CALCIUM TRANSPORT BY INSECT MALPIGHIAN TUBULES

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By

AUSTIN A. BROWNE, B.Sc., M.Sc.

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AUTHOR: Austin A. Browne, B.Sc. (Queen's University), M.Sc. (McMaster University)

SUPERVISOR: Dr. Michael J. O'Donnell

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LAY ABSTRACT

This thesis contributes to our understanding of how insects regulate the calcium content of their blood (haemolymph). Using electrophysiological techniques with improved spatial resolution (from millimeters to micrometers) this thesis sought to determine the sites, mechanisms and regulation of Ca^{2+} transport by insect Malpighian (renal) tubules in order to gain insights into the role of Ca-rich granules (similar to those identified in early stages of human kidney stone formation i.e. nephrolithiasis) within these tissues. Using eight insect species this thesis demonstrates that the Malpighian tubules act as dynamic Ca^{2+} stores that appear to be under neuroendocrine control: actively taking up Ca^{2+} through calcium entry channels, where the majority ($\geq$ 90%) of excess haemolymph Ca^{2+} is sequestered within intracellular stores (Ca-rich granules) during period of excess dietary calcium and passively releasing Ca^{2+} back to the haemolymph during periods of metamorphosis or calcium deficiency (i.e. overwintering).

ABSTRACT

Insects maintain blood (haemolymph) Ca^{2+} concentrations within a narrow range in order to support the health of internal tissues and organs. The Malpighian (renal) tubules play a primary role in haemolymph Ca^{2+} homeostasis by sequestering excess Ca^{2+} within calcified biomineral deposits (Ca-rich granules) often located within type I (principal) tubule cells. Using the classic Ramsay assay, the scanning ion-selective microelectrode technique (SIET), and modifications of these two electrophysiological techniques, this thesis begins to unravel the sites and mechanisms of Ca^{2+} transport by the Malpighian tubules isolated from eight insects, representing seven orders. A segment-specific pattern of Ca^{2+} flux was observed along the length of the Malpighian tubules isolated from *D. melanogaster*, *A. aegypti* and *A. domesticus* and was uniform along the length in the remaining species. The majority ($\geq$ 90%) of Ca^{2+} entering the tubule cells is sequestered within intracellular calcium stores in Ca^{2+}-transporting segments of *D. melanogaster* and *A. domesticus* tubules, consistent with the presence of Ca-rich storage granules in these tubule segments. In addition, this thesis provides the first measurements of basolateral Ca^{2+} flux across single principal and secondary tubule cells of *T. ni*, where Ca^{2+} uptake occurs only across principal cells. Perhaps the most important finding of this thesis is that increasing fluid secretion through manipulation of intracellular levels of cAMP or Ca^{2+} in isolated tubules of *A. domesticus* had opposite effects on tubule Ca^{2+} transport. The adenylyl cyclase-cAMP-PKA pathway promotes Ca^{2+} sequestration whereas both 5-hydroxytryptamine and thapsigargin inhibited sequestration. In contrast, tubules of the remaining species were generally insensitive to cAMP or thapsigargin and

rates of tubule Ca^{2+} transport were often very low. The presence of Ca-rich granules in the cells of the midgut in several of the species with low rates of tubule Ca^{2+} transport provide evidence for a putative role of the midgut in haemolymph Ca^{2+} homeostasis. Taken together, these results suggest that the principal cells of the Malpighian tubules contribute to haemolymph calcium homeostasis through neuroendocrine regulated sequestration of excess Ca^{2+} during periods of high dietary calcium intake. Sequestration of dietary Ca^{2+} by the midgut may reduce Ca^{2+} entry into the haemolymph and therefore Ca^{2+} sequestration by the Malpighian tubules need not be so rapid. Finally, reversible tubule Ca^{2+} transport may allow internal reserves of Ca^{2+} (Ca-rich granules) to be returned to the haemolymph allowing insects to survive prolong periods of Ca^{2+} deficiency (i.e. overwintering).

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THESIS FORMAT AND ORGANIZATION

This thesis follows the standard "sandwich Ph.D. thesis" format approved by McMaster University. Chapter 1 provides a general introduction to the field of study, identifies current gaps in knowledge and outlines the objectives of this thesis. Chapters 2, 3 and 4 represent articles published in peer-reviewed journals prior to the completion of this thesis. Chapter 5 provides a general discussion of the major results of this thesis, their implications to calcium homeostasis in insects and provides future directions for further research.

Chapter 1: General Introduction

Chapter 2: Segment-specific Ca^{2+} transport by isolated Malpighian tubules of *Drosophila melanogaster*: A comparison of larval and adult stages
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Chapter 3: Mechanisms of calcium sequestration by isolated Malpighian tubules of the house cricket *Acheta domesticus*
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Chapter 4: Calcium transport across the basolateral membrane of isolated Malpighian tubules: A survey of several insect orders
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Chapter 5: General Discussion

DECLARATION OF ACADEMIC ACHIEVEMENT

Chapters 2, 3 and 4 of this thesis have been prepared as separate manuscripts and published as peer-reviewed journal articles. Experiments, analysis and manuscript preparation were for the most part an individual effort under the guidance of Dr. Michael O'Donnell, Dr. Graham Scott and Dr. Andrew Donini.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine or serotonin
ASIC	acid sensitive ion channels
BAPTA	1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid
BD	Bellini duct
cAMP	cyclic adenosine monophosphate
CaOx	calcium oxalate
CaSRs	calcium-sensing receptors
cGMP	cyclic guanosine monophosphate
CNG	cyclic nucleotide gated
CNS	central nervous system
CRF	corticotropin-releasing factor
CS	corpuscles of Stannius
CT	calcitonin
DAG	diacylglycerol
DMSO	dimethyl solfoxide
ECaC	epithelial calcium channel
ECF	extracellular fluid
EG	ethylene glycol
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPAC	exchange proteins activated by cAMP
GPCR	G protein-coupled receptors
HA	hydroxyapatite
HLP	hydroxyl-L-proline
HPLC	high-performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
IMCD	inner medullary collecting duct
IP_3	inositol triphosphate
ISME	ion-selective microelectrode
micro-CT	micro-computed tomography
MRC	mitochondrial-rich cell
MT	Malpighian tubule
NaOx	sodium oxalate
NCX	Na^+/Ca^{2+} exchange
NO	nitric oxide
PKA	protein kinase A
PMCA	plasma membrane Ca^{2+}-ATPase
PTH	parathyroid hormone
RNA-Seq	RNA sequencing
RP	Randall's plaque
SIET	scanning ion-selective microelectrode technique
SPCA	secretory pathway Ca^{2+}-ATPase

TRP	transient receptor potential
VHA	V-type H^+-ATPase
XDH	xanthine dehydrogenase

Chapter 1

1. General Introduction

1.1. Extracellular calcium regulation: General strategies

In cells from prokaryotes to eukaryotes, the calcium ion (Ca^{2+}) concentration of the intracellular environment is held approximately 10,000-fold lower (~ 100 nM) than the extracellular environment (~ 1 mM) by a Ca^{2+} homeostatic system. The steep inward Ca^{2+} gradient is maintained by balancing rates of Ca^{2+} influx through pathways such as Ca^{2+} channels with Ca^{2+} efflux through ionomotive pumps and ion exchangers. However, high concentrations of Ca^{2+} in cells causes aggregation of proteins and nucleic acids, alters lipid membrane integrity

and initiates the precipitation of calcium phosphates, thus necessitating evolution of a system to keep intracellular Ca^{2+} activity low (Case et al., 2007). In multicellular organisms the extracellular fluid, in particular the interstitial fluid, bathes all the cells of the body and therefore its ionic composition (Ca^{2+}, Na^{+}, K^{+}, Cl^{-} etc.) is tightly regulated. All animals have essentially two milieus: an external milieu in contact with the external surfaces of the animal and the lumen of the gut, and the internal milieu in contact with the tissues (Larsen et al., 2014). The goal of an extracellular Ca^{2+} homeostatic system is to maintain extracellular fluid Ca^{2+} concentrations within a limited range that support cell and tissue health. In animals, the setpoint for extracellular Ca^{2+} concentration is in the millimolar range.

Generally, there are two patterns for extracellular Ca^{2+} homeostasis: first, an open pattern of regulation where Ca^{2+} is exchanged directly with the environment and second, a closed pattern of regulation where Ca^{2+} is exchanged predominantly between the internal environment and isolated calcium stores. In practice, organisms may not strictly follow one particular pattern or the other, but instead often contain elements of both. Aquatic environments provide a virtually unlimited supply of calcium (although calcium concentrations may vary) and thus aquatic organisms tend to have open patterns of calcium regulation. In contrast, terrestrial animals rely on discontinuous dietary sources of calcium and therefore tend to have more closed patterns of calcium regulation. There are several benefits of a more closed strategy, which rely on internal calcium stores: first, calcium stores accumulated during periods of excess provide a supply of Ca during periods of deficiency. For instance, internal Ca reserves enable terrestrial animals to survive prolonged periods without feeding (i.e. starvation or overwintering). Secondly, calcium is often stored internally in concentrated forms (as Ca-rich granules, for example) that may allow for more rapid calcium mobilization compared to extraction from the environment, especially from dilute freshwaters. Thirdly, readily exchangeable internal calcium stores can 'buffer' Ca^{2+} in extracellular fluids during large changes in environmental calcium. Fourthly, calcium-containing biominerals are frequently used to harden the tissues in which they are deposited providing structural support (skeletons) in addition to storage. Finally, mechanisms involved in calcium sequestration/storage are thought to also play a role in detoxifying non-nutrient metals, such as Mn, Fe, Zn, Cd, Cu, Ni, Ba (Ballan-Dufrancais, 2002, Lipovsek Delakorda et al., 2009, Leonard et al., 2009,

Schofield et al., 1997). In the subsequent sections, I will examine calcium homeostasis in three diverse groups: fish (aquatic vertebrates), mammals (terrestrial vertebrates) and crustaceans (aquatic and terrestrial invertebrates) in order to gain insights that may be applicable to considerations of calcium homeostasis in insects.

1.2. Calcium homeostasis in fish

The fishes are arguably one of the most evolutionarily successful aquatic organisms containing more species (~ 30,000) than any other class of vertebrate (Chapman, 2009). At least part of their success likely stems from their ability to regulate ion and water transport across their specialized gills. The calcium concentration of the surrounding water is the primary factor influencing body calcium regulation in fish either directly or indirectly (through hormone control). Calcium is taken up from the water across the gills and, to a lesser extent, from ingested food/water across the intestine (Flik and Verbost, 1993). Regulated Ca^{2+} transport across gills, intestine, kidney and bone likely contribute to extracellular Ca^{2+} homeostasis. Plasma calcium concentrations are maintained within a limited range, 1.3 to 1.5 mM Ca^{2+}, suggesting extracellular Ca^{2+} is well regulated in fish (Kaneko and Hirano, 1993). In the freshwater tilapia, *O. mossambicus*, acclimation to low Ca^{2+} fresh water (from 0.8 to 0.2 mM Ca) increases the proportion of readily exchangeable $^{45}Ca^{2+}$ in the bone by about 2-fold and total plasma Ca content (from 2.7 to 2.9 mM Ca) as total bone density and Ca content is reduced, suggesting that some calcium stored in the bones of fish can be mobilized during periods of low calcium stress (Flik et al., 1986). Bone may thus provide a calcium 'buffer'

function under conditions of increased whole-body Ca^{2+} turnover. Regulation of extracellular calcium homeostasis is thought to be achieved through a bi-directional control system using circulating hormones with both hyper- and hypocalcemic effects. The pituitary gland produces hypercalcemic hormones (prolactin and somatolactin) and the corpuscles of Stannius (CS) produce a hypocalcemic hormone (stanniocalcin) (Kaneko and Hirano, 1993). In aquatic environments hypocalcemic regulation is considered to be the dominant pathway for extracellular calcium homeostasis given the general excess supply of calcium in waters. Calcium-sensing receptors (CaSRs) are G protein-coupled receptors (GPCR) that are expressed in endocrine glands where they detect changes in extracellular fluid Ca^{2+} concentrations and correspondingly effect the secretion of the calciotropic hormones into the circulation (Vezzoli et al., 2009). CaSRs have been implicated in the regulation of stanniocalcin in the flounder, trout and zebrafish (Greenwood et al., 2009, Radman et al., 2002, Lin et al., 2014). The cellular machinery responsible for Ca^{2+} transport across gills is clearly important for extracellular calcium homeostasis in fish. In zebrafish, transcellular Ca^{2+} uptake by the gills is believed to occur across mitochondrial-rich cells (MRCs), or ionocytes, with apical entry from water through epithelial calcium channels (ECaC) and exit into blood across basolateral membranes via plasma membrane Ca^{2+}-ATPase (PMCA) and Na^{+}/Ca^{2+} exchange (NCX) transporters (Lin and Hwang, 2016). In aggregate, these data suggest that fish maintain their extracellular calcium concentrations predominantly through Ca^{2+} transport across the gills controlled by both hypo- and hypercalcemic hormones involving CaSRs. Although insects lack gills and contain waxy cuticles that minimize water and ion

transport across the body surface, mechanisms of Ca^{2+} absorption across the gut of insects may show some similarities to the mechanisms of Ca^{2+} absorption across the gills of fish.

1.3. Calcium homeostasis in mammals

In contrast to aquatic vertebrates, terrestrial vertebrates rely to a greater extent on internal calcium stores and a more closed regulatory system to maintain extracellular Ca^{2+} homeostasis. On land, the diet is the major source of environmental calcium for tetrapods (terrestrial vertebrates), which absorb most nutrients across the gut. In humans, a relatively small percentage of total body calcium is found in the extracellular fluids (0.1%) with greater amounts in cells (1%) and bones (98.9 %) (Hall, 2011). Ca^{2+} transport across the intestine, kidney and bone contribute to extracellular Ca^{2+} homeostasis. Total serum Ca concentrations are tightly regulated in mammals within a narrow range of about 2.2 to 2.5 mM (Blaine et al., 2015). As in fish, extracellular calcium concentration in mammals is regulated by both hypercalcemic (parathyroid hormone and vitamin D) and hypocalcaemic (calcitonin) circulating hormones (Hall, 2011). Parathyroid hormone (PTH) is the dominant hormone for controlling extracellular Ca^{2+} concentrations in mammals, which regulates Ca^{2+} transport by intestine, kidney and bone. In turn, PTH secretion is controlled by calcium-sensing receptors (CaSRs) that detect changes in extracellular Ca^{2+} concentration and, when stimulated by increases in extracellular Ca^{2+}, decrease PTH secretion (Brown and MacLeod, 2001). In humans and mice, loss-of-function mutations in the CaSR cause hypercalcemia and gain-of-function mutations cause hypocalcemia (Tfelt-Hansen and Brown, 2005). PTH acts to: (1) increase bone Ca^{2+} resorption (releasing Ca^{2+} into the extracellular fluid) (2) increase kidney tubule Ca^{2+}

reabsorption and (3) promote conversion of vitamin D to its active form (1,25-dihydroxycholecalciferol), which increases intestinal Ca^{2+} absorption (Hall, 2011). PTH thus increases plasma Ca^{2+} concentration back to the setpoint (hypercalcemic effects). In contrast, calcitonin, a hypocalcemic hormone secreted by the thyroid gland has, in general, the opposite effects of PTH. Calcitonin secretion by thyroid parafollicular cells (C cells) is also thought to be mediated by CaSRs in response to changes in extracellular Ca^{2+} concentration (Garrett et al., 1995). It worth mentioning that calcitonin has a weak effect on plasma calcium in adults and its actions are most pronounced in children (and mice that constantly grow in size) when bone remodeling is most rapid (Brown and MacLeod, 2001). Finally, it is apparent that Ca^{2+} itself functions as a local and/or systemic Ca^{2+}-lowering "hormone" through its actions on CaSRs (Brown and MacLeod, 2001).

Mechanisms of transepithelial Ca^{2+} transport across intestine and kidney are well understood. In order to maintain extracellular calcium homeostasis the kidney must remove Ca^{2+} from the blood at the same rate it is absorbed into the blood across the intestine. Absorption of Ca^{2+} transport across intestine occurs by transcellular (through cells) and paracellular (between cells) pathways (Blaine et al., 2015). The transcellular pathways for both Ca^{2+} absorption by intestine and Ca^{2+} reabsorption in kidney tubules involve apical Ca^{2+} uptake by epithelial calcium channels (ECaCs) of the transient receptor potential family (TRPV5/TRPV6) and exit into the blood through basolaterally located plasma membrane Ca^{2+}-ATPase (PMCA) and Na^{+}/Ca^{2+} exchange (NCX) transporters (Hoenderop et al., 2005). More recently, activation of the CaSR in renal

tubule cells was found to stimulate TRPV5-mediated currents, suggesting a role for CaSR in detecting Ca^{2+} concentrations in the pro-urine and subsequently influencing renal Ca^{2+} transport (Topala et al., 2009).

During acute/large changes in dietary calcium, large pools of readily exchangeable Ca^{2+} in bone provide a Ca^{2+} 'buffering' function that blunts changes in extracellular Ca^{2+} concentrations. The relative importance of this exchange between bone fluid (fluid contained within the bone lining cells, which form a continuum) and extracellular fluid (ECF) is often underappreciated (Mundy and Guise, 1999). It is worth noting that although there is continuous resorption and formation of bones in adults, there is generally no net gain or loss of calcium from bone (van Os, 1987). Ca^{2+} exchange between bone and ECF provides the first line of defense against changes in plasma Ca^{2+} concentrations even before the PTH and calcitonin hormonal systems can respond (Hall, 2011). During prolonged changes in dietary calcium (or in the complete depletion/saturation of calcium stores) the hormonal feedback systems are most important in maintaining calcium homeostasis.

In aggregate, these results suggest that blood calcium regulation is precisely maintained by a complex regulatory system involving extracellular Ca^{2+} sensors, calciotropic hormones, specialized Ca^{2+} transporting epithelia, Ca^{2+} pumps, channels and exchangers, intracellular and extracellular Ca^{2+} buffers and several internal Ca^{2+} storage sites. It has been suggested that some aspects of the mammalian tripartite (PTH, vitamin D, calcitonin) hormonal system may regulate the formation of calcified structures in invertebrates (Luquet and Marin, 2004). Finally, increasing functional roles of CaSRs in

extracellular Ca^{2+} homeostasis in both fish and mammals lead to the possibility of a similar role of CaSRs in the invertebrates.

1.4. Calcium homeostasis in crustaceans

Invertebrates also maintain extracellular (haemolymph) calcium concentrations, although less precisely than do vertebrates (Taylor, 1987). Rather than Ca-rich endoskeletons, some invertebrates use exoskeletons to provide protection and structural support for internal organs. Rigid exoskeletons physically constrain the size of arthropods and therefore must be shed (molted) during growth. Of the invertebrates, crustaceans represent good models for investigating calcium homeostasis given that their exoskeletons are calcified (containing much calcium carbonate) and therefore must cope with the inevitable loss of calcium during each molting cycle. Furthermore, crustaceans occupy a wide variety of environments ranging from aquatic (marine to freshwater) to terrestrial (shore to forest) species, which allowing some insights into the alterations of Ca transport mechanisms during evolution of terrestrial forms (Wheatly, 1999). Calcium is taken up predominantly across the gills in aquatic species and predominantly across the digestive epithelium in terrestrial species. Regulated Ca^{2+} transport across the gill, digestive epithelium, antennal gland (analog of the kidney) and cuticular hypodermis contribute to extracellular Ca^{2+} homeostasis in crustaceans. During the molting cycle haemolymph Ca^{2+} concentrations are maintained despite large Ca^{2+} fluxes between transient internal calcium stores and the calcified exoskeleton (cuticle). Crustaceans spend most of their time in intermolt with fully calcified cuticles and during this period Ca^{2+} fluxes across Ca^{2+}-transporting epithelia are relatively low as the body is in calcium

homeostasis (Greenaway, 1985). Total haemolymph calcium concentrations are held at about 12 mM Ca throughout the intermolt in a range of decapods (Wheatly, 1999) suggestive of tight haemolymph Ca^{2+} regulation. In the relatively brief premolt period, calcium is resorbed from the cuticle and either lost to the environment (excreted) or stored in amorphous form (Wheatly, 1997). The stored calcium represents 4% to 75% of the calcium required for the complete calcification of the exoskeleton (Luquet and Marin, 2004). Generally, in marine species losses are high compared to the amount stored, whereas in terrestrial species losses are reduced through enhanced storage. At ecdysis, any calcium remaining in the old cuticle is lost when the old cuticle is cast off. Immediately after ecdysis, the new cuticle is calcified, first using calcium mobilized from internal deposits then other sources, including the water (aquatic species) and food (aquatic and terrestrial). The period between ecdysis and full calcification of the new cuticle is a time of increased vulnerability to predation and so internal calcium stores (in amorphous form) may have evolved to decrease the duration of cuticular calcification. In addition, mouthparts are often calcified early allowing many terrestrial species to quickly ingest the old cuticle after it is shed (exuviae) to reclaim any residual calcium contained within it (~ 30% of body Ca in freshwater species, (Wheatly, 1997)).

Regulation of haemolymph calcium concentrations is poorly understood in crustaceans but is believed to involve vertebrate-like hormones, such as vitamin D and calcitonin (Wheatly, 1999). In addition, the molting cycle is under hormonal regulation by ecdysone and given the dramatic changes in calcium transport during the molting

cycle it has been speculated that ecdysone may play an indirect role in calcium homeostasis in crustaceans (Luquet and Marin, 2004).

Epithelial Ca^{2+} transport is believed to be predominantly transcellular (through cells) in crustaceans facilitated by membrane pumps, channels and exchangers. For example, gene expression of both plasma membrane Ca^{2+}-ATPase (PMCA) and Na^{+}/Ca^{2+} exchangers (NCX) in the anterior sternal epithelium (surrounding the $CaCO_3$ stores) of the terrestrial isopod, *P. scaber*, increases during Ca^{2+}-transporting stages (late premolt and intramolt) compared to non-transporting stages (Ziegler et al., 2002). In a subsequent study of the same epithelium, the expression of V-type H^{+}-ATPase (VHA) was also increased during Ca^{2+}-transporting stages and reversed polarity from basolateral to apical localization, correlated to the mineralization-demineralization cycle (Ziegler et al., 2004). Similarily, a proposed model of transcellular Ca^{2+} transport in crustacean epithelial cells (gills, antennal glands and hepatopancreas) indicates the involvement of PMCA, $Na^{+}/Ca^{2+}(H^{+})$ exchange (in various stoichiometries) and verapamil/nifedipine-sensitive calcium channels (Ahearn et al., 2004).

In summary, crustaceans maintain extracellular calcium homeostasis within narrow limits throughout periods of calcium stress (each molt). Like fish and mammals, calcium homeostasis is achieved by the actions of Ca^{2+}-transporting epithelia that contain a similar compliment of Ca^{2+} transport proteins. A greater reliance on internal calcium stores in terrestrial species using Ca-rich granules reflects their attempt to reduce calcium losses during the molt. Mechanisms of Ca^{2+} sequestration and Ca-rich granule formation in crustaceans (invertebrates), and especially in the terrestrial isopods, are likely to be

most similar to those of insects compared to fish or mammals given their closer relations, and in the case of the isopods, their similarity in size.

1.5. Summary of calcium homeostasis: Lessons from comparative physiology (fish, mammals and crustaceans)

Internal storage of Ca^{2+} in mineralized form is an ubiquitous and evolutionarily ancient strategy that allows organisms to maintain the internal calcium environment independently of their external environment. Sources of environmental calcium are less stable in terrestrial environments, compared to aquatic pools, and terrestrial organisms must overcome this additional challenge to internal calcium homeostasis. They have succeeded, in this regard, by elaborating and relying on internal calcium stores to a greater extent than their aquatic counterparts. Extracellular calcium homeostasis is achieved through regulated Ca^{2+} transport across the various Ca^{2+}-transporting epithelia: mainly the intestine, kidney and membranes surrounding internal calcium stores (i.e. bone or the various Ca-rich deposits of invertebrates). Ca^{2+} exchange between bone and extracellular fluid may be analogous to Ca^{2+} exchange between Ca-rich granules and haemolymph of invertebrates. Fish and aquatic crustaceans may also transport Ca^{2+} across the gills. Essentially all cells (and to a greater extent specialized Ca^{2+}-transporting epithelia) contain Ca^{2+} channels, NCX and PMCA transport proteins, suggesting that mechanisms of cellular Ca^{2+} transport (including calcium signaling) are conserved in many vertebrate and invertebrate species (Simkiss, 1996, Griffith, 2017). In crustaceans, precipitation of calcium within biomineralizations is also aided by a proton pump, H^{+}-ATPase, thought to control the local pH and thus solubility of calcium salts at the site of

mineralization (Ziegler et al., 2004). Conversely, acidification of the mineralized microenvironment by a H^+-ATPase during Ca^{2+} mobilization would allow Ca^{2+} to be maintained in its soluble form, which can then be readily transported. The pumps, channels and exchange proteins that facilitate epithelial Ca^{2+} transport are often controlled by the concerted actions of calciotropic hormones that both increase (hypercalcemic) and decrease (hypocalcemic) extracellular Ca^{2+} concentrations (i.e. a bi-directional control mechanism). It appears that the active form of vitamin D plays a role in calcium homeostasis in crustaceans, fish and mammals. Moreover, vitamin D or its polar metabolites show biological activity in yeast, plants, some invertebrates and is also essential for bone homeostasis in amphibians, birds and mammals (Bouillon and Suda, 2014). There is evidence across the animal kingdom that calcium-sensing receptors (CaSRs) located in many Ca^{2+}-transporting epithelia, or remotely, play a role in maintaining systemic/local calcium homeostasis, possibly by modulating calciotropic hormone secretion (as in PTH) and/or regulating important cellular functions (Tfelt-Hansen and Brown, 2005). Taken together, these data suggest that CaSRs and hormones that are conserved in vertebrates may have arisen early in evolution and may play a role in extracellular Ca^{2+} homeostasis in insects.

1.6. Metal storage & detoxification

Calcium plays important roles in cell division, growth and metabolism, secretion, ion transport, blood clotting, programmed cell death (apoptosis), muscle contraction, nerve conduction and other aspects of cell function. Clinical symptoms of acute disturbances in calcium homeostasis are mostly related to changes in nerve and muscle

function (Taylor, 1987). For example, extracellular Ca^{2+} negatively influences neuronal excitability; hypercalcemia results in central nervous system (CNS) depression (Hall, 2011). In addition, chronic renal failure is thought to be a result of cellular Ca^{2+} toxicity brought about by altered Ca^{2+} transport (enhanced Ca^{2+} influx through calcium channels and decreased Ca^{2+} efflux by Ca^{2+}-ATPase pumps) that result in significant elevation in the basal levels of cytosolic calcium (Massry and Fadda, 1993). Further, elevations in cytosolic [Ca^{2+}] are believed to be responsible for neuronal death (excitotoxicity) during cerebral ischemia (e.g. stroke) through effects on ion transporters, such as transient receptor potential (TRP) calcium channels, Na^{+}/Ca^{2+}-exchanger (NCX), acid sensitive ion channels (ASIC) in addition to ionotropic glutamate receptor subtypes (Annunziato et al., 2007). It is clear that dysregulation of Ca^{2+} transport is toxic to cells and tissues of all organisms and therefore cells require metal storage systems. Metal storage systems allow the accumulation of high intracellular concentrations of metal ions without the toxic effects that such accumulations would otherwise entail (Bouillon and Suda, 2014). In other words, many of the mechanisms of metal ion storage also play a role in detoxification of either non-physiological or physiological metal ions that can cause toxicity. There are two main strategies for metal ion storage that also serve to prevent toxicity due to overload of metal ions. Firstly, metal ions may be bound by cytoplasmic proteins (such as metallothionein and ferritin found in vertebrates, plants, some fungi and bacteria) or secondly, metal ions may be sequestered within membrane-bound compartments within the cell (Lyons and Eide, 2007). Physiological ions, such as Ca^{2+}, are generally more abundant than non-nutrient metals in the body and therefore likely

require higher capacity stores. Storing metals within an isolated space (i.e. membrane-bound stores), where the metal is less damaging may allow for higher density calcium storage.

1.7. Mineralized calcium stores – Ca-rich granules

Intracellular mineral deposits that are often amorphous, concentrically layered and spherical in nature occur in virtually every phylum of animals (Simkiss, 1977). These metal-containing granules are generally small, with diameters most commonly between 0.5 and 2 μm in invertebrates (Brown, 1982). The composition of metal-containing granules in invertebrates is highly variable and can contain a variety of elements: including C, O, N, Ca, Mg, Na, K, Cl, P, S, Fe, Zn, Cu, Ni, Pb, Ba and Mn (Ballan-Dufrancais, 2002, Lipovsek Delakorda et al., 2009, Brown, 1982). Based on elemental analysis, calcium-rich granules are thought to be principally composed of calcium carbonates or phosphates embedded within a protein matrix (Brown, 1982). Many factors affect the appearance and composition of the granules: biological cycles, feeding and diet, age and size, environmental background and tolerance (Brown, 1982). The presence of toxic metals within Ca-rich granules has lead others to suggest that granule formation (biomineralization) may represent a cellular route for the detoxification of heavy metal ions through a common mechanism of metal sequestration (Simkiss, 1977). Proposed functions of the granules include: ion stores, high-energy (PO_4) stores, detoxification sites, or acid-base regulators (Simkiss, 1996). Ca-rich granules may provide additional benefits to small animals in two ways. First, mineralization (especially crystallization) necessarily excludes water and therefore provides an efficient means of storing calcium in

concentrated form while minimizing water loss. Small animals have a high surface area:volume ratio and are therefore at greater risk of desiccation than larger animals. Second, the classic problem of internal storage is that of space and small animals will tend to have less available 'space' for internal storage. Thus Ca-rich granules may be particularly beneficial in small animals, such as crustaceans and insects.

Although there is no unified hypothesis for the formation of mineralized calcium storage in animals, some common themes have emerged. Most models of calcium mineralization envision that calcium, in its biologically active ionic form is taken up from an extracellular source (aquatic milieu or body fluids) by specialized Ca^{2+}-transporting cells (or epithelium) where Ca^{2+} is mineralized within specialized vesicles (Weiner and Addadi, 2011). Mineral-bearing vesicles can either remain within the cells or be transported to various subcellular or extracellular compartments or excreted from the body. Often the mineral within the vesicles is transported to the crystallization front within a mineralizing tissue where the initial disordered mineral phase is transformed into a more ordered phase of the mature mineralized tissue (Weiner and Addadi, 2011). Disordered mineral phases dissolve more rapidly than pure crystals and are thus advantageous for temporary calcium storage (Simkiss, 1996). Ca-rich granules are often allowed to accumulate in storage structures, which vary in location and size but generally are more elaborate in terrestrial species. The use of amorphous Ca-rich granules for internal calcium storage is widespread in terrestrial crustaceans and therefore several examples will be described in further detail below:

Some crustaceans attempt to reduce cuticular calcium losses during each molt by temporarily transferring calcium from their calcified cuticles to internal storage sites, which can then be remobilized to calcify the newly formed cuticle following ecdysis. Calcium is stored, frequently as amorphous calcium phosphate/carbonate granules, in various body compartments throughout crustacea: in cells of the hepatopancreas (Brachyura), gastroliths (Astacidea), haemocoel, posterior midgut cecae (Amphipoda) or ventral storage sites as in Isopoda (Greenaway, 1985). In decapods (crayfish and crabs), Ca can be stored as mineralized granules within the haemolymph or hepatopancreas or as gastroliths within the stomach (Luquet and Marin, 2004). For instance, in the freshwater land crab, *Holthuisana transversa*, 66% of the total body calcium of the intermolt is stored within spherical $CaPO_4$-rich granules of the haemolymph leading to a milky appearance of the haemolymph (Sparkes and Greenaway, 1984). This strategy allows total haemolymph Ca concentrations to rise to nearly 2 M during the premolt whereas Ca^{2+} activity fell by only 0.15 mM Ca^{2+}, suggesting extracellular Ca^{2+} is well regulated even with high background levels of bound calcium. In contrast, in almost all terrestrial isopods, such as *P. scaber*, Ca is stored as agglomerations of spherical $CaCO_3$-rich granules within anterior sternites (Fabritius and Ziegler, 2003). In the amphipod, *O. cavimana*, calcium originating from the old cuticle is stored as amorphous calcium carbonate granules (representing 44% of the new cuticle Ca content) within the posterior caecae, which are paired diverticula of the midgut that superficially resemble the Malpighian tubules of insects (Luquet and Marin, 2004). Interestingly, although the same amount of calcium is transported throughout the premolt and postmolt periods, the time

for mobilization of the calcium stores (~ 48 hours) is much more rapid than the time for the stores to be accumulated (~16 days). Taken together, these data suggest that amorphous calcium-rich granules act as dynamic internal calcium stores that may be rapidly mobilized into the haemolymph while maintaining extracellular Ca^{2+} homeostasis.

1.8. Patterns of extracellular calcium regulation in insects

Like other animals, insects maintain the Ca^{2+} concentration of their extracellular fluid (haemolymph) within a narrow range, suggesting haemolymph Ca^{2+} is well regulated. The chief excretory organs of insects, the Malpighian (renal) tubules are thought to play a major role in haemolymph Ca^{2+} homeostasis by eliminating excess Ca^{2+} from the haemolymph by incorporating it into the primary urine (secretion) or by forming calcified granules within the tubule cells (sequestration) (Dube et al., 2000a). The contribution of the gut (midgut or hindgut) to calcium homeostasis has received much less attention. In one study of $^{45}Ca^{2+}$ absorption by isolated midguts of the adult blowfly, *Calliphora vicina*, (Taylor, 1985b) concluded that Ca^{2+} absorption is unregulated and therefore is not a major site of haemolymph calcium homeostasis. Unregulated Ca^{2+} transport by the gut is in contrast to the vitamin D pathway previously described for mammalian intestine. In addition, intestinal Ca^{2+} absorption in fish is negatively regulated by stanniocalcin (Flik and Verbost, 1993). Furthermore, the immunocytological localization of 1,25-dihydroxyvitamin D_3-like molecules and their receptors to the digestive Ca^{2+}-transporting epithelium of the terrestrial crustacean, *Orchestia cavimana*, is also suggestive of regulated Ca^{2+} transport by the gut of terrestrial crustaceans. It is unclear why Ca^{2+} absorption would be unregulated in insects given that calcium in the

diet may exceed requirements, especially in insects feeding on vertebrate blood. The contribution of the hindgut, if any, to haemolymph calcium homeostasis is currently unclear. Likewise, the identity of any haemolymph-bourne calciotropic hormones that influence haemolymph calcium regulation has yet to be uncovered. Working towards a more complete picture of global aspects of extracellular calcium homeostasis in insects, I will describe in subsequent sections the sites and mechanisms of Ca^{2+} transport by Malpighian tubule and their pivotal role in haemolymph calcium homeostasis in insects.

1.9. Haemolymph calcium

In insects, haemolymph is the predominant extracellular organic fluid that bathes all internal organs and contains about 1.5 to 51 mM total calcium, typically higher than the 2-3 mM found in vertebrates (Taylor, 1987). Since acidic amino acids (with anionic character) bind divalent cations, most calcium in haemolymph is bound to protein. Consistent with this idea are values of calcium activity, as measured with Ca^{2+}-selective microelectrodes, in the range of 0.5 to 2.5 mM (Dube et al., 2000b, O'Donnell and Maddrell, 1995, Maddrell et al., 1991, Krueger et al., 1988, Stewart et al., 1994). Increases in dietary calcium have revealed strict regulation of haemolymph calcium. For example, in the silkmoth, *Hyalophora cecropia*, increasing dietary calcium from 56 to 285 mEq l^{-1} had no effect on haemolymph calcium concentration with a mean of 10 mEq l^{-1}(Jungreis et al., 1973). In addition, calcium concentration of the haemolymph was unchanged in the cockroach *Periplaneta americana* when fed a variety of diets, although the calcium content of the fed diets was not explicitly measured (Pichon, 1970). Lastly, haemolymph calcium concentrations (~ 0.5 mM) in the adult fruit fly, *Drosophila*

melanogaster, were unchanged when raised on diets containing 2.5 to 15.6 mM $CaCl_2$ (Dube et al., 2000b). Similarily, in the blowfly, *Calliphora vicina*, haemolymph calcium concentrations rose only 0.6 mM Ca following an increase (from 0 to 12.5 mM $CaCl_2$) in the calcium content of the diet (Taylor, 1985b).

If Ca^{2+} absorption is unregulated then insects are likely concerned mostly with excreting excess Ca^{2+} from the haemolymph, especially when calcium in the diet is in excess of requirements. Hematophagous (blood-feeding) insects tend to imbibe large volumes of blood relative to their body size. Vertebrate blood containing approximately 2 mM Ca^{2+} would more than likely represent a Ca^{2+} load that exceeds any requirements associated with metabolism or reproduction (i.e. deposition in eggs). In addition, during the rapid postprandial diuresis, fluid absorption is enhanced, which concentrates the solutes remaining in the lumen of the gut (Maddrell et al., 1991). Elevated Ca^{2+} concentrations in the gut would likely enhance passive Ca^{2+} entry into the haemolymph. Thus unregulated (and potentially accelerated) Ca^{2+} absorption in blood feeders must be matched with equally high rates of Ca^{2+} removal by excretory mechanisms in order to maintain haemolymph Ca^{2+} homeostasis.

The excretory system in insects consists of the Malpighian tubules and hindgut. The Malpighian tubules remove small solutes (including Ca^{2+}) from the haemolymph and transfer them to the gut lumen within the primary urine. The hindgut is located downstream of the Malpighian tubules where useful solutes and water are reabsorbed leaving wastes behind in the gut lumen to be excreted. As mentioned above, the role of the hindgut in haemolymph Ca^{2+} homeostasis is virtually unexplored. Taken together,

these results indicate that insects have the ability to efficiently regulate their haemolymph calcium concentrations by removing excess Ca^{2+} in the haemolymph by excretion.

1.10. Malpighian tubules are sites of extracellular calcium regulation

Insects are able to accumulate minerals in nearly all organs but the nervous system and muscle tissue (Ballan-Dufrancais, 2002). Thus it is not surprising that calcium-containing minerals are often abundant within the Malpighian tubules of insects. In the fruit fly, *Drosophila melanogaster*, Malpighian tubules contain approximately 30% of the calcium content of the whole fly (Dube et al., 2000b). All 4 tubules isolated from adults of blowfly, *Calliphora vicina,* or larvae of the fruit fly, *Drosophila hydei,* contain approximately 25% and 88% of the calcium content of the entire flies, respectively (Dube et al., 2000b, Taylor, 1985b, Wessing and Zierold, 1992). The calcium content of the Malpighian tubules isolated from *Drosophila hydei* and *Calliphora vicina*, increased approximately 4-fold when fed on a diets containing calcium phosphate (100 mg Kg^{-1} diet) or calcium chloride (12.5 mM) within 9 days and 3.5 days, respectively (Taylor, 1985b, Wessing and Zierold, 1992). The majority of the calcium content within Malpighian tubules is attributable to calcium-containing granules found in the lumen. These granules are relatively small (0.2 to 10 um) spherical concretions composed of an organic matrix on/in which minerals aggregate successively as concentric layers (Wessing et al., 1992). Often these aggregates of calcium-containing granules are so densely populated within the lumen that they can distend the outer diameter of the tubule. Regions where significant granule accumulation occurs often appear opaque (white) in contrast to

the translucent nature of tubular cells. Although the lumen of the tubule can become completely occluded the presence of granules appears not to be detrimental (Maddrell et al., 1991, Wessing et al., 1992, Hazelton et al., 2001, Herbst and Bradley, 1989). In response to an acute increase in dietary $CaCl_2$, calcium content of whole flies (*Calliphora vicina*) more than doubled in 86 hours with approximately 70% of the increase accounted for by the Malpighian tubules (Taylor, 1985b). In addition, the Malpighian tubules were identified as the site of most rapid calcium turnover, with specific activity of the anterior tubules being higher than posterior tubules in response to dietary $^{45}CaCl_2$. These results clearly establish the Malpighian tubules as major sites of haemolymph calcium regulation in insects, where haemolymph calcium regulation is achieved through regulated Ca^{2+} transport between haemolymph and internal calcium stores found within the Malpighian tubules (i.e. Ca-rich granules).

1.11. Relative roles of sequestration and secretion

Excretion is broadly defined as any process that removes a substance from interacting with cells and tissues (Nation, 2001). Thus the role of the Malpighian tubule in calcium homeostasis is clearly excretory. Malpighian tubules may excrete calcium in two ways: by sequestration or by secretion. Sequestration refers to the temporary or permanent storage of calcium in an insoluble form, whereas secretion refers to the incorporation of calcium as a solute into the primary urine. Transepithelial Ca^{2+} secretion can be measured using Ca^{2+}-selective microelectrodes in droplets of fluid secreted by isolated tubules set up in an in vitro assay first developed by J. Arthur Ramsay and hereafter referred to as the Ramsay assay. Ca^{2+} sequestration can be estimated from the

difference between basolateral Ca^{2+} fluxes measured by radioisotopic methods or by the scanning ion-selective electrode technique (SIET), and transepithelial fluxes measured by the Ramsay assay. In the blood sucking insect, *Rhodnius prolixus*, the majority (> 55%) of $^{45}Ca^{2+}$ injected into the haemolymph is accumulated in the cells of the upper Malpighian tubules whereas < 1% is present in the excreta 15 days post-injection (Maddrell et al., 1991). In addition, tubules isolated from fifth-instar insects injected with $^{45}Ca^{2+}$ 9 days earlier release the vast majority of labeled calcium to the haemolymph side with < 1% lost in the secreted fluid *in vitro* over an 80 min period. Taken together, these results suggest the majority of calcium in the diet is sequestered within the upper tubules and therefore retained within the body. In *Drosophila melanogaster*, approximately 85% of Ca^{2+} which enters the tubule is sequestered while 15% is secreted in soluble form into the tubule lumen (Dube et al., 2000a). Although the relative contributions of secretion and sequestration likely vary in insects depending on their requirements for calcium it would appear that sequestration is the dominant mode for haemolymph calcium regulation. A reliance on Ca^{2+} sequestration may be an effective strategy for excreting large quantities of concentrated calcium (and counter ions) with relatively little water loss. Further investigation is required to determine whether stored calcium can be resorbed back into the haemolymph or the calcium flux is unidirectional and only can be deposited. (Maddrell, 1972) used the term ‘storage excretion’ to refer to the former and ‘deposit excretion’ to refer to the later. In conclusion, it appears that the excretory role of the Malpighian tubules is fulfilled mostly by internal sequestration rather than secretion of soluble calcium.

1.12. Segment-specific Ca^{2+} transport

Malpighian tubules of many insect species exhibit distinct morphological segmentation along their length and in several cases Ca-rich granules appear within distinct 'storage' regions of the tubules. Examples can be found in insects from several diverse orders, including Diptera (Wessing et al., 1992, Herbst and Bradley, 1989), Hemiptera (Maddrell et al., 1991) and Orthoptera (Lipovsek Delakorda et al., 2009). It follows, then, that basolateral Ca^{2+} transport is likely to be regionalized along the length of Malpighian tubules from species with diverse tubule morphology. In anterior tubules of both adults and larvae of *Drosophila melanogaster*, basolateral Ca^{2+} influx is specific to the distal calcium storage segments that readily accumulate Ca-rich granules (Dube et al., 2000a). Malpighian tubules of crickets, such as *Acheta domesticus*, are also segmented along their length with a market contrast in ultrastructure between the midtubule segment (which appear opaque due to the presence of numerous Ca-rich granules within the cells) and the distal tubules that are translucent and lack granules (Hazelton et al., 1988). The presence of granules in the midtubule suggests basolateral Ca^{2+} transport is likely to occur there and not in the distal segment. Ca^{2+} transport appears to be segment-specific based on the location of dense granules in Malpighian tubules of the alkali fly (*Ephydra hians*; Herbst and Bradley (1989)), American cockroach (*Periplaneta americana*; Wall et al. (1975)), harvester ant (*Formica polyctena*; Garayoa et al. (1992)), kissing bug (*Rhodnius prolixus*; Maddrell et al. (1991)) and fruit flies (*Drosophila hydei* & *melanogaster*; (Wessing and Zierold, 1999, Dube et al., 2000a)) based on direct Ca^{2+} measurements.

Segment-specific Ca^{2+} transport may allow some insects to store more calcium without impeding fluid and solute secretion by adjacent tubule segments.

1.13. Mechanisms of Ca^{2+} sequestration

Models of Ca^{2+} sequestration within principal cells of Malpighian tubules have been proposed (Wessing and Zierold, 1999, MacPherson et al., 2001, Chintapalli et al., 2012). Most view basolateral Ca^{2+} uptake to occur transcellularly through Ca^{2+} entry channels down a steep (~10^4-fold) Ca^{2+} concentration gradient (Chintapalli et al., 2012, Davies et al., 2014). Although the molecular identities of the channels are currently unknown, likely candidates include members of the L-type, transient receptor potential (TRP) and cyclic nucleotide gated (CNG) Ca^{2+} channel families (Dube et al., 2000a, MacPherson et al., 2001, MacPherson et al., 2005). Calcium-binding proteins are believed to interact with calcium channels and to facilitate transfer of Ca^{2+} through the cytosol to subcellular organelles in order for intracellular Ca^{2+} concentrations to remain low (Lambers et al., 2006). In electron micrographs, intracellular metal-containing granules are often enveloped by membranes of a lysosomal nature (Brown, 1982). In electron micrographs of tubules of the cave cricket, *T. neglectus*, intracellular Ca-rich 'spherites' (granules) are enveloped by ribosome-studded membranes, consistent with the involvement of the lysosome system (Lipovsek Delakorda et al., 2009). In tubules of *D. melanogaster*, the granule membrane is proposed to be of peroxisomal origin containing secretory pathway Ca^{2+}-ATPases (SPCA) and V-type H^+-ATPases that control granule formation by altering Ca^{2+} entry and local pH i.e. the solubility of amorphous calcium salts (Southall et al., 2006, Chintapalli et al., 2012). In *Drosophila hydei*, Ca-rich granules

(Type-I) are believed to be formed by the deposition of amorphous calcium onto a protein matrix composed of proteoglycans and aminoglycans within intracellular vesicles (Wessing and Zierold, 1999). Ultimately, Ca-rich granules may be accumulated, stored (either transiently or permanently), transported to mineralizing tissues, demineralized during increased calcium demand or excreted. For instance, in *Drosophila hydei*, Ca-rich granules (Type-I) are voided in-tact in the first excretions (meconium) of the adult fly (Wessing et al., 1992). It is worth noting that although most insects do not calcify their cuticles, some do, suggesting that this strategy (calcified cuticle) may have originated from the crustaceans. One such example is the face fly, *Musca autumnalis*, which transports calcium from Ca-rich granules stored in the Malpighian tubules to the puparial cuticle directly through the haemolymph beginning in late larval stages (Krueger et al., 1988). As mentioned above, it is reasonable to suspect that some mechanisms involved in Ca-rich granule handling are conserved in both these invertebrate groups. Further study of the mechanisms of Ca^{2+} sequestration by Malpighian tubules is required to further understand the role of calcium-containing granules within this renal epithelium.

1.14. Hypotheses and objectives

In reviewing the literature it is clear that many animals maintain internal calcium stores that aid in extracellular calcium homeostasis. In vertebrates and some invertebrates, endoskeletons and exoskeletons, respectively, are major calcium stores (sources or sinks) of body calcium used to maintain extracellular calcium homeostasis in terrestrial environments. In contrast, the major calcium stores of insects are thought to be contained within the numerous Ca-rich granules frequently found in the Malpighian tubules.

Unfortunately, direct measurements of Ca^{2+} transport by Malpighian tubules are sparse (< 10 studies) and all have employed the Ramsay technique, which has limited spatial resolution (millimeters). A more recent technique developed by Kuhtreiber and Jaffe (1990) known as the scanning ion-selective microelectrode technique (SIET) has much improved spatial resolution (micrometers) allowing basolateral Ca^{2+} transport to be examined in short lengths (specialized segments) of tubules in much greater detail than was previously possible. Given the conserved nature of Ca^{2+} channels, pumps and exchangers across animal kingdoms, mechanisms of epithelial Ca^{2+} transport are expected to have broad relevance in the field of epithelial ion transport. Although haemolymph Ca^{2+} homeostasis and by extension tubule Ca^{2+} transport are likely under hormonal regulation, as in other animals, currently there are no calciotropic hormones that have been identified in insects.

Therefore, the main objective of this thesis is to **determine the sites, mechanisms and regulation of Ca^{2+} transport by insect Malpighian tubules in order to gain insights into the role of Ca-rich granules within these tissues.** Using electrophysiological techniques this thesis will address the main objective by testing the following hypotheses:

1) The sites of Ca^{2+} transport along the length of Malpighian tubules occur within specific segments of the Malpighian tubules, especially segments containing deposits of Ca-rich granules, in insects with diverse tubule morphology.

2) In tubule regions with high rates of Ca^{2+} uptake most Ca^{2+} taken up by the Malpighian tubules will be sequestered within the cells rather than being secreted into the primary urine.
3) Mechanisms of tubule Ca^{2+} transport involve influx through Ca^{2+} channels
4) Basolateral Ca^{2+} transport by Malpighian tubules is regulated by calciotropic hormones acting through second messenger pathways.
5) Mechanisms of tubule Ca^{2+} transport are conserved in insect species from diverse orders.

1.15. Chapter summaries

1.15.1. Chapter 2

In this chapter, hypotheses 1 and 2 were tested. Segment-specific Ca^{2+} transport across the tubules of *Drosophila melanogaster* adults and larvae were evaluated using both SIET, Ramsay assays and modifications of these two techniques. This study revealed Ca^{2+} transport occurs specifically across the distal tubule segments of both larvae and adults, where numerous Ca-rich granules are stored within the tubule lumen (hypothesis 1). Ca^{2+} transport also varies throughout the life cycle; Ca^{2+} is released by distal tubules of larvae, taken up by distal tubules of young adults and is released once again by tubules of adults by 168 h post-eclosion. Early investigations were focused on Ca^{2+} efflux by larval distal tubules and two novel techniques were developed to validate the direction of Ca^{2+} transport and effects of bathing media composition on Ca^{2+} efflux. The first technique, essentially allowing for SIET measurements of tubules bathed in small (~ 3 µl) droplets of haemolymph held under oil, confirmed the direction (efflux) of Ca^{2+} transport

in larval distal tubules. The technique also allows for simultaneous measurements of transepithelial Ca^{2+} transport, in a manner similar to that of the Ramsay assay, and revealed that most Ca^{2+} exiting the tubule cells is lost to the haemolymph side rather than to the primary urine contained within the tubule lumen (hypothesis 2). The second technique involves bathing single distal segments in small droplets (< 0.5 µl) of bathing media held under oil and estimating Ca^{2+} flux from changes in bathing Ca^{2+} concentration over time. Media with elevated Ca^{2+} concentration reduced rates of Ca^{2+} efflux whereas phosphate-free media had the opposite effect. The results of this chapter support the conclusion that Ca-rich granules of the distal tubules of *Drosophila melanogaster* represent dynamic (reversible) internal calcium stores.

1.15.2. Chapter 3

Hypotheses 1 through 4 were examined in this chapter. For the first time, tubule Ca^{2+} transport was investigated in an Orthopteran insect, the house cricket *Acheta domesticus*. SIET measurements of Ca^{2+} flux made along the length of the tubules indicated Ca^{2+} transport was specific to midtubules (hypothesis 1), where Ca-rich granules accumulate within the cells. The relative roles of Ca^{2+} secretion (3%) and sequestration (97%) by midtubules (hypothesis 2) were determined using both Ramsay assays and SIET Ca^{2+} flux measurements. Mechanisms of midtubule Ca^{2+} transport (hypothesis 3) and regulation (hypothesis 4) were examined using calcium channel blockers and by manipulation of putative second messenger pathways (cAMP and Ca^{2+} pathways) using a pharmacological approach. Together, the results of this chapter suggested that the midtubules are dynamic calcium stores, which maintain haemolymph

calcium concentration by manipulating rates of Ca^{2+} uptake (facilitated by L-type calcium channels) and subsequent intracellular sequestration through stimulatory (cAMP) and inhibitory (Ca^{2+}) regulatory pathways.

1.15.3. Chapter 4

The focus of this chapter, which is an extension of Chapter 3, was to test hypotheses 1, 4 and 5. The purpose of this chapter was to determine if the second messenger pathways (cAMP and Ca^{2+}) found in *Acheta* midtubules (Chapter 3) were broadly applicable to other insects from different orders. Thus Chapter 4 examines the sites (hypothesis 1) and effects of cAMP and thapsigargin (hypothesis 4) on basolateral Ca^{2+} transport by tubules of insects from several orders (hypothesis 5). A broad selection of eight readily available insects were selected for the survey: *Drosophila melanogaster* (Diptera), *Aedes aegypti* (Diptera), *Tenebrio molitor* (Coleoptera), *Acheta domesticus* (Orthoptera), *Trichoplusia ni* (Lepidoptera), *Periplaneta americana* (Blattodea), *Halyomorpha halys* (Hemiptera) and *Pogonomyrmex occidentalis* (Hymenoptera). Ca^{2+} transport was segment-specific across tubules of *Aedes aegypti*, and relatively uniform along the length of the whole tubule of the other species. Segment-specific Ca^{2+} transport had been previously demonstrated in tubules of *Drosophila melanogaster* and *Acheta domesticus* in chapters 2 and 3, respectively. SIET measurements revealed that manipulation of second messenger pathways using cAMP and thapsigargin had little effect on rates of basolateral Ca^{2+} transport, suggesting previous effects observed across midtubules of *A. domesticus* were unique to this species. In addition, this study is the first to provide measurements of basolateral Ca^{2+} across single principal and secondary tubule

cells of the cabbage looper, *Trichoplusia ni*, where Ca^{2+} was specifically taken up by principal cells. In aggregate, the lack of effects of cAMP and thapsigargin in most of the species investigated do not support regulatory roles for cAMP- and Ca^{2+}-dependent second messenger pathways and lead to the conclusion that there is no universal mechanism for the control of tubule Ca^{2+} transport among insects.

1.15.4. Summary

Overall, the three results chapters of this thesis have provided evidence that the Malpighian tubules play a key role in haemolymph Ca^{2+} regulation in insects. In some species, accumulations of Ca-rich granules appear to function as readily exchangeable calcium stores that can be filled (net gain of calcium) or emptied (net loss of calcium) depending on calcium demand throughout the lifecycle. Exchange of calcium between haemolymph and Malpighian tubule stores in some species appears to be sufficient to allow these insects to maintain haemolymph calcium concentrations within a narrow range (i.e. to achieve haemolymph calcium homeostasis). Calcium is taken up specifically by principal cells by a transcellular mechanism involving calcium entry channels and is subsequently sequestered within Ca-rich granules when present. Tubule Ca^{2+} entry is believed to be under hormonal control and in the house cricket, *Acheta domesticus*, the ability to effect basolateral Ca^{2+} fluxes by application of serotonin or manipulation of second messenger pathways supports this hypothesis. Taken together, the results of this thesis further our understanding of calcium homeostasis in insects and likely have general relevance to models of epithelial Ca^{2+} transport in other organisms.

Chapter 2

2. Segment-specific Ca^{2+} transport by isolated Malpighian tubules of *Drosophila melanogaster*: A comparison of larval and adult stages

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2.1. Abstract

Haemolymph calcium homeostasis in insects is achieved through the regulation of calcium excretion by Malpighian tubules in two ways: 1) sequestration of calcium within biomineralized granules and 2) secretion of calcium in soluble form within the primary urine. Using the scanning ion-selective electrode technique (SIET), basolateral Ca^{2+} transport was measured at the distal, transitional, main and proximal tubular segments of anterior tubules isolated from both 3rd instar larvae and adults of the fruit fly *Drosophila melanogaster*. Basolateral Ca^{2+} transport exceeded transepithelial secretion by 800-fold and 11-fold in anterior tubules of larvae and adults, respectively. The magnitude of Ca^{2+} fluxes across the distal tubule of larvae and adults were 10 and 40 times larger than fluxes across the downstream segments, respectively, indicating a dominant role for the distal segment in whole animal Ca^{2+} regulation. Basolateral Ca^{2+} transport across distal tubules of *Drosophila* varied throughout the life cycle; Ca^{2+} was released by distal tubules of

larvae, taken up by distal tubules of young adults and was released once again by tubules of adults ≥ 168 hours post-eclosion. In adults and larvae, SIET measurements revealed sites of both Ca^{2+} uptake and Ca^{2+} release across the basolateral surface of the distal segment of the same tubule, indicating that Ca^{2+} transport is bidirectional. Ca^{2+} uptake across the distal segment of tubules of young adults and Ca^{2+} release across the distal segment of tubules of older adults was also suggestive of reversible Ca^{2+} storage. Our results suggest that the distal tubules of *D. melanogaster* are dynamic calcium stores which allow efficient haemolymph calcium regulation through active Ca^{2+} sequestration during periods of high dietary calcium intake and passive Ca^{2+} release during periods of calcium deficiency.

2.2. Introduction

The Ca^{2+} concentration in the haemolymph of insects (0.5 – 2.5 mM) is well-regulated in response to wide variations in dietary calcium intake (Maddrell et al., 1991, Krueger et al., 1988). Haemolymph Ca^{2+} concentration in the adult fruit fly, *Drosophila melanogaster*, for example, is unchanged (~ 0.5 mM) when flies are raised on diets containing 2.5 to 15.6 mM $CaCl_2$ (Dube et al., 2000b). Calcium is generally abundant in the diets of insects and calcium uptake is thus in excess of requirements. Adult *D. melanogaster* ingest approximately 2 µl of food per day (Deshpande et al., 2014) from fruits (such as peaches) containing approximately 6 mg Ca 100 g^{-1}, equivalent to 2.5 nmol of Ca per day (U.S. Department of Agriculture, 2013). Thus an adult fly, containing 3 nmol of Ca when reared on a standard diet, ingests the majority of the whole body Ca content each day (Dube et al., 2000b). In the face of high dietary calcium intake,

haemolymph concentrations may be maintained by reducing calcium uptake across the gut, enhancing calcium removal via excretory organs or through a combination of both. In the blow fly, *Calliphora vicinia*, adults flies fed excess $^{45}Ca^{2+}$ continued to absorb $^{45}Ca^{2+}$ at high rates (1 to 3 nmol min^{-1}) despite large transient increases in calcium content of the haemolymph, leading Taylor (1985b) to conclude that regulation of excretion and not midgut absorption is the primary mechanism of haemolymph calcium homeostasis.

The insect excretory system is composed of the Malpighian tubules and hindgut. The Malpighian tubules filter the blood by actively secreting fluid, which is then discharged into the hindgut where water and useful solutes can be reclaimed leaving wastes behind in the hindgut lumen to be removed in the excreta. Both larvae and adults of *Drosophila melanogaster* have four Malpighian tubules, with one pair of tubules extending anteriorly to surround the midgut and a second pair directed posteriorly to surround the hindgut. The tubules have morphologically and functionally distinct segments: distal, transitional, main and proximal (lower) (Rheault and O'Donnell, 2001, Sozen et al., 1997). The distal segment of the anterior tubule does not transport fluid at measureable rates (Dow et al., 1994). Only anterior tubules possess the enlarged distal segment, which often contains an abundance of small (0.2 to >10 μm diameter) spherical granules (Wessing et al., 1992). Aggregates of granules are often so densely populated within the lumen of this region that they distend the tubule, noticeably increasing its diameter. In addition, regions where significant granule accumulation occurs often appear white (opaque) under light microscopy in contrast to the translucent nature of tubular cells. These lumenal granules are composed of concentric layers of Ca, and Mg and small

amounts of K, along with carbonate, phosphate, chloride and an organic matrix of glycosaminoglycans or proteoglycans (Wessing et al., 1992). The majority of the Ca content of Malpighian tubules is presumed to be a direct result of their Ca-containing granules. In the fruit fly, *Drosophila melanogaster*, Malpighian tubules can contain approximately 30% of the Ca^{2+} content of the whole adult fly and in a related species, *Drosophila hydei*, anterior tubules contain up to 88% of whole body Ca^{2+} in flies reared on a standard diet (Wessing and Zierold, 1992, Dube et al., 2000b). Clearly, the distal segments of adult and larval tubules of *Drosophila* have the potential to regulate haemolymph Ca^{2+} concentration by sequestering excess Ca^{2+} as biomineralized granules within the Malpighian tubules. Posterior tubules, which lack a distal segment containing Ca-enriched granules and contain about half as much calcium as the anterior tubules, also have the capacity to remove calcium from the haemolymph (Dube et al., 2000a). Fluid secreted by the main segment of adult posterior tubules contains calcium (~ 0.2 mM Ca^{2+}) and therefore contributes to significant rates of removal (~ 0.1 pmol min^{-1} Ca^{2+}).

Although multiple studies have addressed the composition of Ca-containing granules within Malpighian tubules of various insects, physiological investigations of Ca^{2+} transport by Malpighian tubules are sparse. Almost all previous studies of Ca^{2+} transport have bathed tubules in radiolabeled ($^{45}Ca^{2+}$) saline and traced radioactivity to whole tubules or tubular fluid (Dube et al., 2000b, Maddrell et al., 1991, Southall et al., 2006). In this study, we have developed techniques aimed at improving the spatial resolution of Ca^{2+} transport measurements in order to investigate specific regions of Malpighian tubules that previously have not been studied in isolation, especially those of

the calcium-enriched distal segment found in both *Drosophila* larvae and adults. Firstly, we have made use of the self-referencing ion-selective electrode technique (SIET), which allows net basolateral Ca^{2+} transport to be rapidly determined with enhanced spatial resolution (~ 5 µm) relative to the whole-tubule resolution of previous studies using $^{45}Ca^{2+}$. Secondly, a novel technique was used to bath single larval distal tubule segments in microdroplets of media under paraffin oil while monitoring the $[Ca^{2+}]$ of the bathing droplets. Release of Ca^{2+} from the distal segment into Ca^{2+}-containing media caused droplet Ca^{2+} concentration to increase, allowing net basolateral Ca^{2+} efflux to be determined more rapidly than with SIET. Thirdly, we have made use of a modified Ramsay secretion assay to determine the rate at which Ca^{2+} is secreted into the primary urine in order to provide insight into the relative contribution of secretion versus sequestration in both larva and adult tubules. Fourthly, a novel technique was developed that allows SIET and Ramsay assays to be performed simultaneously on tubules bathed in microliter droplets of media isolated under paraffin oil. Using this technique basolateral and transepithelial (secretory) Ca^{2+} fluxes were determined simultaneously, permitting more precise assessment of their contributions to whole-tubule Ca^{2+} transport.

2.3. Materials & Methods

2.3.1. *Drosophila* culture

Stocks of *Drosophila melanogaster* of the strain Oregon R were maintained in vials at room temperature (21-23 $^{\circ}$C) on a diet containing (in g l^{-1}): 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 and 50 dry active yeast (Roberts and

Stander, 1998). To prevent mold growth, 7.45 ml l^{-1} of 10% tegosept (butyl 4-hydroxybenzoate; Sigma-Aldrich, St Louis, MO, USA) dissolved in ethanol and 10 ml l^{-1} acid mix (11 parts H_2O, 10 parts propionic acid and 1 part of 85% *o*-phosphoric acid) were also included. Unless specified otherwise, non-wandering 3rd instar larvae or adult females 1-3 days post-eclosion were used in the experiments.

2.3.2. Bathing media

Ca^{2+}-replete saline contained (in mM): 2 $CaCl_2$, 135 NaCl, 20 KCl, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 15 MOPS, 20 Glucose (Dube et al., 2000b). Saline pH was adjusted to pH 7.0 using 1 M NaOH. Salines with lower Ca^{2+} concentrations (i.e. 1 mM Ca^{2+} saline, calibration solutions or Ca^{2+}-free saline) were prepared from Ca^{2+}-replete saline by removing $CaCl_2$ in exchange for $NaCl_2$ in a 3:1 molar ratio to maintain the ionic strength of the solutions. Amino acid replete saline (AARS) was prepared by adding the following amino acids (in mM) to saline containing 1 mM $CaCl_2$: 1.7 Glycine, 7 L-proline, 6.15 L-glutamine, 0.95 L-histidine, 0.55 L-leucine, 4.5 L-lysine, 1.3 L-valine. Phosphate-free saline was made by equimolar substitution of NaH_2PO_4 with NaCl. In most experiments bathing media was used rather than simple salt solutions (saline). Bathing media containing 2 or 3 mM Ca^{2+} were prepared by mixing 1 part of Schneider's insect medium (S1046; Sigma) containing 4 mM Ca^{2+} with 1 part Ca^{2+}-free saline or 1 part 2 mM Ca^{2+} saline, respectively.

2.3.3. Construction and calibration of Ca^{2+}-selective microelectrodes

Calcium ion concentrations were determined in bathing media, haemolymph or

tubule fluid using liquid membrane ion-selective microelectrodes (ISMEs) containing the Ca^{2+} ionophore ETH1001 (Calcium Ionophore 1 - cocktail A; Sigma-Aldrich, St Louis, MO, USA) and fabricated as described below.

Micropipettes were pulled from borosilicate glass tubing (TW150-4; WPI, Sarasota, FL, USA) using a horizontal puller (P-97; Sutter Instruments Co., Novato, CA, USA), and dried on a hot plate at 200°C for 10 min before silanization. The latter process makes the glass surface hydrophobic and facilitates retention of the hydrophobic ionophore cocktails. For silanization of micropipettes for use with SIET, a drop of *N*,*N*-dimethyltrimethylsilylamine (75 µl) was pipetted onto the inside of a 150 mm diameter PYREX Petri dish, which was then inverted over the micropipettes that had been placed on the hot plate. For calcium measurements in secreted fluid or haemolymph samples under paraffin oil, micropipettes were lightly silanized by exposure to 0.4 µl *N*,*N*-dimethyltrimethylsilylamine. The low level of silanization rendered the glass sufficiently hydrophobic to retain the hydrophobic ionophore cocktail and prevent its displacement by capillary rise of aqueous solutions, but not so hydrophobic as to allow capillary rise of paraffin oil when the tip was moved through the oil and into an aqueous droplet (Kocmarek and O'Donnell, 2011). Batches of silanized micropipettes were stored for up to 3 weeks in a sealed container over a desiccant. Micropipettes were then backfilled with a solution containing 100 mM KCl, 1 mM $CaCl_2$ and a short column (~ 50 µm) of cocktail was drawn into the tip by capillary action. Lightly silanized micropipettes were tip-filled with cocktail by applying negative pressure to the butt end using a 20 ml syringe connected through polyvinyl tubing to a half-cell microelectrode holder fitted with a

pressure port (MEH900S; WPI, Sarasota, FL, USA). Reference electrodes for use under paraffin oil were fabricated from filamented borosilicate glass tubing using a vertical puller and backfilled with a 150 mM KCl solution. Reference electrodes for measurements using the scanning ion-selective electrode technique were constructed from 1.5 mm O.D. borosilicate tubing filled with 3 mol l^{-1} KCl containing 3% agar and connected to the ground input of the headstage using an Ag/AgCl half-cell microelectrode holder.

Ca^{2+}-selective microelectrodes were calibrated in saline containing 0.02 – 5 mM Ca^{2+}. Slopes for a 10-fold change in Ca^{2+} concentration were 24 mV between 0.02 and 0.2 mM Ca^{2+} and 28 – 29 mV per decade in saline containing > 0.2 mM Ca^{2+}. All calibration solutions were titrated to pH 7.0 with 1 M NaOH.

2.3.4. Calcium secretion assay

Ca^{2+} concentrations were measured in fluid secreted by Malpighian tubules held under oil. Wells cut into the bottom of a Sylgard®-lined Petri dish held 20 µl droplets of bathing media under paraffin oil. Dissections were performed in bathing media containing 2 mM Ca^{2+}. Pairs of Malpighian tubules were severed from the gut at the common ureter and then transferred to bathing droplets under oil using a fine glass rod (Dow et al., 1994). One end of the pair was drawn out into the oil and used as an anchor for the other tubule that remained within the bathing medium. Fluid secreted by the immersed tubule formed a droplet at the cut ureter which was positioned just outside the bathing droplet. Secreted fluid droplets were removed after 45 min and their diameters (d) measured using an eyepiece micrometer at 800X magnification in order to estimate their volume ($\pi d^3/6$).

Fluid secretion rate (nl min^{-1}) was determined by dividing the volume of secreted fluid (nl) by the time (min) over which it had formed.

Calcium concentrations in secreted fluid droplets were measured using Ca^{2+}-selective microelectrodes connected through chlorided silver wires to a high input impedance (>10^{13} Ω) amplifier and data acquisition system (PowerLab; ADInstruments, Sydney, Australia). Recorded voltages were analyzed using Chart software (version 5).

Calcium concentrations were calculated using the following equation:

$$[Ca^{2+}]_{Sample} = [Ca^{2+}]_{Cal} \cdot 10^{(\Delta V/S)} \quad (1),$$

where $[Ca^{2+}]_{Sample}$ represents the Ca^{2+} concentration in the sample, $[Ca^{2+}]_{Cal}$ represents the Ca^{2+} concentration in one of the calibration solutions, ΔV represents the difference in voltage recorded between the sample and the calibration solution, and S represents the slope of the electrode for a 10-fold change in Ca^{2+} concentration. Secretory Ca^{2+} flux (pmol min^{-1}) was calculated from the product of secretion rate (nl min^{-1}) and Ca^{2+} concentration of secreted fluid (mM).

2.3.5. Measurement of Ca^{2+} flux along the length of Malpighian tubules using SIET

Malpighian tubules were isolated under bathing medium containing 2 mM Ca^{2+} using fine forceps as described previously (Dube et al., 2000a). A single pair of tubules (either anterior or posterior) was removed from its insertion into the gut at the muscular ureter and discarded leaving the remaining pair for experimentation still attached to short

flanking segments of gut. The small segments of gut allowed the preparation to be manipulated and transferred without touching the tubules and also preserved the muscular contractions of the ureter. Tubules were transferred to 6 ml Petri dishes containing 3 ml of either Ca^{2+}-replete or Ca^{2+}-free saline. Adhesion of the distal segment to the bottom of the Petri dish was facilitated by pre-treating the dishes with 70 µl droplets of 62.5 µg ml^{-1} poly-L-lysine hydrobromide (Sigma) that were allowed to air dry. A 5 µl air bubble formed at the tip of a 10 µl pipette submerged in the saline was pressed gently along the distal segment to ensure uniform adhesion of the tissue to the dish. Tissue preparation was complete upon addition of Schneider's insect media (3 ml) to the Petri dish. SIET scans of adult tubules were performed on tubules isolated from adult flies 24 hr post-eclosion.

SIET measurements were made as previously described (Rheault and O'Donnell, 2004) using hardware from Applicable Electronics (Forestdale, MA, USA) and automated scanning electrode technique (ASET) software (version 2.0; Science Wares Inc., East Falmouth, MA, USA). Ca^{2+}-selective microelectrodes were fixed to a 3-axis translational stage with each axis driven by a computer-controlled stepper motor. The microelectrode was first positioned 3–5 µm from the surface of the tubule. After a wait period of 4 s to allow re-establishment of ion concentration gradients that had been disturbed by microelectrode movement, microelectrode voltage was recorded for 0.5 s and the microelectrode was then repositioned 50 µm further out, along a line perpendicular to the long axis of the tubule. The voltage at the outer site was then recorded after a further wait period, and the voltage gradient between the inner and outer limits of microelectrode

excursion was calculated. Voltage gradients were corrected for electrode drift measured at a reference site >1000 μm away from the tissue.

Ca^{2+} concentration gradients (ΔC) in μmol cm^{-3} were calculated using the following equation:

$$\Delta C = C_B \cdot 10^{\Delta V/S} - C_B \quad (2),$$

where C_B is the background Ca^{2+} concentration (mM or μmol cm^{-3}); ΔV is the voltage gradient between the inner and outer limits of microelectrode excursion at each site (μV); and S is the slope (μV) of the microelectrode for a 10-fold change in Ca^{2+} concentration. Diffusive Ca^{2+} fluxes were then calculated from Ca^{2+} concentration gradients using the Fick equation:

$$J_{Ca^{2+}} = D_{Ca^{2+}} \cdot \Delta C/\Delta x \quad (3),$$

where $J_{Ca^{2+}}$ is the net flux of Ca^{2+} (pmol cm^{-2} s^{-1}); $D_{Ca^{2+}}$ is the diffusion coefficient of Ca^{2+} at 25 ºC ($7.9x10^{-6}$ cm^2 s^{-1}) (Smith et al., 1999); and Δx is the excursion distance. By convention, positive fluxes indicate Ca^{2+} efflux from the tubule while negative fluxes are representative of Ca^{2+} influx into the tubule.

2.3.6. Rapid Measurement of Ca^{2+} flux from distal segments under oil

A method was developed to measure Ca^{2+} transport along the entire length of the non-secretory (distal) segment of *Drosophila* Malpighian tubules more rapidly than was possible using SIET (Figure 2.1). The method was based on measuring changes in the Ca^{2+} concentration of small droplets of saline or bathing media (bathing droplet) into which the distal segment was positioned. Glass pins pressed into Sylgard® lining the bottom of a 50 mm Petri dish held bathing droplets of known volumes (0.2 - 0.4 µl) under paraffin oil by surface tension. Volumes of bathing droplets ejected under oil using a micropipette were determined using an eyepiece micrometer as described above. Single anterior tubules removed at the proximal (lower) region from 3rd instar larvae were transferred into the bathing droplet using a fine glass rod. The main and transitional tubular segments were then pulled out into the oil and wrapped around a second glass pin positioned ~ 1 mm away so that only the distal segment remained within the bathing droplet. Ca^{2+} concentration of the bathing droplet was measured using Ca^{2+}-selective microelectrodes immediately following setup and again 60 min thereafter. The measured change in Ca^{2+} concentration (Δ mM) between the two samples and the initial volume of the bathing droplet (µl) were used to calculate changes in Ca^{2+} content (Δ pmol) of the bathing droplet. Ca^{2+} flux (pmol min^{-1}) was then calculated from the change in Ca^{2+} content (Δ pmol) divided by the time interval (Δ min) between measurements.

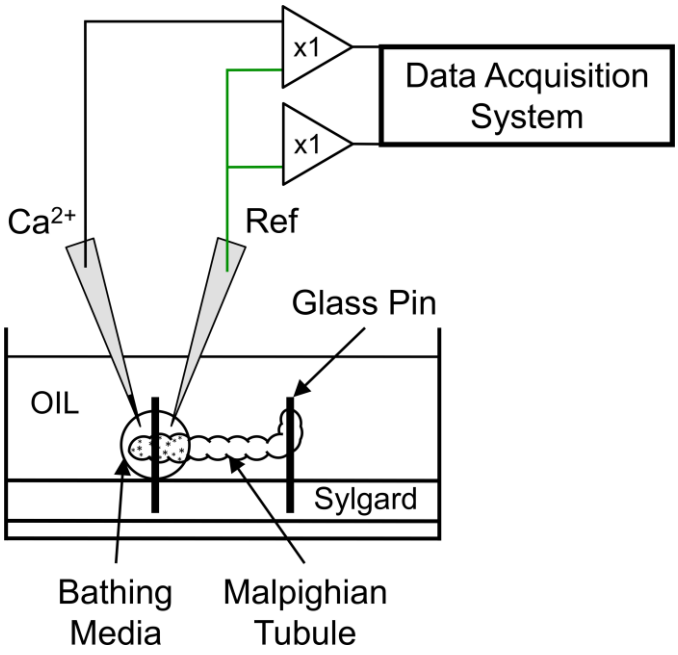

Figure 2.1 Schematic diagram of the apparatus used to measure Ca^{2+} flux across the entire distal segment of larval tubules held under paraffin oil. The diagram is not drawn to scale. Further explanation in the text.

2.3.7. Measurements of Ca^{2+} gradients adjacent to distal segments bathed in haemolymph – combining Ramsay assays with SIET

A modification of our SIET technique was developed to estimate Ca^{2+} gradients adjacent to the surface of Malpighian tubules bathed in pooled droplets of haemolymph isolated under oil (Figure 2.2). These experiments allowed us to test whether a haemolymph-borne factor influenced Ca^{2+} transport. The difference between basolateral Ca^{2+} flux determined by SIET and transepithelial Ca^{2+} flux measured using a modified Ramsay secretion assay represents the sequestration or net release of Ca^{2+} by the tubule.

Larvae were rinsed with dH_2O and dried with tissue paper before haemolymph was collected under paraffin oil by piercing the cuticle with fine forceps. Samples of haemolymph from 15 larvae were pooled and transferred into a small depression (~2 mm long x 1.5 mm wide x 0.5 mm deep) that was melted into the bottom of a poly-L-lysine lined Petri dish using a fine-tipped soldering iron and then filled with paraffin oil. Samples were left for 10 min to allow coagulation of haemocytes. Haemolymph samples free of hemocytes were collected by pipette and transferred to Petri dishes used for the modified Ramsay assay, as follows.

Pairs of Malpighian tubules dissected from 3rd instar larvae by transecting the ureter were transferred to 3 µl droplets of saline containing 2 mM Ca^{2+} held under oil in a Petri dish pre-coated with poly-L-Lysine. One of the tubules was wrapped around a steel pin that had been heated with a soldering iron to allow it to be pushed into the bottom of the dish. After secreted fluid droplets formed at the ureter (indicative of functional tubules) the bathing saline was removed and replaced with clear haemolymph. Ca^{2+}-selective and reference microelectrodes were inserted into the haemolymph

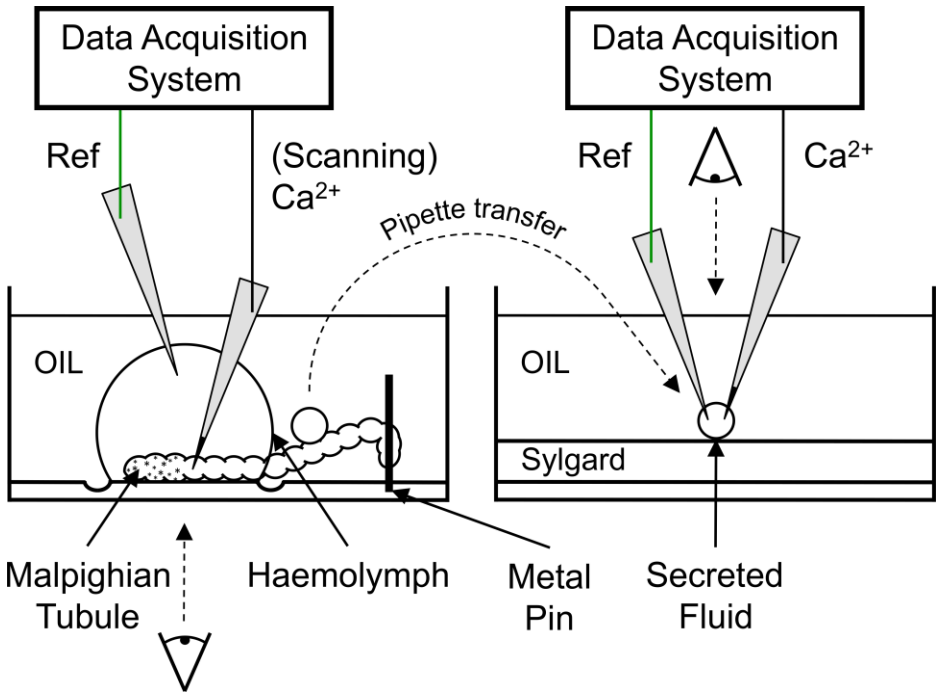

Figure 2.2 Schematic diagram of the apparatus for estimating basolateral and transepithelial Ca^{2+} flux from individual Malpighian tubules bathed in pooled droplets of larval haemolymph held under paraffin oil. Basolateral fluxes were measured by SIET (left schematic) and transepithelial fluxes were determined from measurements of secreted fluid droplet diameters and [Ca^{2+}] (right schematic). Lines-of-sight through a microscope objective are indicated by the straight dashed arrows. The diagram is not to scale. Further explanation in the text.

droplet and the distal segment scanned as described above. SIET measurements were completed at a single site adjacent to each distal segment within 30 min of haemolymph collection, the time equivalent to the secretion period.

Following the application of bathing haemolymph, initial droplets of secreted fluid were removed from the ureter using a fine glass rod and discarded. Secreted fluid droplets were collected after a further 30 min and transferred to a Sylgard®-lined Petri dish filled with paraffin oil. Transepithelial (secretory) Ca^{2+} flux was calculated from the volume, duration of droplet formation and Ca^{2+} concentration of secreted fluid droplets as described previously for the secretion assay.

Net Ca^{2+} flux (pmol min^{-1}) across the entire distal segment was estimated by multiplying the mean flux measured by SIET (pmol cm^{-2} s^{-1}) by 60 sec min^{-1} and by the surface area of the distal segment (cm^2). Surface area of each distal segment was estimated from stereological measurements of images captured at 200X magnification using ImageJ analysis software (version 1.47; National Institutes of Health, Bethesda, MD, USA). The surface area of distal segments were approximated using the formula for the surface area of a cylinder (πdl) where d is diameter and l is length. The diameter of each tubule was obtained by dividing the area of the distal segment captured within the image by the length of tubule in the image excluding the hemispherical distal tip.

2.3.8. Statistical analysis

Data are expressed as means ± SEM for the indicated number of samples (N). Comparisons involving only two groups were made using a student's *t*-test while comparisons of three or more groups were made using one-way ANOVA followed by a

Tukey's range test. Graphing and statistical analysis were performed using Prism software (version 4.0b; GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant if $P<0.05$.

2.4. Results

2.4.1. Secretion of Ca^{2+} by anterior and posterior tubules of adults and larvae

The asymmetrical distribution of anterior and posterior tubules in both larvae and adults and the close association of the distal segments of the anterior tubules with the midgut suggested that each pair might play different roles in calcium homeostasis. We therefore used the Ramsay secretion assay and Ca^{2+}-selective microelectrodes to evaluate the rates of fluid and Ca^{2+} transport by anterior and posterior Malpighian tubules isolated from both larval and adult flies. The rates of fluid secretion did not differ significantly in any of the groups tested (Figure 2.3A). The concentration of Ca^{2+} in the fluid secreted by posterior tubules isolated from adults was 70 % - 350 % higher than that found in either pair of larval tubules (Figure 2.3B). Ca^{2+} flux, calculated as the product of fluid secretion rate and secreted fluid Ca^{2+} concentration, was 2-fold to 4-fold higher in posterior adult tubules than in larval tubules (Figure 2.3C). Total rates of Ca^{2+} secretion by all 4 tubules of larvae and adults were 0.14 pmol min^{-1} and 0.4 pmol min^{-1}, respectively.

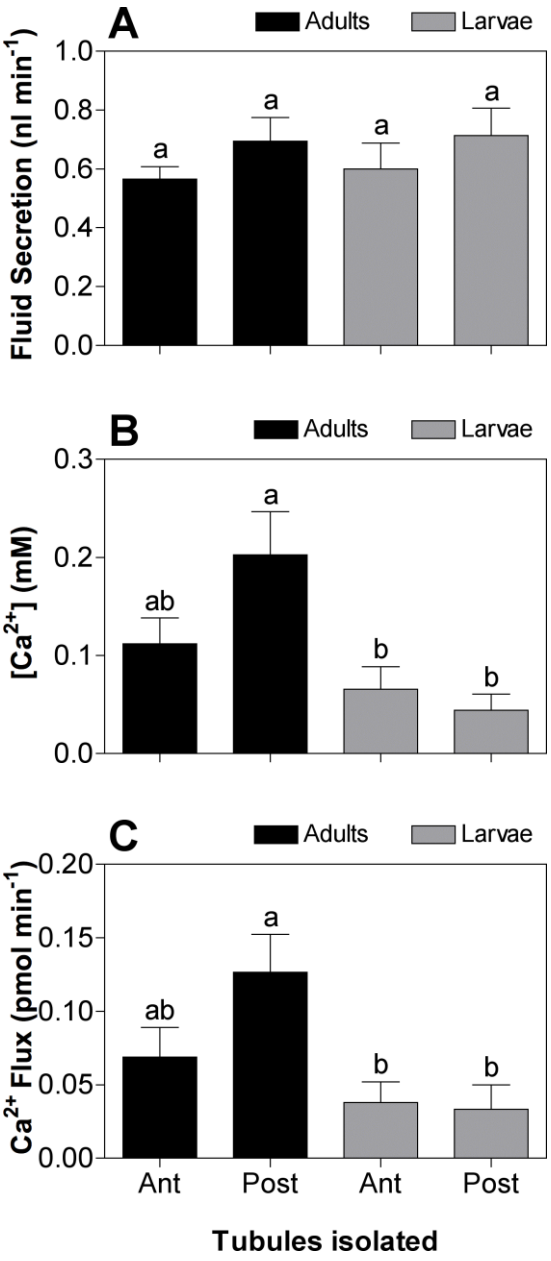

Figure 2.3 (A) Fluid secretion rate (B) secreted fluid Ca^{2+} concentration and (C) transepithelial Ca^{2+} flux of anterior (Ant) and posterior (Post) Malpighian tubules isolated from adults (black bars) and larvae (grey bars). All tubules were bathed in bathing media containing 2 mM Ca^{2+}. For each anterior tubule, distal and main segments were placed in the bathing saline in the Ramsay assay. For the posterior tubule (which lacks a distal segment) the main segment was placed in the bathing saline in the Ramsay assay. Data are expressed as means ± SEM. Bars labeled with different letters are significantly different (one-way ANOVA, $P<0.05$; adults $N=10–11$, larvae $N=12–14$).

2.4.2. Segment-specific Ca^{2+} transport

In order to assess the contribution of each segment to whole tubule Ca^{2+} transport, SIET measurements were made at 4 sites separated by 100 µm in each tubule segment. Ca^{2+} fluxes across the distal segment were 10 - 40 times larger than those in the transitional, main and proximal segments of the anterior Malpighian tubules of both adults and larvae, respectively (Figure 2.4). Although the mean Ca^{2+} flux (+67 ± 22 pmol cm^{-2} s^{-1}, *N*=10) across the distal segment of larval tubules was outward (Figure 2.4A), there was a small number of sites of influx in many larval tubules. In contrast, the mean Ca^{2+} flux for the adult distal segment (-38 ± 7 pmol cm^{-2} s^{-1}, *N*=11) was inward and consisted almost entirely of sites of Ca^{2+} influx (Figure 2.4B). Subsequent experiments revealed sites of Ca^{2+} efflux across distal tubules of older flies, as described below. In summary, the direction of mean Ca^{2+} flux was outward across larval distal tubules and inward across adult distal tubules although sites of both efflux and influx were observed in many tubules of both larval and adult flies. The differences in direction of mean Ca^{2+} fluxes remained even when adult and larval tubules were bathed in identical solutions (bathing medium containing 2 mM Ca^{2+}).

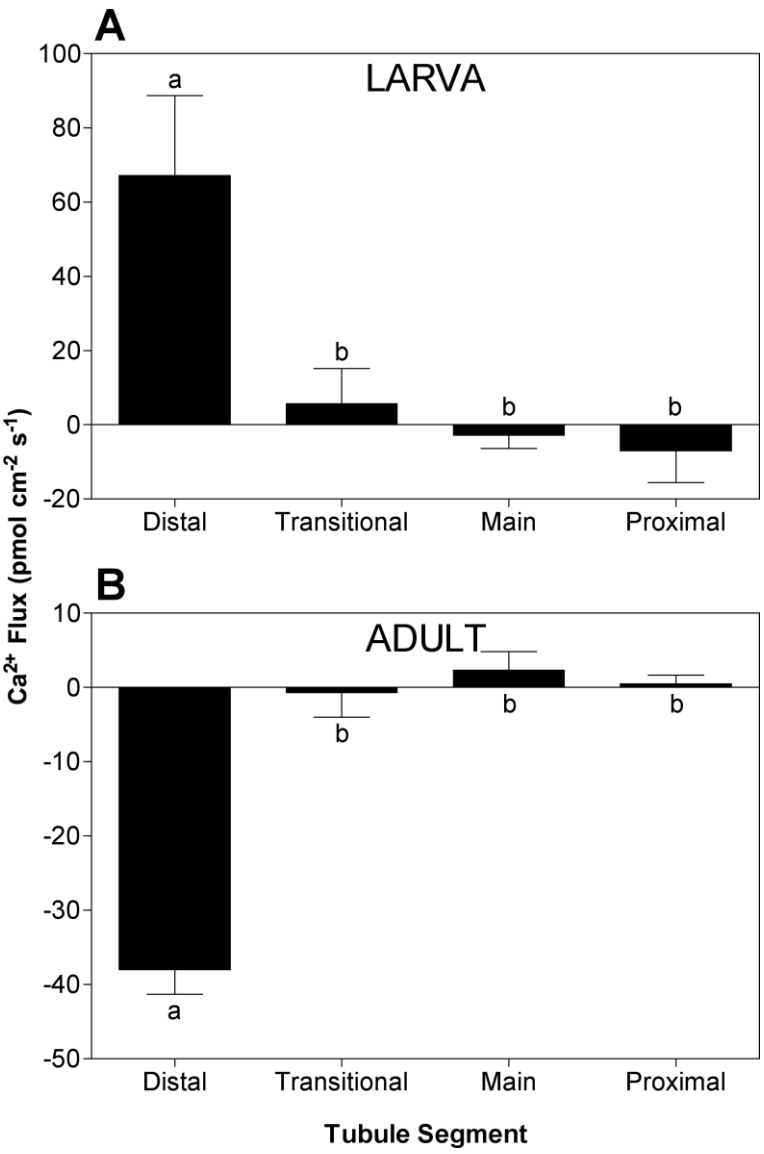

Figure 2.4 Ca^{2+} fluxes across the 4 segments of (A) larval and (B) adult Malpighian tubules bathed in bathing medium containing 3 and 2 mM Ca^{2+}, respectively. Ca^{2+} fluxes were measured across each distal tubule at intervals of 100 µm within 400 µm of the tubule tip. Positive flux values represent net outward-directed basolateral Ca^{2+} transport (efflux) while negative flux values signify net inward-directed basolateral Ca^{2+} transport (influx). Data are expressed as means ± SEM. Bars labeled with different letters are significantly different (one-way ANOVA, $P<0.05$; adults $N=8–9$, larvae $N=10$).

2.4.3. Patterns of Ca^{2+} flux along the length of larval and adult distal tubules

Preliminary SIET measurements of larval distal tubules made at 4 sites separated by 100 µm revealed high variation in Ca^{2+} efflux; the standard error of the mean (SEM) varied from 48 to 59% of the mean. This variability in Ca^{2+} efflux was examined further through scans of 11 larval tubules at higher spatial resolution (12 to 13 sites separated by 30 µm). Along the length of the larval tubule, sites of peak Ca^{2+} efflux were flanked by regions in which the fluxes were smaller (inset Figure 2.5A). A site of peak Ca^{2+} efflux was defined as one which was greater than twice the mean flux value for each distal tubule; at some sites, Ca^{2+} efflux was as much as 8-fold higher than the mean Ca^{2+} flux of the entire distal tubule. In approximately 50% of the tubules there was a single site of peak efflux. In the remaining tubules, there were 2 sites of peak Ca^{2+} efflux separated by 100 - 300 µm. The Malpighian tubule is formed from a single layer of epithelial cells, and the thickness of the wall of the tubule thus reflects the thickness of the epithelial cell layer. Sites of peak Ca^{2+} efflux corresponded to distinct regions where the wall of the tubule appeared thin ($\leq$ 1 µm) and thus the luminal granules were in close proximity to the basal surface of the tubule (Figure 2.5A). By contrast, there were smaller Ca^{2+} effluxes or Ca^{2+} influxes in tubule regions where luminal granules were visibly separated from the basal membrane and the wall of the tubule (i.e. the thickness of the epithelial cell layer) was between 1 and 5 µm thick (Figure 2.5A). Of all sites examined, Ca^{2+} fluxes measured in tubule regions with thin walls (+216 ± 41 pmol cm^{-2} s^{-1}, *N*=33) were significantly higher than those found in regions with thick walls (+57 ± 10 pmol cm^{-2} s^{-1}, *N*=80). Sites of Ca^{2+} influx of up to -96 pmol cm^{-2} s^{-1}, were found less frequently than

Ca^{2+} effluxes in the distal tubules of larvae and occurred almost exclusively in regions with thick walls (19 of 21 influx sites).

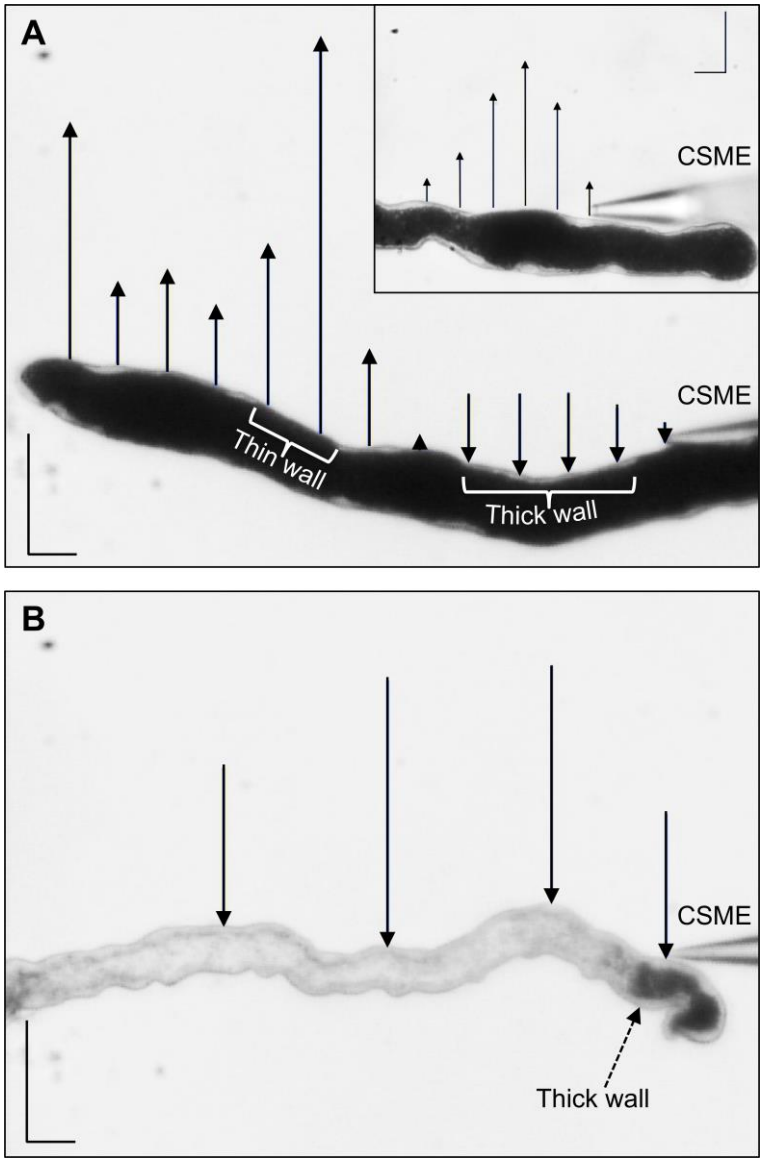

Figure 2.5 Representative Scanning Ion-selective Electrode Technique (SIET) measurements of Ca^{2+} fluxes adjacent to the surface of distal Malpighian tubules isolated from (A) 3rd instar larvae and (B) an adult fly 24 hours post-eclosion. The length and arrowheads represent the magnitude and direction of flux, respectively. Vertical and horizontal scale bars (vertical/horizontal): A, 160 pmol cm^{-2} s^{-1}/30 µm; inset A, 250 pmol cm^{-2} s^{-1}/20 µm; B, 30 pmol cm^{-2} s^{-1}/30 µm. (Inset A) In larval distal tubules, sites of peak Ca^{2+} efflux corresponded to tubule regions where the wall appeared thin (≤ 1 µm), whereas sites of smaller Ca^{2+} efflux occurred in regions where the tubule wall appeared thick (≥ 1 µm). (A) In some cases Ca^{2+} influxes were observed in thick-walled regions of the tubule. (B) In adult distal tubules, the magnitude of Ca^{2+} influxes and tubule wall thickness (~ 3 µm) were uniform within ~ 400 µm of the tubule tip. Ca^{2+}-SME, calcium-selective microelectrode.

In contrast to larval distal tubules, the magnitude and direction of Ca^{2+} fluxes were more consistent along the length of adult distal tubules isolated 24 hours post-eclosion (Figure 2.5B). In tubules bathed in saline containing 3 mM Ca^{2+}, the mean Ca^{2+} flux was directed inward, where Ca^{2+} influx was observed at 86% (37 of 43 sites in 11 tubules) of all measurement sites within approximately 400 µm of the tubule tip. The lumen of adult Malpighian tubules also contained small spherical granules (< 8 µm in diameter) at the distal tubule tips. The abundance of granules declined progressively at distances ≤ 170 µm from the distal tip (Figure 2.5B). Unlike larval tubules, there were no regions with thin walls; i.e. even in regions where the lumen was filled with granules, the wall of the tubule was > 1 µm thick (Figure 2.5B). As described below, the abundance of granules increased in tubules of older flies.

2.4.4. Ca^{2+} flux across distal tubules isolated from adults of different ages

Microscopy of adult Malpighian tubules revealed an increase in the number of granules within the lumen of the distal segment at increasing lengths of time following eclosion (Figure 2.6A). To test the possibility that rates of Ca^{2+} transport across distal tubules might also be influenced by the age of the flies, Ca^{2+} fluxes were measured across distal tubules at increasing lengths of time post-eclosion. Ca^{2+} influx increased from -10 pmol cm^{-2} s^{-1} in tubules from flies collected at 1 hour post-eclosion to a peak rate of -38 pmol cm^{-2} s^{-1} at 24 hr post-eclosion (Figure 2.6B). Subsequently, there was a linear decline (~ 0.3 pmol h^{-1} for an adult distal tubule) in the magnitude of Ca^{2+} influx in the distal segment of tubules isolated from flies between 24 and 168 hours post-eclosion. The

decline in Ca^{2+} influx corresponded to an increase in the number of granules within the lumen of the distal segment (Figure 2.6A). Significant variation in Ca^{2+} flux was observed from tubules isolated from flies ≥ 168 hours post-eclosion; standard errors of the mean (SEM) were up to 1.8-fold higher than the mean. Ca^{2+} efflux was observed in approximately half of all measurement sites taken ≥ 168 hours post-eclosion while Ca^{2+} influx occurred in the remainder. The magnitude of the Ca^{2+} effluxes (+31 ± 10 pmol cm^{-2} s^{-1}, *N*=11) were larger than Ca^{2+} influxes (-23 ± 11 pmol cm^{-2} s^{-1}, *N*=8) and the resulting mean fluxes for both 168 and 192 hours post-eclosion thus indicated Ca^{2+} efflux. The proportion of sites of Ca^{2+} efflux increased from 14 % to 53 % of measurement sites (40 sites in 10 tubules) from distal tubules isolated from flies 24 hours and ≥ 168 hours post-eclosion, respectively.

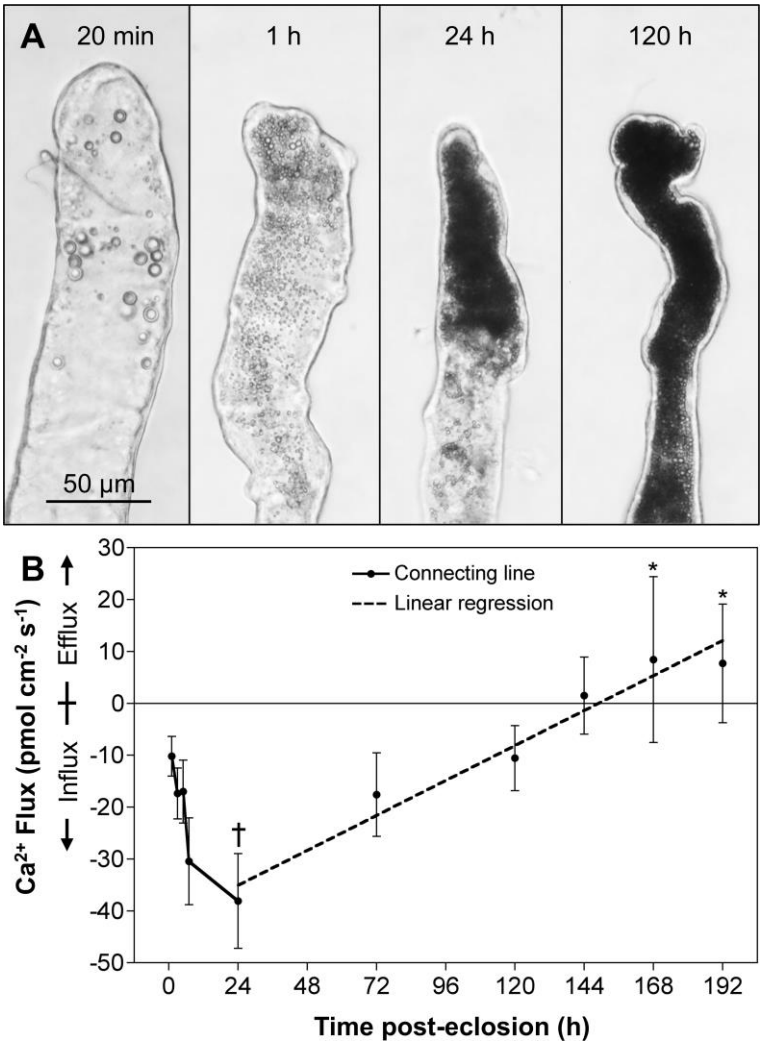

Figure 2.6 Images (A) of the distal segment of tubules isolated from adult flies at the indicated times after eclosion and (B) corresponding Ca^{2+} fluxes. (A) Lumenal granules appear as small (< 8 µm) dark circles. Dense aggregations of granules at 24 h and 120 h block transmitted light and appear black. (B) Tubules were bathed in bathing medium containing 3 mM Ca^{2+}. Data are means ± SEM of measurements taken at 3 sites measured at 100 µm intervals along the distal segment (*N* =9–15 tubules at each time point). A linear regression was performed on the data from 24 to 192 hours post-eclosion (dotted line) and revealed a linear correlation ($y=0.2807x - 41.78$, $r^2 = 0.96$, $p=0.007$). Data points labeled with † are significantly different from 0 hr (Tukey, one-way ANOVA, $P<0.05$, $N=9–12$) while points labeled with * are significantly different from 24 hr post-eclosion (Tukey, one-way ANOVA, $P<0.05$, $N=9–15$).

2.4.5. Effects of metabolic inhibition on Ca^{2+} transport by adult and larval distal tubules

Cyanide and iodoacetic acid were applied to tubules to inhibit aerobic and anaerobic metabolism, respectively. The effects of the metabolic inhibitors on Ca^{2+} fluxes were measured at 4 sites along all tubules separated by 100 µm or at thin-walled regions in adult and larval tubules, respectively. SIET measurements revealed that Ca^{2+} influx by adult distal tubules was reduced from -59 ± 13 pmol cm^{-2} s^{-1} (*N*=7) to +1 ± 1 pmol cm^{-2} s^{-1} (*N*=6) in the presence of cyanide alone, consistent with an active (*i.e.* ATP-dependent) component to Ca^{2+} influx. In contrast, Ca^{2+} efflux by larval distal tubules (+369 ± 53 pmol cm^{-2} s^{-1}, *N*=9) was unchanged in the presence of cyanide alone (+318 ± 32 pmol cm^{-2} s^{-1}, *N*=9) or in combination with iodoacetic acid (+376 ± 53 pmol cm^{-2} s^{-1}, *N*=9).

2.4.6. Ca^{2+} flux from distal segments isolated under paraffin oil: Effects of bathing medium composition on Ca^{2+} transport by the distal tubule of larvae

To determine whether the magnitude of Ca^{2+} flux was altered by changes in bathing solution composition, larval tubules were bathed in saline or media that have been used in previous studies of fluid and ion transport by Malpighian tubules of *D. melanogaster* (Dube et al., 2000a, Dow et al., 1994). Isolation of the distal segment in saline droplets under paraffin oil allowed resolution of small changes in Ca^{2+} flux because Ca^{2+} transport could be measured over longer time intervals than with SIET. As described in the Methods, Ca^{2+} fluxes into or out of the distal segment were inferred from changes in the Ca^{2+} concentration of the bathing droplet. Addition of amino acids to saline containing 1 mM Ca^{2+} (amino acid replete saline, AARS) had little effect on Ca^{2+} fluxes,

whereas Ca^{2+} efflux from distal tubules was significantly reduced when tubules were bathed in Schneider's medium containing 4 mM Ca^{2+} and in saline containing 5 mM Ca^{2+} (Figure 2.7A). We also examined the effects of bathing saline phosphate on Ca^{2+} fluxes because phosphate is known to be present in the luminal granules (Wessing and Zierold, 1992). The removal of phosphate from saline containing 1 mM Ca^{2+} increased Ca^{2+} efflux by approximately 6-fold (Figure 2.7B).

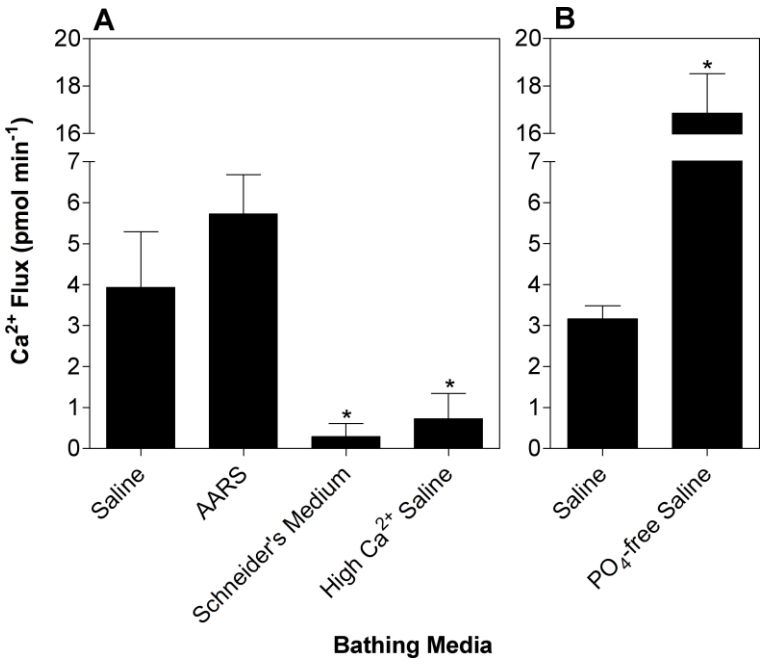

Figure 2.7 Effects of amino acids (amino acid replete saline, AARS), an enriched media (Schneider's) and $CaCl_2$ addition (A) or phosphate removal (B) from bathing saline on Ca^{2+} flux from larval distal tubules bathed under paraffin oil. Schneider's medium contained 4 mM Ca^{2+} and high calcium saline contained 5 mM Ca^{2+}, whereas the remaining salines contained 1 mM Ca^{2+}. Data are expressed as means ± SEM. Bars labeled with * are significantly different from saline in panels A and B, respectively (A, Dunnett, one-way ANOVA, P<0.05, N=6–9; B, unpaired Student's t-test, P<0.05, N=6–12).

2.4.7. Relative roles of basolateral and transepithelial Ca^{2+} transport by larval Malpighian tubules bathed in haemolymph: Combined SIET and Ramsay secretion assays

Bathing larval tubules in haemolymph rather than artificial media more accurately reflects *in vivo* conditions since the haemolymph may contain hormonal factors that influence ion transport. By modifying SIET for use under oil we were able to measure basolateral Ca^{2+} flux from larval distal tubules bathed in haemolymph, as described in the Methods. A modified Ramsay assay was also used in parallel so that paired measurements of basolateral and transepithelial Ca^{2+} flux were made from single tubules in order to more accurately determine their relative contributions to net Ca^{2+} release (i.e. apical vs. basolateral membrane Ca^{2+} release). In order to compare basolateral with transepithelial Ca^{2+} fluxes, basolateral fluxes measured by SIET (mol cm^{-2} s^{-1}) were multiplied by distal tubule surface area (cm^2) and 60 s min^{-1} to convert to the same units as transepithelial fluxes obtained from the Ramsay assay (mol min^{-1}). Surface area (0.0014 cm^2) of larval distal tubules was calculated from measurements of the outer diameter (0.0049 ± 0.0001 cm, N = 17) and length (0.09 ± 0.03 cm, N = 20), which was taken to be the distance from tubule tip to where lumenal granules no longer occupied the entire width of the lumen. Basolateral Ca^{2+} efflux obtained from SIET (+96 ± 18 pmol cm^{-2} s^{-1}, N=16) was equivalent to +7.98 pmol min^{-1} Ca^{2+} across the entire distal segment of larval tubules bathed in haemolymph. The corresponding rate of transepithelial Ca^{2+} secretion (-0.01 ± 0.002 pmol min^{-1}, N=16) was approximately 800-fold lower. This rate of transport can be appreciated by estimating the time required to double the entire haemolymph Ca^{2+} content. In larvae, a haemolymph Ca^{2+} content of ~ 200 pmol can be estimated as the

product of haemolymph Ca^{2+} concentration (~ 1 mM) from this study and the corresponding volume (~ 200 nl) determined previously (Piyankarage et al., 2008). Release of Ca^{2+} by pairs of tubules could thus double the entire haemolymph Ca^{2+} content (~ 200 pmol) in approximately 13 minutes. Similar rates of net Ca^{2+} release (+5.57 pmol min^{-1}) were observed from larval distal tubules bathed in bathing media containing 3 mM Ca^{2+}.

2.4.8. Relative roles of basolateral and transepithelial Ca^{2+} transport by adult Malpighian tubules

Net Ca^{2+} transport by anterior tubules of adults was calculated from SIET and Ramsay assays measurements reported above. Basolateral Ca^{2+} flux across adult distal tubules bathed in bathing media containing 2 mM Ca^{2+} (-38 ± 7 pmol cm^{-2} s^{-1}, *N*=11) was equivalent to -0.8 pmol min^{-1} Ca^{2+} across the entire distal segment. This value was > 11-fold larger than the corresponding rate of Ca^{2+} secretion (-0.07 ± 0.02 pmol cm^{-2} s^{-1}, *N*=10) by whole tubules. Subtracting transepithelial from basolateral Ca^{2+} flux gives the rate of net Ca^{2+} sequestration (-0.73 pmol min^{-1}) by adult distal tubules in bathing media containing 2 mM Ca^{2+}. The significance of this rate of transport can be appreciated by estimating the time required to remove the entire haemolymph Ca^{2+} content. Using estimates of the volume (~ 50 nl) (MacMillan and Hughson, 2014) and Ca^{2+} concentration of the haemolymph of adult flies (~ 0.5 mM) (Dube et al., 2000b), the resulting haemolymph Ca^{2+} content is ~ 25 pmol. At a rate of -0.73 pmol min^{-1}, pairs of tubules could remove the entire haemolymph Ca^{2+} content (~ 25 pmol) in approximately 17 minutes.

2.5. Discussion

Our results have revealed both uptake and release of Ca^{2+} from the distal segment of the Malpighian tubule of *Drosophila melanogaster* larvae and adults. In addition, we have quantified the roles of Ca^{2+} secretion and sequestration in tubules of the larvae, extending a previous study of the adult tubule (Dube et al., 2000a).

2.5.1. Secretion of Ca^{2+} by isolated MTs

Both anterior and posterior tubules of *D. melanogaster* incorporate Ca^{2+} into the primary urine at physiologically relevant rates and thus contribute to haemolymph Ca^{2+} elimination. Ca^{2+} secretion by all 4 tubules of larva (0.14 pmol min^{-1}) and adult flies (0.4 pmol min^{-1}) is sufficient to eliminate the entire Ca^{2+} content of their haemolymph in as little as 24 h or 1 h, respectively. Tubules isolated from adults in this study secreted Ca^{2+} at rates similar to those (~ 0.1 pmol min^{-1} $tubule^{-1}$) previously reported (O'Donnell and Maddrell, 1995, Ruiz-Sanchez and O'Donnell, 2012). The much higher rate of Ca^{2+} secretion by posterior tubules of adults above those of either anterior or posterior tubules of the larvae suggests that adults rely to a greater extent on Ca^{2+} secretion for haemolymph calcium homeostasis than do larvae.

2.5.2. Segment-specific basolateral Ca^{2+} transport: A primary role for the distal tubule

SIET analysis of Ca^{2+} transport provides greatly improved spatial resolution over previous radiotracer methods and has thus allowed Ca^{2+} transport to be examined across each segment of the anterior tubules of *Drosophila*. Specifically, the non-secretory distal

segment of anterior tubules, where Ca-containing granules accumulate in the lumen, have hitherto not been examined separately from other tubule segments. SIET measurements made along the length of anterior Malpighian tubules isolated from larval and adult flies indicate that the distal segment is the main site of basolateral Ca^{2+} transport. Significant accumulations of Ca-enriched granules are also unique to this tubule segment suggesting that basolateral transport of Ca^{2+} influences the local formation of lumenal Ca-containing granules. Segment-specific Ca^{2+} transport has also been demonstrated by Maddrell et al. (1991) in the Hemipteran, *Rhodnius prolixus*, where $^{45}Ca^{2+}$ is selectively accumulated by the distal (upper) tubules. In addition, Ca-containing granules appear localized to distinct regions of the Malpighian tubules in other insects, including the storage segment (lime glands) of the Dipteran, *Ephydra hians*, the midtubule region of the Orthopteran, *Acheta domestica*, and the yellow region of the Lepidopteran, *Calpodes ethlius* (Herbst and Bradley, 1989, Hazelton et al., 1988, Ryerse, 1979). Taken together with the results of the present study, it appears that Ca^{2+} is typically sequestered within a specific region or segment of the tubule, but that the region utilized differs in Malpighian tubules of insects of different orders.

2.5.3. Uptake and release of Ca^{2+} across the basolateral surface of tubules isolated from adults and larvae.

It is generally accepted that the distal tubules of *Drosophila* function to accumulate and store excess haemolymph calcium. To that end, distal tubules of adults take up Ca^{2+} at consistently high rates along their length (~ -38 pmol cm^{-2} s^{-1}). Basolateral Ca^{2+} influx across the entire distal segment was -0.8 pmol min^{-1}, equivalent to -1.6 pmol

min^{-1} per tubule pair. These results suggest that pairs of adult distal tubules can remove the entire Ca^{2+} content of the haemolymph (~ 25 pmol) in as little as 16 mins. Basolateral Ca^{2+} influx depends on a metabolically active transport mechanism since it was abolished by addition of 1 mM cyanide (an inhibitor of aerobic metabolism) to the bathing media.

Measurements of Ca^{2+} flux by the Malpighian tubules of larval *Drosophila* have not been published previously. We hypothesized that tubules isolated from 3rd instar larva would take up Ca^{2+} at high rates just as their adult counterparts do. The distension of the distal segment with luminal concretions is consistent with Ca^{2+} transport into the tubule lumen *in vivo*. Thus the release of Ca^{2+} by larval tubules at high rates (+67 pmol cm^{-2} s^{-1}) *in vitro* was unexpected. Basolateral Ca^{2+} release at the above rate, equivalent to ~ 11 pmol min^{-1} across the entire length of a pair of distal tubules, could double the Ca^{2+} content of the haemolymph (~ 200 pmol) in approximately 18 mins. Ca^{2+} efflux by larval distal tubules was unchanged in the presence of inhibitors of aerobic and anaerobic metabolism, suggestive of a passive transport mechanism, independent of ATP hydrolysis. It is worth noting that Maddrell et al. (1991) also observed Ca^{2+} efflux from *Rhodnius* tubules *in vitro,* yet Ca^{2+} influx when injected with radiolabeled $^{45}Ca^{2+}$ *in vivo*. The Ca^{2+} efflux was interpreted as evidence for Ca^{2+} extrusion pumps located on the basolateral membrane of tubule cells. In larvae and pupae of the face fly, *Musca autumnalis,* calcium stored within the Malpighian tubules is released and transported to the cuticle via the haemolymph in order to harden the puparium (Krueger et al., 1988). Although *Drosophila* do not utilize minerals for cuticular hardening, the ability to sequester and reabsorb calcium in Dipterans may serve to maintain the calcium content of

the haemolymph during rapid growth and pupation.

2.5.4. Ca^{2+} transport across distal tubules is bidirectional/reversible

In larval tubules, sites of peak Ca^{2+} efflux corresponded to tubule regions where the basal membrane appeared thinnest whereas sites of Ca^{2+} influx, or smaller efflux, occurred in regions where the basal membrane was thickest. The presence of both sites of Ca^{2+} efflux and influx in many tubules provide evidence of Ca^{2+} recycling across the basolateral surface of larval distal tubules. Ca^{2+} released from thin regions may re-enter the tubule across an adjacent thick region. In addition, a small number of sites of Ca^{2+} efflux were observed in addition to the predominant Ca^{2+} influx across distal tubules of adults. High rates of Ca^{2+} turnover across the distal tubules may be necessary for rapid regulation of haemolymph calcium and may provide a route to return sequestered Ca^{2+} to the haemolymph during periods of dietary calcium deficiency. In the cave cricket, *Troglophilus neglectus*, Ca-containing granules (spherites) within the mid-tubule accumulate Ca during active life stages above ground whereas the Ca content is reduced during overwintering in caves (Lipovsek Delakorda et al., 2009). Together, the results suggest that Malpighian tubules can build-up calcium stores through sequestration and then release stores to increase haemolymph Ca^{2+} levels.

2.5.5. Ca^{2+} transport across distal tubules varies throughout the life cycle

We observed that Ca-containing granules continually fill the lumen of the distal tubules of larvae from 1st through to the 3rd instar. The granules remain within the tubule tips during wandering and early stages of pupation but are found in the meconium just

prior to eclosion. The meconium is voided during or shortly after emergence, coincident with a 4-fold decrease in the whole-body Ca content of an adult fly (Dube et al., 2000b). As a result tubules of recently emerged adults appear distended but contain very few, if any, lumenal granules. A similar strategy has also been observed in another Dipteran, *Ephydra hians*, where Ca-containing granules accumulate throughout larval life and are discharged *via* the gut at pupariation (Herbst and Bradley, 1989). Practically, this strategy represents an effective means of removing calcium (and other potentially toxic divalent metals) without significant water loss (Wessing and Zierold, 1992). Ca^{2+} influxes rapidly increased within 24 hours of eclosion, coincident with the appearance of more granules within the lumen. Ca^{2+} uptake rates declined in tubules isolated from older flies as the distal tubule progressively filled with lumenal concretions. We suggest that the switch in the direction of mean Ca^{2+} transport from influx to efflux represents the point where the capacity for Ca^{2+} sequestration is exceeded (possibly due to the saturation of lumenal space occupied by granules) resulting in the release of any excess Ca^{2+}.

2.5.6. Influence of bathing media Ca^{2+} and PO_4^{3-} on Ca^{2+} release from larval distal tubules

Ca^{2+} efflux by larval tubules decreased in response to elevated bathing Ca^{2+} concentrations, suggesting the presence of an outwardly directed Ca^{2+} concentration gradient from lumen-to-bath. Increases in bathing Ca^{2+} would tend to reduce this Ca^{2+} gradient allowing less Ca^{2+} to return passively to the bathing media. Since intracellular Ca^{2+} concentrations are typically low (~ 100 nmol l^{-1}; Rosay et al. (1997)) and efflux is passive, Ca^{2+} may move from lumen to haemolymph through the paracellular pathway.

Paracellular efflux from the larval distal segment would require that the concentration of Ca^{2+} in the lumen of the distal segment exceeds that in the haemolymph (~ 1 mM) or that there is a lumen-positive transepithelial potential sufficient to overcome an lumen-directed Ca^{2+} concentration gradient.

Wessing and Zierold (1999) concluded that the granules (type-I) isolated from the distal tubule of *Drosophila hydei* are composed of Ca-phosphate and Ca-bicarbonate/Ca-carbonate. This conclusion was based on elemental analysis indicating significant amounts of both Ca and P in isolated granules, along with the finding that inhibitors of carbonic anhydrase (reducing intracellular bicarbonate/carbonate) inhibited granule formation. The increase in Ca^{2+} efflux by larval distal tubules in response to the removal of phosphate from the bathing saline in our study suggests that Ca^{2+} stored within the tubule is accompanied by phosphate counterions. ^{45}Ca measurements have shown that calcium concretions in the lumen of Malpighian tubules are dynamic structures rather than static precipitates of calcium phosphate (Maddrell et al., 1991). Removal of phosphate from the bathing medium may thus enhance the dissolution of calcium phosphate granules, with a consequent increase in Ca^{2+} concentration in the lumen favouring an increase in Ca^{2+} efflux into the haemolymph. More recently, elemental maps of granules isolated from tubules of the cricket, *Troglophilus neglectus,* have revealed Ca, P and O are co-localized to electron-dense layers, consistent with the presence of calcium-phosphate (Lipovsek Delakorda et al., 2009).

2.5.7. Relative roles of basolateral and transepithelial Ca^{2+} transport by larval MTs

Another benefit of our modification of SIET for use under oil is that it allows SIET to be performed simultaneously with a Ramsay-like secretion assay. The pairing of the techniques helps to resolve the relative roles of basolateral and transepithelial Ca^{2+} transport by single Malpighian tubules with the added benefit of paired results for the purposes of statistical analysis. In anterior larval tubules bathed in haemolymph basolateral Ca^{2+} flux was 800-fold higher than transepithelial Ca^{2+} flux. The relatively high rates of basolateral Ca^{2+} release by anterior tubules of larva suggest that most Ca^{2+} crosses the tubule wall into the haemolymph more readily than it moves down the tubule lumen. There are many contributing factors that may serve to retain Ca-containing granules within the distal tubule (and hence maintain high rates of basolateral Ca^{2+} release while simultaneously limiting the diffusion of Ca^{2+} down the lumen (resulting in relatively low rates of transepithelial Ca^{2+} secretion). Chief among these factors are: the blind-ended nature of the distal tubule, the lack of fluid transport by the surrounding epithelium of the distal segment, the narrowing of the lumen and the bending (180 degrees) of the tubule *in vivo* in the adjacent transitional segment, and the aggregation of granules that can effectively plug the lumen of the distal tubule. In any case, high rates of basolateral Ca^{2+} release by the distal tubules of larvae may be necessary to sustain rapid growth and the relatively low rates of Ca^{2+} secretion might be adaptive, limiting hindgut Ca^{2+} (and possibly other heavy metals) exposure thus avoiding potential interference with vital water recovery processes (Maddrell et al., 1991).

2.5.8. Relative roles of basolateral and transepithelial Ca^{2+} transport by adult MTs

In distal tubules of adults Ca^{2+} is actively taken up across the basolateral membrane of the distal tubule 11 times faster than it lost into the primary urine, suggesting that anterior tubules of adults remove the majority of Ca^{2+} from the haemolymph by sequestration rather than by secretion. Basolateral Ca^{2+} flux exceeded transepithelial Ca^{2+} flux by 7-fold or more in posterior tubules, which lack a distal segment (Dube et al., 2000a). Likewise, distal tubules of *Rhodnius prolixus* take up $^{45}Ca^{2+}$ 12 times faster than it is removed through secretion, implying Ca^{2+} sequestration is the dominant method for the excretion of Ca^{2+} by Malpighian tubules. Pairs of adult anterior tubules sequestered Ca^{2+} at a rate of -1.45 pmol min^{-1} while the posterior tubule pair secreted Ca^{2+} at a rate of -0.26 pmol min^{-1}. The sum of the two, -1.71 pmol min^{-1}, suggests the Malpighian tubules of an adult fly can remove Ca^{2+} at physiologically relevant rates; eliminating the entire Ca^{2+} content of the haemolymph in approximately 15 minutes. It is useful to examine the rates of Ca^{2+} sequestration by the Malpighian tubules of adults in the context of whole-fly calcium handling. As described above, we estimate that Ca^{2+} uptake in the food is ~ 2.5 nmol of Ca per day, equivalent to ~ 1.7 pmol min^{-1}. Work by Taylor (1985b) using isolated midguts from the blow fly, *Calliphora vicina*, indicates that almost all (97 %) of the Ca fed to these flies over several days is absorbed across the gut. It then follows that the average rate of calcium uptake from gut-to-haemolymph in *D. melanogaster* could be as high as ~ 1.7 pmol min^{-1}. These data suggest that the Malpighian tubules have the capacity to maintain the calcium concentration of the haemolymph by sequestering Ca^{2+} despite potentially high rates of dietary Ca^{2+}

absorption.

A model for Ca^{2+} transport and luminal concretion formation in the distal segment has been proposed on the basis of expression profiles for multiple genes that show differences in expression in anterior versus posterior tubules (Chintapalli et al., 2012). Ca^{2+} entry into the cells via a trp-like Ca^{2+} channel in the basolateral membrane is followed by transport into specialized peroxisomes by *SPoCk-C*, the secretory pathway Ca^{2+}/Mn^{2+}-ATPase (Southall et al., 2006). Phosphate may enter the peroxisomes via a Na^{+}:phosphate cotransporter (NaPiT; CG15096) and the bestrophin Cl^{-} channel (*Best2*) contributes to charge balance across the membrane of the peroxisome (Chintapalli et al., 2012). This model of Ca^{2+} transport may be tested in future studies examining the effects of inhibitors of *Best2* (DIDS; (Liu et al., 2015)), *SPoCk-C* (bisphenol;(Gunaratne and Vacquier, 2006)) and NaPiT (phosphonocarboxylic acids; (Loghman-Adham, 1996)) on Ca^{2+} fluxes measured by SIET. Although microarray data in FlyAtlas (Chintapalli et al., 2007) do not distinguish gene expression levels in different tubule segments or in anterior versus posterior tubules, it would also be of interest in future studies using qRT-PCR to compare the expression levels of Ca transport genes in the isolated distal segment of adult versus larval tubules.

The model also proposes that Ca^{2+} transport in the distal segment may be regulated by calmodulin and a calmodulin responsive transcriptional activator (Chintapalli et al., 2012). Further studies on regulation by these genes, or of the effects of the neuropeptide capa1, which has been shown to elevate Ca^{2+} in cells of the distal segment of adult tubules, may help to understand the basis for differences in Ca^{2+}

transport by distal segments of larval versus adult tubules that we have described in this paper.

In summary, this study has introduced two novel techniques to study Ca^{2+} transport by insect Malpighian tubules, specifically the non-secretory distal segments known to contain large quantities of Ca^{2+}-containing granules. In addition, temporal and spatial differences in Ca^{2+} transport were observed and are consistent with the role of the tubules as dynamic regulators of extracellular calcium in insects. Given that the *Drosophila* Malpighian tubule has been proposed as a model for human nephrolithiasis (Miller et al., 2013), we believe that further studies of Ca^{2+} uptake by the distal segment of adult tubules and Ca^{2+} release from the distal segment of the larval tubules will be relevant to understanding fundamental mechanisms of Ca^{2+}-containing granule (kidney stone) formation.

Chapter 3

3. Mechanisms of calcium sequestration by isolated Malpighian tubules of the house cricket *Acheta domesticus*

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3.1. Abstract

Haemolymph calcium homeostasis in insects is achieved by the Malpighian tubules, primarily by sequestering excess Ca^{2+} within internal calcium stores (Ca-rich granules) most often located within type I (principal) tubule cells. Using both the scanning ion-selective microelectrode technique (SIET) and the Ramsay secretion assay this study provides the first measurements of basolateral and transepithelial Ca^{2+} fluxes across the Malpighian tubules of an Orthopteran insect, the house cricket *Acheta domesticus*. Ca^{2+} transport was specific to midtubule segments, where 97% of the Ca^{2+} entering the tubule is sequestered within intracellular calcium stores and the remaining 3% is secreted into the lumen. Antagonists of voltage-gated (L-type) calcium channels decreased Ca^{2+} influx ≥5-fold in cAMP-stimulated tubules, suggesting basolateral Ca^{2+} influx is facilitated by voltage-gated Ca^{2+} channels. Increasing fluid secretion through manipulation of intracellular levels of cAMP or Ca^{2+} had opposite effects on tubule Ca^{2+}

transport. The adenylyl cyclase-cAMP-PKA pathway promotes Ca^{2+} sequestration whereas both 5-HT and thapsigargin inhibited sequestration. Our results suggest that the midtubules of *A. domesticus* are dynamic calcium stores, which maintain haemolymph calcium concentration by manipulating rates of Ca^{2+} sequestration through stimulatory (cAMP) and inhibitory (Ca^{2+}) regulatory pathways.

3.2. Introduction

Cells and tissues require regulation of extracellular calcium concentrations for normal function. The ionized calcium concentration of the haemolymph, the chief extracellular fluid in insects, is within the range of 0.5 to 2.5 mM (Dube et al., 2000b, O'Donnell and Maddrell, 1995, Maddrell et al., 1991). Haemolymph Ca^{2+} concentrations of flies increase <1.5-fold when fed on diets with >6-fold higher calcium concentrations, demonstrating that Dipterans have the capacity for haemolymph calcium regulation (Taylor, 1985b, Dube et al., 2000b). Haemolymph Ca^{2+} concentrations are altered by changes in the rates of Ca^{2+} absorption (input) or Ca^{2+} excretion (removal). Rates of $^{45}Ca^{2+}$ absorption across isolated midguts of the blowfly, *C. vicina,* are unaffected by increases in the calcium content of the diet (from 0 to 12.5 mM $CaCl_2$), leading Taylor (1985b) to conclude that absorption of dietary Ca^{2+} across the midgut is unregulated and therefore modulation of rates of Ca^{2+} excretion is the primary means by which haemolymph calcium regulation is achieved in insects.

The Malpighian (renal) tubules and hindgut together form the functional ‘kidney’ in insects (Maddrell, 1972). Malpighian tubules generate primary urine by transporting ions and osmotically-obliged water from the haemolymph into the tubule lumen. Several

lines of evidence suggest that the tubules play a major role in excreting excess Ca^{2+} from the haemolymph, either by secretion or sequestration. Secretion refers to the transport of Ca^{2+} in soluble form into the primary urine, whereas sequestration refers to the transport of Ca^{2+} into tubule calcium stores. In tubules of adult *D. melanogaster*, ≥85% of the Ca^{2+} which enters the tubules is sequestered and the remaining ≤15% is secreted into the lumen (Dube et al., 2000a, Browne and O'Donnell, 2016). A reliance on Ca^{2+} sequestration may be an effective strategy for eliminating large quantities of concentrated calcium (and counter ions) with relatively little water loss. Measurements of the calcium content of the Malpighian tubules indicate that they are also sites of physiologically relevant calcium storage. All 4 tubules isolated from larvae of *D. hydei* contain approximately 88% of the calcium content of the entire flies (Wessing and Zierold, 1992). The majority of the calcium content within insect Malpighian tubules is correlated with the presence of numerous calcium-rich granules found in the cells and/or lumen (Brown, 1982).

In crickets, these granules are spherical, relatively small (0.2 to 5 µm in diameter) and are composed of concentric layers of electron-dense regions enriched in calcium-phosphate and electron-lucent regions containing scaffolding proteins/carbohydrates (Lipovsek Delakorda et al., 2009). Increases in the calcium content of the granules is correlated with increases in the calcium content of the diet (Wessing and Zierold, 1992) and the granules generally increase in size and/or number with age (Sohal et al., 1976, Browne and O'Donnell, 2016). It is likely, then, that haemolymph calcium regulation is achieved through regulated Ca^{2+} transport between haemolymph and internal calcium stores (Ca-rich granules) found within the Malpighian tubules.

Malpighian tubules of many insect species exhibit distinct morphological segmentation along their length and in several cases Ca-rich granules appear within distinct 'storage' segments of the tubules. Examples can be found in insects from several diverse orders, including Diptera (Wessing et al., 1992, Herbst and Bradley, 1989), Hemiptera (Maddrell et al., 1991) and Orthoptera (Lipovsek Delakorda et al., 2009). It follows, then, that basolateral Ca^{2+} transport is likely to be regionalized along the length of Malpighian tubules from species with diverse tubule morphology. In anterior tubules of both adults and larvae of *D. melanogaster*, basolateral Ca^{2+} influx is specific to the distal calcium storage segments that readily accumulate Ca-rich granules (Browne and O'Donnell, 2016). Malpighian tubules of *A. domesticus* are also segmented with marked contrast between the midtubule segments which appear opaque due to the presence of numerous intracellular Ca-rich granules and the distal tubule segments which are translucent and lack granules (Hazelton et al., 1988). It follows that Ca^{2+} may be taken up primarily across the basolateral membranes of the granule-containing midtubule segments.

Studies of Ca^{2+} transport by Malpighian tubule have been limited to insects of only two orders: Diptera and Hemiptera (Taylor, 1985b, Maddrell et al., 1991, Dube et al., 2000a, Herbst and Bradley, 1989, Southall et al., 2006). In this study, we aim to identify sites and mechanisms of Ca^{2+} transport by isolated Malpighian tubules from an Orthopteran insect, the house cricket *Acheta domesticus*. First, rates of basolateral Ca^{2+} transport were determined within distal and midtubule segments using the scanning ion-selective electrode technique (SIET) to evaluate possible segment-specific Ca^{2+} transport.

Second, rates of Ca^{2+} secretion were determined using Ramsay assays. Together, these measurements allow estimation of the relative contributions of Ca^{2+} secretion and Ca^{2+} sequestration to haemolymph calcium regulation. Finally, we have investigated mechanisms of Ca^{2+} secretion and sequestration and the means by which these processes are modulated by the diuretic factor serotonin and the intracellular second messengers cAMP and Ca^{2+}.

3.3. Materials & Methods

3.3.1. Insects

A colony of *Acheta domesticus* was maintained at 28 ^{o}C with access to water and ground multi-fowl feed containing 0.05% calcium by weight (W-S Feed & Supplies Ltd, Tavistock, ON, Canada) *ad libitum* and held under 12 h light : 12 h dark conditions. First instar nymphs were transferred to translucent 5 L Tupperware® containers and maintained through to adulthood. Malpighian tubules were isolated from adult females 7-8 weeks of age.

3.3.2. Dissection and fluid secretion assay

A segment of gut (from gizzard to colon) containing the entire tubule mass was removed under saline containing (in mM): 2 $CaCl_2$, 100 NaCl, 8.6 KCl, 8.5 $MgCl_2$, 4 $NaHCO_3$, 4 NaH_2PO_4, 25 MOPS, 24 glucose, 10 proline (Coast and Kay, 1994). Saline pH was adjusted to pH 7.2 using NaOH.

Fluid secretion rates of isolated Malpighian tubules were measured using the Ramsay assay (Coast, 1988). The dissected gut was transferred to a dish of fresh saline

and single tubules were pulled free from their insertion at the ampulla. Tubules were then transferred using a fine glass rod to droplets of bathing saline (20 µl) held under water-saturated paraffin oil in a Sylgard®-lined petri dish. The cut end of each tubule was pulled out of the bath so that it contacted the surface of a minuten pin (0.15 mm o.d.) pushed into Sylgard® and coated with poly-L-lysine to facilitate tubule adhesion. Dumont® #5 forceps were used to rupture the tubule wall between the pin and the bathing droplet allowing formation of a secreted fluid droplet. Droplets were removed at 40 min intervals and diameters (d) measured using an eye-piece micrometer. Fluid volume was calculated from the formula for the volume of a sphere ($\pi d^3/6$) and fluid secretion rate (nl min^{-1}) was calculated by dividing the fluid volume (pl) by the time (min) over which the droplet had formed.

3.3.3. Ca^{2+} secretion assay

Calcium concentrations of secreted fluid droplets were measured using Ca^{2+}-selective microelectrodes based on the Ca^{2+} ionophore ETH1001 (Calcium Ionophore 1 – cocktail A; Sigma–Aldrich, St. Louis, MO, USA) as described by Browne and O'Donnell (2016). Briefly, micropipettes pulled from borosilicate glass and made hydrophobic by exposure to N,N-dimethyltrimethylsilylamine were backfilled with a solution containing 150 mM KCl and 1 mM $CaCl_2$ then tip-filled with short (~ 70 µm) lengths of Ca^{2+} ionophore cocktail. Reference microelectrodes for use in the Ramsay assay were fabricated from filamented borosilicate glass capillaries pulled to a fine tip and backfilled with 150 mM KCl.

Ca^{2+} selective microelectrodes were calibrated in solutions mimicking secreted

fluid ion composition (30 mM NaCl and 114 mM KCl) and containing Ca^{2+} at concentrations bracketing the range of interest. Changes in calcium concentration were achieved by exchange of $CaCl_2$ with KCl in a 1:3 molar ratio in order to maintain ionic strength of all solutions. Slopes per 10-fold change in Ca^{2+} concentration were 27 to 29 mV.

Microelectrode voltages were measured with high-impedance (>10^{13} Ω) electrometers connected to a data acquisition system (PowerLab; ADInstruments, Sydney, Australia) and analyzed using Chart 5 software. Calcium concentrations were calculated using the following equation:

$$[\mathrm{Ca^{2+}}]_{Sample} = [\mathrm{Ca^{2+}}]_{Cal} \cdot 10^{(\Delta V/S)} \quad (1)$$

where $[\mathrm{Ca^{2+}}]_{Sample}$ represents the Ca^{2+} concentration in the sample, $[\mathrm{Ca^{2+}}]_{Cal}$ represents the Ca^{2+} concentration in one of the calibration solutions, ΔV represents the difference in voltage between the sample and the calibration solution, and S represents the slope of the electrode for a 10-fold change in Ca^{2+} concentration.

Tubule Ca^{2+} secretion rate (pmol min^{-1}) was calculated from the product of fluid secretion rate (nl min^{-1}) and Ca^{2+} concentration of secreted fluid (mM). To control for differences in tubule length within the bathing saline, a length-specific rate of Ca^{2+} secretion (pmol mm^{-1} min^{-1}) was calculated by dividing tubule Ca^{2+} secretion (pmol min^{-1}) by the total length (mm) of tubule bathed within the saline droplet (Coast and Krasnoff, 1988).

3.3.4. Drugs

Stock solutions of H-89, nifedipine, thapsigargin, 3-isobutyl-1-methylxanthine (IBMX) and forskolin were prepared in dimethyl sulfoxide (DMSO) so that the maximum final concentration of DMSO was ≤2% (v/v). Preliminary measurements indicated Ca^{2+} fluxes were unaffected by tubules bathed in saline containing ≤2% DMSO (v/v). Verapamil, adenosine 3',5'-cyclic monophosphate (cAMP), 5-hydroxytryptamine (5-HT) and NaCN were dissolved directly in bathing saline. All compounds were obtained from Sigma–Aldrich.

3.3.5. Measurement of $[Ca^{2+}]$ in haemolymph

A hind leg was removed from an adult cricket and held under paraffin oil. Light pressure was applied to the femur to extrude small (<1 µl) samples of haemolymph from the wound site. Samples were collected and transferred by pipette to another Petri dish filled with paraffin oil for measurement of haemolymph Ca^{2+} concentrations using Ca^{2+}-selective microelectrodes as described above.

3.3.6. SIET measurements of basolateral Ca^{2+} transport by Malpighian tubules

Single isolated tubules were transferred to 200 µl of bathing saline held within a plastic ring formed with a glue gun on the bottom of a 6 ml Petri dish. Dishes were pre-coated with 75 µl droplets of 62.5 µg ml^{-1} poly-L-lysine hydrobromide (Sigma–Aldrich) and air-dried to facilitate tubule adhesion.

SIET measurements were made using established protocols (Rheault and O'Donnell, 2004) with hardware from Applicable Electronics (Forestdale, MA, USA) and

automated scanning electrode technique (ASET) software (version 2.0; Science Wares Inc., East Falmouth, MA, USA). Microelectrode voltage was recorded at two positions within a two-dimensional plane perpendicular to the long axis of the tubule following a 'move, wait, sample' protocol at each site. Microelectrodes were first positioned within 5 µm of the tissue then moved 50 µm away. After each movement, the microelectrode remained stationary for 3.5 s, to allow ion gradients near the tissue to re-establish following localized stirring by the movement of the probe, followed by voltage measurement ('sampling') for 0.5 s. Voltage differences between the limits of microelectrode excursion were measured at each site. A mean voltage difference was calculated from 5 measurements made at 1 minute intervals at each site, and each measurement was itself the mean of 3 replicate measurements. Voltage differences were corrected for any drift in microelectrode voltage by subtracting a reference voltage difference recorded at a site >1000 µm away from the tissue. The bath electrode for use with SIET was pulled from 1.5 mm O.D. capillary glass, filled with 3 M KCl solution containing 3% agar and connected to the ground input of the headstage using an Ag/AgCl half-cell microelectrode holder.

Voltage differences obtained from the ASET software were converted to concentration differences using the following equation:

$$\Delta C = C_B \cdot 10^{\Delta V/S} - C_B \quad (2)$$

where C_B is the background Ca^{2+} concentration (mM or µmol cm^{-3}); ΔV is the voltage

difference between the inner and outer limits of microelectrode excursion at each site (µV); and S is the slope (µV) of the microelectrode for a 10-fold change in Ca^{2+} concentration. Diffusive Ca^{2+} fluxes were then calculated from Ca^{2+} concentration gradients using the Fick equation:

$$J_{Ca^{2+}} = D_{Ca^{2+}} \cdot \Delta C / \Delta x \quad (3)$$

where $J_{Ca^{2+}}$ is the flux of Ca^{2+} (pmol cm^{-2} s^{-1}); $D_{Ca^{2+}}$ is the diffusion coefficient of Ca^{2+} at 25 ºC (7.9×10^{-6} cm^2 s^{-1}) obtained from Smith et al. (1999); and Δx is the excursion distance (0.005 cm). By convention, positive fluxes indicate Ca^{2+} flux out of the tubule (efflux) and negative fluxes indicate Ca^{2+} flux into the tubule (influx).

3.3.7. Statistical analysis

Graphing and statistical analysis were performed using Prism software (version 4.0b; GraphPad Software Inc., San Diego, CA, USA). Data are presented as means ± SEM for the indicated number of samples (*N*). Comparisons between means were made using Student's t-tests and differences were considered statistically significant if $P < 0.05$.

3.4. Results

3.4.1. Segment-specific basolateral Ca^{2+} transport

The numerous Ca-rich granules observed within the cells of the midtubule, which are notably absent in the distal tubule region, suggested different patterns of Ca^{2+} transport by the two regions. We therefore used SIET to measure basolateral Ca^{2+}

transport in both tubule segments. Preliminary results (data not shown) revealed that within each tubule segment basolateral Ca^{2+} fluxes were consistent along their length. Basolateral Ca^{2+} fluxes measured across distal tubules (0 ± 0.9 pmol cm^{-2} s^{-1}, *N*=8) were negligible relative to those across the midtubule (-33 ± 0.3 pmol cm^{-2} s^{-1}, *N*=8).

3.4.2. Effect of cyclic nucleotides on tubule Ca^{2+}transport

To investigate potential links between fluid secretion and Ca^{2+} transport, tubules were exposed to the second messenger, cAMP, known to stimulate fluid secretion in cricket tubules (Coast et al., 1991, Xu and Marshall, 2000). SIET measurements revealed that basolateral Ca^{2+} influx increased >3.5-fold in the presence of 1 mM cAMP (Figure 3.1A). There was no effect of cAMP on Ca^{2+} secretion by the tubules, as measured in the Ramsay assay (Figure 3.1B).

Although fluid secretion increased 1.5-fold, from 165 ± 7 pl mm^{-1} min^{-1} (*N*=15) to 249 ± 19 pl mm^{-1} min^{-1} (*N*=13), Ca^{2+} concentration of the secreted fluid decreased 2-fold, from 0.28 ± 0.04 mM Ca^{2+} (*N*=15) to 0.14 ± 0.02 mM Ca^{2+} (*N*=13). The reduction in Ca^{2+} concentration of luminal fluid was consistent with increased fluid transport, i.e. dilution of luminal contents. Together, these results indicate that cyclic nucleotides promote Ca^{2+} entry across the basolateral membrane of the tubules but have little effect on Ca^{2+} secretion across the apical membrane.

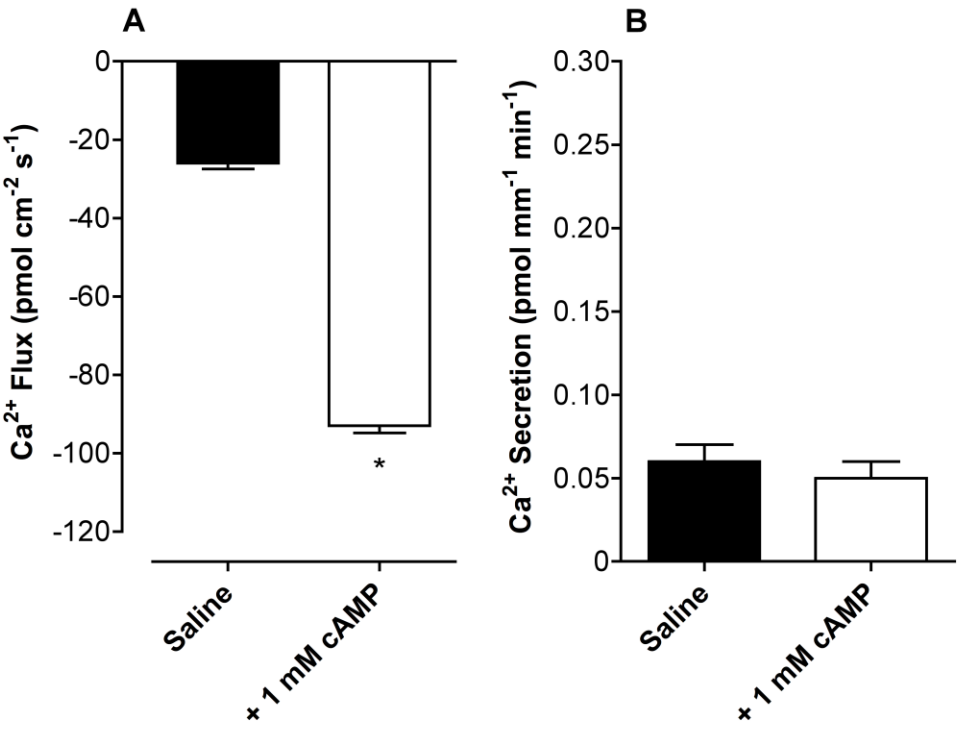

Figure 3.1 Basolateral Ca^{2+} flux (A) and Ca^{2+} secretion (B) by midtubules isolated from *Acheta domesticus* bathed in saline alone (solid bars) or in saline containing 1 mM cAMP (open bars). A) Negative values represent net inward-directed basolateral Ca^{2+} transport (influx) measured by SIET. B) Positive flux values represent lumen-directed Ca^{2+} transport (secretion) measured in the Ramsay assay. Significant differences relative to saline (solid bars) are indicated by an asterisk. *N*=171–321 and *N*=13–15 for A and B, respectively.

3.4.3. Pharmacological manipulation of cAMP-dependent pathways using IBMX, forskolin and H-89

Endogenous cAMP-dependent pathways were manipulated to further investigate the role of cAMP in stimulating basolateral Ca^{2+} influx. Tubules were simultaneously stimulated with forskolin (an activator of adenylyl cyclase) and 3-isobutyl-1-methylxanthine (IBMX; a non-selective inhibitor of phosphodiesterases) in order to maximally increase intracellular cAMP concentrations. Basolateral Ca^{2+} influx increased by 84% in response to 0.1 mM IBMX and 0.1 mM forskolin (Figure 3.2A).

Tubules were exposed to the protein kinase A (PKA) antagonist, H-89, to determine if cAMP-dependent Ca^{2+} influx was mediated by PKA. Basolateral Ca^{2+} influx in cAMP-stimulated tubules decreased by 45% in response to 10 µM H-89 (Figure 3.2B). In aggregate, these results suggest that Ca^{2+} influx across tubules is regulated by an endogenous cAMP-dependent pathway utilizing PKA.

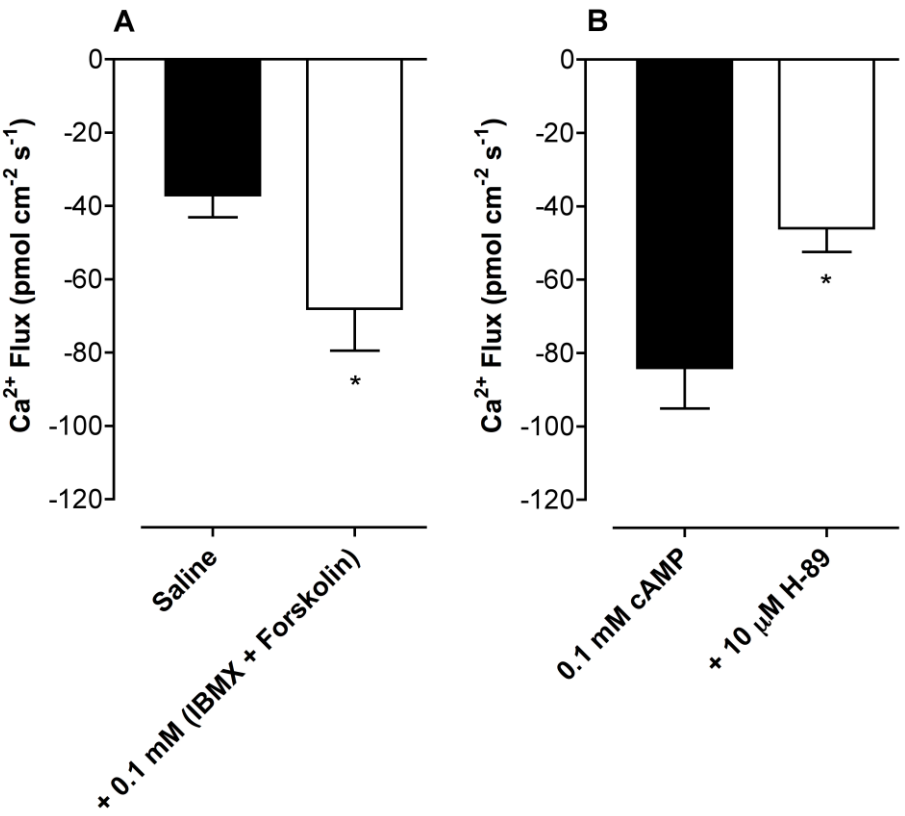

Figure 3.2 Effects of manipulation of endogenous cAMP levels on basolateral Ca^{2+} fluxes. A) Tubules were bathed in saline alone (solid bar) or in combination with both 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.1 mM Forskolin (open bar). B) Tubules were exposed to saline containing 0.1 mM cAMP alone (solid bar) or in combination with 10 µM H-89 (open bar). Saline controls contained 2% DMSO and 0.1% DMSO in A and B, respectively. Significant differences relative to drug-free salines (solid bars) are indicated by an asterisk. *N*=8–9 for both A and B.

3.4.4. Effects of 5-HT and thapsigargin on Ca^{2+}transport

Both serotonin (5-HT) and thapsigargin are stimulants of fluid secretion that act, in-part, by elevating intracellular Ca^{2+} concentrations (Coast, 2011, MacPherson et al., 2001). We therefore assessed whether pharmacological manipulation of intracellular Ca^{2+} concentrations altered Ca^{2+} transport across basolateral and/or apical membranes. Basolateral Ca^{2+} flux decreased >2-fold across tubules exposed to 1 µM 5-HT (Figure 3.3A) and was reversed from influx to efflux across tubules exposed to 10 µM thapsigargin (Figure 3.3B). In tubules stimulated with 0.1 mM cAMP, basolateral Ca^{2+} influx was decreased >6-fold following treatment with 10 µM thapsigargin, suggesting calcium signaling can override stimulation by cAMP (Figure 3.3C). In contrast to the inhibitory effects of thapsigargin and 5-HT on basolateral Ca^{2+} fluxes, Ramsay secretion assays revealed that rates of Ca^{2+} secretion increased 6-fold following thapsigargin treatment, from 0.041 ± 0.007 pmol mm^{-1} min^{-1} (*N*=7) to 0.251 ± 0.031 pmol mm^{-1} min^{-1} (*N*=7), as a result of increases in the rates of both fluid secretion and secreted fluid Ca^{2+} concentration. Fluid secretion increased 2-fold, from 134 ± 5 pl mm^{-1} min^{-1} (*N*=7) to 267 ± 12 pl mm^{-1} min^{-1} (*N*=7) and secreted fluid Ca^{2+} concentration increased 3-fold, from 0.22 ± 0.03 mM Ca^{2+} (*N*=7) to 0.67 ± 0.08 mM Ca^{2+} (*N*=7), in response to 10 µM thapsigargin.

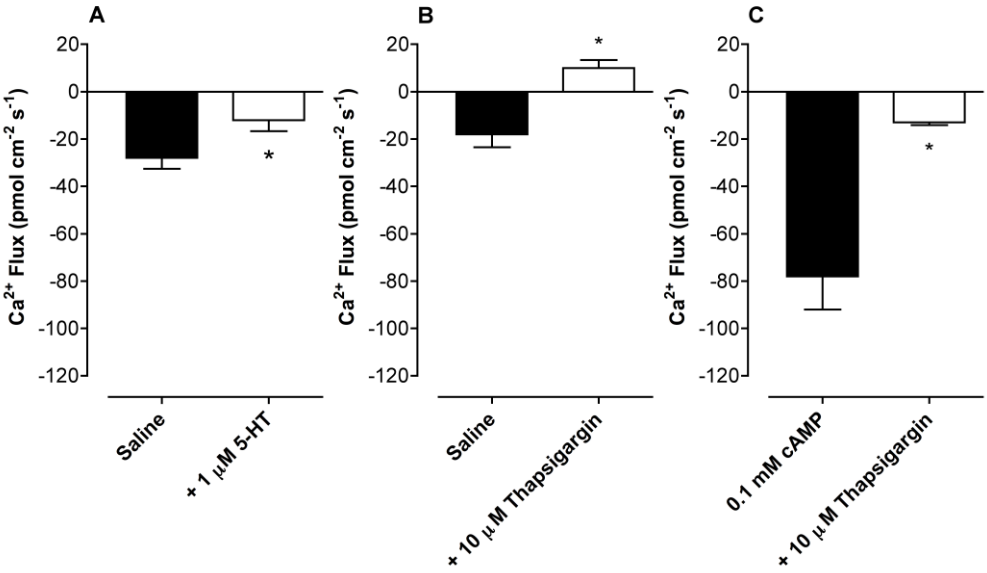

Figure 3.3 Effects of manipulation of intracellular Ca^{2+} levels on basolateral Ca^{2+} flux. A) Tubules were bathed in saline with (open bar) or without (solid bar) 1 µM 5-HT. B) Tubules were bathed in saline with (open bar) or without (solid bar) 10 µM thapsigargin. C) Tubules were bathed in saline containing 0.1 mM cAMP alone (solid bar) or in combination with 10 µM thapsigargin. Saline controls contained 1% DMSO in B and C. Significant differences relative to solid bars are indicated by an asterisk. *N*=10, 7–8 and 10-14 for A, B and C, respectively.

3.4.5. Effects of L-type calcium channel antagonists on basolateral Ca^{2+} transport

Previous studies of tubules from flies have demonstrated inhibition of Ca^{2+} transport by the calcium channel blockers nifedipine, verapamil and diltiazem (Dube et al., 2000a, MacPherson et al., 2001). We therefore used these drugs to test the hypothesis that calcium channels also play a role in Ca^{2+} entry across tubules of the house cricket. Tubules were exposed to 0.1 mM cAMP alone or together with nifedipine, verapamil or diltiazem (0.1 mM). Rates of Ca^{2+} influx by tubules stimulated with 1 mM cAMP (83 ± 8 pmol cm^{-2} s^{-1}, *N*=6) were unaffected by the addition of the drug vehicle (1% DMSO) to the bathing saline (88 ± 11 pmol cm^{-2} s^{-1}, *N*=6). SIET measurements revealed basolateral Ca^{2+} influx was reduced >7-fold and >5-fold by tubules treated with nifedipine and verapamil, respectively (Figure 3.4). In diltiazem-treated tubules, basolateral Ca^{2+} flux was reversed from influx to efflux (Figure 3.4). These data are consistent with Ca^{2+} entry into cAMP-stimulated tubules through voltage-gated (L-type) calcium channels.

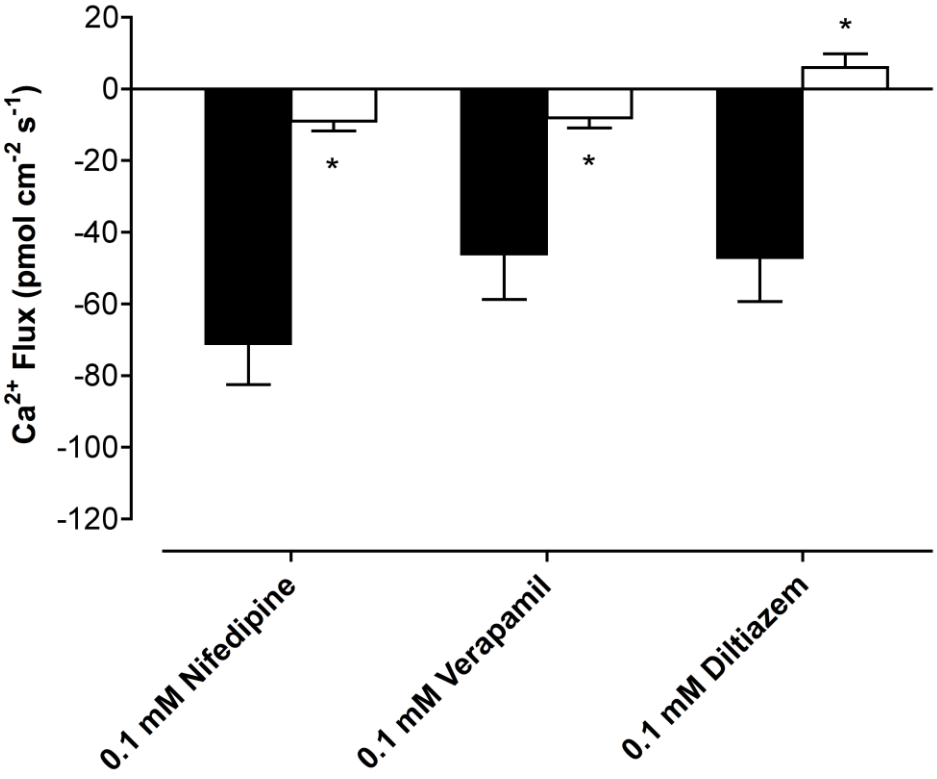

Figure 3.4 Effects of calcium channel blockers on basolateral Ca^{2+} fluxes by tubules bathed in saline containing 0.1 mM cAMP. Tubules were bathed in the absence (solid bars) or presence (open bars) of either nifedipine, verapamil or diltiazem (0.1 mM). Nifedipine experiments included the drug vehicle (1% DMSO). Significant differences relative to drug-free salines (solid bars) are indicated by an asterisk. *N*=7–8 for each drug.

3.4.6. Effects of high bathing [K^+] on basolateral Ca^{2+} transport

Results from Figure 3.4 suggested voltage-gated calcium channels facilitate cAMP-stimulated basolateral Ca^{2+} transport. Changes in bathing saline K^+ concentration have been shown to alter basolateral membrane potential, as measured by conventional microelectrodes, in tubules of *A. domesticus* (Coast et al., 2007). Further, increasing bathing saline K^+ concentration from 4 to 100 mM K^+ was associated with a >40% decrease in basolateral $^{45}Ca^{2+}$ flux across tubules of *D. melanogaster* (Dube et al., 2000a). To investigate the voltage-dependence of basolateral Ca^{2+} influx mechanisms, tubules were bathed in saline containing 8.6 or 86 mM K^+; saline containing 86 mM K^+ was made by equimolar substitution of KCl for NaCl.

Rates of Ca^{2+} influx by tubules bathed in saline containing 8.6 mM K^+ (-27 ± 6 pmol cm^{-2} s^{-1}, *N*=7) decreased 9-fold when bathed in saline containing 86 mM K^+ (-3 ± 3 pmol cm^{-2} s^{-1}, *N*=8).

3.4.7. Rates of Ca^{2+} sequestration by tubules

Ca^{2+} which enters the tubule across the basolateral membrane may be secreted into the tubule lumen or sequestered within the tubule cells. Rates of Ca^{2+} sequestration can be calculated from the difference between basolateral Ca^{2+} transport measured by SIET and Ca^{2+} secretion measured using the Ramsay assay. Estimates of basolateral Ca^{2+} flux (pmol min^{-1}) across single tubules were calculated by multiplying SIET fluxes (pmol cm^{-2} s^{-1}) by the surface area of the tubules (cm^2) and by 60 s min^{-1}. Given that Ca^{2+} flux across the distal tubule is less than 3% of that across the midtubule, only the area of the midtubule was used for these calculations. An estimate of the surface area of the

midtubule (0.013 cm^2) was calculated using the formula for the surface area of a cylinder (πdl) and published estimates of tubule diameter (*d*, 0.0055 cm) and midtubule length (*l*, 0.75 cm) from Hazelton et al. (1988). Ca^{2+} secretion (pmol min^{-1}) by single tubules was estimated by multiplying rates of length-specific Ca^{2+} secretion obtained from the Ca^{2+} secretion assay (pmol mm^{-1} min^{-1}) by the total length of tubules (10 mm) presented by Hazelton et al. (1988). Basolateral Ca^{2+} influx (-26.3 ± 1 pmol cm^{-2} s^{-1}, *N*=171) was equivalent to -20.5 pmol min^{-1} and Ca^{2+} secretion (0.06 ± 0.01 pmol mm^{-1} min^{-1}, *N*=15) was equivalent to 0.6 pmol min^{-1} for tubules bathed in saline. Thus, basolateral Ca^{2+} influx was >30-times larger than the corresponding rate of Ca^{2+} secretion. The difference between the influx of -20.5 pmol min^{-1} and the secretion of 0.6 pmol min^{-1} indicates that Ca^{2+} was sequestered within the tubule cells at the rate of -19.9 pmol min^{-1} (Figure 3.5A). Corresponding calculations for tubules bathed in saline containing 1 mM cAMP revealed that basolateral Ca^{2+} influx (-72.5 pmol min^{-1}) was >140-times larger than the corresponding rate of Ca^{2+} secretion (0.5 pmol min^{-1}) resulting in Ca^{2+} sequestration (-72 pmol min^{-1}) that was >3.5-fold higher than in the absence of cAMP stimulation (Fig 3.5B). Measurements for tubules bathed in saline containing 10 µM thapsigargin revealed that basolateral Ca^{2+} efflux (+7.8 pmol min^{-1}) was >3-fold larger than the corresponding rate of Ca^{2+} secretion (+2.5 pmol min^{-1}) consistent with release of sequestered Ca^{2+} (+10.3 pmol min^{-1}) that was 2-fold lower in magnitude and opposite in direction compared rates of sequestration for tubules bathed under saline alone (Figure 3.5C versus Figure 3.5A).

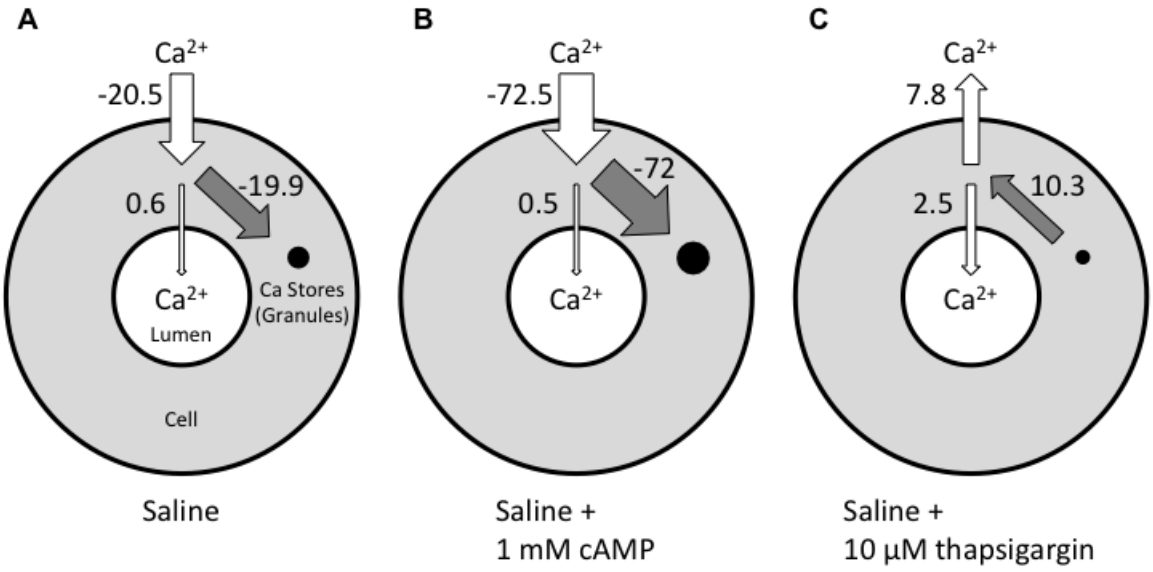

Figure 3.5 Schematic diagram summarizing rates of Ca^{2+} sequestration (filled arrows) by tubules (drawn in cross-section) bathed in the presence of (A) saline (B) 1 mM cAMP or (C) 10 µM thapsigargin. Open arrows represent basolateral Ca^{2+} fluxes and Ca^{2+} secretion. Arrow widths approximate the magnitude of the indicated pathway (all values have units of pmol min^{-1}). Further explanation in the text.

3.5. Discussion

In this study, we show that Ca^{2+} transport by isolated Malpighian tubules of *A. domesticus* is specific to midtubule segments, where 97% of the Ca^{2+} entering the tubule across the basolateral membrane is sequestered within intracellular calcium stores. Antagonists of voltage-gated (L-type) calcium channels decreased Ca^{2+} influx by cAMP-stimulated tubules, suggesting basolateral Ca^{2+} influx is facilitated by voltage-gated Ca^{2+} channels. Using a pharmacological approach, the existence of two signaling pathways were discovered for the control of Ca^{2+} sequestration: 1) an adenylyl cyclase-cAMP-PKA pathway that promotes Ca^{2+} sequestration and 2) a pathway involving intracellular Ca^{2+} where 5-HT and thapsigargin inhibit Ca^{2+} sequestration. Possible first messengers involved in the modulation of Ca^{2+} sequestration are discussed below and a working model of Ca^{2+} transport and related regulatory pathways is provided (Figure 3.6).

3.5.1. Secretion of fluid and Ca^{2+} are not directly linked

The distal segments of *A. domesticus* tubules secrete fluid at high rates despite the lack of basolateral Ca^{2+} influx, indicating that secretion of fluid and Ca^{2+} transport are not linked in this segment. Both 1 mM cAMP and 10 µM thapsigargin increased fluid transport by the midtubule but had very different effects on Ca^{2+} transport. Simultaneous Ca^{2+} efflux and fluid influx during thapsigargin exposure is *prima facie* evidence that basolateral Ca^{2+} and fluid transport are uncoupled and suggests basolateral Ca^{2+} influx is not required for fluid secretion. These data fit well with observations that tubules of *A. domesticus* secrete at control rates in Ca^{2+}-free saline (Coast, 2011). In addition, the magnitude of the increase (6-fold) in Ca^{2+} secretion was much larger than the increase (2-

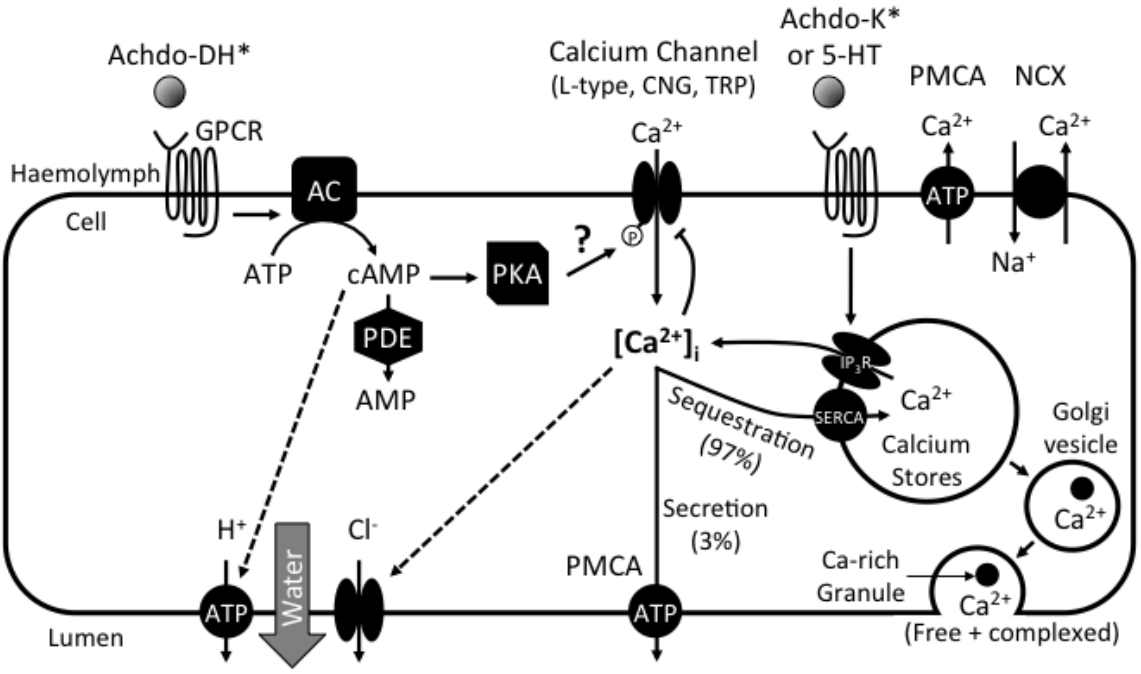

Figure 3.6 A model of Ca^{2+} transport and related regulatory pathways by Malpighian tubules of *A. domesticus*. Asterisks (*) represent possible first messengers based on evidence that *A. domesticus* diuretic hormone (Achdo-DH) and kinins (Achdo-K) increase [cAMP] and $[Ca^{2+}]_i$ in tubules of *A. domesticus*, respectively (Coast and Kay, 1994, Coast, 2011). Dashed arrows represent stimulatory pathways that increase fluid secretion. Abbreviations: AC, adenylyl cyclase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CNG, cyclic-nucleotide gated; GPCR, G-protein coupled receptor; 5-HT, 5-hydroxytryptamine; IP_3R, inositol triphosphate receptor; NCX, Na^+/Ca^{2+}-exchanger; PDE, phosphodiesterase; PKA, protein kinase A ; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; TRP, transient receptor potential

fold) in fluid secretion by tubules exposed to 10 µM thapsigargin, suggesting fluid and Ca^{2+} secretion are not directly linked.

3.5.2. Secretion of Ca^{2+} and the nature of apical calcium transport

It is well established that Malpighian tubules of insects (*A. domesticus, T. oceanicus, D. melanogaster, R. prolixus*) secrete calcium into the primary urine, contributing to the elimination of excess dietary calcium (O'Donnell and Maddrell, 1995, Marshall et al., 1993, Maddrell et al., 1991, Spring and Hazelton, 1987). Ca^{2+} is moved from cell-to-lumen thermodynamically uphill by active Ca^{2+} exporting pumps since both chemical (~1000-fold) and electrical (V_{apical} = +71 mV lumen-positive) gradients across apical membranes of *A. domesticus* tubules oppose Ca^{2+} entry into the lumen (Coast, 2012). Plasma membrane Ca^{2+}-ATPase (PMCA) pumps have been localized to the apical membranes in tubules of the gypsy moth, *L. dispar* (Pannabecker et al., 1995). These data suggest that the spontaneous Ca^{2+} secretion observed in tubules of *A. domesticus* may be facilitated by apically located Ca^{2+}-ATPases.

Intracellular Ca-rich granules found in cricket tubules are believed to be released into the lumen by exocytosis in response to cAMP-stimulation (Spring and Felgenhauer, 1996, Wessing and Zierold, 1999). Secretion of Ca-rich granules would go undetected by our methods since Ca^{2+}-selective microelectrodes only detect calcium in the ionic form. These data are consistent with the lack of effect of 1 mM cAMP on rates of tubule Ca^{2+} secretion using the Ramsay assay. In addition, Ca^{2+} concentrations of secreted fluid decreased 2-fold in response to 1 mM cAMP, suggesting Ca-rich granules of *A. domesticus* do not readily decalcify within the secreted fluid. Taken together, these data

suggest that in response to cAMP-stimulation tubules of *A. domesticus* may secrete Ca-rich granules into the lumen where they remain relatively intact.

Intracellular calcium concentrations can be elevated using the calcium mobilizing agents thapsigargin and A23187. Ca^{2+} secretion by tubules was increased 6-fold following exposure to 10 µM thapsigargin. Even larger increases (10-fold and 15-fold) in Ca^{2+} secretion were described across posterior tubules of *D. melanogaster* in response to application of 2 µM thapsigargin or 1 µM A23187, respectively (Dube et al., 2000a). Further, Ca^{2+} secretion was increased >20-fold across tubules of *D. melanogaster* in response to stimulation with 1 mM cAMP in combination with 100 µM leucokinin-1, a Ca^{2+}-dependent diuretic peptide (O'Donnell and Maddrell, 1995). These results suggest that one consequence of increasing intracellular Ca^{2+} levels is increased secretion of Ca^{2+} into the tubule lumen.

3.5.3. Segment-specific basolateral Ca^{2+} transport – a primary role for midtubules

In order to accumulate calcium within internal stores (granules), calcium must first be taken up by the surrounding tubule epithelia. To that end, basolateral Ca^{2+} influx across midtubule segments is consistent with the presence of numerous intracellular Ca-rich granules observed in ultrastructural investigations (Hazelton et al., 1988, Lipovsek Delakorda et al., 2009). Conversely, the lack of Ca^{2+} transport by the distal tubules is consistent with the absence of granules. Segment-specific Ca^{2+} transport has been demonstrated previously: in specialized tubule segments of the alkali fly, *E. hians* (Herbst and Bradley, 1989), in the upper tubules of the kissing bug, *R. prolixus* (Maddrell et al.,

1991) and in distal tubules of the fruit fly, *D. melanogaster* (Browne and O'Donnell, 2016). Regionalization of Ca^{2+} transport may reflect the distribution of the Malpighian tubules within the hemocele. For example, tubule storage segments in anterior tubules of *D. melanogaster* are distributed such that calcium storage segments (distal tubules) lie adjacent to regions of dietary calcium absorption across the midgut (Chintapalli et al., 2012).

3.5.4. Basolateral Ca^{2+} transport by voltage-sensitive calcium channels and other putative transporters

Malpighian tubules remove excess haemolymph calcium by taking it up across the basolateral membrane in storage segments. Net transport across the basolateral membrane is the result of two competing processes: Ca^{2+} influx and Ca^{2+} efflux. Ca^{2+} influx is thermodynamically favorable as both chemical (~ 10,000-fold) and electrical gradients (V = -41 mV) across the basolateral membrane strongly favor Ca^{2+} entry (Coast et al., 2007). L-type calcium channels have been immunolocalized to basolateral membranes of the principal cells in tubules of *D. melanogaster*, where they presumably facilitate passive Ca^{2+} influx (MacPherson et al., 2001). Simultaneously, the plasma membrane Ca^{2+}-ATPase (PMCA) and the sodium-calcium exchanger (NCX) are proposed to facilitate Ca^{2+} efflux (Simkiss, 1996). A range of chemically-distinct calcium channel blockers, nifedipine (dihydropyridine), verapamil (phenylakylamine) and diltiazem (benzothiazepine), were effective in blocking cAMP-mediated increases in Ca^{2+} influx in our studies, suggesting that Ca^{2+} influx occurs through voltage-gated calcium channels. The voltage-dependence of basolateral Ca^{2+} influx was confirmed using high potassium

(86 mM K^+) saline to depolarize the basolateral membrane potential, resulting in a 9-fold drop in rates of Ca^{2+} influx.

Given that the cyclic nucleotide, cAMP, stimulated basolateral Ca^{2+} influx, it is possible cyclic-nucleotide-gated (CNG) calcium channels may also facilitate basolateral Ca^{2+} entry. Tubules of *D. melanogaster* express both CNG and L-type calcium channels, suggestive of possible roles in basolateral Ca^{2+} uptake (MacPherson et al., 2001). As reported above, our results using high K^+ saline indicated basolateral Ca^{2+} influx was voltage dependent. In contrast, CNG channels show very little voltage-dependence, suggesting they may play a relatively minor role in Ca^{2+} uptake by tubules of *A. domesticus* (Zagotta and Siegelbaum, 1996). Likely owing to their similarity, the L-type calcium channel blockers nifedipine and diltiazem have been shown to block CNG activity at similar doses used in this study (Zufall and Firestein, 1993), suggesting the L-type calcium channel blockers are not specific for L-type calcium channels. Transient receptor potential (TRP) calcium channels have also been proposed to play a role in basolateral Ca^{2+} entry, as they have been immunolocalized to principal cells in tubules of *D. melanogaster* where they mediate intracellular Ca^{2+} signaling events by the diuretic neuropeptide CAP_{2b} (MacPherson et al., 2005). In light of these findings, several types of Ca^{2+} influx channels (L-type, CNG and TRP) may play roles in basolateral Ca^{2+} entry across the tubules of *A. domesticus*.

3.5.5. Relative roles of basolateral and apical Ca^{2+} transport

Regulation of haemolymph calcium appears to be dictated predominantly by Ca^{2+} transport across basolateral membranes. Rates of basolateral Ca^{2+} transport exceeded

apical Ca^{2+} secretion >30-fold in saline, >140-fold in saline containing 1 mM cAMP and >3-fold in saline containing 10 µM thapsigargin. Consistent with these results, rates of whole-tubule basolateral Ca^{2+} transport exceed Ca^{2+} secretion by >11-fold and >7-fold in anterior and posterior tubules of adult *D. melanogaster*, respectively (Browne and O'Donnell, 2016, Dube et al., 2000a).

The physiological relevance of Ca^{2+} transport by tubules can be appreciated by estimating the time required for all tubules (112, Hazelton et al. (1988)) to remove the entire calcium content of the haemolymph. Using estimates of haemolymph volume (~100 µl, MacMillan et al. (2012)) and Ca^{2+} concentration (2.1 ± 0.1 mM, *N*=11) the resulting haemolymph calcium content was ~200 nmol. At rates of Ca^{2+} transport by all 112 tubules exposed to saline (-2 nmol min^{-1}) and 1 mM cAMP (-8 nmol min^{-1}) the tubules could remove the entire haemolymph calcium content within 100 min and 25 min, respectively. These estimates suggest that endocrine factors which elevate intracellular cAMP may contribute to regulation of hemolynph Ca^{2+} concentrations in response to increased dietary Ca^{2+} intake.

3.5.6. An adenylyl cyclase-cAMP-PKA pathway promotes Ca^{2+} sequestration

The stimulatory effect of the second messenger, cAMP, was suggestive of a cAMP-dependent pathway for basolateral Ca^{2+} entry. Activation of adenylyl cyclase with forskolin in combination with the non-selective phosphodiesterase inhibitor, IBMX, increased basolateral Ca^{2+} influx by 84%, whereas application of the protein kinase A (PKA) antagonist, H-89, to cAMP-stimulated tubules decreased Ca^{2+} influx by 45%. Our

results suggest that a first messenger, such as a corticotropin-releasing factor (CRF)-related peptide (Coast and Kay, 1994) may lead to activation of adenylyl cyclase and increases in intracellular cAMP concentration that then activate PKA-dependent phosphorylation of effector proteins that ultimately result in enhanced Ca^{2+} influx. Other second messengers, such as cyclic guanosine monophosphate (cGMP), known to stimulate fluid secretion (Xu and Marshall, 2000, Coast et al., 2007), also have the potential to influence tubule Ca^{2+} transport.

3.5.7. Elevation of intracellular Ca^{2+} inhibits Ca^{2+} sequestration

Pharmacological manipulations of the serotonin receptors on *A. domesticus* tubules suggests that activation of a 5-HT-like G-protein coupled receptor (GPCR) stimulates fluid secretion via the inositol triphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores (Coast, 2011). We found 5-HT decreased Ca^{2+} influx by tubules >2-fold, suggesting that IP_3-mediated release of Ca^{2+} from intracellular stores inhibits Ca^{2+} influx from the haemolymph. Application of the calcium mobilizing agent thapsigargin reversed the direction of basolateral Ca^{2+} transport (from influx to efflux) and increased tubule Ca^{2+} secretion by 4-fold, suggesting that elevations in intracellular calcium inhibit Ca^{2+} sequestration by decreasing Ca^{2+} influx and by promoting the release of Ca^{2+} from sequestered stores within the tubule cells (Figure 3.6).

The efflux of Ca^{2+} from tubule cell-to-bath in response to 10 µM thapsigargin (Figure 3.5C) may result from changes in the relative contribution of putative basolateral membrane Ca^{2+} transporters, such as the Na^+/Ca^{2+}-exchanger. For example, if entry through voltage-dependent Ca^{2+} channels is inhibited in response to thapsigargin, Ca^{2+}

efflux through basolateral Na^+/Ca^{2+}-exchangers and Ca^{2+}-ATPases may be unmasked. The increase in secretion of Ca^{2+} in response to thapsigargin may relate to the depolarizing effects of increases of intracellular Ca^{2+} on transepithelial membrane potentials (Coast and Kay, 1994). Kinins and thapsigargin enhance Cl^- permeability of the apical membrane, thus depolarizing transepithelial potential (O'Donnell et al., 1996).

3.5.8. Control of Ca^{2+} sequestration by cAMP and intracellular Ca^{2+}

Ca^{2+} sequestration by the Malpighian tubules is the dominant mechanism for haemolymph Ca^{2+} homeostasis from at least one insect species from the orders Diptera, Hemiptera and Orthoptera, suggesting mechanisms of tubule Ca^{2+} sequestration may have broad relevance across insect orders. We have identified two opposing second messenger pathways (cAMP and Ca^{2+}) affecting tubule Ca^{2+} sequestration which may play a role in haemolymph Ca^{2+} homeostasis in response to variations in dietary Ca^{2+} intake. Firstly, a cAMP-dependent pathway that enhances Ca^{2+} sequestration could maintain haemolymph calcium concentrations during periods of high dietary calcium intake or feeding. The importance of this pathway is highlighted given that calcium is abundant in many of the foods ingested by insects: hematophagous insects may feed on mammalian and avian blood containing 1 to 3 mM Ca^{2+} (Moore, 1970, Bar, 2009), and phytophagous insects often feed on fruits and vegetables such as peaches and cabbage that contain 6 and 40 mg Ca $100g^{-1}$, respectively (U.S. Department of Agriculture, 2013). Thus, rates of dietary Ca^{2+} absorption into the haemolymph may often be in excess of requirements. In *A. domesticus*, the actions of the corticotropin releasing factor (CRF)-related diuretic neuropeptide, Achdo-DH, can be mimicked by cAMP, suggesting that the CRF-related

diuretic pathway may also play a role in tubule Ca^{2+} transport (Coast and Kay, 1994). Secondly, we identified inhibition of Ca^{2+} sequestration in response to agents which elevate intracellular Ca^{2+} levels (thapsigargin, 5-HT). Inhibition of tubule Ca^{2+} sequestration in response to kinins or 5-HT may contribute to haemolymph Ca^{2+} homeostasis during periods of low dietary calcium intake or starvation.

Chapter 4

4. Calcium transport across the basolateral membrane of isolated Malpighian tubules: A survey of several insect orders

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4.1. Abstract

The Malpighian tubules play a major role in haemolymph calcium homeostasis in insects by sequestering excess Ca^{2+} within biomineralized granules that often accumulate in the tubule cells and/or lumen. Using the scanning ion-selective microelectrode technique (SIET) measurements of basolateral Ca^{2+} transport were determined at several sites along the length of the Malpighian tubules isolated from the following eight insects representing seven orders: *Drosophila melanogaster* (Diptera), *Aedes aegypti* (Diptera), *Tenebrio molitor* (Coleoptera), *Acheta domesticus* (Orthoptera), *Trichoplusia ni* (Lepidoptera), *Periplaneta americana* (Blattodea), *Halyomorpha halys* (Hemiptera) and *Pogonomyrmex occidentalis* (Hymenoptera). Ca^{2+} transport was specific to tubule segments containing Ca-rich granules in *D. melanogaster* and *A. aegypti*, whereas Ca^{2+} transport was relatively uniform along the length of whole tubules in the remaining species. Generally, manipulation of second messenger pathways using cAMP and

thapsigargin had little effect on rates of basolateral Ca^{2+} transport, suggesting previous effects observed across midtubules of *A. domesticus* are unique to this species. In addition, this study is the first to provide measurements of basolateral Ca^{2+} across single principal and secondary tubule cells, where Ca^{2+} uptake occurs only across principal cells. Estimated times for all tubules to eliminate the entire haemolymph Ca^{2+} content in each insect ranged from 6 min (*D. melanogaster*) to 19 hours (*H. halys*) or more, indicating that rates of Ca^{2+} uptake by the Malpighian tubules are not always rapid. Our results suggest that the principal cells of the Malpighian tubules contribute to haemolymph calcium homeostasis by sequestering excess Ca^{2+}, often within specific tubule segments.

4.2. Introduction

Haemolymph calcium concentrations in insects are maintained within narrow limits in order to maintain tissue function. Despite a > 6-fold increase in the calcium content of the diet, haemolymph Ca^{2+} concentrations of flies increase < 1.5-fold, indicating Dipterans have the capacity for haemolymph calcium regulation (Taylor, 1985b, Dube et al., 2000b). Changes in either Ca^{2+} absorption or Ca^{2+} excretion may alter haemolymph Ca^{2+} concentrations and thus absorptive and excretory organs are potential sites for haemolymph calcium regulation. Measurements of $^{45}Ca^{2+}$ transport across isolated preparations of midgut (absorptive gut segment) and Malpighian tubules (excretory organs) led Taylor (1985b) to conclude that regulation of Ca^{2+} excretion and not Ca^{2+} absorption is the primary mechanism for haemolymph calcium regulation in the blowfly, *C. vicina*. Taylor proposed that insects fully absorb all dietary calcium into the haemolymph and that any excesses are then removed by the excretory organs (the

Malpighian tubules and hindgut). To date, the contribution of the hindgut to Ca^{2+} homeostasis remains to be investigated. In contrast, there is much evidence to suggest that the Malpighian tubules are major sites of haemolymph calcium homeostasis. Firstly, Malpighian tubules are sites of high $^{45}Ca^{2+}$ turnover (Taylor, 1985b) and are often sites of significant stores of calcium (containing up to 94% of total body calcium in the fruit fly, *D. hydei*; Wessing and Zierold (1992)). Secondly, spherical granules ~ 0.1 to 10 µm in diameter and rich in calcium, often as phosphates, accumulate within the cells and/or lumen of the Malpighian tubules in many insects (Brown, 1982, Ballan-Dufrancais, 2002). These Ca-rich granules are believed to be the major sites of internal calcium storage, contributing to haemolymph calcium homeostasis in insects. Lastly, in studies measuring Ca^{2+} transport along the entire length of Malpighian tubules, basolateral Ca^{2+} transport is most prominent within specialized tubule regions that contain Ca-rich granules, implicating sites of calcium storage (Ca-rich granules) in tubule Ca^{2+} handling (Browne and O'Donnell, 2016, Browne and O'Donnell, 2018).

Malpighian tubules can remove excess Ca^{2+} from the haemolymph either by secretion or sequestration. Secretion refers to the transport of Ca^{2+} from haemolymph to tubule lumen in soluble form whereas sequestration refers to Ca^{2+} transported into the Malpighian tubules where it is then incorporated into Ca-rich granules as precipitates of calcium. In tubules isolated from adults of *D. melanogaster* and *A. domesticus* 85-91 % and 97 %, respectively, of the Ca^{2+} that enters the tubule cells is sequestered there with the remainder being secreted into the primary urine (Browne and O'Donnell, 2016, Browne and O'Donnell, 2018, Dube et al., 2000a). It follows, then, that Ca^{2+} sequestration

is the dominant mechanism for haemolymph calcium homeostasis in these insects, where increases in dietary Ca^{2+} uptake are followed by increases in Ca^{2+} sequestration in order to maintain haemolymph calcium concentrations within a limited physiological range.

The Malpighian tubules of many insects are morphologically and/or functionally segmented along their length. Segmented Malpighian tubules allow, just as in vertebrate renal tubules, fluid and useful solutes transferred into the tubule lumen in upper regions to be reabsorbed in downstream segments (O'Donnell and Maddrell, 1995). The accumulation of Ca-rich granules within distinct tubule segments suggests that Ca^{2+} is often taken up and sequestered by the Malpighian tubules in a segment-specific manner: within the distal (upper) tubules of *R. prolixus* (Hemiptera; Maddrell et al. (1991)), the distal tubules of *D. melanogaster* and *C. vicina* (Diptera; (Taylor, 1985b, Browne and O'Donnell, 2016)), and the midtubules of *A. domesticus* (Orthoptera; Browne and O'Donnell (2018)). Restricting Ca^{2+} uptake and sequestration to specific segments of the Malpighian tubules may allow internal calcium stores to accumulate large quantities of calcium without hindering osmoregulatory functions. It is thus of interest to determine sites of basolateral Ca^{2+} entry along the length of the Malpighian tubules in species with diverse tubule morphology.

Malpighian tubules lack innervation and thus are controlled by neuropeptide hormones released into the haemolymph from neurohemal areas in response to feeding or other stimuli (Coast and Garside, 2005). Neuropeptides act by binding cell surface receptors that initiate signal transduction pathways mediated by intracellular second messengers, such as cAMP and Ca^{2+}. In midtubules of the house cricket, *A. domesticus*,

basolateral Ca^{2+} uptake is stimulated > 3.5-fold by cAMP and inhibited > 2-fold or reversed by 5-hydroxytryptamine (5-HT) or thapsigargin, respectively (Browne and O'Donnell, 2018). Both 5-HT and thapsigargin increase intracellular Ca^{2+} concentrations in tubule cells Coast (2011). In addition, pharmacological manipulation of the cAMP-dependent pathway using forskolin (an agonist of adenylyl cyclase), 3-isobutyl-1-methylxanthine (IBMX; a non-selective phosphodiesterase antagonist) and H-89 (a selective protein kinase A antagonist) indicated a role for the adenylyl cyclase-cAMP-protein kinase A (PKA) pathway (Browne and O'Donnell, 2018). These results were interpreted as evidence for a bidirectional control mechanism whereby haemolymph calcium homeostasis in this insect is achieved predominantly by manipulating rates of Ca^{2+} sequestration through stimulatory (cAMP) and inhibitory (Ca^{2+}) regulatory pathways (Browne and O'Donnell, 2018).

In this contribution, we measure basolateral Ca^{2+} transport across isolated Malpighian tubules of eight insects from seven orders: *Drosophila melanogaster* (Diptera), *Aedes aegypti* (Diptera), *Tenebrio molitor* (Coleoptera), *Acheta domesticus* (Orthoptera), *Trichoplusia ni* (Lepidoptera), *Periplaneta americana* (Blattodea), *Halyomorpha halys* (Hemiptera) and *Pogonomyrmex occidentalis* (Hymenoptera) using the scanning ion-selective microelectrode technique (SIET). Our aims were, firstly, to probe for regulatory pathways by bathing tubules from each of the insects in saline containing cAMP and thapsigargin, compounds previously found to stimulate or inhibit, respectively, basolateral Ca^{2+} influx across calcium storage segments (midtubules) in the house cricket, *A. domesticus*. Secondly, we determine whether basolateral Ca^{2+} transport

is segment-specific in each insect by measuring basolateral Ca^{2+} transport within each morphologically and/or functionally distinct segment along the length of the Malpighian tubules. Thirdly, a useful specialization of *T. ni* Malpighian tubules, the large (> 50 µm diameter) principal and secondary cells of the ileac plexus region, allowed us to assess cell type-specific Ca^{2+} transport across individual Malpighian tubule cells using SIET. Finally, Ca^{2+} concentrations were determined in haemolymph samples taken from each insect using Ca^{2+}-selective microelectrodes. These values were then related to Ca^{2+} fluxes obtained by SIET in order to estimate the time required to eliminate the entire haemolymph Ca^{2+} content for each insect. Estimation of these elimination times allows us to evaluate the physiological significance of Ca^{2+} uptake by the Malpighian tubules to haemolymph Ca^{2+} regulation in each species.

4.3. Materials & Methods

4.3.1. Insects

All insects were maintained at room temperature (21-23 $^{\circ}$C) under 12 h light : 12 h dark conditions unless stated otherwise.

Drosophila melanogaster (Diptera) adults of the Oregon-R strain were obtained from laboratory cultures maintained at McMaster University, ON, Canada. Adults were maintained in vials on a diet containing (in g l^{-1}): 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 and 50 dry active yeast (Roberts and Stander, 1998). To prevent mold growth, 7.45 ml l^{-1} of 10% tegosept (butyl 4-hydroxybenzoate; Sigma-Aldrich, St Louis, MO, USA) dissolved in ethanol and 10 ml l^{-1} acid mix (11 parts H_2O,

10 parts propionic acid and 1 part of 85% *o*-phosphoric acid) were also included. Adult females 24-30 h post-eclosion were used in all experiments.

Aedes aegypti (Diptera) eggs were obtained from laboratory cultures maintained at McMaster University, ON, Canada and allowed to develop in plastic containers filled with dechlorinated tap water. Larvae were fed a 1:1 solution of liver powder and dry active yeast made up in distilled water *ad libitum*. The rearing media contained 0.4 mM Ca^{2+}, as determined by Ca^{2+}-selective microelectrodes. Fourth instar larvae were used in all experiments.

Tenebrio molitor (Coleoptera) larvae were obtained from PetSmart® (Ancaster, ON, Canada), maintained in translucent 1.2 L Tupperware® containers and fed dry wheat bran supplemented with carrot slices weekly. Larvae 9-10 weeks of age were used in all experiments.

Acheta domesticus (Orthoptera) adults were also obtained from PetSmart® and maintained at 28 $^{\circ}$C with access to water and ground multi-fowl feed containing 0.05% calcium by weight (W-S Feed & Supplies Ltd, Tavistock, ON, Canada) *ad libitum*. Moistened topsoil firmly packed into 250 ml Tupperware® containers provided substrate for egg deposition. First instar nymphs were transferred to translucent 5 L Tupperware® containers and maintained through to adulthood. Malpighian tubules were isolated from adult females 7-8 weeks of age.

Trichoplusia ni (Lepidoptera) larvae were obtained from the Great Lakes Forestry Centre (Sault St Marie, ON, Canada) and reared in groups of 5-10 in 22 ml cups

containing synthetic diet (McMorran, 1965). Fourth instar larvae were used in all experiments.

Periplaneta americana (Blattodea) adults were obtained from Boreal Science (St. Catharines, ON, Canada) and maintained in a 100 L glass aquarium with a sand substrate. Adults were fed ground multi-fowl feed containing 0.05% calcium by weight (W-S Feed & Supplies Ltd, Tavistock, ON, Canada) with access to water *ad libitum*.

Halyomorpha halys (Hemiptera) adults were obtained from laboratory cultures maintained at McMaster University, ON, Canada. Adults were maintained in translucent 5 L Tupperware® container with access to organic lettuce *ad libitum*.

Pogonomyrmex occidentalis (Hymenoptera) adult workers were obtained from Boreal Science and maintained in translucent 5 L Tupperware® containers with access to cotton balls soaked in a 10% (w/v) sucrose solution *ad libitum*.

4.3.2. Salines and Malpighian tubule dissections

Malpighian tubules were dissected under physiological saline solutions specific to each insect (Table 4.1). All compounds were obtained from Sigma–Aldrich. Due to the absence of published saline recipes for *H. halys* and *P. occidentalis*, Malpighian tubule dissections and experiments were performed in salines previously used to bathe Malpighian tubules from the related large milkweed bug *O. fasciatus* (Te Brugge and Orchard, 2008), and the harvester ant *F. polyctena* (Laenen et al., 2001), respectively. Procedures for the isolation of single Malpighian tubules have been described previously for *A. aegypti* (Scott et al., 2004), *D. melanogaster* (Browne and O'Donnell, 2016), *A. domesticus* (Browne and O'Donnell, 2018), *T. molitor* (Wiehart et al., 2002), *P.*

americana (Kay et al., 1992), and *T. ni* (O'Donnell and Ruiz-Sanchez, 2015). Tubules of *H. halys* were dissected following a procedure similar to that of used for *O. fasciatus* (Meredith et al., 1984). Tubules of *P. occidentalis* were isolated by first removing the gaster by transection of the pedicel. The gut and tubules were then extricated from the gaster by gently pulling on the stinger with Dumont® #5 forceps. Single tubules were then pulled free from their insertions into the gut.

Table 4.1 Composition of experimental salines (mmol l^{-1})

	Species							
	Acheta domesticus	*Aedes aegypti*	*Drosophila melanogaster*	*Halyomorpha halys*	*Periplaneta americana*	*Pogonomyrmex occidentalis*	*Tenebrio molitor*	*Trichoplusia ni*
NaCl	100	150	117.5	20	139	-	90	15
KCl	8.6	3.4	20	24	3	27	50	34
$CaCl_2$	2	2	2	2	2	2	2	2
$MgCl_2$	8.5	1	8.5	8.5	2	13	5	30
$NaHCO_3$	4	1.8	10.2	4	10.2	-	6	-
NaH_2PO_4	4	-	4.3	-	4.3	-	4	-
$KHCO_3$	-	-	-	-	-	-	-	10
KH_2PO_4	-	-	-	-	-	-	-	1
Glucose	24	5	20	34	20	138.8	50	10
Maltose	-	-	-	-	-	11.7	-	10
Trehalose	-	-	-	-	5	10.5	-	-
Sucrose	-	-	-	114.6	-	-	-	-
Na_3 citrate	-	-	-	-	-	-	-	5
K_3 citrate	-	-	-	-	-	8	-	-
Na_2 fumerate	-	-	-	-	-	16.8	-	-
Na_2 succinate	-	-	-	-	-	14.4	-	-
Glycine	-	-	-	-	-	-	10	10
Alanine	-	-	-	-	-	2.8	-	10
Proline	10	-	-	-	-	-	10	10
Glutamine	-	-	10	-	10	-	10	10
Valine	-	-	-	-	-	-	-	10
Serine	-	-	-	-	-	-	10	5
Histidine	-	-	-	-	-	-	10	5
MOPS	25	25	8.6	5	8.6	12.1	-	-
pH	7.2	7.1	7	6.9	7.2	7.2	7	7.2
Reference	a	b	c	d	e	f	g	h

[a]Coast & Kay, 1994;[b]Williams & Beyenbach, 1984;[c]Dow, 1994;[d]Brugge & Orchard, 2008;[e]Kocmarek & O'Donnell, 2011;[f]Laenen et al., 2001;[g]Nicolson, 1992;[h]Maddrell & Gardiner, 1976

4.3.3. Drugs

Stock solutions of thapsigargin were prepared in dimethyl sulfoxide (DMSO) so that the maximum final concentration of DMSO was ≤ 1% (v/v). A previous study indicated that Ca^{2+} fluxes were unaffected by tubules bathed in saline containing ≤ 2 % DMSO (v/v) (Browne and O'Donnell, 2018). The second messenger, adenosine 3′,5′-cyclic monophosphate (cAMP), was dissolved in bathing saline.

4.3.4. Ca^{2+}-selective microelectrodes and calibration solutions

Ca^{2+}-selective microelectrodes were fabricated using the Ca^{2+} ionophore ETH1001 (Calcium Ionophore 1 – cocktail A; Sigma–Aldrich) as described by Browne and O'Donnell (2016). Reference microelectrodes for use with SIET (scanning ion-selective microelectrode technique) were pulled from 1.5 mm O.D. capillary glass, filled with 3 M KCl solution containing 3% agar and connected to the ground input of the headstage using an Ag/AgCl half-cell microelectrode holder. Reference microelectrodes for haemolymph sampling were fabricated from filamented borosilicate glass capillaries pulled to a fine tip and backfilled with 150 mM KCl.

Ca^{2+} selective microelectrodes were calibrated in species-specific saline solutions containing Ca^{2+} at concentrations bracketing the range of interest (typically 2.5 and 0.25 mM Ca^{2+}). Changes in calcium concentration were achieved by exchange of $CaCl_2$ with NaCl or KCl in a 1:3 molar ratio in order to maintain ionic strength between experimental and calibration solutions. KCl was exchanged for $CaCl_2$ in calibration solutions for use with *P. occidentalis* due to the lack of NaCl in the saline. Slopes per 10-fold change in Ca^{2+} concentration were 27 to 29 mV.

4.3.5. SIET measurements of basolateral Ca^{2+} transport by Malpighian tubules

Single isolated tubules were transferred to 200 µl of bathing saline held within a plastic ring formed with a glue gun on the bottom of a 6 ml Petri dish. Dishes were pre-coated with 75 µl droplets of 62.5 µg ml^{-1} poly-L-lysine hydrobromide (Sigma–Aldrich) and air-dried to facilitate tubule adhesion.

SIET measurements were made using established protocols (Browne and O'Donnell, 2018) with hardware from Applicable Electronics (Forestdale, MA, USA) and automated scanning electrode technique (ASET) software (version 2.0; Science Wares Inc., East Falmouth, MA, USA). Briefly, microelectrode voltage was recorded at two positions within a 2D plane perpendicular to the long axis of the tubule. Microelectrodes were first positioned within 5 µm of the tissue then moved 50 µm away following a 'move-wait-sample' protocol at each site. Wait and sample times were 3.5 s and 0.5 s, respectively. A mean voltage difference was calculated from 5 measurements made at 1 minute intervals at each site, and each measurement was itself the mean of 3 replicate measurements. Voltage differences were corrected for electrode drift by subtracting a reference voltage difference recorded at a site >1000 µm away from the tissue. Voltage differences obtained from the ASET software were converted to concentration differences using the following equation:

$$\Delta C = C_B \cdot 10^{\Delta V/S} - C_B \quad (1)$$

where C_B is the background Ca^{2+} concentration (mM or µmol cm^{-3}); ΔV is the voltage difference between the inner and outer limits of microelectrode excursion at each site (µV); and S is the slope (µV) of the microelectrode for a 10-fold change in Ca^{2+} concentration. Diffusive Ca^{2+} fluxes were then calculated from Ca^{2+} concentration gradients using Fick's first law of diffusion:

$$J_{Ca^{2+}} = D_{Ca^{2+}} \cdot \Delta C/\Delta x \quad (2)$$

where $J_{Ca^{2+}}$ is the flux of Ca^{2+} (pmol cm^{-2} s^{-1}); $D_{Ca^{2+}}$ is the diffusion coefficient of Ca^{2+} at 25 ºC ($7.9x10^{-6}$ cm^2 s^{-1}) obtained from Smith et al. (1999); and Δx is the excursion distance (0.005 cm). By convention, positive fluxes indicate Ca^{2+} flux out of the tubule (efflux) and negative fluxes indicate Ca^{2+} flux into the tubule (influx).

4.3.6. Measurement of [Ca^{2+}] in haemolymph

Insects were held under paraffin oil and their cuticle ruptured with Dumont® #5 forceps. Droplets of haemolymph exuding from the wound were collected in a pipette and transferred to another Petri dish filled with paraffin oil for measurement of haemolymph Ca^{2+} concentrations using Ca^{2+}-selective microelectrodes. Microelectrode voltages were recorded with high-impedance (>10^{13} Ω) electrometers connected to a data acquisition system (PowerLab; ADInstruments, Sydney, Australia) and analyzed using Chart 5 software. Calcium concentrations were calculated using the following equation:

$$[Ca^{2+}]_{Sample} = [Ca^{2+}]_{Cal} \cdot 10^{(\Delta V/S)} \quad (3)$$

where $[Ca^{2+}]_{Sample}$ represents the Ca^{2+} concentration in the sample, $[Ca^{2+}]_{Cal}$ represents the Ca^{2+} concentration in one of the calibration solutions, ΔV represents the difference in voltage between the sample and the calibration solution, and S represents the slope of the electrode for a 10-fold change in Ca^{2+} concentration.

4.3.7. Estimates of net Ca^{2+} flux

Net Ca^{2+} flux (pmol min^{-1}) by tubules was calculated as the sum of Ca^{2+} fluxes across all tubule segments within each insect. Segment-specific Ca^{2+} flux (pmol h^{-1}) across each tubule segment was estimated by multiplying the Ca^{2+} flux (pmol cm^{-2} s^{-1}) determined by SIET by 3600 sec h^{-1} and the corresponding surface area (cm^2) of the segment. Surface area of tubule segments were calculated using the formula for the surface area of a cylinder (πdl), where d is diameter and l is length. Tubule diameters were estimated from images at 200X magnification using ImageJ analysis software (version 1.47; National Institutes of Health, Bethesda, MD, USA). Tubule lengths were estimated using an eyepiece micrometer at 124X to 800X magnification, where single tubules under saline were fixed to the bottom of 6 ml Petri dishes that had been pre-coated with poly-L-lysine to facilitate adhesion. Weights of animals to the nearest milligram were obtained using an analytical balance.

4.3.8. Statistical analysis

Graphing and statistical analysis were performed using Prism software (version 4.0b; GraphPad Software Inc., San Diego, CA, USA). Data are presented as means ±

SEM for the indicated number of samples (*N*). Comparisons between two groups were made using a student's *t*-test while comparisons of three or more groups were made using one-way ANOVA followed by Tukey's range test. Differences were considered statistically significant if $P < 0.05$.

4.4. Results

4.4.1. Basolateral Ca^{2+} transport by Malpighian tubules from several insect orders and the effects of 1 mM cAMP and 10 µM thapsigargin

In a previous study, basolateral Ca^{2+} influx across midtubules of *A. domesticus* increased > 3.5-fold in the presence of 1 mM cAMP and reversed from influx to efflux across tubules exposed to 10 µM thapsigargin (Browne and O'Donnell, 2018). Along with the use of forskolin and IBMX, these results were interpreted as evidence for a bidirectional control system involving a stimulatory adenylyl cyclase-cAMP-PKA pathway and an inhibitory pathway involving increases in intracellular Ca^{2+}. We therefore extended the results of the previous paper on the midtubules by determining the effects of cAMP and thapsigargin on Ca^{2+} transport across the distal tubules of *A. domesticus*. Rates of Ca^{2+} transport by distal tubules bathed in saline were unchanged in the presence of either 1 mM cAMP or 10 µM thapsigargin (Figure 4.1A). In addition, Ca^{2+} influx by distal tubules bathed in saline were lower than the published value (-26 ± 1 pmol cm^{-2} s^{-1}, *N*=171) for midtubules (Browne and O'Donnell, 2018). The results from the previous study are included in panel A (left three bars) for comparison. Together, these results indicate that the distal tubules do not respond to cAMP and thapsigargin as do the midtubules.

The Malpighian tubules of *A. domesticus* and *P. americana* are morphologically similar: both consist of three (proximal, middle and distal) segments with spherical intracellular granules abundant in the midtubules (which cause them to appear opaque under light microscopy) and a hyaline distal segment lacking intracellular granules. The similarities in morphology suggested Ca^{2+} transport by the tubules would be similar. Unexpectedly, basolateral Ca^{2+} influx reversed (from influx to efflux) within the midtubules following the addition of 1 mM cAMP to the bathing saline (Figure 4.1B). There was also a trend for reversal of Ca^{2+} influx to efflux in the presence of 10 µM thapsigargin, although the difference from controls (saline) was not

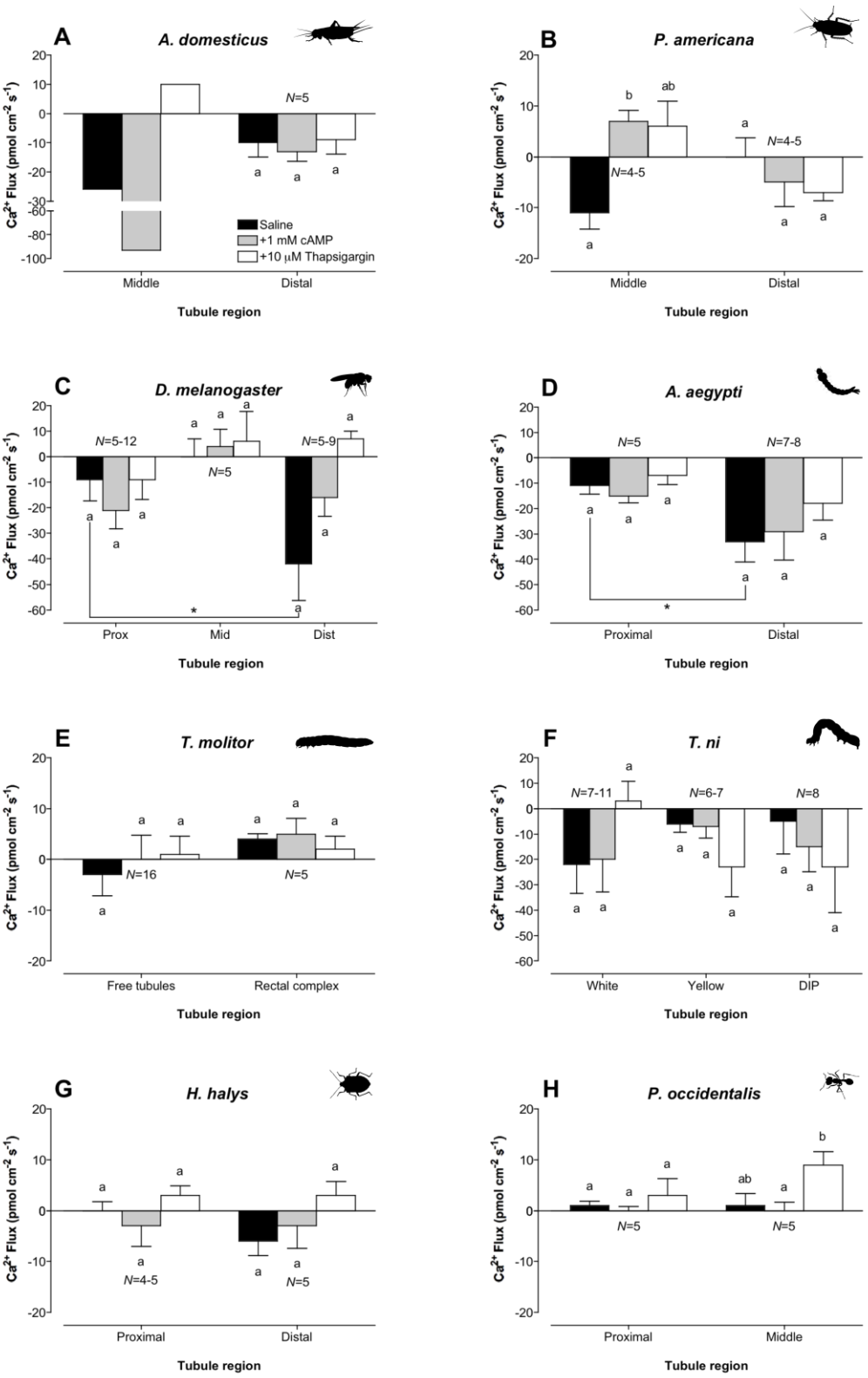
A
A. domesticus
B
P. americana
C
D. melanogaster
D
A. aegypti
E
T. molitor
F
T. ni
G
H. halys
H
P. occidentalis
Ca2+ Flux (pmol cm-2 s-1)
Tubule region
Saline
+1 mM cAMP
+10 μM Thapsigargin
Middle
Distal
Prox
Mid
Dist
Proximal
Free tubules
Rectal complex
White
Yellow
DIP
N=5
N=4-5
N=5-12
N=5-9
N=7-8
N=16
N=7-11
N=6-7
N=8

Figure 4.1 Basolateral Ca^{2+} flux across isolated segments of Malpighian tubules from (A) *A. domesticus*, (B) *P. americana*, (C) *D. melanogaster*, (D) *A. aegypti*, (E) *T. molitor*, (F) *T. ni*, (G) *H. halys* and (H) *P. occidentalis* bathed in saline (solid bars), saline containing 1 mM cAMP (grey bars) and saline containing 10 µM thapsigargin (open bars). In panel A, previously published data for the middle tubule region (left three bars) are included for comparison without error bars to acknowledge that the data has already been published (Browne and O'Donnell, 2018). Data are presented as means ± s.e.m. Significant differences between treatments (saline, cAMP, thapsigargin) within each tubule region for all insects were tested using a one-way ANOVA. For each tubule region, bars labeled with different letters are statistically different ($P<0.05$). For comparisons of different regions bathed in saline, bars labeled with * and connecting lines are significantly different (unpaired Student's *t*-test, $P<0.05$).

significant (Student's *t*-test, p = 0.07). Rates of basolateral Ca^{2+} transport by distal tubules were low (< 8 pmol cm^{-2} s^{-1}) and were unaffected by either cAMP or thapsigargin.

Distal tubules isolated from *D. melanogaster* adults had the highest rates of Ca^{2+} uptake of any species in this study (Figure 4.1C). Basolateral Ca^{2+} influx was higher in distal segments compared to proximal segments. Ca^{2+} influx was unaffected by the presence of either 1 mM cAMP or 10 µM thapsigargin in either proximal, middle (main) or distal segments.

Like *D. melanogaster*, rates of Ca^{2+} uptake were high across tubules of *A. aegypti* larvae (Figure 4.1D). Basolateral Ca^{2+} influx was higher in distal segments compared to proximal segments and was unaffected by the presence of either 1 mM cAMP or 10 µM thapsigargin in both segments. These results suggest that Ca^{2+} transport occurs at high rates across tubules of Dipterans within specific segments that containing Ca-rich granules.

In both Coleopterans and Lepidopterans the distal portions of the Malpighian tubules are closely associated with the rectal epithelium and ensheathed in the perinephric membrane, which isolates them from the haemolymph. The remaining portions of the tubules, proximal to their insertions into the perinephric membrane, are freely exposed to haemolymph. In the free tubules of *T. molitor* larvae, rates of Ca^{2+} transport were uniform along their length (data not shown) coinciding with their uniform appearance. In addition, the magnitude of Ca^{2+} transport across the free tubules was low (< 4 pmol cm^{-2} s^{-1}) and unaffected by the presence of either 1 mM cAMP or 10 µM thapsigargin (Figure 4.1E). Due to difficulties in isolating perirectal tubules from *T. molitor* larvae, Ca^{2+} transport

was measured across the perinephric membrane at the proximal region of the rectal complex where the perinephric membrane is thinnest (Grimstone et al., 1968). Ca^{2+} was consistently released from the rectal complex at low (< 6 pmol cm^{-2} s^{-1}) rates even in the presence of either 1 mM cAMP or 10 µM thapsigargin.

In contrast to Coleopterans, the free tubules of Lepidopteran larvae are highly segmented. We therefore measured basolateral Ca^{2+} transport within the distal ileac plexus (DIP), yellow and white tubule segments of *T. ni*. Ca^{2+} was taken up across each tubule segment at similar rates (Fig 1F). In addition, there were no effects of either 1 mM cAMP or 10 µM thapsigargin on rates of basolateral Ca^{2+} uptake in any of the tubule segments.

To our knowledge, our study is the first physiological investigation of the Malpighian tubules of *H. halys*. Three distinct segments were observed under light microscopy: a short (5.6 ± 0.2 mm, *N*=7) translucent proximal segment with a relatively smooth basal surface, a short (8.8 ± 1 mm, *N*=7) translucent middle segment with a nodular basal surface, and a longer (30.6 ± 0.3 mm, *N*=6) translucent distal segment containing conspicuous green pigment and with a highly nodular basal surface. Preliminary SIET experiments indicated Ca^{2+} fluxes across the proximal and middle tubule segments were equivalent (data not shown) and therefore only proximal and distal tubules were further examined. SIET measurements revealed that basolateral Ca^{2+} fluxes were negligible (zero) across the proximal segment and small (< 6 pmol cm^{-2} s^{-2}) across the distal segment (Figure 4.1G). In both tubule segments there was no effect of cAMP or thapsigargin on Ca^{2+} transport.

It was noted during dissections that the lumen of *P. occidentalis* tubules were frequently filled with dense granules (appearing opaque under light microscopy) that were not present within the tubule cells, which instead appeared transparent. If these lumenal granules contain calcium salts their presence may influence Ca^{2+} transport across the basolateral surface. Measurements of basolateral Ca^{2+} transport indicated Ca^{2+} was released from the tubules at very low rates ($\leq$ 1 pmol cm^{-2} s^{-1}) in both proximal and middle segments bathed in saline (Figure 4.1H). In proximal segments, there was no effect of cAMP or thapsigargin on basolateral Ca^{2+} fluxes. In contrast, basolateral Ca^{2+} effluxes were modestly increased in the presence of thapsigargin in distal segments relative to tubules exposed to cAMP (Figure 4.1H). Together, these results suggest that Ca^{2+} transport is outwardly-directed in the Malpighian tubules of *P. occidentalis* and therefore they do not contribute to the removal of excess Ca^{2+} from the haemolymph.

All the above results, together, suggest that Ca^{2+} is most often taken up by the Malpighian tubules of insects when bathed in their species-specific saline solutions. In *A. domesticus* and the Dipterans (*A. aegypti* and *D. melanogaster*) basolateral Ca^{2+} transport was specific to the opaque calcium storage segments (midtubule and distal tubule segments, respectively). Generally, there was no effect of cAMP on tubule Ca^{2+} transport in most of the insects investigates with the exception of the midtubule of *P. americana*, where cAMP reversed the direction of transport from Ca^{2+} influx to efflux. Thapsigargin was also without effect in each species when compared to tubules held under control (saline) conditions.

4.4.2. Cell-type specific basolateral $Ca2^{+}$ transport by tubules of *T. ni*

The presence of large (> 50 µm diameter) secondary cells found in the distal Malpighian tubules of *T. ni* allowed for Ca^{2+} flux measurements that were specific to individual cells. SIET measurements made adjacent to principal and secondary cells found in the middle or distal ileac plexus regions of *T. ni* tubules revealed that basolateral Ca^{2+} influxes were specific to principal cells (Figure 4.2). Rates of basolateral Ca^{2+} transport by secondary cells were no different than a value of zero. These results suggest that tubule Ca^{2+} transport occurs by a transcellular route through principal cells.

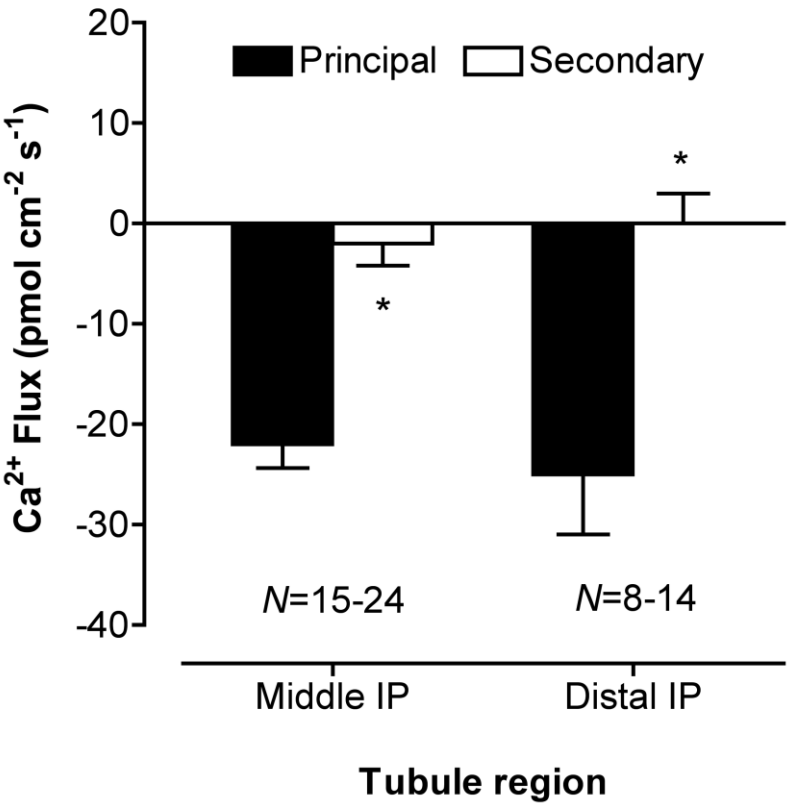

Figure 4.2 Basolateral Ca^{2+} flux across principal cells (solid bars) and secondary cells (open bars) found in the middle and distal ileac plexus (IP) regions of the Malpighian tubules of *T. ni* larvae. Data are presented as means ± s.e.m. Bars labeled with * are significantly different from principal cells in either tubule region (unpaired Student's *t*-test, $P<0.05$).

4.4.3. Haemolymph Ca^{2+} concentrations

To improve our understanding of the role of the Malpighian tubules in haemolymph calcium regulation we measured the calcium activity of haemolymph samples taken from each insect. Haemolymph calcium concentrations ranged from 0.6 to 5.1 mM Ca^{2+} with a mean value of 2.4 ± 0.5 mM Ca^{2+} (N=8), as measured by Ca^{2+}-selective microelectrodes (Figure 4.3). In particular, haemolymph calcium concentrations were lowest in *A. aegypti* and highest in *P. americana*.

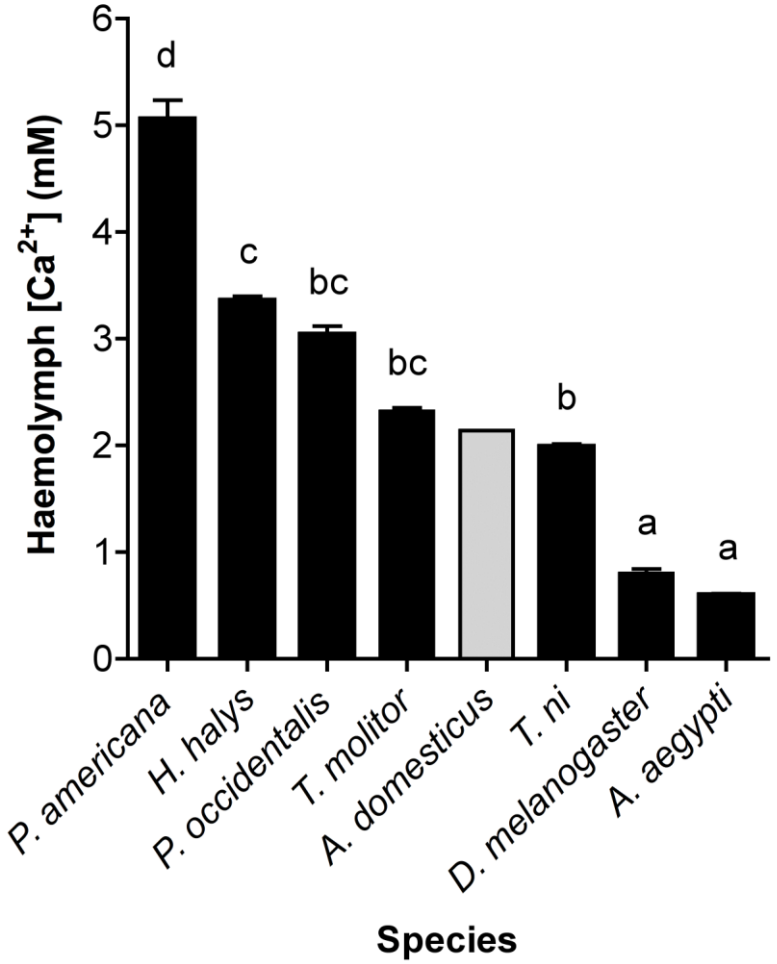

Figure 4.3 Concentrations of Ca^{2+} in samples of haemolymph collected from several insects. A published value of haemolymph Ca^{2+} concentration (grey bar) reported for *A. domesticus* was included for comparison (Browne and O'Donnell, 2018). Data are presented as means ± s.e.m. Bars labeled with different letters are statistically different (one-way ANOVA, $P<0.05$).

4.4.4. Haemolymph Ca^{2+} elimination times

Integrating SIET measurements of basolateral Ca^{2+} flux along the entire length of the tubules and accounting for the number of tubules (from 4 to ~ 150) allowed us to assess rates of Ca^{2+} transport by all tubules in each insect. By comparing 'whole insect' rates of tubule Ca^{2+} transport with estimates of the haemolymph Ca^{2+} content, the time for all tubules to eliminate the entire haemolymph Ca^{2+} content could be estimated (Table 4.2). As described above, Ca^{2+} fluxes across each segment were calculated as the product of mean Ca^{2+} flux (pmol cm^{-2} s^{-1}) measurements taken under saline conditions (solid bars from Figure 4.1), the surface area (cm^2) of each tubule segment and 3600 sec h^{-1}. Using *D. melanogaster* as an example, Ca^{2+} flux across the proximal segment was -32 pmol h^{-1} = (-9 pmol cm^{-2} s^{-1})(0.001 cm^2)(3600 sec h^{-1}). Equivalent calculations for the middle and distal segments result in Ca^{2+} fluxes of 0 pmol h^{-1} and -151 pmol h^{-1}, respectively. Each segment Ca^{2+} flux was then multiplied by the number of specific segments in the insect and summed with all other segments to obtain a total, representing the rate of Ca^{2+} transport by all tubules. For instance, *D. melanogaster* has 4 proximal, 4 main and 2 distal segments. The resulting Ca^{2+} fluxes for all proximal (-128 pmol h^{-1} = -32 pmol h^{-1} x 4) middle (0 pmol h^{-1}) and distal (-302 pmol h^{-1} = -151 pmol h^{-1} x 2) segments sum to -430 pmol h^{-1}. The rate of Ca^{2+} flux by all tubules (-430 pmol h^{-1}) was then related to haemolymph Ca^{2+} content in order to estimate elimination time. Haemolymph Ca^{2+} content for *D. melanogaster* (40 pmol) was the product of haemolymph volume (0.05 µL) and haemolymph Ca^{2+} concentration (0.8 mM Ca^{2+}, see Figure 4.3). In the absence of published measurements of haemolymph volume of other species, volumes were

estimated as a percentage of the body mass (Maddrell, 1981). Finally, the time to eliminate Ca^{2+} from the haemolymph (0.1 hours) was calculated by dividing the haemolymph Ca^{2+} content (40 pmol) by the Ca^{2+} flux (-430 pmol h^{-1}) by all tubules and multiplying by -1 so that negative fluxes (i.e. Ca^{2+} flux from haemolymph to tubule) correspond to positive elimination times.

Table 4.2 Key values used to estimate the time to eliminate Ca^{2+} from the haemolymph by the Malpighian tubules of several insects.

Species (tubule region)	Segment length (mm)	Segment diameter (µm)	Segment surface area (mm^2)	Segment Ca^{2+} flux (pmol h^{-1})	Number of tubules	Ca^{2+} flux by all tubules (pmol h^{-1})	Body mass (mg)	Haemolymph Volume (µL)	Haemolymph Ca^{2+} content (pmol)	Time to eliminate Ca^{2+} from the haemolymph (h)
D. melanogaster (Prox)	0.8 ± 0.03 (*N*=10)		0.001	-32						
(Mid)	1.4 ± 0.05 (*N*=10)	48 ± 2 (*N*=10)	0.002	0	4	-430		0.05[a]	40	0.1
(Dist)	0.5 ± 0.06 (*N*=10)		0.001	-151						
A. aegypti (Prox)	2.5 ± 0.1 (*N*=10) 0.8[Ψ]	51 ± 1 (*N*=10)	0.001	-40	5	-3765		1.65[b]	990	0.3
(Dist)	1.8	105 ± 6 (*N*=10)	0.006	-713						
T. molitor (Dor)	18.4[f]		0.049	-529						
(Lat)	31.5[f]	85 ± 4 (*N*=10)	0.084	-907	6	-12312	65 ± 4 (*N*=10)	6.5 (0.065 g x 10 %[b])	15080	1.2
(Ven)	21.3[f]		0.057	-616						
A. domesticus (Mid)	6.1 ± 0.2 (*N*=10)	57 ± 2 (*N*=10)	0.011	-990	112[c]	-118944	522 ± 22 (*N*=9)	95 (0.522 g x 18.2 %[d])	191100	1.6
(Dist)	1.2 ± 0.1 (*N*=14)	43 ± 2 (*N*=10)	0.002	-72						
T. ni (WR)	7[g]	102 ± 3 (*N*=10)	0.022	-1742						
(YR)	7[g]	102 ± 3 (*N*=10)	0.022	-475	6	-17730	69 ± 6 (*N*=10)	24 (0.069 g x 34.5 %[d])	47760	2.7
(IP)	13[g]	100 ± 5 (*N*=10)	0.041	-738						
P. americana (Mid)	23.0 ± 1.0 (*N*=7)	62 ± 3 (*N*=10)	0.045	-1782	≈ 170[e]	-300560	1090 ± 98 (*N*=7)	194 (1.09 g x 17.8 %[d])	981640	3.3
(Dist)	1.3 ± 0.1 (*N*=10)	41 ± 3 (*N*=10)	0.002	14						
H. halys (Prox+Mid)	14.4 ± 1 (*N*=7)	106 ± 5 (*N*=10)	0.048	0	4	-8640	154 ± 10 (*N*=9)	48 (0.154 g x 30.9 %[d])	161280	18.7
(Dist)	30.6 ± 2.9 (*N*=6)	104 ± 3 (*N*=10)	0.100	-2160						
P. occidentalis (Prox)	7.2 ± 0.2 (*N*=9) 0.7[Ψ]	54 ± 1 (*N*=10)	0.001	4	5	180	8 ± 1 (*N*=10)	≈ 0.5[h]	1520	-8.4*
(Mid+Dist)	6.5	44 ± 2 (*N*=10)	0.009	32						

[a] MacMillan & Hughson, 2014; [b] Donini & O'Donnell, 2005; [c] Hazelton et al., 1988; [d] Maddrell, 1981; [e] Wall et al., 1975; [f] Nicolson, 1992; [g] O'Donnell & Ruiz-Sanchez, 2015; [h] Van Kerkhove et al., 1989

[Ψ] lengths of proximal segments of *A. aegypti* and *P. occidentalis* were taken to be 30% and %10 of the total tubule lengths, respectively

* Negative elimination time represents the time to double haemolymph Ca^{2+} content

4.5. Discussion

Previous studies of the Malpighian tubules of *D. melanogaster* (Dube et al., 2000a, Browne and O'Donnell, 2016) and *A. domesticus* (Browne and O'Donnell, 2018) have shown pronounced localization of basolateral Ca^{2+} transport to specialized tubule segments containing Ca-rich granules. In this study, we found regional differences in tubule Ca^{2+} transport in only one other insect, *A. aegypti*, whereas there were no regional differences in Ca^{2+} transport in tubules of *T. molitor*, *T. ni*, *P. americana*, *H. halys* and *P. occidentalis*. Clearly, Ca^{2+} transport is segment-specific across tubules of some species, and relatively uniform along the length of the whole tubule of other species.

In a previous study, rates of Ca^{2+} transport across midtubules of the house cricket, *A. domesticus*, were altered in opposing ways by modulators of cAMP-dependent (cAMP) and Ca^{2+}-dependent (thapsigargin) second messenger pathways, suggestive of a bidirectional control mechanism for tubule Ca^{2+} transport (Browne and O'Donnell, 2018). Since these second messenger pathways have been extensively studied in the context of osmoregulation in crickets (Clark and Spring, 1992, Xu and Marshall, 2000) and other insects (Wiehart et al., 2003, Beyenbach and Piermarini, 2011, Davies et al., 2013), it was hypothesized that the Ca^{2+} regulation pathways identified in *A. domesticus* (Browne and O'Donnell, 2018) would be broadly applicable in insects. In this study, manipulation of second messenger pathways using 1 mM cAMP and 10 µM thapsigargin had no effect on Ca^{2+} fluxes across the tubules of *D. melanogaster*, *A. aegypti*, *T. molitor*, *T. ni*, *H. halys* or distal tubules of *A. domesticus*, suggesting that the effects of cAMP and thapsigargin

are specific to the midtubules of *A. domesticus* and thus alternative modes of control may be operative in tubules of other species.

Unexpectedly, rates of tubule Ca^{2+} transport were very low in many of the species examined. Calculations described below suggest that rates of Ca^{2+} transport in tubules of *H. halys* and *P. occidentalis* are likely too low to be physiologically relevant. Malpighian tubules of these insects may not play a significant role in haemolymph calcium regulation and thus other tissues may be involved.

4.5.1. Segment-specific, uniform and cell type-specific patterns of basolateral Ca^{2+} transport

In the insects where basolateral Ca^{2+} transport has been directly measured within sub-segments along the length of the Malpighian tubules (*D. melanogaster* (Dube et al., 2000a, Browne and O'Donnell, 2016), *R. prolixus* (Maddrell et al., 1991) and *A. domesticus* (Browne and O'Donnell, 2018)) a segment-specific pattern of Ca^{2+} transport has emerged. The sites of Ca^{2+} uptake correspond to tubule regions that accumulate dense granules enriched in salts of calcium (frequently as phosphates in amorphous form; (Maddrell et al., 1991, Ballan-Dufrancais, 2002)) giving these 'calcium storage' regions an opaque appearance under light microscopy. In this study, Ca^{2+} transport was segment-specific across the tubules of the mosquito, *A. aegypti*, where Ca^{2+} is taken up across the distal tubules at higher rates than proximal tubules. High rates of Ca^{2+} influx across the distal segment are consistent with ultrastructural investigations that have revealed an abundance of Ca-rich granules that occupy a large proportion of the cytosol within principal cells of this region (Bradley et al., 1990). Further, low rates of basolateral Ca^{2+}

influx were apparent in proximal tubules at sites near to their insertions into the gut where the tubules cells appear transparent, a region with apparently fewer Ca-rich granules. A similar spatial pattern of Ca^{2+} transport is observed in the larvae of the related Dipteran, *D. melanogaster*, where rates of Ca^{2+} transport were highest across distal tubules (where Ca-rich granules are accumulated) and were much lower in proximal tubules that contain fewer Ca-rich granules (Browne and O'Donnell, 2016). In contrast, the direction of Ca^{2+} transport (Ca^{2+} efflux) by distal tubules of *D. melanogaster* was opposite (Ca^{2+} influx) to that of the corresponding region of *A. aegypti* tubules. In addition, the Ca-rich granules are accumulated within the lumen of the distal tubules in *D. melanogaster*, whereas they are accumulated within the principal cells in *A. aegypti*. It is apparent, then, that although there are some similarities in the spatial patterns of tubule Ca^{2+} transport in some species there may also be concurrent fundamental differences in Ca^{2+} sequestration within the tubules, even among closely related species.

In contrast, there were no regional differences in rates of basolateral Ca^{2+} transport across tubules of *T. molitor*, *T. ni*, *P. americana*, *H. halys* and *P. occidentalis*. In other words, Ca^{2+} transport across the tubules of these insects is uniform along their length despite, in some instances, stark morphological and/or functional segmentations. The sites of Ca^{2+} transport in tubules of *D. melanogaster*, *R. prolixus* and *A. domesticus* correlate with the opaque regions observed in the otherwise transparent tubules (Browne and O'Donnell, 2016, Maddrell et al., 1991, Browne and O'Donnell, 2018). In tubules of *T. molitor* and *H. halys*, there are no obvious opaque regions along the length, consistent with uniform Ca^{2+} transport. The appearance of *P. occidentalis* tubules is also uniform

along their length, where the tubule cells are transparent and the lumen is mostly filled with opaque deposits. Uniform Ca^{2+} transport is consistent with the uniform appearance of the tubule cells under light microscopy. In *P. americana*, the results were unexpected given that the morphology of the tubules superficially resembles that of *A. domesticus*, where Ca^{2+} transport was previously shown to be specific to the midtubules. It is worth noting that the diets of the species investigated vary considerably: crickets and cockroaches are generalists (omnivores), mosquito larvae feed on decomposing organic matter, fruit flies feed on rotting fruit, ants feed on plant nectar, mealworms feed on stored grains, the caterpillar consumes foliage and the stink bug is saprophagous. The source of dietary calcium for each insect is likely to influence the need for tubule Ca^{2+} storage and hence influence tubule Ca^{2+} transport as measured by SIET. In addition, the ionic composition of the bathing saline, specifically with regard to phosphate concentrations, have been previously demonstrated to dramatically influence rates of tubule Ca^{2+} transport by distal tubules of *D. melanogaster* (Browne and O'Donnell, 2016). It is not surprising, then, that tubule Ca^{2+} transport can vary considerably among species.

The large principal and secondary cells of the distal ileac plexus region of the Malpighian tubules of the cabbage looper, *T. ni*, allow for Ca^{2+} flux measurements across single tubule cells using SIET. We found that Ca^{2+} is taken up across only the principal cells of *T. ni* tubules. Although the secondary cells are sites of high rates of reabsorption of the monovalent cations Na^{+} and K^{+} (O'Donnell and Ruiz-Sanchez, 2015), there is no evidence of reabsorption of the divalent cation Ca^{2+}. There are several lines of evidence that suggest Ca^{2+} taken up by Malpighian tubules occurs specifically across principal

cells. Firstly, calcium channel blockers (nifedipine, verapamil and diltiazem) abolished basolateral Ca^{2+} uptake by cAMP-stimulated midtubules of *A. domesticus*, an insect with tubules that lack secondary cells (Spring et al., 2007, Browne and O'Donnell, 2018). Secondly, an antibody raised to an epitope common to two *D. melanogaster* L-type calcium channel genes (*Dmca1A* and *Dmca1D)* binds to the basolateral membrane of the principal cells in the main segments of *D. melanogaster* tubules, as does a fluorescently-labeled L-type calcium channel blocker (verapamil) (MacPherson et al., 2001). Together, these results suggest basolateral Ca^{2+} entry occurs through calcium channels located within principal cells of the Malpighian tubules that accumulate opaque Ca-rich granules.

4.5.2. cAMP and thapsigargin affect tubule Ca^{2+} transport in midtubules of *A. domesticus,* but not in tubules of other species

In a previous investigation, cAMP stimulated Ca^{2+} influx whereas thapsigargin resulted in a switch from Ca^{2+} influx to efflux by the midtubule of *A. domesticus*. By contrast, neither cAMP or thapsigargin at the concentrations used in the previous study had any effect on Ca^{2+} fluxes across the distal tubule of *A. domesticus*. The low rates of Ca^{2+} transport across the distal tubule and the lack of effect of either cAMP or thapsigargin on Ca^{2+} transport by the distal segment are consistent with the absence of Ca-rich granules in this tubule segment. Further, cAMP and thapsigargin had no effect on the tubules of *D. melanogaster*, *A. aegypti*, *T. molitor*, *T. ni*, *H. halys* and *P. occidentalis* compared to controls (saline). It is possible that basolateral Ca^{2+} transport by Malpighian tubules in these species may be under the control of other second messengers, such as cyclic guanosine monophosphate (cGMP) or nitric oxide (NO). Together, these results

suggest that cAMP and Ca^{2+} second messenger pathways play a role in midtubule Ca^{2+} transport in *A. domesticus* but have little effect on Malpighian tubules of many insects from other orders.

4.5.3. Malpighian tubules rapidly eliminate haemolymph Ca^{2+} in some but not all species

Haemolymph Ca^{2+} elimination times for both *D. melanogaster* (0.1 h) and *A. aegypti* (0.3 h) were very brief, suggest that the Malpighian tubules alone have the capacity to rapidly eliminate excess haemolymph Ca^{2+} in these Dipterans. Rapid rates of haemolymph Ca^{2+} removal may allow these insects to maintain haemolymph Ca^{2+} concentrations during acute changes in calcium absorption (e.g. during feeding). In these Dipterans, the majority of the Ca^{2+} transport occurs across the distal tubule where Ca-rich granules are abundant. Our elimination times are consistent with previous estimates of the time (~ 1.6 h) required for all four tubules of *D. melanogaster* to transport the amount of Ca^{2+} equivalent to the Ca^{2+} content of the whole fly (Dube et al., 2000a). Similarly, all tubules of the blowfly (*C. vicina;* Diptera) can turnover the whole-body Ca content in ~ 2 h (Taylor, 1987). It would appear that tubules of Dipterans rapidly eliminate Ca^{2+} from the haemolymph. High rates of haemolymph Ca^{2+} sequestration may be a response to rapid Ca^{2+} absorption of excess calcium in the diet or may be a consequence of mechanisms which mitigate the effects of exposure to toxic divalent cations (Dube et al., 2000a).

Haemolymph Ca^{2+} elimination times for *T. molitor*, *A. domesticus*, *T. ni*, *P. americana*, *H. halys* and *P. occidentalis* were 1.2 hours or more, indicating that rates of

Ca^{2+} uptake by the Malpighian tubules are not always rapid. In the kissing bug, *R. prolixus*, $^{45}Ca^{2+}$ is accumulated by the Malpighian tubules at a more or less uniform rate, sufficient to eliminate Ca^{2+} from ~ 0.5 µl of haemolymph each day, for at least 12 days following a blood meal (Maddrell et al., 1991). If the tubules of *R. prolixus* were to continue to eliminate Ca^{2+} from the haemolymph at this rate, it would take approximately 30 days to completely eliminate Ca^{2+} from the haemolymph (a volume of ~15 µl). High rates of Ca^{2+} uptake by the Malpighian tubules are apparently not required in this species despite the tubules' major role in calcium handling (Maddrell et al., 1991). High rates of Ca^{2+} uptake by the Malpighian tubules may not be necessary when rates of Ca^{2+} absorption are low (*i.e.* between meals or overwintering) or in infrequent feeders, such as *Rhodnius*.

Lengthy times to eliminate haemolymph Ca^{2+} content raise the possibility that the Malpighian tubules of some species do not have the capacity to excrete excess Ca^{2+} from the haemolymph at physiologically relevant rates. The very low rates of Ca^{2+} transport, in addition to the minimal effects of cAMP and thapsigargin, also raise the possibility that other tissues play a role in regulating haemolymph Ca^{2+} levels, either by absorbing less Ca^{2+} from the midgut lumen, or excreting excess Ca^{2+} across regions of the midgut or hindgut. The presence of intracellular Ca-containing granules in the midgut of the predatory stink bug, *B. tabidus* (Heteroptera), suggests that the midgut of stink bugs may play a role in haemolymph calcium homeostasis (Guedes et al., 2007). In ants, granules rich in calcium phosphate are abundant within the cells and lumen of the midgut suggesting that at least some Ca^{2+} taken up by the midgut cells is sequestered there,

eventually being released into the lumen across the apical membrane by a merocrine secretion mechanism (Ballan-Dufrancais, 2002). In aggregate, these results suggest that the Malpighian tubules of *D. melanogaster*, *A. aegypti*, *T. molitor*, *A. domesticus*, *T. ni* and *P. americana* play a role in haemolymph calcium homeostasis, whereas tissues other than the Malpighian tubules (possibly the midgut) may contribute significantly to haemolymph calcium homeostasis in *H. halys* and *P. occidentalis*.

Chapter 5

5. General Discussion

This thesis has examined the mechanisms and control of Ca^{2+} secretion and sequestration, the relationship of Ca^{2+} transport to fluid secretion, the contributions of principal and secondary cells to tubule Ca^{2+} transport, and changes in Ca^{2+} transport throughout the life cycle. Although I have not examined the role of the midgut, results of my research and research by other groups suggest that the midgut may be involved in haemolymph and whole animal Ca regulation. In addition, it is worth considering the relevance of my studies to the use of Malpighian tubules as a model for human nephrolithiasis.

5.1. Calcium is abundant in the diet of many insects

Most evidence suggests that Ca^{2+} is abundant in the diets of insects and therefore Ca^{2+} excretion is necessary for haemolymph Ca^{2+} homeostasis. Following a meal, Ca^{2+} is rapidly absorbed into the haemolymph across the midgut (Taylor, 1985a); excess haemolymph Ca^{2+} is then removed by the Malpighian tubules. Many insects of relevance to humans feed on plant tissues (often production crops) or vertebrate blood (i.e. disease transmission). I will consider four examples of insects in which considerations of feeding rates, dietary calcium content and haemolymph volume demonstrate a need for elimination or sequestration of excess calcium.

In plant feeders with an affinity for fruit, such as *Drosophila melanogaster*, it has been estimated that the fly consumes about 3 times its entire body Ca content each day (Dube et al., 2000a). Absorption of dietary Ca at a rate of 9.6 nmol per day (Dube et al., 2000a) would increase the haemolymph Ca^{2+} concentration by 120 to 192 mM Ca^{2+} (haemolymph volume = 0.08 to 0.05 µl; (Folk et al., 2001, MacMillan and Hughson, 2014)) in the absence of Ca^{2+} excretion assuming all Ca is absorbed in ionized form. Clearly, then, adults of *Drosophila melanogaster* require high rates of Ca^{2+} removal from the haemolymph in order to prevent large increases in haemolymph Ca^{2+} concentration (i.e. maintain haemolymph Ca^{2+} balance).

Lepidopteran larvae are also plant feeders with a preference for plant foliage. The caterpillar, *Hyalophora cecropia*, consumes tree leaves at a relatively high rate of 0.8 mg biomass eaten/mg larval biomass/day (Scriber, 1977). If we assume that larvae of the related Lepidopteran studied in this thesis, *Trichoplusia ni*, (~ 20 mg dry weight; chapter 4) feed at a similar rate on a diet of cabbage (containing 40 mg Ca per 7.8 g (dry weight); (U.S. Department of Agriculture, 2013)), then *T. ni* may consume 2000 nmol Ca each day. The haemolymph Ca^{2+} content of *T. ni* is 48 nmol (chapter 4), suggesting Lepidopteran larvae may also need to remove excess Ca^{2+} from the haemolymph during feeding.

Crickets feed on either plant or animal tissues (omnivorous) and were used in chapters 3 and 4 of this thesis. In the first 10 days of adulthood *Acheta domesticus* consume about 34 mg of food per day (Clifford and Woodring, 1990). If fed a diet of lettuce containing 18-36 mg Ca per 100 g (wet weight)(U.S. Department of Agriculture,

2013), the resulting Ca load, assuming all Ca is absorbed, would be 153 - 306 nmol per day. The haemolymph Ca^{2+} content of *Acheta domesticus* is estimated to be 190 nmol (chapter 4), suggesting there is a need for haemolymph Ca^{2+} excretion during this period. If no Ca excretion occurs and we assume all Ca consumed enters the haemolymph in ionized form (Ca^{2+}) then the haemolymph Ca^{2+} concentration would increase by 3.2 mM each day (volume = 95 µl, $[Ca^{2+}]_{haemolymph}$ = 2.1 mM; chapter 4). This would more than double the haemolymph Ca^{2+} concentration, from 2.1 to 5.3 mM Ca^{2+}, over a 24 hr period. Clearly, then, there is a need for haemolymph Ca^{2+} excretion in adults of *Acheta domesticus*.

Blood feeding insects, such as the kissing bug, *Rhodnius prolixus*, can consume several times their own body weight (up to 10 times the body weight in *Rhodnius*; (Orchard, 2006)) on vertebrate blood containing more than 1.5 mM Ca (Maddrell et al., 1991). Within a typical 15 min feeding, 5^{th} stage *Rhodnius prolixus* weighing about 40 mg (Maddrell, 1964), may thus consume a ~ 400 µl blood meal, equivalent to a 600 nmol Ca load. For a haemolymph volume of 6 µl (Gringorten and Friend, 1979) this load would result in haemolymph Ca concentration of 100 mM if all the Ca in the meal was transferred into the haemolymph! Blood feeders, then, likely face an even greater excess of dietary calcium than do most other insects and therefore excretion of excess haemolymph Ca^{2+} may play a large role in haemolymph Ca^{2+} homeostasis during the rapid digestion of the meal.

Together, these results suggest that many insects enjoy excess Ca in their diets and therefore excretion of excess Ca^{2+} (predominantly by the Malpighian tubules) is thus necessary for haemolymph Ca^{2+} homeostasis.

5.2. Most Ca^{2+} is sequestered within the tubule cells rather than incorporated into the primary urine

The Malpighian tubules contribute to Ca^{2+} excretion by secreting Ca^{2+} in soluble form or sequestering Ca^{2+} within biomineralized granules. The relative roles of Ca^{2+} secretion and sequestration in the excretion of excess Ca^{2+} from the haemolymph have been evaluated in only two insects: the fruit fly, *Drosophila melanogaster* (chapter 2, Dube et al. (2000a)) and the house cricket, *Acheta domesticus* (chapter 3). In both cases, the majority ($\geq$ 85%) of the Ca^{2+} entering the tubules is sequestered while the remainder ($\leq$ 15%) is secreted into the primary urine. In tubules of *Acheta*, the proportion of sequestered Ca^{2+} is increased to over 99% during stimulation by the second messenger, cAMP, suggesting that the tubules have the capacity to sequester essentially all of the Ca^{2+} they take up (chapter 3). A reliance on Ca^{2+} sequestration rather than Ca^{2+} secretion may have the following advantages: 1) sequestration allows for the accumulation of Ca reserves that enable terrestrial animals to survive prolonged periods without feeding (Lipovsek et al., 2016). 2) tubule Ca reserves may allow for more rapid calcium mobilization compared to extraction from the environment (Luquet and Marin, 2004). 3) sequestered Ca^{2+} that is readily exchangeable may 'buffer' rapid changes in Ca^{2+} in extracellular fluids (Mundy and Guise, 1999). 4) sequestration prevents toxic effects due to excessive intracellular Ca^{2+} (Annunziato et al., 2007) or heavy metal (Simkiss, 1977)

exposure (i.e. a detoxification role). High levels of calcium can block sodium channels and inhibit depolarization of nerve and muscle fibers, thus increasing the threshold for depolarization (Armstrong and Cota, 1999). Increasing external calcium concentration 10-fold has about the same effect as hyperpolarizing the membrane by 15 mV, thus taking nerve fibers further from threshold and making them harder to excite (Frankenhaeuser and Hodgkin, 1957).

The secretion of Ca^{2+} in complexed form (Ca-rich granules) may explain the relatively low rates of Ca^{2+} secretion calculated from measurements of fluid secretion rate and dissolved Ca^{2+} concentration in Ramsay assays. Ca-rich granules appear to be released directly into the tubule lumen in both the anterior tubules of the fruit fly, *Drosophila melanogaster* (Wessing and Zierold, 1999), and the midtubules of the house cricket, *Acheta domesticus* (Hazelton et al., 2001). In *Acheta*, stimulating midtubules with exogenous cAMP is thought to result in the release of granules into the lumen across the apical membrane (Hazelton et al., 2001). Granule release in response to stimulation with cAMP suggests that the secretion of granules may be under hormonal control. In chapter 3, cAMP was found to increase basolateral Ca^{2+} uptake by midtubules of *Acheta*. Taken together, these results suggest that there may be some coordination between basolateral and apical Ca^{2+} transport mechanisms. In either case, the secretion of Ca-rich granules (each representing a portion of tubule Ca^{2+} stores) would free up capacity for extra Ca^{2+} storage in the tubules, thus allowing further Ca^{2+} sequestration. A mechanism of tubule Ca^{2+} excretion, whereby Ca^{2+} is taken across the basolateral membrane, sequestered within Ca-rich granules within the tubule cells and subsequently secreted across the apical

membrane is consistent with low rates of Ca^{2+} secretion as measured by Ca^{2+}-selective microelectrodes and Ramsay assays (chapters 2 and 3). Note that Ca^{2+}-selective microelectrodes (based on Ca^{2+}-selective ionophores) detect only free Ca^{2+} ions in solution (Messerli and Smith, 2010) and therefore any Ca^{2+} contained within Ca-rich granules in the secreted fluid would go undetected.

Secretion of Ca-rich granules may be most important when the size of the tubules (and therefore the Ca^{2+} storage capacity) is constrained. For instance, the capacity of calcium stores in larvae would appear to be expandable as they continually feed (larval tubules increase in size through each successive instar, Ryerse (1979)) whereas stores of adults have more rigid space constraints (adult tubules are generally of fixed dimensions, Ryerse (1979)) and thus the removal of Ca-rich granules may be most important in adults.

If rates of Ca^{2+} sequestration exceed those of Ca^{2+} secretion, the tubules will fill with Ca-rich granules. Ca-rich granules are rapidly accumulated and appear to fill the lumen of the distal tubules of *Drosophila* (Wessing et al., 1992), within 48 hours of eclosion (chapter 2). The tubules may thus reach the limits of their capacity for Ca^{2+} storage, and at this point the removal of some Ca^{2+} from the tubules would be necessary in order for further tubule uptake of Ca^{2+} from the haemolymph to occur. In feeding adults, continual Ca^{2+} uptake by the Malpighian tubules is most likely required to maintain haemolymph Ca^{2+} homeostasis, whereas Ca^{2+} absorbed from the midgut may be more easily accommodated by expansion of haemolymph volume in rapidly growing larvae (Ryerse, 1979). Clearly, one strategy for removing stored Ca^{2+} from the Malpighian tubules is by periodically releasing large quantities of Ca-rich granules from

the tubules during metamorphosis. In *Drosophila*, granules are released from the distal tubules during pupal-adult metamorphosis, eventually being excreted in the meconium (chapter 2, Wessing et al. (1992)). Fluid transport by the Malpighian tubules is minimal during metamorphosis and thus the secretion of Ca-rich granules during this period may not require concomitant fluid secretion (potentially greatly reducing water loss, Wessing et al. (1992)).

The results from chapters 2 and 3 indicate that the majority of excess dietary Ca^{2+} is sequestered by the Malpighian tubule cells within biomineralized Ca-rich granules that function to maintain haemolymph Ca^{2+} balance throughout the life cycle. The secretion of Ca-rich granules may also allow sequestered Ca^{2+} to be removed from the body in highly concentrated form, avoiding intracellular Ca^{2+} toxicity and potentially reducing water loss.

5.3. Tubule Ca^{2+} transport is likely to be regulated independently of fluid secretion

Malpighian tubules transfer fluid from haemolymph-to-tubule lumen down an osmotic gradient generated by secretion of ions (predominantly K^+ or Na^+ and Cl^-) (Larsen et al., 2014). Fluid secretion is therefore principally controlled by modulating the secretion of these ions using neuroendocrine factors (hormones) released into the haemolymph (Spring, 1990). There is some evidence for a link between fluid and Ca^{2+} transport. For example, decreases in extracellular (using EGTA) or intracellular (using BAPTA) bathing Ca^{2+} concentrations reduces rates of fluid secretion by tubules of *Drosophila melanogaster* (Dube et al., 2000b, O'Donnell et al., 1996). Consistent with its

role as a second messenger, these results suggest tubule cells must have an adequate supply (either from the bathing media or intracellular stores) of Ca^{2+} for proper function. In addition, cAMP increases both fluid and tubule Ca^{2+} transport in tubules of *Acheta domesticus* (chapter 3,(Coast et al., 1991, Clark and Spring, 1992)). However, several lines of evidence suggest that tubule Ca^{2+} transport is independent of fluid secretion in the house cricket, *Acheta domesticus*. Firstly, tubules of *Acheta* secrete fluid at control rates in the absence of Ca^{2+} in the bathing media, suggesting that tubules of *Acheta* are able to rely solely on internal Ca^{2+} stores (possibly Ca-rich granules) for intracellular signaling related to modulation of ion and fluid transport rather than relying on Ca^{2+} entry from the haemolymph (Coast, 2011). Secondly, rates of fluid secretion are highest across distal tubule segments (Kim and Spring, 1992), where little-to-no basolateral Ca^{2+} transport occurs (chapter 3). Thirdly, in the presence of the Ca^{2+} mobilizing agent, thapsigargin, Ca^{2+} is released from the tubules (chapter 3), whereas the secretion of fluid (fluid uptake) is enhanced (Coast, 2011). The ability of Ca^{2+} to be transported in the opposite direction of fluid secretion is *a priori* evidence that the two transport pathways are independent of one another. Furthermore, serotonin decreased basolateral Ca^{2+} uptake by midtubules (chapter 3). In contrast, preliminary experiments indicated that 1 µM serotonin increased fluid secretion by more than 2-fold (A. Browne, unpublished data). The diuretic actions of serotonin on the tubules of *Acheta domesticus*, *Locusta migratoria*, *Rhodnius prolixus* and *Aedes aegypti* are well known (Coast, 2011, Coast, 1995, Barrett and Orchard, 1990, Clark and Bradley, 1997). Fourthly, the extensive vacuolization of the principal cells in response to cAMP stimulation do not appear to facilitate fluid transport across the cell

(Hazelton et al., 2002). Vacuolization is more likely to influence Ca^{2+} transport since many of the vacuoles contain Ca-rich granules. Fifthly, the lack of effect of cAMP and thapsigargin (in general both increase fluid secretion) on Ca^{2+} transport by Malpighian tubules in most of the insects investigated in chapter 4, suggest that Ca^{2+} and fluid secretion are not directly linked in several species.

In aggregate, it is clear that tubule Ca^{2+} transport can be modulated independently of fluid secretion suggesting that tubule Ca^{2+} transport is under separate neuroendocrine control. It remains to be determined whether calciotropic and diuretic hormones are distinct or whether multifunctional hormones may act on both of these distinct pathways simultaneously.

5.4. Tubule Ca^{2+} sequestration may be reversible

Malpighian tubules are sites of internal calcium storage that result from Ca^{2+} sequestration. A long standing question has been whether these calcium stores that result from Ca^{2+} sequestration can be resorbed back into the haemolymph (reversible Ca^{2+} sequestration), or whether these stores can only be accumulated and not subsequently released across the basolateral membrane into the haemolymph (irreversible Ca^{2+} sequestration). (Maddrell, 1972) used the term ‘storage excretion’ to refer to the former and ‘deposit excretion’ to refer to the later. Reversal of Ca^{2+} sequestration would be useful during periods of dietary Ca^{2+} deficiency. There are several lines of evidence from this thesis and other published works that suggest that Ca^{2+} sequestration and/or the underlying Ca^{2+} transport by Malpighian tubules result in ‘storage excretion’ (i.e. reversible calcium storage).

In chapter 2, basolateral Ca^{2+} transport was found to vary throughout the life cycle of *Drosophila melanogaster*, with Ca^{2+} efflux observed across distal tubules of larva and Ca^{2+} influx across distal tubules of adults. These results imply that the Malpighian tubules of *Drosophila* have the capacity for both uptake and release of Ca^{2+}. In addition, metabolic inhibition using cyanide and iodoacetic acid (inhibitors of aerobic and anaerobic metabolism, respectively) reversed Ca^{2+} influx to Ca^{2+} efflux by distal tubules of adult flies, whereas they were without effect on larval distal tubules, suggesting that mechanisms of Ca^{2+} influx are distinct from those of Ca^{2+} efflux. One explanation for the opposing directions of tubule Ca^{2+} transport is that Ca^{2+} sequestration is 'OFF' in late larval stages and subsequently switched 'ON' in adults. Similarily, fluid secretion by the Malpighian tubules is switched 'OFF' during pupation and resumes in adults (Ryerse, 1979). To further examine potential sites of Ca^{2+} transport along the length of the distal tubules, basolateral Ca^{2+} transport was measured with high spatial resolution in distal tubules of *Drosophila* larvae using SIET. Sites of both Ca^{2+} influx and Ca^{2+} efflux were found within single isolated distal tubules, suggesting Ca^{2+} may be recycled along the length of the distal storage segment. If sites of Ca^{2+} influx and Ca^{2+} efflux are modulated independently, as the metabolic inhibition results suggest, Ca^{2+} transport by the distal tubule may be readily reversed by regulation of either influx or efflux pathways.

In chapter 3, incorporating pharmacological antagonists into the bathing media surrounding midtubules of the house cricket, *Acheta domesticus*, indicated that basolateral Ca^{2+} fluxes (as measured by SIET) were reversible. Midtubules bathed in the presence of the 0.1 mM diltiazem (a calcium channel blocker) or 10 µM thapsigargin (a specific

antagonist of the endoplasmic reticulum Ca^{2+}-ATPase) reversed the direction of basolateral Ca^{2+} transport from Ca^{2+} influx to efflux. The majority (97%) of the Ca^{2+} entering the midtubule cells of *Acheta* is sequestered within intracellular stores. These data suggest that blocking Ca^{2+} uptake through calcium channels, either directly or indirectly, unmasks an underlying Ca^{2+} extrusion mechanism that is able transport Ca^{2+} from tubule cell to haemolymph. Basolateral plasma membrane Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchangers are believed to facilitate this resorption of Ca^{2+} (Simkiss, 1996).

Ca^{2+} uptake is the dominant direction of transport in isolated tubules bathed in species-specific salines, as described in Chapter 4. Ca^{2+} influx by the Malpighian tubules contributes to the removal of Ca^{2+} from the haemolymph and may provide insects with an efficient means of excreting excess dietary Ca^{2+} absorbed across the midgut (Taylor, 1985b). In order to maintain haemolymph calcium concentrations rates of dietary Ca^{2+} absorption are presumably closely matched to rates of Ca^{2+} excretion by the Malpighian tubules. In Dipterans, dietary Ca^{2+} absorption is unregulated (Taylor, 1985b) and therefore the Malpighian tubules may play a relatively larger role in Ca^{2+} excretion compared to tubules of insects where Ca^{2+} absorption may be restricted/regulated, as discussed further below in relation to the potential role of the midgut. Consistent with this idea, the tubules of *A. aegypti* larvae had the highest rates of Ca^{2+} influx by tubules bathed in saline. Dietary calcium is likely absorbed across the midgut in larvae of *A. aegypti*, given their relatively impermeable cuticle and the lack of Ca^{2+} transport observed by the anal papillae, which are in direct contact with the external environment (Donini and O'Donnell, 2005). In their natural environment, larvae develop in small pools of

freshwater that are prone to dilution during rainfall. High rates of Ca^{2+} sequestration by the Malpighian tubules may contribute to tubule calcium stores (Ca-rich granules) that allow larvae to survive very dilute freshwater environments for extended periods.

In addition to Ca^{2+} influxes, Ca^{2+} effluxes were recorded less frequently and were of lesser magnitude. Rates of both Ca^{2+} influx and efflux are suggestive of bi-directional transport. For example, spontaneous Ca^{2+} efflux is proposed to occur from cells at rest due to basolateral Ca^{2+} efflux transporters (plasma membrane Ca^{2+}-ATPases, PMCA; and Na^{+}/Ca^{2+}-exchangers, NCX) present in many animal cells (Simkiss, 1996). In contrast, Ca^{2+} influx is thought to be facilitated by basolateral calcium channels of the L-type, cyclic nucleotide gated (CNG) and/or transient receptor potential (TRP/TRP-like) families in tubules of *D. melanogaster* and *A. domesticus* (Davies et al., 2014, Browne and O'Donnell, 2018). The evidence based on patterns of Ca^{2+} influx and efflux at different times (*Drosophila*) and regions (*Drosophila* and *Acheta*) are consistent with independent control. The implications of such a mechanism would allow tubule calcium stores to accumulate during periods of calcium excess and allow tubule calcium stores to be reclaimed during periods of calcium deficiency, albeit at a reduced rate.

Evidence for reversible Ca^{2+} sequestration also comes from other published works. During the active life phase of the cave cricket, *Troglophilus neglectus*, essential elements are accumulated within Ca-rich granules of the midtubules (Lipovsek Delakorda et al., 2009). In contrast, during overwintering (a time of natural starvation) in caves the elements of the granules are progressively exploited for use in basic metabolic processes. The abundance of autophagic structures within the tubule cells correlated with the

progressive exploitation of mineral (Lipovsek et al., 2016), suggesting autophagic structures may be responsible for decalcification of Ca-rich granules. Essentially the same observations were made in tubule cells of the herald moth, *Scoliopteryx libatrix,* that overwinters in natural and artificial subterranean habitats (Lipovsek et al., 2017). These data are consistent with the presence of Ca-rich granules in the house cricket, *Acheta domesticus*, and suggest that Ca^{2+} within the granules can be reclaimed during periods of Ca^{2+} deficiency (e.g. overwintering). Further, seasonal changes in the mineral content of laminated spherites (granules) in the midgut gland of the arachnid, *Gyas annulatus* (Lipovšek et al., 2002), and the reversible Ca^{2+} transport by the epithelia surrounding Ca^{2+} stores in crustaceans (Wheatly, 1997) indicates that Ca^{2+} sequestration is a dynamic, reversible, phenomenon among several arthropods.

In aggregate, these results suggest that Ca^{2+} transport by epithelia surrounding internal calcium stores of insects (Malpighian tubules) are dynamic; changing throughout the life cycle to adapt to changing environmental calcium conditions (e.g. changing dietary sources or feeding regimes) allowing insects to accumulate Ca^{2+} stores during periods of calcium excess and to reclaim some of the stored Ca^{2+} back into the haemolymph to maintain haemolymph Ca^{2+} homeostasis during periods of calcium deficiency (i.e. during starvation or overwintering).

5.5. Mechanisms of Ca^{2+} sequestration by Malpighian tubules and their control by hormones

Models of tubule Ca^{2+} sequestration propose Ca^{2+} entry to occur through basolateral calcium channels (of either the L-type, CNG or TRP families) down a steep

concentration gradient (Browne and O'Donnell, 2018, Chintapalli et al., 2012, Davies et al., 2014). Ion uptake for mineralization purposes often involves entry through membrane transporters such as ion exchangers and ion channels that allow ions to enter the cell and then to follow a pathway, often through the endoplasmic reticulum to the site of mineralization (Weiner and Addadi, 2011). The detailed mechanisms of the formation of Ca-rich granules within the tubule cells are unclear but have been proposed to involve the ER-Golgi complex (Wessing and Zierold, 1999), specialized peroxisomes containing Ca^{2+}-ATPases (Southall et al., 2006), and increased protein expression of V-type H^{+}-ATPases (Chung et al., 2016).

Although no calciotropic hormones have been identified in insects to date, the stability of haemolymph Ca^{2+} concentrations and the presence of calciotropic hormones in other arthropods suggest that calciotropic hormones are likely to be present in insects where they presumably function to maintain haemolymph Ca^{2+} homeostasis. Malpighian tubules lack innervation and their rates of fluid and ion transport are controlled by hormones released into the haemolymph by neurosecretory cells of the central or peripheral nervous systems (Spring, 1990). Almost all studies of neuropeptides that act on the Malpighian tubules have been investigated within the context of their actions on diuresis. For example, many ‘diuretic’ hormones were identified by using high-performance liquid chromatography (HPLC) to separate peptide material from neural tissue (frequently head extracts) and subsequently testing the isolated fractions for diuretic activity, typically using the Ramsay assay (Spring, 1990).

Although we found evidence in chapter 3 (discussed above) that Ca^{2+} and fluid transport are not directly linked (potentially being regulated independently) there are important insights that can be gained from the studies of neuroendocrine regulation of diuresis. In this context, regulation of excretion is achieved by several families of neuropeptides (putative hormones) including the corticotrophin-releasing factor related diuretic hormones (CRF-related DHs), the kinins, calcitonin-like diuretic hormones (CT-like DH), tachykinin-related peptides, CAPA peptides and biogenic amines (serotonin and tyramine), all of which initiate diuretic or anti-diuretic effects on fluid secretion (Larsen et al., 2014). These factors represent 1st messengers that, upon binding their specific tubule cell surface receptors (frequently G-protein coupled receptors, GPCRs; Johnson et al. (2005)), initiate intracellular 2nd messenger signaling cascades that affect ionomotive pumps, channels and exchangers directly involved in ion and fluid secretion. In general, CRF-like neuropeptides act through an adenylyl cyclase-cAMP pathway (Kay et al., 1991, Nässel, 2002), kinins act through elevations in intracellular Ca^{2+} (Coast et al., 1990, Coast, 1996), CT-like neuropeptides act through an adenylyl cyclase-cAMP pathway (Coast et al., 2001, Coast et al., 2005), CAPA peptides act through Ca^{2+}-nitric oxide-cGMP pathway (Davies et al., 2013) and serotonin (5-HT) acts through an adenylyl cyclase-cAMP pathway (Barrett and Orchard, 1990). Identification of 2nd messenger pathways involved in tubule Ca^{2+} uptake and sequestration may provide insights into the formation of Ca-rich granules.

In chapter 3, two 2nd messenger pathways were identified with opposing effects on basolateral Ca^{2+} uptake by midtubules of *Acheta domesticus*. An adenylyl cyclase-cAMP-

PKA pathway (cAMP-pathway) stimulated Ca^{2+} uptake by tubules whereas thapsigargin (which increases intracellular Ca^{2+}) inhibited Ca^{2+} uptake. A cAMP-PKA pathway is also present in Malpighian tubules of *Rhodnius prolixus* (Grieco and Lopes, 1997) and *Aedes aegypti* (Tiburcy et al., 2013), resulting in modulation of specific ionomotive pumps (Na^+/K^+-ATPase and V-type H^+-ATPase, respectively).

These data suggest that haemolymph Ca^{2+} homeostasis, at least in the midtubules of *Acheta domesticus*, is achieved through the actions of two antagonistic calciotropic hormones (i.e. a bi-directional control mechanism) that likely modulate Ca^{2+} pumps, channels or exchangers. Consistent with this idea, tubule fluid secretion is also thought to be controlled by the actions of diuretic and anti-diuretic hormones (Coast et al., 2002). Antagonistic (bi-directional) control of tubule fluid secretion by both diuretic (Tenmo-DH_{37} and Tenmo-DH_{47}) and anti-diuretic (Tenmo-ADF) neuropeptides was, for the first time, demonstrated in the mealworm, *Tenebrio molitor* (Wiehart et al., 2002). There is apparently even more complexity in the control of diuresis, given that at least 2 diuretic neuropeptides are present in at least 7 insects: *Diploptera punctata, Manduca sexta, Hyles lineata, Tenebrio molitor* (Furuya et al., 2000), *Acheta domesticus* (Spring, 1990), *Drosophila melanogaster* (Hewes and Taghert, 2001) and *Aedes aegypti* (Sajadi et al., 2018). Multiple hormones may act antagonistically, synergistically or cooperatively to achieve haemolymph ion (and fluid) homeostasis (O'Donnell and Spring, 2000).

Putative first messengers of the stimulatory pathway include neuropeptides known to act through a cAMP-pathway on tubule cells, such as diuretic neuropeptides of the CRF and CT families (Coast, 2009). Furthermore, putative first messengers of the

inhibitory pathway include neuropeptides known to act through intracellular Ca^{2+}, such as the diuretic neuromodulator serotonin or the kinin family of neuropeptides (Coast, 2011, Browne and O'Donnell, 2018).

In chapter 3, when both stimulatory (cAMP) and inhibitory (Ca^{2+}) pathways controlling tubule Ca^{2+} transport in *Acheta domesticus* were activated simultaneously (using both cAMP and thapsigargin, respectively) the inhibitory pathway (Ca^{2+}) was found to be dominant. Consistent with our observations, (Clark and Spring, 1992) found that increases in intracellular Ca^{2+} (using the Ca^{2+} ionophore A23187) could override cAMP signaling in the control of fluid secretion by *Acheta* midtubules. Taken together, these results suggest that haemolymph Ca^{2+} homeostasis is achieved by the Malpighian tubules of *Acheta domesticus* through the coordinated actions of at least two calciotropic hormones that act antagonistically; one stimulatory factor acting through cAMP and another inhibitory factor acting through intracellular Ca^{2+}. The inhibitory pathway can override the stimulatory pathway, just as in fluid secretion, with the implication being that there is an inherent risk associated with eliminating Ca^{2+} from the haemolymph too rapidly, especially considering this insect has more than 100 tubules.

In chapter 4, there were only minor effects of cAMP or thapsigargin on Ca^{2+} uptake by *Acheta domesticus* distal tubule, suggesting that the bi-directional control system contributing to haemolymph Ca^{2+} homeostasis is specific to the *Acheta* midtubule. There are several possible explanations for the unexpected lack of effects observed in the SIET data. The simplest explanation is that 2nd messenger pathways other than cAMP or Ca^{2+} are involved in modulating tubule Ca^{2+} transport. For instance, the 2nd messenger,

cGMP, has been found to increase fluid secretion by midtubules of crickets (Coast et al., 2007, Xu and Marshall, 2000). Preliminary experiments revealed that the cGMP analog, 8-Br-cGMP, stimulated rates of Ca^{2+} uptake by midtubules of *Acheta domesticus* to the same extent as cAMP when either were applied to the tubules at a concentration of 1 mM (A. Browne, unpublished data). These results suggest that a calciotropic hormone acting through the cGMP-pathway is present in *Acheta* and that this hormone acts in conjunction (cooperatively or synergistically) with a hormone acting through the cAMP-pathway to stimulate tubule Ca^{2+} uptake. It is worth noting that cGMP has an anti-diuretic effect in other species, such as *Tenebrio molitor* (Wiehart et al., 2002), *Aedes aegypti* (Massaro et al., 2004) and *Rhodnius prolixus* (Quinlan et al., 1997).

Although several neuropeptides result in increases in intracellular cAMP, they may target different downstream effectors. For instance, the diuretic hormones Anoga-DH_{31} (CT-like) and Anoga-DH_{44} (CRF-like) both elevate intracellular cAMP levels in tubule cells but only the former results in increased Na^{+} secretion (natriuresis) (Coast et al., 2005). It has been proposed by Davies et al. (2014) that various exchange proteins activated by cAMP (EPAC) may target activation of multiple effectors from a single 2nd messenger. There is also evidence of cross-talk between second messenger pathways (cAMP and cGMP) through protein kinases and phosphodiesterases that may result in diverging effects of cAMP (Davies et al., 2014).

In general, insects appear to have more hormones for precisely controlling diuresis than was previously thought (Maddrell, 2009). Therefore it is not surprising that chapter 3 provides evidence for the presence of more than one putative calciotropic hormone. In

chapter 4, control of Ca^{2+} transport in tubules of multiple species appears not to be mediated by the 2nd messenger, cAMP, or by elevations in intracellular Ca^{2+}. It is possible that multiple hormones acting through multiple 2nd messenger pathways may be required to influence tubule Ca^{2+} transport and the endocrine pathways involved may be species-specific. Further, as outlined below, the midgut may play a role in haemolymph Ca^{2+} homeostasis in some species that result in lower rates of tubule Ca^{2+} transport. In these species, control of tubule Ca^{2+} transport may be of lesser importance.

Taken together, these results suggest that calciotropic neuropeptides are likely to facilitate haemolymph Ca^{2+} homeostasis in ways similar to those identified for the control of fluid secretion by the Malpighian tubules. It is well known that insect neuropeptides appear to be multifunctional (e.g. many diuretic neuropeptides are also myotropic, Nässel (2002)). It is conceivable, therefore, that neuropeptides implicated in diuresis may also play putative roles in tubule Ca^{2+} transport. In midtubules of the house cricket, *Acheta domesticus*, evidence suggests that CRF or CT-like neuropeptides (cAMP-pathway) stimulate Ca^{2+} sequestration, whereas the biogenic amine, serotonin, or neuropeptides of the kinin family (Ca^{2+}-pathway) inhibit Ca^{2+} sequestration. It has become clear that, just as in the neuroendocrine control of fluid and ion transport by the Malpighian tubules, tubule Ca^{2+} transport and by extension haemolymph Ca^{2+} homeostasis are likely to be species-specific.

5.6. Tubule Ca^{2+} transport is specific to principal cells

The large secondary cells found in the ileac plexus region of *T. ni* tubules allow Ca^{2+} flux measurements to be made adjacent to single cells using SIET (chapter 4). The

published work found in chapter 4 is the first to examine basolateral Ca^{2+} transport by single Malpighian tubule cells (both principal and secondary) of an insect. The results indicated that principal cells take up Ca^{2+} whereas neighbouring secondary cells appear not to transport Ca^{2+}. Several lines of evidence suggest tubule Ca^{2+} transport is specific to principal cells. Firstly, the metal-containing granules characteristic of principal (Type I) cells are absent or scarce in secondary (Type II) cells in ultrastructural investigations of Malpighian tubules of flies (*Drosophila*, Wessing et al. (1999); *Musca domestica*, Sohal (1974); *Sarcophaga ruficornis*, Pal and Kumar (2013)), the larvae of the Mecopteran hanging fly (*Terrobittacus implicatus*, Liu and Hua (2018)), the locust (*Schistocerca gregaria,* Garrett et al. (1988)), and cave cricket (*Troglophilus neglectus,* Lipovsek Delakorda et al. (2009)). Secondly, an antibody raised against an epitope common to two L-type calcium channels in *D. melanogaster* tubules (*Dmca1A* and *Dmca1D*) and a fluorescently-labeled L-type calcium channel blocker (Verapamil) were specifically colocalized to the basolateral membrane of only principal cells in the main segments of *D. melanogaster* tubules (MacPherson et al., 2001). Thirdly, tubules that lack secondary cells are able to sequester Ca^{2+} within storage segments (*A. domesticus*, Browne and O'Donnell (2018); *R. prolixus*, Maddrell et al. (1991)). Fourthly, application of the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, to tubules of *Drosophila melanogaster* results in increases in $[Ca^{2+}]_i$ in both principal and secondary (stellate) cells (Rosay et al., 1997). When tubules were subsequently bathed in the absence of external calcium, the thapsigargin-induced $[Ca^{2+}]_i$ increases are abolished only in principal cells, suggesting that plasma membrane calcium channels contribute to thapsigargin-induced

$[Ca^{2+}]_i$ increases specifically in principal cells. Secondary cells may rely on Ca^{2+} release from intracellular calcium stores rather than influx through calcium channels. Lastly, differences in Ca^{2+} handling by the two tubule cell types (Rosay et al., 1997) suggests basolateral Ca^{2+} transport occurs through the principal cells (transcellular pathway) rather than between them (paracellular pathway). Taken together, these results confirm that the tubule principal cells are the chief Ca^{2+} transporting cells of the Malpighian tubules and therefore play the major role in removing excess Ca^{2+} from the haemolymph.

5.7. Changes in calcium supply and demand may explain temporal patterns in tubule Ca^{2+} transport

Haemolymph Ca^{2+} homeostasis involves balancing calcium supply and demand with excesses being stored within the Malpighian tubules. Changes in either dietary supply or metabolic demand are likely to influence tubule calcium storage and by extension tubule Ca^{2+} transport. Insects living in a terrestrial environment are not guaranteed a reliable source of dietary Ca^{2+} and thus Ca^{2+} uptake from the environment is likely to change over time (chapter 2). For instance, in holometabolous insects, larvae and adults often feed on different diets (a strategy that helps to reduce competition between the life stages) that may vary in Ca^{2+} content. Conversely, adult stages of some Lepidoptera do not feed at all (Heath et al., 1971). Demand for calcium during the life of an insect is also unlikely to remain constant. Demand for haemolymph Ca^{2+} is likely to be highest during rapid growth, in and around metamorphosis (a rapid transformation) and/or during egg development. All of which is to say that Ca^{2+} transport by Malpighian tubules

is likely to change over time, allowing insects to maintain haemolymph Ca^{2+} homeostasis during periods of Ca^{2+} stress, both throughout the life cycle and within each life stage.

Throughout the life cycle, differences in Ca^{2+} transport suggest that tubule Ca^{2+} changes over time. In chapter 2, the direction of Ca^{2+} transport was opposite in distal tubules from larvae (Ca^{2+} efflux) compared to adults (Ca^{2+} influx) of *Drosophila melanogaster*. Preliminary experiments also indicated Ca^{2+} efflux in earlier (2^{nd}) instars and no transport was observed across the distal tubules of pupa despite obvious distension (> 100 µm diameter) of the tubule diameter by lumenal granules (A. Browne, unpublished data). These results suggest that larval distal tubules readily accumulate Ca-rich granules throughout development (i.e. Ca^{2+} stores are always full) and Ca^{2+} efflux may indicate high rates of Ca^{2+} exchange necessary for rapid growth. Granules are found in the meconium (rectum) of late stage pupa and are voided following emergence of adults. The meconium contains approximately 75% of the total Ca content of the larval body, suggesting that in the early stages of adult life *Drosophila* possess a calcium deficit (Dube et al., 2000b).

Within the adult life stage, changes in tubule Ca^{2+} transport over time are dramatic following pupal-adult metamorphosis. In chapter 2, the rate of Ca^{2+} influx across the distal tubules of *Drosophila* is rapidly increased to a peak rate at 24 hours post-eclosion corresponding to the appearance of Ca-rich granules within the tubule lumen. Thus tubule calcium stores are emptied during pupal-adult metamorphosis (possibly for detoxification purposes) but are then rapidly refilled following emergence. Ca^{2+} uptake then slows (decreases at a steadily declining rate), eventually reversing direction (from influx to

efflux). It is unclear why tubule Ca^{2+} stores begin to be depleted in aging (24 to 192 hours post-eclosion) adults. It may be that Ca^{2+} within the Malpighian tubules is released back into the haemolymph to be used in egg production. Oviposition generally starts on the second day after eclosion (~ 24 to 48 hours), increases to a peak rate at about 6-10 days (144 to 240 hours) and then declines thereafter (Robertson and Sang, 1944). Commencement of oviposition approximately 24 hours post-eclosion corresponds well with the start of the declining rate of Ca^{2+} uptake by adult tubules in chapter 2. Calcium is abundant in the eggs of insects (Przelecka et al., 1986, Taylor, 1984). As the ovaries accumulate Ca there is a corresponding decrease in the Ca content of the Malpighian tubules isolated from the blowfly, *Calliphora vicina* (Taylor, 1984), suggesting that calcium is mobilized from tubule Ca stores to eggs during development. Large numbers of eggs laid each day (> 50 at peak; (Robertson and Sang, 1944)) by the related Dipteran, *Drosophila melanogaster*, would suggest that a significant Ca^{2+} demand occurs during egg development.

A similar temporal pattern of Ca^{2+} uptake occurs across the Malpighian tubules of *Rhodnius prolixus* following a blood meal (Maddrell et al., 1991). Ca^{2+} uptake is increased several fold across the upper Malpighian tubules within the first 4 days following a blood meal, declining thereafter but proceeding for at least 9 days. The rapid increase in Ca^{2+} uptake is associated with an increase in the Ca^{2+} content of the Malpighian tubules and the presence of Ca-rich granules abundant within the cells (Maddrell et al., 1991). These data suggest that, in both *Rhodnius* and *Drosophila*, rates

of Ca^{2+} uptake and sequestration by the Malpighian tubules can respond rapidly to changes in dietary supply or demand for calcium.

It is worth noting that parasites of insects often disrupt/modify the internal ionic balance of an insect and therefore have the potential to influence haemolymph Ca^{2+} homeostasis. Consistent with this hypothesis, parasitism of the caterpillar, *Diatraea saccharalis*, by the parasitic wasp, *Cotesia flavipes*, increases the abundance Ca-rich granules in the columnar cells of the midgut (de Oliveira Pinheiro et al., 2008). Rossi et al. (2014) provide evidence that parasitism by *Cotesia* reduces relative food consumption by *Diatraea* but enhances utilization of digested food such that no difference in final weight were observed between the parasitized and non-parasitized larvae. Enhanced Ca^{2+} sequestration by the midgut may be an adaptive response to accelerated Ca^{2+} absorption or reduced Ca^{2+} sequestration by the Malpighian tubules. In addition, mosquitoes (*Aedes aegypti*) susceptible to infection by the heartworm, *Dirofilaria immitis*, drastically reduces the abundance of Ca-rich granules in the Malpighian tubules (Bradley et al., 1990). The precise mechanism for the reduction in tubule granules is unclear but, given the previous example, could involve enhanced Ca^{2+} sequestration by the midgut of mosquito larvae.

Lastly, the Ca-rich granules themselves appear to be altered over time. For example, annual changes in granule composition in a cave cricket (Lipovsek Delakorda et al., 2009) and also in a cave arachnid (Lipovšek et al., 2002), suggest granule constituents (i.e. calcium) can be resorbed and utilized during periods of deficiency (overwintering). Formation of Ca-rich granules by tubule cells is thought to be actively controlled by Ca^{2+}

transport across the cell surface and within intracellular membrane-bound subcellular compartments. Since Ca-rich granules are directly influenced by tubule Ca^{2+} transport seasonal changes in granule composition are *a prior* evidence for seasonal changes in tubule Ca^{2+} transport.

In summary, temporal patterns of tubule Ca^{2+} transport between and within life stages may be explained by changes in the dietary source of Ca^{2+} and by changes in the demand for haemolymph Ca^{2+} during growth, reproduction or metamorphosis. Tight control of haemolymph Ca^{2+} homeostasis in insects, as is the case in other animals, is likely the result of homeostatic mechanisms that continually monitor haemolymph Ca^{2+} (possibly through calcium-sensing receptors) and therefore can continually respond to changes in environmental calcium throughout the life cycle by modulating Ca^{2+} transport by the Malpighian tubules.

5.8. Potential role of the midgut in haemolymph Ca^{2+} homeostasis

Evidence from other animals, such as humans, fish and crustaceans suggest that intestinal Ca^{2+} absorption is a hormonally regulated process that contributes to extracellular Ca^{2+} homeostasis (Hall, 2011, Flik and Verbost, 1993, Wheatly, 1999). In insects, few studies have investigated Ca^{2+} absorption across the midgut. Net $^{45}Ca^{2+}$ absorption (from lumen to bath) occurs at high rates (2 to 3 nmol min^{-1}) across isolated midguts of the blowfly, *Calliphora vicina* (Taylor, 1985a). Known stimulants (dopamine, serotonin, octopamine, proctolin) of the midgut epithelium were without effect on Ca^{2+} transport and uptake rates remained high in elevated bath Ca^{2+} concentrations, leading

(Taylor, 1985b) to conclude that Ca^{2+} absorption is unregulated in the blowfly. If this conclusion is applicable to other insects, especially closely related Dipterans, then rates of Ca^{2+} excretion by the Malpighian tubules must be equal to rates of Ca^{2+} absorption by the midgut in order for Ca^{2+} concentrations of the haemolymph to be maintained. One might expect, then, that rates of tubule Ca^{2+} uptake would be even higher in insects which ingest calcium-rich diets and/or are rapid feeders (i.e. blood feeders or larvae). In contrast, rates of tubule Ca^{2+} uptake were very low in many of the species investigated (chapter 4), and haemolymph Ca^{2+} turnover times (taking into account the number and morphology of all the Malpighian tubules in each insect) were also very long (> 18 hours) in some species (*H. halys* and *P. occidentalis*). In addition, (Maddrell et al., 1991) found tubule Ca^{2+} excretion to be quite slow (> 10 days) in the blood feeding bug, *Rhodnius prolixus*. Low rates of Ca^{2+} uptake or slow haemolymph Ca^{2+} turnover by the Malpighian tubules may be a consequence of other tissues that function to limit increases in haemolymph calcium activity. Alternatively, these insects may have been in calcium balance and thus in a period when haemolymph calcium turnover would be expected to be relatively slow. There are multiple lines of evidence that suggest that the midgut may sequester Ca^{2+} and therefore aid in haemolymph Ca^{2+} homeostasis. During homeostasis, rates of Ca^{2+} excretion by the Malpighian tubules are presumably linked to rates of Ca^{2+} absorption, thus a decrease in Ca^{2+} absorption would lower the requirement for Ca^{2+} excretion.

Ultrastructural investigations of the midgut epithelia have revealed concentric metal-containing granules similar in shape and size to those often found in the Malpighian tubules of various Lepidopteran larvae (Ryerse, 1979, Turbeck, 1974, Waku

and Sumimoto, 1971) . Unlike the Malpighian tubules, the midgut is significantly remodeled during metamorphosis (Ryerse, 1979), and most larval midgut cells are replaced by new differentiated cells, so any calcium present within the degenerating midgut cells of larvae would naturally be excreted following ecdysis. In some Lepidopteran larvae, spherical granules are present in the apical cytoplasm of columnar cells during the molting cycle (Turbeck, 1974). The appearance of the granules first at the apical membrane would suggest that Ca^{2+} is sequestered within the granules following absorption across the apical membrane of midgut cells. Net $^{45}Ca^{2+}$ absorption occurs across isolated midguts of the giant silkmoth, *Hyalophora cecropia*, with minimal back flux (Wood and Harvey, 1976). Following application of the radiotracer the authors reported a significant delay (~ 45 min) prior to reaching tracer steady state, which was interpreted as evidence for a rather large pool of calcium in the transport route across the midgut (Wood and Harvey, 1976). Conceivably, the Ca-rich granules of the midgut cells could represent such a large pool of exchangeable calcium.

Although many aspects of the mechanisms of midgut granule formation are unknown (as with their formation within the Malpighian tubules) they are believed to have an excretory function (Maddrell et al., 1991). Since the midgut granules are found predominantly in cells showing evidence of degeneration (corresponding to the molting cycle) their formation may ultimately be an indirect consequence of molting hormones. In contrast, the midgut cells of adult Lepidopterans typically contain few or no spherical granules, consistent with their intermittent feeding on liquid diets (e.g. flower nectar) or lack of feeding entirely (Heath et al., 1971). Adults may not encounter high levels of Ca^{2+}

in their diets and thus not require midgut Ca^{2+} sequestration to aid in haemolymph Ca^{2+} homeostasis.

In the silkworm, *Bombyx mori*, following degeneration of the larval midgut cells, the newly formed pupal midgut cells (regenerative cells) rapidly accumulate Ca-containing granules, which first appear near the basal membrane but then fill most of the cytoplasm (Waku and Sumimoto, 1971). The histological staining method used in this study suggested the granules are composed of calcium phosphate or carbonate and not calcium oxalate (calcium oxalate monohydrate crystals are found in the Malpighian tubules). In late pupal stages the Ca-containing granules are frequently observed in the midgut lumen suggesting they are discharged by exocytosis. The midgut of the adult moth is devoid of the Ca-containing granules suggesting that granules are likely excreted within the meconium of the recently emerged adult. These data were interpreted as evidence that during larval-pupal metamorphosis Ca^{2+} from haemolymph is temporarily accumulated and stored in the cells of the pupal midgut in addition to the accumulation within the Malpighian tubules. Similar storage granules have been observed in columnar and around goblet cell cavities in the midgut of the velvetbean caterpillar, *Anticarsia gemmatalis* (Gomes et al., 2012), and the tobacco hornworm, *Manduca sexta* (Dow et al., 1984), although these granules appear to be rich in P and Mg with lesser amounts of Ca. The midgut of Lepidopterans may thus serve as internal calcium stores that contribute to haemolymph calcium homeostasis during metamorphosis.

In contrast to Lepidopteran larvae, midgut granules are present in adult life stages of some insects. For instance, in the lantern bug, *Pyrops candelaria* (Homoptera),

spherical granules accumulate with age within the midgut cells of adults (Cheung and Marshall, 1982). Similar Ca-rich granules are also found in the Malpighian tubules of *Pyrops*, suggesting both tissues simultaneously play a role in Ca^{2+} sequestration. In another adult Homopteran, the balsam woolly adelgi, *Adelges piceae*, Ca-rich granules are stored within the cells of the anterior midgut, where they accumulate with age (Jarial, 1998). Midgut granules of *Adelges* appear to be extruded into the lumen. It is worth noting that *Adelges* lack Malpighian tubules and thus metal ion storage by the midgut may be an anatomical adaptation to their sap-sucking behavior.

In the Hymenoptera, concentric granules rich in calcium, protein and polysaccharides are present in the apex of the digestive (columnar) cells of the posterior midgut in the adult honey bee, *Apis mellifera* and stingless bee *Melipona quadrifasciata anthidioides* (Hymenoptera) (Da Cruz-Landim and Serrão, 1996). The results of this study were interpreted as evidence for excretory and detoxification roles of the Ca-rich granules that ultimately allow for internal ion homeostasis. In the harvester ant, *Formica polyctena*, larvae do not contain Ca-rich granules in either the midgut or Malpighian tubules, but midgut granules appear during larval-nymphal metamorphosis (Ballan-Dufrancais, 2002). The midgut is the main site of bioaccumulation of physiological cations (Ca, K, Mg, Mn, and Zn) and/or toxic metal cations (V, Co, Cu, As, Pd, Cd, Sn, Sb, Ba, Pt, Hg, and Pb). Generally, when cells of the midgut were found to store a particular cation that cation was also found in the Malpighian tubules but at lower concentrations, suggesting that cation bioaccumulation by the midgut limits cation haemolymph exposure. In other words, the sequestration of metals by the midgut in some

cases is sufficient and thus removal by the Malpighian tubules is not necessary or is reduced.

Concentric granules are also present in the anterior midgut of the stick insect, *Carausius morosus* (Beadle and Gahan, 1969). Moreover, stick insects have appendices of the midgut composed of a blind-ended tubules, which are distinct from the Malpighian tubules, connected to a proximal ampulla that empties into the posterior midgut lumen. Cationic dyes are sequestered (believed to be precipitates of calcium salts) within the ampulla, which are then gradually eliminated into the midgut lumen (Shelomi and Kimsey, 2014). These specialized midgut appendices likely contribute, along with the Malpighian tubules, to haemolymph calcium homeostasis in Phasmatodea (phasmids) that are known for their highly constrained body plan.

In aggregate, these data suggest that in multiple species the midgut plays a role in internal Ca^{2+} storage (excretion) by sequestering Ca^{2+} within intracellular Ca-rich granules that are similar in structure and composition to those frequently found within the Malpighian tubules. In actively feeding life stages Ca^{2+} sequestration by the midgut may function as an initial barrier to Ca^{2+} entry into the haemolymph (in addition to the established role of the midgut in detoxification of toxic metals; (Ballan-Dufrancais, 2002, Lauverjat et al., 1989, Lemaitre and Miguel-Aliaga, 2013)) and therefore limit excessive increases in haemolymph [Ca^{2+}] that would require removal by the Malpighian tubules. Cells of the Malpighian tubules and midgut containing Ca-rich granules often appear to have the capacity to extrude the Ca-rich granules from the cytosol into the lumen of the tubule or gut, respectively. Ultimately, these lumenal granules from either route (tubules

or gut) will be passed into the hindgut lumen where they will presumably be eliminated in the excreta. During periods of metamorphosis (molting or pupating), just as in crustaceans (i.e. cuticle hardening with Ca salts), is an opportune time for large-scale internal Ca^{2+} movement. It is not surprising, then, that large accumulations of Ca-rich granules (representing stores of excess calcium) are frequently lost from both the Malpighian tubules and midgut during these periods. Both midgut and Malpighian tubules are in contact with haemolymph and thus calciotropic hormones released into the haemolymph could potentially regulate Ca^{2+} transport by both of these tissues. In addition, enteric neurons projecting to the midgut may provide an addition mode of regulation (Schoofs et al., 2014).

In contrast to the insect orders described above, it appears that the midgut of Dipterans, especially in larvae, plays a limited role in Ca^{2+} sequestration. In the adult house fly, *Musca domesticus* (Diptera) concentric granules resembling those of their Malpighian tubules are not observed in the epithelial cells of the midgut; instead electron-dense granules are present in lysosomes (Sohal, 1974). Elemental analysis reveals that they contain the elements Ca, Fe, Cu, Cl, P, and S with Ca having among the lowest relative concentration of the tested elements (Sohal et al., 1977). These granules are not extruded into the lumen, but are instead steadily accumulated such that the granules may occupy approximately 50% of the cytosol in some 40 day old flies. In another Dipteran, *Drosophila melanogaster*, the larval midgut is devoid of Ca-rich granules (Ballan-Dufrancais, 2002) but instead accumulates Fe, Zn and Cu along a relatively short length of the middle midgut (Lemaitre and Miguel-Aliaga, 2013).

It is important to note that for those species in which Ca-rich granules are present in the midgut cells, transport into the cells may occur across either the apical or basolateral membrane. In other words, the source of the Ca^{2+} within the granules could either be from the midgut lumen or the haemolymph. Ca^{2+} is readily absorbed from lumen-to-bath with minimal back flux in isolated midguts of the blowfly, *Calliphora vicina* (Diptera)(Taylor, 1985a), and caterpillar, *Hyalophora cecropia* (Lepidoptera)(Wood and Harvey, 1976), suggesting Ca^{2+} uptake by midgut cells occurs predominantly across the apical membrane.

I have argued that for an excretory role of the midgut that would work in conjunction with the Malpighian tubules to maintain haemolymph Ca^{2+} homeostasis in insects. From the literature it is clear that in some species (and at different life stages within a given species) the midgut plays a lesser role in Ca^{2+} sequestration. Since both midgut and Malpighian tubules have complimentary functions (excretion of excess Ca^{2+}) the relative roles of the midgut or Malpighian tubule in Ca^{2+} excretion would be expected to vary. It follows, then, that species with limited Ca^{2+} sequestration by midgut would compensate with higher rates of Ca^{2+} sequestration by the Malpighian tubules. Consistent with this hypothesis, Taylor (1985b) found that Ca^{2+} absorption is unregulated in perfused larval midguts of the Dipteran, *Calliphora vicina*. The absence of Ca-rich granules in the midguts of Dipterans (Sohal et al., 1977), suggests that the Dipteran midgut plays a minor role in Ca^{2+} sequestration (especially in larvae) and therefore rates of Ca^{2+} sequestration by the Malpighian tubules may be higher than in insects with midgut Ca^{2+} sequestration. In chapter 4, tubules of the Dipterans (*Aedes aegypti* larvae and *Drosophila melanogaster*

adults) showed the highest rates of Ca^{2+} uptake among the species examined. It is known that most (> 80 %) of Ca^{2+} taken up by the tubule cells is sequestered (chapter 2 and 3), suggesting high rates of Ca^{2+} sequestration by the Malpighian tubules of Dipterans (Dube et al., 2000a). Moreover, rates of Ca^{2+} efflux by distal tubules of *Drosophila melanogaster* larvae were even higher than rates of Ca^{2+} influx by distal tubules of adults (chapter 2). Despite the efforts to date, the role of the midgut in Ca^{2+} homeostasis remains unclear. Thus physiological investigations of Ca^{2+} transport by midguts of insect species that contain Ca-rich granules would help to clarify their contribution, if any, to extracellular Ca^{2+} homeostasis.

In summary, these results suggest that in some insects Ca^{2+} is sequestered within the midgut epithelia within Ca-rich granules that are eventually released into the hindgut lumen and excreted. In this way Ca^{2+} absorption is reduced and therefore Ca^{2+} sequestration by the Malpighian tubules need not be so rapid. The formation of Ca-rich granules is thought to be a highly regulated process, therefore it is more likely than not that the midgut has some capacity for regulated Ca^{2+} absorption when Ca-rich granules are present.

5.9. Haemolymph volume estimates

Haemolymph volume of single insects is often estimated through weighing by difference: single insects are weighed before and after tearing the cuticle and absorbing the extracted haemolymph using blotting paper in the so called ‘blotting technique’ (Cohen et al., 1986, Nicolson et al., 1974). Haemolymph volumes of *D. melanogaster* adult females based on the blotting technique range from 0.050 to 0.078 µl (Folk et al.,

2001, Folk and Bradley, 2003). Alternatively, 0.056 µl of haemolymph were extracted from *D. melanogaster* adult females using a high-throughput method that involves applying positive pressure to the body wall (MacMillan and Hughson, 2014). It is worth noting that all haemolymph extraction techniques cannot extract the total haemolymph volume from an insect and therefore represent underestimates of the total haemolymph volume. Using the range of values for *D. melanogaster* results in negligible changes to the calculated haemolymph Ca^{2+} elimination times (15 min to 30 min) as described in Chapter 2 or (0.1 hours to 0.15 hours) as in Chapter 4.

A mean haemolymph volume of 46.2 µl for adults of the house cricket, *A. domesticus,* has also been obtained by the blotting technique (Chung et al., 1994). A similar value of 40.8 µl is reported for the field cricket, *G. pennsylvanicus,* using a similar technique (MacMillan and Sinclair, 2011). In these studies extracted haemolymph volume was used as an approximation of haemolymph volume. Higher values (75 to 200 µl) have been reported for *G. pennsylvanicus* using the inulin dilution technique (MacMillan et al., 2012). The value of 46.2 µl is likely an underestimate and the value of ~100 µl may represent an overestimate given that adult females of *G. pennsylvanicus* are generally larger (323 to 1056 mg; (Souroukis and Murray, 1994)) than females of *A. domesticus*. Using the range of haemolymph volumes (40 µl to 100 µl) estimates for *A. domesticus* results in negligible changes to the calculated haemolymph Ca^{2+} elimination times (40 min to 100 min) as described in Chapter 3 or (0.7 hours to 1.6 hours) as in Chapter 4. In summary, crude estimates of the haemolymph volume of the study insects allows useful

comparisons of the approximate time for all Malpighian tubules to eliminate the haemolymph Ca^{2+} content in multiple species.

5.10. Comparative physiology: Relevance to human nephrolithiasis

Kidney stones affect about 9% of people in the United States (Scales et al., 2012). As in other animals, the composition of renal stones are variable and are believed to be influenced by factors such as diet, sex and geographic distribution (Ramello et al., 2001). The most prevalent stones are those principally composed of calcium oxalate (40-60%) but others contain calcium phosphate (20-60%), uric acid (5-10%), struvite (5-15%), cystine (1-3%) or often contain mixtures of constituents such as calcium oxalate/calcium phosphate (35-40%) (Moe, 2006). In contrast, granules of arthropods typically consist of calcium carbonates or calcium phosphates (Ballan-Dufrancais, 2002). There appear to be four main pathways for kidney stone formation (nephrolithiasis): 1) growth over white interstitial hydroxyapatite (HA) plaque, known as Randall's plaque 2) growth over Bellini duct (BD) plugs 3) formation within inner medullary collecting ducts (IMCD) and 4) formation in free solution within the calyces or renal collecting system (Evan et al., 2015). Calcium oxalate (CaOx) stone formation often occurs in patients with no symptoms of renal disease, so called idiopathic CaOx stone formers, and formation typically follows the first pathway. Spherical granules made of alternating layers of matrix and mineral are present in the initial stages of Randall's plaque (RP) formation occurring in the basement membranes of the thin loops of Henle (Evan et al., 2003). Accumulation of these granules and plaque initially form within the interstitium but at

some point breach the overlying urothelium exposing the granules, more importantly their mineralizing matrix, to urine which appears to create nucleations that form the base of an overgrowth stone (Evan et al., 2015).

Under light and transmission electron microscopy, renal granules are strikingly similar to the Ca-rich granules of crustaceans and insects described in the introductory chapter. The initial stages of stone formation are poorly understood, limiting therapeutic approaches to nephrolithiasis. Ryall (2008) pointed out the similarity of the renal granules of human and invertebrate origin and suggested that biomimetic models ought to be exploited to investigate the processes involved in the formation of Ca-rich granules in renal epithelia. Ca-rich granules that resemble those found in the Malpighian tubules of insects are also present in samples of Randall's plaque early in the development of renal stones (Chi et al., 2015). The similarity in structure suggest similar mechanisms of formation and function. Therefore, the Malpighian tubules of insects may represent good biomimetic models of human nephrolithiasis (Dow and Romero, 2010). There are several lines of evidence that suggest Malpighian tubules are similar to the human renal tubules. Firstly, both function as excretory tissues that generate a primary urine from the extracellular fluid (Maddrell, 1981, Hall, 2011). Secondly, both are segmented along their length allowing the composition of the primary urine to be precisely controlled through modification by downstream segments (Maddrell, 1981, Hall, 2011). Thirdly, tubule cells of both origins contain similar membrane transport proteins, such as Ca^{2+} channels, PMCA and NCX (Chintapalli et al., 2012, Blaine et al., 2015). Insect and mammalian models also contain calcium binding proteins (Chintapalli et al., 2012, Lambers et al.,

2006). Finally, the formation and composition of the primary urine in both systems are under hormonal control (Spring, 1990, Hall, 2011). It is not surprising, then, that the fruit fly, *Drosophila melanogaster*, has been marketed as a "powerful translational model of human disease, including nephrolithiasis" (Miller et al., 2013). A 'good' model is one that resembles human physiology as closely as possible in the relevant aspects, not necessarily in absolute terms. The evidence thus far suggests that the shared and therefore 'relevant' phenomenon is the formation of physiological Ca-rich granules within the renal/Malpighian tubules (Ryall, 2008). Narrowing our focus to the fundamental principals of kidney stone formation (the granules) is likely to reveal powerful insights that are applicable across the animal kingdom, including humans and insects. In this regard, Malpighian tubules yield a relatively unobstructed view (Malpighian tubules are relatively transparent) of this process in living tissue in real-time (Hirata et al., 2012), which is not possible in other animal models. In fact, it may also improve our understanding of ectopic calcification in other human tissues, such as occurs in vascular calcification (Leopold, 2015). A brief review of the literature (>15 papers published between 2013 and 2018) reveals that *Drosophila* is quickly becoming a model for human nephrolithiasis.

Although *Drosophila* tubules naturally produce granules composed of calcium carbonates or phosphates, they can produce CaOx crystals (akin to CaOx kidney stones) when fed on diets enriched with the lithogenic agents: sodium oxalate (NaOx), hydroxyl-L-proline (HLP) or ethylene glycol (EG)(Chen et al., 2011). Since these lithogenic agents do not represent a dietary calcium load but result in Ca-rich crystals, it follows that Ca^{2+}

must also be in excess in the diet where both (cation and anion) are ultimately sequestered by a common pathway within the Malpighian tubules. Melamine also increases crystal formation in *Drosophila* tubules but unlike the other lithogenic agents melamine increases the abundance of spherical crystals (resembling the physiological Ca-rich granules) that are of mixed (not CaOx-rich) composition (Chen et al., 2012). Consistent with our results of Ca^{2+} transport by distal Malpighian tubules of adult flies, crystals induced by NaOx, EG, HLP and melamine appear most often within the distal tubule segments (Chen et al., 2011, Chen et al., 2012). Crystal morphology in the NaOx group was small and extensive, whereas they were larger and had "polyangular" shape in the EG diet (Chung and Turney, 2017). It should be emphasized here that most calcium stored in the Malpighian tubules is believed to be in the form of amorphous (not crystalline) Ca-rich granules (Maddrell et al., 1991, Ballan-Dufrancais, 2002, Krueger et al., 1987). In contrast, the pathological crystals formed during dietary administration of toxic lithogenic agents often appear crystalline (octahedral CaOx dihydrate crystals are a clear example) and lack the concentric layering (organic matrix) of the physiological Ca-rich granules (Chen et al., 2011). In the xanthine dehydrogenase *Drosophila* model (described further below), the pathological crystals are much larger than the physiological granules and their excess often results in the distension of the tubule walls and correlates with a dramatically shortened lifespan (Chi et al., 2015). In a preliminary experiment, Ca^{2+} secretion was reduced in anterior Malpighian tubules of *Drosophila melanogaster* larvae when bathed in saline containing 0.33 mM NaOx suggesting that Ox^{2-} influences Ca^{2+} transport by the Malpighian tubules (A. Browne, unpublished data). Presumably,

bathing tubules in the presence of NaOx would promote NaOx crystal formation in principal cells and thus reduce the amount of Ca^{2+} available within the cell to be secreted across the apical membrane. At present, the mechanisms of formation of the physiological Ca-rich granules or pathophysiological Ca-rich crystals are poorly understood and may represent distinct pathways for mineralization.

One advantage to the study of the pathophysiological CaOx-rich crystals is that they exhibit a birefringence and thus are readily observed under polarized light microscopy (Dow and Romero, 2010). Consequently, most studies of nephrolithiasis in *Drosophila* involve feeding the flies toxic lithogenic agents followed by the visualization of crystal abundance (calculated from various stereological measurements) using polarized light microscopy. Because of its simplicity and speed, the visualization of crystals in Malpighian tubules (Wu et al., 2014) or excrement (Ali et al., 2016) of *Drosophila* in response to co-administration of lithogenic and potential litholitic agents in the diet has been used as a high-throughput method to screen for potential litholitic agents for use in the treatment of human nephrolithiasis. At least 16 potential plant extracts (Wu et al., 2014), 5 neutroceuticals (Ali et al., 2016) and a synthetic plant metabolite (Abd El-Salam et al., 2018) have been found to reduce the presence of CaOx crystals within the Malpighian tubules of *Drosophila*. Presumably these drug leads will be pursued further in mammalian models to verify their litholitic effects prior to any clinical trials in humans. Finally, another advantage of the *Drosophila* CaOx model of nephrolithiasis is that the flies are suitable for micro-computed tomography (micro-CT) scanning, which allows CT-dense (CaOx) crystals to be localized in 3D at high resolution (800 nm) and

radiodensity measurements to be determined *in situ* (Hirata et al., 2012, Chen et al., 2018). No CT-dense crystals are observed in control flies, allowing specific comparisons to be made.

A different approach is to exploit the powerful genetic tools developed for *Drosophila* to investigate putative genes involved in tubule CaOx crystal formation. Genetic knock down of *dPrestin* gene (encoding an electrogenic Cl^-/Ox^{2-} exchanger) by RNAi in *Drosophila* tubules reduce crystal formation in flies fed a NaOx-rich diet, suggesting that *dPrestin* (abundantly expressed in midgut, Malpighian tubules and hindgut) plays a role in oxalate excretion by the tubules and ultimately CaOx crystal formation (Hirata et al., 2012). The effect of *dPrestin*-dependent CaOx crystal formation occurs to a greater extent in anterior tubules (that contain the distal segment) compared to distal tubules, suggesting that the distal tubules play a role in CaOx crystal formation. More recently, *dPrestin* has been associated with excretion of CaOx from late prepupal salivary glands of *Drosophila melanogaster* (Robert et al., 2016). These results highlight the importance of investigating the transport of the major cation (Ca^{2+}) and the counter anion (Ox^{2-}) as both are likely to influence the mineralization of the end product (CaOx crystals).

In other studies, genetic knockdown and pharmacological inhibition (allopurinol) of xanthine dehydrogenase (XDH, an enzyme that converts xanthine to urate) increases crystal formation correlated with an increase in xanthine and hypoxanthine levels in whole flies (Chi et al., 2015). The resulting crystals were found to be principally composed of hydroxyapatite, a common constituent of human kidney stones (and bones)

(Evan et al., 2003). The authors also used dietary, pharmacological and genetic approaches to identify a role for zinc in modulating crystal formation: Zn feeding increased crystal formation whereas reductions in dietary Zn using a Zn-specific chelator reduced crystal formation, and knockdown of tubule Zn transporters by RNAi reduced crystal formation and increased whole body Zn levels (Chi et al., 2015).

Insights into the mechanisms of nephrolithiasis in *Drosophila* have also been gained through use of RNA sequencing (RNA-Seq) to identify potential genes differentially expressed during dietary NaOx or EG treatment compared to a control diet (Chung and Turney, 2017). Of the 58 differentially expressed genes in the NaOx group and 20 of the EG group there were no overlapping genes, suggestive of distinct pathways for NaOx and EG-induced crystal formation. In particular, the expression of DH_{44} was increased in response to EG feeding. DH_{44} encodes a corticotropin-releasing factor (CRF) like peptide hormone involved in desiccation and starvation tolerance through a cAMP-dependent pathway in *Drosophila melanogaster* (Cannell et al., 2016). In addition DH_{44} acts specifically on principal cells (Cabrero et al., 2002). Taken together, these results suggest that DH_{44} may play a role in tubule mineralization by principal cells. Alternatively, increases in DH_{44} expression may be a response to ionic or osmotic disturbances resulting from EG exposure (discussed below). The results in chapter 3 suggested hormonal control of Ca^{2+} sequestration through a cAMP-dependent pathway that acts to enhance Ca^{2+} sequestration specifically across tubule principal cells. These results are thus in agreement with ours and suggest that DH_{44} may play a role in stimulating tubule biomineralization. In chapter 4, cAMP had no effect of Ca^{2+} transport

by *Drosophila* tubules, suggesting DH_{44} may influence the transport of the anion (for example, Ox^{2-}) rather than the transport of the cation (Ca^{2+}).

Proteomic changes in Malpighian tubules of *Drosophila* following treatment with dietary NaOx or EG compared to a control diet allows putative proteins involved in crystal formation to be identified (Chung et al., 2016). Among the 57 differentially abundant proteins common to both NaOx and EG groups but not the control group, Vha100-1 (Vacuolar H^+-ATPase 100 kD subunit 1) was pointed out as an interesting candidate protein because its human homologue, Hsap\ATP6V0A4, is known to cause metabolic acidosis (due to reduced renal H^+ secretion) and is often accompanied by nephrolithiasis (Chung et al., 2016). In fact, one perceived limitation of the EG model of nephrolithiasis is that EG is well known to cause metabolic acidosis in humans (Knauf and Preisig, 2011) and thus EG-induced CaOx crystal formation in *Drosophila* may be a consequence of metabolic acidosis or an underlying pathology of tubule H^+ transport. These data suggest that a V-ATPase plays a role in Ca^{2+} sequestration in Malpighian tubules of *Drosophila*.

Lastly, insights into the mechanisms of tubule nephrolithiasis have also been gained by bathing tubules in media containing putative lithogenic agents (a more direct and rapid approach than dietary methods). Crystals are more rapidly (< 15 min) visualized *in vitro* by exposing tubules directly to NaOx compared to the appearance of granules > 6 hours following dietary exposure to NaOx (Hirata et al., 2012). Additionally, this method avoids the potential biotransformation of test compounds associated with digestion occurring in the foregut and the potential sequestration by an intestinal barrier.

5.11. Future Work

Although substantial progress has been made in recent years, our understanding of calcium homeostasis in insects is incomplete and further work is required if we are to understand how insects regulate haemolymph Ca^{2+} and the role played by Ca-rich granules in this regard. Similarities in Ca-rich granules in vertebrate renal tubules and Malpighian tubules suggests that studies of Ca^{2+} transport and Ca-rich granule formation are likely to have broad relevance.

One of the key findings of this thesis is that two distinct second messenger pathways control tubule Ca^{2+} transport in *Acheta domesticus* midtubules. These results suggest that two calciotropic hormones are present in *Acheta* that act through cAMP or Ca^{2+} second messenger pathways, respectively. The presence of calciotropic hormones has not been investigated to date. There are several ways to identify putative calciotropic hormones. First, one could take the classic approach used to identify putative diuretic hormones; test fractions of neuronal tissue separated by HPLC in a biological assay. We found in both *D. melanogaster* and *Acheta domesticus* that basolateral Ca^{2+} transport exceeded Ca^{2+} secretion, thus screening putative neuropeptide isolates in a biological assay measuring basolateral Ca^{2+} transport would be ideal. SIET is not conducive to high-throughput screening as only a single tubule can by assayed at a time (in series). A superior approach would be to use the assay developed in chapter 2 that allows for basolateral Ca^{2+} transport measurements of multiple tubules simultaneously (in parallel). Alternatively, neuropeptides that affect the formation of pathological CaOx-rich stones could be pursued using the emerging *Drosophila* models of nephrolithiasis described

above (Dow and Romero, 2010). Secondly, a genetic approach could be taken to identify putative calciotropic hormones. Using a *Drosophila* genetic model of human nephrolithiasis, DH_{44} has been identified as a putative hormone of nephrolithiasis by comparing tubule expression profiles (transcriptomes) of flies fed lithogenic agents compared to those on a control diet (Chung and Turney, 2017). Thus DH_{44} appears to be a multi-functional neuropeptide, implicated in both diuresis and nephrolithiasis. The effect of DH_{44} on tubule Ca^{2+} transport could easily be investigated using SIET in any, or all, of the species investigated in chapter 4. A similar approach could be taken to identify putative calciotropic hormones controlling tubule Ca^{2+} transport rather than pathological tubule nephrolithiasis. For example, comparisons of tubule transcriptomes/proteomes of flies fed Ca-restricted diets (possibly using Ca^{2+} chelators) compared to flies fed a control diet (control diets are Ca-rich; containing about 4.5 mM Ca^{2+}, chapter 2) would likely reveal novel mechanisms of control. Thirdly, since neuroendocrine factors acting though cAMP and intracellular Ca^{2+} were indicated in *Acheta domesticus* midtubules, diuretic hormones acting through these second messenger pathways could be tested for the effects on basolateral Ca^{2+} transport by the midtubules of *Acheta*. The CRF-related neuropeptide, Achdo-DH, known to act through cAMP (Coast and Kay, 1994) and the kinins, Achdo-K1 through KV (Coast et al., 1990), acting though intracellular Ca^{2+} could also be tested in any of the biological assays. Since the peptide sequences of the neuropeptides are known and are relatively short (the kinins are ≤ 9 peptides long, (Coast et al., 1990)) they can be synthesized relatively inexpensively.

As outlined in the introductory chapter, vitamin D (or its polar metabolites) show biological activity in yeast, plants and some invertebrates, and is also essential for bone homeostasis in amphibians, birds and mammals. The implication is that the most active form of vitamin D, 1,25$(OH)_2$D, is likely to influence haemolymph Ca^{2+} homeostasis in insects. Therefore this compound may be tested for its effect on basolateral Ca^{2+} transport by the Malpighian tubules. In addition, there is evidence across the animal classes that calcium-sensing receptors (CaSRs) located in many Ca^{2+}-transporting epithelia (including renal tubules) play a key role in extracellular Ca^{2+} regulation (chapter 1). Currently it is unknown whether the Malpighian tubules express CaSRs. If they do, they would likely have the ability to 'sense' Ca^{2+} directly. Alternatively, CaSRs are possibly located in the central nervous system where they modulate calciotropic hormone release. In either case, the Malpighian tubules clearly have the ability to respond directly to changes in bathing Ca^{2+} concentrations. Increases in bathing Ca^{2+} concentration (0.5 to 32 mM Ca^{2+}) were found to increase rates of basolateral Ca^{2+} uptake by isolated distal tubules of adult *Drosophila melanogaster* (A. Browne, unpublished data), consistent with putative Ca^{2+} sensing by the Malpighian tubules directly. In addition, data mining may be used to identify putative CaSR genes from vertebrate sequences. Conversely, there are pharmacological agonists selective for CaSRs known as "calcimimetics", such as NPS R-467 (Radman et al., 2002) that could be used to probe for the presence of such a receptor in the Malpighian tubules.

Future work could also evaluate the model of Ca-rich granule formation proposed for *Drosophila* (Chintapalli et al., 2012) using pharmacological/genetic knockdown

approaches. In their model Ca^{2+} enters tubule cells through basolateral Ca^{2+} channels, which is then transported into specialized peroxisomes by the secretory pathway Ca^{2+}/Mn^{2+}-ATPase isoform SPoCk-C (Southall et al., 2006). Phosphate may enter the peroxisomes via a Na^{+}:phosphate cotransporter (NaPiT; CG15096) and the bestrophin Cl^{-} channel (Best2) contributes to charge balance across the peroxisomal membrane (Chintapalli et al., 2012). This model of Ca^{2+} transport may be tested by examining the effects of inhibitors of Best2 (such as DIDS (Liu et al., 2015)), SPoCk-C (bisphenol; (Gunaratne and Vacquier, 2006)) and NaPiT (phosphonocarboxylic acids; ((Loghman-Adham, 1996)) on basolateral Ca^{2+} fluxes measured by SIET.

The lack of effect of cAMP and thapsigargin in most of the insects surveyed in chapter 3 suggested other second messenger pathways may influence tubule Ca^{2+} transport in these species. Therefore future work could investigate the effects of other second messengers, such as cyclic guanosine monophosphate (cGMP), nitric oxide (NO) and diacylglycerol (DAG) on basolateral Ca^{2+} transport across the distal tubules of insects other than *Acheta domesticus*. The effects of these putative second messenger pathways on basolateral Ca^{2+} fluxes could also be investigated across single principal cells of the Malpighian tubules isolated from the cabbage looper, *Trichoplusia ni*.

In summary, future explorations into the mechanisms of control over tubule Ca^{2+} transport are likely to yield insights into the mechanisms of Ca-rich granule formation occurring in the tubule cells, which have broad implications to the study of biomineralization (occurring in essentially all animals) including human nephrolithiasis for which insect models are currently viewed with a great deal of interest.

6. References

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