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RESEARCH ARTICLE

Strategies of ionoregulation in the freshwater nymph of the mayfly *Hexagenia rigida*

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ABSTRACT

This study investigated ionoregulatory strategies used by freshwater (FW) nymphs of the mayfly *Hexagenia rigida*. Like other FW organisms, *H. rigida* nymphs maintain hemolymph ion levels (in mmol l^{-1}: Na^+ ~102; Cl^- ~84; K^+ ~6; pH ~7.35) far in excess of their surroundings. This appears to be accomplished by the combined actions of the alimentary canal, Malpighian tubules (MTs) and tracheal gills. The alimentary canal contributes in a region-specific manner, a view supported by: (1) spatial differences in the activity of basolateral Na^+/K^+-ATPase (NKA) and apical V-type H^+-ATPase (VA) and (2) region-specific Na^+ and K^+ flux rates. Both indicate a prominent role for the hindgut (rectum) in K^+ reabsorption. MTs also exhibit region-specific differences in Na^+ and K^+ flux rates that are coupled with an organized but tortuous architecture. NKA and VA activities were highest in MTs versus all other organs examined. Tracheal gills were found to be sites of Na^+ uptake, but no difference in Na^+ uptake was found between gills taken from different regions of the abdomen or spatially along individual gills. This is likely because each gill exhibited a dense population of NKA and/or VA immunoreactive cells (putative ionocytes). Data provide new insight into how FW mayfly nymphs regulate salt and water balance using the alimentary canal, MTs and tracheal gills as well as the first direct evidence that tracheal gills acquire ions from FW.

KEY WORDS: Na^+/K^+-ATPase, V-type H^+-ATPase, Rectum, Gill, Gastrointestinal tract, Malpighian tubule

INTRODUCTION

Mayflies are considered the oldest winged insects, dating back to the Carboniferous and Permian times (Brittain, 1982). Mayfly nymphs are prominent in freshwater (FW) ecosystems worldwide, and because of their prevalence in unpolluted water as well as their importance in aquatic food webs, they have become popular indicators of ecosystem health (Brittain, 1982; Clifford, 1982). Typically, the aquatic nymph stage of a mayfly dominates the lifespan, lasting up to two and a half years before they emerge as short-lived sexually mature adults (Brittain, 1982; Clifford, 1982). This prolonged aquatic stage exposes nymphs to both short- and long-term cyclic changes in environmental parameters such as temperature, pH, dissolved oxygen and water ion content, all of which present challenges to the regulation of salt and water balance. Despite this, the ionoregulatory strategies used by mayfly nymphs are not clearly defined.

The early developmental stages of insect species that reside in FW must regulate obligatory ion loss and tissue hydration in order to maintain ionoregulatory homeostasis. Salt and water balance is maintained by the integrated function of ionoregulatory organs, many of which are associated with the alimentary canal (Dow, 1986; O'Donnell, 2008). The alimentary canal possesses functionally distinct regions, which, anterior to posterior, include the foregut, midgut (containing the gastric caeca and further subdivided regions) and hindgut, the latter of which is subdivided into the ileum and rectum (Dow and Harvey, 1988; Zhuang et al., 1998; Shanbhag and Tripathi, 2005, 2009). The midgut is an important site for mineral, water and nutrient absorption (Dow, 1986; Linser et al., 2009). However, Malpighian tubules (MTs; gut diverticula located at the midgut–hindgut junction) produce an iso-osmotic fluid (primary urine) that passes into the hindgut (Beyenbach et al., 2010), and following this, the rectum selectively reabsorbs ions, water and nutrients from feces and MT-derived fluid (Phillips, 1981; Jonusaite et al., 2013).

In addition to the alimentary canal, mayfly nymphs also possess an elaborate cuticular extension most commonly referred to as tracheal gills. Tracheal gills run along both sides of the lower abdomen and differ morphologically between species (Wingfield, 1939). Although the ventilatory and respiratory function of tracheal gills can vary from species to species (Wingfield, 1939), these structures all appear to possess cells that exhibit the ultrastructural characteristics of ionocytes (Wichard and Komnick, 1971). Therefore, putative tracheal gill ionocytes were called ephemerid chloride cells (CCs) because they resembled the CCs (ionocytes) of the teleost fish gill epithelium (Wichard and Komnick, 1971). Histochemical observations of sodium and chloride precipitation in association with ephemerid CCs as well as alterations in ephemerid CC number in association with changes in water ion content suggest that ephemerid CCs contribute to salt absorption across the body surface of FW mayfly nymphs (Wichard and Komnick, 1971; Wichard et al., 1973). If this is the case, the tracheal gill is an ionoregulatory organ that may contribute significantly to the regulation of salt and water balance in FW mayfly nymphs. But direct evidence to support this contention is currently lacking.

The objective of this study was to define the ionoregulatory strategies of a FW-residing mayfly nymph, and provide a foundation upon which a more complete mechanistic understanding of FW mayfly nymph ionoregulation can be built. To achieve this, an integrative approach was taken using nymphs of the mayfly *Hexagenia rigida* McDunnough 1924. Saouter et al. (1991) have previously described some of the biological and ecological characteristics of *H. rigida* nymphs that make them useful models for understanding FW mayfly nymph physiology, including a burrowing lifestyle of 10–24 months in the upper layers of FW lentic sediments as well as a high tolerance of modifications in

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abiotic factors. Therefore, the present study examined organ-specific activity and immunolocalization of the ion transport enzymes Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA), as well as organ- and region-specific ion flux rates of Na^+ and/or K^+ using the scanning ion-selective electrode technique (SIET). Of particular importance was to directly examine whether tracheal gills of a FW mayfly nymph acquire ions from surrounding water, and in this regard act as an ionoregulatory organ as was suggested almost 50 years ago (Wichard and Komnick, 1971).

MATERIALS AND METHODS

Animals

Nymphs of the mayfly *H. rigida* (16–35 mm in length) were obtained from the culture maintained by the Ontario Ministry of the Environment and Climate Change (MOECC, Etobicoke, ON, Canada). MOECC culture conditions for *Hexagenia* spp. are defined by standard operating procedures (MOECC, 2016). Nymphs were maintained in the Department of Biology at York University in aquaria containing a 3:1 ratio of room temperature (21°C) aerated dechlorinated municipal tap water (approximate composition in μmol l^{-1}: $[Na^+]$ 590; $[Cl^-]$ 920; $[Ca^{2+}]$ 760; $[K^+]$ 43; pH 7.35) and uncontaminated field-collected control sediment (8 cm depth; also supplied by the MOECC; chemistry available upon request). Aquaria were exposed to a 12 h:12 h light:dark photoperiod. Nymphs were fed twice a week by adding a 10 ml aliquot of food to the tank that contained Cereal Grass Media (Ward's Science, Rochester, NY, USA; 37.5 mg ml^{-1} dechlorinated water) and ground TetraFin Goldfish Flake Food (Tetra Holdings US, Blacksburg, VA, USA; 25 mg ml^{-1} dechlorinated water). All studies were performed on larvae of similar size (25 mm in length).

Hemolymph Cl^-, Na^+ and K^+ activities and pH

To collect hemolymph, a nymph was placed on tissue paper (to absorb surface moisture), and then the articular membrane at the base of a leg was punctured with fine forceps. This resulted in the formation of a pool of hemolymph from which a sample was collected using a micropipette. Hemolymph (0.5–1.0 μl) was either transferred to an oil dish for immediate analysis (i.e. Na^+, K^+ and H^+) or stored at −80°C until further analysis (2 μl for Cl^- assay). Hemolymph $[Cl^-]$ was measured using a colorimetric technique (Zall et al., 1956). Ion-selective microelectrodes (ISMEs) were used to measure K^+, Na^+ and H^+ composition of the collected hemolymph droplets as previously described (Jonusaite et al., 2011). The following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses) were used: K^+ ionophore I cocktail B (100 mmol l^{-1} KCl), Na^+ ionophore II cocktail A (100 mmol l^{-1} NaCl) and H^+ ionophore I cocktail B (100 mmol l^{-1} NaCl/100 mmol l^{-1} sodium citrate, pH 6.0). The ISMEs were calibrated in the following solutions: K^+, 1.5 mmol l^{-1} KCl/298.5 mmol l^{-1} NaCl and 15 mmol l^{-1} KCl/285 mmol l^{-1} NaCl; Na^+, 30 mmol l^{-1} NaCl/270 mmol l^{-1} LiCl and 300 mmol l^{-1} NaCl; H^+, 150 mmol l^{-1} NaCl containing 1 mmol l^{-1} Hepes at pH 7.5 and 8.5. The ISME slopes (mV) for a 10-fold change in ion concentration were (means±s.e.m.): 53.00±0.87 (N=12) for K^+ and 59.78±0.61 (N=12) for Na^+. To measure pH, standard buffer solutions of pH 7 and 10 were used where the slope of the ISME was 151.74±4.37 (N=12). Measurements were recorded with an ML165 pH Amp connected to a PowerLab 4/30 (ADInstruments, Colorado Springs, CO, USA) data acquisition system and the voltage recordings were analyzed using LabChart 6 Pro software (ADInstruments). Hemolymph ion activities were calculated with the following equation as described by Donini et al. (2007):

$$a_h = a_c \times 10^{\Delta V/S}, \quad (1)$$

where a_h is the hemolymph ion activity, a_c is the ion activity in one of the calibration solutions, ΔV is the difference in voltage between the hemolymph and the calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity. For pH, Eqn 1 was modified substituting 1000 for 10 and utilizing the slope over a 1000-fold change in ion activity.

Organ-specific NKA and VA activity

The activity of NKA and VA were measured in the anterior midgut (AMG), posterior midgut (PMG), MTs, hindgut (HG) and tracheal gills. The tracheal gills were removed by cutting them at the proximal end, where they attach to the abdomen. To isolate different organs of the alimentary canal, the entire alimentary canal was removed from the animal and discrete structural cues were used to distinguish where one region transitioned into another. Tissue was then cut at these regions and the entire organ (i.e. AMG, PMG, MT or HG) was isolated for further analysis. Upon collection, dissected organs were placed in collection tubes and immediately frozen in liquid nitrogen. Thereafter, tissue was stored at −80°C until use. Preparation of tissue for enzyme analysis and determination of NKA and VA activity was undertaken according to methods originally described by McCormick (1993) and modified by Jonusaite et al. (2011). For inhibition of mayfly nymph NKA and VA activity, 5 mmol l^{-1} of ouabain and 1 μmol l^{-1} of bafilomycin were used, respectively. These concentrations were determined to be optimal after examining various concentrations of the enzyme inhibitors.

Immunohistochemical localization of NKA and VA

The upper body and lower abdomen of mayfly nymphs were fixed in Bouin's solution (3 h, room temperature). Fixed tissues were further processed, embedded and sectioned (4–5 μm thick) according to Chasiotis and Kelly (2008). Fixed tissue was sectioned on the sagittal plane. Immunohistochemical localization of NKA was carried out using a mouse monoclonal antibody raised against the α-subunit of avian NKA (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) at 1:10 dilution, as described in detail by Jonusaite et al. (2013). The specific localization of VA was achieved using a guinea pig polyclonal serum antibody raised against the V_1 complex of the VA of *Manduca sexta* (1:1000 in ADB; kind donation from Dr Weiczorek, University of Osnabruk, Germany). To visualize NKA and VA, tetramethylrhodamineisothiocyanate-labeled goat anti-mouse secondary antibody (1:400 in ADB; Jackson ImmunoResearch Laboratories) and AlexaFluor 488-conjugated goat anti-guinea pig secondary antibody (1:400 in ADB; Jackson ImmunoResearch Laboratories) were used, respectively. Tissues were rinsed again in PBS and mounted in ProLong Gold Antifade reagent (Invitrogen Canada, Burlington, ON, Canada). Images were captured using an Olympus IX81 inverted microscope (Olympus Canada, Richmond Hill, ON, Canada) equipped with an X-CITE 120XL fluorescent Illuminator (X-CITE, Mississauga, ON, Canada). All images were assembled using Adobe Photoshop CS2 software (Adobe Systems Canada, Toronto, ON, Canada).

Organ-specific Na^+ and K^+ flux rates along the alimentary canal

SIET measurement of K^+ and Na^+ voltage gradients was used to determine organ-specific Na^+ and K^+ flux rates along the alimentary

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canal. The SIET system and protocol utilized in this study is described in detail elsewhere (Donini and O'Donnell, 2005). The entire alimentary canal with head attached was isolated in physiological saline (composition in mmol l^{-1}: 5 KCl, 74 NaCl, 1 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 8.6 Hepes, 20 glucose, 10 glutamine, pH 7.0; adapted from Leonard et al., 2009). K^+ voltage gradients were measured in the following discrete regions of the alimentary canal – AMG, PMG, MTs and rectum – using morphological criteria as described above and methods outlined by Jonusaite et al. (2013). In the case of the MTs, a further spatial examination of voltage gradients was examined in areas defined as the trunk and coiled region. The K^+ ISME slope (mV) for a 10-fold change in ion concentration was 56.3±0.40 (mean±s.e.m., n=37) when calibrated in 1 and 10 mmol l^{-1} solutions of KCl and *N*-methyl-D-glucamine (136.3 mmol l^{-1} and 127.3 mmol l^{-1}, respectively). Na^+ voltage gradients were recorded in modified saline (composition in mmol l^{-1}: 73 *N*-methyl-D-glucamine, 5 KCl, 1 NaCl, 1 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 8.6 Hepes, 20 glucose, 10 glutamine, pH 7.0) calibrated in 5 and 50 mmol^{-1} solutions of NaCl and *N*-methyl-D-glucamine (132.3 mmol l^{-1} for the 5 mmol l^{-1} Na^+ solution and 87.3 mmol l^{-1} for the 50 mmol l^{-1} Na^+ solution). The Na^+ ISME slope (mV) for a 10-fold change in ion concentration was 58.9±0.68 (mean±s.e.m., N=41) when calibrated in 5 and 50 mmol l^{-1} solutions of NaCl and *N*-methyl-D-glucamine.

Na^+ flux across the tracheal gill

SIET measurement of Na^+ voltage gradients as described above (see Materials and methods, Organ-specific Na^+ and K^+ flux rates along the alimentary canal) were used to determine Na^+ flux rates across tracheal gills isolated from different regions of the abdomen (i.e. anterior, middle and posterior) as well as spatially along the central axis of individual tracheal gills. Gills were isolated from nymphs by cutting them at the base where they attached to the abdomen. The cut region was then anchored in a drop of petroleum jelly (Hargell Inc., Toronto, ON, Canada) that was held in a Petri dish filled with dechlorinated water.

Calculating Na^+ and K^+ flux rates from SIET-measured voltage gradients

Voltage gradient readings captured using ASET 2.0 software were converted to concentration gradients using the following equation:

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

where ΔC is the concentration gradient between the two points measured in µmol l^{-1} cm^{-3}; C_B is the background ion concentration, calculated as the average of the concentration at each point measured in µmol l^{-1}; ΔV is the voltage gradient obtained from ASET in µV; and S is the slope of the electrode. After obtaining the concentration gradients, a flux value for the corresponding gradients was derived using Fick's law of diffusion:

$$J_I = D_I(\Delta C)/\Delta x, \quad (3)$$

where J_I is the net flux of the ion in pmol cm^{-2} s^{-1}; D_I is the diffusion coefficient of the ion (1.92×10^{-5} cm^2 s^{-1} for K^+, 1.55×10^{-5} cm^2 s^{-1} for Na^+); ΔC is the concentration gradient in pmol cm^{-3}; and Δx is the distance between the two points measured in cm.

Statistics

All data are expressed as means±s.e.m. (n), where n is the number of biological replicates. A one-way ANOVA followed by a Tukey's or Dunn's comparison test (on log-transformed values in the case of gill data) was used to statistically compare biological replicates, and data were considered to be significantly different when P<0.05. All statistical analyses were conducted using SigmaStat 3.5 software (Systat Software, San Jose, CA, USA).

RESULTS

Mayfly nymph hemolymph ion composition and pH

The major inorganic ions in the hemolymph of *H. rigida* nymphs residing in FW were Na^+ and Cl^-, with mean values of 101.50±2.53 mmol l^{-1} (n=24) and 83.83±2.58 mmol l^{-1} (n=22), respectively. Potassium levels were comparatively lower, ranging from 3.81 to 10.27 mmol l^{-1} (mean=5.95±0.41 mmol l^{-1}, n=23). Hemolymph pH ranged from 6.31 to 8.03, with a mean value of 7.35±0.08 (n=24).

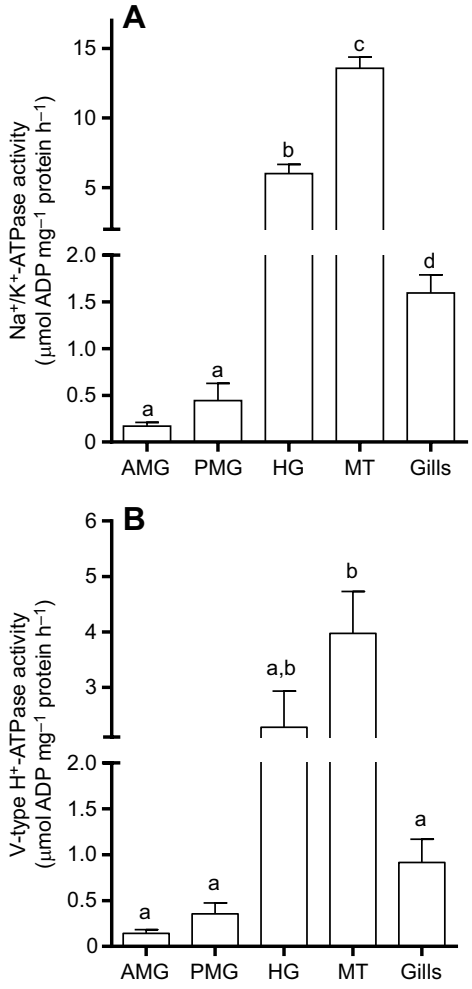

Fig. 1. Organ-specific ion transport enzyme activity in freshwater-residing mayfly (*Hexagenia rigida*) nymphs. The activity of (A) Na^+/K^+-ATPase (NKA) and (B) V-type H^+-ATPase (VA) along the alimentary canal and in the tracheal gills of mayfly nymphs. All data are expressed as means±s.e.m. (n=3–5). Different lowercase letters denote a significant difference (P<0.05) between alimentary canal regions and tracheal gills as determined by a one-way ANOVA followed by a multiple comparison test. Discrete regions of the alimentary canal (from anterior to posterior) are the anterior midgut (AMG), posterior midgut (PMG), hindgut (HG) and Malpighian tubules (MTs).

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NKA and VA activity along the alimentary canal and in tracheal gills of the mayfly nymph

The alimentary canal of *H. rigida* nymphs can be divided into discrete regions (i.e. AMG, PMG, HG and MTs) based on clear morphological distinctions. The activity of NKA and VA in the AMG and PMG were quantitatively similar at 0.17 and 0.45 µmol ADP mg^{-1} protein h^{-1} for NKA and 0.14 and 0.21 µmol ADP mg^{-1} protein h^{-1} for VA, respectively (Fig. 1A, B). NKA activity was significantly higher in the HG versus midgut regions of mayfly nymphs (Fig. 1A). A similar trend in HG versus midgut VA activity was also observed, but this was not found to be significantly different (Fig. 1B). The MTs exhibited higher NKA (13.57 µmol ADP mg^{-1} protein h^{-1}) and VA activity (3.96 µmol ADP mg^{-1} protein h^{-1}) than any other region of the alimentary canal (Fig. 1A,B). Tracheal gill NKA activity was ~1.60 µmol ADP mg^{-1} protein h^{-1} and VA activity was ~0.92 µmol ADP mg^{-1} protein h^{-1} (Fig. 1A,B).

Immunolocalization of NKA/VA and ion flux rates along the alimentary canal of the mayfly nymph

Immunohistochemical localization of NKA and VA in discrete regions of the alimentary canal revealed staining of VA on the apical and NKA on the basolateral side of epithelia irrespective of location (Fig. 2). What differed was the intensity of immunofluorescence, which was comparatively modest in the AMG (Fig. 2B) and PMG (Fig. 2C), but prominent in the rectal region of the hindgut (Fig. 2D).

SIET measurements adjacent to the hemolymph-facing surface of the AMG detected Na^+ flux (secretion, from the bathing medium to the gut lumen) of approximately –313 pmol cm^{-2} s^{-1} (Fig. 3A,D) and an average K^+ flux (absorption, from the gut lumen to the bathing medium) of 190 pmol cm^{-2} s^{-1} (Fig. 3A,E). A high rate of Na^+ secretion was observed across the PMG, averaging around –866 pmol cm^{-2} s^{-1} (Fig. 3B,D), and this was coupled with an increase in K^+ absorption, measuring around 282 pmol cm^{-2} s^{-1} (Fig. 3B,E). Comparatively high levels of K^+ absorption were also observed across the rectum (264 pmol cm^{-2} s^{-1}, Fig. 3C,E), but Na^+ flux rates across the rectum were lower than those observed across the PMG at –448.26 pmol cm^{-2} s^{-1} (Fig. 3C,D).

Morphology, immunolocalization of NKA/VA and ion flux rates of the mayfly nymph MTs

In *H. rigida* nymphs, MTs exhibit an organized but tortuous morphology comprising structurally distinct regions (Fig. 4A–D). Each MT originates as one of five trunks (Fig. 4A) that protrude from the junction between the PMG and HG. The MT trunks branch into thin conducting tubes, which lead to proximal coiled regions

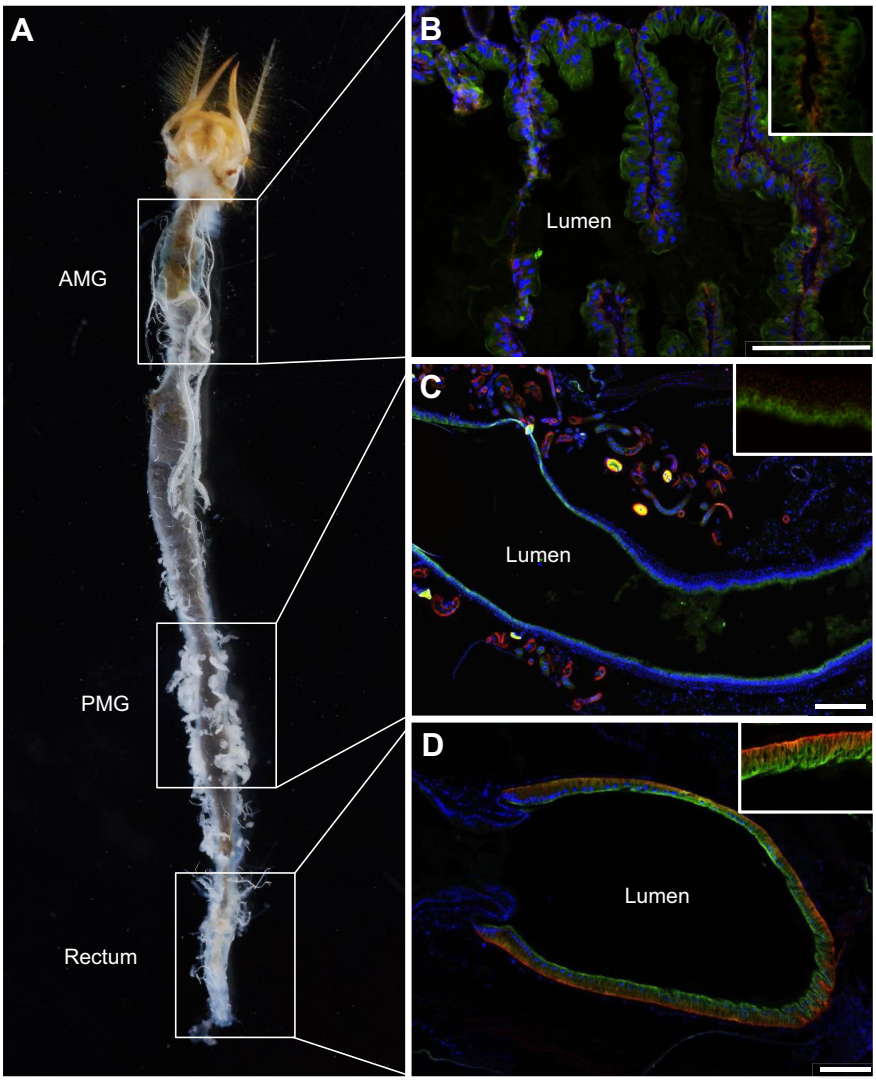

Fig. 2. Immunolocalization of Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA) in different regions of the alimentary canal of freshwater-residing mayfly (*Hexagenia rigida*) nymphs. (A) The entire alimentary canal (still attached to the head), used as a frame of reference to show which regions (white boxes) were examined for NKA (red) and VA (green) immunoreactivity (ir). (B) Anterior midgut (AMG). (C) Posterior midgut (PMG). (D) Rectum. In B–D, insets are included showing NKA- and VA-ir to illustrate comparative intensities of ir between regions. All regions were found to exhibit VA-ir on the apical and NKA-ir on the basolateral regions of epithelia. In C, little to no immunostaining of either ATPase is seen in the PMG, while in D the rectum is seen to possess high levels of apical VA and basal NKA. Nuclei are stained with DAPI (blue). Scale bars, 100 µm.

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comprising coiled blind-ended finger-like projections (Fig. 4A–D). Immunohistochemical examination of the MTs in cross-section revealed basolateral NKA and apical VA immunoreactivity (Fig. 4E).

The MTs of larval *H. rigida* were found to exhibit region-specific rates of Na^+ and K^+ transport (Table 1). The distally protruding coiled region (finger-like extension) of MTs typically exhibited K^+ flux rates that averaged ∼−28 pmol cm^{-2} s^{-1} (i.e. K^+ secretion). However, various sites along these two regions also sporadically demonstrated K^+ absorption at rates that averaged ∼182.5 pmol cm^{-2} s^{-1}. In contrast, the trunk connecting the protruding regions of the MT to the gut was always found to be a site of K^+ absorption, with average rates of ∼123 pmol cm^{-2} s^{-1}. The transport of Na^+ was always found to be secretory across the MT regions measured in this study, and ranged from ∼−241 to −358 pmol cm^{-2} s^{-1} (Table 1).

Immunolocalization of NKA/VA and Na^+ transport across mayfly nymph tracheal gills

Immunofluorescence microscopy showed pronounced immunostaining of both NKA and VA in the gill epithelia (Fig. 5A–C), and a pattern of staining that is indicative of cell-specific enrichment of these enzymes (Fig. 5C). More specifically, some regions of the gill epithelium did not exhibit staining for either enzyme, while some cells had both ATPases and others appeared to express only one of the enzymes (Fig. 5C). In the latter case, cells that exhibited VA staining only were particularly obvious (Fig. 5C).

Na^+ flux across the tracheal gill of mayfly nymphs indicated uptake (i.e. Na^+ absorption) from FW to the hemolymph (Fig. 5D–F). Na^+ flux rates averaged ∼60 pmol cm^{-2} s^{-1}. There was no significant difference in the magnitude of Na^+ transport across gill filaments isolated from the anterior, middle or posterior abdomen (Fig. 5D). In addition, there was no significant difference observed in Na^+ flux along the length of a single gill filament (proximal to distal, Fig. 5E), although an increasing trend was observed towards the distal portion of the gill filament (Fig. 5F).

DISCUSSION

Overview

This study provides a detailed look at ionoregulatory strategies used by the nymph of a FW mayfly. This is significant because mayfly nymphs play an important role in FW ecology worldwide, yet their ability to maintain salt and water balance in hyposmotic surroundings has yet to be defined. Mayfly nymphs, like those of stoneflies, caddisflies and damselflies, possess tracheal gills, which were first suggested to serve a respiratory function (Thorpe, 1933). Later, the idea that the tracheal gill may also be an ionoregulatory structure responsible for ion absorption from FW was proposed by Wichard and Komnick (1971). In the present study, we provide the first direct evidence to support the contention that tracheal gills acquire ions directly from surroundings by showing that tracheal gills of FW *H. rigida* nymphs absorb Na^+ from FW. To what degree the tracheal gill contributes to ion acquisition and ionoregulatory homeostasis in FW mayfly nymphs will require further study. However, observations made in the present study introduce the idea that Na^+ acquisition across the tracheal gill may be, at least in part, balancing Na^+ 'losses' across discrete regions of the alimentary canal and select regions of the MTs. Otherwise it would seem counterintuitive for a FW organism to allow the secretion of Na^+.

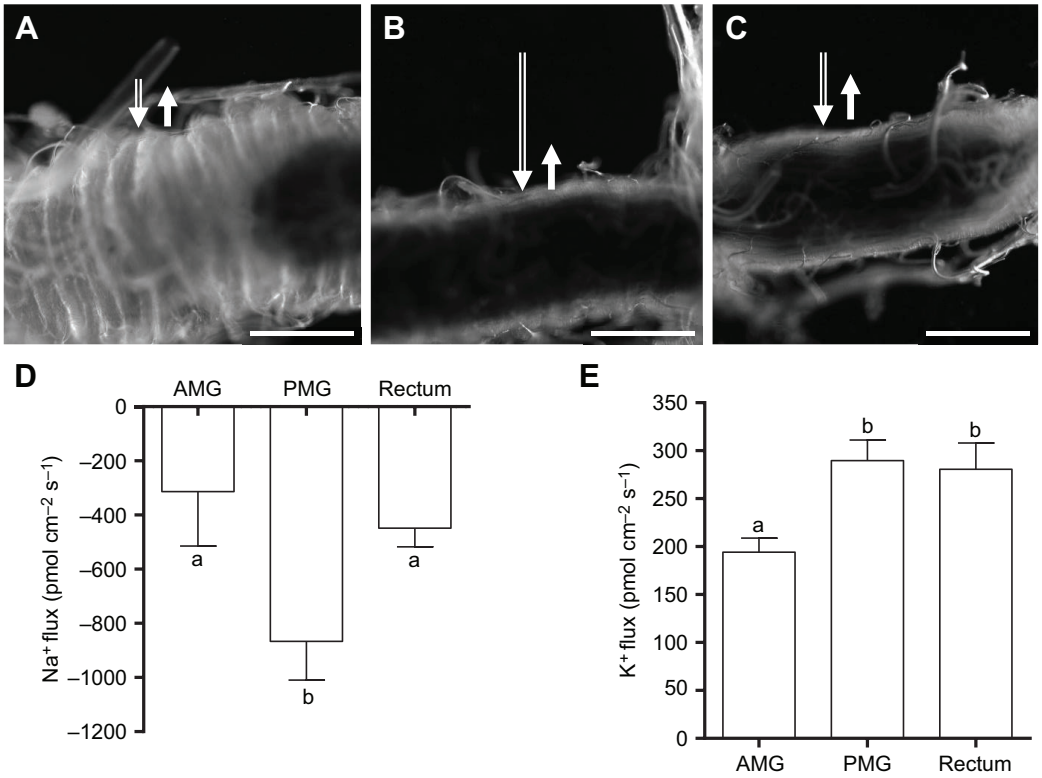

Fig. 3. Na^+ and K^+ flux across discrete regions of the freshwater-residing mayfly (*Hexagenia rigida*) nymph alimentary canal. Bright-field images of the (A) anterior midgut (AMG), (B) lower posterior midgut (PMG) and (C) rectum complete with arrows indicating direction and mean magnitude of ion flux (double-lined arrow, Na^+; solid arrow, K^+). (D) Mean Na^+ flux (secretion, bathing medium to gut lumen). (E) Mean K^+ flux (absorption, gut lumen to bathing medium). Data in D and E are expressed as means±s.e.m. (n=11–16); different lowercase letters denote a significant difference (P<0.05) between regions as determined by a one-way ANOVA followed by a multiple comparison test. Scale bars, (A–C) 200 μm.

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Nonetheless, in the regions of the alimentary canal and MTs where Na^+ secretion takes place, K^+ absorption occurs and this strategy undoubtedly contributes to ionoregulatory homeostasis.

FW mayfly nymph hemolymph ion composition

Hemolymph ion composition of the FW-residing *H. rigida* nymph is qualitatively consistent with the hemolymph ion composition of other aquatic insect larvae (Sutcliffe, 1962; Jonusaite et al., 2011, 2016), and quantitatively similar to that of other species of Ephemeroptera (Sutcliffe, 1962). We are unaware of any previous report on hemolymph ion composition or pH for *H. rigida* nymphs.

Table 1. Region-specific differences in Na^+ and K^+ flux rates (pmol cm^{-2} s^{-1}) along the Malpighian tubules of the freshwater larval mayfly *Hexagenia rigida*

	Trunk	Coiled region
Na^+	−240.77±15	−357.58±60
K^+	123.26±25.08	−28.34±10.73
		182.50±29

Bathing medium to lumen (≡hemolymph to lumen) ion flux is represented by negative values.
Lumen to bathing medium (≡lumen to hemolymph) ion flux is represented by positive values.

The alimentary canal as an ionoregulatory organ in the FW mayfly nymph

Region-specific NKA and VA activity along the alimentary canal of *H. rigida* was conspicuous, and immunohistochemical localization of the enzymes revealed basolateral staining of NKA and apical staining of VA in all regions. Particularly prominent was increasing activity of both enzymes towards the posterior region of the alimentary canal, resulting in a 20- to 30-fold increase in HG NKA versus AMG and PMG, respectively, and a 13- to 14-fold increase in VA. Immunohistochemical observations of the HG revealed that the bulk of NKA- and VA-immunoreactivity (ir) was located in the HG rectal region, with little to no enzyme-ir in the ileum, suggesting that the high HG NKA and VA activity can be attributed to the rectum. These observations are consistent with those of Jonusaite et al. (2013), who examined the spatial distribution of NKA and VA activity along the alimentary canal of larval *Chironomus riparius* and reported an ∼20-fold increase in HG NKA activity versus AMG and PMG, as well as an ∼15-fold increase in HG VA activity versus AMG and PMG. They are also consistent with the observations of Khodabandeh (2006) who reported a substantial increase in HG NKA activity relative to the combined foregut/midgut region in two species of aquatic Odonata larvae. This characteristic of alimentary

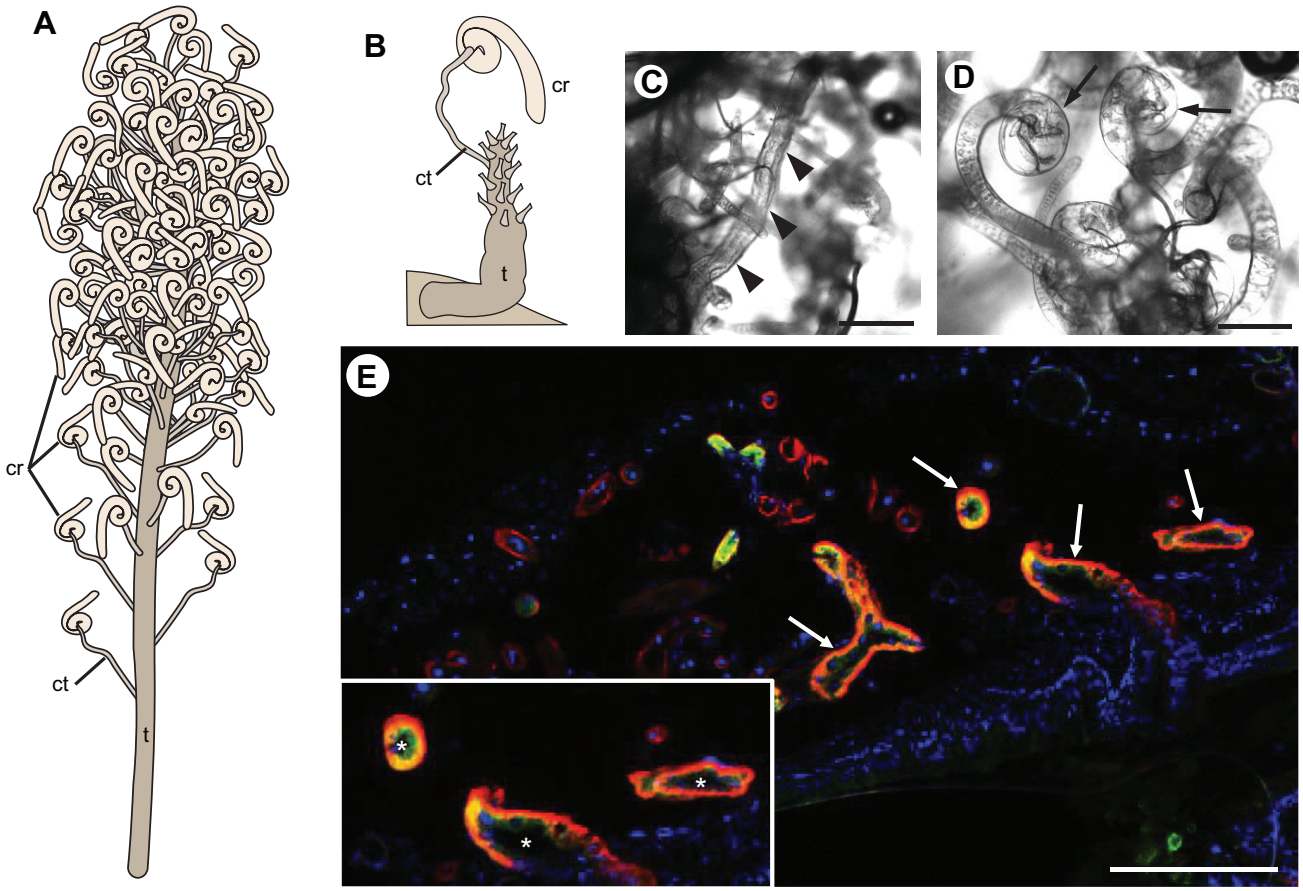

Fig. 4. Freshwater-residing mayfly (*Hexagenia rigida*) nymph Malpighian tubule (MT) morphology as well as Na^+/K^+-ATPase (NKA) and V-type-H^+-ATPase (VA) immunoreactivity (ir). (A) One of five identical mayfly nymph MTs and its branching and tortuous organization. (B) Magnified image of a coiled region with connecting duct, many of which can be seen in A. A bright-field image of (C) one of the five main trunks of mayfly nymph MTs (black arrowheads), which extend from the posterior midgut and give rise to (D) multiple blind-ended coiled tubules (depicted by arrow). (E) NKA- (red) and VA-ir (green) in cross-sectioned MTs. NKA and VA localize to the basolateral and apical membranes of the MT epithelial cells respectively (white arrows), which can be easily seen in the magnified inset, where a white asterisk denotes the MT lumen. In panels A and B, t is trunk, ct is connecting tube, and cr is coiled region. In E, nuclei are stained with DAPI (blue). Scale bars, 200 μm.

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canal NKA activity in aquatic insects can also be seen in terrestrial insects (Peacock, 1977, 1981; Tolman and Steele, 1976), suggesting a common role for NKA along the alimentary canal irrespective of surroundings. In contrast, little is known about the spatial distribution of VA along the alimentary canal of insects, and in particular differences between midgut regions and the HG. Nevertheless, VA has been associated with alkalization of FW larval mosquito midgut (Zhuang et al., 1998) as well as with acidic regions of the midgut in terrestrial insects (Overend et al., 2016). In addition, Jonusaite et al. (2013) highlighted a role for VA in K^+ reabsorption across the rectum of *C. riparius* larvae, and given the similarities of region-specific VA activity in *C. riparius* versus *H. rigida*, it seems likely that VA will play a comparable role in the rectum of mayfly nymphs. This is further supported by observations of K^+ absorption across the rectum of *H. rigida*, as was seen in *C. riparius* (Jonusaite et al., 2013).

Measurement of Na^+ flux across different regions of the mayfly nymph alimentary canal suggested that the anterior and posterior midgut, as well as the rectum, are responsible for Na^+ secretion. This may be problematic for the FW mayfly nymph because of all the circulating ions in extracellular fluid, Na^+ exhibits the steepest hemolymph to water electrochemical gradient. As such, passive Na^+ loss must be offset by strategies of Na^+ conservation or acquisition. Therefore, the question becomes, how does Na^+ secretion across different regions of the alimentary canal help the FW mayfly nymph maintain ionoregulatory homeostasis? One possible explanation is that a major role for the alimentary canal is to ensure the reabsorption of K^+. The result is that some Na^+ is lost, but this loss is compensated for by Na^+ acquisition across the tracheal gill (see Discussion, The tracheal gill as an ionoregulatory organ in the FW mayfly nymph). We are unaware of any other study that has examined Na^+ transport across the midgut or hindgut of an aquatic

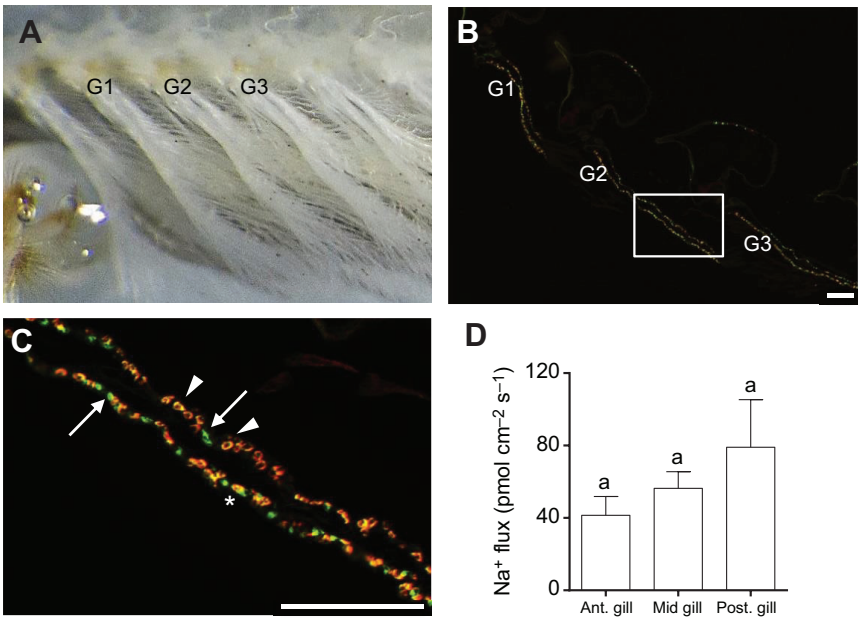

Fig. 5. Immunolocalization of Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA) in the tracheal gills of the freshwater-residing mayfly (*Hexagenia rigida*) nymph and Na^+ transport across different gills as well as different regions of the same gill. (A) The tracheal gills (G1–G3) that were fixed, sectioned and stained to reveal NKA (red) and VA (green) immunoreactivity (ir) as seen in B. (C) A region of the image shown in B (white box) magnified to reveal NKA-ir and VA-ir in ionocytes. Note that NKA and VA are found in the same cells (white arrowheads) or VA is found alone (white arrows). Some ionocytes (white asterisk) appear to exhibit NKA-ir and VA-ir in different regions of the same cell. (D) Na^+ flux across tracheal gills taken from different abdominal regions (anterior, middle and posterior). (E) The six different regions of the same tracheal gill (RI–RVI; region of measurement indicated by hashed line along central axis of tracheal gill) that were used to examine (F) spatial differences in Na^+ flux across the tracheal gill. In D and F, all data are expressed as means±s.e.m. (n=8–9). No significant difference was found between different gills (D) or different regions of the same gill (F). Scale bars, (B,C) 50 μm.

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larval insect. Therefore, it is difficult to reason whether the observations made in the present work will be unique to FW mayfly nymphs (or other aquatic insects that possess tracheal gills), or whether this might be a general strategy. That said, the exchange of Na^+ and K^+ (similar to what we observe in the mayfly nymph) has been shown in the posterior midgut of the terrestrial adult mosquito *Aedes aegypti*, where Na^+ is absorbed into the hemolymph while K^+ is secreted into the gut lumen for up to 2 h after a blood meal (Pacey and O'Donnell, 2014). Mechanistically, this transport is driven by basal NKA and apical VA along with the composition of the midgut lumen contents and the resulting electrochemical gradients for Na^+ and K^+. This becomes evident 24 h after a blood meal, when both Na^+ and K^+ are absorbed (Pacey and O'Donnell, 2014). Hence, it is worth pursuing measurements of feeding state and gut lumen content in mayfly nymphs to gain insight into the mechanism driving the observed Na^+ secretion and K^+ absorption.

The MTs as an ionoregulatory organ in the FW mayfly nymph

The morphology of ephemerid MTs is complex and the ultrastructure of ephemerid MT cells is consistent with an ionoregulatory function (Nicholls, 1983; Gaino and Rebora, 2000a,b; Giglio and Brandmayr, 2017). In turn, this is consistent with the role of MTs as an ionoregulatory organ of insects (Pannabecker, 1995; Donini et al., 2008; Jonusaite et al., 2017). Amongst the organs examined in the present study, NKA and VA activity were at their highest in the MTs, a trend that was particularly conspicuous in the case of NKA. Observations of high enzyme activity were also well reflected by MT NKA- and VA-ir, which was prominent in the basolateral and apical regions of MT cells, respectively. Although the present work is the first to examine the organ-specific activity of NKA and VA in a mayfly nymph, high MT NKA activity relative to other regions of the alimentary canal have been reported for FW nymphs of the stonefly *Paragnetina media* (Kapoor, 1980). In contrast, NKA and VA activity in the MTs of FW *C. riparius* larvae are equivalent to levels found in their midgut and ∼20 times lower than the activity observed in their HG (Jonusaite et al., 2013). Furthermore, although VA-ir is prominent in the MTs of FW *A. aegypti* larvae, NKA-ir is confined to the small intercalating stellate cells (Patrick et al., 2006). When comparing the structure of *H. rigida* MTs (see Fig. 4) with those of *C. riparius* and *A. aegypti* (see Jonusaite et al., 2013, 2017), a clear distinction can be seen in architectural complexity, with the convoluted arrangement of *H. rigida* MTs contrasting with the simple tube arrangement of *C. riparius* and *A. aegypti* MTs. Taken together, the above observations suggest that the role of MTs in the early developmental stages of aquatic insects that possess tracheal gills (e.g. mayfly or stonefly nymphs) may be different from those that possess anal papillae (e.g. *C. riparius* and *A. aegypti*). Interestingly, MTs of insects (including *C. riparius* and *A. aegypti*) are well known to secrete a fluid that is iso-osmotic to the hemolymph; however, it was suggested that the trunk of mayfly nymph MTs has a reabsorptive function aiding in production of hypo-osmotic fluid (Gaino and Rebora, 2000a). This idea is based on ultrastructural studies of the mayfly nymph MT trunk which reported that the cells making up this region are morphologically similar to the cells lining the lumen of the HG (Nicholls, 1983; Martoja and Ballan-Dufrançais, 1984; Gaino and Rebora, 2000a). In insects, the primary site of reabsorption of ions, which in FW insects results in a hypo-osmotic fluid to be excreted, is the HG (Cochran, 1985; Phillips et al., 1986; Bradley, 1987). This suggests that MTs of mayfly nymphs play a prominent role in reabsorption of ions which may be reflected in the differences in structure and NKA and VA expression between mayfly nymph MTs and those of other FW insect larvae.

To gain further insight into this idea, the present study measured K^+ and Na^+ fluxes at select regions of the MTs that were relatively easy to access with the SIET probe. These measurements detected K^+ absorption but also Na^+ secretion across the trunk region of mayfly nymph MTs, suggesting that the ionoregulatory function of the trunk may be more complex than just simply reabsorptive. Furthermore, observations of Na^+ and K^+ flux rates measured across the MT trunk are very similar to those measured across the mayfly nymph HG, suggesting that in addition to the structural similarities as noted by previous studies, functional similarities between the MT trunk and the HG are also evident. Similar observations of Na^+ flux are seen across the coiled region of the MT, but in the coiled region both K^+ absorption and K^+ secretion were observed. It seems likely that ion transport across this region of the MT, at least, may be regionalized in a highly specific manner, perhaps reflecting cellular heterogeneity and differences in the ionoregulatory function of MT regions and/or cells. For example, not examined in this study is the conducting tube that connects the coiled MT region to the trunk. This area has been reported to have basal channels in *Ecdyonumus diaspar* (a heptageniid mayfly), which is suggestive of a reabsorption function (Nicholls, 1983). Also, the presence of specialized channels in the cells of the coiled/spiral region of *E. diaspar* tubules suggests the removal of ions from fluid into the hemolymph, which would facilitate the production of hypo-osmotic fluid for excretion in FW nymphs (Nicholls, 1983). In short, the complex physiology of mayfly nymph MTs will require further species-specific examination, but the present study has been able to show both reabsorptive as well as secretory functions in this region of the alimentary canal of *H. rigida*.

The tracheal gill as an ionoregulatory organ in the FW mayfly nymph

To the best of our knowledge, ion absorption across the tracheal gill has never been directly measured until now. In the present study, Na^+ flux was directly measured across gills taken from different regions of the abdomen (anterior to posterior) as well as spatially along the central axis of individual gills (proximal to distal). This approach revealed Na^+ absorption irrespective of gill location (i.e. which gill was measured) or location along the gill, and this is consistent with the need for FW mayfly nymphs to acquire salts from the surrounding water. Direct observations of Na^+ absorption are also consistent with the presence of NKA- and VA-ir cells (putative ionocytes) in the gill, which, in turn, fits with measured levels of NKA and VA activity, which were quite high relative to anterior regions of the alimentary canal. The NKA- and VA-ir cells in the gill are most likely the ephemerid CCs. We can draw this conclusion because ephemerid CCs exhibit the ultrastructural characteristics of ionocytes (Wichard and Komnick, 1971) and gill/epidermal ionocytes of other FW animal species exhibit similar intense VA- and/or NKA-ir (e.g. Ehrenfeld and Klein, 1997; Wilson et al., 2000). An interesting observation is that the average calculated single-point flux rate of Na^+ (∼60 pmol cm^{-2} s^{-1}) across the tracheal gill of a FW mayfly nymph is lower than that seen across ionoregulatory epithelia of other FW insect larvae. For example, single-point flux rates of Na^+ across the anal papillae of FW larval *C. riparius* and *A. aegypti* (which interface directly with FW like the tracheal gill) have been reported as ∼127 and ∼300 pmol cm^{-2} s^{-1}, respectively (Donini and O'Donnell, 2005; Nguyen and Donini, 2010). The high rate of Na^+ absorption

reported for *A. aegypti* likely reflects their rearing medium, which was distilled water. However, Nguyen and Donini (2010) held *C. riparius* larvae in FW of the same mineral composition as the FW used in this study. But single-point flux rates of ions are only part of the story. When the surface area of mayfly nymph tracheal gills is taken into consideration and compared with area-corrected Na^+ flux across the anal papillae of *C. riparius* (using the same methodological approach as Nguyen and Donini, 2010), a different story emerges. Specifically, total Na^+ flux across the tracheal gills of *H. rigida* is ~800 pmol s^{-1} compared with 0.5 pmol s^{-1} as reported for *C. riparius* anal papillae (Nguyen and Donini, 2010).

A final observation of Na^+ flux across the tracheal gills is that no significant difference in rates could be determined between gills taken from different regions of the abdomen. This reflects the abundance of NKA- and VA-ir cells seen irrespective of gill location and differs from the gills of select other aquatic arthropods (i.e. some species of crustacean) that are functionally differentiated depending on their location (for review, see Péqueux, 1995). However, Na^+ measurements taken along the central axis of each gill showed slightly higher rates of Na^+ flux in the middle region of the gill (RIV) as well as in the distal regions (RV and RVI). Although the change in flux was not significant, the increased Na^+ absorption towards the middle of the gill coincides with more concentrated pigmented spots on the gills (image not shown), and these areas have previously been associated with the presence of CCs (Wichard et al., 1973).

Conclusions and perspectives

Mayfly nymphs such as those of *H. rigida* are important organisms in FW ecosystems, where their biomass provides trophic support for fishes, amphibians, birds and mammals (Saouter et al., 1991). Despite this, the ability of these animals to cope with a central challenge to life in FW, the maintenance of salt and water balance, remains poorly defined. Our current knowledge of FW mayfly nymph ionoregulation is drawn largely from inference and the meticulous observations of ionoregulatory organ (or putative ionoregulatory organ) structure and ultrastructure (Wichard and Komnick, 1971; Wichard et al., 1973). The present study advances our knowledge of mayfly biology in so far that it outlines the ionoregulatory strategies of FW *H. rigida* nymphs. But a considerable amount of work will be required before we have a clear mechanistic understanding of *H. rigida* nymph ionoregulatory physiology. In addition, it will be of great interest to consider the ionoregulatory strategies and/or mechanisms of nymphs of other species of mayfly.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: F.N., S.J., T.W., A.D., S.K.; Methodology: F.N., S.J., A.D., S.K.; Validation: F.N., A.D., S.K.; Formal analysis: F.N., A.D., S.K.; Investigation: F.N., S.J., A.D., S.K.; Resources: T.W., A.D., S.K.; Writing - original draft: F.N., S.J., A.D., S.K.; Writing - review & editing: F.N., S.J., T.W., A.D., S.K.; Visualization: F.N., S.J., A.D., S.K.; Supervision: S.J., A.D., S.K.; Project administration: A.D., S.K.; Funding acquisition: A.D., S.K.

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