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RESEARCH ARTICLE

Water and ion transport across the eversible vesicles in the collophore of the springtail *Orchesella cincta*

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ABSTRACT

Springtails (Collembola) are ancient close relatives of the insects. The eversible vesicles are their unique paired transporting organs, which consist of an epithelium located inside a tube-like structure called the collophore on the first abdominal segment. The vesicles can be protruded out of the collophore and several lines of evidence indicate that they have a vital function in water uptake and ion balance. However, the amount of water absorbed by the vesicles and which other ions apart from Na^+ are transported remain unknown. Using *Orchesella cincta* as a model, we developed protocols for two assays that enabled us to study water and ion movement across the eversible vesicles in whole living springtails. Using an inverse Ramsay assay we demonstrate that the eversible vesicles absorb water from a droplet applied onto their surface. Using the scanning ion-selective electrode technique (SIET), we show that the vesicles absorb Na^+ and Cl^- from the bathing medium, secrete NH_4^+, and both absorb and secrete K^+. H^+ is secreted at a low level in the anterior part and absorbed at the posterior part. We did not detect transport of Ca^{2+} at significant levels. The highest flux was the absorption of Cl^-, and the magnitude of ion fluxes was significantly lower in fully hydrated springtails. Our data demonstrate that the eversible vesicles are a transporting epithelium functioning in osmo- and ionoregulation, nitrogenous waste excretion and probably also acid–base balance.

KEY WORDS: Collembola, Transporting epithelium, Osmoregulation, Excretion, Water absorption, Scanning ion-selective electrode technique

INTRODUCTION

Springtails (Collembola) are a basal lineage of Hexapoda, which is a group of terrestrial arthropods that also includes the insects (Hopkin, 1997; Misof et al., 2014). Compared to the insects, springtails lack some of the advanced adaptations to terrestriality, including the Malpighian tubules for excretion and ion balance. Besides being important for understanding insect evolution, springtails also play a key role in the soil and act as a model for ecotoxicology, and tolerance to cold and drought (e.g. Cannon and Block, 1988; Rusek, 1998; Roelofs et al., 2009; Worland et al., 2010; Holmstrup et al., 2015; Faddeeva-Vakhrusheva et al., 2016). Despite their importance, many basic aspects of springtail physiology remain unknown.

A defining characteristic of juvenile and adult springtails is the collophore (ventral tube) (Hoffmann, 1905; Imms, 1906), a tube-like structure in the centre of the ventral side of the first abdominal segment. The collophore originates from a pair of limbs that fuse together during embryogenesis (Imms, 1906; Uemiya and Ando, 1987; Konopova and Akam, 2014). The terminal part of the collophore is formed by the eversible vesicles, which are a special transporting epithelium. The vesicles are normally closed inside the tube, but can be everted by haemolymph pressure and retracted by muscles located inside the collophore (Eisenbeis, 1976).

Although the collophore is important for the springtails, its relevance to physiological processes of the animal remains unclear (Konopova and Akam, 2014; Favret et al., 2015). The organ is multifunctional, secreting, for instance, a substance with adhesive properties (Favret et al., 2015). However, most evidence to date indicates that its primary role is in uptake of water and transport of ions carried out by the eversible vesicles (e.g. Nutman, 1941; Noble-Nesbitt, 1963b; Eisenbeis, 1982; Eisenbeis, 1974; Eisenbeis, 1976; Eisenbeis and Wichard, 1975a,b; Eisenbeis and Wichard, 1977).

The role of the collophore in water balance has long been suspected (Noble-Nesbitt, 1963b). When a dehydrated springtail encounters free water (e.g. in moist soil), the vesicles are everted and remain in contact with the water while the animal is drinking with its mouth. That fluid passes through the vesicles has been demonstrated by using vital dyes as indicators (Nutman, 1941). In the 1980s Eisenbeis and colleagues developed an assay that enabled estimation of the amount of water that is absorbed (Eisenbeis, 1982; Jaeger and Eisenbeis, 1984). Dehydrated springtails were allowed to walk on a wet filter paper and observed as they everted the vesicles. The rate at which water was taken up was calculated from the increase in animal weight after the experiment. Each springtail in these experiments had to be observed using a pocket lens to confirm that only the eversible vesicles and not the mouth were used for water uptake. Springtails seen drinking with their mouth were excluded from calculations. Because of the small size of the animals (about 5 mm) and the fact that they typically stand with their heads close to the ground (our observation) it cannot be ruled out that some of the water considered to be absorbed by the vesicles was actually taken up by mouth. Absorption by the integument, including as water vapour (e.g. Bayley and Holmstrup, 1999; Kærsgaard et al., 2004), cannot be controlled for in this assay. Results from the earlier experiments (Eisenbeis, 1982; Jaeger and Eisenbeis, 1984) indicated that the eversible vesicles are efficient in absorption of water; however, because of the limitations of the assay, the evidence that significant amounts of water are taken up exclusively by the eversible vesicles is still lacking.

Studies by electron microscopy on a range of springtails showed that the eversible vesicles have ultrastructural characteristics of epithelia that also transport ions (Eisenbeis, 1974; Eisenbeis and Wichard, 1975a,b; Eisenbeis and Wichard, 1977). However, only the uptake of radioactively labelled sodium has been demonstrated (Noble-Nesbitt, 1963b). The likely transport of chloride ions was indicated by histological staining with silver salts as markers

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(Eisenbeis and Wichard, 1975a,b). It is unknown if other ions are transported.

Here, we studied the springtail *Orchesella cincta*, which has previously been adapted as a model for ecotoxicology and developmental genetics (Konopova and Akam, 2014; Faddeeva-Vakhrusheva et al., 2016). We had two main goals. First, to establish a method for quantification of fluid transport specifically by the eversible vesicles. Second, to develop a protocol for electrophysiological measurement of ion transport across the eversible vesicles of *Orchesella* and to examine whether transport of selected ions by the vesicles indicates a role in osmo- and ionoregulation (Na^+, K^+, Cl^-, Ca^{2+}), nitrogenous waste excretion (NH_4^+) and acid–base balance (H^+).

To quantitatively measure uptake of water by the eversible vesicles, we adapted the classical Ramsay assay, which was developed as an *in vitro* assay of fluid secreted by insect Malpighian tubules (Ramsay, 1954). The original assay uses dissected tubules immersed in saline under paraffin oil and the rate of fluid secretion is calculated from the increasing size of a droplet secreted by the tubule. We show that an inverse Ramsay assay can be used for whole, living *Orchesella*, in which changes in the size of a droplet in contact with the eversible vesicles are measured. Next, we present measurement of ion fluxes across the eversible vesicles using the scanning ion-selective electrode technique (SIET). We chose SIET because it enabled us to record ion transport rates in whole living springtails and at several different locations on the body in a single preparation. SIET uses ion-selective microelectrodes to measure ion concentrations in the unstirred layer of the measuring medium directly adjacent to the transporting cells and then further away (Piñeros et al., 1998; O'Donnell, 2009). From the differences in concentration of ions, fluxes are calculated, thus providing values for ion absorption (influx) or secretion (efflux). We show that the eversible vesicles absorb water from a droplet and transport a range of physiologically relevant ions. Our results demonstrate that the eversible vesicles are a multifunctional transporting epithelium and an important component of springtail osmoregulatory and excretory physiology.

MATERIALS AND METHODS

Animals

Orchesella cincta (Linnaeus 1758) were maintained in Petri dishes with a base of plaster of Paris and fed with algae (*Pleurococcus* sp.) and yeast, as described previously (Konopova and Akam, 2014). The animals were kept and experiments carried out at room temperature (23°C). Adults, mixed males and females, were used for all measurements. Animals used in the experiments were first mildly dehydrated. They were removed from the culture, i.e. from the dishes that were kept continuously wet (distilled water or diluted algal medium added with a plastic Pasteur pipette) and transferred into a dry dish with food (algal medium dried out, yeast kept dry) and kept there for 1–3 (mostly 2) days. The dry (experiments) and wet (culture) dishes were kept in the same room and thus, under the same relative humidity of 30%. Springtails on dry dishes stopped laying eggs under this regime.

Inverse Ramsay assay

Adult springtails were briefly anaesthetized with CO_2 and immersed in heavy paraffin oil (Caledon Laboratory Chemicals, Georgetown, Canada) in a Sylgard-coated dish (Sylgard 184, World Precision Instruments, Sarasota, FL, USA). They were restrained between minutien pins to keep them in place and to isolate the collophore from moving appendages. Care was taken so that the integument remained intact without haemolymph leaks. The collophore was brought into contact with a droplet of fluid mimicking ground water (modelled after Schouten and van der Brugge, 1989) (in mmol l^{-1}): 0.1 KCl, 0.2 NaCl, 0.3 NH_4Cl, 0.17 $CaCl_2$, 0.15 $MgSO_4$, 1.7 KNO_3, 1 HEPES, pH=6.0. The diameter (d) of the droplet that was pipetted onto the everted vesicles (experiment) or freely into the oil (control) was measured at 800× magnification using a calibrated ocular micrometer. The absorption rate (AR, nl min^{-1}) by the vesicles was calculated from the change in droplet volume ($4/3\pi r^3$, where r is the droplet radius) over 30 min. This rate was divided by the absorptive area of the vesicles (0.110 mm^{-2}), which was determined for *Orchesella* previously (Eisenbeis, 1982), to estimate the rate per unit area; note that we did not measure this if our animals were the same size (e.g. body length) as the animals in the previous study.

SIET

The hardware, software and methodology for acquiring SIET data and calculating ion fluxes have been described previously (Donini and O'Donnell, 2005; O'Donnell and Ruiz-Sanchez, 2015; Kolosov et al., 2018a). Briefly, SIET measurements were made with hardware from Applicable Electronics (Forestdale, MA, USA) and Automated Scanning Electrode Technique (ASET) software (version 2.0; Science Wares, Falmouth, MA, USA). Micropipettes were pulled on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co., Novato, CA, USA) from 1.5 mm borosilicate glass (World Precision Instruments Inc., Sarasota, FL, USA). At each measurement site, the ion-selective microelectrode was vibrated perpendicular to the tissue surface between two positions separated by 50 µm. The measured voltage gradients between the two points were converted into concentration gradients using the following equation:

$$\Delta C = C_B \times 10^{\left(\frac{\Delta V}{S}\right)} - C_B, \quad (1)$$

where ΔC is the concentration gradient between the two points measured in µmol cm^{-3}; C_B is the background ion concentration, calculated as the average of the concentrations 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.

Fluxes were estimated from the measured concentration gradients using Fick's law:

$$J_I = \frac{D_I \Delta C}{\Delta x}, \quad (2)$$

where J_I is the net flux of the ion in pmol cm^{-2} s^{-1}; D_I is the diffusion coefficient (Robinson and Stokes, 1968) of the ion (1.55×10^{-5} cm^2 s^{-1} for Na^+ and Cl^-; 1.92×10^{-5} cm^2 s^{-1} for K^+; 1.19×10^{-5} cm^2 s^{-1} for Ca^{2+}, 9.4×10^{-5} cm^2 s^{-1} for H^+ and 2.09×10^{-5} cm^2 s^{-1} for NH_4^+); ΔC is the concentration gradient in µmol cm^{-3}; and Δx is the distance between the two points measured in cm. Microelectrodes were constructed with the following ionophores (Sigma-Aldrich, St Louis, USA) [backfill and calibrating solutions (in mmol l^{-1}) indicated in brackets]: Na^+ ionophore X cocktail (3.5% Na^+ ionophore X, 0.6% potassium tetrakis chlorophenyl borate, 95.9% nitrophenyl octyl ether), (150 NaCl backfill, 0.15/1.5 NaCl calibrations); K^+ ionophore I, cocktail B (150 KCl backfill, 1.5/15 KCl calibrations); Cl^- ionophore I, cocktail A (150 KCl backfill, 0.15/1.5 NaCl calibrations); Ca^{2+} ionophore I, cocktail A (100 $CaCl_2$ backfill, 0.15/1.5 $CaCl_2$ calibrations); H^+ ionophore I, cocktail B (100 NaCl/100 Na-Citrate pH=6 backfill, 1 HEPES, pH=6/7 calibrations); NH_4^+ ionophore I, cocktail A (100 NH_4Cl backfill, 0.15/1.5 NH_4Cl calibrations). For measurements of Na^+, K^+ and Ca^{2+}, the bathing medium was that described above for the inverse Ramsay assay. To avoid interference

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of competing ions with ionophores employed in SIET, the original recipe had to be modified for the measurement of NH_4^+ ions (0.1 mmol l^{-1} KNO_3 and 1.7 mmol l^{-1} KCl were replaced with N-methyl-D-glucamine chloride to avoid K^+ interference with NH_4^+ ionophore) and Cl^- ions (1.7 mmol l^{-1} KNO_3 was replaced with 0.85 mmol l^{-1} K_2SO_4 to avoid NO_3^- interference with Cl^- ionophore). H^+ was measured in the medium adapted for NH_4^+ measurements. Because protons may diffuse freely or in association with buffers in the saline, proton transport rates were corrected for buffering using equations described in Messerli et al. (2006).

The scanning was carried out as follows: each scan started at the anteriormost part of one of the paired vesicles and continued at 25 µm intervals until the posterior-most part. Protocol 1: if no regional signal heterogeneity was observed (Na^+, Cl^-, NH_4^+), three to five values surrounding the signal maximum were averaged and used as a representative n=1 for the biological replicate. Protocol 2: if anterior-posterior heterogeneity was observed (H^+), three to five values surrounding signal maxima were averaged independently in the anterior and posterior lobes of the vesicle. Protocol 3: if anterior-posterior heterogeneity was present only in some of the samples, approaches 1 or 2 were followed depending on whether that particular sample exhibited the heterogeneity.

Measurement of haemolymph ion concentration

Springtails were immersed in paraffin oil and one or two antennae were cut off at the midway point. Haemolymph was collected at the cut end of the antenna using a glass microcapillary pulled to a fine point. Samples were expelled under oil in a separate dish and analysed with ion-selective microelectrodes as described previously (Naikkwah and O'Donnell, 2011; Kolosov et al., 2018b). Collecting samples from the antennae is a convenient method that provides relatively clean haemolymph as compared to sampling from around the collophore, which contains fat body. The haemolymph composition is expected to be similar in different parts of the body.

Preliminary results indicated that an unknown factor interfered with Na^+ ionophore X-based electrodes in haemolymph samples as reported previously (Kolosov et al., 2018b). Therefore, Na^+ ionophore III-based electrodes were used to avoid this. A solid-state Cl^- microelectrode was employed (construction described in Donini and O'Donnell, 2005) as Cl^- ionophore I ionophore is affected by other anions abundant in the haemolymph (e.g. HCO_3^-). The K^+ and Ca^{2+} microelectrodes were based on the ionophores and backfill solutions described above for SIET. Calibration solutions were as follows: Na^+, 15 mmol l^{-1} and 150 mmol l^{-1} NaCl; K^+, 1.5 mmol l^{-1} and 15 mmol l^{-1} KCl; Cl^-, 150 mmol l^{-1} and 15 mol l^{-1} KCl; Ca^{2+}, 0.15 and 1.5 mmol l^{-1} $CaCl_2$; H^+, 1 mmol l^{-1} HEPES pH=8 and pH=9.

Statistics

Significant differences in (1) transport of Cl^- between fully hydrated and mildly dehydrated springtails and (2) H^+ flux between anterior and posterior collophore regions were determined using a Student's t-test in SigmaPlot (version 11), with a P<0.05 limit.

Image processing

Brightness and contrast in micrographs was adjusted using Adobe Photoshop CC 2017.1.1.

RESULTS

An inverse Ramsay assay quantifies the water absorbed specifically by the eversible vesicles of living *Orchesella*

The original Ramsay assay that we adapted here uses dissected organs immersed in paraffin oil. Our setup uses whole living adult *Orchesella* (Fig. 1A). Because the cuticle on the body of springtails is highly hydrophobic (Gundersen et al., 2017), the animals immerse easily into the oil. They survive the immersion well and stay alive for several hours under oil. Unlike the rest of the body, the cuticle on the eversible vesicles is hydrophilic and together with the claws they represent the only parts of the animal that are wettable (Fig. 1B) (Noble-Nesbitt, 1963a). The quantification of fluid absorption by the vesicles is based on measuring the decrease in size of a droplet that is dispensed onto their surface.

To find out at what rate the eversible vesicles likely absorb water in nature we let them absorb a medium that mimics the composition of ground water (Schouten and van der Brugge, 1989) and which *Orchesella* is likely to encounter in its natural habitat. A 1 µl droplet of the medium was dispensed onto the everted vesicles of springtails that had been mildly dehydrated (see Materials and Methods). Previous studies indicated that the main absorption period after the vesicles come into contact with water is 10–30 min (Eisenbeis, 1982). We monitored the change in size of the droplet after 30 min and from the values calculated a rate of fluid uptake of 2.55±0.40 nl min^{-1} (N=8; mean±s.e.m.). Expressed per absorptive area of the vesicles, the rate was 23.18 nl mm^{-2} min^{-1} (see Materials and Methods). All springtails used in this experiment remained alive after they were removed from the oil. As a control, 1 µl droplets of the medium were kept under oil without contact with the springtail; none of the five droplets that we observed changed in size after 30 min. This demonstrates that the decrease in size detected in the experiment did not take place by diffusional loss of water into the oil. Taken together, the inverse Ramsay assay is a suitable method for precise measurement of the rate of fluid absorption by the eversible vesicles.

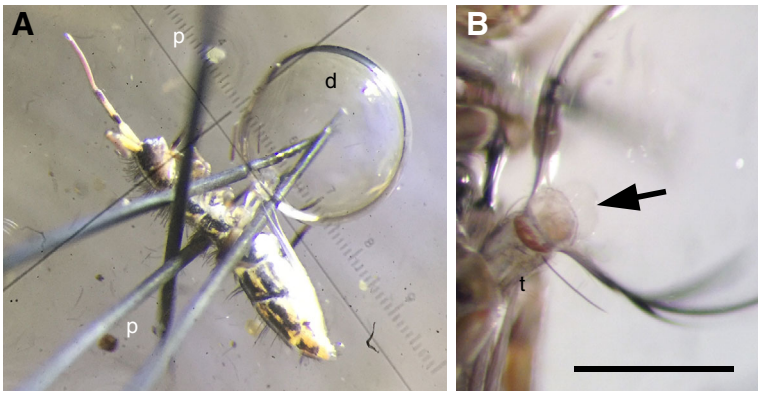

Fig. 1. Measurement of water uptake by the eversible vesicles of *Orchesella cincta* using an inverse Ramsay assay. (A) Adult *Orchesella* were immersed in paraffin oil and a droplet of fluid was dispensed onto the everted vesicles. The springtails usually everted the vesicles under the oil themselves or were forced to do so by gentle pressure on the abdomen. (B) The cuticle on the vesicles is hydrophilic and attached well to the droplet. The everted vesicles are marked with an arrow. Anterior is to the top left in A and to the top in B. d, droplet of the measuring fluid; p, pins; t, tube of the collophore. Scale bar: 0.5 mm.

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SIET detects significant fluxes of Na^+, Cl^-, K^+, H^+ and NH_4^+ but not Ca^{2+} ions across the eversible vesicles of *Orchesella*

Animals were restrained for SIET as for the inverse Ramsay assay, but were bathed in medium mimicking ground water instead of paraffin oil. Because of their hydrophobic cuticle the springtails tended to float on the surface of the medium and were restrained with bent minutien pins (as shown in Fig. 2A,A′). The springtails remained alive during the recording, moved occasionally and fully recovered after the experiment.

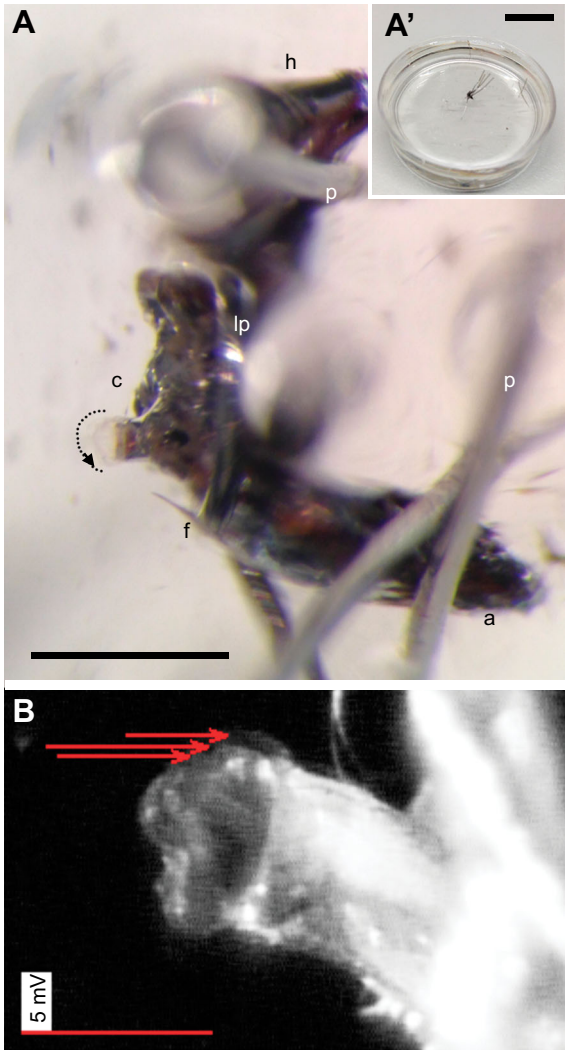

Fig. 2. Preparation for SIET measurement of ion fluxes across the eversible vesicles of *Orchesella*. (A) Adult *Orchesella* were immersed into the measuring medium. The collophore protrudes through the loop made from a pin. The animal in this photograph moved immediately after it was returned to the culture; after a few minutes of rest it crawled away. The dotted line with an arrow around the vesicles indicates how the scanning was done from anterior to posterior. The inset (A′) shows the dish with the preparation. (B) Representative scan from SIET. Uptake of Cl^- in the anterior part of the eversible vesicles is shown. The length and direction of the arrows indicate the magnitude and direction of the flux, respectively. The magnitude is expressed as the difference in voltage between recording by the microelectrode close to the cells of the vesicles and then farther away (marker 5 mV). The values were then used for calculation of concentration difference, which was converted into the net flux (pmol cm^{-2} s^{-1}). Head to the top and ventral side to the left in all photographs. a, abdomen; c, collophore; f, furca; h, head; lp, pin making a loop; p, pins. Scale bars: 1 mm (A); 1 cm (A′).

We used SIET to determine if we could detect the transport of Na^+, Cl^-, K^+, Ca^{2+}, NH_4^+ and H^+ across the vesicles (Fig. 2B). All our experimental animals were mildly dehydrated (see Materials and Methods), as in the inverse Ramsay assay. The microelectrode recordings were carried out at several locations around the sphere of one of the everted vesicles from anterior to posterior (Fig. 2A). Vesicles remained everted during the whole time of recording, as monitored by a camera on the stereomicroscope that was attached to the SIET rig. Significant fluxes of all selected ions except Ca^{2+} were detected across the vesicles (Fig. 3). To check whether our microelectrodes detect ions specifically in the proximity of the everted vesicles rather than anywhere around the animal, we also made recordings of Na^+ on two separate preparations of the collophore with retracted vesicles and on a special abdominal appendage called the furca that is used for jumping (Hopkin, 1997). No measurable flux was detected, but it is worth noting that due to the hydrophobicity of the cuticle (including the tube of the collophore and furca) there was no direct contact with the medium to allow exchange of ions under these conditions.

Na^+ and Cl^- ions were absorbed by the vesicles of all animals and across all anterior to posterior locations on the vesicles that we examined. Mean values were −104 and −1541 pmol cm^{-2} s^{-1}, for Na^+ and Cl^-, respectively (Fig. 3A). K^+ ions were taken up by half of

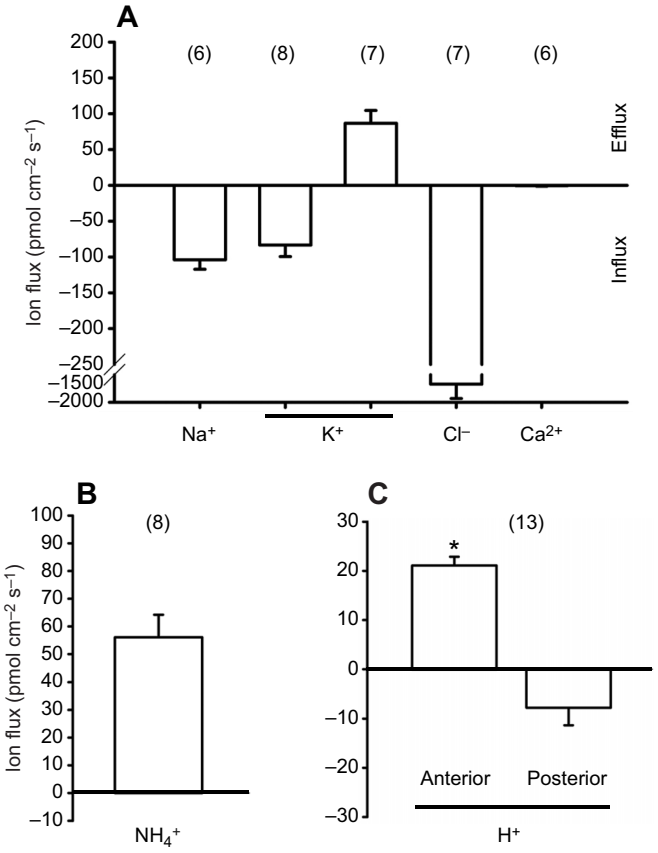

Fig. 3. Ion fluxes across the eversible vesicles of *Orchesella* measured by SIET. Fluxes of ions implicated in osmo- and iono-regulation (A), nitrogenous waste excretion (B) and acid–base balance (C) were measured on mildly dehydrated springtails (see Materials and Methods). The asterisk in C indicates significant difference between anterior and posterior as determined by a Student's *t*-test (t=297, P<0.001). Values are means±s.e.m. Numbers in brackets above the bars indicate the number of animals examined (N). Negative values indicate ion uptake (influx), positive values indicate ion loss (efflux).

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the animals, but secreted by others (Fig. 3A). Both influx and efflux of K^+ occurred in all anterior to posterior locations on the vesicles, suggesting that unidirectional movement is not regionalized. The magnitudes of influx, −83±16 pmol cm^{-2} s^{-1}, and efflux, 86±18 pmol cm^{-2} s^{-1}, were similar. These results were obtained on animals examined during the same round of measurement (the same day), one animal measured after the other using the same rig and taken from the same dish. The distribution of animals demonstrating K^+ influx or efflux was random within the group (e.g. one animal showed influx, the following one efflux, the next one influx). NH_4^+ ions were secreted by all animals at all anterior to posterior locations on the vesicles (Fig. 3B). The values were 56±8.1 pmol cm^{-2} s^{-1}. H^+ ions were secreted in the anterior half of the vesicles (efflux 21±1.8 pmol cm^{-2} s^{-1}) and simultaneously absorbed (influx −7.8±3.6 pmol cm^{-2} s^{-1}) across the posterior half (Fig. 3C) in all animals. In summary, these experiments demonstrate that the eversible vesicles transport a range of ions and the notably highest fluxes in our experiments were of Cl^-.

Uptake of Cl^- ions is lower in fully hydrated springtails

Next, we examined whether the mild dehydrations of our experimental animals had any effect on the ion fluxes. As an example, we chose the Cl^- ion, for which we recorded the highest flux across the vesicles, and compared the flux between fully hydrated springtails and those that were dehydrated for 3 h at ∼30% RH. We found that the eversible vesicles of fully hydrated *Orchesella* still absorbed Cl^- ion, but this influx was ∼13 times (significantly) lower (Fig. 4). Thus, mild dehydration of the whole animal leads to more pronounced fluxes of Cl^- across the eversible vesicles.

Haemolymph concentrations of Na^+, Cl^- and K^+ indicate that the eversible vesicles transport these ions against the concentration gradient

Finally, we determined whether the exchange of ions between the eversible vesicles and external medium takes place against the concentration gradient, thus likely by an active transport. We compared the concentrations of Na^+, Cl^- and K^+ in the haemolymph and in the medium. Haemolymph concentrations were measured using ion-selective microelectrodes and concentrations in the medium were calculated from the recipe. The concentration of all these three ions in the haemolymph is higher than in the measuring medium (Table 1). This indicates that the uptake of such ions takes place against a concentration gradient.

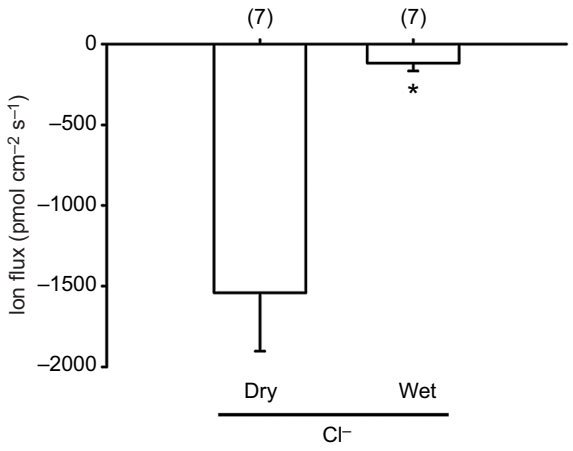

Fig. 4. The level of *Orchesella* hydration affects the amount of Cl^- uptake by the eversible vesicles. Springtails kept at optimal humid conditions ('wet') showed significantly lower uptake of Cl^- by their eversible vesicles compared to animals previously exposed to mild dehydration ('dry'). The data for mildly dehydrated animals are from Fig. 3A. Values are means±s.e.m. Numbers in brackets above the bars indicate the number of animals examined (*N*). Asterisk indicates significant difference between dry and wet as determined by Student's *t*-test (*t*=5.09, *P*<0.001).

DISCUSSION

Eversible vesicles in the collophore of *Orchesella* are potent water-absorbing organs

By employing the inverse Ramsay assay we found that the pair of eversible vesicles of mildly dehydrated *Orchesella* absorbs water at a rate of 23.18 nl mm^{-2} when expressed per absorptive area of the vesicles. How does this value compare (1) to the absorption rate measured on springtails previously and (2) to the transport rate across fluid transporting epithelia of insects?

Previous quantification of water absorption by eversible vesicles of *Orchesella* and closely related *Tomocerus* was based on weighing springtails before and after they were allowed to absorb fluid from a wet filter paper (Eisenbeis, 1982; Jaeger and Eisenbeis, 1984). The absorption rate, given by weight gain over a period of time, was expressed per mm^2 of absorptive area of the vesicles. The rates from the filter paper absorption-weighing assay for *Orchesella* were 8 times higher for distilled water and 5 times higher for 25 mmol l^{-1} NaCl (solutions with lower and higher osmolarity than the medium used in the present experiments) compared with our results. Our absorption rate is closest to the minima previously obtained for 25 mmol l^{-1} NaCl (Eisenbeis, 1982). Why are the results from the two assays different? While the assay introduced by Eisenbeis and colleagues simulates natural conditions better, our inverse Ramsay assay is specific. It measures only the fluid taken up by the eversible vesicles and excludes drinking and absorption by other tissues. The method used to dehydrate the springtails (thus, likely the level of dehydration) also differs between the two assays: dehydration by keeping the springtails in laboratory conditions without access to water for a few days in our assay (see Materials and Methods) versus several cycles of dehydrations at relative humidity 33% followed by rehydration in the previous assay (Eisenbeis, 1982). Other experimental conditions also may differ.

How does the rate of absorption by the eversible vesicles compare to transport by other epithelia? The Ramsay assay on tissue immersed in paraffin was used for measurement of the rate of secretion from the Malpighian tubules. A single Malpighian tubule has a basal (unstimulated) secretory rate of typically less than 1 nl min^{-1} (e.g. Ramsay, 1954; Dow et al., 1994; Beyenbach, 2003), but up to 15 nl min^{-1} in some species (Kolosov et al., 2018a) (compare with absorption by the eversible vesicles 2.55 nl min^{-1}). Expressed per size of secretion area, the maximal rate of secretion by the Malpighian tubules measured on a wide range of insects is about 0.05–13 nl mm^{-2} min^{-1} (Philips, 1981), typically less than

Table 1. Comparison of ion concentration in the haemolymph and medium

	Concentration (mmol l^{-1})	
Ion	Haemolymph	Medium (calculated)
Na^+	131.8±5.3 (16)	0.20
Cl^-	178.3±6.9 (13)	0.94
K^+	5.9±0.7 (21)	1.80

Haemolymph ion concentrations were measured with ion-selective microelectrodes. Values are means±s.e.m. (*N*). Ion concentrations in the measuring medium were calculated from the chemical composition in the protocol.

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half the absorption rate for the eversible vesicles of *Orchesella* measured in the current study (23.18 nl mm^{-2} min^{-1}). The Ramsay assay was also used to quantify secretion by the 'fastest fluid-secreting cell known', the cells in the Malpighian tubules of *Rhodnius prolixus* (Maddrell, 1991). After the blood-meal, the secretion rate increases up to 46 nl mm^{-2} min^{-1} (expressed per the area of the secretory part) (Bradley, 1983). Based on these approximate comparisons, it appears that the eversible vesicles of *Orchesella* transport water at approximately half the speed measured in the tubules of a blood-feeding hemipteran specifically adapted for rapid fluid secretion.

The eversible vesicles transport ions at levels comparable to other transporting epithelia of insects

Our microelectrode recordings across the eversible vesicles using SIET detected significant fluxes of Na^+, K^+, Cl^-, NH_4^+ and H^+. In the transporting epithelia of insects, the magnitudes of fluxes measured by SIET typically range from 10 to 1000 pmol cm^{-2} s^{-1} (e.g. Donini and O'Donnell, 2005; Nguyen and Donini, 2010; Naikkhwah and O'Donnell, 2012; Pacey and O'Donnell, 2014; Paluzzi et al., 2014; Robertson et al., 2014; O'Donnell and Ruiz-Sanchez, 2015; D'Silva et al., 2017; Kolosov et al., 2018a). The fluxes that we obtained for Na^+, Cl^-, K^+ and NH_4^+ (−104, −1541, −83/+86, +56 pmol cm^{-2} s^{-1}, respectively; Fig. 3A,B) are of comparable magnitude. The influx of Na^+ recorded is consistent with previous observations showing that the eversible vesicles pick up radiolabelled Na^+ from ground water (Noble-Nesbitt, 1963b). The influx of Cl^- is particularly remarkable. For comparison, the anal papillae of mosquitoes absorb Cl^- *in vivo* at a rate of 230 pmol cm^{-2} s^{-1} (Donini and O'Donnell, 2005).

Chloride uptake exceeded the sum of the uptake rates of the measured cations. The uptake of cations that we have not measured (e.g. Mg^{2+}) or the efflux of other anions (sulphate, phosphate, carbonates, organic anions) across the eversible vesicles may partially offset the imbalance. Alternatively, the difference may reflect non-steady state conditions during recording, with Cl^- uptake measured sooner after transfer of the animals to the experimental chamber, or small differences in hydration status, which can greatly alter ion uptake.

The eversible vesicles function in osmo- and ionoregulation, excretion and likely acid–base balance

Our data provide support for the function of the eversible vesicles in osmo- and ionoregulation. Na^+, Cl^- and K^+ are among the major ions in the haemolymph. These were the most intensively transported ions in our mini screen using SIET (Fig. 3A). We suggest that when the springtail encounters free water in the soil, it uses the eversible vesicles to replenish ions and maintain internal homeostasis (Fig. 5). Half of the animals in our experiments absorbed K^+ and half of them excreted it. This might reflect as yet unknown physiological differences in K^+ homeostasis between the animals. It is consistent with previous observations showing that K^+ transport by epithelia is affected by the hydromineral status of the animal (e.g. Naikkhwah and O'Donnell, 2011; O'Donnell and Ruiz-Sanchez, 2015). The uptake of Na^+, Cl^- and K^+ likely takes place by an active transport, because their concentrations measured in the haemolymph were higher than in the external medium (Table 1). The active uptake of Na^+, Cl^- and K^+ then probably osmotically draws water into the cells (Maddrell, 1969). This may also bring in small organic molecules, such as urea or glycerol (Schreiber and Eisenbeis, 1985).

The concentrations of Na^+, Cl^- and K^+ in *Orchesella* haemolymph were measured previously (Klein et al., 2008) and the relative values that we obtained for our springtails were 0.86, 1.56 and 0.85 times different, respectively. The observed differences could be caused by different methodology used in the previous study (Na^+ and K^+ determined by optical emission spectrometry, Cl^- by micropotentiometry on samples diluted in hydrazine monohydrate; compared with our approach in the Materials and Methods).

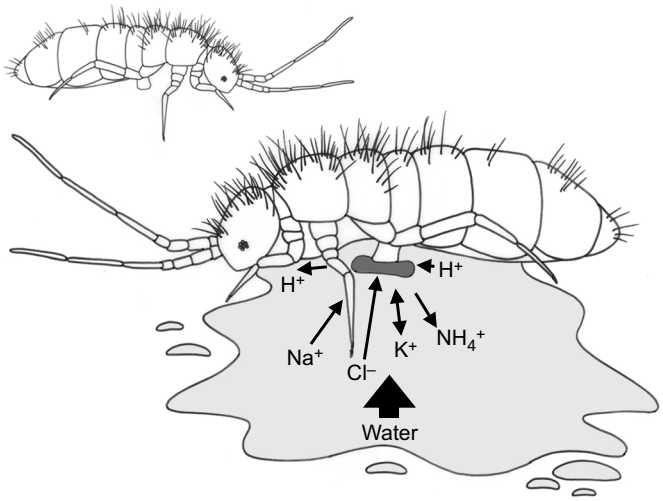

Fig. 5. How the eversible vesicles of springtails are used. Springtails on a dry surface (top) keep their eversible vesicles inside the tube of the collophore. When the springtail encounters water (bottom), either free water in moist soil or a droplet (typically in laboratory conditions), the vesicles are everted. Water is absorbed and ions are exchanged: Na^+, Cl^- are absorbed, K^+ absorbed or secreted, NH_4^+ secreted, H^+ secreted at the anterior part of the vesicles and absorbed at the posterior part. The length of the arrow corresponds only roughly to the magnitude of the ion flux.

Previous studies on *Orchesella* and *Tomocerus* have shown that the maximum rate of water absorption occurred from distilled water (Eisenbeis, 1982). The same pattern has been demonstrated for water uptake by the coxal vesicles of the jumping bristletails (Archaeognatha) *Petrobius brevistylus* (Houlihan, 1976) and *Trigoniophthalmus alternatus* (Eisenbeis, 1983). These observations suggest that water can be absorbed by a mechanism, such as the standing gradient hypothesis (Diamond and Bossert, 1967), that does not involve co-transported ions. Recycling of Na^+ and Cl^-, and differential expression of aquaporins in the vesicle epithelial membranes could allow for water uptake in the absence of net ion uptake. However, the data presented in our study indicate that absorption of water could be explained on the basis of solute-coupled water flow as we found a link between dehydration and increased Cl^- uptake (Fig. 4). Further studies on springtails with defined levels of dehydration will clarify this.

Several physiological mechanisms contribute to osmoregulation in springtails. When *Tomocerus* and *Orchesella* were exposed to drought in laboratory experiments they did not show any ability to osmoregulate (Verhoef, 1981; Verhoef and Prast, 1989). Osmotic pressure of the haemolymph rose during dehydration and decreased during rehydration. But during slow dehydration in nature, *Orchesella* regulated haemolymph osmotic pressure (Verhoef, 1981). Maintaining the stable ion concentrations of the haemolymph during dehydration in *Orchesella* involves both ion excretion and ion storage (Verhoef and Prast, 1989). Springtails inhabiting marine littoral zones, such as *Isotoma viridis* and *Hypogastrura viatica*, hyper-regulate haemolymph osmotic and ionic concentrations when exposed to low salt concentrations in the soil water (Witteveen et al., 1987). The intertidal species *Anurida*

maritima is physiologically tied to salt environment and behaves as an osmoconformer that is unable to survive fresh water conditions; to live at high salinities it produces free amino acids (FAA), such as glutamine, alanine and proline, which function as osmolytes in the haemolymph (Witteveen et al., 1987).

Similarly, accumulation of FAA and other osmolytes (sugars and polyols) was also found in soil-inhabiting (i.e. non-marine) springtails exposed to drought (Bayley and Holmstrup, 1999; Holmstrup et al., 2001; Kærsgaard et al., 2004; Holmstrup and Bayley, 2013; Holmstrup et al., 2015). The osmolytes increase the overall osmolarity of the animals and thus prevent water loss, and in springtails with highly permeable cuticle they even enable absorption of water vapour. *Orchesella*, as a representative of surface-dwelling (epedaphic) springtails, has a largely impermeable cuticle and does not produce osmolytes (sugars and polyols) in response to desiccation stress (Kærsgaard et al., 2004). This suggests that this species would be more dependent on water balance by the eversible vesicles. Determining the functional roles of the eversible vesicles in osmoregulation of springtails with diverse ecological adaptations will require experiments that combine measurements of osmolarity and haemolymph composition with measurements of ion transport across the vesicles.

An observation not anticipated from previous studies was the secretion of NH_4^+ (Fig. 3B). This suggests that the eversible vesicles also function in nitrogen (metabolic) waste excretion. Generally, terrestrial insects excrete nitrogen waste as urea or uric acid, and aquatic insects excrete primarily NH_3/NH_4^+, collectively known as ammonia (O'Donnell and Donini, 2017). While excreting ammonia is energetically advantageous, accumulation of this molecule in tissues is toxic. In aquatic animals, ammonia is carried away by the surrounding water. Perhaps the springtails use the opportunity to release NH_4^+ into the free water in the soil while water and ions are being absorbed (Fig. 5).

Measurements on whole body excretion rates of NH_4^+ were previously done by an alternative method (Sjursen and Holmstrup, 2004) and indicated that this might be the most important nitrogen waste product of springtails, as in other soil animals. It will be of interest in future studies to examine effects of variations in dietary nitrogen load and hydration status on NH_4^+ excretion by the vesicles.

Finally, we found that the eversible vesicles transport H^+ ions, although at relatively low levels (Fig. 3C and discussed above). This result suggests that the epithelium might also function in acid–base homeostasis (pH), similarly to the anal papillae of mosquito larvae (Donini and O'Donnell, 2005). We showed that H^+ is secreted in the anterior part and that there is a lower level of H^+ absorption in the posterior part. The difference in direction of the flux suggests that there is a functional specialization between the anterior and posterior part of the vesicles. It is worth noting that the H^+-selective microelectrode will detect transport of any ion that can buffer protons, such as HCO_3^-. Thus, the detected regional heterogeneity of H^+ transport may indicate transport of different ions. It is also worth noting that the anterior half of the collophore and the vesicles contain the ventral groove (linea ventralis). This groove in the cuticle extends from the labial glands in the head and contains fluid excreted by the glands (called urine), which contains uric acid (Hoffmann, 1905; Verhoef et al., 1979, 1983). Possibly, the urine in the anterior of the vesicles influences the H^+ concentration.

The eversible vesicles in the collophore are an important part of springtail physiology

The eversible vesicles are a multifunctional epithelium (Imms, 1906; Hopkin, 1997). The absorption of water is clearly important as it helps to speed up rehydration of animals living in the soil where water content might be changeable. Arctic and Antarctic springtails use dehydration as a strategy to survive extremely low temperature (Cannon and Block, 1988; Worland et al., 2010; Sørensen and Holmstrup, 2011). When favourable conditions return, and the springtails need to intake an amount of water, they 'drink' with both their mouth and the vesicles (William Block, British Antarctic Survey, personal communication).

A peculiar feature of springtail physiology is the absence of Malpighian tubules (Humbert, 1974), a key insect organ for osmo- and ionoregulation, acid–base balance and excretion. How can springtails cope without them? In insects, the Malpighian tubules (secretory epithelium) together with the hindgut (absorptive epithelium) make the 'functional kidney'. Although springtails excrete nitrogen waste by accumulating it in the midgut epithelium that is removed at each moult (springtails continue moulting as adults) (Humbert, 1974, 1978), previous research suggests that the labial glands and the eversible vesicles form the 'kidney' (Verhoef et al., 1979, 1983). Whether the secretion from the labial glands is re-absorbed at the eversible vesicles (Verhoef et al., 1983) is unclear. This will be the subject of future research.

The protocols for the inverse Ramsay assay and SIET developed here will enable future studies on the eversible vesicles. Together with molecular genetic methods, they will help to identify what specific transporters (such as ATPases, water and ion channels) function in the organs (Chintapalli et al., 2013). SIET and the methods developed in this paper may also be used in studies on the absorption of soil pollutants, such as toxic metals, by springtails (Hopkin, 1997; Roelofs et al., 2009).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.K.; Methodology: B.K., D.K., M.J.O.; Validation: B.K., D.K.; Formal analysis: D.K.; Investigation: B.K., D.K., M.J.O.; Resources: B.K., D.K., M.J.O.; Writing - original draft: B.K.; Writing - review & editing: B.K., D.K., M.J.O.; Visualization: B.K., D.K.; Supervision: M.J.O.; Project administration: B.K., D.K., M.J.O.; Funding acquisition: M.J.O.

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