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RESEARCH ARTICLE

The rectal complex and Malpighian tubules of the cabbage looper (*Trichoplusia ni*): regional variations in Na^+ and K^+ transport and cation reabsorption by secondary cells

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ABSTRACT

In larvae of most Lepidoptera the distal ends of the Malpighian tubules are closely applied to the rectal epithelia and are ensheathed within the perinephric membrane, thus forming the rectal complex. The cryptonephric Malpighian tubules within the rectal complex are bathed in fluid within a functional compartment, the perinephric space, which is separate from the haemolymph. In this study, the scanning ion-selective electrode technique (SIET) was used to measure transport of Na^+ and K^+ across the rectal complex and across multiple regions of the Malpighian tubules of larvae of the cabbage looper *Trichoplusia ni*. Measurements were made in an intact preparation in which connections of the tubules upstream to the rectal complex and downstream to the urinary bladder and gut remained intact. SIET measurements revealed reabsorption of Na^+ and K^+ across the intact rectal complex and into the bath (haemolymph), with K^+ fluxes approximately twice as large as those of Na^+. Analyses of fluxes in larvae with empty guts, found in recently moulted larvae, versus those with full guts highlighted differences in the rates of K^+ or Na^+ transport within tubule regions that appeared morphologically homogeneous, such as the rectal lead. The distal rectal lead of larvae with empty guts reabsorbed K^+, whereas the same region secreted K^+ in tubules of larvae with full guts. SIET measurements of the ileac plexus also indicated a novel role for secondary (type II) cells in cation reabsorption. Secondary cells reabsorb K^+, whereas the adjacent principal (type I) cells secrete K^+. Na^+ is reabsorbed by both principal and secondary cells, but the rate of reabsorption by the secondary cells is approximately twice the rate in the adjacent principal cells.

KEY WORDS: Lepidoptera, Principal cell, Larvae, K^+ flux, Na^+ flux, SIET

INTRODUCTION

In all Coleoptera and in larvae of most Lepidoptera, the excretory system has been modified to form a rectal complex in which the distal ends of the Malpighian tubules (MTs) are closely applied to the rectal epithelia and ensheathed within the perinephric membrane. The tubules within the rectal complex are thus termed cryptonephric Malpighian tubules (cMTs) and are bathed in fluid within a functional compartment, the perinephric space (PNS), which is separate from the haemolymph. Although the term 'rectal complex' is used for both Coleoptera and Lepidoptera, the latter evolved much later, contemporary with the evolution of flowering plants, and are more closely related to Hymenoptera and Diptera (Misof et al., 2014). The rectal complexes of Coleoptera and Lepidoptera are thus likely to represent examples of convergent evolution.

In the Coleoptera, generation of high osmotic pressures through transport of KCl into the lumen of the cMTs in the rectal complex of tenebrionid beetles is clearly an adaptation for water recovery from the contents of the rectal lumen (Grimstone et al., 1968; Ramsay, 1964) and for water vapour absorption (Machin, 1983; O'Donnell and Machin, 1988). Early studies of lepidopteran larvae suggested that the rectal complex was primarily a means for regulating haemolymph Na^+ and K^+ levels in response to high throughputs of these ions through the gut in actively feeding and growing larvae (Ramsay, 1976). Subsequent studies revealed that the rectal complex also serves to recover water from the faeces in the rectal lumen (Reynolds et al., 1985). Water recovered from the faeces is recycled to increase the water content of food in the gut.

Water recovery from the hindgut is necessary because K^+ and base equivalents are actively secreted at high rates into the midgut of larval Lepidoptera (Chamberlin, 1990; Moffett and Koch, 1992). The accompanying osmotic water flow increases the water content of the anterior midgut (Reynolds and Bellward, 1989) and raises the pH of the contents to 10 or above (Dow, 1992). The large volume of the gut lumen relative to the haemocoel and high rates of feeding have led to proposals for recycling, in which secretion of K^+, base equivalents and fluid into the midgut are matched by reabsorption of ions and fluid from the posterior gut (Moffett, 1994; Reynolds et al., 1985). The cycle is completed by the return of K^+, base equivalents and fluid to the haemolymph across the downstream free segments of the MTs (Moffett, 1994).

Larvae conserve water when fed drier food and lose more water in the faeces when fed moister food (Reynolds and Bellward, 1989). Water content of faecal pellets that enter the rectum decreases over time, whereas that of pellets within the ileum does not, indicating that the rectum is the major site of water reabsorption. The ultrastructure of the rectal epithelium is consistent with a role in active ion transport from rectal lumen into the PNS (Reynolds and Bellward, 1989). Identification of two aquaporins in the rectal epithelium and cMTs suggest that salt transport drives osmotic flow of water from the rectal lumen across the rectal epithelium and then into the lumen of the cMTs (Azuma et al., 2012).

Two hormones have been implicated in controlling the rate of fluid absorption by the lepidopteran rectal complex. *Manduca sexta* diuretic hormone (Mas-DH), which stimulates fluid secretion by adult MTs of *M. sexta*, acts as an antidiuretic on the rectal complex of the larvae because it increases fluid absorption from the rectum (Audsley et al., 1993). It is proposed that Mas-DH stimulates a bumetanide-sensitive $Na^+/K^+/2Cl^-$ co-transporter on the basal

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membrane of the cMTs. An antidiuretic factor purified from extracts of the larval brain–corpora cardiaca–corpora allata complex exerts an antidiuretic effect that is not inhibited by bumetanide. The increase in absorption in response to this antidiuretic factor is sensitive to Cl^- channel blockers or removal of Cl^- from the lumen side of the rectal complex, suggesting that the factor stimulates a Cl^- pump associated with the complex (Liao et al., 2000).

Previous studies of the lepidopteran rectal complex have assessed Na^+ and K^+ transport by collecting fluid from isolated MTs set up in the Ramsay assay (Irvine, 1969), or by collecting fluid from cuts at the proximal (white) MT or distally, where the rectal lead emerges from the rectal complex (Ramsay, 1976). Everted sac preparations have been used to measure transport of fluid, but not ions, across the rectal complex (Audsley et al., 1993). In this study, we have used the scanning ion-selective electrode technique to measure transport of Na^+ and K^+ across the rectal complex and across multiple regions of the MTs *in situ*, so that connections upstream to the rectal complex and downstream to the urinary bladder and gut remain intact. Our measurements reveal that there are important regional differences in the rates of transport within tubule regions with similar morphology, and that the rate and direction of transport may differ in tubules from larvae with empty versus full guts. Scanning ion-selective electrode technique (SIET) data also reveal differences in Na^+ and K^+ transport by the principal (type I or primary) cells and secondary (type II) cells in the ileac plexus MTs.

RESULTS

The regions of the rectal complex and MTs in the cabbage looper (*Trichoplusia ni*) are indicated in Fig. 1. The MTs within the rectal complex are enveloped by the perinephric membrane and are termed cryptonephridial Malpighian tubules (cMTs). They are contained within a compartment, termed the perinephric space (PNS), between the rectal epithelium and the perinephric membrane. A short rectal lead emerges from the rectal complex and extends ∼1 mm to the highly convoluted region of the tubule known as the ileac plexus. The total length of the ileac plexus in 4th instar larvae is ∼13 mm. The ileac plexus is divided into three regions on the basis of morphology and ion transport characteristics. Whereas the rectal lead is translucent and colourless and appears to be composed of a single cell type, there are two cell types in the ileac plexus. The distal ileac plexus, within ∼3 mm of the rectal lead, is distinguished by the relative abundance of type II (secondary or stellate) cells, which constitute approximately 25% of the cells and are discussed in detail below. Approximately 75% of the cells in the distal ileac plexus are principal (type I) cells, which are relatively opaque with yellow cytoplasm. The middle ileac plexus consists of a region ∼7 mm in length between the distal and proximal ileac plexus. Secondary cells are less common in this region, constituting ∼15% of the total. The proximal ileac plexus, ∼3 mm in length, is distinguished on the basis of its Na^+ transport characteristics, as described below. Approximately 10% of cells in this region are secondary cells. More anterior, near the junction of the midgut and the ileum, the tubule straightens out and is termed the yellow region. The yellow region extends anteriorly for ∼7 mm and is then reflected 180 deg and continues for another ∼7 mm as a second straight region, termed the 'white region' because of the uric acid crystals, which impart a milky white colour. The white regions of three of the six tubules on each side of the larvae join to form a short (∼500 µm) common ureter that empties into a urinary bladder, which, in turn, empties into the gut. The maximum dimension of the ellipsoidal bladder, which was usually filled with fluid, was ∼600 µm. On occasion, the bladders were seen to empty their contents in ∼10 s, but this emptying was infrequent and was not studied further.

SIET measurements are reported below in posterior to anterior order, first for the rectal complex and then for each successive downstream region of the MT, ending with the urinary bladder. All SIET recordings were made on MTs *in situ*, with intact connections between the rectal complex and rectal lead posteriorly and the white MT, urinary bladder and gut anteriorly. Measurements on *in situ* tubules, as opposed to those isolated for the Ramsay assay (Irvine, 1969), allow for transport of ions and water between the rectal lumen and the rectal complex and for transfer of fluid and ions from the tubules of the rectal complex to downstream segments of the tubule. Preliminary SIET measurements indicated that there were

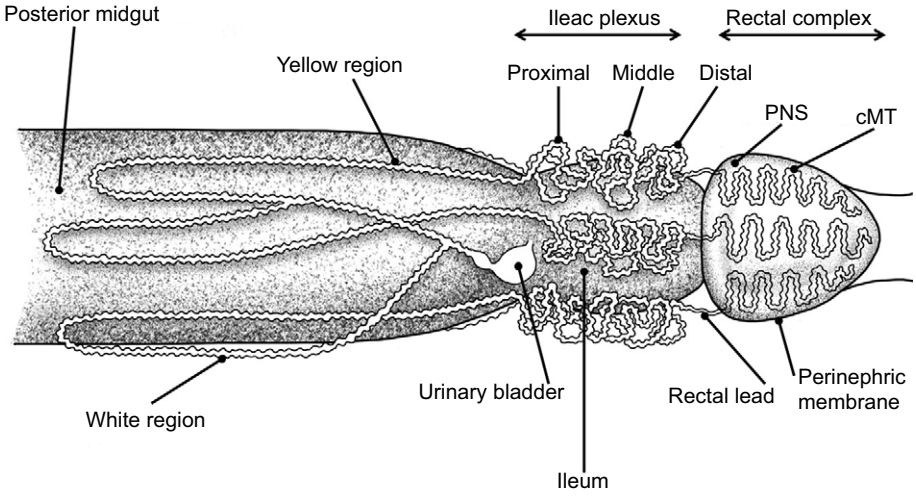

Fig. 1. Diagram showing the anatomical relations of the gut and Malpighian tubules in larvae of the cabbage looper, *Trichoplusia ni*. The cryptonephridial Malpighian tubule (cMT) is applied to the surface of the rectum and is enveloped by the perinephric membrane. The cMT is thus enclosed within the perinephric space (PNS). The cMTs, rectal epithelium (not shown) and perinephric membrane together form the rectal complex. The rectal lead connects the cMT to the ileac plexus, which consists of distal, middle and proximal regions. The straight region of tubule anterior to the ileac plexus and overlying the posterior midgut is the yellow region and the second straight region running posteriorly along the midgut is the white region. The white regions of three MTs on each side of the midgut join to form a common ureter which leads into the urinary bladder. Fluid collected in the urinary bladder is periodically discharged into the gut. Further details are described in the text.

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differences in Na^+ and K^+ transport in actively feeding larvae, in which the gut was full, versus recently moulted larvae, in which the gut contained clear fluid but not food. Differences in the ionic composition of the rectal contents might influence ion transport by the cMTs, and we therefore measured the concentrations of K^+ and Na^+ in samples of fluid expelled from the rectum. The K^+ concentration in droplets of rectal contents expelled from larvae with empty guts (97.5±3.3 mmol l^{-1}; N=14) was significantly higher (Student's t-test; P<0.001) than in droplets from larvae with full guts (38.1±5.3 mmol l^{-1}; N=10). By contrast, the Na^+ concentration in the same droplets of rectal contents expelled from larvae with empty guts (2.8±0.3 mmol l^{-1}) was significantly lower (P<0.001) than in the droplets from larvae with full guts (7.6±0.8 mmol l^{-1}). In the following sections, SIET measurements for each region are reported for larvae with empty and full guts.

SIET scans of the rectal complex at multiple sites separated by 40–50 µm revealed reabsorption (i.e. from the rectal complex into the haemolymph) of both Na^+ and K^+ (Fig. 2A,B and Fig. 3). Rates of K^+ reabsorption were almost double those of Na^+ (Fig. 3). For both ions, the rates of reabsorption in larvae with empty guts (Fig. 3A,C) were similar to those in larvae with full guts (Fig. 3B,D).

Preliminary SIET measurements indicated two functional regions within the rectal lead. Across the short distal rectal lead, within 125–200 µm of the rectal complex, there was a low rate of K^+ reabsorption and negligible net Na^+ transport in larvae with empty guts (Fig. 3B,D). By contrast, the distal rectal lead in larvae with full guts was a region of Na^+ and K^+ secretion (i.e. from haemolymph to MT lumen; Fig. 2C and Fig. 3A,C). The proximal rectal lead, within ~600 µm of the ileac plexus, was a region of K^+ secretion (Fig. 2D) and Na^+ reabsorption (Fig. 2E) in larvae with either empty or full guts (Fig. 3).

In Fig. 3, fluxes were measured by SIET only near the more numerous principal (primary or type I) cells of the ileac plexus; transport across secondary (type II) cells, known as stellate cells in MTs of dipterans, is described in a following section. K^+ was secreted at higher rates by the principal cells of the distal ileac

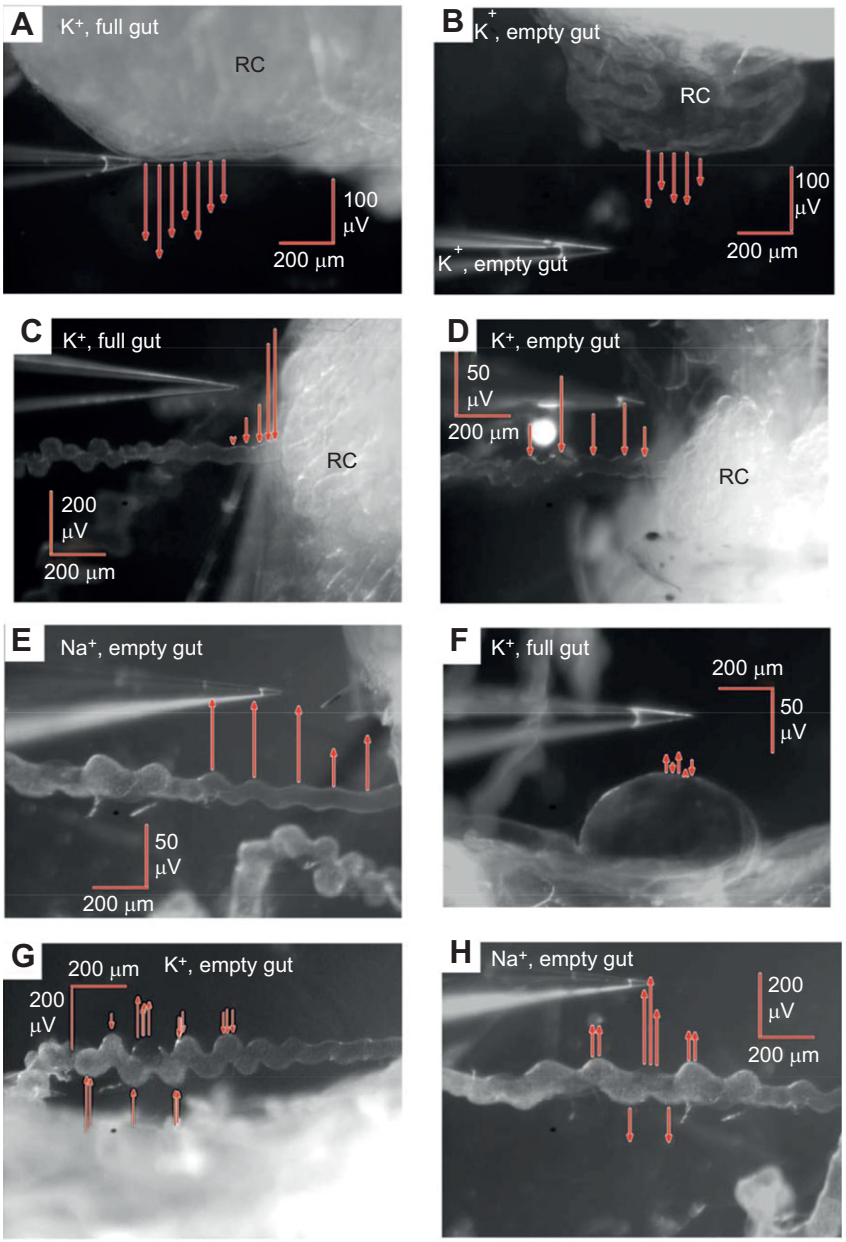

Fig. 2. Representative scans of the rectal complex and Malpighian tubules showing the voltages recorded by K^+-selective or Na^+-selective microelectrodes using the scanning ion-selective electrode technique. In each panel, the arrowhead indicates the direction of ion flux and the length of the arrow corresponds to the magnitude of the voltage gradient recorded over the 50 µm excursion distance, as described in the Materials and methods. K^+ fluxes were measured across the rectal complex (RC) of larvae with a full gut (A) and an empty gut (B). In B, the cryptonephridial tubules within the rectal complex are clearly visible. K^+ secretion across the distal rectal lead of a larva with a full gut (C) is highest close to the rectal complex. K^+ is secreted along the length of the proximal rectal lead (D) in larvae with empty guts, whereas Na^+ is reabsorbed across the same region (E). K^+ transport across the urinary bladder (F) of a larva with a full gut is negligible. K^+ is reabsorbed at the secondary cell (*) of the distal ileac plexus in a larva with an empty gut (G), whereas K^+ is secreted by the principal cells upstream and downstream. Na^+ is reabsorbed at both a secondary cell (*) and the principal cells of the distal ileac plexus in a larva with an empty gut (H).

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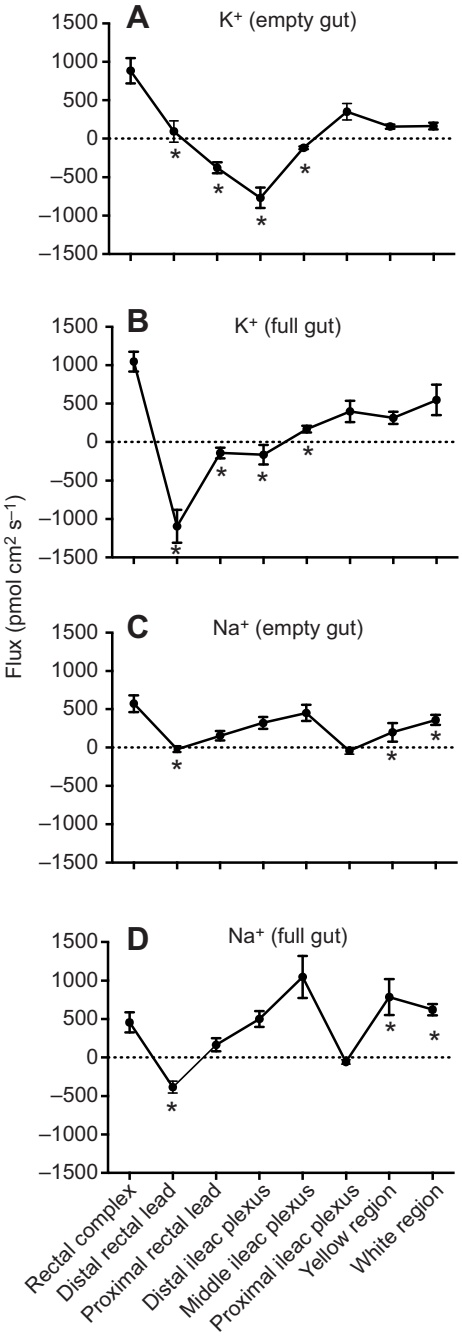

Fig. 3. K^+ and Na^+ fluxes across the rectal complex and Malpighian tubules. K^+ fluxes (A,B) and Na^+ fluxes (C,D) of larvae with empty guts (A,C) or full guts (B,D). Each point shows the mean±s.e.m. for 5–15 larvae. Positive values indicate ion reabsorption (from tubule lumen to haemolymph) and negative values indicate secretion (from haemolymph to tubule lumen). Significant differences (P<0.05; unpaired Student's t-test) between values measured in the same region in larvae with full versus empty guts are indicated with asterisks.

plexus of larvae with empty guts, relative to those with full guts (Fig. 3A,B). Na^+ was reabsorbed across the principal cells of the distal ileac plexus in larvae with empty or full guts (Fig. 3B,D).

K^+ was secreted by the middle ileac plexus of larvae with empty guts, whereas it was reabsorbed by the same region in larvae with full guts (Fig. 3A,B). Na^+ was reabsorbed by the middle ileac plexus of larvae with empty or full guts (Fig. 3C,D).

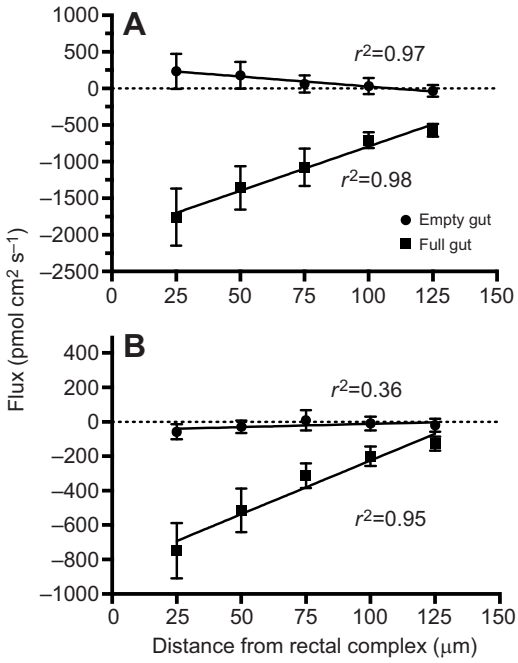

Fig. 4. K^+ and Na^+ fluxes across the distal rectal lead measured at 25 µm intervals from the rectal complex. The lines were fitted by linear regression and the r^2 values are noted. Each point is the mean±s.e.m. for 5 larvae.

The proximal ileac plexus was distinguished on the basis of a 1–2 mm region in which Na^+ was secreted at low rates, in larvae with empty or full guts (Fig. 3B,D). By contrast, K^+ was reabsorbed in the proximal ileac plexus of both groups of larvae (Fig. 3A,C).

Both Na^+ and K^+ were reabsorbed across the yellow and white regions in both groups of larvae (Fig. 3). Rates of K^+ reabsorption were similar in both yellow and white regions and in larvae with empty or full guts. The rate of Na^+ reabsorption was approximately four times higher in the yellow region in larvae with full guts relative to those with empty guts (Fig. 3C,D). The rate of Na^+ reabsorption was approximately 70% higher in the white region of the tubule in larvae with full guts relative to those with empty guts (Fig. 3C,D).

Rates of K^+ transport across the distended urinary bladders in larvae with empty guts (20±27 pmol cm^{-2} s^{-1}, N=6) and full guts (−28±20 pmol cm^{-2} s^{-1}, N=5) did not differ significantly from zero (Fig. 2F). Similarly, the rate of Na^+ transport across the distended urinary bladders in larvae with empty guts (2±8 pmol cm^{-2} s^{-1}, N=6) did not differ significantly from zero and the rate across larvae with full guts (−19±13 pmol cm^{-2} s^{-1}, N=5) was near zero.

The data presented in Fig. 3 for the distal rectal lead are the mean values of five measurements made at 25 µm intervals within 125 µm of the rectal complex. Linear regression analyses (Fig. 4) showed a strong correlation between K^+ flux and distance from the rectal complex for the distal rectal leads in larvae with empty or full guts. Rates of K^+ secretion were higher closest to the rectal complex in the distal rectal leads of larvae with full guts, whereas reabsorption of K^+ was higher close to the rectal complex in the distal rectal leads of larvae with empty guts. Na^+ secretion in larvae with full guts showed a similar trend to K^+ (Fig. 4). For larvae with empty guts, Na^+ fluxes in the distal rectal lead were small and not strongly influenced by proximity to the rectal complex (r^2=0.36)

SIET measurements also revealed dramatic differences in the directions and/or rates of K^+ and Na^+ transport by the principal (type I) versus secondary (type II) cells in the ileac plexus. Both K^+ and Na^+ are reabsorbed by the secondary cells. In larvae with empty

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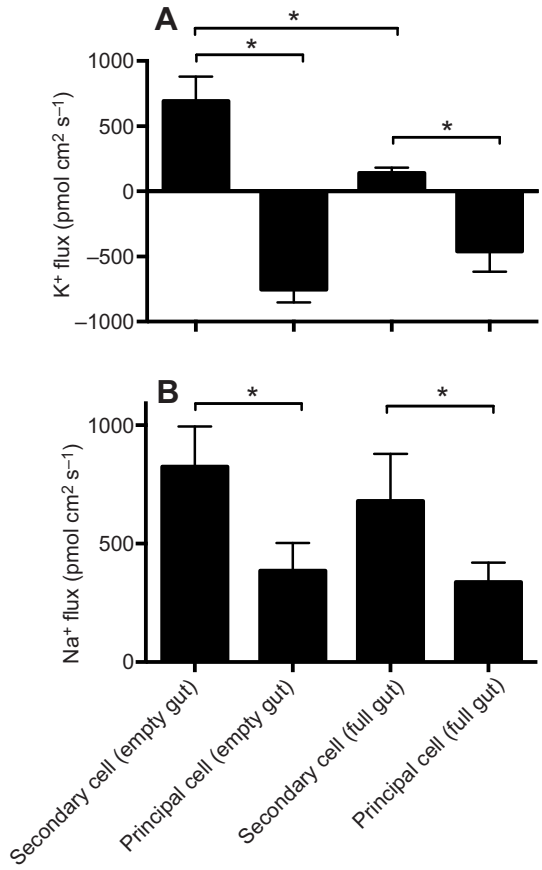

Fig. 5. K^+ and Na^+ fluxes at secondary cells and principal cells in larvae with empty or full guts. K^+ (A) and Na^+ (B) fluxes were measured in the distal ileac plexus across secondary cells and adjacent principal cells in the same tubule, as described in the Materials and methods. Each bar shows the mean+s.e.m. K^+ fluxes were measured in 20 tubules of 11 larvae with empty guts and 11 tubules from 4 larvae with full guts. Na^+ fluxes were measured in 7 tubules of 5 larvae with empty guts and 9 tubules from 6 larvae with full guts. Significant differences (P<0.05) between bars linked by square brackets are indicated by asterisks. Differences were determined using paired or unpaired Student's *t*-tests.

guts, K^+ reabsorption by the secondary cells is of approximately equal magnitude (~700 pmol cm^{-2} s^{-1}) to the secretion of K^+ by the adjacent primary cells upstream and downstream of each secondary cell (Fig. 2G and Fig. 5). K^+ was also reabsorbed at a lower rate (~140 pmol cm^{-2} s^{-1}) by secondary cells in the ileac plexus of larvae with full guts (Fig. 5). The rate of Na^+ reabsorption by the secondary cells is approximately twice the rate of Na^+ reabsorption by the adjacent primary cells (Fig. 2H and Fig. 5). Na^+ is reabsorbed at similar rates by secondary cells in the ileac plexus of larvae with empty or full guts (Fig. 5).

DISCUSSION

Our study is the first to use the scanning ion electrode technique to measure the transport of Na^+ and K^+ by the rectal complex and MTs of a lepidopteran larva. Rates of transport were measured with the rectal complex and tubules *in situ*, so that anterior and posterior connections between the tubules and the gut remain intact. Transport of Na^+ and K^+ in downstream regions of the tubules may thus be influenced by the input of fluid and ions upstream, from the rectal lumen, across the rectal epithelium and into the PNS and cryptonephridial tubules. Conversely, transport by the rectal complex may be influenced not only by input of fluids and ions from the rectal lumen, but also by the tidal flow of fluid (Ramsay, 1976) in which periodic changes in hydrostatic pressure force fluid from the lumen of the free tubules back into the cryptonephridial tubules, antidromic to the normal orthodromic flow of secreted fluid. Such tidal flows, brought about by contractions of the rectal musculature, were observed but not quantified by Ramsay in studies of the rectal complex of *Pieris brassicae* and *Manduca sexta* (Ramsay, 1976).

Our data provide novel insights into four aspects of the rectal complex and free MTs of *T. ni* larvae. To aid understanding of the regional variations of K^+ and Na^+ transport, schematic diagrams summarizing the results for larvae with empty and full guts are provided in Fig. 6.

Reabsorption of Na^+ and K^+ by the rectal complex

SIET measurements clearly show reabsorption of Na^+ and K^+ across the intact rectal complex and into the bath (haemolymph), with K^+ fluxes approximately twice as large as those of Na^+ (Fig. 6). Reabsorption of Na^+ and K^+ was seen both in larvae with full guts and those with empty guts. Our measurements of Na^+ and K^+ concentrations in the contents of the rectum confirmed that both ions are present in the fluids contained within the rectum, both in larvae with full guts and in larvae with empty guts. We suggest that the proximal source of ions for efflux from the rectal complex is the PNS and that Na^+ and K^+ from the rectal lumen are transported into the PNS across the rectal epithelium, which shows the apical and basal infoldings and high density of mitochondria characteristic of transporting epithelia (Reynolds and Bellward, 1989). Measurements of Na^+ and K^+ concentration in the perinephric fluid in *M. sexta* larvae in response to Na^+ or K^+ loading also lead to the conclusion that the rectal epithelium is capable of active Na^+ and K^+ transport (Ramsay, 1976). Previous studies have emphasized the role of the downstream segments of the MTs in K^+ reabsorption (Irvine, 1969; Moffett, 1994). Our SIET measurements suggest that potassium and sodium ions that are reabsorbed from the rectal lumen into the PNS may return to the haemolymph through one of two routes: (1) through the downstream segments of the MTs, or (2) directly across the perinephric membrane of the rectal complex.

Reabsorption of Na^+ and K^+ across the rectal complex of *T. ni* is in stark contrast to the generally accepted view of K^+ secretion in the tenebrionid rectal complex. The perinephric membranes of the tenebrionid rectal complex consist of as many as 40 thin cellular layers interrupted by blister-like cells known as leptophragmata, which are presumed to be the site of K^+ uptake from the haemolymph. There are no leptophragmata in the lepidopteran rectal complex, in which the perinephric membranes are formed of thin, flattened cells that are nearly devoid of cytoplasm (Azuma et al., 2012; Ramsay, 1976). Although the perinephric membrane is unlikely to be a site of active transepithlial ion transport (Ramsay, 1976), the concentrations of Na^+ and K^+ in the PNS are two to three times greater than those in the haemolymph (Ramsay, 1976), suggesting that passive diffusion may mediate the fluxes out of the rectal complex of lepidopteran larvae. Aquaporins are present in the rectal epithelium and the cryptonephric MTs but not in the perinephric membranes (Azuma et al., 2012). Taken together with the results of the present study, it appears that the Na^+ and K^+, but not water, can be recovered from the PNS by transfer across the perinephric membrane and into the haemolymph. In the tenebrionid rectal complex, double-barrelled ion-selective microelectrodes have been used to measure K^+ and Na^+ concentrations in the PNS and the electrical potential between the PNS and the bathing saline (Machin and O'Donnell, 1991; O'Donnell and Machin, 1991). Comparable

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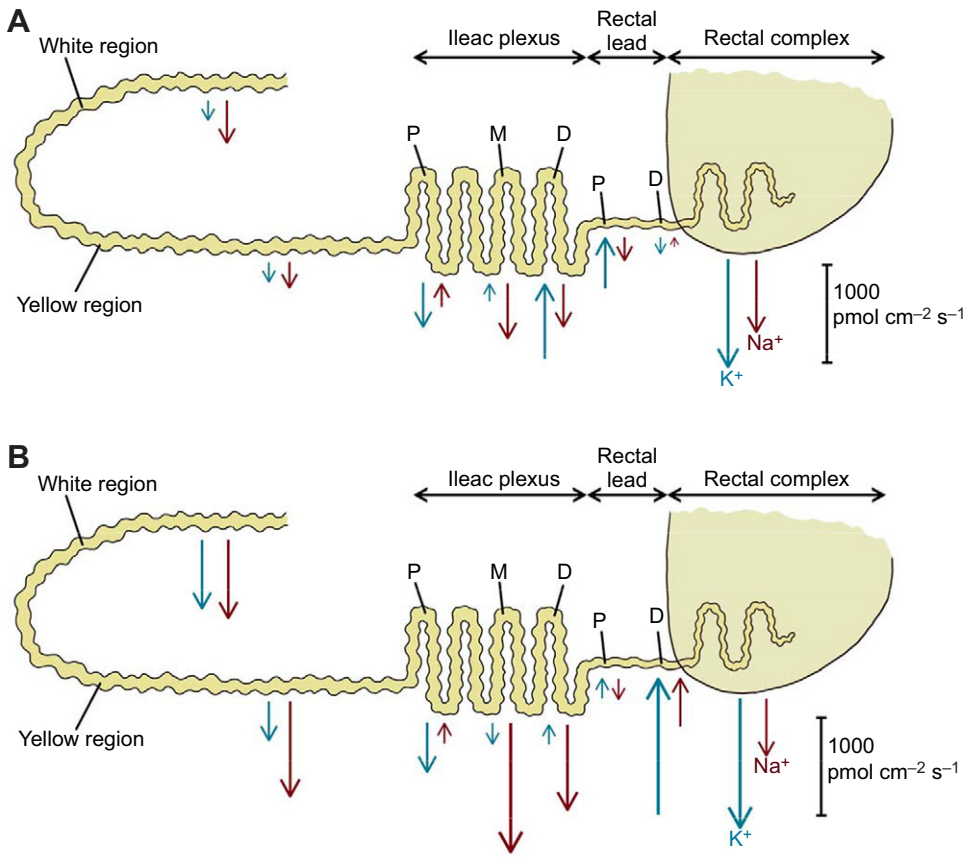

Fig. 6. Schematic diagrams summarizing regional variations in K^+ and Na^+ transport by the rectal complex and Malpighian tubules of *T. ni* larvae. K^+ transport (blue arrows) and Na^+ transport (red arrows) in larvae with (A) empty guts and (B) full guts. Arrows directed towards the tissue denote secretion and arrows directed outward denote reabsorption. The length of the arrows corresponds to the magnitude of ion flux at each site, using the data for mean fluxes from Fig. 3. P, proximal; M, middle; D, distal.

measurements in the rectal complex of *T. ni* and calculations of the electrochemical gradients between the PNS and the bathing saline will be of use in assessing the active or passive nature of K^+ and Na^+ transport across the PNS.

Differences in Na^+ and K^+ transport within regions of apparently uniform structure

Analyses of larvae with empty versus full guts highlighted the differences in the rates of K^+ or Na^+ transport within tubule regions that appeared morphologically homogeneous. Although the distal and proximal rectal leads are relatively straight and translucent and appear to comprise a single cell type, K^+ was reabsorbed in the distal rectal lead but secreted in the proximal rectal lead in larvae with empty guts, whereas Na^+ was secreted across the distal rectal lead and reabsorbed across the proximal rectal lead in larvae with full guts (Fig. 6). In larvae with full guts, there was a clear gradient of Na^+ and K^+ secretion along the length of the distal rectal lead, with maximal values closes to the rectal complex (Fig. 4). In addition, the rate of K^+ secretion in rectal leads of larvae with full guts was much higher in the distal relative to the proximal rectal lead. Previous studies of isolated tubules of larvae of *Calpodes ethlius* have shown that both the rate of fluid secretion by the rectal lead and the composition of the secreted fluid are affected by the potassium concentration or the potassium: sodium concentration ratio of the bathing medium (Irvine, 1969). Higher rates of Na^+ and K^+ secretion may contribute to higher rates of fluid secretion by the distal rectal lead, perhaps contributing to higher rates of fluid and ion recycling in the excretory system of feeding larvae.

Differences in the directions and/or rates of Na^+ or K^+ transport were also apparent within the ileac plexus. Although the principal cells of the middle ileac plexus reabsorb Na^+ at ~500 pmol cm^{-2} s^{-1} in larvae with empty guts and ~1000 pmol cm^{-2} s^{-1} in those with full guts, the principal cells of the proximal ileac plexus secrete Na^+ at 41 and 57 pmol cm^{-2} s^{-1} in larvae with empty and full guts, respectively (Fig. 3). By contrast, K^+ is secreted across the principal cells of the middle ileac plexus and reabsorbed across the principal cells of the proximal ileac plexus in tubules of larvae with empty guts.

Variations in ion transport within tubule regions that appear morphologically uniform have been seen previously in other species. The first recorded instance of a physiological discontinuity in a tubule epithelium with apparently uniform cell type was seen in the lower MT of *Rhodnius prolixus*, in which the 30% of the length closest to the junction with the gut is involved in KCl reabsorption (Maddrell, 1978). Higher rates of secretion of the organic cation tetraethylammonium are seen in the region of the lower tubule of *Drosophila melanogaster* that is closest to the ureter (Rheault and O'Donnell, 2004). The implication of these previous studies and of our data for *T. ni* tubules is that tubule regions that appear morphologically similar may nonetheless have qualitatively and quantitatively different transport functions.

Changes in direction or rate of Na^+ and K^+ transport in specific tubule regions of larvae with full versus empty guts

Our data also reveal bidirectional transport of K^+ in specific tubule regions in preparations of larvae with full or empty guts. The distal rectal lead of larvae with empty guts absorbs K^+, whereas the same region secretes K^+ in tubules of larvae with full guts (Figs 3 and 6). There was also a reversal of the direction of K^+ transport in the middle ileac plexus; in larvae with empty guts, this region secretes K^+, whereas in larvae with full guts, the same region reabsorbs K^+ (Figs 3 and 6). These differences may relate to differences in the

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concentrations of K^+ and Na^+ in the rectal lumen. For example, if K^+ is reabsorbed from the rectum by the cryptonephridial tubules, the higher concentration of K^+ (97.5 mmol l^{-1}) in the rectal contents of larvae with empty guts may provide a source of K^+ for reabsorption downstream in the rectal lead. However, it is also important to note that larvae with empty guts had recently completed moulting. Thus, the differences that we have seen in Na^+ and K^+ transport in specific tubule regions in larvae with empty versus full guts may relate not just to the ionic composition of the gut contents but to differences in the hormonal state of intermoult larvae relative to those recently moulted. In *Drosophila* tubules, for example, the moulting hormone 20-hydroxyecdysone is implicated in organization and fluid secretion (Gautam et al., 2014).

Na^+ and K^+ reabsorption by secondary cells

Our measurements reveal that secondary (type II) cells in the ileac plexus reabsorb K^+, whereas the adjacent primary (type I) cells secrete K^+. Na^+ is reabsorbed by both principal and secondary cells, but the rate of reabsorption by the secondary cells is approximately twice that of the principal cells.

Although reabsorption of Na^+ by secondary (type II or stellate) cells of the blowfly *Calliphora erythrocephala* was proposed by Berridge and Oschman (1969), there has been no direct evidence for Na^+ reabsorption by secondary cells before the results of the present study. Stellate cells in tubules of *Drosophila melanogaster* are the site of kinin-stimulated Cl^- secretion through Clc-a channels (Cabrero et al., 2014) and are an important site of water transport via aquaporins (*Drosophila* integral protein, DrIP) (Kaufmann et al., 2005). Immunohistochemical studies have provided evidence for multiple Na^+ and K^+ transporters in stellate cells of dipterans. The Na^+/K^+-ATPase is localized to basolateral membrane of stellate cells in the tubules of *Aedes aegypti* (Patrick et al., 2006) and its expression increases in larvae adapted to high salinity (Patrick and Gill, 2003). The inwardly rectifying K^+ channel AeKir1 is also found in the basolateral membrane of the stellate cells of *A. aegypti* tubules and mediates 46% of transepithelial K^+ secretion when haemolymph K^+ concentration is elevated to 34 mmol l^{-1} (Piermarini et al., 2015). In addition, antibodies against a sodium–proton exchanger (NHE2) label stellate cells in the *Drosophila* tubule (Day et al., 2008) and a member of the NHA exchanger family, NHA2, is found primarily in the apical membrane of stellate cells in *Aedes* tubules (Xiang et al., 2012). NHA2 may mediate transport of Na^+ and K^+ from cell to lumen. Alternatively, gap junctions may functionally couple stellate and principal cells (Beyenbach et al., 2010; Calkins et al., 2015; Piermarini and Calkins, 2014), thus allowing principal cells to secrete K^+ that enters stellate cells through AeKir1 (Piermarini et al., 2015). In contrast to our extensive knowledge of stellate cells in tubules of dipterans, little is known of secondary cell transporters and their functions in lepidopteran tubules. Given the predominance of principal cells in the ileac plexus of *T. ni* tubules, it would appear that the net function of this region is K^+ secretion and Na^+ reabsorption. Because Na^+ and K^+ are also reabsorbed in downstream segments such as the white and yellow tubules, cation reabsorption by the secondary cells at first glance seems superfluous. However, Na^+ and/or K^+ reabsorption by the secondary cells may be correlated with other processes unrelated to fluid secretion or haemolymph Na^+ and K^+ homeostasis. Secondary cells of *T. ni* are richly endowed with a multidrug-resistant protein (MRP) presumed to be implicated in excretion of toxins (Labbé et al., 2011). In excretory tissues of many animals, MRPs are implicated in excretion of potentially toxic organic anions, and multiple other organic anion transporters are also present (Wright and Dantzler, 2004). Small, hydrophilic organic anions are typically transported by Na^+-dependent transporters in MTs (Linton and O'Donnell, 2000; Ruiz-Sanchez and O'Donnell, 2006). If Na^+-dependent organic anion transporters are present in the secondary cells of *T. ni* tubules, then maintenance of a Na^+ gradient may require extrusion of Na^+ from the cell, perhaps via an Na^+/K^+-ATPase, as in dipterans. Similarly, K^+ leakage through channels may be important for maintenance of an inside negative membrane potential, perhaps to drive uptake of organic cations through conductive pathways (Rheault et al., 2005). It will be of interest in future studies to examine the effects of diuretic factors on K^+ and Na^+ fluxes across the secondary cells. A recent study used binding of a fluorescently tagged kinin to reveal the presence of kinin receptors on the basolateral membrane of the secondary cells in tubules of the larvae of the silk moth *Bombyx mori* and the tobacco hornworm *Manduca sexta* (Halberg et al., 2015).

Although mechanisms of K^+ reabsorption have been examined in the tubules of the haematophagous insect *Rhodnius prolixus* (Haley and O'Donnell, 1997), to our knowledge there are no previous studies of K^+ reabsorption by MTs of phytophagous species and no studies of Na^+ reabsorption by the tubules of any insect. The mechanisms of Na^+ and K^+ reabsorption, the influence of alterations in dietary water and ion content and the possible roles of diuretic and antidiuretic factors can be examined in future studies making use of the Ramsay assay, ion-selective microelectrodes and SIET. The secondary cells in tubules of 4th and 5th instar of *T. ni* are particularly amenable to further studies using SIET because of their large size. Previous studies have also highlighted the importance of the white region of MTs in acid–base balance (Moffett, 1994). Alkalinization of the midgut to pH 10 or above requires secretion of base equivalents into the midgut; current models propose that base equivalents are recovered by the ileum and possibly the rectum, and are returned to the middle and posterior midgut by the MTs, in particular the white region (Moffett, 1994). Future studies using pH-selective microelectrodes and SIET will be of use in defining the mechanisms of acid–base transfer across the tubules of lepidopteran larvae.

MATERIALS AND METHODS

Insects and dissection

A colony of *Trichoplusia ni* (Hübner 1800) (Lepidoptera, Noctuidae) was established from eggs supplied by the Great Lakes Forestry Centre (Sault St Marie, ON). Larvae were reared in groups of 5–10 in 22 ml cups containing synthetic diet (McMorran, 1965) which contained 59 mmol l^{-1} K^+ and 18 mmol l^{-1} Na^+. Larvae (4th instar) were dissected and analysed under physiological saline which was developed previously for studies of MTs of other Lepidoptera (*Manduca sexta* and *Pieris brassicae*; Maddrell and Gardiner, 1976) and contained (in mmol l^{-1}), NaCl 15, KC1 30, $CaCl_2$ 2, $MgCl_2$ 30, $KHCO_3$ 10, $KHPO_4$ 5, glucose 10, maltose 10, sodium citrate 5, glycine 10, alanine 10, proline 10, glutamine 10, valine 10, serine 5, histidine 5. Saline pH was adjusted to 7.2 with KOH. Individual larvae were placed under saline on a Sylgard-lined 5 cm Petri dish and were pinned through the head and one of the anal prolegs using 0.15-mm-diameter minutien pins. For SIET measurements of the rectal complex, the larva was positioned on its side and a lateral incision was made through the last two abdominal segments. The cuticle was reflected and secured with a 3rd minutien pin so that the dorsal surface of the rectal complex was oriented perpendicular to the bottom of the dish. For SIET measurements of the rectal lead and MTs *in situ*, the larvae was positioned dorsal side up and a mid-dorsal incision was made from the 3rd segment to the last segment. Transverse cuts were made underneath the gut anteriorly and posteriorly so as to separate a flat sheet of cuticle from the gut and MTs. Forceps (no. 5 Dumont) were used to separate the trachea from each spiracle so that the cuticle could be pulled anteriorly and removed. The gut and adherent MTs

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remained intact. For SIET analysis, the tracheal connections between the gut and the tubules were removed using forceps and glass probes. The probes were made from10 cm lengths of 3-mm-diameter coloured glass rods, which had been pulled over a flame to diameters of ~50 µm. The ends of the probes were fire polished. To avoid the influence of Na^+ and K^+ transport by the gut on the gradients in unstirred layer [Na^+] and [K^+] on the surface of the MTs, 3–6 mm lengths of tubule were pulled away from the gut and looped loosely around a minutien pin so that the long axis of the loop was at right angles to the long axis of the gut. Care was taken to avoid damage to the tubules and only tubules that were connected posteriorly to the rectal complex and anteriorly to the urinary bladder and midgut were used for SIET measurements.

Measurement of K^+ and Na^+ concentration in the rectal contents

The concentration of K^+ and Na^+ in samples expelled from the rectum of larvae held under paraffin oil were measured using ion-selective microelectrodes. Fabrication of Na^+-selective and K^+-selective microelectrodes for use under paraffin oil has been described previously (Naikkhwah and O'Donnell, 2011). Larvae were held under paraffin oil in a Sylgard-lined Petri dish and gentle pressure was applied with the index finger to compress the posterior region. With the aid of a dissecting microscope, a blunt glass probe was inserted to a depth of ~200 µm into the anus. Droplets of rectal contents (~1 µl) formed on the glass probe and were pulled away from the larva and deposited on the bottom of the dish. The droplets were then collected by pipette and transferred to another dish for measurement of K^+ and Na^+ concentrations. Na^+ and K^+ concentrations were measured under paraffin oil by positioning ion-selective and reference electrodes in the droplet and measuring the potential change relative to that in droplets of calibration solutions. K^+-selective and Na^+-selective microelectrodes were calibrated in NaCl–KCl mixtures in which the sum of both cation concentrations was 150 mmol l^{-1}. Ion concentrations in the samples were calculated from the equation:

$$[\mathrm{Ion}]_{\mathrm{sample}} = [\mathrm{Ion}]_{\mathrm{c}} 10^{(\Delta V/S)}, \quad (1)$$

where $[\mathrm{Ion}]_{\mathrm{sample}}$ is the ion concentration of the droplet of rectal contents, $[\mathrm{Ion}]_{\mathrm{c}}$ is the concentration in a calibration drop, ΔV is the change in potential (mV) between the sample and the same calibration drop and S is the slope (mV) for a tenfold change in ion concentration. All experiments were performed at room temperature, 23°C.

SIET measurement of K^+ and Na^+ fluxes

Net transport of ions across an epithelium produces small differences in ion concentration in the unstirred layer adjacent to the epithelium surface. SIET makes use of an ion-selective microelectrode that is moved between two positions within the unstirred layer; the measured difference in ion concentration between the two positions is converted into a corresponding ion flux across the tissue using the Fick equation. Hardware and software used for SIET have been described previously (Naikkhwah and O'Donnell, 2012). K^+-selective microelectrodes were backfilled with 150 mmol l^{-1} KCl and were tip-filled with K^+ ionophore I, cocktail B (Fluka Selectophore, Sigma-Aldrich, St Louis MO); selectivity of these electrodes for K^+ exceeds that for Na^+ by a factor of 7900. Na^+-selective microelectrodes were backfilled with a solution containing 100 mmol l^{-1} NaCl and 10 mmol l^{-1} MOPS buffer, pH 7.0, and were tip-filled with a cocktail based on Na^+ ionophore VI (Schueler et al., 2011) or Na^+ ionophore X (Jayakannan et al., 2011; Messerli et al., 2008); selectivity of these electrodes for Na^+ exceeds that for K^+ by factors of 100 and 200, respectively.

Each SIET measurement consisted of a series of computer-controlled movements of the ion-selective microelectrode. Measurements of K^+ and Na^+ concentration differences were made by moving the K^+-selective or Na^+-selective microelectrode tip perpendicularly from the MT or rectal complex surface between two points separated by 50 µm. Initially, the ion-selective microelectrode tip was positioned within 5 µm of the tissue surface, representing the inner limit of the 50 µm excursion of the microelectrode. Positioning was followed by a 4.0 s wait period, during which no measurements were made, to allow the re-establishment of ion gradients at the tissue surface following the localized disturbance of the microelectrode movement. A wait time of 3 s is sufficient to allow the gradients to fully re-establish (Naikkhwah and O'Donnell, 2012). The voltage at the microelectrode tip was then recorded during a sample period of 0.5 s following the wait period. The microelectrode was then moved 50 µm further out from the tissue at 200 µm s^{-1} and another wait and sample period was completed. The move, wait and sample cycle encompassing recordings at both extremes of the microelectrode excursion was completed in ~9.5 s and was repeated three times, requiring a total of ~28.5 s. The mean voltage gradient at each tissue site was calculated from the three replicates and each scan consisted of a measurement of the voltage gradient at five or more sites within the region of interest along the MT or rectal complex. Including the additional time for movement of the microelectrode between sites, scans were completed within ~5 min. Sites in the middle and proximal ileac plexus, yellow region and white region were separated by 100–200 µm. Sites in the distal rectal lead were separated by 25 µm and those within the proximal rectal lead and rectal complex by 50–100 µm. For comparison of fluxes across the principal and secondary cells of the distal ileac plexus, measurements were made typically at three sites separated by 10–25 µm on each secondary cell and also at four or more sites on four or more adjacent principal cells upstream and downstream of the secondary cell. To correct for any drift in microelectrode voltage, measurements were also made at a reference site well outside the unstirred layer, at a distance of 400–1000 µm from the tissue. Mean voltage differences at the reference site were subtracted from those at the measurement sites before ion concentration differences and fluxes were calculated. Typical voltage differences recorded by SIET at the measurement sites were 7-fold to 50-fold greater than voltage differences at reference sites of ±5 µV for K^+ and ±10 µV for Na^+.

Statistics

All data are reported as means±s.e.m. (N) where N is the number of tubules or rectal complexes. Graphing of data and statistical tests were done using Prism v6 (GraphPad Software, La Jolla, CA). Differences between the same tubule region or rectal complex in larvae with empty versus full guts were assessed using unpaired Student's t-tests. Significance of differences in fluxes between secondary cells and adjacent principal cells in the same tubule were determined using paired Student's t-tests. Significance of differences in fluxes between secondary cells or principal cells in larvae with empty guts versus the same cell type in larvae with full guts fluxes were determined using unpaired Student's t-tests.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.J.O. and E.R.-S. conceived the study, performed the experiments, analysed the data and wrote the manuscript.

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