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Osmotic regulation of the amphipod *Gammarus chevreuxi* (Sexton, 1912)

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Abstract

This study investigated osmotic regulation in the amphipod, *Gammarus chevreuxi*, and is the first publication of this organism's osmotic regulatory curves. Unlike most estuarine amphipods studied to date which are hyperosmotic regulators, *G. chevreuxi* is a hyper-hyposmotic regulator. The hyperosmotic gradient maintained by *G. chevreuxi* in dilute media is very low (~120mmol.L^{-1}), which has been interpreted as evidence of freshwater ancestry among the Crustacea. Salinity acclimation did not appear to affect heart rate or pleopod beat frequency. The gill cells responsible for ion uptake appeared to be smaller in high salinities or distorted. The apical membrane in concentrated media shows signs of damage beneath the cuticle. These findings demonstrate strong osmoregulatory ability in *G. chevreuxi* and suggest descent from a freshwater lineage of amphipods.

Keywords**:** osmoregulation, ion regulation, *Gammarus chevreuxi*, gill ultrastructure, haemolymph, sodium, calcium, magnesium, salinity acclimation, heart rate

Introduction

Crustaceans have successfully invaded environments that can be described as; brackish, freshwater, estuarine, and hypersaline. This involved physiological adaptations to maintain ion and water homeostasis. Organisms can protect their cells from osmotic stresses by osmotic regulation of the extracellular fluids relative to the osmolality of the environment (Lockwood 1962; Pequeux 1995). Stenohaline animals do not have the mechanisms to maintain a gradient between the extracellular fluid and the media. Most estuarine crustaceans are euryhaline and exhibit a hyper-isosmotic pattern of regulation; maintaining haemolymph hyperosmotic at low salinities by active uptake of ions but conforming at higher salinities. Examples of amphipods inhabiting brackish or estuarine environments exhibiting hyperosmotic regulation include: *Gammarus duebeni* (Lockwood 1963), *Dikerogammarus villosus*, *G. zaddachi, G. tigrinus* (Brooks 2006) and *Corophium volutator* (McLusky 1968). An alternative pattern of osmotic regulation is hyper-hyposmotic regulation, for example the diadromous crab, *Eriocheir sinensis* (Roast et al. 2002), the semi-terrestrial talitrids (Spicer et al. 1987; Morritt 1989) and many estuarine or intertidal palaemonid shrimps (Freire et al. 2003; Augusto et al. 2009). These organisms maintain a hyposmotic gradient relative to the media in high salinities. Examples of hyperosmotic regulation show that the evolution hyper-hyposmotic regulation is not a necessity for the invasion estuarine environments. The hyper-hyposmotic pattern however, is prevalent in crustaceans inhabiting freshwater or terrestrial habitats. The palaemonid shrimps present an exception to this statement, for example the brackishwater shrimp, *Palaemon pandaliformis* and the diadromous, *Macrobrachium olfersii* (Freire et al. 2003) and the intertidal *P. northropi* (Augusto et al. 2009).

The investigation of a species pattern of osmotic regulation can tell us about its ancestral physiology. In this study, the osmotic regulatory pattern of *Gammarus chevreuxi* was investigated. This species is also an exception to the general hyperosmotic regulation exhibited in brackish and estuarine environments and this could indicate that this lineage has freshwater ancestors.

Gammarus chevreuxi (Sexton, 1912) was described at the Marine Biological Association, Plymouth, UK, using specimens from brackish ditches at Chelson Meadows, Plymouth. The organism was first studied in Mendalian crosses because of its short generation times and its eye pigmentation phenotypes. *Gammarus chevreuxi* was one of the first model organisms to demonstrate genetic control of timing of the eye pigment trait (Ford and Huxley 1927). In the laboratory *G. chevreuxi* can reproduce at a range of salinities (0 – 35 ‰), suggesting that osmotic regulation occurs (Sexton and Clark 1936). The animal has since been identified in similar habitats in western Europe where salinity is usually 11 ‰ or below (Subida et al. 2005). Lowenstein (1934) demonstrated that *G. chevreuxi* acclimated to 25% seawater consumed 20% less oxygen than when acclimated to 100% seawater. This data and the fact that most estuarine amphipods are hyperosmotic regulators led to the original prediction that *G. chevreuxi* also follows this pattern.

This study addressed three questions. 1) What pattern of osmotic regulation is exhibited by *G. chevreuxi*? 2) Is the heart rate of *G. chevreuxi* affected by acclimation to different salinities? 3) How does the ultrastructure of the gill epithelia respond under acclimation to 5 ‰ and 35 ‰? The size of *G. chevreuxi* (10-14 mm) necessitated the use of a sensitive instrument to determine haemolymph ion

concentrations. Heart rate was chosen as a proxy for metabolic rate as employed by Calosi *et al.* (2005). Gill ventilation may covary with this trait so pleopod beat frequency was also measured. Transmission electron microscopy was used to examine the ion transporting gill epithelia, which has been shown to respond to salinity (Lockwood and Inman 1973; Shires et al. 1994; Shires et al. 1995). This is the first study to determine the osmo-regulatory pattern of *G. chevreuxi.*

Materials and methods

Collection and acclimation

Amphipods were collected from the Plym estuary, Plymouth, Devon. The gravel bottom was kick sampled at low tide, 1st October, 2011. The amphipods were returned to the laboratory within one hour in water from the habitat and sorted into stock aquaria (300-400 individuals per aquaria, volume = 5L). The stock aquaria were acclimated stepwise to the relevant salinity and temperature in the laboratory was 15°C. Five days later the animals were identified and transferred into three salinity treatments, in triplicate (~100 individuals per aquaria, volume = 2L). The salinities were; 2 ‰; 10 ‰ and 35 ‰. A 25 ‰ treatment in duplicate was introduced, 15th October 2011 (~50 individuals per aquaria, volume = 2L), this treatment was only sampled for haemolymph ion concentrations. Amphipods were fed *ad libitum* with slices of carrot. Artificial seawater was used during the experiment using ocean salts (Aquarium systems, France) and changed weekly. Salinity was recorded 3-4 times per week using a refractometer and adjusted as necessary (experimental salinities ±SD were; 2 ‰ = 2.02 ±0.33; 10 ‰ = 9.90 ±0.47; 25 ‰ = 24.88 ±0.64; 35 ‰ = 34.74 ±0.85). Sampling was rotated to reduce time effects. A Star:Oddi DST CTD logger was deployed for 48 h, 27th October 2011, at the collection site to measure tidal salinity variation. Prior to deployment the logger was tested in the laboratory to determine sensor drift.

Determination of heart rate and pleopod beat frequency

Heart rate (HR) and pleopod beat frequency (PB) were determined by observation under low magnification (< x10) using a light microscope with transmitted light. The individual was shaken three times, horizontally on the worktop, prior to observations to elicit maximum HR. The traits were measured as efficiently as possible to avoid increases in temperature due to the heat from the lamp. It was not possible to observe HR without transmitted light. Ten *G. chevreuxi* from each aquarium were observed. After recording HR and PB, individuals were frozen until observations were completed. Then they were dried to constant mass at 80°C to measure dry mass.

Determination of haemolymph ion concentrations

Haemolymph samples were collected with a 10 µL Hamilton Syringe (33 gauge, flat-tipped needle) from mature individuals (≥ 10 mm). The puncture was made into the pericardial cavity between two of the thoracic dorsal plates. Samples were pooled (3-5 individuals) on a microscope slide, kept above ice. 0.5 µL was diluted with 1 mL of ELGA water, 10-15 samples were collected from each aquarium. Samples were refrigerated until analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES). To account for the use of artificial seawater; several samples were prepared for the four treatments, so that haemolymph and media ion

concentrations could be plotted against one another. Haemolymph and water samples were analysed for sodium, magnesium and calcium, using a Varian-725ES ICP-OES instrument. The operating conditions used were: forward power = 1.4 kW, plasma flow was 15 $L.min^{-1}$, auxiliary flow was 1.5 $L.min^{-1}$ and the nebuliser flow was 0.68 $L.min^{-1}$. The viewing height was set to 8 mm above the load coil and the read time was 4 s. Data were recorded in $mg.L^{-1}$ and expressed as $mmol.L^{-1}$.

Gill preparation for transmission electron microscopy (TEM)

To examine the ultrastructure of the gill epithelium, four individuals were acclimated to 5 ‰ and four acclimated to 35 ‰. After a period of 48 h, they were killed in their treatment water by adding approximately 2.5% glutaldehyde and fixed for 1 h. The procedures for fixing were based on techniques used by Lovell et al. (2005). After several rinses in cacodylate buffer (0.1 M, pH = 7.2) and a rinse in distilled water the fourth gill (right side) of *G. chevreuxi* was removed by pulling on the coxal plate. The gill was secondarily fixed with the pereopod and plate attached in 1% osmium. After 2 h the sample was rinsed in buffer and dehydrated through a graded ethanol series. Once in 100% ethanol the gill was transferred through graded concentrations of low viscosity agar resin, until 100% infiltration. Gills were set in Beem capsules and polymerised overnight at 70°C. The set gills were cross-sectioned with a Reichert-Jung Ultracut and a Microstar diamond knife. Ultrathin sections were picked up using 200-mesh thin bar copper grids. The sections were stained, firstly with a saturated ethanol solution of uranyl acetate and secondly with Reynolds lead citrate (15 min each stain). The processed images were taken with a Jeol 1200 EXII TEM and images captured with an SIS Mega view III.

Statistical analysis

Data Analysis was carried out using the software package, SPSS 19. HR and PB were first compared with mass using linear regression, neither variable showed any relationship, so mass was not included in further tests, the residual distribution was not significantly different to normal (HR: mass, $R^2_{adj} = 0$, $P > 0.05$; PB: mass, $R^2_{adj} = 0$, $P > 0.05$). Regression between HR and PB showed covariance between the two traits (HR: PB; $R^2_{adj} = 0.30$, $P < 0.05$). Therefore, PB was used as a covariate in ANCOVA to compare mean HRs between treatments. Residuals were tested for normality with the Kolmogorov-Smirnov test ($P > 0.05$). Mean haemolymph sodium, magnesium and calcium were plotted against the respective ion concentrations of the four treatments. Haemolymph data were checked for within treatment differences using ANOVA for sodium and calcium. Residuals for magnesium differed from normality so Kruskal-Wallis tests and Mann-Whitney U for the 25 ‰ treatment were used to compare within treatment means.

Results

Salinity profile of the collection site

Figure 1a shows the location where the *G. chevreuxi* for this study were sampled and the salinity logger was deployed. *Figure 1b* illustrates the salinity increases associated with high tide in the Plym estuary, this population of *G. chevreuxi* experiences a salinity pulse, when salinities reach values of 30 ‰ for approximately 4 h.

Heart rate and pleopod beat

Mean heart rates (±95% CI) were; 210.30 (± 6.22), 230.7 (± 6.08) and 224.8 (± 8.41) bpm for the 2 ‰, 10 ‰ and 35 ‰ treatments respectively. Median PB were; 233.5, 258.5 and 251.5 bpm for the 2 ‰, 10 ‰ and 35 ‰ treatments respectively. These data are presented in *figure 2.* 2-way ANCOVA of HR, using the factors salinity and replicate with PB as a covariate, identified within treatment differences ($F_{6,89}$ = 7.07, P < 0.05, see *table one*).

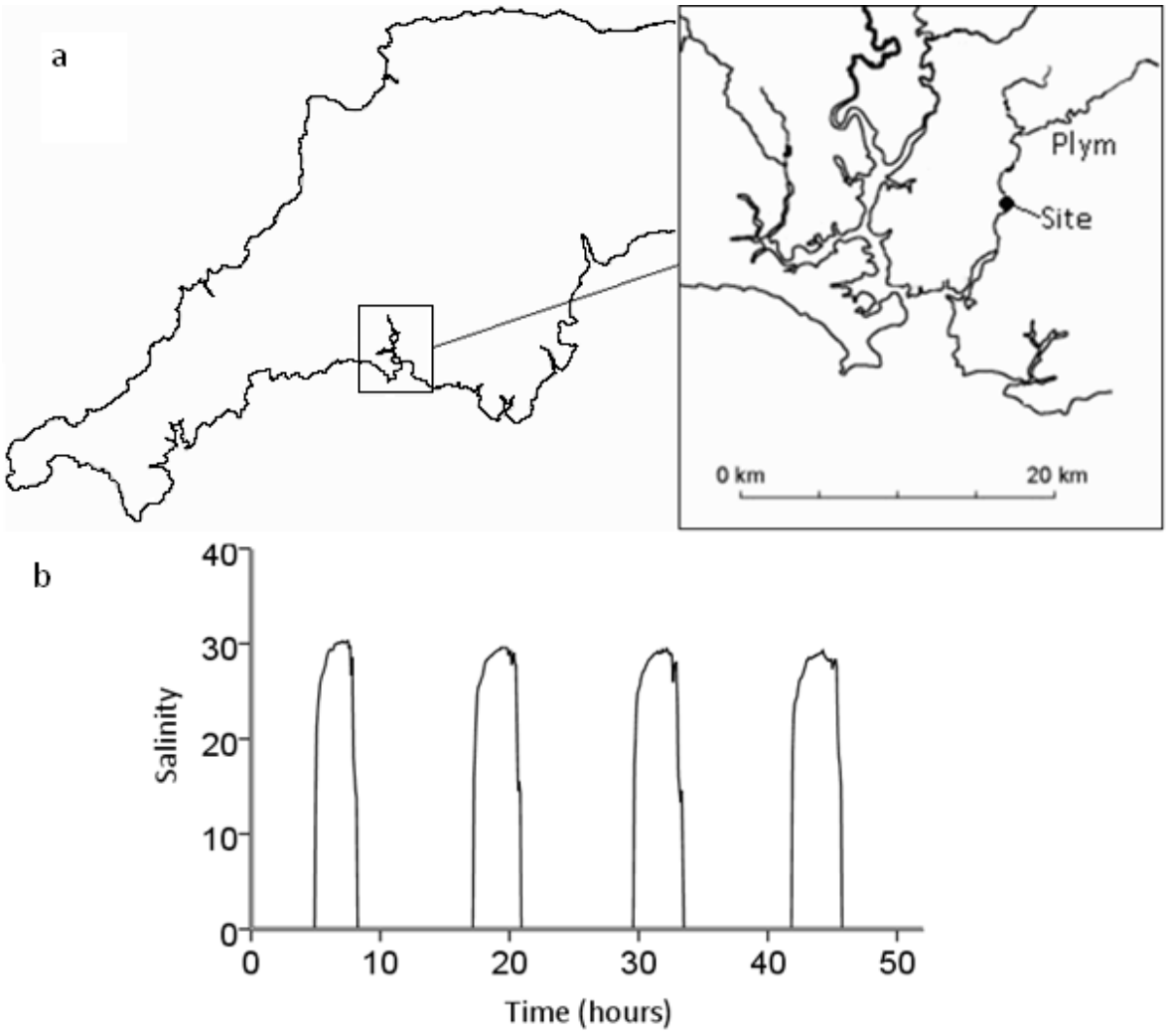


Figure 1: a) Southwest England (left) Plym and Tamar estuaries (right) identifying the *site* where *Gammarus chevreuxi* were collected and where the salinity logger was deployed. b) Salinity variation at the Plym estuary collection site, 27th October 2011. Note the sharp rise in salinity at high tide and sharp decline < 4h later.

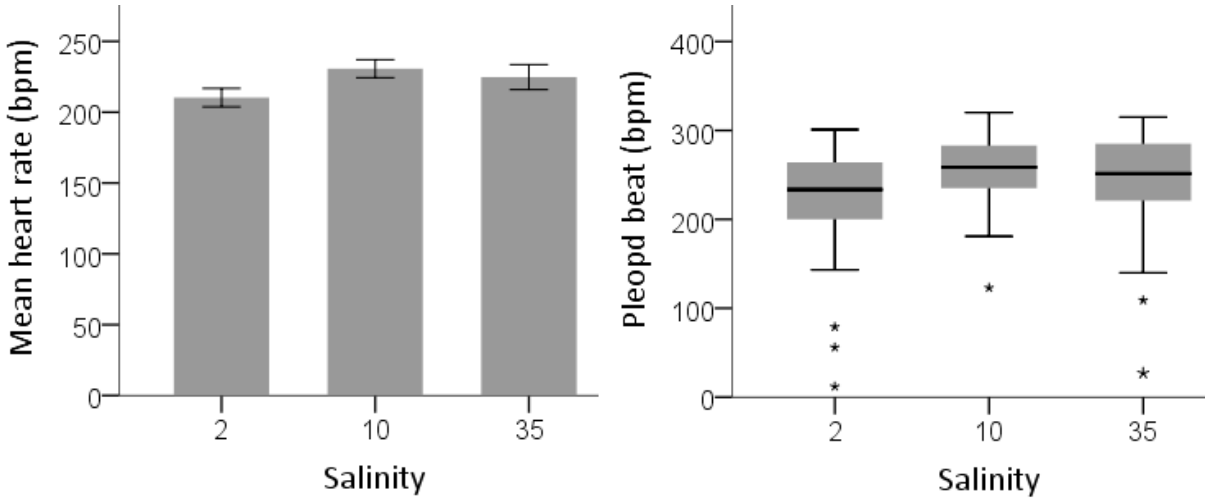
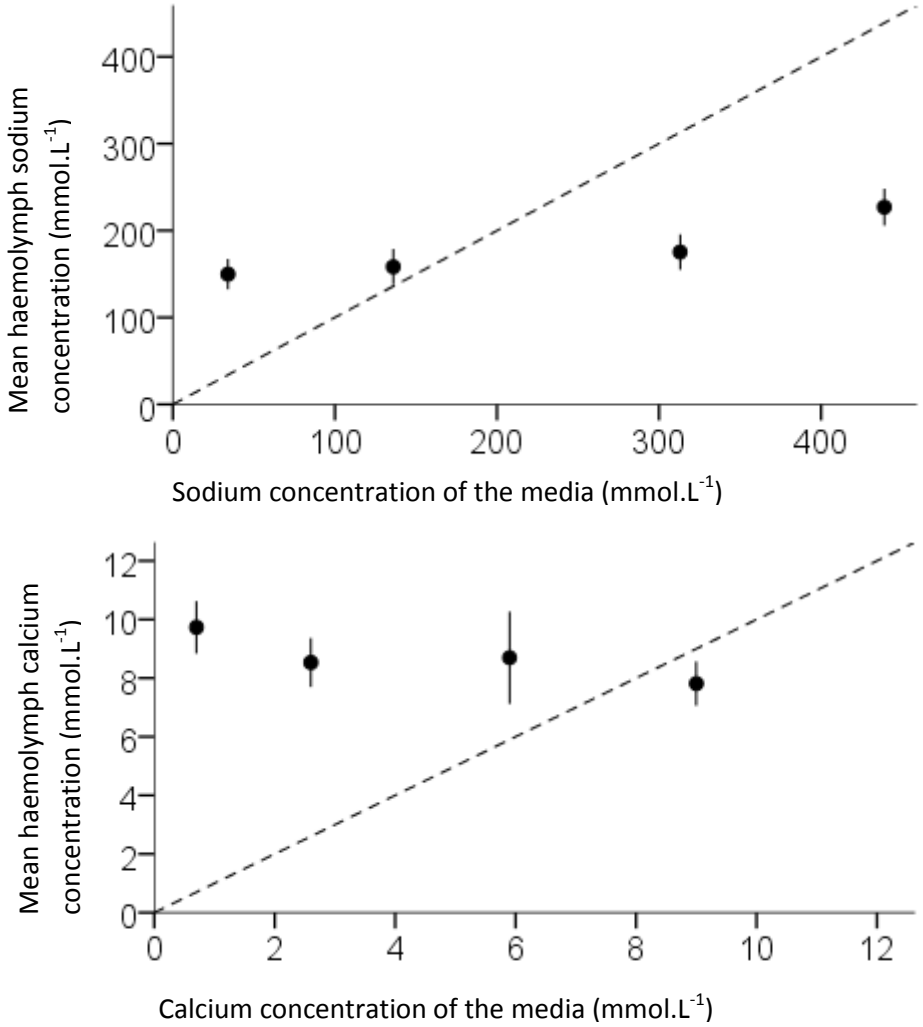


Figure 2: Left, mean heart rates (± 95% CI) of *Gammarus chevreuxi* acclimated to three salinities (2‰; 10 ‰ and 35 ‰). Note, ANCOVA identified within treatment differences ($F_{6,89}$ = 7.07, P < 0.05, see *table 1*). Right, median pleopod beat (± 25% quartiles) of *G. chevreuxi* acclimated to three salinities (as above).



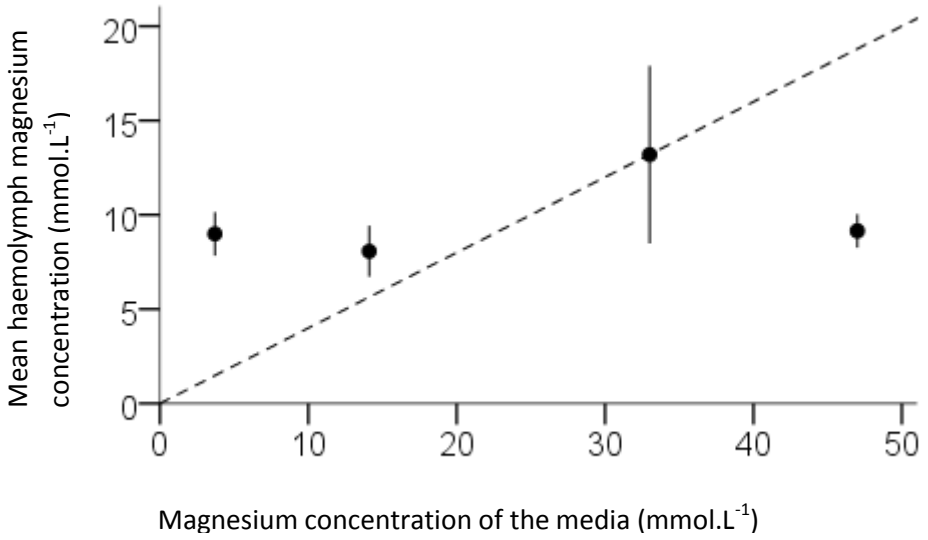


Figure 3: Haemolymph ion regulatory curves for sodium, calcium and magnesium (top to bottom) for *G. chevreuxi* acclimated to four salinities (2 ‰, 10 ‰, 25 ‰ and 35 ‰). Points represent pooled biological replicates from the respective treatments (N = 40 – 45, except in 25 ‰ where N = 19). Dashed line is the isosmotic line.

Haemolymph ion concentrations

Replicate data for sodium, magnesium and calcium concentrations were pooled by treatment to construct the ion regulatory curves, *figure 3*. The sodium and magnesium curves show hyper-hyposmotic regulation of the haemolymph. Calcium was regulated between 7.5-10 mmol.L^{-1} in all four treatments. Sodium concentrations between replicates were significantly different (ANOVA: $F_{10,132} = 6.28$, $P < 0.001$, see *table 1*). SNK *post-hoc* tests showed that all replicates within the four treatments fell within homogenous subsets. No replicates were significantly different for calcium (ANOVA: $F_{10,132} = 1.79$, $P > 0.05$, see *table 1*). Kruskal-Wallis tests between treatment replicates showed that magnesium concentrations in the 2 ‰ treatment were significantly different ($P < 0.05$). Mann-Whitney U test showed that the two replicates in the 25 ‰ treatment had significantly different means ($P < 0.01$).

TEM gill preparations

The coxal gills of *G. chevreuxi* are leaf-shaped, flattened sacks covered by a thin cuticle approximately 0.25 µm thick, as has been observed in *G. oceanicus* (Milne and Ellis 1973), *G. duebeni* (Lockwood et al. 1972) and *Sternomoera yezoensis* (Kikuchi et al. 1993). Two layers of epithelial cells are orientated back to back with haemolymph vessels and spaces in between. *Figures 4* and *5* show the gill epithelia of *G. chevreuxi* acclimated to 5 ‰ artificial seawater. *Figure 4* shows a poorly defined basal infolding system (BIS) and haemolymph space, which was similar between both salinities. The cell boundaries are easily identified at 5 ‰, particularly the baso-lateral cell boundaries. *Figure 5* shows the apical infolding system (AIS) and a boundary between two neighbouring cells at 5 ‰. The microvilli tips extend to the cuticle. Adherent junctions are present and the cells show some interdigitation at the apical cell boundary. *Figure 6* shows the gill epithelia of *G. chevreuxi* acclimated to 35 ‰ artificial seawater. The AIS and a cell boundary can be seen in the image. The cell boundaries are less defined due to extensive interdigitation between

neighbouring cells which extended to the baso-lateral boundaries. The microvilli are not in contact with the cuticle and there are signs of apical membrane damage in the sub-cuticular space. This was apparent in all the images from this treatment. More mitochondria are present in the 35 ‰ treatment. Mean distances (±SD) between the tips of apical microvilli and the cuticle were 0.086 µm ± 0.10 and 1.99 µm ± 1.00 in the 5 ‰ and 35 ‰ treatments respectively. The mean widths (±SD) of the microvilli were 0.054 µm ± 0.012 and 0.062 µm ± 0.016 in the 5 ‰ and 35 ‰ treatments respectively, not supporting a reduction in cell volume.

Discussion

The osmotic regulatory curves show that adult *G. chevreuxi* are hyper-hyposmotic regulators. This pattern may not be present throughout ontogeny, for example, *G. duebeni,* exhibits hyper-hyposmotic regulation in embryos between stages five and seven of development, possibly associated with the gills taking up regulatory function from the dorsal organ (Morritt and Spicer 1995).

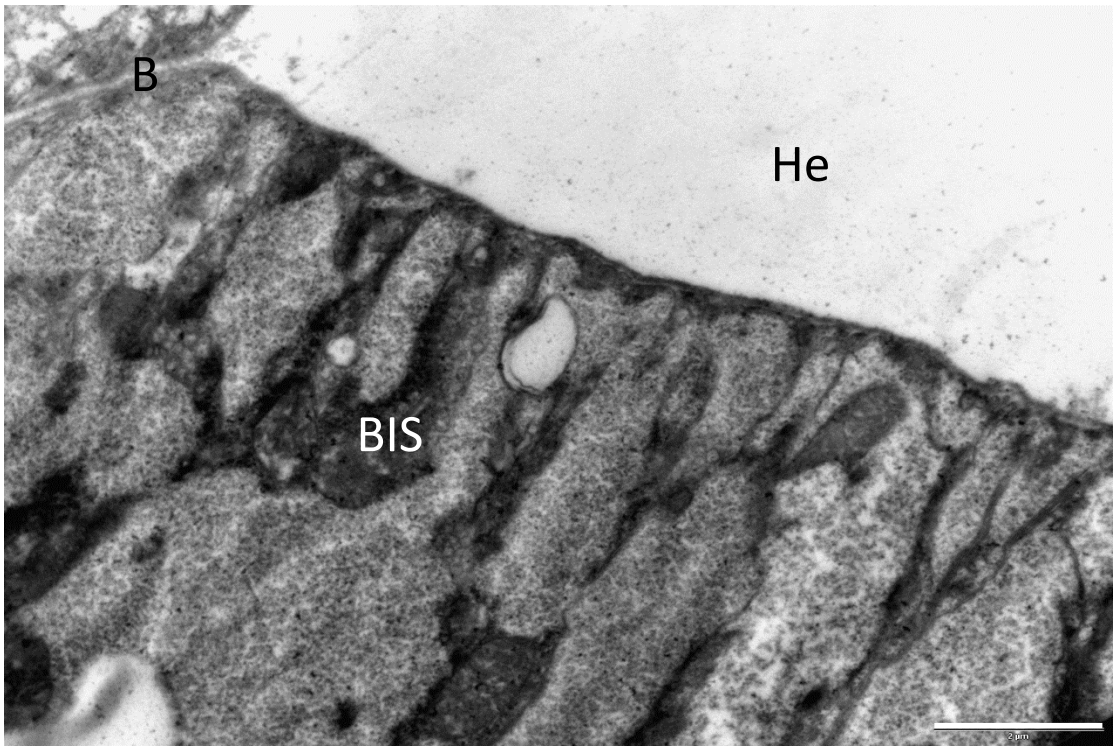


Figure 4: TEM showing the basal infolding system, BIS, of the gill epithelium of *G. chevreuxi* acclimated to 5 ‰. The infolding is not very distinct, which was also the case images from the 35 ‰ treatment. Note the distinct cellular boundary, B, in the top left of the image. The heamolymph space is labelled He and the scale bar = 2 µm.

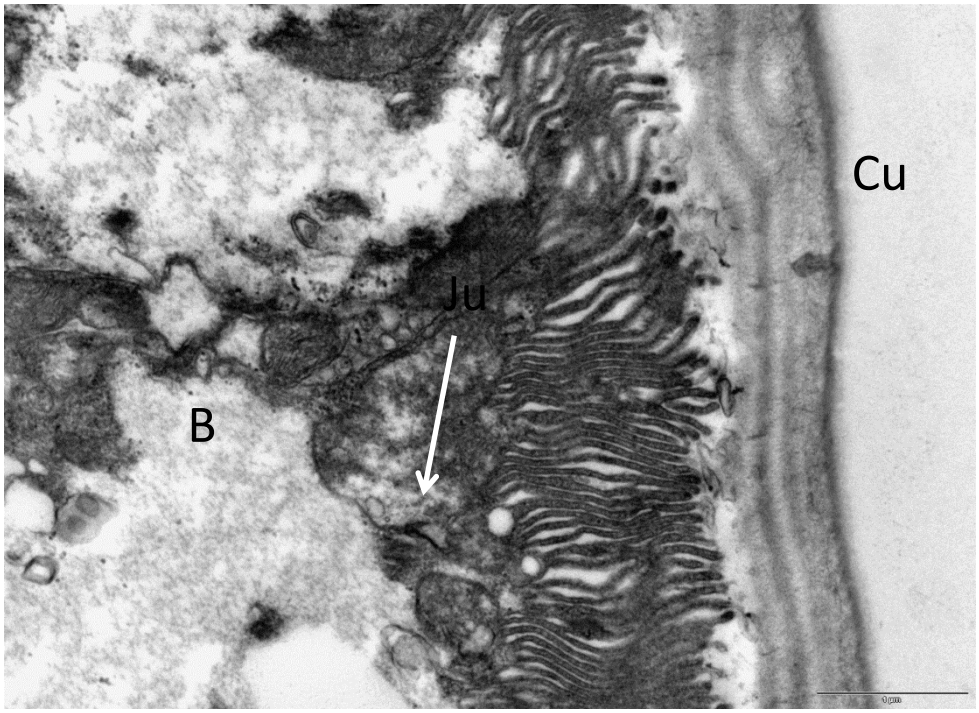


Figure 5: TEM showing the apical infolding system, AIS, of the gill epithelium of *G. chevreuxi* acclimated to 5 ‰. The cell boundary, B, is fairly clear in the apical region and the adherent junction, Ju, apparent just beneath the AIS. The cuticle is labelled Cu and the scale bar = 1 µm.

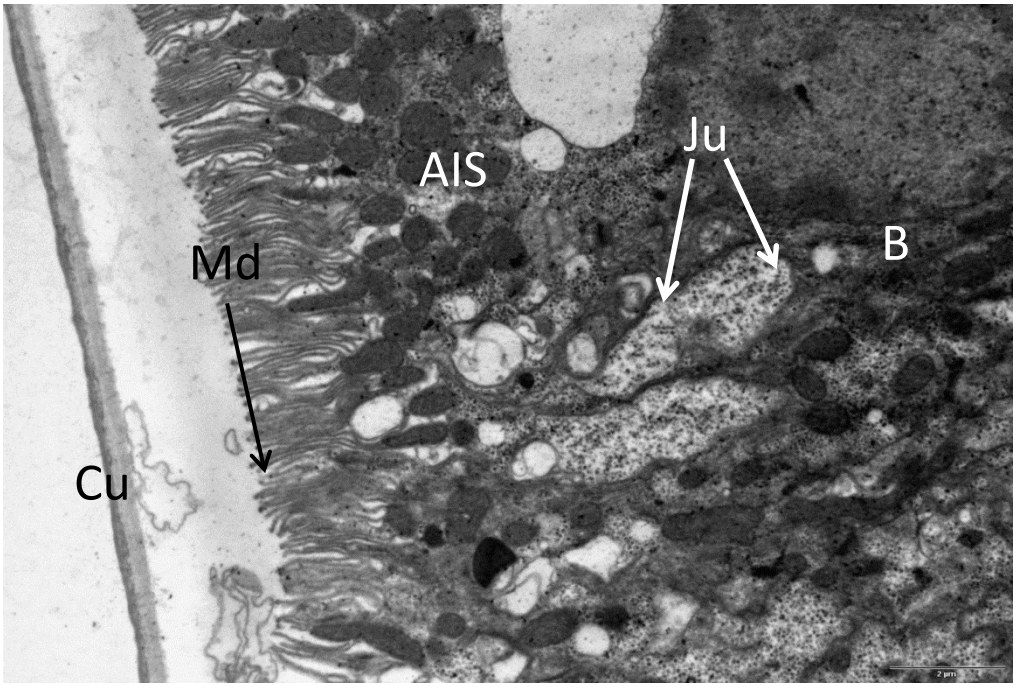


Figure 6: TEM showing the apical infolding system, AIS, of the gill epithelium of *G. chevreuxi* acclimated to 35 ‰. The cell boundary, B, is less distinct due to interdigitation between neighbouring cells but cellular adherent junction, Ju, is still apparent. Note the numerous mitochondria and the deep sub-cuticular space. Apical membrane damage, Md, was apparent in all images from the 35 ‰ treatment. The cuticle is labelled Cu and the scale bar = 2 µm.

Table 1: Two-way ANCOVA to test mean heart rates of *Gammarus chevreuxi* acclimated to three salinities (2 ‰, 10 ‰, 25 ‰ and 35 ‰, top row). ANOVA results to check for within treatment differences in haemolymph ion concentrations; sodium, middle row and calcium, bottom row. Note, SNK *post hoc* tests showed that all differences were at treatment level for sodium.

Test	Source	d.f.	SS	MS	*F*	P
2-way ANCOVA	Pleopod	1	3014.56	3014.56	15.17	0.001
Heart rate	Replicate	6	8433.671	1405.61	7.07	0.001
salinity by	Salinity	-	-	-	-	-
replicate	Residual	80	15901.64	198.77		
	Total	89	39765.29			
1-way ANOVA	Replicate	10	212713.44	21271.34	6.28	0.001
Haemolymph	Residual	132	446906.27	3385.65		
Sodium	Total	143	5160754.36			
1-way ANOVA	Replicate	10	179.38	17.94	1.79	0.068
Haemolymph	Residual	132	1323.54	10.03		
Calcium	Total	143	12966.96			

There are few examples of estuarine hyper-hyposmotic regulating amphipods, most studied to date are hyperosmotic regulators. *G. pulex* is a freshwater species that has a hyperosmotic pattern but does not tolerate high salinities the gradient maintained in low salinities is less than 200 $mmol.L^{-1}$ sodium (Lockwood 1962; Brooks 2006). A rare example of an amphipod that exhibits hyper-hyposmotic regulation is the freshwater species *G. fossarum,* which can tolerate higher salinities but suffers high mortality (Dorgelo 1977). The osmoregulatory curve for this species is similar to *G. chevreuxi* except at higher salinities, where the hyposmotic gradient is not as large. Therefore, *G. chevreuxi* exhibits an uncommon pattern of osmoregulation among amphipods.

Low ion concentrations in dilute media suggest freshwater decent, because in dilute media there is an energetic benefit to having a small sodium gradient; 1) because ions are lost at a slower rate 2) the rate of active uptake can be reduced to maintain homeostasis (Lockwood 1962). In terms of comparison to other Amphipods, hyposmotic regulation could also be interpreted as a freshwater adaptation (Lockwood 1962; Little 1990; Schubart and Diesel 1999). However many palaemonid shrimps that are estuarine, intertidal or freshwater are hyper-hyposmotic regulators, which would seem to counter the statements made by the authors' cited above (Freire et al. 2003; Augusto et al. 2009). Lockwood (1962), still offers the most satisfactory explanation to this apparent controversy. There are a variety of mechanisms that govern haemolymph ion concentrations *id est:* permeability of the body surface; excretion; active uptake; the critical concentration of the media at which uptake mechanisms are activated and drinking. Therefore, the pattern alone does not allow us to make assumptions about evolutionary history but the size of the gradient must also be considered.

For haemolymph sodium the gradient maintained by *G. chevreuxi* at 2 ‰ was approximately 120 mmol.L^{-1} this is low even when compared to terrestrial talitrids such as *Orchestia gammarellus,* which in near freshwater maintains a gradient of 300 mmol.L^{-1} sodium, but compares well to the semi terrestrial species *Arcitalitrus dorrieni* that maintains a gradient of 150 mmol.L^{-1} in low salinities (Morritt 1989). Whence, for sodium concentration the pattern exhibited by *G. chevreuxi* compares better to terrestrial amphipods than estuarine amphipods. In all four treatments the concentrations of calcium in the haemolymph approximated that of 30-35 ‰ seawater, suggesting that this ion is stably regulated. Calcium is an important ion for all animals and particularly for crustaceans because of its association with the exoskeleton (Spicer and Taylor 1987). The magnesium ion was regulated in a similar manner to sodium by *G. chevreuxi*, although within treatment differences were found.

Ion transport across the gill epithelia involves ion channels, symporters and ATPase transporters. Electrophysiological studies on the split gill lamella of *C. maenas* (hyperosmotic) and *E. sinensis* (hyper-hyposmotic) have demonstrated different mechanisms between the two crabs (Onken and Reistenpatt 1998; Freire et al. 2008). The key difference is the mechanism generating negative cellular potential, which drives the other transporters. *C. maenas* excludes potassium ions and *E. sinensis* pumps hydrogen ions with V-type H^+ATPase. The evidence suggests the latter mechanism applies to Crustaceans exhibiting hyper-hyposmotic regulation. The copepod *Eurytamora affinis* has made many independent invasions of freshwater. Lee *et al.* (2011) have shown *in vitro* that within twelve generations *E. affinis* increases the expression of V-type H^+ATPase supporting the role this enzyme plays in adaptation to freshwater. Based on this evidence negative potential in the gill epithelial cells of *G. chevreuxi* is likely to use V-type H^+ATPase. Measurement of the activity of this transporter would show whether the mechanisms are applicable among other groups of crustaceans.

Heart rate and pleopod beat were not related to mass, which was also observed by Calosi et al. (2005) in *Talitrus saltator.* The data suggest that maximum HR is unaffected by salinity acclimation. This could be explained by two reasons. Firstly, the gradients maintained between the haemolymph and media are fairly low (120-200 mmol.L^{-1}), possibly too small to observe a response. Secondly, maximal HR is possibly finite regardless of salinity acclimation. It was not possible, however, to observe the HR of a resting animal, even the use of an electrocardiograph would cause procedural responses in HR. In light of these observations metabolic rate should have been measured by oxygen utilisation.

Unfortunately there is no replication of the TEMs to confirm the membrane damage and cellular distortion, apparent in gill epithelial cells acclimated to 35 ‰, but the images lend support to freshwater ancestry. Some of the differences observed between treatments can be explained by a reduction in cell volume at 35 ‰. For instance, more mitochondria observed in the high salinity images are likely associated with cell shrinkage, concentrating the cell contents. However, the measurements of microvilli show they were the same width between treatments which does not suggest a reduction in cell volume, so it is also possible that the haemolymph vessels and spaces are smaller. Caution must be taken when TEMs are interpreted as the procedure for fixing can alter the structure of the cells creating

artefacts in the images. However as the gills were processed simultaneously any artefacts would be equally present in both treatments. In 35 ‰ seawater *G. chevreuxi* has haemolymph that is hypotonic to the media, which would cause body fluid loss to the media. Cell boundaries exhibited complex interdigitations at 35 ‰ and this could be a cellular response to paracellular fluid movements. The gills are responsible for hyper-regulation but not hypo-regulation which is thought to occur in the gut (Pequeux 1995; Schubart and Diesel 1999; Freire et al. 2008). So the withdrawal of the microvilli could be indicative that the cells are not actively transporting ions, this would also be expected to reduce permeability (Shires et al. 1994). The population studied here experiences seawater salinities for < 4 h per tidal cycle the epithelial distortion and cellular interdigitation are beneficial to restrict water loss, via the gills, during high tide.

Shires *et al.* (1994) examined the gill epithelia of *G. duebeni* acclimated to freshwater but transferred to seawater to reverse the direction of the osmotic gradient. Gills were fixed at 1 h, 5 h and 16 h after transference to seawater. The cells at 1 h began to take on a distorted appearance sharing many of the features described above for *G. chevreuxi* at 35 ‰. However, after 16 h the cells of *G. duebeni* regain their former shape. This is because *G. duebeni* is a hyperosmotic regulator and transference to 35 ‰ seawater temporarily reverses the gradient, after 16 h the haemolymph conforms to osmotic pressure of the media. *G. chevreuxi*, on the other hand remains hyposmotic with respect to the media so the gill epithelia retains its contorted shape despite acclimation. Shires *et al.* (1994) drew attention to batteries of microtubules associated with the septate junctions in the gill epithelia of *G. duebeni*. Although adherent junctions can be identified, the images are not of sufficient resolution to determine the presence of microtubules.

Gammarus chevreuxi is a hyper-hyposmotic regulator, maintaining a low hyperosmotic sodium gradient at 2 ‰ (34 mmol.L^{-1} Na^{+}) of ~120 mmol.L^{-1} and a larger hyposmotic gradient at 35 ‰ (439 mmol.L^{-1} Na^{+}) of ~200 mmol.L^{-1}. It can be predicted that this animal generates negative cellular potential to drive ion transport using V-type H^{+}ATPase, based on the experiments reviewed by Onken and Reistenpatt (1998) and this would be interesting to determine. From amphipods studied to date, this is an unusual pattern of osmoregulation among amphipods, but fits well with many euryhaline prawns such as; *Macrobrachium olfersii, M. potiuna, Palaemon northropi* and *P. pandaliformis* (Freire et al. 2003). No responses were observed in HR or PB to salinity acclimation, it is not clear whether this is a procedural issue or that there is a physiological explanation. The ultrastructure of the gill epithelia show a retraction of the microvilli from the Cuticle and interdigitation between cells under acclimation to 35 ‰ seawater and signs of apical membrane damage, replication is required to confirm these observations. The combination of results suggests that the osmotic apparatus of *G. chevreuxi* is better adapted to freshwater or brackish environments, as noted by Subida *et al.* (2005). The evidence provided in this study, and by other authors, supports the overall conclusion that *G. chevreuxi* is descended from a freshwater lineage of amphipods that have subsequently adapted a tolerance to saline water.

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