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# Calcium transport across the basolateral membrane of isolated Malpighian tubules: a survey of several insect orders

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**Abstract.** The Malpighian tubules play a major role in haemolymph calcium homeostasis in insects by sequestering excess $Ca^{2+}$ within the biomineralized granules that often accumulate in the tubule cells and/or lumen. Using the scanning ion-selective microelectrode technique, measurements of basolateral $Ca^{2+}$ transport are determined at several sites along the length of the Malpighian tubules isolated from the 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 is specific to tubule segments containing Ca-rich granules in *D. melanogaster* and *A. aegypti*, whereas $Ca^{2+}$ transport is relatively uniform along the length of whole tubules in the remaining species. Generally, manipulation of second messenger pathways using cAMP and thapsigargin has little effect on rates of basolateral $Ca^{2+}$ transport, suggesting that previous effects observed across midtubules of *A. domesticus* are unique to this species. In addition, the present 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 range from 6 min (*D. melanogaster*) to 19 h (*H. halys*) or more, indicating that rates of $Ca^{2+}$ uptake by the Malpighian tubules are not always rapid. The results of the present study suggest that the principal cells of the Malpighian tubules contribute to haemolymph calcium homeostasis by sequestering excess $Ca^{2+}$, often within specific tubule segments.

**Key words.** *Acheta*, *Aedes*, calcium, *Drosophila*, ionoregulation, Malpighian tubule, secretion, sequestration, transport.

## Introduction

Haemolymph calcium concentrations in insects are maintained within narrow limits to maintain tissue function. Despite a more than six-fold increase in the calcium content of the diet, haemolymph $Ca^{2+}$ concentrations of flies increase by less than 1.5-fold, indicating that Dipterans have the capacity for haemolymph calcium regulation (Taylor, 1985; 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) lead Taylor (1985) to conclude that the regulation of $Ca^{2+}$ excretion and not $Ca^{2+}$ absorption is the primary mechanism for haemolymph calcium regulation in the blowfly *Calliphora vicina*. Taylor (1985) proposes that insects fully absorb all dietary calcium into the haemolymph and that any excesses are then removed by the excretory organs (i.e. the Malpighian tubules and hindgut). To date, the contribution of the hindgut to $Ca^{2+}$ homeostasis remains to be investigated. By contrast, there is much evidence to suggest that the Malpighian tubules are major sites of haemolymph calcium homeostasis. First, Malpighian tubules are sites of high $^{45}Ca^{2+}$

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turnover (Taylor, 1985) and are often sites of significant stores of calcium (containing up to 94% of total body calcium in the fruit fly, *Drosophila hydei*) (Wessing & Zierold, 1992). Second, spherical granules approximately 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 considered 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 & O'Donnell, 2016, 2017).

Malpighian tubules can remove excess $Ca^{2+}$ from the haemolymph either by secretion or sequestration. Secretion refers to the transport of $Ca^{2+}$ from the haemolymph to the 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 *Drosphila melanogaster* and *Acheta 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 (Dube *et al.*, 2000a; Browne & O'Donnell, 2016, 2017). Accordingly, $Ca^{2+}$ 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 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. As in vertebrate renal tubules, segmented Malpighian tubules allow fluid and useful solutes that are transferred into upper regions of the tubule lumen to be reabsorbed in downstream segments (O'Donnell & 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 *Rhodnius prolixus* (Hemiptera) (Maddrell *et al.*, 1991), the distal tubules of *D. melanogaster* and *C. vicina* (Diptera) (Taylor, 1985; Browne & O'Donnell, 2016) and the midtubules of *A. domesticus* (Orthoptera) (Browne & O'Donnell, 2017)). 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 neurohaemal areas in response to feeding or other stimuli (Coast & 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 by more than 3.5-fold by cAMP and inhibited by more than two-fold or reversed by 5-hydroxytryptamine (5-HT) or thapsigargin, respectively (Browne & O'Donnell, 2017). 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) indicate a role for the adenylyl cyclase–cAMP–protein kinase A (PKA) pathway (Browne & O'Donnell, 2017). These results are interpreted as evidence for a bidirectional control mechanism where 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 & O'Donnell, 2017).

In the present study, 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). First, we probe for regulatory pathways by bathing tubules from each of the insects in saline containing cAMP and thapsigargin, which are compounds previously found to stimulate or inhibit, respectively, basolateral $Ca^{2+}$ influx across calcium storage segments (midtubules) in the house cricket *A. domesticus*. Second, 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. Third, a useful specialization of *T. ni* Malpighian tubules, the large (> 50 μm in diameter) principal and secondary cells of the ileac plexus region, allows us to assess cell type-specific $Ca^{2+}$ transport across individual Malpighian tubule cells using SIET. Finally, $Ca^{2+}$ concentrations are determined in haemolymph samples taken from each insect using $Ca^{2+}$-selective microelectrodes. These values are then related to $Ca^{2+}$ fluxes obtained by SIET with the aim of estimating 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 with respect to haemolymph $Ca^{2+}$ regulation in each species.

## Materials and methods

### *Insects*

All insects were maintained under a 12 : 12 h light/dark photocycle at 21–23 °C unless stated otherwise.

*Drosophila melanogaster* (Diptera) adults of the Oregon-R strain were obtained from laboratory cultures maintained at McMaster University (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

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ferric sulphate and 50 dry active yeast (Roberts & Stander, 1998). To prevent mold growth, 7.45 mL $L^{-1}$ of 10% tegosept (butyl 4-hydroxybenzoate; Sigma-Aldrich, St Louis, Missouri) 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 at 24–30 h post-eclosion were used in all of the experiments.

*Aedes aegypti* (Diptera) eggs were obtained from laboratory cultures maintained at McMaster University (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 of the experiments.

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

*Acheta domesticus* (Orthoptera) adults were also obtained from PetSmart and maintained at 28 °C with access to water and ground multi-fowl feed containing 0.05% calcium by weight (W-S Feed & Supplies Ltd, 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 at 7–8 weeks of age.

*Trichoplusia ni* (Lepidoptera) larvae were obtained from the Great Lakes Forestry Centre (Canada) and reared in groups of 5–10 in 22-mL cups containing a synthetic diet (McMorran, 1965). Fourth-instar larvae were used in all experiments.

*Periplaneta americana* (Blattodea) adults were obtained from Boreal Science (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) with access to water *ad libitum*.

*Halyomorpha halys* (Hemiptera) adults were obtained from laboratory cultures maintained at McMaster University (ON). Adults were maintained in a 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*.

### *Salines and Malpighian tubule dissections*

Malpighian tubules were dissected under physiological saline solutions specific to each insect (Table 1). All compounds were obtained from Sigma-Aldrich. As a result of the absence of available 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 *Oncopeltus fasciatus* (Te Brugge & Orchard, 2008), and the harvester ant *Formica 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 & O'Donnell, 2016), *A. domesticus* (Browne & O'Donnell, 2017), *T. molitor* (Wiehart *et al.*, 2002), *P. americana* (Kay *et al.*, 1992) and *T. ni* (O'Donnell & Ruiz-Sanchez, 2015). Tubules of *H. halys* were dissected in accordance with a procedure similar to that 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 #5 forceps (Dumont, Switzerland). Single tubules were then pulled free from their insertions into the gut.

### *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 & O'Donnell, 2017). The second messenger cAMP was dissolved in bathing saline.

### *$Ca^{2+}$-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 & O'Donnell (2016). Reference microelectrodes for use with SIET were pulled from 1.5 mm outer diameter 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 to maintain ionic strength between experimental and calibration solutions. KCl was exchanged for $CaCl_2$ in calibration solutions for use with *P. occidentalis* as a result of the lack of NaCl in the saline. Slopes per 10-fold change in $Ca^{2+}$ concentration were 27 to 29 mV.

### *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.

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**Table 1.** Composition of experimental salines (mmol $L^{-1}$).

| | Species | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Saline components | *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 *et al.* (1994).
[d] Te Brugge & Orchard (2008).
[e] Kocmarek & O'Donnell (2011).
[f] Laenen *et al.* (2001).
[g] Nicolson (1992).
[h] Maddrell & Gardiner (1976).

SIET measurements were made in accordance with established protocols (Browne & O'Donnell, 2017) using hardware from Applicable Electronics (Forestdale, Massachusetts) and automated scanning electrode technique (ASET) software, version 2.0 (Science Wares Inc., East Falmouth, Massachusetts). Briefly, microelectrode voltage was recorded at two positions within a two-dimensional plane perpendicular to the long axis of the tubule. Microelectrodes were first positioned within 5 μm of the tissue then moved 50 μm away in accordance with a 'move-wait-sample' protocol at each site. Wait and sample times were 3.5 and 0.5 s, respectively. A mean voltage difference was calculated from five measurements made at intervals of 1 min at each site, and each measurement was itself the mean of three 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 equation:

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

where $C_B$ is the background $Ca^{2+}$ concentration (mmol $L^{-1}$ or μmol $cm^{-3}$); $\Delta V$ 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 °C ($7.9 \times 10^{-6}$ $cm^2$ $s^{-1}$) obtained from Smith *et al.* (1999); and $\Delta x$ 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).

### *Measurement of [$Ca^{2+}$] in haemolymph*

Insects were held under paraffin oil and their cuticle ruptured with #5 forceps (Dumont). Droplets of haemolymph exuding

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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 ($> 10^{13}\ \Omega$) electrometers connected to a data acquisition system (PowerLab; ADInstruments, Australia) and analyzed using CHART, version 5 (ADInstruments). Calcium concentrations were calculated using the 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; $\Delta V$ 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.

*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 s $h^{-1}$ and the corresponding surface area ($cm^2$) of the segment. The surface area of tubule segments was calculated using the formula for the surface area of a cylinder ($\pi dl$), where $d$ is diameter and $l$ is length. Tubule diameters were estimated from images at × 200 magnification using IMAGEJ, version 1.47 (National Institutes of Health, Bethesda, Maryland). Tubule lengths were estimated using an eyepiece micrometer at × 124 to × 800 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.

*Statistical analysis*

Graphing and statistical analysis were performed using PRISM, version 4.0b (GraphPad Software Inc., San Diego, California). Data are presented as the mean ± SEM for the indicated number of samples ($n$). Comparisons between two groups were made using Student's $t$-test, whereas comparisons of three or more groups were made using one-way analysis of variance followed by Tukey's range test. $P < 0.05$ was considered statistically significant.

## Results

*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 by > 3.5-fold in the presence of 1 mM cAMP and reversed from influx to efflux across tubules exposed to 10 µM thapsigargin (Browne & O'Donnell, 2017). Along with the use of forskolin and IBMX, these results were interpreted as providing 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 study 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 (Fig. 1A). In addition, $Ca^{2+}$ influx by distal tubules bathed in saline was lower than the published value ($-26 \pm 1$ pmol $cm^{-2}$ $s^{-1}$, $n = 171$) for midtubules (Browne & O'Donnell, 2017). The results from the previous study are shown in Fig. 1(A) (left three bars) for comparison. Together, these results indicate that the distal tubules, in contrast to the midtubule, do not respond to cAMP and thapsigargin.

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 that $Ca^{2+}$ transport by the tubules would be similar. Unexpectedly, basolateral $Ca^{2+}$ influx reversed (from influx to efflux) within the midtubules after the addition of 1 mM cAMP to the bathing saline (Fig. 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 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 the present study (Fig. 1C). Basolateral $Ca^{2+}$ influx was higher in distal segments compared with 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.

Similar to *D. melanogaster*, rates of $Ca^{2+}$ uptake were high across tubules of *A. aegypti* larvae (Fig. 1D). Basolateral $Ca^{2+}$ influx was higher in distal segments compared with 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

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**Fig. 1.** Basolateral $Ca^{2+}$ flux across isolated segments of Malpighian tubules from (A) *Acheta domesticus*, (B) *Periplaneta americana*, (C) *Drosophila melanogaster*, (D) *Aedes aegypti*, (E) *Tenebrio molitor*, (F) *Trichoplusia ni*, (G) *Halyomorpha halys* and (H) *Pogonomyrmex occidentalis* bathed in saline (solid bars), saline containing 1 mM cAMP (grey bars) and saline containing 10 μM thapsigargin (open bars). (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 & O'Donnell, 2017). Data are presented as the mean ± SEM. Significant differences between treatments (saline, cAMP, thapsigargin) within each tubule region for all insects were tested using a one-way analysis of variance. For each tubule region, bars labelled with different lowercase letters are statistically different ($P < 0.05$). For comparisons of different regions bathed in saline, bars labelled with an asterisk (*) and connecting lines are significantly different (unpaired Student's *t*-test, $P < 0.05$).

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thapsigargin (Fig. 1E). As a result of 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.

By contrast to Coleopterans, the free tubules of Lepidopteran larvae are highly segmented. We therefore measured basolateral $Ca^{2+}$ transport within the distal ileac plexus, 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, the present study is the first physiological investigation of the Malpighian tubules of *H. halys*. Three distinct segments were observed under light microscopy: a short ($5.6 \pm 0.2$ mm, $n = 7$) translucent proximal segment with a relatively smooth basal surface; a short ($8.8 \pm 1$ mm, $n = 7$) translucent middle segment with a nodular basal surface; and a longer ($30.6 \pm 0.3$ mm, $n = 6$) translucent distal segment containing conspicuous green pigment and with a highly nodular basal surface. Preliminary SIET experiments indicated that $Ca^{2+}$ fluxes across the proximal and middle tubule segments were equivalent (data not shown) and therefore only proximal and distal tubules were examined further. 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 (Fig. 1G). In both tubule segments, there was no effect of cAMP or thapsigargin on $Ca^{2+}$ transport.

During dissections, the lumen of *P. occidentalis* tubules were noted to be 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 (Fig. 1H). In proximal segments, there was no effect of cAMP or thapsigargin on basolateral $Ca^{2+}$ fluxes. By contrast, basolateral $Ca^{2+}$ effluxes were modestly increased in the presence of thapsigargin in distal segments relative to tubules exposed to cAMP (Fig. 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 taken 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 investigated, 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 compared with tubules held under control (saline) conditions.

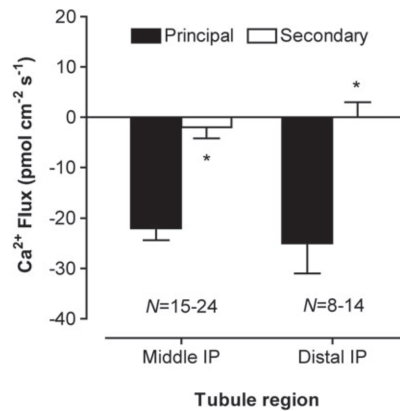


**Fig. 2.** Basolateral $Ca^{2+}$ flux across principal cells (solid bars) and secondary cells (open bars) found in the middle and distal ileac plexus (IP) regions of the Malpighian tubules of *T. ni* larvae. Data are presented as the mean ± SEM. Bars labelled with an asterisk (*) are significantly different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

### *Cell-type specific basolateral $Ca^{2+}$ 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 (Fig. 2). Rates of basolateral $Ca^{2+}$ transport by secondary cells were no different from a value of zero. These results suggest that tubule $Ca^{2+}$ transport occurs by a transcellular route through principal cells.

### *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 $2.4 \pm 0.5$ mM $Ca^{2+}$ ($n = 8$), as measured by $Ca^{2+}$-selective microelectrodes (Fig. 3). In particular, haemolymph calcium concentrations were lowest in *A. aegypti* and highest in *P. americana*.

### *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 approximately 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

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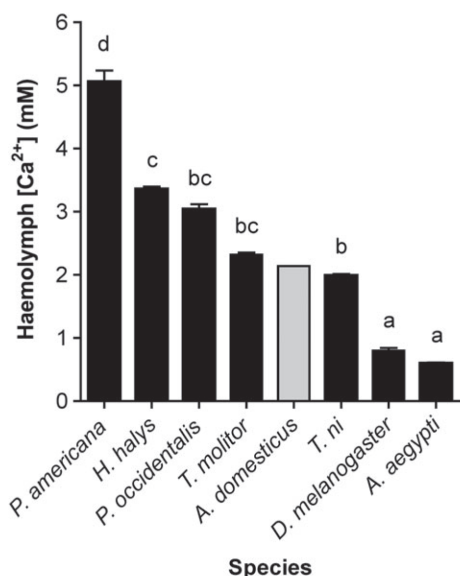


**Fig. 3.** Concentrations of $Ca^{2+}$ in samples of haemolymph collected from several insects. A published value of haemolymph $Ca^{2+}$ concentration (grey bar) reported for *Acheta domesticus* is included for comparison (Browne & O'Donnell, 2017). Data are presented as the mean ± SEM. Bars labelled with different lowercase letters are statistically different (one-way analysis of variance, $P < 0.05$).

for all tubules to eliminate the entire haemolymph $Ca^{2+}$ content could be estimated (Table 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 (Fig. 1, solid bars), the surface area ($cm^2$) of each tubule segment and 3600 s $h^{-1}$. 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 s $h^{-1}$). Equivalent calculations for the middle and distal segments result in $Ca^{2+}$ fluxes of 0 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 that represented the rate of $Ca^{2+}$ transport by all tubules. For example, *D. melanogaster* has four proximal, four main and two distal segments. The resulting $Ca^{2+}$ fluxes for all proximal ($-128$ pmol $h^{-1}$ = $-32$ pmol $h^{-1}$ × 4) middle (0 pmol $h^{-1}$) and distal ($-302$ pmol $h^{-1}$ = $-151$ pmol $h^{-1}$ × 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 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 $Ca^{2+}$) (Fig. 3). In the absence of published measurements of haemolymph volume for 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 h) 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.

## Discussion

Previous studies of the Malpighian tubules of *D. melanogaster* (Dube *et al.*, 2000a; Browne & O'Donnell, 2016) and *A. domesticus* (Browne & O'Donnell, 2017) report a pronounced localization of basolateral $Ca^{2+}$ transport to specialized tubule segments containing Ca-rich granules. In the present study, we find regional differences in tubule $Ca^{2+}$ transport in only one other insect, *A. aegypti*, whereas there are 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, as well as 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* are 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 & O'Donnell, 2017). Because these second messenger pathways are the subject of extensive studies in the context of osmoregulation in crickets (Clark & Spring, 1992; Xu & Marshall, 2000) and other insects (Wiehart *et al.*, 2003; Beyenbach & Piermarini, 2011; Davies *et al.*, 2013), it is hypothesized that the $Ca^{2+}$ regulation pathways identified in *A. domesticus* (Browne & O'Donnell, 2017) would be broadly applicable in insects. In the present study, manipulation of second messenger pathways using 1 mM cAMP and 10 μM thapsigargin has 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 are very low in many of the species examined. The calculations that we describe below suggest that rates of $Ca^{2+}$ transport in tubules of *H. halys* and *P. occidentalis* are likely too low to be physiologically relevant. The Malpighian tubules of these insects may not play a significant role in haemolymph calcium regulation and thus other tissues may be involved.

### *Segment-specific, uniform and cell type-specific patterns of basolateral $Ca^{2+}$ transport*

In insects where basolateral $Ca^{2+}$ transport is measured directly within sub-segments along the length of the Malpighian tubules, such as in *D. melanogaster* (Dube *et al.*, 2000a; Browne & O'Donnell, 2016), *R. prolixus* (Maddrell *et al.*, 1991) and *A. domesticus* (Browne & O'Donnell, 2017), a segment-specific pattern of $Ca^{2+}$ transport emerges. 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 the present study, $Ca^{2+}$ transport is segment-specific across the tubules of the mosquito *A.*

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

| Segment (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) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Drosophila 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 | | | | | | |
| *Aedes 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 | | | | | | |
| *Tenebrio molitor* (Dor) | 18.4[c] | | | 0.049 | −529 | | | | | | |
| (Lat) | 31.5[c] | | 85 ± 4 (*n* = 10) | 0.084 | −907 | 6 | −12312 | 65 ± 4 (*n* = 10) | 6.5 (0.065 g × 10%[d]) | 15080 | 1.2 |
| (Ven) | 21.3[c] | | | 0.057 | −616 | | | | | | |
| *Acheta domesticus* (Mid) | 6.1 ± 0.2 (*n* = 10) | | 57 ± 2 (*n* = 10) | 0.011 | −990 | 112[e] | −118944 | 522 ± 22 (*n* = 9) | 95 (0.522 g × 18.2%[d]) | 191100 | 1.6 |
| (Dist) | 1.2 ± 0.1 (*n* = 14) | | 43 ± 2 (*n* = 10) | 0.002 | −72 | | | | | | |
| *Trichoplusia ni* (WR) | 7[f] | | 102 ± 3 (*n* = 10) | 0.022 | −1742 | | | | | | |
| (YR) | 7[f] | | 102 ± 3 (*n* = 10) | 0.022 | −475 | 6 | −17730 | 69 ± 6 (*n* = 10) | 24 (0.069 g × 34.5%[d]) | 47760 | 2.7 |
| (IP) | 13[f] | | 100 ± 5 (*n* = 10) | 0.041 | −738 | | | | | | |
| *Periplaneta americana* (Mid) | 23.0 ± 1.0 (N = 7) | | 62 ± 3 (*n* = 10) | 0.045 | −1782 | ≈ 170[g] | −300560 | 1090 ± 98 (*n* = 7) | 194 (1.09 g × 17.8%[d]) | 981640 | 3.3 |
| (Dist) | 1.3 ± 0.1 (*n* = 10) | | 41 ± 3 (*n* = 10) | 0.002 | 14 | | | | | | |
| *Halyomorpha 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 × 30.9%[d]) | 161280 | 18.7 |
| (Dist) | 30.6 ± 2.9 (*n* = 6) | | 104 ± 3 (*n* = 10) | 0.100 | −2160 | | | | | | |
| *Pogonomyrmex occidentalis* (Prox) | 7.2 ± 0.2 | 0.7[ψ] | 54 ± 1 (*n* = 10) | 0.001 | 4 | 5 | 180 | 8 ± 1 (*n* = 10) | ≈ 0.5[h] | 1520 | −8.4[i] |
| (Mid+Dist) | (*n* = 9) | 6.5 | 44 ± 2 (*n* = 10) | 0.009 | 32 | | | | | | |

[a]MacMillan & Hughson (2014).
[b]Donini & O'Donnell (2005).
[c]Nicolson (1992).
[d]Maddrell (1981).
[e]Hazelton *et al.* (1988).
[f]O'Donnell & Ruiz-Sanchez (2015).
[g]Wall *et al.* (1975).
[h]Van Kerkhove *et al.* (1989).
[i]Negative elimination time represents the time to double haemolymph $Ca^{2+}$ content.
[ψ]Lengths of proximal segments of *A. aegypti* and *P. occidentalis* were taken to be 30% and 10% of the total tubule lengths, respectively.

*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 reveal an abundance of Ca-rich granules occupying a large proportion of the cytosol within principal cells of this region (Bradley *et al.*, 1990). Furthermore, low rates of basolateral $Ca^{2+}$ influx are apparent in proximal tubules at sites near to their insertion into the gut where the tubules cells appear transparent, comprising 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 are highest across distal tubules (where Ca-rich granules are accumulated) and are much lower in proximal tubules that contain fewer Ca-rich granules (Browne & O'Donnell, 2016). By contrast, the direction of $Ca^{2+}$ transport ($Ca^{2+}$ efflux) by distal tubules of *D. melanogaster* is 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*. Accordingly, although there are some similarities in the spatial patterns of tubule $Ca^{2+}$ transport in some species, it is apparent that there may also be concurrent fundamental differences in $Ca^{2+}$ sequestration within the tubules, even among closely related species.

By contrast, there are 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 (Maddrell *et al.*, 1991; Browne & O'Donnell, 2016, 2017). 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 obtained are unexpected given that the morphology of the tubules superficially resembles that of *A. domesticus*, where $Ca^{2+}$ transport 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, is reported to dramatically influence rates of tubule $Ca^{2+}$ transport by distal tubules of *D. melanogaster* (Browne & O'Donnell, 2016). It is not therefore not surprising that tubule $Ca^{2+}$ transport can vary considerably between 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 find 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 & Ruiz-Sanchez, 2015), there is no evidence of reabsorption of the divalent cation $Ca^{2+}$. There are several lines of evidence to suggest that $Ca^{2+}$ taken up by Malpighian tubules occurs specifically across principal cells. First, calcium channel blockers (nifedipine, verapamil and diltiazem) abolish basolateral $Ca^{2+}$ uptake by cAMP-stimulated midtubules of *A. domesticus*, an insect with tubules that lack secondary cells (Spring *et al.*, 2007; Browne & O'Donnell, 2017). Second, 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 fluorescence-labelled L-type calcium channel blocker (verapamil) (MacPherson *et al.*, 2001). Together, these results suggest that basolateral $Ca^{2+}$ entry occurs through calcium channels located within the principal cells of the Malpighian tubules accumulating opaque Ca-rich granules.

### *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 is reported to stimulate $Ca^{2+}$ influx, whereas thapsigargin results in a switch from $Ca^{2+}$ influx to efflux by the midtubule of *A. domesticus*. By contrast, neither cAMP, nor thapsigargin at the concentrations used in the previous study have 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. Furthermore, cAMP and thapsigargin have no effect on the tubules of *D. melanogaster*, *A. aegypti*, *T. molitor*, *T. ni*, *H. halys* and *P. occidentalis* compared with 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 or nitric oxide. 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 the Malpighian tubules of many insects from other orders.

### *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) are very brief, suggesting that the Malpighian tubules alone have the capacity to rapidly eliminate excess haemolymph $Ca^{2+}$ in these

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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 (approximately 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 approximately 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 that 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* are 1.2 h 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 that is sufficient to eliminate $Ca^{2+}$ from approximately 0.5 μL of haemolymph each day, for at least 12 days after a bloodmeal (Maddrell *et al.*, 1991). If the tubules of *R. prolixus* 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 approximately 15 μL). High rates of $Ca^{2+}$ uptake by the Malpighian tubules are apparently not required in this species despite the major role of tubules 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 *Brontocoris 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*.


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