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Ontogeny of osmoregulation in embryos of intertidal crabs (*Hemigrapsus sexdentatus* and *H. crenulatus*, Grapsidae, Brachyura): putative involvement of the embryonic dorsal organ

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Summary

This study examined whether the existence of hyperosmotic internal fluids in embryos of euryhaline crabs (*Hemigrapsus sexdentatus* and *H. crenulatus*) in dilute seawater reflects osmotic isolation due to impermeability of the egg envelope, as proposed for other decapods, or active osmoregulation. When ovigerous crabs with eggs at gastrula stage were transferred from 100% seawater (osmolality 1000 mmol kg^{-1}) to 50% seawater, embryogenesis and hatching of zoea were completed normally, but were delayed. Hatching failed if the transfer to 50% seawater occurred before gastrulation, and embryogenesis was abnormal in 25% seawater. In 100% seawater, embryos at all stages were internally hyperosmotic by 150–250 mmol kg^{-1}. On transfer to 50% seawater, osmolality initially decreased but remained 200–350 mmol kg^{-1} hyperosmotic to the medium for several weeks until hatching. High efflux rates of tritium-labelled water ($t_{1/2}$ 16–75 min) and ^{22}Na ($t_{1/2}$ 109–374 min) from *H. crenulatus* embryos were inconsistent with the osmotic isolation hypothesis. It is concluded that post-gastrula embryos were actively hyper-osmoregulating. The diffusional water permeability of the embryos decreased during development while the sodium efflux rate increased 10-fold. Very rapidly exchanging pools of water and sodium ($t_{1/2}$ a few seconds to minutes) probably corresponded to peri-embryonic fluid and implied that the egg envelope was a negligible barrier to diffusion of water and salts. Higher Na^+/K^+-ATPase activities in late embryos of *H. crenulatus* incubated in 50% seawater than in embryos incubated in full strength seawater were consistent with an acclimation response. An area of the embryonic surface located over the yolk in the region of the embryonic dorsal organ stained with $AgNO_3$. Staining appeared at gastrulation, persisted throughout development and was lost at hatching. Deposits of AgCl between the outer and inner membranes, identified by X-ray microanalysis, suggest that the dorsal organ was a site of chloride extrusion. A model for osmoregulation in post-gastrula embryos is proposed: osmotic uptake of water is balanced by excretion of water and salts via the dorsal organ and salt loss is balanced by active uptake over the general embryonic ectoderm.

Key words: crab, *Hemigrapsus*, osmoregulation, excretion, development, water permeability, sodium transport, embryonic dorsal organ, silver staining.

Introduction

Hemigrapsus sexdentatus (formerly *H. edwardsii*) and *H. crenulatus* are euryhaline intertidal crabs endemic to New Zealand. The adults of both species are strong hyperosmotic regulators, consistent with their exposure to rainfall at low tide and their frequent occurrence near to freshwater streams and, in the case of *H. crenulatus*, within estuaries (Hicks, 1973; Jones, 1976; Bedford and Leader, 1977; McLay, 1988). Ovigerous females brood their embryos (i.e. between cleavage and hatching as zoea) externally, within eggs attached ventrally to the abdominal pleopods. Thus, the adults and their embryos are potentially exposed to similar osmotic regimes. Studies of other hyper-regulating decapods, including homarid lobsters (Charmantier and Aiken, 1987; Charmantier et al., 1988; Charmantier and Charmantier-Daures, 1991; Charmantier, 1998), grapsid crabs (Charmantier et al., 2002), and astacid freshwater crayfish (Susanto and Charmantier, 2000; Susanto and Charmantier, 2001) have concluded that the capacity for osmoregulation is absent in embryonic stages. Although the embryonic and peri-embryonic fluids of *Homarus* and *Astacus* have been observed to be hyperosmotic to the external medium, this state is believed to be maintained by the low permeability of the membranes of the egg envelope to water and salts, thus protecting the embryos from osmotic and diffusive exchanges with the external medium (Charmantier and Charmantier-Daures, 2001).

We have shown previously that the embryos of both species, at all stages between gastrulation and hatching, survive acute

transfer to seawater diluted by as much as 100-fold (~10 mmol kg^{-1} osmolality) for several days without significant mortality or obvious impairment of development (Taylor and Seneviratna, 2005). During this time, the internal osmolality of these eggs (i.e. the embryo, peri-embryonic fluid, and external membranes) was maintained hyperosmotic to the external medium, displaying the characteristics of strong osmoregulation. In contrast, cleavage stages osmoconformed within 6 h and experienced higher mortality in dilute seawater.

Interpretation of the osmotic behaviour of *Hemigrapsus* embryos in terms of the above osmoprotection hypothesis implies that the egg envelope becomes impermeable to water and solutes a few days after extrusion as has been proposed for two other grapsid crabs (Bas and Spivac, 2000). Such absolute impermeability ostensibly conflicts with the requirements for exchange of respiratory gases, ammonia and other excretory products during development, with the observed increases in water and electrolyte contents during embryogenesis in these crabs (Seneviratna, 2003) and other decapodan eggs (Pandian, 1970), and with qualitative observations on the solute permeability of homarid eggs (Yonge, 1937; Yonge, 1946). However, quantitative measurements of water and solute fluxes and permeabilities that are required to critically evaluate osmoprotection *versus* osmoregulation hypotheses, are unavailable for crustacean eggs.

Steady state hyperosmotic regulation requires both the active uptake of salts from the external medium and a route for the elimination of water gained osmotically. Gills, important in the osmoregulation of adult crabs (Mantel and Farmer, 1983; Pequeux, 1995), are absent from the embryonic stages of crabs and the renal (antennal) organs are not fully formed until larval stages (Anderson, 1973). In contrast to the proposed situation in decapods, embryonic osmoregulation has been demonstrated in euryhaline gammarid and talitrid amphipods, and is believed to involve the embryonic dorsal organ the ultrastructure of which is suggestive of ion transport and it stains characteristically with silver ions (Meschenmoser, 1989; Morritt and Spicer, 1995; Morritt and Spicer, 1996). Embryonic dorsal organs have also been reported in brachyuran embryos (Anderson, 1973; Fioroni, 1980) but have not been linked to an osmoregulatory function. Dorsal organs, also referred to as nuchal or neck organs, are present in other larval and adult crustaceans, notably in the nauplii of branchiopods. Their homology with embryonic dorsal organs is uncertain but they are believed to function as either ionoregulatory or sensory organs (Martin and Laverack, 1992; Aladin and Potts, 1995).

To examine the longer term osmotic homeostasis of *Hemigrapsus* embryos, and its significance for normal embryogenesis, we observed embryonic development and hatching success, and changes in the osmolality and volume of the eggs during chronic exposures of both species to dilute seawater, commencing either during the osmoconforming cleavage stages or after gastrulation, when we hypothesised that osmoregulation begins. To further investigate the basis of apparent hyper-osmoregulation we measured steady state fluxes of isotopically labelled water and sodium ions in *H. crenulatus* eggs. Demonstration of turnover times that are short in relation to the duration of the hyperosmotic state, would favour active rather than passive maintenance of this condition. We measured the activity of Na^+/K^+-ATPase in *H. crenulatus* chronically exposed to either normal or diluted seawater as a possible indicator of osmoregulatory acclimation. We describe, using light and electron microscopy, the selective staining with silver ions of a discrete patch on the surface of *Hemigrapsus* embryos and discuss its possible importance in embryonic osmoregulation.

Materials and methods

Ovigerous crabs carrying early embryos were collected on the east coast of South Island, New Zealand at the start of their spawning periods. *Hemigrapsus sexdentatus* H. Milne Edwards 1837 [formerly *H. edwardsii* Hilgendorf 1882 (see McLay and Schubart, 2004)] were obtained from Glenafric Beach, Waipara in April and *Hemigrapsus crenulatus* (H. Milne Edwards 1837) from the Avon-Heathcote Estuary, Christchurch in August to September. The crabs were tagged for identification and transferred to recirculating seawater aquaria at 15°C under a 12 h:12 h light:dark cycle, provided with a gravel base and rocky refugia, fed on mussel pieces, and subjected to simulated tidal emersion:immersion on a 6.2 h:6.2 h cycle. The osmolality of the seawater was adjusted every few days to either 1000 mmol kg^{-1} (nominally '100% seawater', salinity ~35), 500 mmol kg^{-1} ('50% seawater') or 250 mmol kg^{-1} ('25% seawater'), and the water was changed completely every 2 weeks.

Effect of chronic exposure to dilute seawater on embryonic development

For observations of developmental stage and viability, ten ovigerous crabs of each species with embryos either at stage 1 or at stage 2 (see below) were placed into each of the three tidal aquaria (100%, 50% and 25% seawater) and observed until the embryos hatched or were aborted. Crabs were removed briefly, a small sample of the eggs was detached from the pleopods for microscopical examination, and the crab replaced. The developmental stage of embryos is reported on a five-step scheme (Taylor and Seneviratna, 2005) (Fig. 1). The approximate timings of these stages in 100% seawater at 15°C are for *H. sexdentatus*: (1) cleavage, 0–5 days; (2) gastrula, 6–28 days; (3) eyespot, 29–37 days; (4) four-lobe, 38–50 days; (5) two-lobe to hatching zoea, 51–62 days. For *H. crenulatus* the timings are: (1) cleavage, 0–2 days; (2) gastrula, 3–18 days; (3) eyespot, 19–27 days; (4) four-lobe, 28–36 days; (5) two-lobe to hatching zoea, 37–43 days.

Effect of chronic exposure to dilute seawater on volume, osmolality, and Na^+/K^+-ATPase activity of embryos

For measurement of egg osmolality and volume, ovigerous crabs with eggs at either stage 1 or stage 2 were introduced to each of the three seawater concentrations. At set times after the

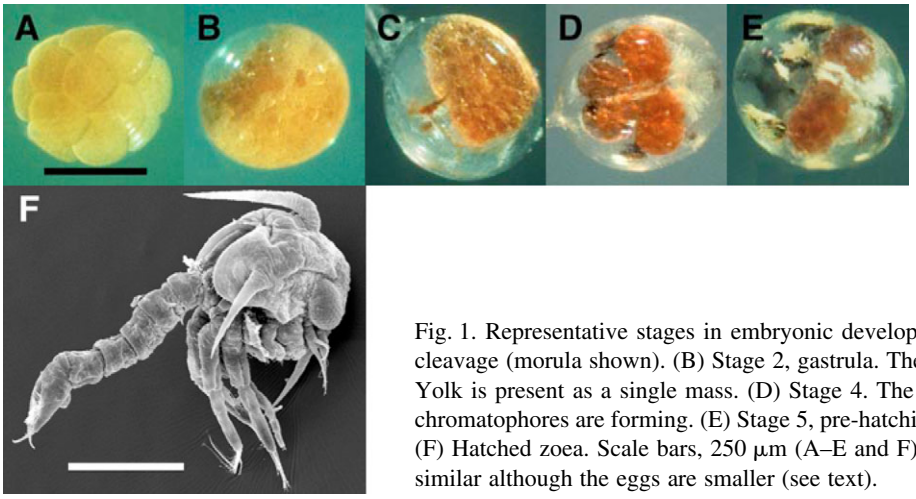

Fig. 1. Representative stages in embryonic development of *Hemigrapsus sexdentatus*. (A) Stage 1, cleavage (morula shown). (B) Stage 2, gastrula. The clear zone is the embryo. (C) Stage 3, eyespot. Yolk is present as a single mass. (D) Stage 4. The yolk forms four lobes, the heart is beating, and chromatophores are forming. (E) Stage 5, pre-hatching. The yolk has now been reduced to two lobes. (F) Hatched zoea. Scale bars, 250 μm (A–E and F). The steps in development of *H. crenulatus* are similar although the eggs are smaller (see text).

estimated time of egg extrusion (*H. sexdentatus*, 1, 4, 6, 11, 21, 31, 41, 51, 61, 71, 81 days; *H. crenulatus*, 1, 4, 8, 13, 19, 26, 32, 38, 44 days; or until the eggs had all hatched or were aborted), crabs were removed for measurements. Crabs were not re-sampled and the measurements were extended over two breeding seasons. Egg volume (V) was calculated from the largest (D) and smallest (d) diameters of 25 eggs from each crab using a stereomicroscope equipped with a micrometer eye-piece ($V=\pi d^2D/6$). The osmolalities of 10 μl sub-samples from ~0.2 g of homogenized eggs (Taylor and Seneviratna, 2005) and of the seawater were determined by vapour pressure osmometry (Wescor 5100, Logan, UT, USA).

The Na^+/K^+-ATPase activity of embryos was measured separately using *H. crenulatus* only. Groups of ovigerous crabs were transferred at stage 2 to the 100% and 50% seawater tidal systems. Crabs in 100% seawater were sampled immediately and the remainder maintained at 15°C and removed at either stage 4 or stage 5. The Na^+/K^+-ATPase activity was measured on ~0.4 g of homogenized eggs (less than five samples in each stage and salinity combination) as described previously (Taylor and Seneviratna, 2005) and expressed as pmol P_i embryo^{-1} min^{-1}.

Morphological observations

Live embryos were examined using a stereomicroscope (Zeiss Stemi 2000c) and photographed using a digital camera (Sony DSC-S75) mounted on the microscope. Embryos were also aldehyde-fixed for preparation of semi-thin (~1 μm) resin sections: 2 h under vacuum at room temperature, overnight at 4°C, in 3% glutaraldehyde, 1.2% paraformaldehyde, 0.05% tannic acid, 0.05% saponin, 0.1 mol l^{-1} sodium cacodylate buffer in 50% seawater, pH 7.3, osmolality 1300 mmol kg^{-1}, post-fixed for 4 h at 4°C in 1% osmium tetroxide, 0.2 mol l^{-1} sodium cacodylate, dehydrated in graded ethanol and propylene oxide series and embedded in epoxy resin (Spurr, 1969). Sections were stained with 1% Toluidine Blue in a 1% borax solution, observed using a Zeiss Axioskop 2 microscope, and images captured digitally (Zeiss Axiocam).

Silver staining

Eggs were detached from ovigerous crabs and rinsed in distilled water (changed twice) for about 30 s. They were then transferred to 5 g l^{-1} $AgNO_3$ for 5 min, again rinsed in distilled water and exposed to sunlight for 10-15 min. Light microscope observations were made on unfixed silver-stained embryos in seawater and on epoxy sections.

Scanning electron microscopy and X-ray microanalysis

Silver-stained and unstained eggs with embryos at all developmental stages of both *H. sexdentatus* and *H. crenulatus* were examined using the scanning electron microscope (SEM). Eggs were fixed in 2.5% glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.3) containing 0.3 mol l^{-1} sucrose for 48 h at 4°C, washed in buffer overnight, dehydrated in a graded ethanol series, transferred *via* a graded ethanol/amyl acetate series to liquid CO_2, and critical-point dried. The eggs were mounted on aluminium stubs using carbon-impregnated double-sided adhesive tabs. In order to view the silver deposit, the outer egg membrane was carefully ruptured with the tip of a fine scalpel blade. Finally, the samples were sputter coated with 60 nm gold/palladium and observed and photographed in the SEM (Leica S440) at an accelerating voltage of 15 kV. Energy dispersive X-ray microanalysis was carried out on the silver-stained patch in the SEM {Link Pentafect detector, ISIS Software v3.34 [Oxford Instruments (UK) Ltd, High Wycombe, UK], 20 kV, 600 pA probe current} using the spot analysis mode to generate spectra (2 min) and X-ray dot mapping to show the distribution of silver within the patch area.

Water and sodium turnover in H. crenulatus eggs

Tritiated water fluxes

Eggs with embryos at each of the five stages were pooled from four to five ovigerous crabs and sub-sampled for measurement of the mean volume and mass of individual eggs. They were then equilibrated for 24 h in batches of ~100 in 2 ml of seawater labelled with tritiated water (2 MBq ml^{-1}) in a covered solid watch-glass. Groups of four to eight eggs were removed with

minimal fluid carry over and washed in ~5 ml of unlabelled seawater. The seawater was gently stirred and changed periodically during the washing period. After 0.5, 1, 2, 4, 8, 10, 25, 50, 75, 90 and 115 min, eggs were pipetted onto a filter paper. As soon as adherent fluid had been absorbed (a few seconds), they were transferred with forceps to scintillation vials containing 1.0 ml of aqueous scintillation fluid (Ready-Solv MP, Beckman Instruments, Inc., Fullerton, CA, USA). For the zero time measurement, the eggs were transferred directly from the labelling medium to the filter paper. The activity of tritium was measured by liquid scintillation counting (Beckman 5800) and corrected for quenching. Five or more replicate groups of eggs were measured for each developmental stage, at each wash time. The radioactivity of the labelling medium was also measured. Mean egg volume was measured on sub-samples of the same batch. Measurements were made at room temperature (~20°C).

^{22}Na efflux

Sodium efflux from *H. crenulatus* eggs was measured using a protocol similar to that used for tritium. After estimating their mean volume and mass, eggs were equilibrated for 24 h in ^{22}Na-labelled seawater (200 kBq ml^{-1}). Five or more replicates were performed for each developmental stage and wash time. Radioactivity was measured by liquid scintillation counting (Beckman 5800) without quench correction.

Water and sodium contents

The total exchangeable water and sodium contents (C nl or C nmol, respectively) of single eggs were estimated from their radioactivity at time zero (A_0; d.p.m. egg^{-1}).

$$C = A_0/A_m ,$$

where A_m is the radioactivity of the labelling seawater (d.p.m. nl^{-1} or d.p.m. nmol^{-1}).

Compartmental analysis of tracer washout data

^{3}H and ^{22}Na radioactivities of the eggs generally did not decline as first order exponentials. Thus, washout curves were analysed as bi-exponential decay curves of the form:

$$A_t = P.\exp(at) + Q.\exp(bt) ,$$

where A_t is the total radioactivity (expressed as either nl egg^{-1} of ^3H_2O or nmol egg^{-1} of ^{22}Na, at the radioactivity concentration of the loading medium) remaining at time t (min), and P and Q represent radioactivities of 'fast' and 'slow' pools, declining with rate constants of a and b (min^{-1}), respectively. For initial analysis, an iterative non-linear curve fitting routine (Marquardt method) supplied with the FigP graphing package (Biosoft, Cambridge, UK) was used to determine P, Q, a and b. Mean data were weighted inversely by their standard errors. Where all four parameters were statistically significant (P<0.05 that they were non-zero), these values are reported, along with standard errors of the estimates and their corresponding half times ($t_{1/2}$=ln(0.5)/a or ln(0.5)/b). Where a rapidly exchanging pool was present but its rate constant (a) was too high in relation to the sampling intervals to be determined by this method, Q and b were determined by linear regression of ln(At) *versus* time when none of the fast pool remained (>2 min for ^3H_2O, >25 min for ^{22}Na). The size of the fast pool (P) was then determined by subtraction of Q from the specific activity at time zero (C=P+Q).

Calculation of water and sodium efflux rates and permeabilities

These estimates are reported for the slow pool only (assumed to represent the embryo, see Discussion section) relative to the surface area of the egg envelope (A, cm^2), assumed to be a sphere of diameter equal to the mean of the largest and smallest diameters. The diffusional permeability constant of water is:

$$P_w = Qb10^{-6}/(60A) \text{ cm s}^{-1} .$$

The permeability constant for sodium influx was calculated assuming that the eggs were in steady state turnover in seawater. Thus, the permeability constant for sodium is:

$$P_{Na} = Qb10^{-9}/(60A[Na]_{ext}) \text{ cm s}^{-1} ,$$

where ($[Na]_{ext}$ is the sodium concentration in seawater (0.49×10^{-3} mol cm^{-3}). The mass-specific efflux of sodium,

$$J_{OUT(Na)} = Qb10^{-6}60/M \text{ mmol kg}^{-1} \text{ h}^{-1} ,$$

where M is the mass of one egg, estimated by weighing a known number of eggs from the same batch.

Data analysis

Data are presented as means ± s.e.m. Changes in the volume of eggs with development time in the control series (100% seawater) were analysed using single factor analysis of variance (ANOVA). The effects of salinity (% seawater) and development time or stage on egg ATPase activity and volume were analysed using two-factor ANOVA. In the latter case, to avoid missing cells resulting from differential survival in 50% and 25% seawater of stage 1 embryos, separate ANOVAs were performed (see Results). Tukey HSD tests were employed for *post-hoc* comparison of means. Differences were considered statistically significant where P<0.05.

Results

Development and hatching success in dilute seawater

Hyposaline exposure commencing before gastrulation

Ovigerous *H. sexdentatus* that were introduced to the 100% seawater tidal system with embryos at stage 1 (cleavage) had incubated essentially all of their eggs to stage 5 (two yolk lobes, pre-hatch) at 60 days and these all hatched to motile zoea after ~62 days (cf. Fig. 1; see Materials and methods). When stage 1 ovigerous crabs were transferred to the 50% seawater system, cleavage proceeded normally but embryogenesis was progressively retarded from gastrulation onwards. Although there was a higher incidence of non-viable embryos, the majority reached stage 5 after about 70 days when development was arrested. In about 10% of the eggs, the envelope ruptured but

the hatched larvae appeared incapable of swimming. In 25% seawater, development did not proceed beyond gastrulation and the crabs aborted their clutches of disintegrating embryos between 30 and 40 days. Similar effects were observed in *H. crenulatus* that were introduced into the tidal system at stage 1. In 100% seawater, embryogenesis occurred normally and all had successfully hatched at 37 days. In 50% seawater, development invariably proceeded to completion but was delayed. In this case, the majority of the eggs hatched around 43 days but, as for *H. sexdentatus*, the motility of the larvae was impaired. In 25% seawater, gastrulation of *H. crenulatus* was unsuccessful and the eggs were aborted between 18 and 30 days.

Hyposaline exposure commencing after gastrulation

Delaying the introduction to dilute seawater until after gastrulation markedly improved the viability of the embryos. Although retarded similarly to those commenced at cleavage, embryos of both species successfully hatched as active larvae in 50% seawater (by 70 days for *H. sexdentatus* and by 43 days for *H. crenulatus*). The yolk reserves in late hatching zoea were noticeably reduced (and white rather than yellow) compared with those from 100% seawater. In 25% seawater, embryogenesis continued apparently normally until about stage 3 (at ~40 days and ~24 days, respectively) but then ceased, and the eggs were subsequently aborted.

Osmoregulation

The mean osmolalities of the embryos (homogenised whole eggs) carried by *H. sexdentatus* and *H. crenulatus* maintained in the 100% seawater tidal systems were consistently hyperosmotic (150–250 mmol kg^{-1}) to that of the seawater (997–1005 mmol kg^{-1}) throughout development (Fig. 2). On transfer to 50% seawater (498–505 mmol kg^{-1}) and 25% seawater (248–260 mmol kg^{-1}), the osmolality of the eggs of both species decreased during the first few days but stabilised hyperosmotic to the external medium by several 100 mmol kg^{-1}. Embryonic osmolality decreased more slowly when the period of hyposaline exposure commenced after gastrulation (stage 2) compared with those started during cleavage (stage 1) and, in the case of *H. crenulatus* the plateaus were higher (Fig. 2). Stage 1 embryos of *H. sexdentatus* became almost iso-osmotic with 25% seawater after 11 days. The final upturn in osmolality of both species introduced to 25% seawater at stage 1 corresponded to a period of abnormal morphology, and possibly autolysis, just prior to their abortion. The general hyper-osmoticity exhibited by the embryos was statistically highly significant. Thus at each sample time, in all 12 treatment series shown in Fig. 2A,B, the mean value of osmolality, the lower 95% confidence limit, and indeed every replicate, was higher than the simultaneously measured osmolality of the external seawater.

Egg volume

The mean volumes of the eggs carried by control ovigerous crabs maintained in 100% seawater from stage 1 (Fig. 3A,C) increased significantly at successive sample times (one way ANOVA, *H. sexdentatus*, $F_{8,81}$=998, P<0.001; *H. crenulatus*,

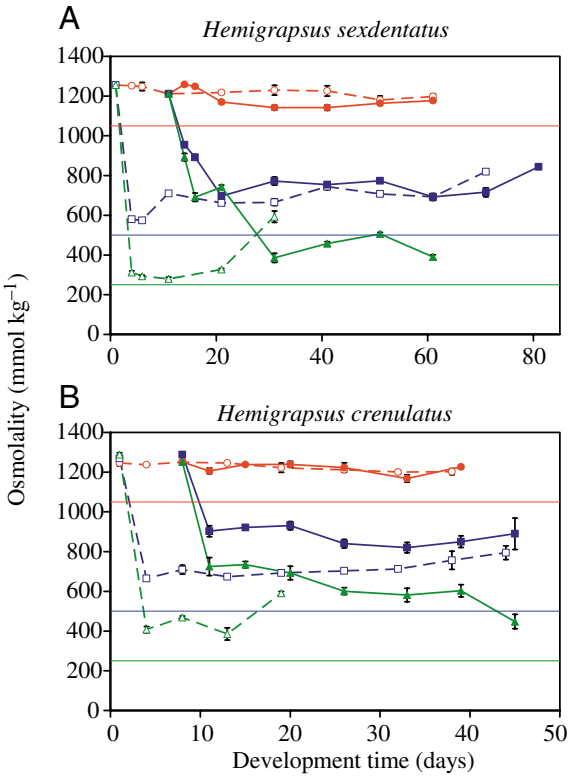

Fig. 2. Time course of changes in the internal osmolality of embryos (homogenised eggs) sampled from ovigerous crabs (A, *Hemigrapsus sexdentatus*; B, *H. crenulatus*) reared in dilutions of seawater: red, 100% (osmolality 1000 mmol kg^{-1}); blue 50% (osmolality 500 mmol kg^{-1}); green 25% (osmolality 250 mmol kg^{-1}). The abscissa shows the estimated elapsed time from egg extrusion. The hyposaline exposure was commenced either during cleavage (1 day after extrusion, broken lines and open symbols) or after gastrulation (11 days and 8 days respectively after extrusion, solid lines and closed symbols). The final measurement was made just before hatching (for embryos in 100% seawater and those exposed to 50% seawater as gastrulae) or before abortion (25% seawater and those commenced during cleavage). Horizontal coloured lines show the osmolalities of the corresponding external seawaters. Values are means ± s.e.m., N=5–7 (A) and 3–5 (B).

$F_{7,30}$=952, P<0.001). Between cleavage (stage 1) and hatching (stage 5), egg volumes of *H. sexdentatus* increased from 19.7±0.36 nl (diameter ~0.34 mm) to 35.1±0.35 nl (~0.41 mm) and eggs of *H. crenulatus* more than doubled in volume from 7.1±0.23 nl (~0.24 mm) to 18.3±0.12 nl (~0.33 mm). For crabs introduced to 50% and 25% seawater at stage 1, egg volumes were significantly higher than the corresponding values in 100% seawater during gastrula stages (i.e. up to about 21 days and 13 days, respectively) (two-factor ANOVA for *H. sexdentatus* 100% *versus* 50% seawater, significant effects of time $F_{7,144}$=878, salinity $F_{1,144}$=148, and interaction $F_{7,144}$=24.2; 100% *versus* 25% seawater, significant effects of time $F_{4,90}$=23.4, salinity $F_{1,90}$=3596, and interaction $F_{4,90}$=260; for *H. crenulatus* 100% *versus* 50% seawater significant effects of time $F_{6,40}$=726, no significant effect of salinity $F_{1,40}$=0.3, but a

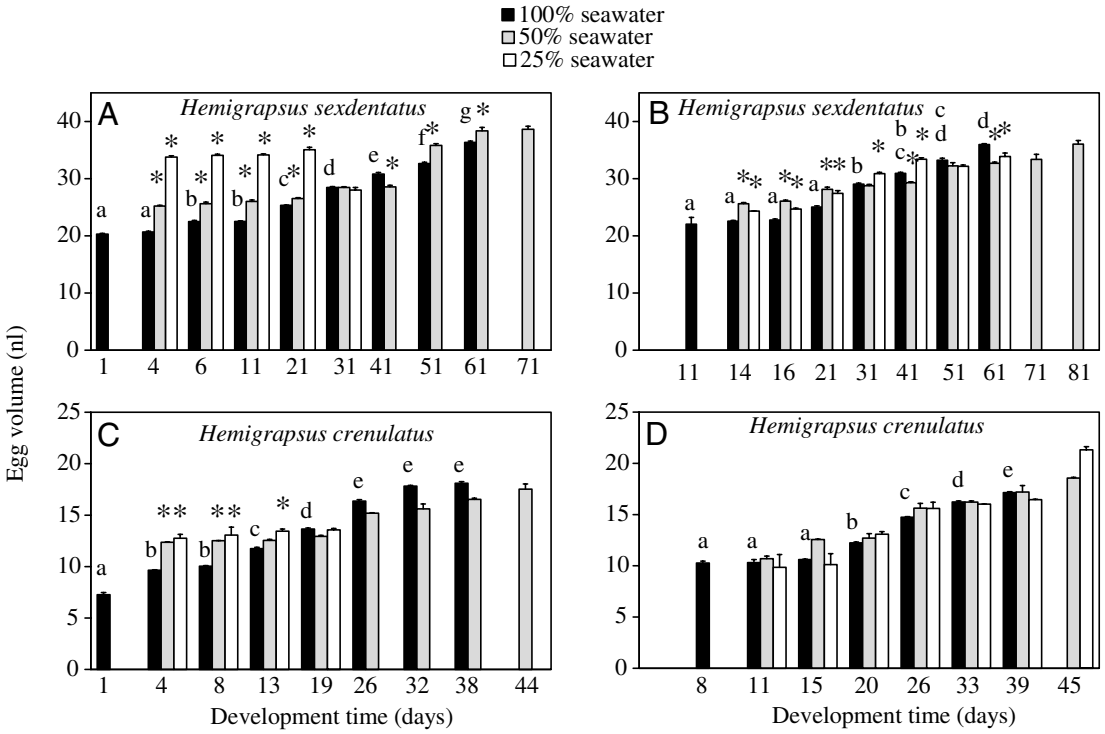

Fig. 3. Changes in the volume of eggs sampled from ovigerous crabs (A,B, *Hemigrapsus sexdentatus*; C,D, *H. crenulatus*) reared in dilutions of seawater (black bars, 100% seawater; grey bars, 50% seawater; white bars, 25% seawater). The hyposaline exposure was commenced either during stage 1 (cleavage, A,C) or at stage 2 (early gastrula, B,D). The abscissa shows the estimated elapsed times from egg extrusion. The final measurement was taken just before hatching (for embryos in 100% seawater and for embryos exposed to 50% seawater after gastrulation) or before abortion (25% seawater and those commenced during cleavage). Values are means ± s.e.m., N=10 (A) or 5 (B–D). Letters above the 100% seawater bars indicate means that are significantly different (one-way ANOVA, Tukey test). Asterisks above the 50% and 25% seawater bars indicate values that are significantly different from those in 100% seawater (two-way ANOVA, Tukey tests).

significant interaction $F_{6,40}$=109; 100% *versus* 25% seawater significant effects of time $F_{3,2}$=32.0, salinity $F_{1,22}$=107, and interaction $F_{3,22}$=14.8; P<0.001 for each significant effect, see Fig. 3 for *post-hoc* contrasts between means). Smaller but significant elevations of egg volume relative to 100% seawater were also noted during the gastrula stages of *H. sexdentatus* eggs that were introduced to dilute seawater at stage 2 (Fig. 3B; two-factor ANOVA significant effects of time $F_{6,84}$=513, salinity $F_{2,84}$=20.6, and the interaction $F_{12,84}$=27.0; P<0.001) but not for *H. crenulatus*. Towards the end of embryonic development, eggs exposed to dilute seawater were sometimes of significantly lower volume than at those in 100% seawater at the same times. However, by this time the hyposaline-exposed embryos were retarded and a valid comparison is difficult.

Exchangeable water content and water efflux rates of H. crenulatus eggs

Exchangeable water (EW) contents and efflux rates were determined using tritium-labelled seawater. The volume of EW per egg increased during development from about 6 nl at stage 1 (cleavage) to about 18 nl at stage 5 (pre-hatching). During stages 1 and 2 (cleavage to gastrula), the EW comprised a small component that turned over extremely rapidly and a larger more slowly exchanging pool (Fig. 4, Table 1). The fast pool was very small in the eyespot and four-lobe stages (stages 3 and 4) but increased at stage 5. In the two trials using stage 1 embryos, the efflux rate constants for the fast pool were 302 h^{-1} and 116 h^{-1} and were immeasurably high in stages 2 and 3, representing half times for exchange of less than 1 min). At stage 5 the fast pool efflux rate constants were more than an order of magnitude lower at 7–11 h^{-1} ($t_{1/2}$=2.3–6.5 min; Table 1).

Efflux rate constants for the more slowly exchanging water compartment were in the range 0.5–2.6 h^{-1} with half exchange times of 0.25 to 1.25 h. The efflux rate constant of the slow compartment decreased, and $t_{1/2}$ increased, by approximately a factor of three between cleavage and hatching due mainly to the increase in egg volume. The diffusive water permeability of the slow pool was variable but tended to decrease during development with a mean value for all stages of $0.91\pm0.09\times10^{-6}$ cm s^{-1} (N=10).

Exchangeable sodium and sodium efflux rates of H. crenulatus eggs

Exchangeable sodium contents and sodium efflux rates of *H. crenulatus* eggs into 100% seawater were measured using ^{22}Na as a tracer. The exchangeable sodium content of individual eggs increased from about 1 nmol to 4 nmol between cleavage and hatching (Table 2). Sodium efflux was resolved into a rapidly

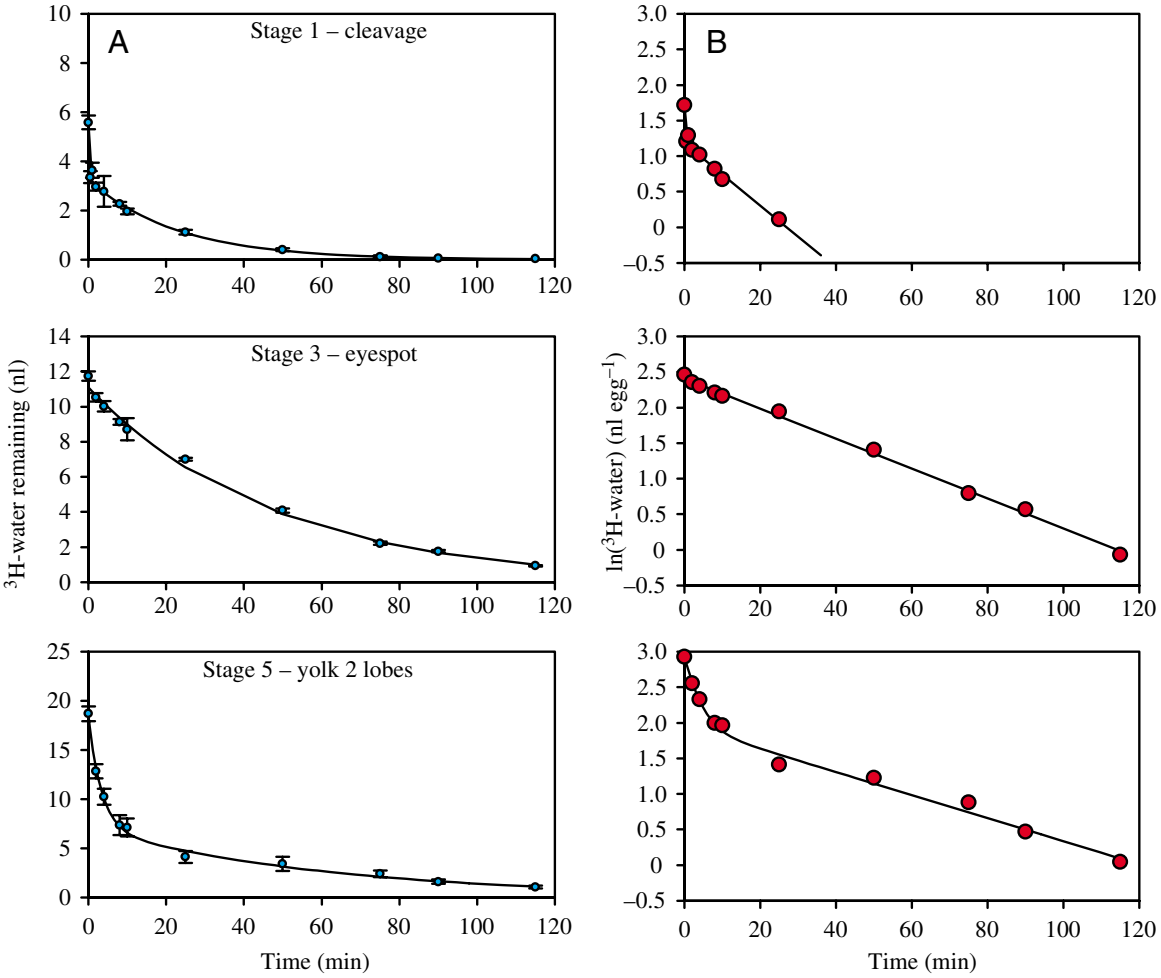

Fig. 4. Representative efflux curves for labelled water at different developmental stages of *Hemigrapsus crenulatus*. Eggs were pooled from five ovigerous crabs, batch-labelled in tritiated seawater for 24 h, and washed in groups of 4–8 in unlabelled 100% seawater for varying times. (A) Egg radioactivity (expressed as nl exchangeable water at the radioactive concentration of the loading medium) is shown on a linear scale; (B) the natural logarithm of the radioactivity is plotted. Each point is the mean (± s.e.m. in the linear plot) of 5–8 groups of eggs sampled at each time. Trend lines were fitted by single or bi-exponential regression (see Materials and methods).

exchanging pool and a slowly exchanging pool at all stages (Fig. 5, Table 2). Significant estimates were obtained for the fast pool rate constants only at stages 1 and 5, with $t_{1/2}$ values of a few seconds and a few minutes, respectively. As for water, there was a large (>30-fold) decrease in the sodium efflux from the fast pool in stage 5 eggs. The slow pool data indicated that the embryos of *H. crenulatus* were relatively permeable to sodium at all stages, with efflux rate constants of 0.11–0.68 h^{-1} and half times for sodium turnover of 1–6 h. In contrast to water fluxes, and the fast sodium pool, the mass-specific sodium efflux from the slow pool increased about 10-fold between cleavage and hatching (Table 2) and was associated with a corresponding increase in the permeability constant (from 0.14×10^{-7} to 1.89×10^{-7} cm s^{-1}).

Effect of dilute seawater on Na^+/K^+-ATPase activity of H. crenulatus embryos

The mean initial Na^+/K^+-ATPase activity of embryos in 100% seawater at stage 2 was 0.50±0.17 pmol P_i embryo^{-1} min^{-1} (N=10). Their activity increased sixfold to 3.25±0.31 pmol P_i embryo^{-1} min^{-1} (N=5) at stage 4 and by a further factor of four to 12.89±0.39 pmol P_i embryo^{-1} min^{-1} (N=5) at stage 5. At stage 4, the mean activity of embryos from the 50% seawater system was 6.61±0.60 pmol P_i embryo^{-1} min^{-1} (N=10), double that in 100% seawater (P<0.01). The value for embryos at stage 5 in 50% seawater was 11.79±0.84 pmol P_i embryo^{-1} min^{-1} (N=5) and not significantly different from that in 100% seawater (two-factor ANOVA, significant effect of stage $F_{1,21}$=131, P<0.0001, salinity not significant $F_{1,21}$=3.0, significant interaction $F_{1,21}$=29.3, P=0.002; Tukey tests).

Silver staining of embryos

Optical microscopy following the silver nitrate staining procedure revealed an oval brownish patch on the surface of embryos of *H. sexdentatus* and *H. crenulatus* at all stages of development except during cleavage (stage 1). The patch was irregular in outline, generally with a more densely stained rim,

represented 5–8% of the surface area of the egg, and was always positioned over the yolk at the opposite pole from the embryo (Fig. 6). A discrete area of staining was not present in cleavage and blastula stages but smaller spots of variable size and intensity were distributed over the whole surface of the egg and in some cases cell outlines were emphasised (Fig. 6A).

Semi-thin resin sections of silver-stained and fixed embryos showed that the densely staining material was interposed between the outer and inner membranes of the egg (Fig. 6G). This was confirmed by scanning electron microscopy (Fig. 7). Although the external surface of intact eggs was smooth and without trace of the deposit, removal of the outer envelope revealed a rather thick amorphous residue dried onto the surface of the inner membrane with a similar size and shape to that observed optically. The 'spotty' staining of blastulae was also observed in the electron microscope (Fig. 7C), each spot appearing as a small crater rimmed by residue. Energy dispersive X-ray spectra and elemental mapping identified silver and chloride as present in the patch (Fig. 8), strongly suggesting that it represented a precipitate of silver chloride.

Discussion

Physiological and morphological observations reported in this paper emphasise that gastrulation is an important event in the ontogeny of hyposaline tolerance by *H. sexdentatus* and *H. crenulatus* embryos. We showed previously (Taylor and Seneviratna, 2005) that post-gastrula stages survived acute transfer to seawater diluted 100-fold and remained hyperosmotic to the external medium for several days. In the present study, embryonic osmolality initially decreased on transfer to dilute seawater but was then maintained hyperosmotic to the medium for several weeks. Measurements of water and sodium fluxes now indicate that this situation did not result simply from a slow rate of osmotic and diffusive equilibrium across the egg envelope as required by the osmoprotection hypothesis (Charmantier and Charmantier-Daures, 2001). Rather, the embryos actively maintained this state in the face of salt and water fluxes; that is, they actively hyper-osmoregulated.

Water and sodium contents

The exchangeable water contents of *H. crenulatus* eggs in normal seawater increased from about 6 nl to 18 nl, between

Table 1. *Exchangeable water contents and efflux rates for Hemigrapsus crenulatus eggs in 100% seawater (~36 salinity) at 20°C*

				Exchangeable water content (nl egg^{-1})			Efflux rate constant (h^{-1})		$t_{1/2}$ (min)		Permeability (10^{-7} cm s^{-1})	
		Series	Egg volume (nl)	Total	Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow
Stage 1	Cleavage	1	7.1±0.2	5.6±0.2	2.3±0.2	3.3±0.0	302±91	2.64±0.06	0.14	15.8	1096	13.3
		2		5.9±1.2	1.7±0.3	2.7±0.1	116±23	1.99±0.05	0.36	20.8	302	8.2
Stage 2	Gastrula	1	10.6±0.1	8.6±0.3	0.7	7.9±0.2		1.27±0.01		32.9		10.1
		2		9.3±2.8	3.0	6.3±0.9		1.60±0.07		26.0		12.8
Stage 3	Eyespot	1	14.8±0.1	11.7±0.3	0.6	11.1±0.5		1.27±0.02		32.9		11.3
		2		10.2±0.2	0.8	9.4±0.3		0.84±0.02		49.0		7.9
Stage 4	Four lobes	1	17.2±0.1	12.9±0.3	0	12.9±0.8		0.90±0.04		46.3		8.3
		2		9.5±0.2	0	11.0±0.7		0.54±0.03		75.1		4.8
Stage 5	Two lobes	1	18.3±0.1	18.7±0.7	11.5±1.1	7.1±0.9	18±4	0.96±0.01	2.31	42.8	159	5.3
		2		16.7±1.9	5.8±1.5	10.6±1.5	7±2	0.84±0.14	6.53	48.0	30	7.4

Table 2. *Exchangeable sodium contents and turnover rates for eggs of Hemigrapsus crenulatus at different developmental stages in 100% seawater (~36 salinity) at 20°C*

			Exchangeable sodium content (nmol egg^{-1})			Efflux rate constant (h^{-1})		$t_{1/2}$ (min)		Efflux rate (mmol kg^{-1} h^{-1})	Permeability (10^{-7} cm s^{-1})	
		Series	Total	Fast	Slow	Fast	Slow	Fast	Slow	Slow	Fast	Slow
Stage 1	Cleavage	1	1.20±0.06	1.0±0.1	0.20±0.01	122±11	0.23±0.04	0.3	179	6.6	348	0.137
Stage 2	Gastrula	1	0.86±0.04	0.3	0.57±0.02		0.37±0.02		112	26.9		0.618
		2	0.84±0.05	0.5	0.35±0.03		0.38±0.07		109	14.6		0.352
Stage 3	Eyespot	1	1.60±0.11	0.4	1.18±0.03		0.21±0.02		202	18.3		0.503
		2	1.32±0.01	0.1	1.18±0.05		0.38±0.03		111	43.7		1.098
Stage 4	Four lobes	1	1.76±0.02	0.2	1.52±0.02		0.11±0.01		374	12.9		0.352
		2	1.72±0.09	0.4	1.32±0.10		0.25±0.05		169	19.9		0.576
Stage 5	Two lobes	1	3.93±0.17	2.4±0.3	1.62±0.3	4.6±1.0	0.68±0.10	9.1	61	63.4	18.4	1.893

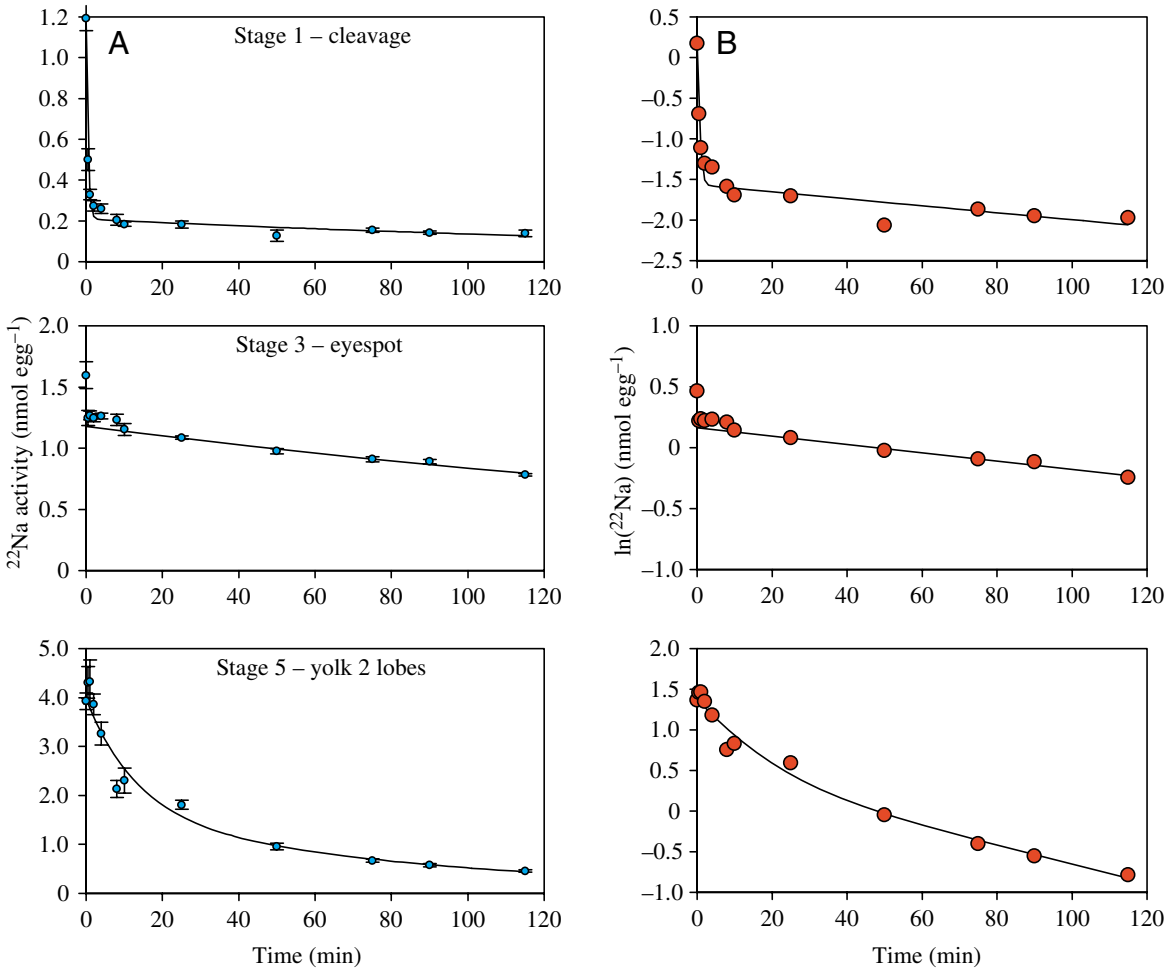

Fig. 5. Representative efflux curves for ^{22}Na at different developmental stages of *Hemigrapsus crenulatus*. Eggs were pooled from five ovigerous crabs, batch-labelled in ^{22}Na-labelled seawater for 24 h, and washed in groups of 4–8 in unlabelled 100% seawater for varying times. (A) Egg radioactivity (expressed as nmol exchangeable sodium at the radioactive concentration of the loading medium) is shown on a linear scale; (B) natural logarithm of the radioactivity is plotted. Each point is the mean (± s.e.m. in the linear plot) of 5–8 groups of eggs sampled at each time. Trend lines were fitted by single or bi-exponential regression (see Materials and methods).

cleavage and hatching. Slightly larger measured volumes of the eggs (Table 1, Fig. 3) presumably reflect the volume occupied by yolk and other solid material. Exchangeable sodium contents (increasing from about 1–4 nmol) were comparable with analyses by atomic absorption spectroscopy (Seneviratna, 2003). Water and sodium in the eggs therefore appear to have fully equilibrated with the tracers in the 24 h loading period. Each pool was resolved into an extremely rapidly exchanging component that varied in size during development and a more slowly exchanging component. The rapidly exchanging fractions are necessarily located superficially and presumably correspond to peri-embryonic fluid situated between the embryo and the outer egg envelope. The outer membrane of the egg therefore appears to be a negligible barrier to water and ion movements.

Turnover of water and salts

The more slowly exchanging pools are believed to correspond to the embryo itself. Half times for efflux of the slow pools of water (0.25–1.25 h) and sodium ions (1–6 h) were much shorter than the periods during which post-gastrula stages of *H. crenulatus* eggs remained hyperosmotic to full-strength and dilute seawater. This implies that post-gastrula embryos actively hyper-osmoregulate with the effluxes of water and sodium balanced by equal influx rates. Although isotopically measured water fluxes primarily represent diffusive exchange (Potts and Parry, 1964; Rasmussen and Andersen, 1996) under the anisosmotic conditions observed here, net uptake of water by osmosis (and excretion by another route) would have taken place continuously. Additionally, at least one primary active transport step is required to drive the steady state flux of sodium through the embryo.

Water permeability of the embryo

The rate-limiting permeability barrier for turnover of the embryonic (slow) water pool probably resides within the ectoderm or the associated inner membrane. In *Carcinus maenas* gastrulae the inner membrane is chitinous (Cheung,

1966). It is secreted by the embryo and appears to be equivalent to the embryonic cuticle, later becoming a multiple layer as a result of embryonic moults (Goudeau and Lachaise, 1983). Diffusional water permeabilities for a range of cells and aquatic organisms, determined using isotopically labelled water, have been compiled by several authors (Potts and Parry, 1964; Stein, 1967; Prosser, 1973; Taylor, 1989; Rasmussen and Andersen, 1996). Normalised to the surface of *H. crenulatus* eggs the diffusional permeability constant of the slow pool (~10^{-6} cm s^{-1}; Table 1) was many orders of magnitudes lower than reported for internal cells such as erythrocytes and squid axons (10^{-2}–10^{-4} cm s^{-1}) (Stein, 1967) and also lower than the permeabilities of most adult euryhaline and freshwater crustaceans (10^{-4}–10^{-5} cm s^{-1}) (Potts and Parry, 1964; Taylor, 1989). The present values are comparable with animals generally regarded as rather impermeable e.g. the larvae of the freshwater insect *Sialis lutaria* (Shaw, 1955), goldfish (Potts and Parry, 1964) and shed fish eggs (Prescott and Zeuthen, 1953; Potts and Eddy, 1973). However, such comparisons obscure the importance of surface/volume ratio. When the water permeabilities of adult crustaceans are expressed in terms of their hourly exchange fractions (Taylor, 1989; Rasmussen and Andersen, 1996), the rate constants of *H. crenulatus* embryos (1–3 h^{-1}; Table 1) are positioned in the middle of the range. Clearly, the relatively low water permeability of *H. crenulatus* embryos does not eliminate the requirement to osmoregulate, although it is certainly an adaptation that reduces the metabolic cost of osmoregulation. The rate of water turnover decreased somewhat during development, implying a corresponding decrease in water

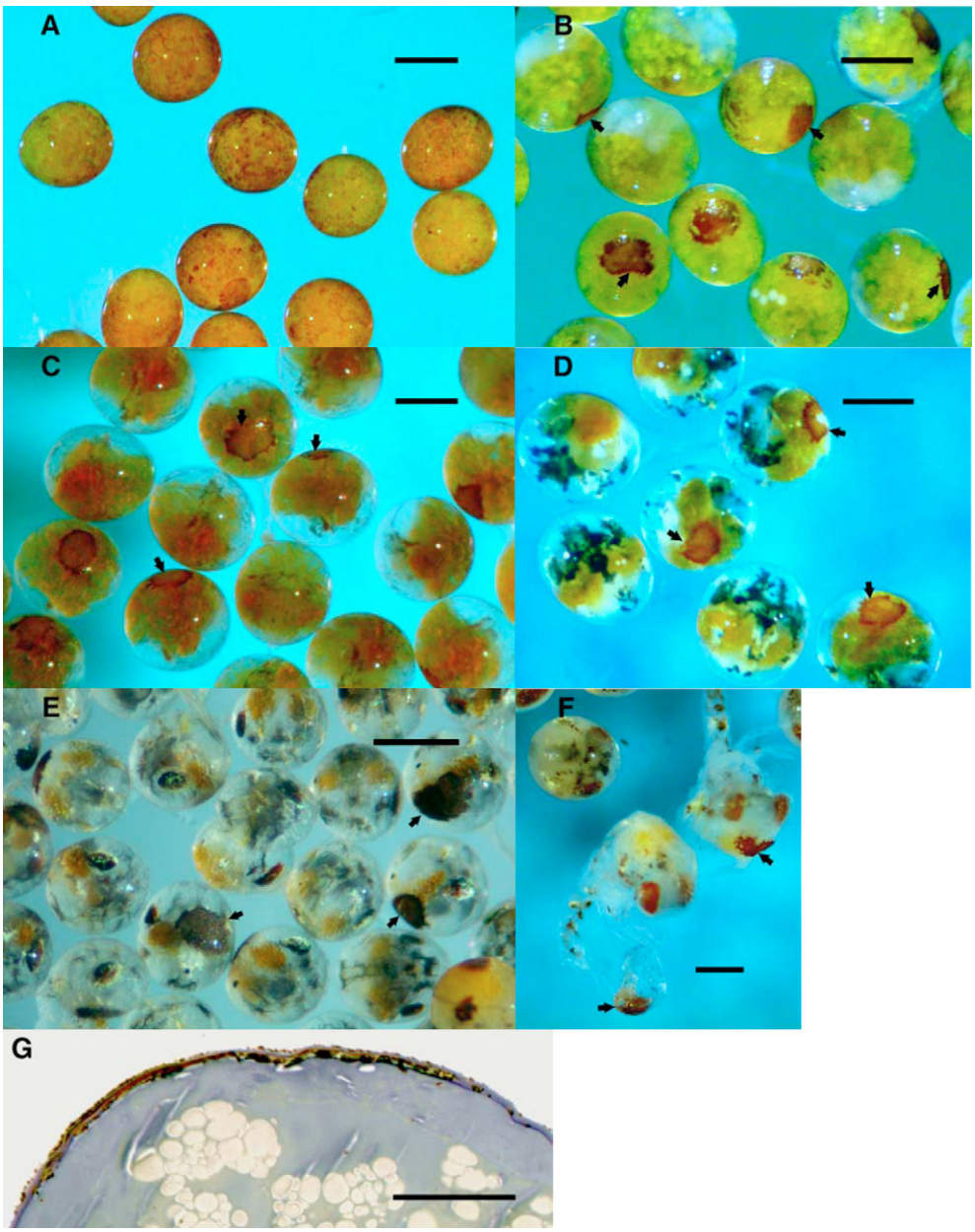

Fig. 6. Silver staining of live embryos of *Hemigrapsus sexdentatus* and *H. crenulatus*. Eggs were washed in distilled water, stained briefly with $AgNO_3$, washed again in distilled water and returned to seawater. (A–F) Appearance *in vivo*. In post-gastrula embryos a silver deposit is observed over the yolk (arrows). It is hypothesised that this patch corresponds to the embryonic dorsal organ and is responsible for water and salt excretion. Scale bars, 250 μm. (A) *H. sexdentatus*, stage 1, blastulae. Weak mottled silver staining over the whole embryo occasionally highlighting cell boundaries. (B) *H. crenulatus*, stage 2, gastrulae. (C) *H. sexdentatus*, stage 3, eyespots formed. (D) *H. sexdentatus*, stage 4–5, two yolk lobes. (E) *H. crenulatus*, stage 5, two yolk lobes, pre-hatching. (F) *H. sexdentatus*, Zoea. Some larvae hatched during staining. Free larvae did not stain but in those that hatched after staining, the silver deposit was cast off with the exuviae at the final embryonic moult (arrows). (G) Semi-thin epoxy section of $AgNO_3$ stained embryo of *H. sexdentatus* at stage 4 showing the dorsal surface and the yolk. A black deposit of silver is present between the outer and inner membranes. Scale bar, 25 μm.

permeability and further energetic saving, perhaps resulting from multiplication of the inner membrane.

Sodium turnover

Mass-specific sodium efflux rates from the embryonic pool of *H. crenulatus* increased tenfold between cleavage (6.6 mmol kg^{-1} h^{-1}) and hatching (63.4 mmol kg^{-1} h^{-1}; Table 2), indicating increasing active ion transport, and were associated with increased Na^+/K^+-ATPase activity of embryos (present data) (Taylor and Seneviratna, 2005). Sodium efflux rates for a range of cells and animals were documented by Potts and Parry (Potts and Parry, 1964). Turnover rates observed in *H. crenulatus* embryos were higher than in most freshwater animals (e.g. *Asellus* 0.4 mmol kg^{-1} h^{-1}; *Eriocheir* 2 mmol kg^{-1} h^{-1}), spanned the range exhibited by marine animals and euryhaline osmoregulators (e.g. *Carcinus maenas* 25 mmol kg^{-1} h^{-1} in seawater, 14 mmol kg^{-1} h^{-1} in 40% seawater; *Nereis diversicolor* 10.9 mmol kg^{-1} h^{-1} in seawater, 5.5 mmol kg^{-1} h^{-1} in freshwater), and even exceeded steady state turnover of many internal tissues (e.g. frog muscle 8.7 mmol kg^{-1} h^{-1}; *Sepia* axons 25 mmol kg^{-1} h^{-1}; rat diaphragm 117 mmol kg^{-1} h^{-1}). It is concluded that the outer layers of *H. crenulatus* embryos do not provide isolation from ionic exchanges with the external seawater and the requirement to osmoregulate.

Tolerance of dilute seawater by developing embryos

Early ovigerous females of *H. edwardsii* have been observed in rock pools with salinity <5 near freshwater streams and ovigerous *H. crenulatus* may be collected from estuarine channels where river and seawaters mix. The previously demonstrated tolerance of post-gastrula embryos to acute changes in osmolality over a very wide range is therefore adaptive to conditions that may be encountered in their habitat (Taylor and Seneviratna, 2005). Prior to gastrulation, cleavage and blastula stages of *H. sexdentatus* and *H. crenulatus* were osmoconformers and were less tolerant of dilution. Similarly, embryos of the estuarine grapsids *Chasmagnathus granulata* and *Cyrtograpsus angulatus* acquired greater tolerance of extreme salinities (3–44) 2 or 3 days following egg extrusion (Bas and Spivak, 2000). These authors attributed the increased tolerance to a reduction in egg permeability but in *H. crenulatus* water permeability did not change appreciably at this time. A plausible alternative interpretation is that the increased survival was associated with gastrulation and the acquisition of the capacity to hyper-osmoregulate.

In the longer term trials reported here the embryos tolerated a narrower range of salinity. Normal embryogenesis and hatching of *H. sexdentatus* and *H. crenulatus* occurred in 100% and 50% seawater (36 to 18 salinity) but not in 25% seawater (9 salinity). Although adult crabs are capable of indefinite survival below 10% seawater (Hicks, 1973; Bedford and Leader, 1977) (H.H.T., unpublished observations), ovigerous crabs presumably must remain within regions of the shore or estuary where the average salinity is higher. Similarly, complete development of *C. granulata* and *C. angulatus* only occurred between salinity 12 and 40 (Bas and Spivak, 2000), and in the estuarine ocypodid *Macrophthalmus hirtipes*, between salinity 18 and 35 (Jones and Simons, 1982). Interestingly, although exposure of embryos to 25% and 50%

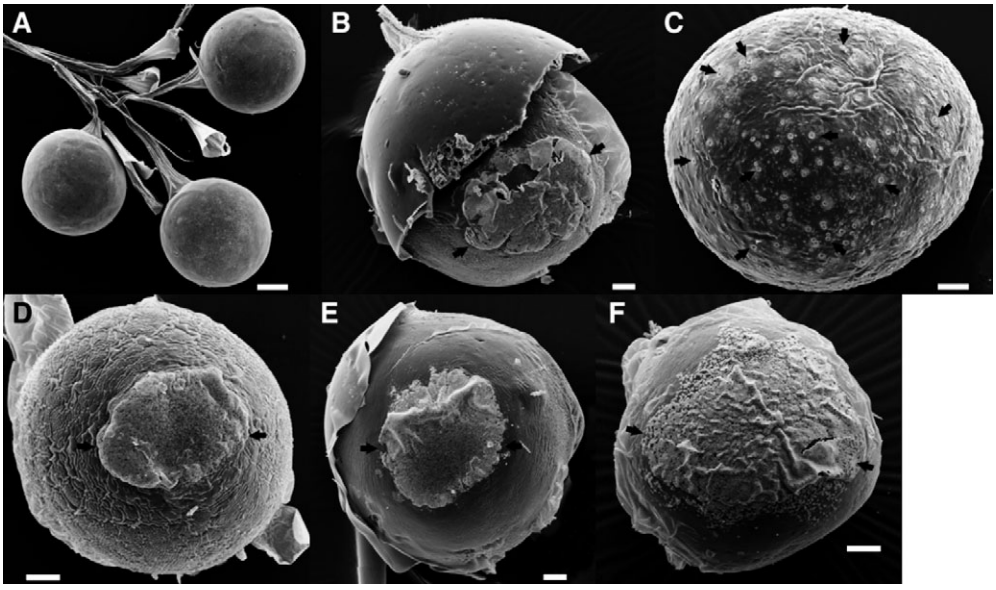

Fig. 7. Scanning electron microscopy of embryos of *Hemigrapsus sexdentatus* and *H. crenulatus* fixed after $AgNO_3$ treatment. AgCl deposits are indicated by arrows. (A) *H. sexdentatus* stage 4 with outer membrane intact. No silver deposit is visible. (B) *H. sexdentatus* stage 4 after partial removal of outer membrane. A thick amorphous deposit of AgCl adheres to the surface of the inner membrane. In C–F the outer membrane has been removed. (C) *H. sexdentatus* at stage 1, blastula. Spots of AgCl are distributed over the whole embryo, typically encircling small holes or craters. (D) *H. crenulatus* at stage 2, (E) *H. sexdentatus* at stage 3, and (F) *H. crenulatus* at stage 4. Scale bar, 25 μm (note that some shrinkage has occurred during processing).

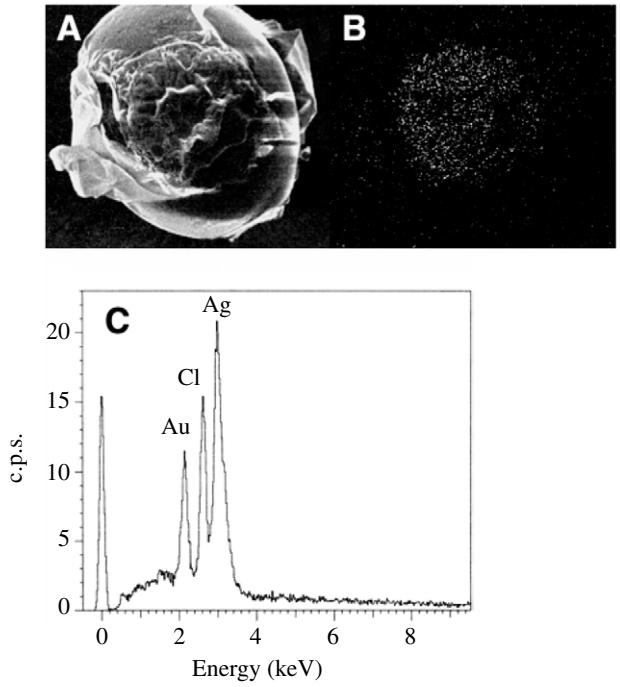

Fig. 8. X-ray microanalysis of the $AgNO_3$-stained area on an embryo of *Hemigrapsus sexdentatus* at stage 4. (A) Secondary electron image. (B) Corresponding mapping of the energy peak for silver atoms showing its localisation over the deposit on the inner membrane. (C) Energy spectrogram showing Ag and Cl atoms are major constituents of the material in the patch (the Au peak is from the sputter coat).

seawater before gastrulation was not obviously detrimental in the short term (present study) (Taylor and Seneviratna, 2005) when exposure was prolonged, development and hatching were subsequently impaired. Possibly, the osmotic stress disrupted critical morphogenetic events around cleavage and gastrulation and this contributed to their failure to develop normally. In the light of the greater vulnerability of pregastrula embryos of intertidal grapsids to osmotic shock, it is important to investigate whether ovigerous crabs exhibit behavioural or other mechanisms to protect the egg clutch during this short period.

The progressive increase in the volume of the *Hemigrapsus* spp. eggs incubated in seawater (Fig. 3) accords with observations on other Brachyura (Wear, 1974; Valdes et al., 1991). Little additional swelling occurred in 50% and 25% seawater, implying that egg volume was regulated, i.e. the embryos possess mechanisms for excreting water gained osmotically. Interestingly, swelling was observed in the eggs that were introduced to dilute seawater during cleavage and failed to gastrulate normally. This also suggests that egg volume depends on embryonic physiological processes rather than the properties of the envelope.

Acclimation to dilute seawater

A link between activities of the Na^+/K^+-ATPase in branchial and other epithelia and the osmoregulatory capacity of larval and adult crustaceans is well-established and many authors have demonstrated that this primary active transporter is up-regulated in response to lowered external salt concentrations (Holliday, 1985; Wheatley and Henry, 1987; Thuet et al., 1988; Holliday et al., 1990; Bouaricha et al., 1991; Bouaricha et al., 1994; Charmantier et al., 2001; Towle et al., 2001). The present observation of doubled Na^+/K^+-ATPase activity in *H. crenulatus* embryos at stage 4 that had been chronically exposed to 50% seawater, taken together with observations of increased Na^+/K^+-ATPase activity during acute (24 h) hyposaline exposures (Taylor and Seneviratna, 2005) suggest that the capability to mount an osmoregulatory acclimation response is present even in the earliest embryonic stages.

Sites of uptake and excretion of water and salts in embryos

Silver staining has been used to identify putative osmoregulatory epithelia in a number of insects (Krogh, 1939) and crustaceans (Koch, 1934; Ewer and Hatlingh, 1952; Talbot et al., 1972; Barra et al., 1983; Felder et al., 1986; Dickson and Dillaman, 1991; Kikuchi and Matsumasa, 1993; Lindhjem et al., 2000; Haond et al., 2001). The appearance of the silver-staining patch on the surface of the embryo at gastrulation coincided with the acquisition of hyper-osmoregulatory capacity, tempting the suggestion that the patch is concerned with ion transport. Silver-stained areas often correspond to epithelia involved in active ion uptake although the mechanism of staining is uncertain. Ag^+ may bind to Na^+ transporters because of similarities between these two ions (Koch, 1934; Krogh, 1939). Conversely, epithelia concerned with salt extrusion may stain with silver ions by precipitating AgCl at sites of Cl^- efflux, e.g. the neck organ of hypo-regulating *Artemia salina* nauplii (Conte et al., 1972) and the apical pit of marine teleosts (Philpott, 1965). Identification of considerable deposits of AgCl within the silver stained-patch on *H. sexdentatus* and *H. crenulatus* eggs implicates this region in chloride extrusion. Furthermore, the location of AgCl between the outer and inner membranes of the egg indicates that at this location the inner membrane is permeable to Cl^- ions and the outer membrane to Ag^+ ions.

The essential features of all multicellular hyperosmoregulators are, firstly, a site on the body surface for the active uptake of salts from the external medium into a hyperosmotic extracellular compartment and secondly, a mechanism for the delivery of extracellular fluid and salts (i.e. urine) to the exterior, to compensate for the osmotic water uptake. In adult decapod crustaceans these requirements typically are met by ion uptake into the haemolymph by branchial ionocytes and ultrafiltration *via* the antennal organs (Mantel and Farmer, 1983; Pequeux, 1995). The same general scheme has been applied even to very simple animals such as a freshwater coelenterate (Marshall, 1969) or embryos of freshwater molluscs (Beadle, 1969a; Beadle, 1969b; Beadle and Beadle, 1969; Taylor, 1977) in which extracellular fluid is expelled from the enteron and the blastocoel, respectively. What are the corresponding structures in hyper-regulating crab embryos?

In post-gastrula decapod embryos the extracellular space is

delimited by the embryonic and the extra-embryonic ectoderm, which extend over and enclose the yolk (Anderson, 1973). In decapods (e.g. *Palaemonetes*, *Leander*, *Crangon*, *Homarus*, *Astacus*, *Palinurus*, *Galathea*, *Eupagurus*, and another grapsid *Leptograpsus*) the embryonic dorsal organ is a thickening of the extraembryonic ectoderm in the dorsal midline at the opposite pole to the developing embryo (Anderson, 1973; Fioroni, 1980). It is believed to be concerned with the histolysis of the extraembryonic ectoderm during the formation of the body wall (Anderson, 1973). Precipitation of AgCl in this region indicates that the embryonic dorsal organs of *H. sexdentatus* and *H. crenulatus* allow the passage of electrolytes to the exterior. We propose that continuous osmotic entry of water into the extracellular compartment generates a small internal hydrostatic pressure that drives the paracellular exit of extracellular fluid; i.e. the embryonic dorsal organ serves as a simple filtration-type excretory organ. Whether cells in this region have podocyte-like specialisations for ultrafiltration or simply form a discontinuous barrier permitting leakage, and whether there are mechanisms for reclaiming useful molecules, are topics for future investigation. A hypothetical scheme for osmoregulation in *Hemigrapsus* embryos is presented in Fig. 9. The site of salt uptake is unknown but is perhaps a property of the general ectoderm. The dorsal organ persists until hatching, by which time the antennal organs are formed and are presumably functional (Anderson, 1973).

Embryonic dorsal organs (and dorsolateral organs) have been described in hyper-regulating non-decapodan crustaceans (Anderson, 1973; Fioroni, 1980; Martin and Laverack, 1992), including isopods (Strömberg, 1972), amphipods (Meschenmoser, 1989; Morritt and Spicer, 1995; Morritt and Spicer, 1996) and branchiopods (Aladin and Potts, 1995). Their ultrastructure is often suggestive of active ion transport and in *Orchestia* the dorsal organ stains with silver (Meschenmoser, 1989). In contrast to *Hemigrapsus* embryos, these dorsal organs may be concerned with salt uptake rather than salt loss. Similarly, the neck organ of hypo-osmoregulating *Artemia* nauplii, apparently a homologous structure (Fioroni, 1980; Martin and Laverack, 1992) is involved in salt excretion (Conte et al., 1972; Ewing et al., 1974; Hootman et al., 1972; Hootman and Conte, 1975; Lee and Watts, 1994).

Water and salt balance during cleavage

Pregastrula stages are hyper-osmoconformers maintaining a small positive osmolality difference (~150 mmol l^{-1}) between the medium and the water (Taylor and Seneviratna, 2005). Given their relatively high permeability to water, there is a requirement for water excretion in cleavage stages also. Possibly, the mottled deposits of AgCl observed in blastulae indicate salt release at multiple loci by temporary rupture of cell junctions between blastomeres as observed in pulmonate embryos (Taylor, 1977).

Conclusions

In summary, the present investigations have provided further evidence for the development of osmoregulatory function from the earliest embryonic stages of crabs. The generality of these conclusions for decapods should now be investigated. Previous studies on embryos of homarid lobsters and freshwater crayfish emphasised the osmoprotective role and apparently low permeability of the egg envelopes. Although the permeabilities of crab embryos in the present study were low compared with larger aquatic animals, they were certainly too high to provide effective osmotic isolation from the external medium. It was suggested (Taylor and Seneviratna, 2005) that the envelopes of homarid and astacid embryos may be more permeable to salts and/or water than previously supposed. These embryos also possess embryonic dorsal organs (Fioroni, 1980; Martin and Laverack, 1992). Comparative studies on the development, ultrastructure and fate of embryonic dorsal organs among crustaceans, immunocytochemical investigations of the appearance and localisation of membrane transporters, and their transcriptional control during embryogenesis, clearly

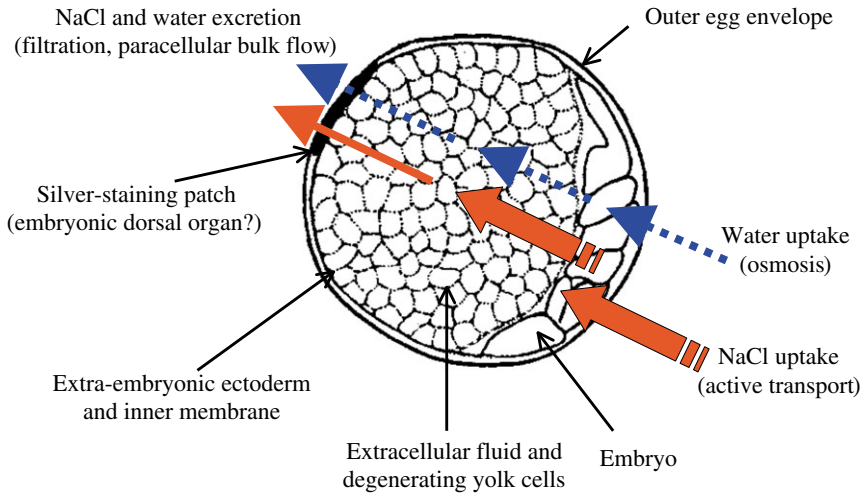

Fig. 9. A hypothetical model accounting for steady state water and salt turnover, hyper-osmoregulation and silver staining of post-gastrula embryos of *Hemigrapsus*. Active uptake of sodium and chloride takes place across the embryonic ectoderm into a hyperosmotic extracellular compartment by bounded embryonic and extra-embryonic ectoderm. Continuous osmotic entry of water into the extracellular compartment generates a small internal hydrostatic pressure causing both water and salts to leak out between the cells. In embryos vital stained with $AgNO_3$, Cl^- ions are precipitated as AgCl between the outer and inner membranes. After gastrulation this paracellular flow occurs primarily in the region of the embryonic dorsal organ, which therefore functions as a simple filtration-type excretory organ.

would help to elucidate the specific adaptations required to achieve water and salt balance of these critical life stages.

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