© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

RESEARCH ARTICLE

Multiple functions of ion transport by the nuchal organ in embryos and neonates of the freshwater branchiopod crustacean *Daphnia magna*

Carolyn Morris and Michael O'Donnell*

ABSTRACT

The nuchal organ, also referred to as the dorsal organ or neck organ, is a dorsal structure located posteriorly to the compound eye, between the bases of the second antennae of embryonic and neonate branchiopod crustaceans such as the water flea, *Daphnia magna*. The ultrastructure of the nuchal organ is similar to ion-transporting tissues in other crustaceans, including abundant mitochondria and extensive amplification of apical and basal plasma membranes through microvilli and infoldings, but direct evidence for ion transport is lacking. We used the scanning ion-selective electrode technique to measure transport of Na^+, K^+, H^+, Cl^-, NH_4^+ and Ca^{2+} across the nuchal organ and body surface of embryos and neonates bathed in dechlorinated Hamilton tap water. Influx of Na^+ and efflux of H^+ and NH_4^+ was found to occur across the nuchal organ of both embryos and neonates. We propose that the efflux of K^+ and Cl^- across the nuchal organ in embryos is related to the expansion of the haemocoel and release of intracellular solutes into the extracellular space during development. K^+ is taken up across the nuchal organ later during development, coincident with expansion of the intracellular compartment through the development of gills and other organs. Ca^{2+} influx across the nuchal organ and body surface of neonates but not embryos is presumably related to calcification of the exoskeleton. Increases in the levels of Na^+ and Ca^{2+} in the water within the brood chamber suggest maternal provisioning of ions for uptake by the embryos. Our data thus support roles for the nuchal organ in ionoregulation, pH regulation and nitrogenous waste excretion.

KEY WORDS: pH regulation, Nitrogen excretion, Acid–base balance, Freshwater ionoregulation, Calcification

INTRODUCTION

The gill is the predominant organ for ionoregulation in euryhaline crustaceans, and the structures of the transporting cells and mechanisms involved have been well characterized in large species such as the blue crab, *Callinectes sapidus*, and the Chinese mitten crab, *Eriochier sinensis* (Henry et al., 2012; Larsen et al., 2014). Smaller crustaceans such as branchiopods pose technical challenges and have been less well studied, but in adult brine shrimp, the branchiae are thought to be the main sites of ion uptake. Classic work involving staining with $AgNO_3$ and use of $KMnO_4$ to oxidize the transporting cells indicates that the first 10 pairs of branchiae are the sites of ion excretion in adult *Artemia salina* in hypertonic conditions, and are probably the site of ion uptake in hypotonic media (Conte, 1984; Croghan, 1958). Hyperosmotic regulation in embryonic ostracods (seed shrimp) is proposed to reflect both salt reserves in the yolk of the egg and the absorption of salts by special cells located in the non-calcified zone of the inner shell layer (Aladin and Potts, 1996). In the freshwater copepod *Eurytemora affinis*, structures termed Crusalis organs are thought to function as osmoregulatory organs (Johnson et al., 2014); the organs consist of ionocytes on the penultimate leg segment of each swimming leg and are enriched in ion transport enzymes.

Another ionoregulatory structure, termed the nuchal organ (also referred to as the neck organ or dorsal gland), is present in a wide variety of crustaceans, both larval and adult, including branchiopods, copepods and malacostracans (Martin and Laverack, 1992). In branchiopods, the nuchal organ is the preferred term to describe structures that contain mitochondria-rich ion transporting cells that are probably involved in salt uptake in freshwater and salt excretion in saline water (Aladin and Potts, 1995). In the nauplius larva of the brine shrimp *Artemia salina*, there is direct evidence for salt excretion by the nuchal organ in larvae preloaded with ^{22}Na (Russler and Mangos, 1978).

Although a role for the nuchal organ in ion uptake by freshwater species has been inferred from ultrastructural studies, direct evidence for uptake is lacking. In the freshwater cladoceran *Daphnia magna*, the nuchal organ appears as an expanded portion of a dorsal ridge which runs from the front to the back of the head in first instar juveniles. In electron micrographs, the perimeter of the nuchal organ is delineated by a densely staining portion of cuticle which separates the thin cuticle covering of the nuchal organ from the thicker and less densely staining surrounding cuticle. The cells that form the nuchal organ fill much of the haemocoelic space between the cuticle and the gut, and are differentiated from surrounding squamous epidermal cells by greater apical–basal depth, extensive amplification of plasma membranes through apical microvilli and basal infoldings, and abundant mitochondria (Halcrow, 1982).

It has been suggested that the nuchal organ is most useful whilst juveniles remain in the brood chamber, particularly in the earlier stages of embryonic development when the thoracic appendages move very little (Halcrow, 1982). Cladocerans incubate their eggs in brood chambers formed by the carapace, except when laying resting eggs (Aladin and Potts, 1995), and the brood chamber remains open to the environment in most brackish and freshwater genera, including *Daphnia*. The egg membrane must therefore be impermeable until the larval organs of osmoregulation have developed. These include both the nuchal organ and the maxillary gland for water excretion. In the free-swimming neonate, the nuchal organ probably functions for about 12 h only (Halcrow, 1982).

Department of Biology, McMaster University, Hamilton, ON, Canada L8S 4K1.

*Author for correspondence (odonnell@mcmaster.ca)

M.O.D., 0000-0003-3988-6059

Received 25 July 2019; Accepted 18 October 2019

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

The nuchal organ disappears at the first embryonic moult when the animal begins to feed and the epipodites of the thoracic appendages probably assume a role in ion uptake (Aladin and Potts, 1995).

In this study, we used the scanning ion-selective electrode technique (SIET) to provide the first direct measurements of the transport of Na^+, K^+, Cl^-, H^+, Ca^{2+} and NH_4^+ across the nuchal organ and across regions of the body surface away from the organ in both embryonic and neonate *Daphnia magna*. Because development of the embryo is associated with formation of the heart and the haemocoel, changes in ion transport may be related not just to the need to replenish ions lost to the environment in freshwater but also to the conversion of the intracellular volume, typically with a Na^+/K^+ ratio much less than 1, into extracellular space with a Na^+/K^+ ratio much greater than 1. There may also be changes in ion transport associated with metabolism of yolk proteins into amino acids and subsequent synthesis of new proteins during the formation of tissues in the neonate such as the thoracic appendages and cuticle. Metabolism during development may thus lead to the formation of organic ions that may alter total cation and anion levels in the newly formed extracellular compartment. Lastly, in view of evidence that isolated embryos do not equilibrate with calcium in the environment and that calcium is transferred to the embryo from the mother (Giardini et al., 2015), we used ion-selective microelectrodes to determine whether embryos are exposed to concentrations of Ca^{2+} and other ions in the brood chamber that differ from the concentrations in the surrounding water.

MATERIALS AND METHODS

Daphnia culture

A starter culture of *Daphnia magna* Straus was obtained from a commercial supplier and maintained at room temperature (23°C) in aerated 20 l tanks of dechlorinated Hamilton tap water (DHTW). The water was sourced from Lake Ontario water, containing (in mmol l^{-1}): 1 Ca, 0.6 Na, 0.70 Cl, 0.3 Mg and 0.05 K, with titration alkalinity of 2.1 mequiv l^{-1}, hardness of ∼140 mg l^{-1} as $CaCO_3$ equivalents, and pH ∼8.0 (Hollis et al., 2001; Leonard et al., 2014). *Daphnia* were fed a 2:2:1 mixture of *Spirulina* powder: *Chlorella* powder:yeast 3 times per week.

SIET measurements

SIET measurements were made with hardware from Applicable Electronics (Forestdale, MA, USA) and Automated Scanning Electrode Technique (ASET) software (ASET-LV4, Science Wares, Falmouth, MA, USA). Micropipettes were pulled from 1.5 mm borosilicate glass (World Precision Instruments Inc., Sarasota, FL, USA) to tip diameters of ∼3 µm on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co., Novato, CA, USA). Na^+-selective microelectrodes were backfilled with 150 mmol l^{-1} NaCl and tip filled with a cocktail consisting of 3.5% Na ionophore X, 0.6% potassium tetrakis (4-chlorophenyl) borate and 95.9% 2-nitrophenyl octyl ether (Jayakannan et al., 2011). Na^+ ionophore X has a high selectivity for Na^+ over Ca^{2+} (>3000-fold) and for Na^+ over K^+ (∼400-fold). Ion-selective microelectrodes for the other ions were constructed with the following ionophores (Sigma-Aldrich, St Louis, MO, USA), with backfill and calibration solutions (in mmol l^{-1}) indicated in parentheses: K^+ ionophore I, cocktail B (150 KCl backfill, 0.5/5 KCl calibration); Ca^{2+} ionophore I, cocktail A (100 $CaCl_2$ backfill, 0.1/1/10 $CaCl_2$ calibration); H^+ ionophore I, cocktail B (100 NaCl/100 sodium citrate at pH 6 backfill, 1 Hepes, 0.6 $NaHCO_3$, 1 $CaCl_2$ at pH 6.5, pH 8.3 calibration); NH_4^+ ionophore I, cocktail A (100 NH_4Cl backfill, 0.1/1 NH_4Cl calibration); Cl^- ionophore I, cocktail A (150 KCl backfill, 0.5/5 NaCl calibration). Because Cl^--selective microelectrodes based on chloride ionophores are known to be sensitive to organic anions that may be released from tissues (Chao and Armstrong, 1987; Del Duca et al., 2011; Kondo et al., 1989; Messerli et al., 2008), SIET measurements of Cl^- flux were also made with a solid-state Cl^- microelectrode (Donini and O'Donnell, 2005) that is insensitive to organic anions such as bicarbonate and acetate (Saunders and Brown, 1977). The solid-state Cl^- microelectrode consisted of the fine tip (∼10 µm diameter) of a chlorided silver wire glued into the barrel of a glass micropipette with hot melt glue so that a piece of wire approximately 50 µm long and 10 µm in diameter protruded from the micropipette tip. To further reduce the exposed surface area of silver at the tip, the solid-state microelectrode was coated with a layer of petroleum jelly (∼5 µm thick), which was then partially removed at the tip by wiping with a small piece of tissue paper so that the exposed chlorided silver wire was reduced to approximately 10 µm in diameter and 5–10 µm in length, thus allowing finer spatial resolution for measurement of Cl^- concentration.

Measurements of Na^+, K^+, Cl^- and Ca^{2+} flux were made in DHTW. Measurements of K^+ flux were also made in DHTW containing 1 mmol l^{-1} KCl. NH_4^+ flux was measured in DHTW containing 0.1 mmol l^{-1} NH_4Cl. Preliminary measurements of H^+ flux were also made in DHTW. However, because protons may diffuse freely or in association with buffers in the saline, proton transport rates must be corrected for buffering using equations described in Messerli et al. (2006). For these experiments, a synthetic Hamilton tap water containing similar levels of Na^+, Cl^- and Ca^{2+} and known buffer concentrations was made using (in mmol l^{-1}): 0.6 $NaHCO_3$, 1 $CaCl_2$ and 1 Hepes, adjusted to pH 8. Measurements of Na^+ transport kinetics were done in water containing six concentrations of NaCl from 0.07 to 2.62 mmol l^{-1}, and 0.5 mmol l^{-1} $CaCl_2$, and Michaelis–Menten curves were fitted to the mean flux at each concentration. Measurements of Ca^{2+} transport kinetics were done in water containing 0.04–1.56 mmol l^{-1} $CaCl_2$ and 1 mmol l^{-1} NaCl. We began with 0.04 mmol l^{-1} and added $CaCl_2$ from a stock solution to approximately double the concentration for each step increase. Five concentration steps were sufficient to reach plateau values for the flux for some animals, whereas six or seven concentration steps were required for others. We therefore determined the Michaelis–Menten parameters for each animal and the mean values presented in the Results are thus the means of the K_m and V_{max} values for each animal.

Ion flux was measured in embryos and neonates, corresponding to developmental stages 5 and 6, respectively (Kast-Hutcheson et al., 2001). Stage 5 is late in embryonic maturation; the second embryonic membrane has ruptured, and the second antennae are partially extended. The antennal setae are poorly developed and the tail spine is folded against the carapace. This stage occurs 45–50 h after deposition into the brood chamber. Stage 6 corresponds to a fully developed neonate, >48 h after deposition into the brood chamber. The organism is free swimming (i.e. emerged from the brood chamber), the setae on the second antennae setae are developed and the tail spine is fully extended from the carapace.

Embryos were collected under water with the aid of a stereomicroscope. Two pairs of forceps were used to pry apart the carapace of the adult so that the embryos spilled out of the brood chamber. Neonates were collected from a Petri dish containing 10–20 gravid adults. The dish was examined at 15 min intervals and the neonates were collected by suction using a plastic transfer pipette. Embryos and neonates were placed on their sides in

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

a small depression made in petroleum jelly on the bottom of a 2 cm Petri dish. The cuticle of neonates adhered to the petroleum jelly and the lateral surface was thus uppermost, with the nuchal organ visible along the dorsal edge. For the embryos, bands of petroleum jelly were manipulated with fine forceps or a pin to form a small chamber enclosing the embryo positioned on its side so that approximately half the body surface, including the nuchal organ, was visible from above through a stereomicroscope. SIET measurements were made at the centre of the nuchal organ and at locations 20 µm anterior and posterior to the centre. At each measurement site, computer-controlled stepper motors moved the ion-selective microelectrode between an inner position within 3–5 µm of the nuchal organ and an outer position 30 or 50 µm further away along a line perpendicular to the tissue surface. Three replicate measurements were made at each site, and the mean voltage difference between the two limits of excursion was converted into a concentration difference using Eqn 1:

$$\Delta C = C_{\mathrm{B}} \cdot 10^{(\Delta V/S)} - C_{\mathrm{B}}, \quad (1)$$

where ΔC is the concentration difference between the two points (in µmol cm^{-3}); C_{B} is the background ion concentration (in µmol cm^{-3}), calculated as the average of the concentrations at each point measured; ΔV is the voltage difference between the two limits of excursion obtained from ASET-LV4 (in mV); and S is the slope of the electrode (in mV) for a 10-fold change in ion concentration.

Flux was estimated from the measured concentration gradients using Fick's law:

$$J_{\mathrm{I}} = D_{\mathrm{I}} \Delta C / \Delta x, \quad (2)$$

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+}; 9.31×10^{-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 inner and outer excursion limits (in cm). Positive values of J_{I} denote efflux, from the tissue surface to the water, and negative values denote influx into the embryo or neonate.

Flux at each of the three sites was averaged, and then a mean value for the three sites was calculated. The typical interval between removal of the embryo from the brood chamber and the first flux measurement was 2–3 min. To determine whether there were changes over time associated with the securing of the embryos and neonates in the Petri dish, five sets of measurements at 3 min intervals were made for each preparation. To determine whether ion flux occurred at sites other than the nuchal organ, control measurements were made along the postero-lateral surface of the carapace, >100 µm from the nuchal organ.

Measurement of brood chamber ion concentrations

Ion-selective microelectrodes fabricated as described above were used to measure the concentrations of Na^+, K^+, NH_4^+, Ca^{2+}, H^+ and Cl^- in the brood chamber of *Daphnia* in three different states: without eggs in the brood chamber, with eggs, and with embryos. Adult *Daphnia* were secured with petroleum jelly to the bottom of a Petri dish filled with DHTW and a micromanipulator was used to position the microelectrode tip in the brood chamber. A second type of liquid membrane Cl^- selective microelectrode based on 2% Cl^- ionophore II, 0.03% tridodecylmethylammonium chloride and 97.97% 2-nitrophenyl octyl ether (Messerli et al., 2008) was used in some of the measurements of the brood chamber water, as discussed below. Potential differences between the ion-selective microelectrode and a reference electrode consisting of a Ag/AgCl pellet connected to the bath through an agar bridge containing 150 mmol l^{-1} KCl in 4% agar were measured using a high-impedance electrometer (pH AMP, ADInstruments, Bella Vista, NSW, Australia) connected to a data acquisition system (Powerlab) running LabChart software.

Statistics

Graphing and statistical tests of significance were done in GraphPad Prism 6 (San Diego, CA, USA). Changes in ion flux at the nuchal organ over time and between embryos and neonates at the same time points were assessed with two-way ANOVA followed by Šidák's multiple comparisons test. Differences between the magnitude of ion flux at sites away from the nuchal organ and zero were assessed with a one-sample *t*-test. Differences were considered significant if $P<0.05$.

RESULTS

Na^+ influx at the nuchal organ of embryos and neonates

The nuchal organ in embryos and neonates of *D. magna* is located near the base of the second antennae, opposite the rostrum and overlapping the anterior portion of the heart (Fig. 1A,B). Neonates were readily distinguishable from embryos by the polygonal patterning of the cuticle, well-developed setae on the second antennae, a more pronounced dorsal ridge and a prominent tail spine, which extended away from the carapace (Fig. 1). Na^+ influx was localized to the nuchal organ, declining to near-zero values at the junction of the nuchal organ and the surrounding cuticle (Fig. 1C). The influx of Na^+ at the nuchal organ was sustained for five sets of measurements made at 3 min intervals in both embryos and neonates (Fig. 2). There were no significant differences in magnitude of the flux between embryos and neonates at each time point, and there were no significant changes over time (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). One-sample *t*-tests indicated that Na^+ flux at sites away from the nuchal organ was not significantly different from zero in embryos (4.4±4.0 pmol cm^{-2} s^{-1}, N=6) or neonates (−22.4±13.8 pmol cm^{-2} s^{-1}, N=6).

Analysis of transport kinetics revealed that the maximum rate of Na^+ influx (V_{max}) across the nuchal organ of embryos was 518.1±25 pmol cm^{-2} s^{-1} and the bath Na^+ concentration at which transport was half-maximal (K_{m}) was 0.433±0.06 mmol l^{-1} (Fig. 3). The latter value is below the measured Na^+ concentration in the DHTW used in these experiments (0.74 mmol l^{-1}). Comparison of Figs 2 and 3 indicated that flux in neonates measured with microelectrodes based on Na^+ ionophore X in DHTW water containing 0.74 mmol l^{-1} Na^+ was ∼−320 pmol cm^{-2} s^{-1}, similar to the value of −315 pmol cm^{-2} s^{-1} predicted using 0.74 mmol l^{-1} and the Michaelis–Menten parameters derived from measurements in water containing 0.07–2.62 mmol l^{-1} NaCl and 0.5 mmol l^{-1} $CaCl_2$ (Fig. 3).

K^+ flux at the nuchal organ

There were pronounced changes in K^+ flux at the nuchal organ during development. In DHTW containing 1 mmol l^{-1} KCl, K^+ influx at the nuchal organ of neonates (∼−50 pmol cm^{-2} s^{-1}; Fig. 4A) was approximately one-quarter of the magnitude of Na^+ influx. By contrast, there was an efflux of K^+ from the nuchal organ of embryos of ∼80 pmol cm^{-2} s^{-1}. One-sample *t*-tests indicated that

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

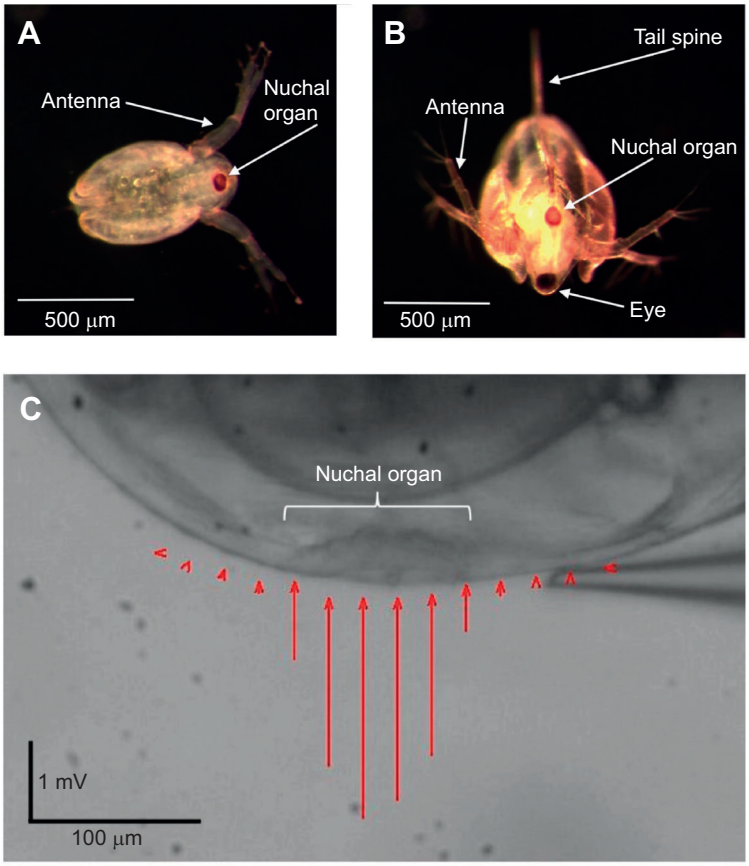

Fig. 1. The nuchal organ in *Daphnia magna*. (A) Embryo and (B) neonate preparations stained with 1% silver nitrate solution to enhance visibility of the nuchal organ. (C) Voltage differences measured with a Na^+-selective microelectrode positioned at 14 locations over or near the nuchal organ. The tip of each arrow indicates the location of the microelectrode tip at the inner excursion limit during measurements using the scanning ion-selective electrode technique (SIET). The length of each arrow corresponds to the voltage difference between the inner and outer excursion limits when the microelectrode was moved orthogonal to the tissue surface from the inner excursion limit to a position 50 μm further away. The outline of the nuchal organ is indicated by the white bracket.

K^+ flux at sites away from the nuchal organ was not significantly different from zero in embryos (4.1±2.3 pmol cm^{-2} s^{-1}, N=6) or neonates (−0.5±1.5 pmol cm^{-2} s^{-1}, N=6). K^+ flux was also measured at the nuchal organ of neonates bathed in DHTW without any added K^+. This water contained 0.04 mmol K^+ and there was an influx of K^+ of −10±2.2 pmol cm^{-2} s^{-1} (N=5), approximately 20% of the influx seen in water containing 1 mmol l^{-1} K^+. For five embryos bathed in DHTW without any added K^+, the water near the nuchal organ contained 0.078 mmol K^+ and there was a K^+ efflux of 59.4±10.2 pmol cm^{-2} s^{-1} across the nuchal organ, approximately 75% of the efflux seen in water containing 1 mmol l^{-1} K^+.

Cl^- efflux at the nuchal organ of embryos and neonates

Measurements of Cl^- flux at the nuchal organ with microelectrodes based on Cl^- ionophore I, cocktail A, indicated a sustained efflux of Cl^- in both embryos and neonates (Fig. 4B). There were no significant differences in the magnitude of Cl^- flux between embryos and neonates at each time point, and there were no significant changes over time (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). Cl^--selective microelectrodes based on Cl^- ionophore I are known to be sensitive to organic anions, and we therefore measured Cl^- flux at the nuchal organ with solid-state Cl^- microelectrodes (Fig. 4C). These measurements also revealed a sustained efflux of Cl^- at the

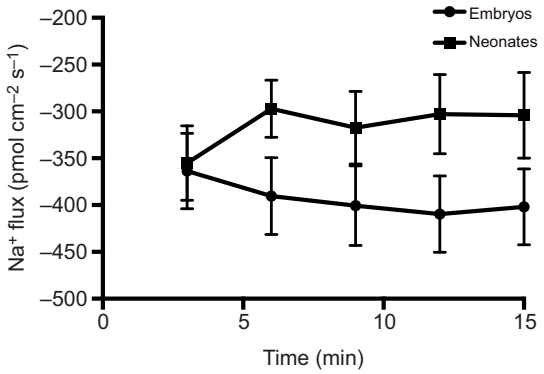

Fig. 2. Na^+ flux at the nuchal organ. Data (means±s.e.m.) are shown for embryos (N=10) and neonates (N=7) measured at 3 min intervals. In this and all subsequent figures, negative values correspond to influx and positive values correspond to efflux (i.e. from nuchal organ to water).

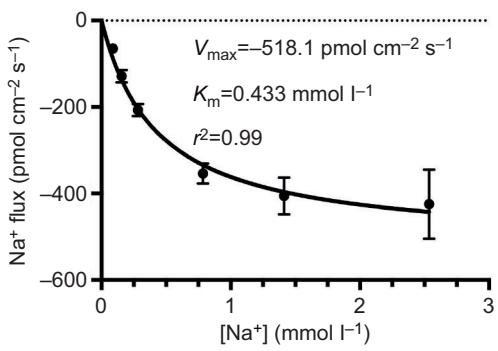

Fig. 3. Na^+ flux at the nuchal organ as a function of water Na^+ concentration. Each point shows the mean±s.e.m. for N=6 embryos. The water contained NaCl at the indicated concentration plus 0.5 mmol l^{-1} $CaCl_2$. The solid line represents the fit to the Michaelis–Menten equation by non-linear regression analysis.

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

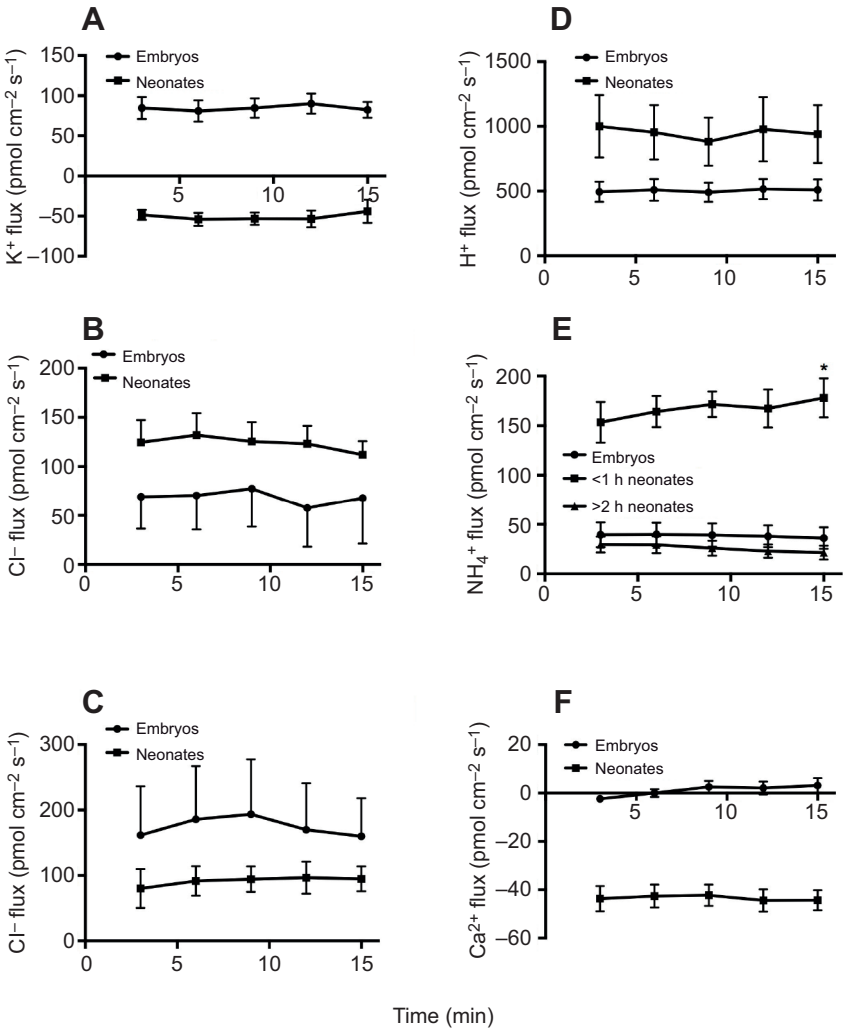

Fig. 4. K^+, Cl^-, H^+, NH_4^+ and Ca^{2+} flux at the nuchal organ. (A) K^+ flux at the nuchal organ of embryos (*N*=8) and neonates (*N*=8). (B) Cl^- flux at the nuchal organ of embryos (*N*=10) and neonates (*N*=12) measured with microelectrodes based on Cl^- ionophore I, cocktail A. (C) Cl^- flux at the nuchal organ of embryos (*N*=5) and neonates (*N*=7) with solid-state Cl^- microelectrodes. (D) H^+ flux at the nuchal organ of embryos (*N*=10) and neonates (*N*=9). (E) NH_4^+ flux at the nuchal organ of embryos (*N*=8), neonates <1 h post-emergence (*N*=6) and neonates >2 h post-emergence (*N*=10). The asterisk denotes a significant difference between flux at the indicated time point relative to that at 3 min (2-way repeated measures ANOVA followed by Šidák's multiple comparisons test). (F) Ca^{2+} flux at the nuchal organ of embryos (*N*=7) and neonates (*N*=8). Measurements were obtained at 3 min intervals and data are shown as means ±s.e.m. In B and C, error bars (s.e.m.) for some points have been omitted for clarity.

nuchal organ; there were no significant differences in the magnitude of Cl^- flux between embryos and neonates at each time point, and there were no significant changes over time (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). Moreover, there were no significant differences between the magnitude of Cl^- flux measured with solid-state Cl^- microelectrodes relative to that based on Cl^- ionophore I in embryos or neonates (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). This last result indicates that Cl^- flux at the nuchal organ was not due to interference by organic anions on the Cl^--selective microelectrodes based on Cl^- ionophore I. One-sample *t*-tests indicated that Cl^- flux at sites away from the nuchal organ was not significantly different from zero in embryos (3.2±5.7 pmol cm^{-2} s^{-1}, *N*=6) or neonates (−2.0±9.4 pmol cm^{-2} s^{-1}, *N*=6).

H^+ efflux at the nuchal organ

There was an efflux of H^+ from the nuchal organ of embryos of 3.2±1.1 pmol cm^{-2} s^{-1} (*N*=7) after 15 min in DHTW. One-sample *t*-tests indicated that H^+ flux at sites away from the nuchal organ was ∼3% of that at the nuchal organ, but was significantly different from zero (0.08±0.02 pmol cm^{-2} s^{-1}, *N*=6). Although most protons diffuse in association with a buffer in relatively hard water such as DHTW, it was not possible to correct for buffer effects because of uncertainties regarding the precise concentrations of carbonate, bicarbonate and dissolved organic matter. H^+ flux was therefore measured in a synthetic Hamilton tap water of known ionic and buffer composition and the raw flux was corrected for buffering using the equations of Messerli et al. (2006). There were no significant changes in corrected H^+ flux over time (Fig. 4D), but the larger mean H^+ efflux in neonates relative to embryos was close to significance (*P*=0.06; two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). One-sample *t*-tests indicated that H^+ flux at sites away from the nuchal organ of embryos in synthetic Hamilton tap water was less than 1% of that at the nuchal organ, but was significantly different from zero (8.1±2.0 pmol cm^{-2} s^{-1}, *N*=6). H^+ flux at sites away from the nuchal organ of neonates was not significantly different from zero (−4.7±6.3 pmol cm^{-2} s^{-1}, *N*=6).

NH_4^+ efflux at the nuchal organ

Preliminary measurements indicated that there were dramatic changes in the magnitude of NH_4^+ efflux from the nuchal organ during development. Measurements were therefore made in neonates within 1 h of emergence from the adult and at >2 h after emergence.

There were no significant changes in NH_4^+ efflux from the nuchal organ of embryos or >2 h neonates over time. However, NH_4^+ efflux from neonates within 1 h of emergence from the brood chamber was 4- to 5-fold greater than that in embryos or in neonates >2 h after emergence (Fig. 4E) and there was a significant increase

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

in the efflux at 15 min relative to that at 3 min in neonates <1 h after emergence (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). One-sample *t*-tests indicated that NH_4^+ efflux at sites away from the nuchal organ of neonates <1 h after emergence was less than 1% of that at the nuchal organ, but was significantly different from zero (1.0±0.4 pmol cm^{-2} s^{-1}, N=6). NH_4^+ flux at sites away from the nuchal organ of embryos was not significantly different from zero (1.5±0.9 pmol cm^{-2} s^{-1}, N=6).

Ca^{2+} transport across the body surface and nuchal organ of embryos and neonates

In contrast to transport of other ions, Ca^{2+} transport was not confined to the nuchal organ. In embryos, there was a small influx of Ca^{2+} at the nuchal organ at 3 min (Fig. 4F), but the value at the nuchal organ (−2.4±1.3 pmol cm^{-2} s^{-1}, N=7) was not significantly different from that away from the nuchal organ (−5.2± 1.5 pmol cm^{-2} s^{-1}, N=7). Ca^{2+} influx across the nuchal organ in embryos was not sustained, and was not significantly different from zero after the first measurement at 3 min (Fig. 4F). Ca^{2+} influx increased dramatically in neonates relative to embryos. However, the influx over the nuchal organ at 3 min (−43.6± 5.2 pmol cm^{-2} s^{-1}, N=8) was not significantly larger than that over the body surface at sites away from the nuchal organ (−27.7± 8.7 pmol cm^{-2} s^{-1}, N=6), consistent with Ca^{2+} uptake over the entire exoskeleton. Flux at sites away from the nuchal organ for all ions measured is summarized in Table S1.

Neonates were exposed to five to seven Ca^{2+} concentrations between 0.04 and 1.56 mmol l^{-1} for analysis of transport kinetics (Table S2). The Michaelis–Menten parameters calculated by non-linear regression for each neonate (N=6) were: K_m=0.146± 0.040 mmol l^{-1} and V_{max}=−68.5±15.3 pmol cm^{-2} s^{-1}. The mean R^2 value for the non-linear regression equations was 0.93± 0.02 (range 0.84 to 1.00).

Ion concentrations in the brood chamber

The concentrations of K^+, Na^+, NH_4^+ and Ca^{2+} in the brood chamber were 2- to 4-fold higher than those in the bathing water for *Daphnia* with or without eggs (Fig. 5A–D). For *Daphnia* with embryos in the brood chamber, the concentrations of K^+ and NH_4^+ were 24% and 126%, respectively, above those in the water, whereas the concentrations of Na^+ and Ca^{2+} were within 5% of those in the bath water. Chloride concentrations in the brood chamber were 3- to 4-fold higher than those in the bathing water for *Daphnia* with or without eggs (Fig. 5E). The larger size of the tip of the solid-state Cl^- electrode relative to the liquid membrane ion-selective microelectrodes precluded measurement of Cl^- concentration within the brood chamber of *Daphnia* containing embryos. Attempts to measure brood chamber Cl^- concentration with a liquid membrane Cl^- microelectrode based on Cl^- ionophore I, cocktail A were discontinued because there was evidence that some component within the brood chamber interfered with the microelectrode. The time required to respond to a change in Cl^- concentration increased from a few seconds to >15 min after the microelectrode tip had been positioned within the brood chamber (N=17; data not shown). Measurements with microelectrodes based on chloride ionophore II (N=8, data not shown) were also unsuccessful; the response time increased and the slope of the microelectrode decreased after sampling of the brood chamber. The pH within the brood chamber did not differ significantly from that in the bath in *Daphnia* with or without eggs or embryos (Fig. 5F).

DISCUSSION

Our results provide direct evidence for a role of the nuchal organ in Na^+ uptake, pH regulation and ammonia excretion (Fig. 6). We suggest below that transport of K^+ and Cl^- across the nuchal organ may be related to the formation and expansion of the haemocoel as the circulatory system develops. Our results also show influx of Ca^{2+} across the cuticle of neonates but not embryos, consistent with calcification of the exoskeleton through deposition of calcium salts.

Contribution of Na^+ influx at the nuchal organ to ionoregulation

Our results indicate a sustained influx of Na^+ at the nuchal organ of both embryos and neonates. Kinetic analysis of the influx suggests that the K_m (0.433 mmol l^{-1}) was slightly below the level of Na^+ in the water in which the animals were reared. The significance of this influx to ionoregulation in the embryos and neonates can be appreciated through estimates of haemolymph volume and nuchal organ surface area. Approximating the embryo shape as an ellipsoid with major and minor axes of 0.700 and 0.430 mm (Fig. 1), respectively, gives a volume of $4/3\pi\times0.350\times0.215\times0.215$=0.068 mm^3. It has been estimated that haemolymph volume in adult *D. magna* corresponds to 61% of animal volume (Kobayashi and Nezu, 1986). An upper limit of haemolymph volume in the embryo is thus 0.61×0.068=0.041 µl. Based on a diameter of the nuchal organ (Fig. 1) of 80 µm, its area is 5×10^{-5} cm^2. Transport across this area at rate of ∼−320 pmol cm^{-2} s^{-1} (Fig. 2) is equivalent to 0.057 nmol h^{-1}. Our measurements of Na^+ concentration in adult *D. magna* (C.M. and M.J.O., unpublished data) indicate a value of 51 mmol l^{-1} for animals reared in DHTW; the estimated haemocoel Na^+ content is thus: 0.051×0.041=2.1 nmol. Complete replacement of Na^+ in the haemocoel of the embryo could be achieved by ∼37 h of transport (2.1/0.057) across the nuchal organ, a similar magnitude to the time required (∼24 h) for development from the time of appearance of the nuchal organ (coincident with appearance of the antennae; Kast-Hutcheson et al., 2001; Mittmann et al., 2014) through to the emergence of a free-swimming neonate. A smaller haemolymph volume as a proportion of animal volume in embryos and neonates relative to adults will decrease the time required for transport of the entire haemocoel content of Na^+. Clearly, haemolymph volume is negligible in the early embryo before the haemocoel is formed, and there may be significant Na^+ content of the egg, in which case the nuchal organ's ionoregulatory role may be partly to replenish passive losses of Na^+ across other body surfaces. Later in development, there may be additional uptake of Na^+ across the epipodites (gills) of the thoracopods (thoracic appendages), which are present in embryos by the time the second antennae have elongated (Mittmann et al., 2014). It has been suggested that the nuchal organ is most useful during the juvenile's stay in the brood chamber, and that it functions for <<12 h in the neonate, before the nuchal organ switches from ion transport to cuticle secretion (Halcrow, 1982).

A previous study of Na^+ uptake by *Daphnia* investigated ion exchange across the whole animal (Bianchini and Wood, 2008) and therefore multiple sites (gut, gill) and mechanisms may have contributed. These authors proposed that a vacuolar-type H^+-ATPase sensitive to bafilomycin in the apical membrane of cells involved in uptake from the water by neonates creates an electrical gradient favouring Na^+ uptake through channels sensitive to the drug phenamil. Such a proposal is consistent with our findings of outwardly directed H^+ flux and inwardly directed Na^+ flux at the nuchal organ. The K_m for Na^+ uptake by whole neonates in that study (0.351 mmol l^{-1}; Bianchini and Wood, 2008) is similar to the K_m for Na^+ uptake at the nuchal organ (0.433 mmol l^{-1}). Further

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

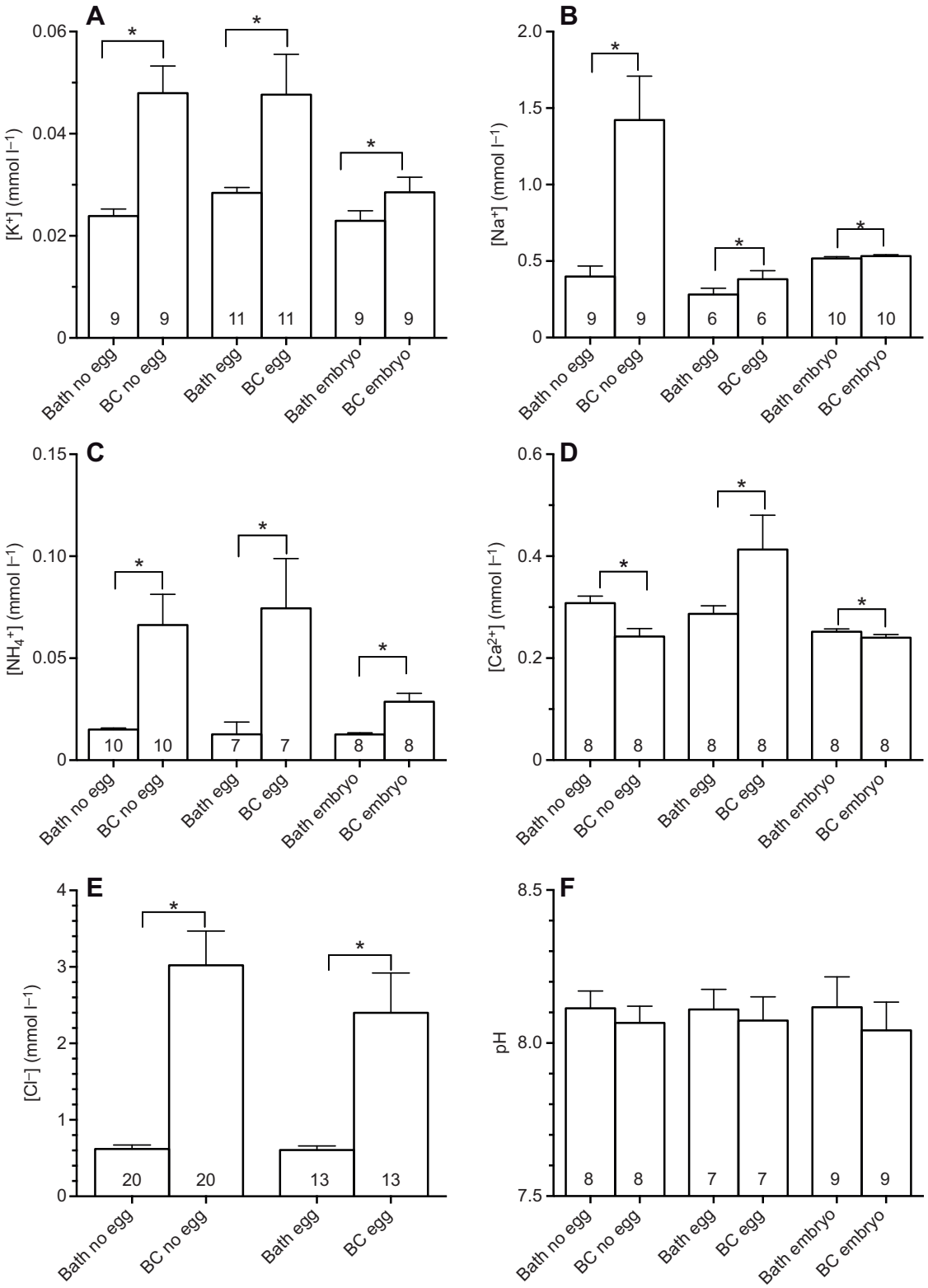

Fig. 5. Ion concentration and pH in the brood chamber of adult *Daphnia* with no eggs, with eggs or with embryos. Ion concentrations (A, K^+; B, Na^+; C, NH_4^+; D, Ca^{2+}; E, Cl^-) and pH (F) were measured in dechlorinated Hamilton tap water in the bath >1 mm away from the *Daphnia* and compared with measurements when the ion-selective microelectrode tip was positioned within the brood chamber (BC). Data are means±s.e.m. Asterisks indicate significant differences (P<0.05) in means as measured by a paired t-test. The number of animals in each condition is given within each bar.

studies of the nuchal organ using SIET will allow the role of specific transporters in Na^+ influx across a single epithelium to be assessed through the application of transport inhibitors and toxins. Silver, for example, causes mortality at extremely low concentrations through inhibition of sodium uptake pathways (Bianchini and Wood, 2003), and it will be of interest in future studies to examine the influence of

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

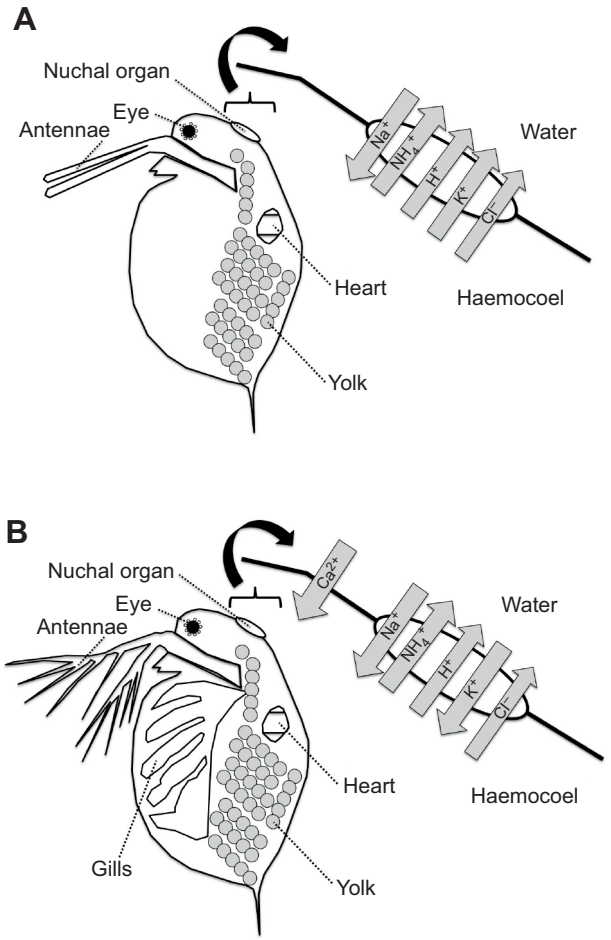

Fig. 6. Schematic diagram summarizing ion flux across the nuchal organ and body surface of *D. magna*. (A) Embryo and (B) neonate. The nuchal organ is the site of influx of Na^+ and efflux of H^+, NH_4^+ and Cl^-. Transport of these ions across the carapace or dorsal ridge is negligible. The nuchal organ is also the site of K^+ efflux in embryos and K^+ influx in neonates. Ca^{2+} transport across the body surface and nuchal organ is negligible in embryos, but there is influx of Ca^{2+} across the body surface in neonates.

silver on Na^+ influx across the nuchal organ. Whole-animal studies have also shown that both the epithelial Na^+ channel blocker phenamil and the vacuolar H^+-ATPase inhibitor bafilomycin A1 inhibit Na^+ uptake in *Daphnia* neonates (Bianchini and Wood, 2008), and that the Na^+ channel and Na^+:H^+ exchange inhibitor amiloride blocks Na^+ uptake in adults (Glover and Wood, 2005). The latter study also revealed complex relationships between ambient Ca^{2+} levels and Na^+ uptake, with Ca^{2+} inhibiting Na^+ uptake at low Na^+ levels, but stimulating Na^+ uptake at high Na^+ levels. Acidic pH severely inhibits sodium influx in adults when calcium concentration is high (Glover and Wood, 2005). Additional studies using SIET will allow analysis of the interrelationships of water pH and hardness on Na^+ uptake by the nuchal organ.

Roles of the nuchal organ in acid–base balance and nitrogen excretion

The magnitude of H^+ efflux from the nuchal organ of embryos and neonates bathed in synthetic Hamilton tap water was approximately 300-fold larger than the uncorrected flux calculated from the measured H^+ concentration values at two points within the unstirred layer. This difference between corrected and uncorrected H^+ flux is a common finding in media containing significant levels of buffers. In a study of mammalian gastric oxyntic cells, buffers enhanced the diffusion of protons by a factor of 2249 (i.e. 1374 by 1 mmol l^{-1} Hepes and 875 by 5 mmol l^{-1} HCO_3^-; Demarest and Morgan, 1995). The large flux of H^+ across the nuchal organ suggests a significant role in acid–base balance, particularly as H^+ flux across the body surface was only 1% of that at the nuchal organ. An efflux of H^+ could be used to drive Na^+ uptake through a Na^+–H^+ exchanger. Alternatively, efflux of H^+ could indicate the activity of a vacuolar H^+-ATPase known to be implicated in Na^+ uptake, or hydration of metabolic CO_2 passing out through the nuchal organ, followed by hydration of CO_2 and dissociation of carbonic acid into H^+ and HCO_3^- through the actions of carbonic anhydrase.

The efflux of NH_4^+ across the nuchal organ of both embryos and neonates may be a consequence of catabolism of protein from yolk granules into amino acids for energy production in embryos and neonates. Given the large H^+ efflux across the nuchal organ, the NH_4^+ gradient measured with SIET could be a consequence of diffusion trapping of NH_3 that has diffused across the nuchal organ. It is worth noting in this context that ammonia excretion in adult *Daphnia* is enhanced at low environmental pH relative to the rate of excretion at circumneutral pH (Al-Reasi et al., 2013).

Although our measurements indicated efflux of ammonia across the nuchal organ, it must be noted that ammonium ionophore I, cocktail A is only 4 times more selective for NH_4^+ than for K^+. Efflux of K^+ from the nuchal organ of embryos may thus lead to an overestimate of apparent NH_4^+ efflux, and influx of K^+ across the nuchal organ of neonates will lead to an underestimate of apparent NH_4^+ efflux. A corrected flux can be estimated by accounting for the effects of K^+ on the NH_4^+-selective microelectrode during SIET measurements. For embryos, the corrected NH_4^+ flux is 55% of the uncorrected value, whereas interference from K^+ at the nuchal organ of neonates results in a small underestimate (2%) of the NH_4^+ flux (see Appendix).

Transport of K^+ and Cl^- across the nuchal organ

Most freshwater animals require uptake of both Na^+ and Cl^- to replace passive loss of these ions. The efflux of Cl^- across the nuchal organ was, therefore, an unexpected finding. We suggest that Cl^- efflux reflects displacement of extracellular Cl^- by production of other anions such as bicarbonate. *Daphnia pulex* is known to have both elevated levels of bicarbonate in the haemolymph (20.9 mmol l^{-1}) and an elevated extracellular pH of 8.33 (Weber and Pirow, 2009). If similar conditions apply to *D. magna*, then both bicarbonate and negative charges on circulating amino acids, peptides and proteins could lead to an anion surplus, favouring efflux of Cl^- across the nuchal organ. Efflux of K^+ from embryos bathed in water containing 1 mm K^+ may also be a consequence of developmental processes. Development of an egg into an embryo requires the formation of extracellular space (typically with $Na^+/K^+ >> 1$). If there were no change in the volume of cytoplasm (with $Na^+/K^+ << 1$), formation of extracellular fluid would require uptake of both Na^+ and K^+. We suggest that intracellular volume is converted into extracellular volume, and that release of cytoplasmic K^+ into the extracellular environment may thus lead to excess K^+ in the extracellular space during early development and expansion of the haemocoel. Later in development, there is an influx of K^+ across the nuchal organ of neonates. This influx is coincident with tissue development (e.g. gills, gut, epidermal cells) that re-expands total intracellular volume, necessitating uptake of K^+. Although we have no data indicating changes in total intracellular volume during development, the smaller number of yolk granules in neonates relative to embryos is consistent with breakdown of the yolk and release of ions.

Journal of Experimental Biology (2019) 222, jeb211128. doi:10.1242/jeb.211128

Influx of Ca^{2+} across the body surface in neonates

Our SIET measurements indicating negligible Ca^{2+} transport across the nuchal organ or body surface of embryos is consistent with an earlier study which used radioactively labelled calcium (^{45}Ca) to trace calcium from mothers to embryos (Giardini et al., 2015). That study demonstrated that calcium is transferred to the embryo from the mother, and that isolated embryos do not equilibrate with calcium in the environment.

SIET measurements indicated Ca^{2+} influx in neonates, but there was no difference in the magnitude of the Ca^{2+} flux across the nuchal organ relative to sites away from the nuchal organ, when the Ca^{2+}-selective microelectrode tip was positioned over the posterior regions of the carapace. *Daphnia* require dissolved calcium to harden the new carapace post-moult, and previous studies have shown that the necessary Ca^{2+} is acquired by uptake from the environment (Tan and Wang, 2009). Our measurements of Ca^{2+} kinetics derived a K_m of 0.146 mmol l^{-1}, considerably below the levels in the relatively hard water (dechlorinated Hamilton tap water) used for rearing *D. magna* in this study. The efficiency of Ca^{2+} uptake presumably aids rapid calcification of the cuticle.

Further studies using SIET will aid analysis of Ca^{2+} transport across the body surface of *Daphnia* in low-Ca^{2+} waters, particularly in neonates, as juveniles are more sensitive to calcium deficiency than adults (Hessen et al., 2000). Such studies could examine the influence of water chemistry (pH, HCO_3^-, hardness) and temperature on Ca^{2+} uptake, and the possible impacts of freshwater acidification from anthropogenic increases in atmospheric CO_2.

Maternal provisioning of ions

Increases in the concentration of K^+, Na^+ and Cl^- in the empty brood chamber of *D. magna* above the corresponding values in the surrounding water indicate that these ions are released from the female. Care was taken to avoid touching the surface of the brood chamber when positioning the microelectrode tip, and the finding of lower Ca^{2+} concentrations in the brood chamber of *D. magna* without eggs suggests that elevated concentrations of the other ions are not simply the result of leakage following damage to the wall of the brood chamber. One caveat is that for measurements with the solid-state Cl^- microelectrode, the larger tip size relative to that of the liquid membrane ion-selective microelectrodes made it more likely that the surface of the brood chamber was contacted by the microelectrode tip during measurements of Cl^- concentration. The concentration of each ion species within the brood chamber will reflect the rate of ion release from the female, uptake or release by the egg or embryo, and convective and/or diffusive exchange of the brood chamber water with the water outside the female. When eggs are present in the brood chamber, the increases in concentration of Na^+, K^+ and Cl^- in the brood chamber could result from release from either the female or the egg, but irrespective of the source of ions in the brood chamber, these increases would tend to reduce any passive loss of ions from the developing eggs. The increased concentration of NH_4^+ in the brood chamber above that in the bathing water, by contrast, creates a larger gradient opposing efflux of NH_4^+ out of the egg or embryo if ammonia is transported as the ion (but not if excretion is occurring as the gas NH_3, which is then trapped as the ion by combining with H^+ to form NH_4^+). Measurements of Ca^{2+} concentration in the brood chamber revealed a complex pattern of changes. Although the concentration of Ca^{2+} in the brood chamber of *D. magna* was slightly lower than the bathing water around *D. magna* with no eggs in the brood chamber, the increase in Ca^{2+} concentration above that in the bathing water when eggs were present is consistent with a previous suggestion of maternal provisioning that was based on the flux of Ca^{45} (Giardini et al., 2015). However, this raises the question of how Ca^{2+} is taken up through the egg membranes, which are assumed to be impermeable to ions to minimize ion loss by the eggs before development of ion-transporting organs. Although we saw only transient uptake of Ca^{2+} by isolated embryos, it is conceivable that uptake is sustained in the ionic and hormonal milieu within the brood chamber. It will be of interest in future studies to determine whether maternal provisioning of Ca^{2+} through release of Ca^{2+} into the brood chamber is of greater significance for *Daphnia* reared in soft water, given that effects of low Ca on growth rate are most apparent during the first days after hatching, reflecting the higher Ca demands of the early juveniles (Hessen et al., 2000). It is worth noting that eggs of land isopods (suborder Oniscidea) are brooded in a fluid-filled maternal marsupium until a few days following the second embryonic moult and that there is evidence for maternal control of the marsupial environment (Surbida and Wright, 2001). Eggs of *Armadillidium vulgare* possess a well-developed dorsal organ underlying a broad silver-staining saddle on the vitelline membrane. Like the nuchal organ of *Daphnia*, the dorsal organ has been implicated in ion regulation and acid excretion, but it also plays a role in calcium provisioning (Wright and O'Donnell, 2010).

APPENDIX

Correcting NH_4^+ flux for interference by K^+ on NH_4^+-selective microelectrodes

Correction for this interference requires estimation of the concentration of K^+ at the inner and outer limits of microelectrode excursion. By re-arranging Fick's equation (see Eqn 2 in Materials and Methods) to solve for ΔC, a K^+ efflux in embryos in DHTW of 59.4 pmol cm^{-2} s^{-1} corresponds to ΔC of 0.0155 mmol l^{-1}. The mean K^+ concentration in the unstirred layer near the nuchal organ in DHTW was 0.078 mmol l^{-1}, so the K^+ concentration at the inner and outer limits of microelectrode excursion can thus be estimated as 0.078+(0.0155/2)=0.86 mmol l^{-1} and 0.078−(0.0155/2)=0.70 mmol l^{-1}, respectively. For embryos in K^+ in DHTW containing 0.1 mmol l^{-1} NH_4^+, the concentration of NH_4^+ near the nuchal organ was 0.14 mmol l^{-1} and the uncorrected NH_4^+ efflux was 36 pmol cm^{-2} s^{-1}, corresponding to ΔC of 0.0086 mmol l^{-1} NH_4^+, and uncorrected NH_4^+ concentrations at the inner and outer excursion limits of 0.144 and 0.136 mmol l^{-1}. The selectivity coefficient for NH_4^+ microelectrodes based on ammonium ionophore I is 0.25. The corrected NH_4^+ concentration at the inner and outer limits of electrode excursion is thus 0.144−(0.25×0.086)=0.123 mmol l^{-1} and 0.136−(0.25×0.070)=0.118 mmol l^{-1}. The corrected ΔC is therefore 0.0047 mmol l^{-1} and the corrected NH_4^+ efflux is 19.8 pmol cm^{-2} s^{-1}, approximately 55% of the uncorrected value. Corresponding calculations for neonates within 1 h of emergence indicate that interference from K^+ produces only a small underestimate (2%) of the NH_4^+ efflux.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.M., M.J.O.; Methodology: C.M., M.J.O.; Formal analysis: C.M., M.J.O.; Investigation: C.M., M.J.O.; Writing - original draft: C.M., M.J.O.; Writing - review & editing: C.M., M.J.O.; Supervision: M.J.O.; Project administration: M.J.O.; Funding acquisition: M.J.O.

Funding

This study was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to M.J.O. (RGPIN-2015-05359).

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.211128.supplemental

References

Al-Reasi, H. A., Yusuf, U., Smith, D. S. and Wood, C. M. (2013). The effect of dissolved organic matter (DOM) on sodium transport and nitrogenous waste excretion of the freshwater cladoceran (Daphnia magna) at circumneutral and low pH. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **158**, 207-215. doi:10.1016/j.cbpc.2013.08.004

Aladin, N. V. and Potts, W. T. W. (1995). Osmoregulatory capacity of the Cladocera. *J. Comp. Physiol. B* **164**, 671-683. doi:10.1007/BF00389810

Aladin, N. V. and Potts, W. T. W. (1996). The osmoregulatory capacity of the Ostracoda. *J. Comp. Physiol. B* **166**, 215-222. doi:10.1007/BF00263985

Bianchini, A. and Wood, C. M. (2003). Mechanism of acute silver toxicity in *Daphnia magna*. *Environ. Toxicol. Chem.* **22**, 1361-1367. doi:10.1002/etc.5620220624

Bianchini, A. and Wood, C. M. (2008). Sodium uptake in different life stages of crustaceans: the water flea *Daphnia magna* Strauss. *J. Exp. Biol.* **211**, 539-547. doi:10.1242/jeb.009175

Chao, A. C. and Armstrong, W. M. (1987). Cl(–)-selective microelectrodes: sensitivity to anionic Cl-transport inhibitors. *Am. J. Physiol. Cell Physiol.* **253**, C343-C347. doi:10.1152/ajpcell.1987.253.2.C343

Conte, F. P. (1984). Structure and function of the crustacean larval salt gland. In *International Review of Cytology* (ed. G. H. Bourne, J. F. Danielli and K. W. Jeon), Vol. 91, pp. 45-106. Elsevier.

Croghan, P. (1958). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae. *J. Exp. Biol.* **35**, 234-242.

Del Duca, O., Nasirian, A., Galperin, V. and Donini, A. (2011). Pharmacological characterisation of apical Na^+ and Cl^- transport mechanisms of the anal papillae in the larval mosquito *Aedes aegypti*. *J. Exp. Biol.* **214**, 3992-3999. doi:10.1242/jeb.063719

Demarest, J. R. and Morgan, J. L. M. (1995). Effect of pH buffers on proton secretion from gastric oxyntic cells measured with vibrating ion-selective microelectrodes. *Biol. Bull.* **189**, 219-220. doi:10.1086/BBLv189n2p219

Donini, A. and O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti:* application of self-referencing ion-selective microelectrodes. *J. Exp. Biol.* **208**, 603-610. doi:10.1242/jeb.01422

Giardini, J.-L., Yan, N. D. and Heyland, A. (2015). Consequences of calcium decline on the embryogenesis and life history of *Daphnia magna*. *J. Exp. Biol.* **218**, 2005-2014. doi:10.1242/jeb.123513

Glover, C. N. and Wood, C. M. (2005). Physiological characterisation of a pH- and calcium-dependent sodium uptake mechanism in the freshwater crustacean, Daphnia magna. *J. Exp. Biol.* **208**, 951-959. doi:10.1242/jeb.01426

Halcrow, K. (1982). Some ultrastructural features of the nuchal organ of *Daphnia magna* Straus (Crustacea: Branchiopoda). *Can. J. Zool.* **60**, 1257-1264. doi:10.1139/z82-169

Henry, R. P., Lucu, Č., Onken, H. and Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. *Front. Physiol.* **3**, 431. doi:10.3389/fphys.2012.00431

Hessen, D. O., Alstad, N. E. and Skardal, L. (2000). Calcium limitation in *Daphnia magna*. *J. Plankton Res.* **22**, 553-568. doi:10.1093/plankt/22.3.553

Hollis, L., Hogstrand, C. and Wood, C. M. (2001). Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. *Arch. Environ. Contam. Toxicol.* **41**, 468-474. doi:10.1007/s002440010273

Jayakannan, M., Babourina, O. and Rengel, Z. (2011). Improved measurements of Na^+ fluxes in plants using calixarene-based microelectrodes. *J. Plant Physiol.* **168**, 1045-1051. doi:10.1016/j.jplph.2010.12.006

Johnson, K. E., Perreau, L., Charmantier, G., Charmantier-Daures, M. and Lee, C. E. (2014). Without gills: localization of osmoregulatory function in the copepod *Eurytemora affinis*. *Physiol. Biochem. Zool.* **87**, 310-324. doi:10.1086/674319

Kast-Hutcheson, K., Rider, C. V. and LeBlanc, G. A. (2001). The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environ. Toxicol. Chem.* **20**, 502-509. doi:10.1002/etc.5620200308

Kobayashi, M. and Nezu, T. (1986). Variation of hemoglobin content in *Daphnia magna*. *Physiol. Zool.* **59**, 35-42. doi:10.1086/physzool.59.1.30156087

Kondo, Y., Bührer, T., Frömter, E. and Simon, W. (1989). A new double-barrelled, ionophore-based microelectrode for chloride ions. *Pflügers Arch.* **414**, 663-668. doi:10.1007/BF00582133

Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W. H. and Weihrauch, D. (2014). Osmoregulation and excretion. *Compr. Physiol.* **4**, 405-573. doi:10.1002/cphy.c130004

Leonard, E. M., Banerjee, U., D'Silva, J. J. and Wood, C. M. (2014). Chronic nickel bioaccumulation and sub-cellular fractionation in two freshwater teleosts, the round goby and the rainbow trout, exposed simultaneously to waterborne and dietborne nickel. *Aquat. Toxicol.* **154**, 141-153. doi:10.1016/j.aquatox.2014.04.028

Martin, J. W. and Laverack, M. S. (1992). On the distribution of the crustacean dorsal organ. *Acta Zool.* **73**, 357-368. doi:10.1111/j.1463-6395.1992.tb01108.x

Messerli, M. A., Robinson, K. R. and Smith, P. J. (2006). Electrochemical sensor applications to the study of molecular physiology and analyte flux in plants. In *Plant Electrophysiology* (ed. A.G. Volkov), pp. 73-107. Springer.

Messerli, M. A., Kurtz, I. and Smith, P. J. S. (2008). Characterization of optimized Na^+ and Cl^- liquid membranes for use with extracellular, self-referencing microelectrodes. *Anal. Bioanal. Chem.* **390**, 1355-1359. doi:10.1007/s00216-007-1804-z

Mittmann, B., Ungerer, P., Klann, M., Stollewerk, A. and Wolff, C. (2014). Development and staging of the water flea *Daphnia magna* (Straus, 1820; Cladocera, Daphniidae) based on morphological landmarks. *EvoDevo* **5**, 12. doi:10.1186/2041-9139-5-12

Robinson, R. A. and Stokes, R. H. (1968). *Electrolyte Solutions*, 2nd edn. London: Butterworths.

Russler, D. and Mangos, J. (1978). Micropuncture studies of the osmoregulation in the nauplius of *Artemia salina*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **234**, R216-R222. doi:10.1152/ajpregu.1978.234.5.R216

Saunders, J. H. and Brown, H. M. (1977). Liquid and solid-state Cl–sensitive microelectrodes. Characteristics and application to intracellular Cl-activity in *Balanus* photoreceptor. *J. Gen. Physiol.* **70**, 507-530. doi:10.1085/jgp.70.4.507

Surbida, K.-L. and Wright, J. C. (2001). Embryo tolerance and maternal control of the marsupial environment in *Armadillidium vulgare* (Isopoda: Oniscidea). *Physiol. Biochem. Zool.* **74**, 894-906. doi:10.1086/324474

Tan, Q.-G. and Wang, W.-X. (2009). The regulation of calcium in *Daphnia magna* reared in different calcium environments. *Limnol. Oceanogr.* **54**, 746-756. doi:10.4319/lo.2009.54.3.0746

Weber, A. K. and Pirow, R. (2009). Physiological responses of *Daphnia pulex* to acid stress. *BMC Physiol.* **9**, 9. doi:10.1186/1472-6793-9-9

Wright, J. C. and O'Donnell, M. J. (2010). *In vivo* ion fluxes across the eggs of *Armadillidium vulgare* (Oniscidea: Isopoda): the role of the dorsal organ. *Physiol. Biochem. Zool.* **83**, 587-596. doi:10.1086/651583