOVA, $P< 0.05$, K^+ N=10 NH_4^+ N=10).

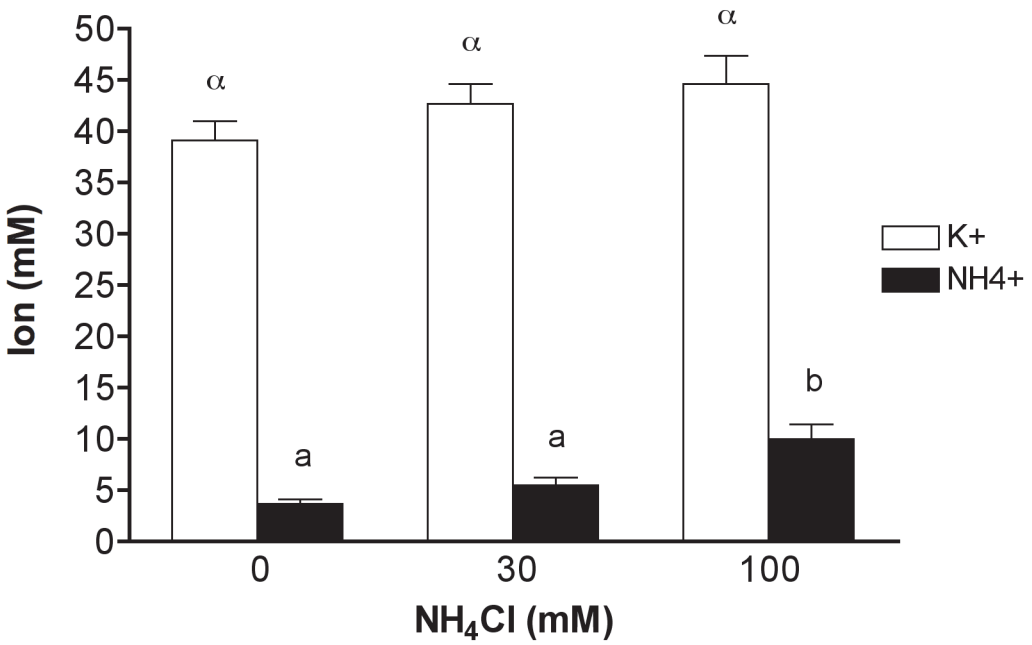

Figure 12.

fluid samples to measure potassium and ammonium under paraffin oil. Measurements of transepithelial movement of ions (pmol min^{-1}), or flux, was calculated from the product of fluid secretion rate (nl min^{-1}) and ion concentration (mM). The mean rate of secretion by Malpighian tubules obtained from both adults and larvae was 0.44 ± 0.01 nl min^{-1} (N = 214). These results support the idea that adult *Drosophila* and their larvae are both quite tolerant of high levels of dietary ammonia.

17.3 Measurement of NH_4^+ in Fluid Secreted by Malpighian Tubules

With *Drosophila* indicating high tolerance for dietary ammonia, we hypothesized that a possible mechanism underlying the tolerance may be active clearance of ammonium from the secretion rate (nl min^{-1}) and ion concentration (mmol l^{-1}). We investigated whether the Malpighian tubules could transport ammonium in conditions that were closest to physiological. When Malpighian tubules (MTs) were bathed in haemolymph-like saline containing 25 mM KCl and 40 mM NaCl, the rate of secretion was unaffected by increasing concentrations of NH_4^+ of up to 40 mM (Fig. 13A). Note that the mean rate was low at 0.21 nl min^{-1} compared to the typical 0.4 nl min^{-1} for Malpighian tubules bathed in *Drosophila* saline containing 20 mM K^+ (Appendix 3, Figure A2) and by others (Riegel et al., 1999, Ianowski and O'Donnell, 2004). In haemolymph-like saline, increasing concentrations of bathing NH_4^+ resulted in secreted fluid that contained roughly equivalent concentrations of NH_4^+ to that of the corresponding bath (Fig. 13B). At concentrations from 0-10 mM NH_4^+, ammonium concentrations of secreted fluid were above the ammonium concentration provided in the bathing saline, while at concentrations from 20-40 mM NH_4^+ ammonium concentrations of the secreted fluid were below the level in the bathing saline. Even

Figure 13. Effects of bathing saline [NH_4^+] on (A) fluid secretion rate (nl min^{-1}) (B) K^+ and NH_4^+ concentrations in secreted fluid (mM) and (C) K^+ and NH_4^+ fluxes (pmol min^{-1}) of tubules bathed in *Drosophila* saline containing 25 mM KCl and 40 mM NaCl. Flux was calculated as the product of FSR and $[Ion]_{SF}$. Dotted lines represent KCl concentration of the saline, while solid red lines indicate the NH_4^+ concentration of the particular experimental saline. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for $NMDG^+$. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0053x – 0.956, where x represents uncorrected ammonium concentrations. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=7-11 NH_4^+ N=7-11).

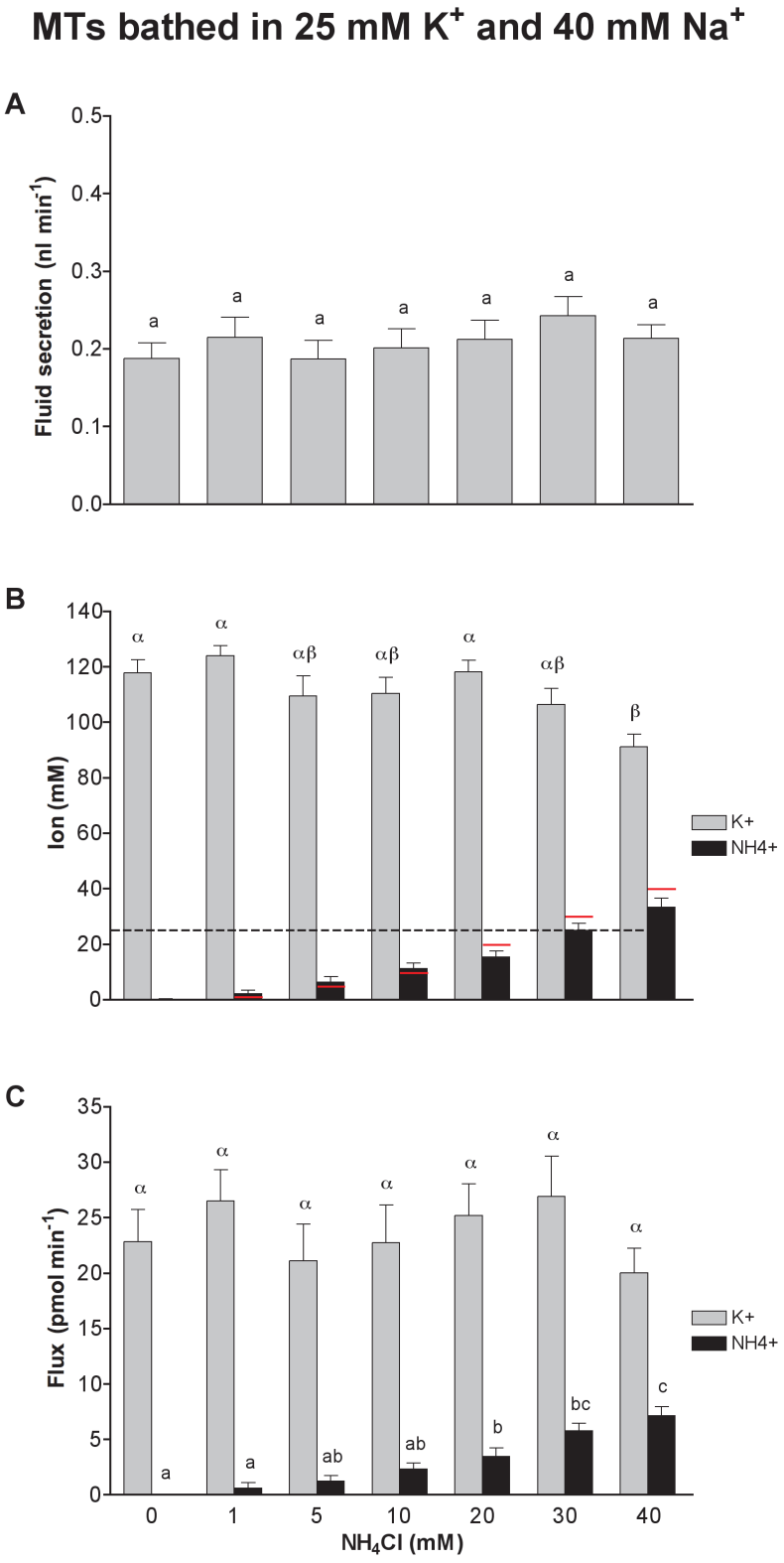
MTs bathed in 25 mM K+ and 40 mM Na+
A
Fluid secretion (nl min-1)
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
a
a
a
B
Ion (mM)
140
120
100
80
60
40
20
0
α
α
αβ
αβ
α
αβ
β
K+
NH4+
C
Flux (pmol min-1)
35
30
25
20
15
10
5
0
α
α
α
α
α
α
α
a
a
ab
ab
b
bc
c
0
1
5
10
20
30
40
NH4Cl (mM)

Figure 13.

with slight differences between bathing saline and secreted fluids, ammonium concentrations of secreted fluid were not significantly different from ammonium concentrations of bathing salines in all treatments. On the other hand, potassium concentrations in the secreted fluid declined slightly, with a significant decrease observed at 40 mM NH_4^+. In saline containing similar concentrations of K^+ (25 mM) and NH_4^+ (30 mM) the secreted fluid contained a 4-fold higher concentration of K^+ than NH_4^+, indicating that the epithelial transporters have a preference for K^+. Flux of ammonium across the Malpighian tubules was significantly increased at doses ≥ 20 mM NH_4^+ (Fig. 13C). In contrast, potassium flux was independent of [NH_4Cl]. The mean potassium flux (23.6 pmol min^{-1}) was higher than ammonium fluxes in all ammonium treatments. In aggregate, these results suggest that Malpighian tubules transport ammonium but with a preference for potassium.

With NH_4^+ known to compete for transport at the K^+ site of various membrane K^+ transporters that can be found in the Malpighian tubules, we hypothesized that elevated potassium may compete with ammonium, decreasing its transport (Garvin et al., 1985). When tubules were bathed in saline containing 40 mM KCl, the rate of fluid secretion was unaffected by increasing concentrations of NH_4^+ of up to 30 mM (Fig. 14A). The secreted fluid had ammonium concentrations that were slightly less than equivalent to the ammonium concentrations of the corresponding bath (Fig. 14B). The concentration of NH_4^+ in the secreted fluid was below that in the bath by a mean 2.3 mM NH_4^+, excluding the NH_4^+-free control. Ammonium concentrations of secreted fluid were not significantly different from ammonium concentrations of bathing salines, except in the 5 and 10 mM NH_4^+ treatments, which were found to be significantly different. K^+ concentration in the secreted fluid was unaltered by changes in bathing saline NH_4^+

Figure 14. Effects of bathing saline [NH_4^+] on (A) fluid secretion rate (nl min^{-1}) (B) K^+ and NH_4^+ concentrations in secreted fluid (mM) and (C) K^+ and NH_4^+ fluxes (pmol min^{-1}) of tubules bathed in *Drosophila* saline containing 40 mM KCl. Flux was calculated as the product of FSR and $[Ion]_{SF}$. Dotted lines represent KCl concentration of the saline, while solid red lines indicate the NH_4^+ concentration of the particular experimental saline. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. A potassium selectivity coefficient of -0.94 was used to correct for potassium interference. Potassium and ammonium measurements were made independently, thus fluid secretion rates of each experiment are displayed. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=7-10 NH_4^+ N=10-12).

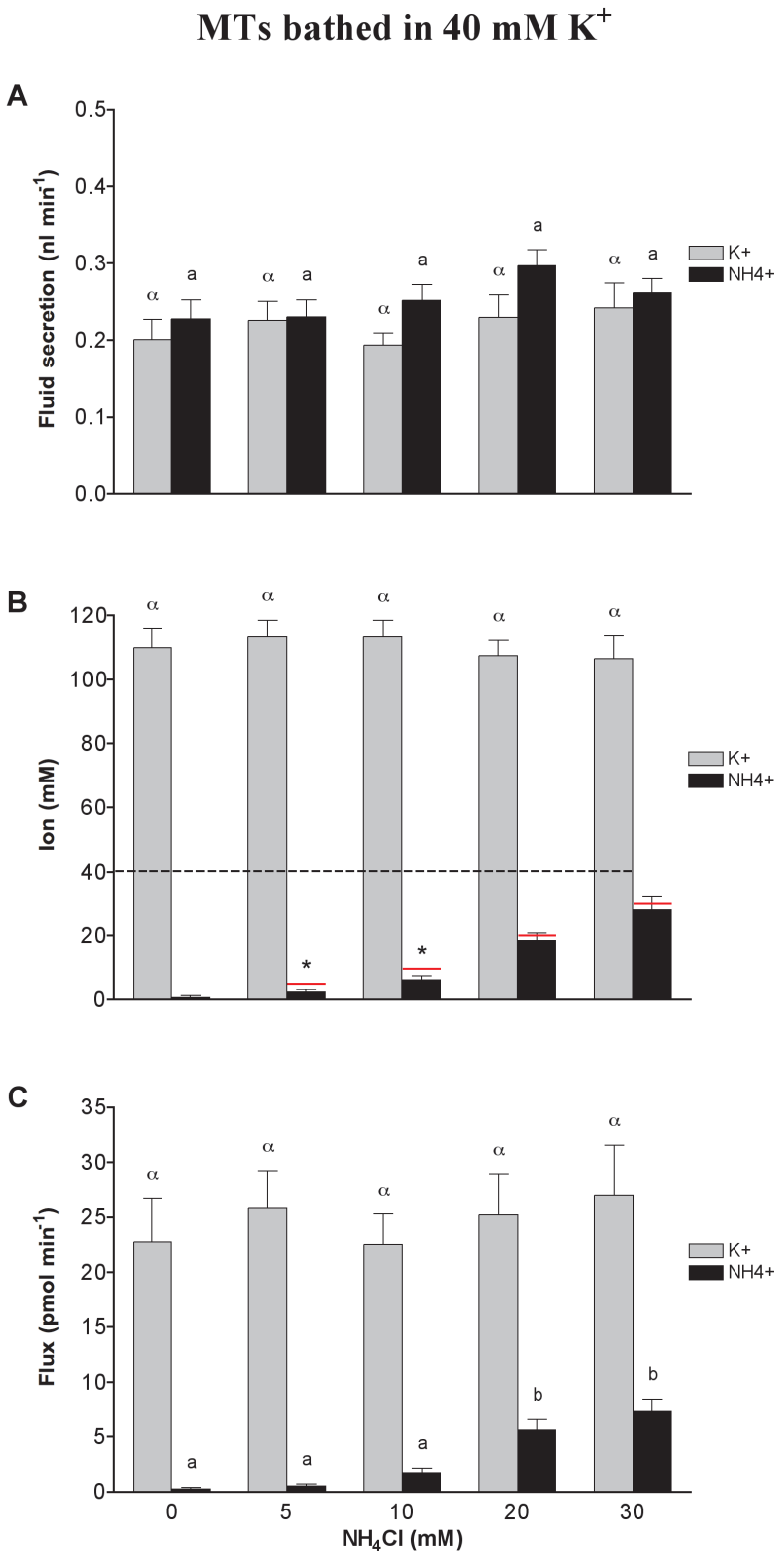
MTs bathed in 40 mM K+
A
Fluid secretion (nl min-1)
0.5
0.4
0.3
0.2
0.1
0.0
α
a
K+
NH4+
B
Ion (mM)
120
100
80
60
40
20
0
*
C
Flux (pmol min-1)
35
30
25
20
15
10
5
0
b
0
5
10
20
30
NH4Cl (mM)

Figure 14.

concentration; the mean value was 110 mM. Flux of ammonium across the tubules was significantly increased at doses ≥ 20 mM NH_4^+ (Fig. 14C). In contrast, potassium fluxes were unchanged with a mean value of 24.7 pmol min^{-1}. Taken together, the data show that increasing bathing saline potassium concentration from 25 mM to 40 mM has little effect on ammonium secretion by the Malpighian tubules.

It was hypothesized that low potassium concentrations in the bathing saline may enhance NH_4^+ transport on K^+-transporters due to reduced competition between the two species for the K^+ site of a common K^+-transporter. In addition, preliminary experiments suggested Malpighian tubules could tolerate K^+-free bathing saline (Appendix 6). Increased transport of NH_4^+ during hypokalemia has been observed in vertebrate models, hence we investigated this effect in an invertebrate model organism (Wall, 2003). Malpighian tubules were bathed in saline containing 5 mM K^+ with increasing concentrations of NH_4^+ of up to 30 mM. The rate of fluid secretion was unaffected by ammonium treatment (Fig. 15A). Mean rates were 0.16 nl min^{-1} for the ammonium experiment and 0.19 nl min^{-1} for the potassium experiment, which were performed separately. Interestingly, the ammonium concentration of the secreted fluid was significantly elevated in all experiments above the ammonium concentration provided in bathing solutions (Fig. 15B). Secreted fluid was above equivalency by a mean 14.2 mM NH_4^+, excluding the NH_4^+-free control as it was essentially zero (-0.06 mM NH_4^+). On the other hand, potassium concentrations in the secreted fluid were low (mean of 64.8 mM K^+) compared to bathing saline containing 25 mM K^+ (mean of 110.3 mM K^+). Ammonium flux increased significantly at 20 mM NH_4^+, while potassium fluxes were consistent at about half (12.8 pmol min^{-1}) the mean value found in 25 mM KCl

Figure 15. Effects of bathing saline [NH_4^+] on (A) fluid secretion rate (nl min^{-1}) (B) K^+ and NH_4^+ concentrations in secreted fluid (mM) and (C) K^+ and NH_4^+ fluxes (pmol min^{-1}) of tubules bathed in *Drosophila* saline containing 5 mM KCl. Flux was calculated as the product of FSR and $[Ion]_{SF}$. Dotted lines represent KCl concentration of the saline, while solid red lines indicate the NH_4^+ concentration of the particular experimental saline. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. A potassium selectivity coefficient of -0.94 was used to correct for potassium interference. Potassium and ammonium measurements were made independently, thus fluid secretion rates of each experiment are displayed. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=12-16 NH_4^+ N=10-12).

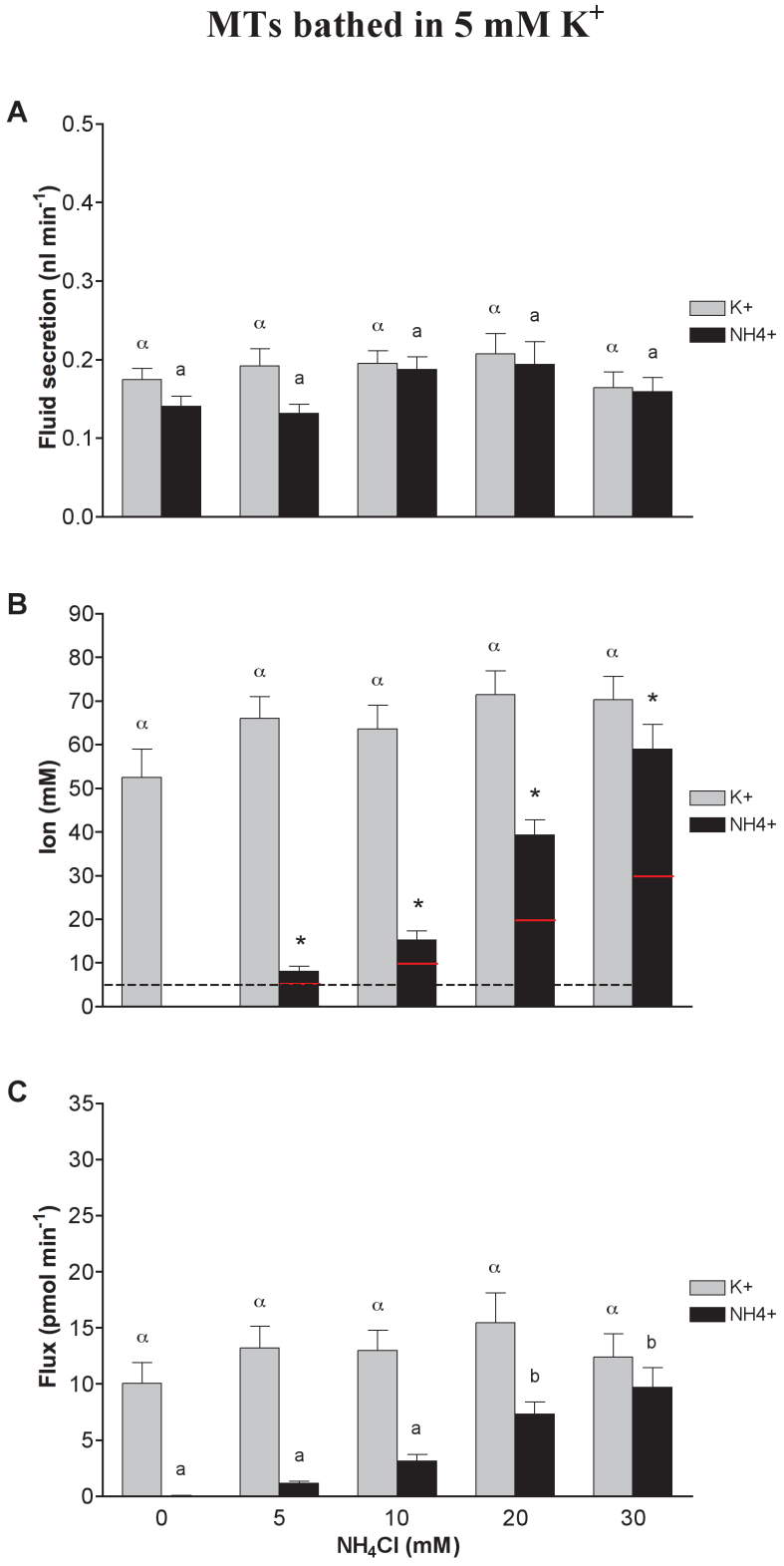
MTs bathed in 5 mM K+
A
Fluid secretion (nl min-1)
0.0
0.1
0.2
0.3
0.4
0.5
α
a
K+
NH4+
B
Ion (mM)
0
10
20
30
40
50
60
70
80
90
*
C
Flux (pmol min-1)
0
5
10
15
20
25
30
35
b
0
5
10
20
30
NH4Cl (mM)

Figure 15.

saline (Fig. 15C). These results revealed that lowering potassium concentrations in the bathing saline greatly increased the concentration of ammonium in secreted fluid, suggesting some level of competition for transport between the two ions, although ammonium fluxes were similar to those found in 25 mM KCl salines.

In low K^+ saline (from 20 to 2 mM K^+) the ionic strength of the secreted fluid was not maintained. For instance, ammonium concentrations increased by 18 mM NH_4^+, while potassium concentrations decreased by 62 mM K^+. Thus it appeared that ionic strength was reduced by 44 mM. Others have observed that Malpighian tubules bathed in K^+-free *Drosophila* saline secreted fluid that contained 150 mM Na^+ and essentially 0 mM K^+ (Linton and O'Donnell, 1999). These results suggest that ionic strength of secreted fluid would be maintained during hypokalemia and that secretion of alternate ions, chiefly sodium or perhaps chloride, may be enhanced during hypokalemia in order to make up the 44 mM deficit observed in our experiments. Specifically, sodium was thought to play a role since secreted fluid contains both sodium (~ 30 mM) and potassium (~ 120 mM) for Malpighian tubules bathed in *Drosophila* saline containing 20 mM KCl (O'Donnell and Maddrell, 1995). Thus Malpighian tubules were bathed in K^+-free *Drosophila* saline, in order to determine if sodium concentrations in secreted fluid would be enhanced (Appendix 5, Figure A4). In addition, the increase in secreted fluid ammonium concentration for tubules bathed in low K^+ saline was expected to be exaggerated in K^+-free conditions. In the presence of increasing concentrations of NH_4^+, the rate of fluid secretion by Malpighian tubules bathed in K^+-free saline was found to be significantly reduced at concentrations $\geq$ 40 mM NH_4^+ (Appendix 5, Figure A4 panel A). Fluid secretion rates were quite low, around 0.1 nl min^{-1} in tubules bathed in 20 mM NH_4^+ or more. As a result, the volume of

fluid secreted by the Malpighian tubules over a 45 min period was below 5 nl. It was observed during these experiments that these small droplets appeared to be shrinking in size over time. The issue of shrinking droplets will be discussed further below. Secreted fluid from tubules bathed in K^+-free saline contained much higher concentrations of sodium (~150 mM) than tubules bathed in saline containing 20 mM KCl (~30 mM) (Appendix 5, Figure A4 panel B). There also appeared to be a decline in sodium concentrations with increasing ammonium concentrations in the bathing saline, indicating that during conditions of low potassium, sodium may compete with ammonium for transport across the Malpighian tubules. Caution should be taken during the interpretation of sodium results as they represent single values. Potassium concentrations in secreted fluid remained below 50 mM K^+ across all ammonium treatments, while ammonium concentrations increased with increasing bathing ammonium concentrations, as expected. Potassium fluxes were consistently low (below 4 pmol min^{-1}) irrespective of the bathing ammonium concentration (Appendix 5, Figure A4 panel C). Sodium fluxes were the highest of the ions but decreased with increasing bathing ammonium concentrations. Ammonium fluxes increased with increasing bathing ammonium to approximately 20 nl min^{-1} when the bath contained 100 mM NH_4^+. Together, these results suggested that during K^+-free conditions, sodium transport is enhanced within Malpighian tubules in order to maintain osmolarity of secreted fluid and may then compete with ammonium for transport.

In K^+-free saline containing high concentrations of ammonium, the health of the tubules was compromised as indicated by very low secretion rates. Thus in order to further investigate the effect of hypokalemia on ammonium secretion the tubules were bathed in decreasing levels of potassium from 20 to 2 mM in the presence of 30 mM NH_4^+. Potassium and ammonium

measurements were made independently in this experiment. During potassium measurements, K^+ depletion had little affect on secretion rate, although tubules bathed in 20 mM K^+ were found to secrete at a significantly higher rates compared to tubules bathed in 2, 5 or 10 mM K^+ (Fig. 16A). The rate of fluid secretion did not significantly change with [K^+] during ammonium sampling with a mean value of 0.18 nl min^{-1}. The influence of decreasing bathing potassium concentrations on both potassium and ammonium secretion was investigated. In general, decreasing potassium concentrations increased ammonium levels in the secreted fluid (Fig. 16B). Ammonium concentrations were significantly higher in secreted fluid than the 30 mM NH_4^+ provided in the bath for salines containing both 2 and 5 mM KCl. For example, in the presence of 30 mM NH_4^+ tubules bathed in 2 mM KCl secreted fluid containing 46 mM NH_4^+ whereas tubules bathed saline containing 20 mM KCl secreted fluid containing 29 mM NH_4^+. The decreased levels of potassium in the bathing saline were associated with a decrease in potassium concentrations found in the secreted fluid. Ammonium fluxes were unaffected by potassium concentrations with a mean value of 7.1 pmol min^{-1} (Fig. 16C). Conversely, potassium fluxes significantly decreased across potassium treatments. In combination, the data suggest that hypokalemia enhances the ability of Malpighian tubules to concentrate ammonium in secreted fluid and that ammonium likely competes with potassium for transport.

Figure 16. Effects of bathing saline [K^+] on (A) fluid secretion rate (nl min^{-1}) (B) K^+ and NH_4^+ concentrations in secreted fluid (mM) and (C) K^+ and NH_4^+ fluxes (pmol min^{-1}) of tubules bathed in *Drosophila* saline containing 30 mM NH_4^+. Flux was calculated as the product of FSR and $[Ion]_{SF}$. Dotted lines represent KCl concentration of the saline, while solid red lines indicate the NH_4^+ concentration of the particular experimental saline. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. A potassium selectivity coefficient of -0.94 was used to correct for potassium interference. Potassium and ammonium measurements were made independently, thus fluid secretion rates of each experiment are displayed. Data are expressed as mean ± S.E.M. with bars not sharing a letter or an asterisk indicating significance (one-way ANOVA or unpaired *t*-test, $P< 0.05$, K^+ N=8-10 NH_4^+ N=10-12).

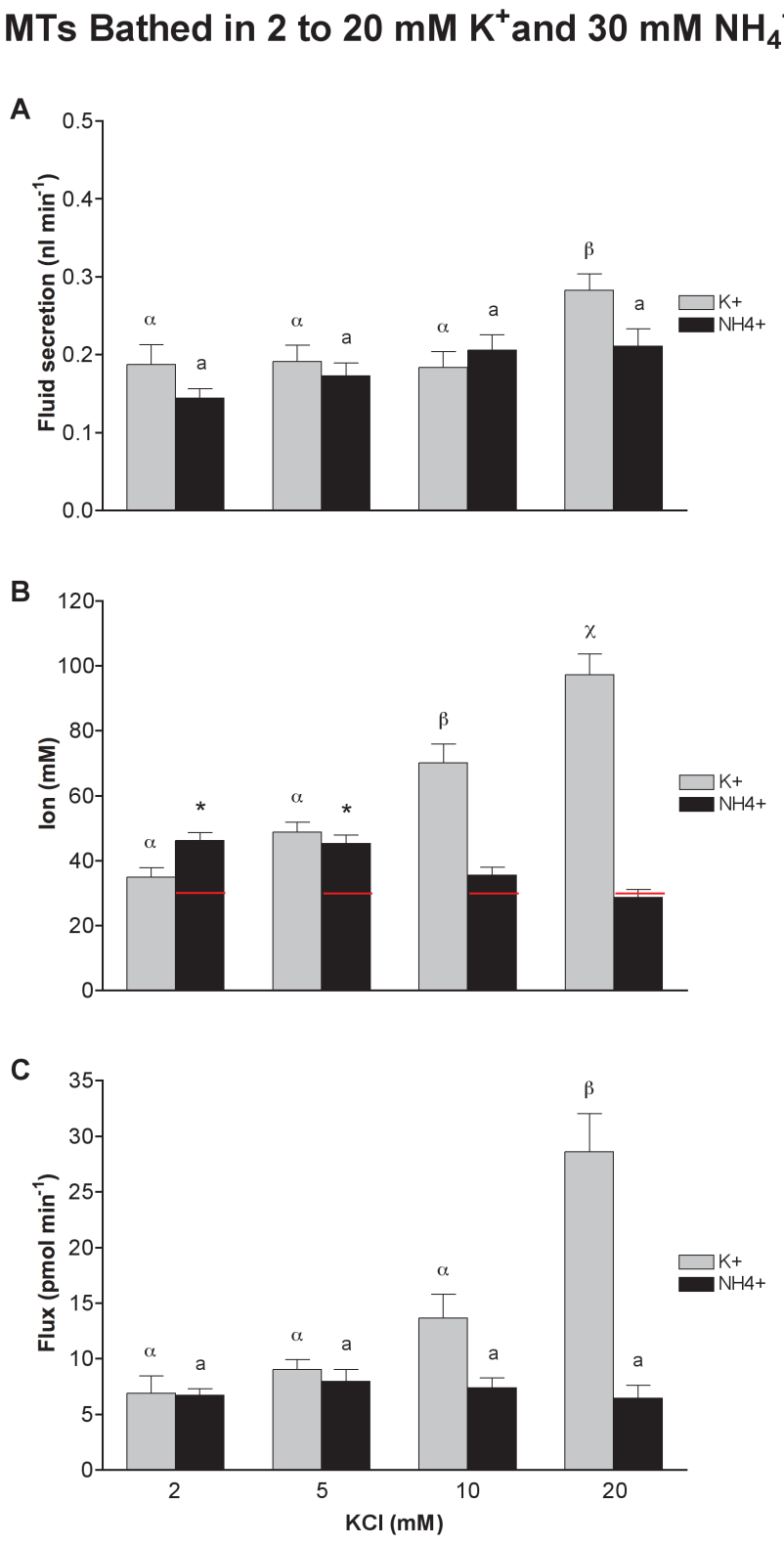
MTs Bathed in 2 to 20 mM K+and 30 mM NH4+
A
Fluid secretion (nl min-1)
0.5
0.4
0.3
0.2
0.1
0.0
α
a
α
a
α
a
β
a
K+
NH4+
B
Ion (mM)
120
100
80
60
40
20
0
α
*
α
*
β
χ
K+
NH4+
C
Flux (pmol min-1)
35
30
25
20
15
10
5
0
α
a
α
a
α
a
β
a
K+
NH4+
2
5
10
20
KCl (mM)

Figure 16.

Chapter 4
Discussion

Transport of ammonia has been studied across many gut segments of various insects. On the contrary, very little is known about the contribution of the Malpighian tubules to the excretion of ammonia. This thesis outlines the characterization of a novel NH_4^+-selective microelectrode and its use as a novel tool for NH_4^+ measurement in insect dietary media, haemolymph and fluid secreted by the Malpighian tubules of *Drosophila melanogaster* larvae. Ion-selective microelectrodes provide significant advantages to the study of ion transport and are well suited to measure ion activity in small volumes, even within single cells.

18 A novel NH_4^+-selective Microelectrode with Improved Na^+ Selectivity

The novel ionophore TD19C6 is an improvement on the classical NH_4^+-selective ionophore nonactin, owing to its improved sodium selectivity (Suzuki et al., 2000). As a result, ammonium measurements can now be performed in the presence of physiological sodium concentrations. With the improved sodium selectivity of the novel ammonium microelectrode, ammonium could potentially be measured in seawater. Considering a log $K_{NH4,Na}$ of -3.58, sodium interference of seawater containing 500 mM Na^+ would be equivalent to the signal produced by 0.13 mM NH_4^+. Significant potassium interference still remained, necessitating its removal from experimental solutions or correction for K^+ effects on NH_4^+-microelectrode potential. Others have chosen to perform experiments in K^+-free or low K^+ conditions, such as in freshwater (Donini and O'Donnell, 2005). We initially chose to do the same, bathing the Malpighian tubules in K^+-free

Drosophila saline, but the reduced FSR lead to small volumes of secreted droplets. In addition, water loss from droplets under paraffin oil and consequent increases in ion concentrations made errors too large to measure ammonium with confidence. The alternative, to correct for potassium interference, allows for ammonium measurement in solutions containing physiological levels of sodium and potassium with the novel ionophore TD19C6. Ammonium could be measured to within 0.3 mM down to about 1 mM NH_4^+ in solutions containing 100 to 140 mM KCl. Due to more accurate measurement of ammonium in the presence of high concentrations of potassium, one possible application of the novel microelectrode is intracellular measurement of ammonium. Using ammonium-selective microelectrodes based on nonactin, intracellular ammonium measurements below 5 mM are considered estimates rather than measurements (Fresser et al., 1991). Using the potassium correction we have improved the detection to approximately 1 mM NH_4^+. Ammonia concentrations in blood of insects can be quite low, with the 0.8 mM observed in *Manduca sexta* and 1.1 mM observed in *Schistocerca gregaria* being similar to our value of 1.5 mM found in *Drosophila* (Stagg et al., 1991, Weihrauch, 2006). With the detection limit of the ammonium electrode (~ 1 mM) close to the observed physiological haemolymph ammonium concentrations, there is the possibility that the blood of some insects and likely mammals (~ 20 μM) may not be sampled with confidence (Magnusson et al., 1994).

19 Effect of K^+ on $K_{NH4,K}$

Selectivity coefficients are traditionally determined using methods that fall under two main groups, separate solution methods or mixed solution methods (Umezawa, 2000). Separate solutions methods involve the comparison of activities or potentials from pure solutions of

principal and interfering ions. The more ideal mixed solutions methods involve solutions containing both principal and interfering ions. A selectivity coefficient for a given electrode is only a constant parameter if Nernstian slopes are observed (Bakker, 1997). Thus in ideal conditions, selectivity coefficients are expected to be relatively consistent in pure solutions of varying activities of the principal or interfering ions. Using the single solutions method, activities of both principal and interfering ions are easily accounted for in the Nicolsky-Eisenman equation as activity coefficients for pure chloride salt solutions are readily available (Ammann, 1986). In practise, a single selectivity coefficient is sufficient to correct for interference independent of the activities of either principal or interfering ions, although electrode responses in mixed (biological) solutions may not be reflective of the ideal pure solutions. Our results revealed that a single $K_{NH4,K}$, determined using the single solutions method, resulted in consistent error of ammonium measurements. Empirical adjustment of $K_{NH4,K}$ in order to maintain accuracy of ammonium measurements revealed that $K_{NH4,K}$ is not constant, rather increasing KCl concentrations from 100 to 150 mM decreased $K_{NH4,K}$ from -0.94 to approximately -0.99. The changes in selectivity may be due to differences in ionic strength of solutions used to determine selectivity coefficients (100 mM) compared to solutions containing 100 mM KCl (140 mM) and 150 mM KCl (190 mM). Increasing ionic strength tends to decrease activity coefficients and thus decrease activity observed by the microelectrodes (Ammann, 1986). Consistent with our hypothesis, others have concluded that changes in electrode measurements due to changes in ionic strength (from 120 to 200 mM) can be almost wholly accounted for by activity considerations (Mohan and Bates, 1975). If the activity coefficient of potassium were preferentially depressed compared to ammonium, as a result of increasing K^+ concentrations while NH_4^+ concentrations remain consistent between experiments, then $K_{NH4,K}$ would be expected to decrease. For example, an increase in ionic strength (in pure solutions) from 100 mM

to 200 mM would decrease the potassium activity coefficient from 0.768 to 0.717 (Hamer and Wu, 1972). In solutions containing 100 mM KCl with an enhanced ionic strength (from 100 to 200 mM) potassium concentrations would be falsely reduced by 5.1 mM K^+. Consequently, $K_{NH4,K}$ would be decreased. The magnitude of the decrease cannot be determined (using separate solutions method calculations) because the potential created by the NH_4^+-selective microelectrode in a solution containing 94.9 mM K^+ cannot be predicted without the use of $K_{NH4,K}$ itself.

20 Effect of NH_4^+ on $K_{NH4,K}$ and the Modified Mixed Solution Method (MMSM)

Using the MMSM, $K_{NH4,K}$ were found to decrease with increasing NH_4^+ concentrations within solutions containing 25 or 120 mM KCl, even at constant ionic strength. These results were unexpected because it was presumed that $K_{NH4,K}$ would remain consistent in solutions containing varying concentrations of the principal ion NH_4^+. Others have noted that selectivity coefficients are dependent on the method used and on the activities of the principal and interfering ions, thus one should allow for certain variation in selectivity coefficients (Ammann, 1986). In the presence of constant ionic strength of 160 mM, others have found that activity coefficients of Na^+, K^+ and Ca^{2+} using macroelectrodes are relatively constant, suggesting that within a given technique selectivity coefficients should be fairly consistent (Mohan and Bates, 1975). We suggest here that potassium interference on the ammonium microelectrode ($[NH_4^+]_{Uncorrected} - [NH_4^+]_{Corrected}$) is greater at low ratios of $[NH_4^+]$:$[K^+]$, which results in less negative values for log $K_{NH4,K}$ and thus an apparent reduction in selectivity of the ammonium microelectrode. The true potassium selectivity coefficient may be consistent, but is increasingly masked due to poor

sensitivity of the ammonium electrode in the presence of high concentrations of interfering ions. The equation used to determine selectivity coefficients (based on a re-arrangement of the Nicolsky-Eisenman equation so as to solve for the selectivity coefficient) assumes a Nernstian response for both principal and interfering ions (Appendix 2). Although the response of the ammonium microelectrode deviates from Nernstian in solutions containing high $[K^+]$ due to K^+ interference, the use of standard solutions accounts for differences in sensitivity. An advantage of our empirical method is that errors affecting potentials, from any interfering ions are taken into account, not only potassium interferences although the vast majority of interference is expected to be due to K^+. Along with reduced $[NH_4^+]$:$[K^+]$, reduced activity coefficients may also play a role in the declining selectivity coefficients.

21 Effect of Decreasing K^+ Activity in Solutions of Constant $[K^+]$

In experiments with solutions containing high concentrations of KCl, potassium concentrations measured using the K^+-selective microelectrode were often found to decline with increasing concentrations of NH_4^+ even in the presence of constant ionic strength. It may be that ammonium decreases the activity coefficient of potassium leaving less potassium to be detected by the K^+-selective microelectrode, even if the potassium concentration in solutions was constant. Sodium was also present in test solutions, as it was used as a substitute for NH_4^+. Thus it may also be that sodium increased the activity coefficient of potassium and as sodium was removed with increasing ammonium concentrations, the increase in potassium activity was abolished. Finally, it is worth considering that the activity coefficient of the anion (Cl^-) may be altered and thus indirectly affect the activity coefficient of the cations within the solutions. Recently, an improved

model was introduced that replaces the traditional Nicolsky-Eisenman equation by a new equation that accounts for interfering ions of different charge (Bakker et al., 1994). The new equation was not used in this study because the concentration of interfering ions of differing charge (Ca^{2+}) are low in haemolymph (~ 0.5 mM) and secreted fluid (~ 0.5 mM) of *Drosophila* (Dube et al., 2000b). It had been suggested that the effect may have been due to differences in activity coefficients of the three species. Activity coefficients for pure 100 mM solutions of NH_4^+, K^+ and Na^+ are very similar: 0.765, 0.766 and 0.776, respectively (Robinson and Sinclair, 1934, Verrall, 1975). At ionic strengths above 100 mM the activity coefficients of the three ions diverge (Ammann, 1986). Thus a mixture of the three species at ionic strengths used during experiments (140 - 196.8 mM) may explain the decreases observed in $K_{NH4,K}$.

22 Evidence for Active NH_4^+ Transport by Malpighian Tubules of *Drosophila*

Malpighian tubules bathed in saline simulating haemolymph (25 mM K^+ and 40 mM Na^+) secreted ammonium at concentrations approximately equal to those in the bathing saline. The transepithelial potential, from bath to lumen is approximately 30 mV lumen-positive (O'Donnell et al., 1996). Thus secretion of NH_4^+ into the more positive lumen is unfavorable. Assuming a Nernstian response of the microelectrode, a 30 mV change in potential represents a 3-fold change in concentration. Therefore if NH_4^+ were to distribute across the Malpighian tubules according to the transepithelial potential only, NH_4^+ concentrations found in the secreted fluid (lumen) would be approximately 3-fold below bathing NH_4^+ concentrations. Since NH_4^+ concentrations of the secreted fluid were well above those expected in a model of passive transport, the results suggested a model of active secretion of ammonium across the Malpighian

tubules. Active transport involves membrane transport proteins that utilize energy sources to translocate molecules. Currently, mechanisms of ammonia transport in insects are unclear. When a short pulse of 20 mM NH_4Cl was applied to Malpighian tubules of *Drosophila hydei*, the initial intracellular alkalinization was small (or absent in one third of the tubules tested), while subsequent intracellular acidification had a much larger amplitude and rapidly decayed (Bertram and Wessing, 1994). These results were interpreted as evidence that the basolateral membrane of *Drosophila* Malpighian tubules had a rather high apparent permeability to peritubular applied NH_4^+. The mechanism(s) underlying the high apparent NH_4^+ permeability of tubular cells are currently unknown, although protein-mediated ammonia transport is suspected. Proteins that directly transport ammonia have been identified in other organisms: ammonia transport proteins (Amt, in bacteria and plants), methylammonium/ammonium permeases (MEPs, in yeast) and Rhesus proteins (Rh) in vertebrates (Khademi et al., 2004, Wright and Wood, 2009). Models of Rh/Amt/MEP transport suggest passive transport of NH_3, although Amt/MEP proteins recruit NH_4^+ at the entrance of the channel, while Rh proteins lack an external binding site (Winkler, 2006, Lupo et al., 2007, Akgun and Khademi, 2011). The driving force for Rh-mediated ammonia transport is currently unclear. Electrical or chemical gradients can drive ammonia transport but these gradients are location-depend, thus localization of Rh proteins within Malpighian tubules is necessary to determine how Rh-mediated NH_3 transport in facilitated. Expression of a Rh50-like glycoprotein has been found to increase in midgut, fat body and Malpighian tubules of the mosquito *Aedes albopictus* following a blood meal (Wu et al., 2010). Expression of a Rh-like protein isolated from *Manduca sexta* (RhMS) was also found to be high in the Malpighian tubules of the insect (Weihrauch, 2006). These results suggest that perhaps insects have Rh proteins that facilitate ammonia secretion by the Malpighian tubules.

Ammonia must be transported across basolateral and apical membranes during the process of secretion from bath to lumen. Ammonium provided in the bathing media may be taken into principal cells of the Malpighian tubules through an ouabain-sensitive Na^+/K^+-ATPase or bumetanide-sensitive $Na^+:K^+:2Cl^-$ cotransporter (Ianowski and O'Donnell, 2004). Entry of NH_4^+ across the basolateral membrane likely would result in intracellular acidification, thus a "bicarbonate shuttle" may exist to buffer intracellular H^+ loading (Weiner and Verlander, 2011). In mammalian thick ascending loop kidney ST-1 cells, inhibition of a sodium bicarbonate cotransporter (NBCn1) blunted uptake of the ammonia analog [^{14}C]methylammonium by the cells (Lee et al., 2010). When expressed in Xenopus oocytes, NBCn1 increased carrier-mediated ^{14}C-MA transport (Lee et al., 2010). These results suggest a sodium bicarbonate cotransporter facilitates ammonia transport through a "bicarbonate shuttle" mechanism. Once in the intracellular milieu, NH_4^+ may cross the apical membrane through amiloride-sensitive K^+/H^+ or Na^+/H^+ exchange driven by a bafilomycin-sensitive H^+-ATPase (Linton and O'Donnell, 1999). Although only the basolateral Na^+/K^+-ATPase and apical H^+-ATPase are active transporters, the others utilize potential/chemical gradients created by the active transporters that may drive secondary or tertiary active transport of ammonium.

Another possibility is that NH_4^+ is not transported at all, with NH_3 being the species predominantly transported. An acid trapping mechanism, previously described, may explain how ammonium could be passively concentrated within secreted fluid (Wall, 2003). Measurements of pH in fluid secreted by Malpighian tubules bathed in the presence of ammonium would allow for the determination of NH_3 gradients from bath to lumen. Further study is required to

determine if the NH_3 gradients are large enough to account for the observed NH_4^+ concentrations in the secreted fluid.

23 NH_4^+ and K^+ may Compete for Transport Across Malpighian Tubules, with NH_4^+/K^+ Transporters Preferring K^+

There does appear to be some debate as to which species of ammonia (NH_3 or NH_4^+) is predominantly transported. Evidence of an electrogenic nature of ammonia transport in *Xenopus laevis* oocytes and stretch receptor neurones of *Astacus astacus* are consistent with NH_4^+ transport (Fresser et al., 1991, Burckhardt and Burckhardt, 1997). In addition, consistent intracellular pH changes upon addition of NH_4Cl to solutions bathing cells have been documented: attributing the initial alkalization to the rapid entry of NH_3 and the subsequent slow acidification to the entry of NH_4^+, suggesting both species play a role (Fresser et al., 1991, Nagaraja and Brookes, 1998, Nakhoul et al., 2001). As well as single cells, NH_3 and NH_4^+ have been implicated in transepithelial movement of ammonia in studies involving tissues. In perfused trout white muscle both pH and electrical gradients affected total ammonia efflux, indicating that the membranes were permeable to both NH_3 and NH_4^+ (Wang et al., 1996). On the other hand, Rh/Amt/MEP proteins known to transport ammonia favour gaseous ammonia based on molecular dynamic analysis of the proteins (Khademi et al., 2004). A combination of active and passive transport of NH_3 and NH_4^+ may be responsible for ammonia transport in Malpighian tubules. For instance, ammonia could be actively transported across the basolateral membrane of the Malpighian tubules, followed by Rh-mediated (passive) transport across the apical membrane or visa versa. Our results suggest that NH_4^+ is actively secreted across the Malpighian tubules rather than NH_3 based on the competition observed between potassium and ammonium. Simple

diffusion or active transport of NH_3 would not be counteracted by K^+, which indicates that K^+ transporters are likely involved in NH_4^+ transport. Intracellular measurement of ammonium in tubular cells may aid in an explaining the mechanisms of ammonia transport by Malpighian tubules.

The evidence for competition between ammonium and potassium for transport, suggest that a basolateral Na^+/K^+-ATPase or $Na^+:K^+:2Cl^-$ cotransporter may play a role in ammonium transport by Malpighian tubules (Ianowski and O'Donnell, 2004). Consistent with this hypothesis is evidence for NH_4^+ substituting for K^+ on either transporter (Garvin et al., 1985, Evans and Turner, 1998). In addition, NH_4^+ uptake by the Na^+/K^+-ATPase in the kidneys of rats increased during hypokalemia via dietary K^+ restriction (Wall et al., 2002). These results are consistent with our observations of enhanced NH_4^+ concentrations in fluid secreted by Malpighian tubules bathed in hypokalemic conditions. Apparent affinity of NH_4^+ for the extracellular K^+ binding site was approximately five to six times lower than that of K^+ on the Na^+/K^+-ATPase in rat inner medullary collecting duct (IMCD) cells (Wall and Koger, 1994). The observed preference for K^+ over NH_4^+ by the Malpighian tubules is consistent with a model containing higher affinities for K^+ than NH_4^+ by the Na^+/K^+-ATPase. The same group also calculated fluxes of NH_4^+ and K^+ to be 2.2 and 26.8 pmol mm^{-1} min^{-1} across the IMCD cells, respectively. The roughly 12-fold greater K^+ flux is consistent with our 10-fold greater K^+ flux (2.3 and 22.8 pmol min^{-1}) found in Malpighian tubules bathed in saline with comparable NH_4^+ and K^+ concentrations (10 mM NH_4^+ and 25 mM K^+). It should be noted that the physiological significance of the Na^+/K^+-ATPase in NH_4^+ transport has been questioned in due to much higher extracellular K^+ concentrations than NH_4^+ concentrations that predominate under most physiological conditions (Wall, 2003). The

effect of hypokalemia on ammonium secretion may be of more use to insects in their natural milieu, as they may encounter periods of dietary potassium restriction. Ammonium secretion by Malpighian tubules may also be accomplished through a Na^+:K^+:2Cl^- cotransporter (NKCC). Bumetanide, an inhibitor of the cotransporter, has been used to successfully reduce the initial rate of intracellular acidification elicited by 1 mM NH_4^+ to one third of the control rate in rodent astrocytes (Nagaraja and Brookes, 1998). Similar results were found in mouse medullary thick ascending limb of Henle, cells which are virtually impermeable to NH_3 (Kikeri et al., 1989). Like the Na^+/K^+-ATPase, greater NKCC1-mediated NH_4^+ uptake occurs with a lower extracellular K^+ concentration in rat IMCD cells (Wall, 2003). In aggregate, these results suggest that both a Na^+/K^+-ATPase and Na^+:K^+:2Cl^- cotransporter should be incorporated in models of epithelial ammonium transport.

Another mechanism of ammonium secretion by the tubules may involve metabolic production and passive release preferentially into the lumen. In mammals, chronic metabolic acidosis and hypokalemia increase renal synthesis of NH_4^+, which is preferentially secreted into the lumen of the proximal tubule (Tannen, 1977, DuBose et al., 1991). Metabolic production of ammonia in insects has also been observed. In *Manduca sexta* larvae, no transepithelial active net transport was found in the hindgut, rather metabolic ammonia was preferentially released (86% of total) from the apical side (Weihrauch, 2006). In addition, when larvae of a cabbage armyworm *Mamestra brassicae* were fed on the leaves of their host plant the amount of excreted NH_4^+-Nitrogen was from 5 to 17-fold greater than that ingested, suggesting a large portion of the NH_4^+ in the frass was metabolically derived (Kagata and Ohgushi, 2011). Ammonia can be derived from a variety of amino acids through a two-step process known as transdeamination (Wright,

1995). In mosquitoes, the amino acids proline and glutamine (two amino acids that can result in ammonia through deamination) may act as temporary nitrogen sinks in order to avoid the toxicity associated with ammonia (Scaraffia et al., 2005). Once acute ammonia toxicities following a blood meal have been avoided, the amino acids may be catabolized by a variety of tissues releasing ammonia for excretion or utilized as an energy substrate during flight (Scaraffia and Wells, 2003) . Together, these results indicate that metabolic production and selective release may play a significant role in the transport of ammonia, from a variety of nitrogen sources.

24 Links Between NH_4^+, K^+ and H^+ - Ammonia Metabolism, Hypokalemia and Acid-Base Balance

There are two primary relationships that link these three ions. The first involves the pH dependent equilibrium between NH_3 and H^+ governed by the reaction $NH_3 + H^+ \leftrightarrow NH_4^+$. The pKa for the reaction is ~ 9, thus at physiological pH (~ 7) the vast majority (~ 99%) is present in the ionic form NH_4^+. The second relationship is due to the nearly identical hydrated radii of NH_4^+ and K^+ (Knepper et al., 1989, Martinelle and Haggstrom, 1993). In mammals, both potassium depletion and acidosis result in increased ammonia excretion (Tannen, 1977). Increases in ammonia excretion in response to acidosis has also been observed in various vertebrate groups including birds, amphibians, freshwater teleosts and marine elasmobranchs (Wright, 1995). At rest, renal ammonia excretion accounts for 50-70% of net acid excretion in humans, thus ammonia can play a crucial role in acid-base homeostasis (Weiner and Verlander, 2011). In locusts, ammonium secretion provides an effective means of increasing acid elimination without increasing the pH gradient against which the hindgut proton pump must work (Phillips et al., 1994). In general, expression of renal ammoniagenic enzymes and epithelial transporters

increased in response to both hypokalemia and metabolic acidosis, suggesting a common mechanism in hypokalemia and metabolic acidosis (Han, 2011). In insects, removal of potassium from solutions bathing *Drosophila* Malpighian tubules results in the acidification of luminal fluid (Bertram and Wessing, 1994). Acute removal of sodium from the bathing media did not alter the pH of luminal fluid, thus the luminal acidification appears to be specific to potassium (Bertram and Wessing, 1994). An acidification of luminal fluid would be expected to enhance ammonia secretion by the tubules, which is consistent with our observations of enhanced ammonium concentrations in luminal fluid during hypokalemia. It may be the case, in Malpighian tubules, that during hypokalemia the basolateral Na^+/K^+-ATPase has reduced functionality due to reduced intracellular potassium concentrations (Linton and O'Donnell, 1999). Consequently, apical cation/H^+ exchange may become reduced resulting in enhanced luminal acidification, which in turn may enhance ammonia secretion across the apical membrane of the tubules (Maddrell and O'Donnell, 1992). Further investigation is necessary to validate such a mechanism and to find the key modulator of ammoniagenesis (K^+ or H^+).

25 Evidence for NH_4^+ Tolerance and Excretion by *Drosophila*

Increases in NH_4^+ concentration in the diet of *Drosophila* in the presence of adults/larvae confirmed previous observations that adult flies and especially larvae experience high levels of environmental ammonia in laboratory culture (Borash et al., 1998). With high ammonia exposure in the diet, the renal tissues of the insect were expected to play a role in the possible distribution, metabolism/detoxification and excretion of ammonia (Dow and Davies, 2006, O'Donnell, 2009). Thus *Drosophila* larvae may represent a good model system for the purpose of studying

ammonium transport in renal tissues. Since *Drosophila* are exposed to high levels of ammonia (~20 mM following 8 days of culture) they were expected to be tolerant of its toxic effects in order to persist. Survival on media containing 100 mM NH_4Cl and no significant change in the rate of fluid secretion of Malpighian tubules bathed in saline containing up to 100 mM NH_4Cl, suggesting that both adult and larval *Drosophila,* and their renal tissues are quite tolerant of ammonia. It should be noted that there were signs of toxicity, as development appeared to be delayed and the size of adult flies appeared to be reduced when cultured on media containing 100 mM NH_4Cl.

A comparison of the ammonium and potassium concentrations in the media in the absence and presence of flies suggested a net uptake of K^+ and net efflux of NH_4^+ from the bodies of the flies. The decrease of K^+ concentration of the diet is likely due to uptake of potassium by the flies for growth requirements and storage in intracellular compartments. Increases in ammonium concentrations of the diet may be due to the conversion of protein in the diet to ammonia during cellular metabolism (Campbell, 1991). The subsequent metabolic ammonia may then be excreted unchanged or in the form of other nitrogenous compounds. In *Drosophila*, uric acid is converted by urate oxidase to allantoin and secreted by the Malpighian tubules (O'Donnell et al., 1983, Wallrath et al., 1990). Although the primary excretory product of *Drosophila* is allantoin, our results suggest they may also excrete small amounts of ammonia. The increase in dietary ammonium concentrations may also be explained by conversion of nitrogenous compounds to ammonium by bacteria present in the fat body or gut of the insects or perhaps in the diet itself. Symbiotic relationships have been observed in cockroaches and termites, where the bacteria are thought to supplement the nitrogen-deficient diets of these insects (Doolittle et al., 2008, Lopez-

Sanchez et al., 2009). Our flies experience high levels of nitrogen in their diet, thus may not require a symbiotic relationship with nitrogen-releasing bacteria and therefore decomposition within the food is likely the main source of bacterially-derived ammonium.

26 Evidence for Haemolymph [NH_4^+] Regulation

Haemolymph NH_4^+ concentrations significantly increased from 3.7 to 10 mM NH_4^+ in larvae reared on media containing 100 mM NH_4Cl compared to larvae reared on standard media. The increase suggested that mechanisms of haemolymph ammonia regulation may be overrun at high levels of environmental ammonia. Since *Drosophila* were able to maintain lower levels of ammonia in their blood despite high levels in their diet (~20 mM), it was concluded that haemolymph ammonia is well regulated in this species. Based on whole body analysis, *Drosophila* reared on food containing 370 mM NH_4Cl had internal concentrations of ammonia that were 10-fold less than the external media (Borash et al., 2000). The 10-fold difference between haemolymph (10 mM) and media (100 mM) of larvae rear on 100 mM NH_4Cl diets in our experiment is also consistent with a model of strict haemolymph ammonia regulation.

Low haemolymph concentrations may be maintained through rapid secretion by the Malpighian tubules. Alternatively, haemolymph ammonium may be converted to less toxic nitrogenous compounds, such as amino acids, for temporary storage (Scaraffia et al., 2005). Note that haemolymph potassium concentrations obtained from flies reared on standard media were not consistent between experiments as seen by the comparison of Fig. 11 & 12. The observed differences are likely due to changes in haemolymph extraction technique. Haemolymph

contains haemocytes that are presumed to have high intracellular K^+ concentrations (Wyatt, 1961). Rupture of haemocytes followed by subsequent potassium release into haemolymph fluid may explain the elevated potassium concentrations observed between the two experiments.

27 General Conclusions

The development of highly specific ion-selective microelectrodes for application in epithelial transport is an ongoing challenge. To that end, this thesis contributes in two ways 1) through the development of a novel NH_4^+-selective microelectrode suitable for measurement of ammonium in the presence of physiological levels of sodium and potassium, and 2) by demonstrating the potential of these microelectrodes through their use in potentiometric determination of NH_4^+ in nanoliter samples of insect dietary media, blood and primary urine. We have concluded that with the novel NH_4^+-selective microelectrode with improved Na^+ selectivity, NH_4^+ could be measured to within 0.3 mM down to about 1 mM NH_4^+ in solutions containing 100 to 140 mM KCl. This represents an improvement from previous nonactin-based microelectrodes where values of intracellular NH_4^+ concentrations below 5 mM are considered estimates rather than measurements. With improved Na^+ selectivity ($K_{NH4,Na}$) from approximately 100 to 3800 fold less selective for Na^+ compared to NH_4^+, NH_4^+ measurements may now be performed in the presence of very high sodium concentrations.

Measurements of ammonium in the diet of cultured *Drosophila* revealed that the flies are exposed to high environmental ammonia (~ 20 mM in our experiments) and therefore likely represent a good model for the purpose of studying ammonia transport throughout this extremely

useful insect. During dietary exposure to high levels of ammonia (100 mM NH_4Cl) the larvae were able to maintain 10 fold lower concentrations in their blood. In light of the toxicity of ammonia, it would be vital for the insect to maintain low levels in their blood. Mechanisms that involve active secretion by Malpighian and metabolic conversion to less toxic nitrogenous metabolites or amino acids were discussed.

To our knowledge, we provide the first evidence that Malpighian tubules of *Drosophila* larvae secrete NH_4^+. The NH_4^+ concentrations of the secreted fluid were well above those expected in a model of passive transport suggesting the observed secretion was active in nature. Further, competition between K^+ and NH_4^+ imply the presence of ammonium/potassium transporters in Malpighian tubules, which is consistent with a 'hitchhiking' hypothesis involving NH_4^+ transport at the K^+ site of various K^+ transport proteins that have been thoroughly investigated in renal tissues. In future, pharmacological inhibitors may reveal mechanisms of ammonia transport in insects.

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Appendices

28 Appendix 1: The $CH_3NH_3^+$-selective Microelectrode

Sodium was a major interfering ion of the methylammonium-selective microelectrode as indicated by a slope of 32 mV down to 1 mM methylammonium (Table 1). Lowering the NaCl concentration to 15 mM allowed for the detection of methylammonium down to 1 mM. Methylammonium detection was sufficient down to 1 mM in the presence of 100 mM LiCl. No useful slopes were obtained in *Drosophila* saline due to the high concentrations of sodium present (117.5 mM). Selectivity coefficients indicated significant interference from both potassium and ammonium, with selectivity coefficients of -0.55 and -1.10, respectively (Appendix 1). With the absence of functional methylammonium detection in *Drosophila* saline, in addition to significant potassium and ammonium interference, the use of the methylammonium microelectrode was discontinued.

Figure A1. Selectivity coefficients ($\log_{10}$) of the methylammonium-selective microelectrode. Values were obtained by the separate solutions method using pure 0.1 M chloride salt solutions of the principle and interfering ions. Composition of the membrane of the microelectrode is listed below the column. KtkClPB, Potassium tetrakis(4-chlorophenyl)borate; NPOE, 2-Nitrophenyl octyl ether.

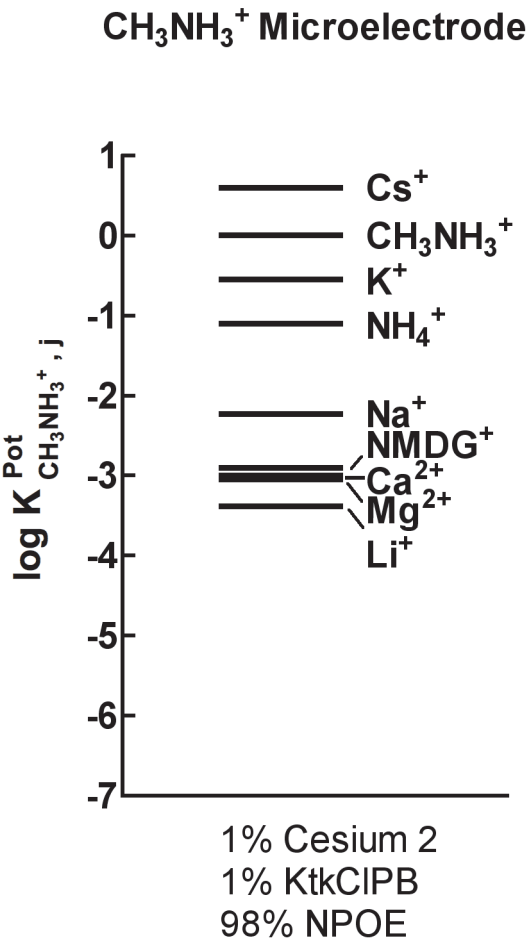
$CH_3NH_3^+$ Microelectrode
1
0
-1
-2
-3
-4
-5
-6
-7
$\log K^{Pot}_{CH_3NH_3^+, j}$
Cs^+
$CH_3NH_3^+$
K^+
NH_4^+
Na^+
$NMDG^+$
Ca^{2+}
Mg^{2+}
Li^+
1% Cesium 2
1% KtkClPB
98% NPOE

Figure A1.

29 Appendix 2: Using the Nicolsky-Eisenman Equation to Correct Measured Concentrations for Interference

$EMF = E_o + s \log [a_i + (K_{ij})(a_j)]$,

where EMF= electromotive force (mV), E_o= reference potential (mV), s = slope, a_i =primary ion activity, a_j = interfering ion activity and K_{ij} = selectivity coefficient for an i-selective electrode towards j (Ammann, 1986).

Using the example of K^+ interference on NH_4^+ microelectrodes, where all measurements are referenced to a calibration solution containing 15 mM NH_4^+ and 135 Na^+, we can calculate the corrected NH_4^+ concentration in the droplet ($NH_4^+{}_c$) as follows:

$EMF_1 = Eo + s \log [NH_4^+{}_c + (K_{NH4,K})(K^+)]$

$EMF_2 = Eo + s \log [15 + (K_{NH4,K})(0)]$

$\Delta V = EMF_1 - EMF_2 = s \log [NH_4^+{}_c + (K_{NH4,K})(K^+)] - s \log [15 + (K_{NH4,K})(0)]$

$= s \log [(NH_4^+{}_c + (K_{NH4,K})(K^+))/15]$

By re-arrangement, solving for $NH_4^+{}_c$:

$\Delta V/s = \log [(NH_4^+{}_c + (K_{NH4,K})(K^+))/15]$

$10\text{^}(\Delta V/s) = (NH_4^+{}_c + (K_{NH4,K})(K^+))/15$

$(15)(10\text{^}(\Delta V/s)) = NH_4^+{}_c + (K_{NH4,K})(K^+)$

$[NH_4^+{}_c] = (15)(10\text{^}(\Delta V/s)) - (K_{NH4,K})(K^+)$

i.e. $NH_4^+{}_{Corrected} = NH_4^+{}_{uncorrected} - (K_{NH4,K})(K^+)$

For example, if ΔV = -0.2 mV relative to 15 NH_4^+, s = 54.9 mV/decade and $\log K_{NH4,K}$ = -0.94, (*i.e.* $K_{NH4,K}$ = 0.115), and K^+ =120 mM,

$-0.2 = 54.9 \log[((NH_4^+{}_c + (0.115)(120))/15] = 54.9 \log ((NH_4^+{}_c + 13.8)/15)$

$NH_4^+{}_c = (15)(10\text{^}(-0.2/54.9)) - 13.8 = 14.9 - 13.8 = 1.1$ mM

Selectivity coefficients are usually reported as $\log K_{NH4,K}$, so it is necessary to convert them as in $10\text{^}(\log K_{NH4,K})$ and use the arithmetic value of the selectivity coefficient in the equation.

30 Appendix 3: Comparison of Salines Substituted with $NMDG^+$ or NaCl

Figure A2. Comparison of (A) fluid secretion rate (nl min^{-1}) and (B) secreted fluid K^+ and NH_4^+ concentrations of *Drosophila* saline substituted with either $NMDG^+$ or NaCl. Potassium selectivity coefficients used to correct for potassium interference were obtain from the equation y = - 0.0027x – 0.994, where x represents uncorrected ammonium concentrations. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (unpaired *t*-test, $P< 0.05$, $NMDG^+$ $N=6$ NaCl $N=4$).

Comparison of Salines Substituted with NMDG or NaCl

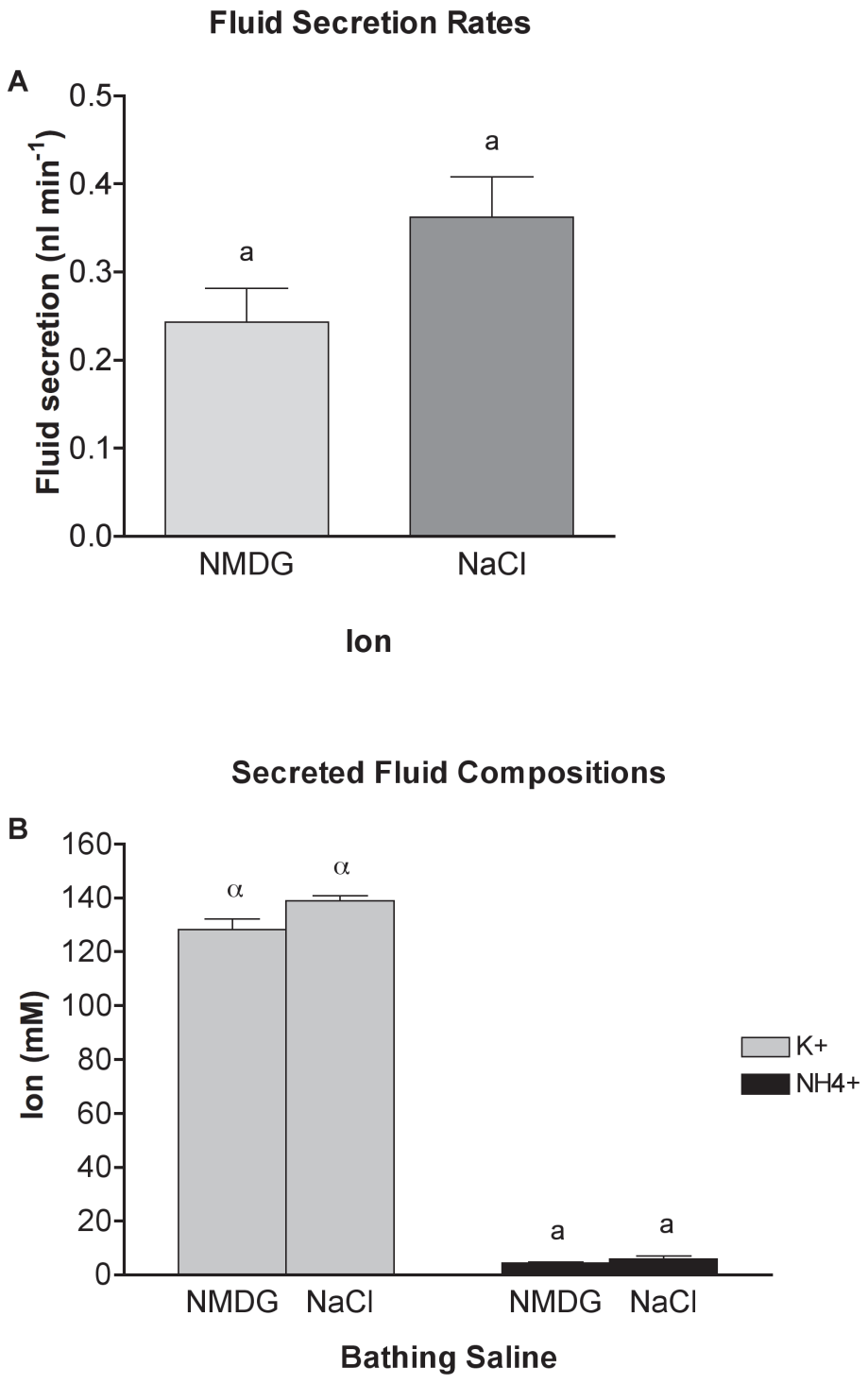

Figure A2.

31 Appendix 4: Tolerance of Malpighian Tubules to NH_4^+

Figure A3. Effect of bathing saline [NH_4^+] on the rate of fluid secretion by Malpighian tubules isolated from (A) adult and (B) 3rd instar larvae in the presence of 20 mM KCl *Drosophila* saline. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (one-way ANOVA, $P < 0.05$, Adults N=7-14 Larvae N=12-31).

Tolerence of Malpighian Tubules to NH_4Cl

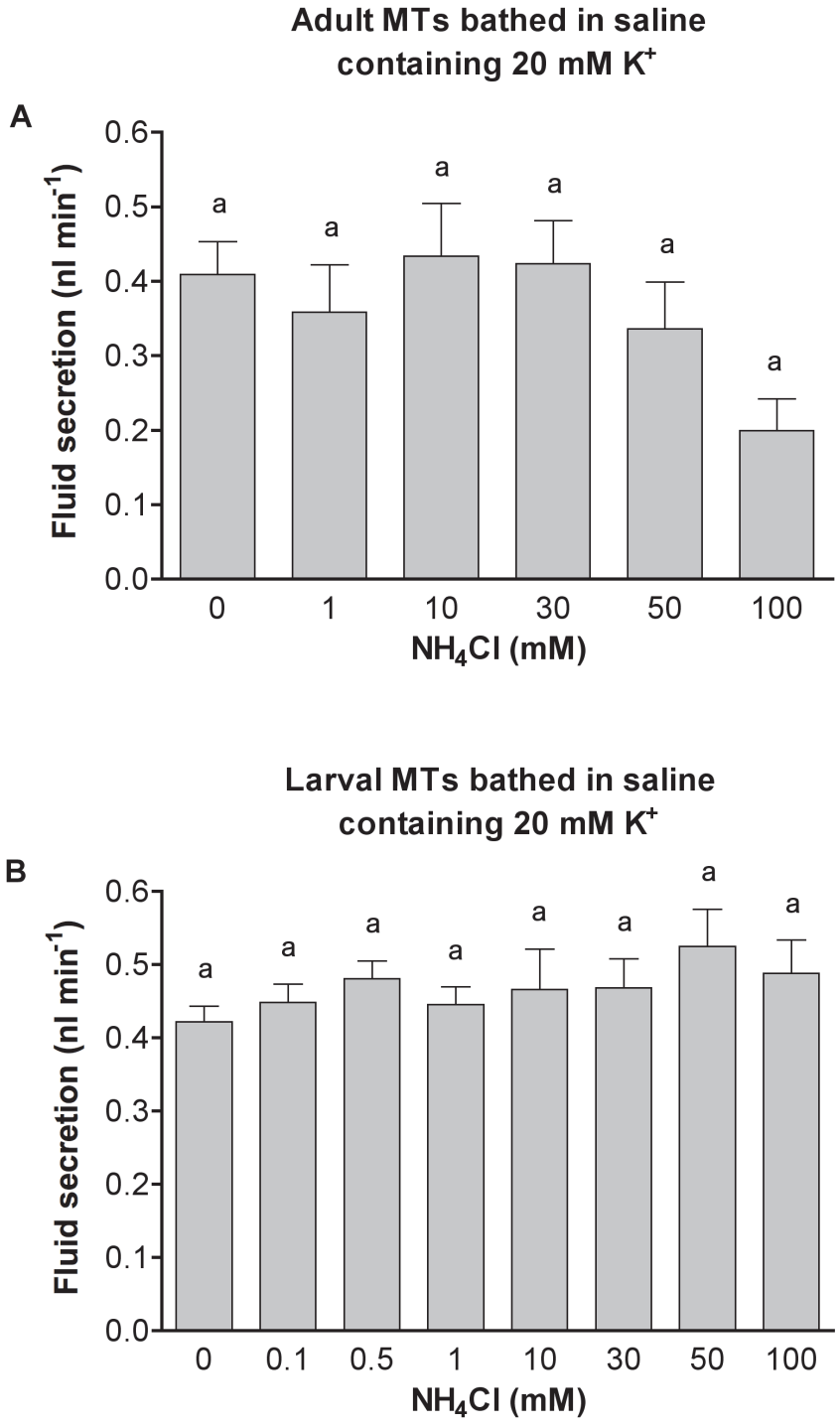

Figure A3.

32 Appendix 5: Malpighian Tubules Bathed in K^+-free Saline

Figure A4. Effects of bathing saline [NH_4^+] on (A) fluid secretion rate (nl min^{-1}) (B) K^+ and NH_4^+ concentrations in secreted fluid (mM) and (C) K^+ and NH_4^+ fluxes (pmol min^{-1}) of tubules bathed in *Drosophila* saline containing 0 mM K^+. Flux was calculated as the product of FSR and $[Ion]_{SF}$. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. A potassium selectivity coefficient of -0.94 was used to correct for potassium interference. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (one-way ANOVA, $P< 0.05$, K^+ N=4-14 Na^+ N=1-7 NH_4^+ N=4-14).

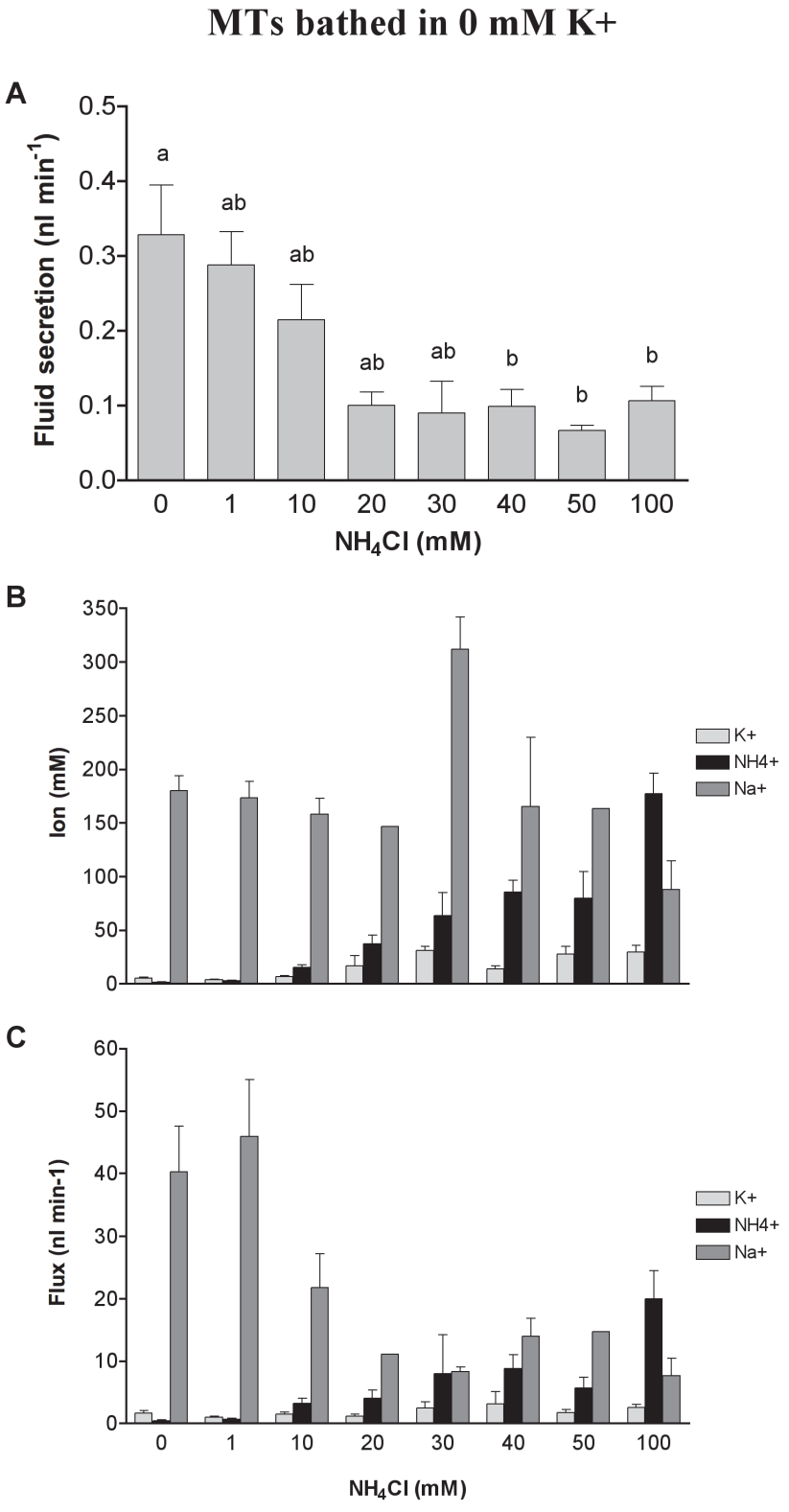
MTs bathed in 0 mM K+
A
Fluid secretion (nl min-1)
0.5
0.4
0.3
0.2
0.1
0.0
a
ab
ab
ab
ab
b
b
b
0
1
10
20
30
40
50
100
NH4Cl (mM)
B
Ion (mM)
350
300
250
200
150
100
50
0
K+
NH4+
Na+
C
Flux (nl min-1)
60
50
40
30
20
10
0
K+
NH4+
Na+
0
1
10
20
30
40
50
100
NH4Cl (mM)

Figure A4.

33 Appendix 6: Secretion Rate of Malpighian Tubules Bathed in 0 or 20 mM K^+

Figure A5. Rates of fluid secretion by Malpighian tubules bathed in *Drosophila* saline containing 0 or 20 mM K^+, in the absence of NH_4Cl. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (one-way ANOVA, $P< 0.05$, 0 mM K^+ N=7 20 mM K^+ N=7).

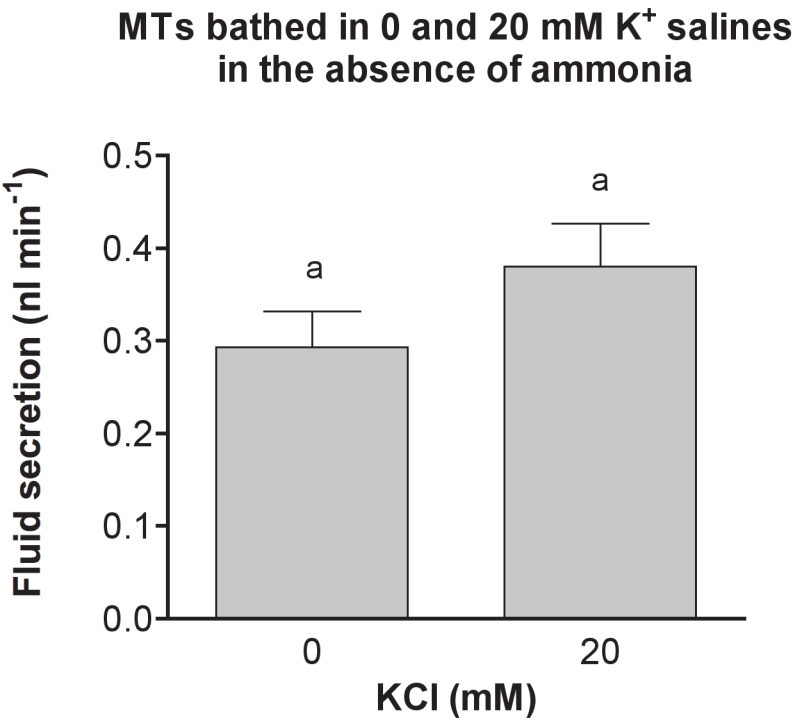
MTs bathed in 0 and 20 mM K+ salines
in the absence of ammonia
Fluid secretion (nl min-1)
0.5
0.4
0.3
0.2
0.1
0.0
a
a
0
20
KCl (mM)

Figure A5.

34 Appendix 7: Water Loss from Small Droplets of Saline Under Oil

Preliminary results indicated that tubules bathed in K^+-free saline had similar rates of fluid secretion to tubules bathed in standard saline, in the absence of ammonia (Appendix 6). Thus it appeared that K^+-free saline would be a viable option for reducing potassium interference in tubular experiments. In the presence of NH_4^+ and K^+-free bathing saline, the rate of fluid secretion declined and droplets collected after 45 minutes were approximately 4.5 nl or less for tubules bathed in 20 mM NH_4^+ or more. These small droplets appeared to be shrink over time thus we measured the volume of simulated droplets (of similar volumes) over time. The volume of saline droplets of approximately 1 nl were significantly reduced within 3 to 4.5 hrs under paraffin oil (Appendices 7A & 7B). In order to determine whether simply water or water and ions were being lost from the droplets, concentration of potassium was measured in simulated droplets under oil. Potassium concentrations in droplets of 1 nl volumes increased from their initial concentration of 20 mM K^+ to roughly 40 mM K^+ within 2 hrs and droplets of 6 nl increased by approximately 2 mM within 1 hr (Appendices 8A & 8B). These results indicated that water was being lost from the droplets, leaving behind ions resulting in concentrated solutions. It should be noted that these experiments were performed in paraffin oil hydrated with deionized water. As a result of the water loss experiments a standard was set for all future experiments utilizing the Ramsay assay: for volumes of secreted fluid $\leq$ 6 nl the measurements needed to be completed within 1 hr of collection.

Figure A6. Effect of water loss on the volume of small droplets under oil. Volumes of small saline droplets were measured over time (A) with the experiment repeated in (B). Volumes were estimated using an eye-piece micrometer at 800x magnification. Data are expressed as mean ± S.E.M. with bars not sharing a letter indicating significance (one-way ANOVA, $P< 0.05$, A $N=5$ B $N=5$).

Effect of Water Loss on Volume of Small Droplets

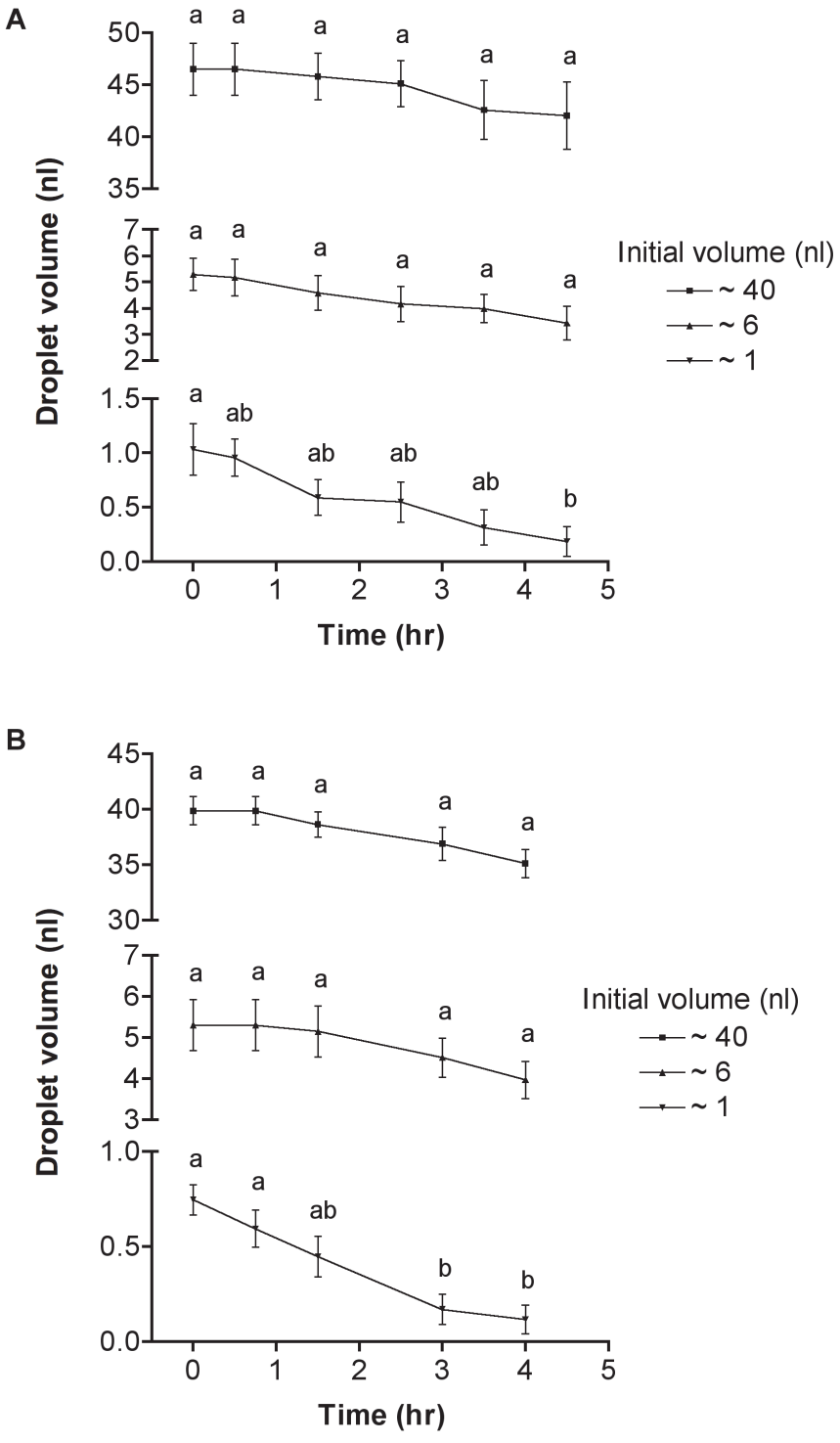

Figure A6.

35 Appendix 8: Increasing Concentration of Small Droplets of Saline Under Oil

Figure A7. Potassium concentration of small *Drosophila* saline droplets measured (A) from a single droplet repeatedly and (B) from separate droplets under oil. Droplets were made from 20 mM K^+ *Drosophila* saline containing no ammonium. Each data point reflects a single measurement.

Effect of Water loss on Concentration of Small Droplets Under Oil

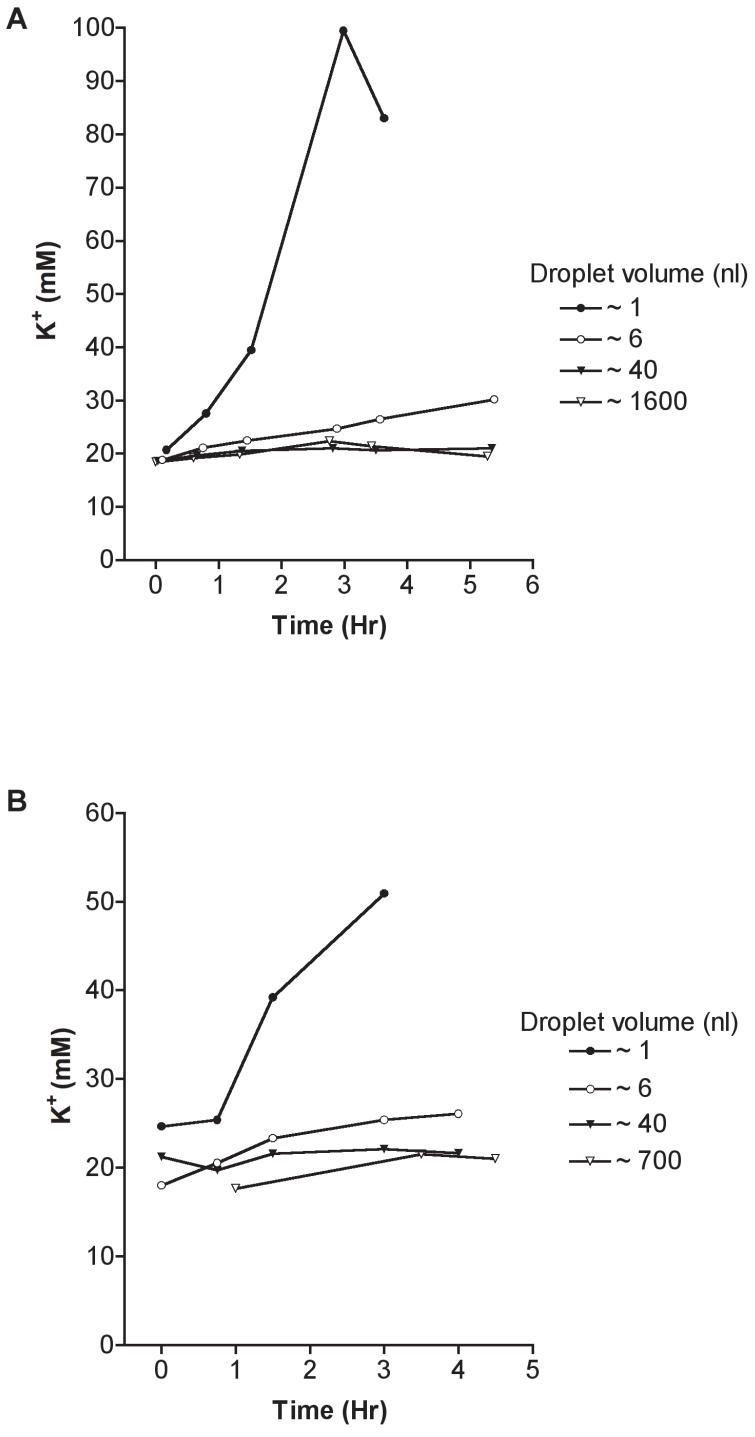

Figure A7.