Ontogeny of osmoregulation in the brackishwater amphipod *Gammarus chevreuxi*

Manuela Truebano*, Stuart D. Robertson, Sam J.S. Houston and John I. Spicer

Marine Biology and Ecology Research Centre, School of Biological and Marine Sciences, University of Plymouth, Plymouth, Drake Circus, Plymouth PL4 8AA, UK.

*Corresponding author: manuela.truebanogarcia@plymouth.ac.uk

Abstract

Osmoregulation is a key regulatory function in animals inhabiting brackish waters or areas subject to considerable salinity change, such as estuaries. While our understanding of osmoregulation in adult crustaceans is relatively good, our knowledge of how osmoregulatory ability develops during ontogeny is not well documented. In indirect developers, improvement in osmoregulatory capacity during ontogeny appears to coincide with a major metamorphosis. This is consistent with the 'incomplete adult hypothesis', which assumes that early developmental stages are 'incomplete individuals' operating less efficiently than individuals at the older stages. Evidence for this is not clear in direct developers. Consequently, we tested the 'incomplete adult hypothesis', by characterising the ontogeny of osmoregulation of the euryhaline amphipod, *Gammarus chevreuxi*, a species which undergoes direct development. We investigated the structure and function of putative osmoregulatory tissues, together with the regulation of key osmoregulatory genes. Embryos were examined at key developmental stages: before the dorsal organ (DO), a putative osmoregulatory structure, appeared (<48 hpf), before the gills appeared but the DO was present (9 dpf), and after both DO and gills were present (14-18 dpf). Adult *G. chevreuxi* exhibited a pronounced hyper-hypo-osmoregulatory pattern, matched by a strong pattern of haemolymph ion regulation. At a salinity of 35, eggs were hyposmotic to the external medium (989 mOsm Kg^{-1}) in the DO and gill stages (mean±SD= 584±80.5 and 744±103 mOsm Kg^{-1} respectively) with less of a difference with the medium before DO development (mean±SD= 810±91 mOsm Kg^{-1}). At a salinity of 2, eggs from all stages were hyperosmotic to the external medium (52 mOsm Kg^{-1}), with the pre-DO stage being closest to the isosmotic line

(mean±SD= 330±29, 510±55 and 502±55 mOsm Kg^{-1} for pre-DO, DO and gill respectively). Differences between the stages diminished at salinity 15. Thus, the adult hyper-hypo-osmoregulatory pattern was present before the ontogeny of the DO and gills, although it improved during ontogeny. Expression of Na^{+}/K^{+}-ATPase transcripts was detected throughout ontogeny, further supporting the idea that ion transporting activity may occur before the formation of osmoregulatory organs. The ontogeny of osmoregulatory function in the gammaridean amphipod *G. chevreuxi* is therefore consistent with the incomplete adult hypothesis.

Key words: Crustacea; Development; Dorsal organ; Embryo; Hyper-hypo-osmoregulation; Na^{+},K^{+}-ATPase

Introduction

Understanding the ontogeny of physiological function and regulation is essential to predicting how and when selection pressures may operate on a developing individual in any given, or changing, environment (Adolph, 1968; Burggren, 2018; Burggren and Warburton, 2005). Such understanding is important in predicting survival through to the reproductively-active adult stage, and consequently Darwinian fitness (Mueller et al., 2015; Spicer et al., 2018). Burggren (2005) suggested that the focus on adult stages in ecophysiological studies is underpinned by the assumption that increasing anatomical complexity invariably leads to increasing physiological complexity. In this view, the early developmental stages would be seen as incomplete individuals operating less efficiently than individuals at the older stages, the so called ‘incomplete adult hypothesis’ (Adolph, 1968; Spicer and Gaston, 1999). According to this hypothesis, adults would display the most well-regulated physiological processes. The corollary of this is the ‘physiological competency hypothesis’, which states that ‘at every stage, the complement of properties and regulations is complete…for operation of the body’ (Adolph, 1968). In this case, the most complex or best-developed physiological regulations or functions would not always be expected to be restricted to, or even present in, the adult stages (Spicer and Gaston, 1999)

Osmoregulation is an important regulatory function that many aquatic animals perform, particularly if they inhabit brackish waters or areas subject to considerable salinity change, such as estuaries (Beadle, 1957; Charmantier et al., 2008; Gilles, 1975; Krogh, 1939; Potts and Parry, 1964). Coastal and estuarine crustaceans have received considerable attention in this regard, and tend to show a strong osmoregulatory ability, at least in the adult stage (Lignot and Charmantier, 2015;

26 Lockwood, 1962; Rivera-Ingraham and Lignot, 2017; Robertson et al., 2010;
27 Schoffeniels and E., 1970; Thabet et al., 2017). Such osmoregulatory functions are
28 carried out by combinations of the following structures: the gills, the gut, the antennal
29 gland, and extrabranchial ion exchange tissues (Freire et al., 2008; Henry et al.,
30 2012; Lignot and Charmantier, 2015).

31 While osmoregulation in adult crustaceans has been widely studied, our knowledge
32 of how osmoregulatory ability, and the structures that provide that function, develop
33 during ontogeny, is not well documented (Anger, 2003; Charmantier, 1998;
34 Charmantier and Charmantier-Daures, 2001; Lignot and Charmantier, 2015).

35 The ontogeny of whole animal osmo-and iono-regulation has been investigated
36 mainly in crustaceans that undergo indirect development, such as crabs (Augusto et
37 al., 2009; Brown and Terwilliger, 2007; Charmantier and Charmantier-Daures, 1994),
38 shrimps (Bouaricha et al., 1994; Cieluch et al., 2005; Felder et al., 1986), crayfish
39 (Susanto and Charmantier, 2002) and lobsters (Charmantier and Aiken, 1987; Dall,
40 1970). Some attention has also been paid to the structures, and biochemical
41 mechanisms, associated with developing osmoregulatory ability, such as the
42 ephemeral crustacean larval salt gland (Conte, 1984), and the onset of Na^+-K^+-
43 ATPase and carbonic anhydrase activity (Lignot and Charmantier, 2015). This work
44 lends support to the incomplete adult hypothesis, where improvement in
45 osmoregulatory capacity appears to coincide with a major metamorphosis. Such
46 support is more equivocal in direct developing groups such as the peracarids:
47 mysids, isopods and amphipods. Peracarids lay and brood their eggs in a
48 marsupium, a modification of the ventral groove, within which semi- and fully
49 terrestrial species, such as the amphipods (Morritt and Spicer, 1996a) and isopods
50 (Hornung, 2011) can regulate the osmotic concentration of the exosomatic water

51 those eggs are immersed in. Even though the osmotic concentration of the marsupial
52 fluid of the semi-terrestrial amphipod *Orchestia gammarellus* is tightly regulated, all
53 embryonic stages display a strong hyper-hypo-regulation (Morritt and Spicer, 1999).
54 Interestingly, the regulation is much weaker in immediately post hatch individuals
55 (Morritt and Spicer, 1999). In this species, new hatchlings are retained within the
56 marsupium for a further 10 days until they (re)develop the adult pattern of
57 osmoregulation (Morritt and Spicer, 1996b). This does not support the incomplete
58 adult hypothesis.

59 There is some evidence for maternal control of osmolality in the marsupium of
60 aquatic peracarid crustaceans but only for two species, the mysid *Praunus flexuosus*
61 (Mclusky and Heard, 1971) and the isopod *Sphaeroma serratum* (Charmantier and
62 Charmantier-Daures, 1994). There is no published information for amphipods,
63 although preliminary experiments investigating the osmolality of water within the
64 marsupium of *Marinogammarus marinus* and *Gammarus chevreuxi* kept in different
65 salinities, point to no maternal control of marsupium water osmolality. This is
66 perhaps not surprising given the more open nature of the gammarid marsupium, and
67 the fact that the pleopods beating in the posterior part of the marsupium ensure a
68 constant replenishment of groove water (Dahl, 1978). Therefore, it is unlikely that
69 there is effective maternal osmotic control in aquatic amphipods, similar to that found
70 in more semi- and fully terrestrial species.

71 Our knowledge of embryonic osmoregulation in fully aquatic isopods and amphipods
72 is limited to one species of each. Embryos of the euryhaline isopod *Sphaeroma*
73 *serratum* displayed a weak osmoregulatory ability throughout development but
74 began to improve early after hatching (Charmantier and Charmantier-Daures, 1994).
75 The ontogeny of osmoregulation in the euryhaline brackishwater amphipod

76 *Gammarus duebeni* is more complex than that of the semi-terrestrial amphipod *O.*
77 *gammarellus*, and the aquatic isopod *S. serratum* (Morritt and Spicer, 1995). Early
78 embryos of *G. duebeni* (Stage 2, characterised by a prominent dorsal organ)
79 displayed a hyper-iso-osmotic pattern of regulation of perivitelline fluid when
80 exposed to a range of environmental salinities. This regulation was present before
81 the appearance of the coxal gills possibly *via* extra-embryonic structures, namely the
82 vitelline membrane and/or the dorsal organ (DO) (Morritt and Spicer, 1995). This
83 pattern was also observed for the haemolymph of new hatchlings but, interestingly,
84 stage 5-7 embryos (pre-hatch but undergoing marked organogenesis) displayed a
85 transient hyper-hypo-osmotic pattern of regulation, similar to the embryonic pattern
86 in the semi-terrestrial *O. gammarellus*. This development and loss of what might be
87 regarded as the most complex form of osmoregulation (i.e. hyper-hypo-regulation),
88 does not support the incomplete adult hypothesis. Clearly, there are differences in
89 the patterns and ontogeny of osmoregulation between euryhaline embryonic isopods
90 and amphipods, and differences between semi-terrestrial and aquatic amphipods,
91 but current knowledge does not allow for generalisations. Furthermore, there is little
92 information on the structures, and no information on the molecular basis, responsible
93 for these regulations or their ontogenies.

94

95 Therefore, the aim of this study was to test the incomplete adult hypothesis of
96 Adolph, (1968) and Spicer and Gaston (1999) by 1) characterising the ontogeny of
97 osmoregulation of a congeneric euryhaline amphipod, at different levels of biological
98 organisation, and 2) compare and contrast the picture that emerges specifically with
99 *G. duebeni* and *O. gammarellus*, the only other amphipod species for which we have
100 comparable information. Consequently, we investigated the ontogeny of the structure

101 and function of putative osmoregulatory tissues, together with the regulation of key
102 osmoregulatory genes, in the brackishwater amphipod *Gammarus chevreuxi*. We
103 predicted that, like in *G. duebeni,* the most complex pattern of regulation (hyper-
104 hypo-) would appear early in embryonic development and then revert to the less
105 complex hyper-iso- regulation before or around hatching. *Gammarus chevreuxi* was
106 chosen as it is a euryhaline species, exposed to freshwater (salinity $\approx$ 0) and full
107 strength sea water (salinity >30) within a tidal cycle, its embryonic development is
108 well characterised, it is lab-hardy (Sexton, 1928), and its transcriptome has recently
109 been sequenced (Collins et al., 2017; Truebano et al., 2013).

110

111 1. **2. Materials and Methods**

112

113 1.1. Collection and husbandry of animals

114 Amphipods were collected during low tide using a kick net (mesh size = 500 μm)
115 from the Plym estuary, Devon (50° 23' 24'' N, 4° 5' 7'' W). A Star:Oddi DST CTD
116 logger was deployed for 48 h at the collection site to measure tidal salinity variation.
117 This population experiences a salinity pulse, when salinities reach values of above
118 30 for approximately 4 h during the tidal cycle. Amphipods were returned to a
119 temperature-controlled laboratory and sorted into stock aquaria containing diluted
120 sea water (vol. = 25 L, T =15 °C, S = 15±1, light = 12h:12h L:D cycle). Amphipods
121 were held in the stock aquaria in pre-exposure conditions for a minimum of two
122 weeks, and fed carrot *ad libitum*. Water changes were performed weekly. After four
123 weeks, pre-copula pairs were isolated from the stock populations and transferred to
124 individual aquaria (vol. = 0.5 L) maintained under the same conditions as the stock
125 aquaria. Males were removed immediately once the pairs had separated and eggs

126 were visible in the marsupium of the female. Individual eggs of different
127 developmental stages, hatchlings or adults were removed as required to supply the
128 experiments described below.

129

130 ## 1.2. Ontogeny of putative osmoregulatory organs

131

132 ### 1.2.1. Morphological observations

133 Each day after fertilization, eggs from a proportion of the females were brushed out
134 of the marsupium using a fine paint brush as described by (Morritt and Spicer, 1995),
135 and placed in double filtered autoclaved sea water adjusted to the same conditions
136 as those used for the pre-exposure period. Eggs were observed and photographed
137 under a high powered light microscope coupled to an Allied Vision Pike 210C real
138 time digital camera (Allied Vision Technology, Germany) and the timing of the
139 appearance and development of putative osmoregulatory structures recorded.

140

141 ### 1.2.2. Scanning electron microscopy (SEM) of embryos

142 To further characterise the structure and position of the DO, embryos (>14 dpf) were
143 examined under SEM. Eggs were fixed in 2.5 % glutaraldehyde in diluted sea water
144 for 12 h and rinsed twice for 15 min in sodium cacodylate buffer (0.1 M, pH 7.2) at 4 ˚
145 C. Eggs were then placed in 30 % ethanol, and carefully dechorionated using two
146 fine dissecting needles. They were then further dehydrated through a graded ethanol
147 series ranging from 30 % through 50, 70, 90 and 100 % and critically point dried in
148 an Emitech K850 critical point drier (Quorum Technologies Ltd., UK). Fully dried

149 samples were coated with gold and examined using a JEOL JSM 5600 LV scanning
150 electron microscope (Jeol, Japan).

151

152 1.2.3. Staining of ion regulatory tissue and permeable areas in embryos

153 Silver staining was used to identify areas of active ion uptake on embryos. Attempts
154 to stain embryos at stages before the DO is visible (< 6 dpf) resulted in cell lysis.
155 Accordingly, the location of putative ion regulatory tissues and permeable areas was
156 determined for eggs 6, 9, 14 and 18 dpf. These stages were selected based on initial
157 morphological observations and mark the appearance and development of structures
158 with putative osmoregulatory function (i.e. DO and gills). Immediately upon removal
159 from the brood pouch, eggs were briefly rinsed twice in deionised water, transferred
160 to a 5 g L^{-1} $AgNO_3$ solution for 5-7 min and washed again for 10 min in deionised
161 water. Observations of staining were made under a high powered light microscope
162 and digital images obtained as described above.

163

164 *1.3.* Ontogeny of osmo- and iono-regulation

165

166 1.3.1. Osmoregulatory patterns in embryos

167 The osmolality of homogenised eggs was measured at different stages of
168 development exposed to three salinity treatments (S= 2, 15 and 33) for 24 h. This
169 exposure time was shorter than used for the adults primarily to ensure the exposures
170 of quite discrete and different periods in embryonic development. Although we have
171 no information for embryonic amphipods, we know that salinity acclimation in adults
172 can be very rapid, with most of the change occurring during the first few hours of

173 transfer, and new steady states achieved well within 48 h (Bolt, 1983; Dorgelo,
174 1974).

175 Samples were selected at stages representing embryos in which the DO had not
176 started to develop (<48 hpf), the DO was present but not the gills (9 dpf), and both
177 the DO and gills were present (14 -18 dpf). For each salinity treatment and stage,
178 batches of eggs were carefully removed from the brood pouch and photographed as
179 previously described. The length and width of each egg was measured using ImageJ
180 (Schneider et al., 2012) and used to calculate egg volume as an oblate ellipsoid.
181 Pools of eggs (n = 20 per salinity) were washed twice in milliQ water, and
182 homogenised with a manual homogeniser. The osmolality of the homogenates (0.03
183 – 0.05 µL) was determined using a direct-reading nanolitre osmometer (Clifton
184 Technical Physics) (Morritt and Spicer, 1996). Variation between replicates was < 35
185 mOsm Kg^{-1}. Homogenates, rather than haemolymph or perivitelline fluid, have been
186 used previously to investigate the ontogeny of osmoregulation in crab embryos
187 (Seneviratna, 2006).

188

189 1.3.2. Osmo- and iono- regulatory patterns in adults

190 Analysis of haemolymph osmolality and key ionic concentrations (sodium (Na^+),
191 calcium (Ca^{2+}) and magnesium (Mg^{2+})) was performed for large adult *G. chevreuxi*
192 (>10 mm length) exposed to one of four salinity treatments (S = 2, 10, 25 and 35) for
193 seven days. Haemolymph was extracted by inserting the needle of a microsyringe
194 (vol.= 10 µL) directly into the heart, dorsally through the second and third dorsal
195 plates of the pereon. The osmolality of untreated pooled haemolymph samples (n =
196 12-15 individuals) was measured in duplicate using a Vapro 5520 vapour pressure
197 osmometer (Wescor, USA) fitted with a reduced volume sample holder (2 µL). as

198 described above. The ionic content of the haemolymph was estimated as follows:
199 Immediately after extraction, 0.5 µL haemolymph (3-5 individuals) was pooled in a
200 microcentrifuge tube, diluted in 1 mL milliQ water, and refrigerated before analysis.
201 Ionic concentrations in adult haemolymph and corresponding treatment media were
202 measured using inductively coupled plasma optical emission spectroscopy (ICP-
203 OES). Samples were analysed for Na^+, Mg^{2+} and Ca^{2+} ions, using a Varian 725ES
204 ICP-OES instrument (Varian, Australia) fitted with a V-groove nebuliser coupled with
205 a Sturman-Masters spray chamber, calibrated using four standards and one blank.
206 Operating parameters were set to a forward power of 1.4 kW, plasma flow of 15 L
207 min^{-1}, auxiliary flow of 1.5 L min^{-1} and nebuliser gas flow of 0.68 L min^{-1}, a viewing
208 height of 8 mm above the load coil and a read time of 4 s.
209

210 1.3.3. Gene expression analysis of Na^+/K^+-ATPase

211 The transcriptome of *G. chevreuxi* at different embryonic stages has been recently
212 sequenced, assembled and annotated as described in Truebano et al (2016). Six
213 transcripts were putatively identified as Na^+/K^+-ATPases beta (four transcripts) or
214 alpha (two transcripts) subunits. Of these, we found differences in expression
215 between early and late developmental stages in two mRNA transcripts putatively
216 identified as Na^+/K^+-ATPase alpha subunit (**GeneBank accession no.**
217 **HADC01011431.1 and HADC01011432.1,** length: 4398 and 4317 bp respectively).
218 To investigate whether the two transcripts are differentially expressed during
219 ontogeny across the developmental stages studied here, expression analysis was
220 carried out in eggs at different developmental stages using qPCR. Expression
221 patterns of both transcripts were investigated in pools of embryos pre-DO (<16 cells,
222 <48 hpf), DO present (9 dpf), gills present (18 dpf) and adults. The <16-cell embryos

223 were included in order to determine whether there is evidence of iono-regulatory
224 capacity in the earliest stages of development. Total RNA was isolated from three
225 pools of 50 embryos per each of the four developmental stages (n=12) or three pools
226 of 10 adults using the Reliaprep RNA tissue Miniprep System (Promega,
227 Southampton, UK) following manufacturer's instructions. RNA purity and
228 concentrations were measured using a NanoDrop 2000 Spectrophotometer (Thermo
229 Scientific, Loughborough, UK) and integrity was assessed using gel electrophoresis.
230 200 ng total RNA was reversed transcribed using the High-Capacity cDNA Reverse
231 Transcription Kit (Applied Biosystems, California, USA). Samples were amplified in
232 triplicate in 10 µL reactions containing 2 µL cDNA (1:10 dilution) in the presence of
233 SYBR Green (iTaq Universal SYBR Green Supermix, BioRad, Hertfordshire, UK) in
234 a StepOne Real-Time PCR system (Life Technologies, Paisley, UK) according to
235 manufacturers' instructions. A melt curve was added to each run. Ct values were
236 normalised to the geometric mean of 18S ribosomal subunit (18S) and elongation
237 factor (EFα) after checking their stability via geNorm (Vandesompele, et al., 2002).
238 Data are presented as ΔCt ($Ct_{reference}$-Ct_{target}). Fold changes were calculated we $2^{\Delta Ct}$.
239 Primers were designed by qStandards (EFα and 18S, Edgware, UK) or Primerdesign
240 Ltd (Na^{+}/K^{+}-ATPase, Southampton, UK) (Table 1).

241

242 Table 1. Primer information for two target Na^{+}/K^{+}-ATPase transcripts (named long
243 and short for convenience) and the reference genes elongation factor (EFα) and 18S
244 ribosomal subunit (18S).

245

Target gene	Primer Sequence	Amplicon length (bp)	Tm (°C)	Efficiency (%)	R^2
Na^{+}/K^{+}-ATPase long	F: gccacaaaaatgagtgatagcg R: tctcccttgaaagtacggttatc	92	75.3	90-100	-

Na^+/K^+-ATPase short	F: tttactgataataccttggatactgtg R: ttcgccttcttcttcgaatcac	97	70.3	90-100	-
EFα	F: caaccgtctgtacatgaaggct R: accgaaggtccagatcttcatgg	163	87.3	103	0.999
18S	F: tgaacgaaagttagaggatcgaagg R: cggattgatggttggcatcgt	77	80.7	98	0.999

246

247

248

249 **2. Results**

250

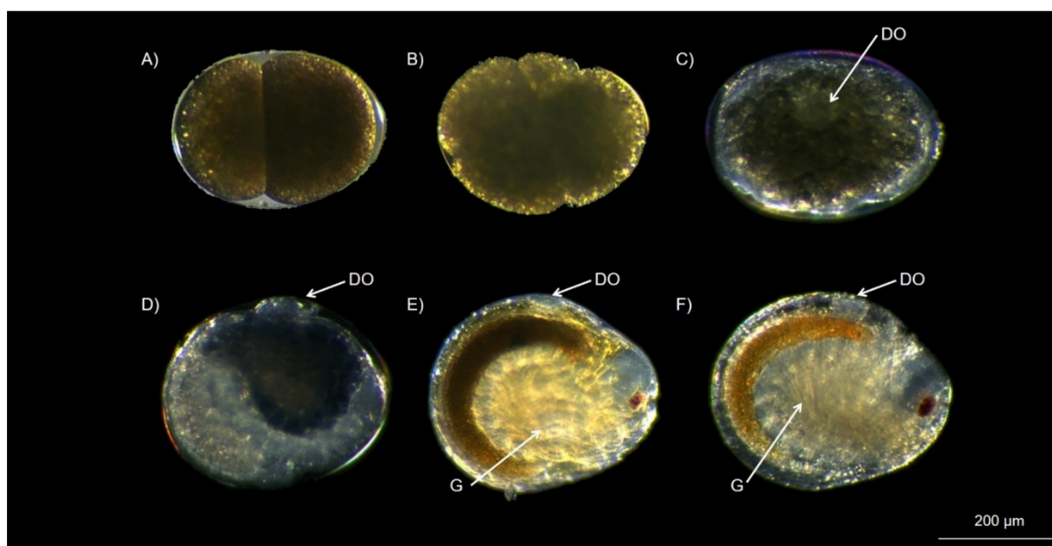
251 2.1. Ontogeny of putative osmoregulatory organs

252

253 2.1.1. Morphological observations

254 Embryogenesis (fertilization to hatching) under the experimental conditions (T =

255 15°C, S = 15) took 400-450 h in *Gammarus chevreuxi* (Figure 1).



256

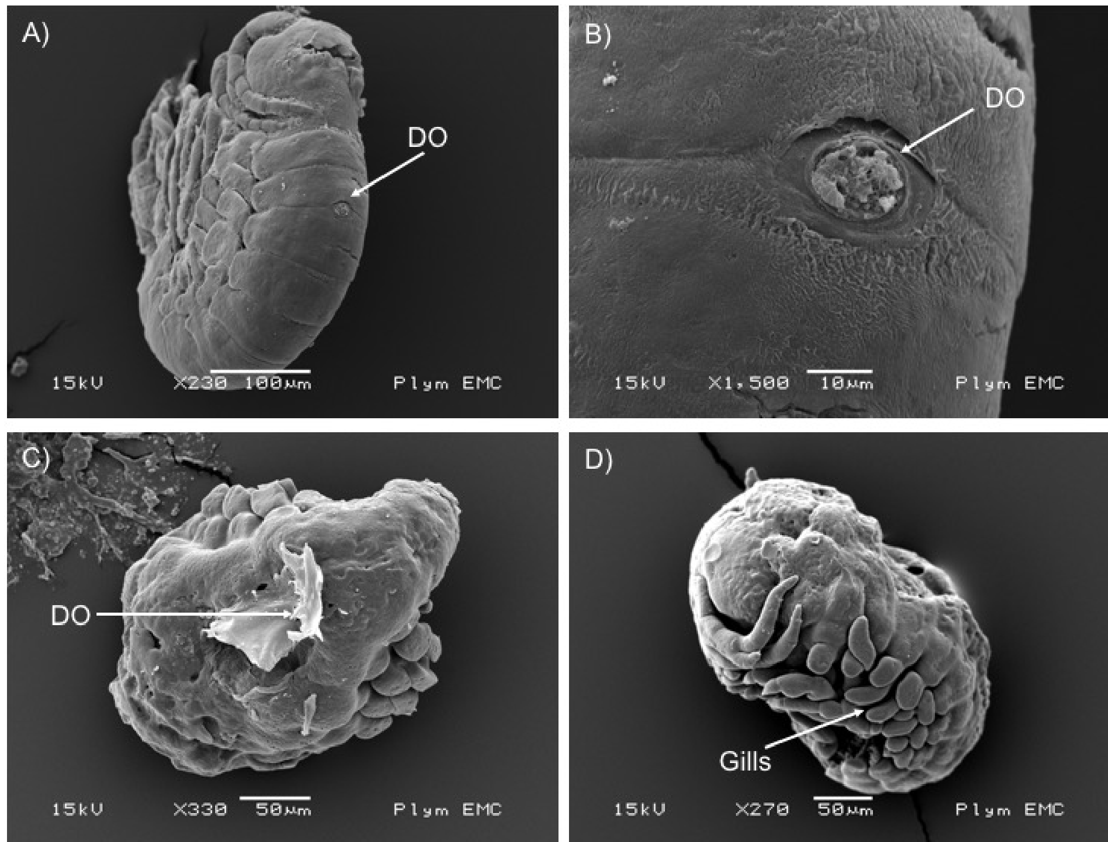
257 Figure 1. Light microscopy images of *Gammarus chevreuxi* embryos developed in

258 the marsupium of females and removed for examination at A) 2-cells, B) <16 cells,

259 C) 6 dpf, D) 9 dpf, E) 14 dpf and F) 18 dpf. DO and G indicate embryonic dorsal
260 organ and gills respectively. Scale bar = 200 μm.
261

262 The first cell division (2-cell stage) occurred within 10 hpf (Figure 1A), fertilization
263 being defined as the time at which the mating pair separates. Cell boundaries are
264 clearly visible under light microscopy until approximately the 16-cell embryo (Figure
265 1B), after which time individual cells are not easily identifiable. The timing of the
266 initial aggregation of cells forming the DO cannot be identified visually using light
267 microscopy during early development. However, 5-6 days after fertilization, the DO is
268 easily identifiable unaided under light microscopy as an aggregation of cells located
269 dorsally (e.g. 6 dpf, Figure 1C). It becomes a well-defined structure that remains
270 located on the anterior dorsal surface through development (Figure 1D, E, F). Upon
271 fixing and dechorionation of the embryos, the DO appears the only point of
272 attachment between the egg membrane and the embryo.
273

274 2.1.2. Scanning electron microscopy (SEM) of embryos



275

276 Figure 2. Scanning electron microscopy images of dechorionated *Gammarus*
277 *chevreuxi* embryos fixed at 14 dpf indicating, A) the position of the embryonic dorsal
278 organ, B) the dorsal organ, C) the association between the dorsal organ and the
279 chorion, and D) the position of the gills. DO and G indicate embryonic dorsal organ
280 and gills respectively. Scale bars (10-100 µm) and magnifications (x230-1500) are
281 shown for each image.

282

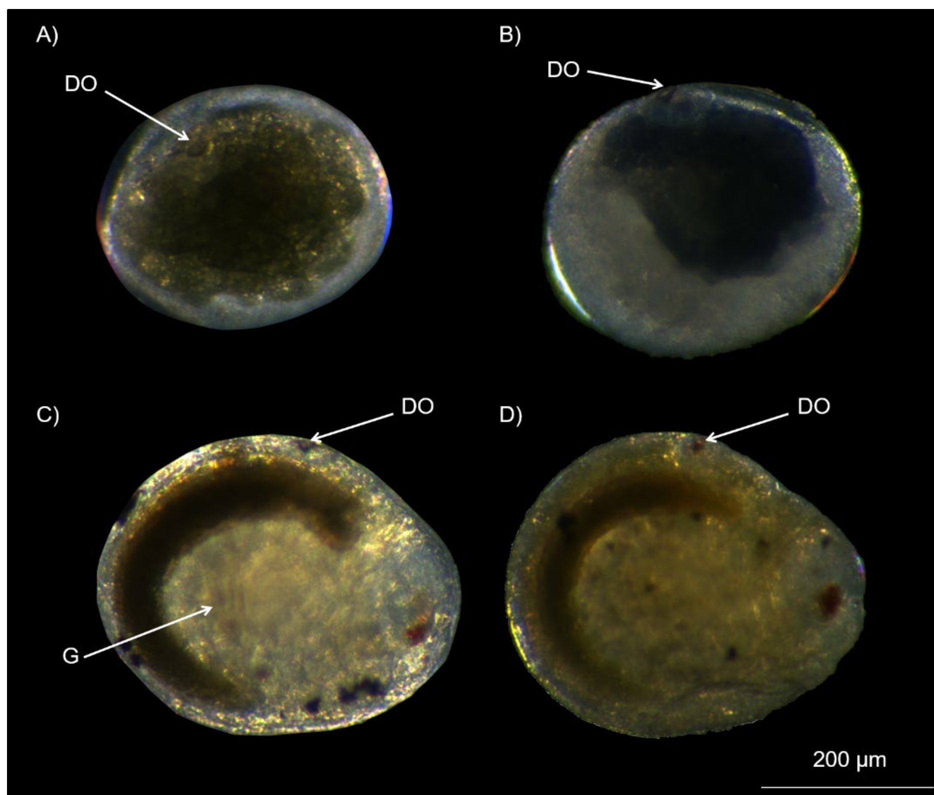
283 SEM of 14 dpf individuals clearly shows the DO as an oval structure (approx. 15 µm
284 diameter), located on the dorsal surface of the embryo between the second and third
285 cuticular segments of the pereon (Figure 2A, B). Figure 2C shows a fragment of the
286 egg membrane apparently attached to the area of the DO, providing further evidence
287 that this is the only point of attachment between the embryo and the egg membrane.
288 The gills are also observable in the same individual (Figure 2D), confirming the co-

289 occurrence of both organs in the late stages of development. As the gills are located
290 beneath the coxal plates, their development is difficult to visualise in live embryos.
291 Therefore, it is possible that rudimentary gills develop earlier than suggested here.
292 The gills are clearly visible under light microscopy at the late stages (>12 dpf, Figure
293 2F, G).

294

295 2.1.3. Staining of ion regulatory tissue and permeable areas in embryos

296



297

298 Figure 3. Light microscopy images of *Gammarus chevreuxi* embryos developed in
299 the female marsupium. Embryos were stained with $AgNO_3$ at A) 6 dpf, B) 9 dpf, C)
300 14 dpf, and D) 18 dpf. Arrows with accompanying letter indicate silver stained areas,
301 corresponding with the locations of the embryonic dorsal organ (DO) and gills (G).
302 Scale bar = 200µm.

303

304 Silver-stained embryos at 6, 9, 14 and 18 dpf present an oval shaped dark area with
305 a darker outer ring that can be identified on the surface of the embryo (Figure 3),
306 corresponding with the position of the DO, as shown in Figure 2. The stained area is
307 likely to be a silver precipitate, which is indicative of active ion uptake. At 14 dpf,
308 weak staining of the gills is observed. Note that the lower intensity does not
309 necessarily indicate a lower active uptake of ions, but could equally indicate a
310 decrease in permeability.

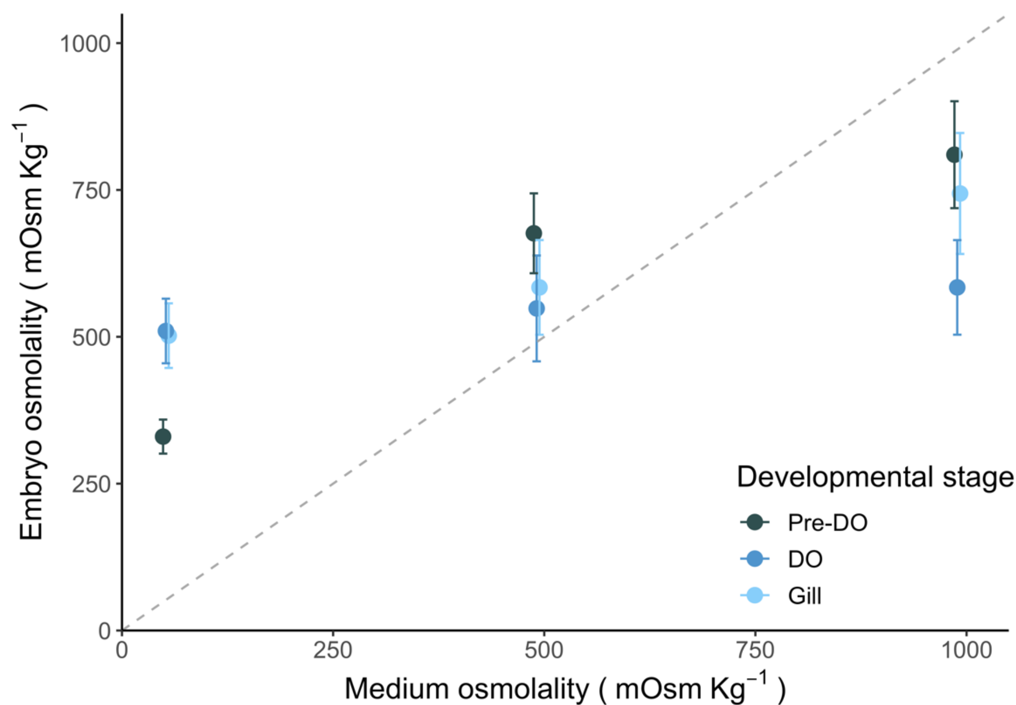
311

312 ## 2.2. Ontogeny of osmo- and iono-regulation

313

314 ### 2.2.1. Osmoregulatory patterns in embryos

315



316

317

318 Figure 4. Osmolality of *Gammarus chevreuxi* homogenate of embryos removed from
319 the mother at three different stages of development (i.e. pre-dorsal organ (pre-DO,
320 dark grey), dorsal organ present (DO, dark blue), and gills present (Gill, light blue))
321 and subsequently exposed to salinities of 2, 15 and 33 for 24 h *in vitro*. Points
322 represent mean concentrations and standard deviations of three biological replicates
323 per treatment, each consisting of pools of 25 embryos. Broken line represents the
324 isosmotic line.

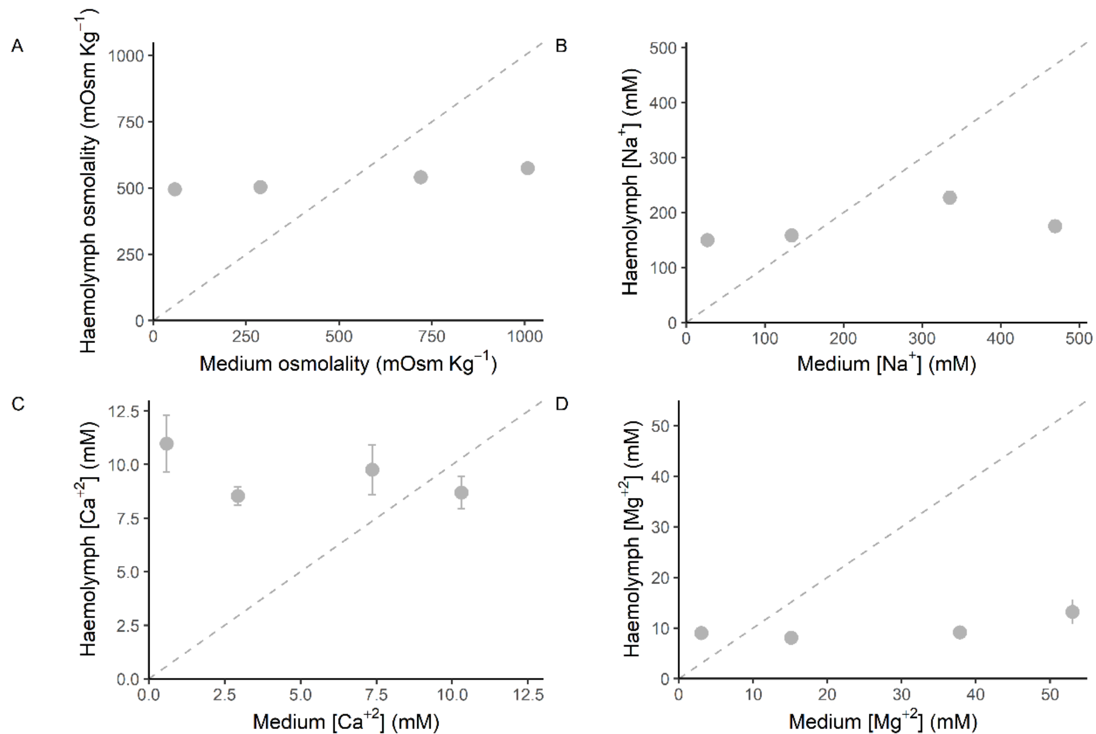
325

326 For *G. chevreuxi* embryos, extraction of periembryonic fluid was not possible until
327 approximately 8 dpf. Therefore, in order to compare osmoregulatory capacities of
328 embryos before and after the development of the DO and gills, osmoregulatory
329 curves were produced using osmolality of embryo homogenates, from embryos
330 removed from the marsupium and exposed to different salinities *in vitro* (S= 2, 15
331 and 33). The osmolality of the homogenate is expressed as a function of the
332 corresponding external osmolality (Figure 4). All embryonic stages investigated
333 showed some degree of hyper-hypo-osmoregulation. At a salinity of 35, eggs were
334 hyposmotic to the external medium (989 mOsm Kg^{-1}) by several hundred mOsm Kg^{-1}
335 in the DO and gill stages (mean±SD= 584±80.5 and 744±103 mOsm Kg^{-1}
336 respectively) (Figure 4 B,C), with less of a difference with the medium before DO
337 development (mean±SD= 810±91 mOsm Kg^{-1}) (Figure 4A). At a salinity of 2, eggs
338 from all stages were hyperosmotic to the external medium (52 mOsm Kg^{-1}), with the
339 pre-DO stage being closest to the isosmotic line (mean±SD= 330±29, 510±55 and
340 502±55 mOsm Kg^{-1} for pre-DO, DO and gill respectively). Differences between the
341 stages diminished at salinity 15.

342

343 2.2.2. Osmo- and iono-regulatory patterns in adults

344



345

346

347 Figure 5. Osmotic and ionic regulation in adult *Gammarus chevreuxi* acclimated to
348 four salinities (S= 2, 10, 25 and 35) for seven days. Haemolymph (A) osmolality (B)
349 sodium (Na^+) concentration, (C) calcium (Ca^{2+}) concentration and (D) magnesium
350 (Mg^{2+}) concentration. Points represent mean concentrations and standard errors of
351 pooled biological replicates from the respective treatments (n= 12, 15, 15 and 15 for
352 salinities 2, 10, 25 and 35 respectively (A); n= 40, 44, 40 and 19 for salinities of 2,
353 10, 25 and 35 respectively (B-D)). Broken lines represent isosmotic lines.

354

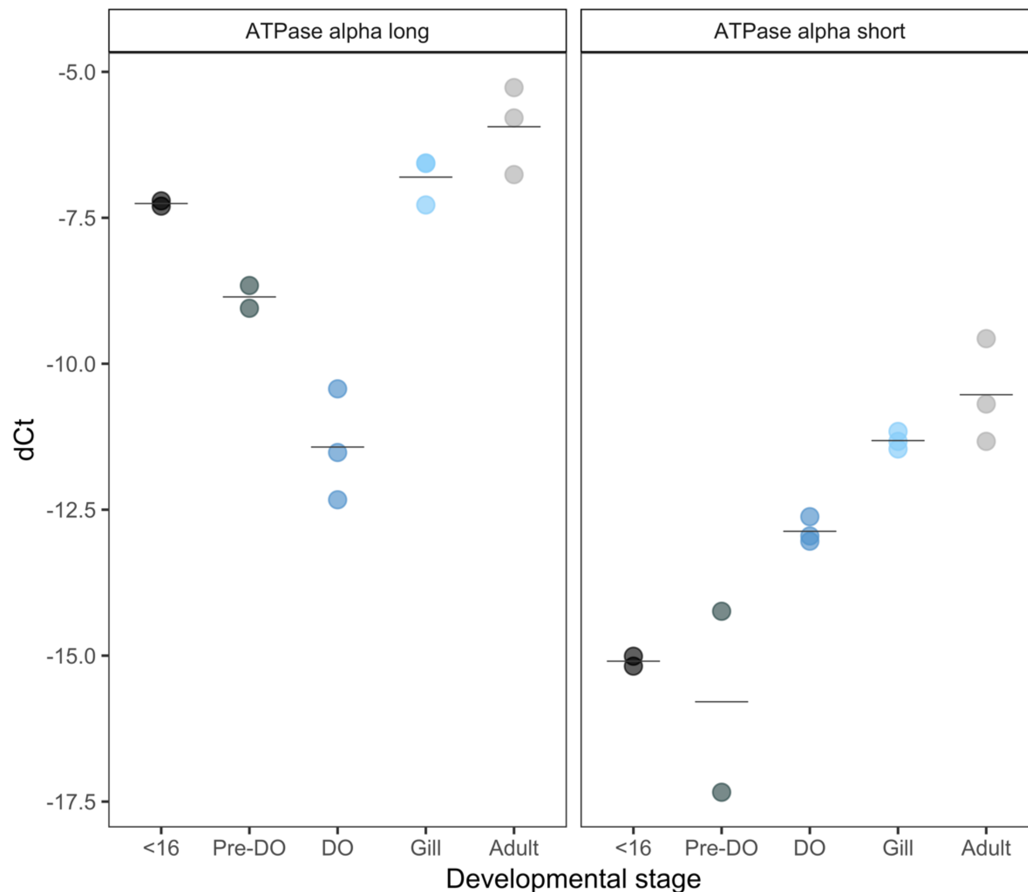
355 Regulation of osmolality and selected key ions in the haemolymph of amphipods
356 exposed to different environmental salinities is presented in Figure 5. In each case, a

357 hyper-hypo-regulation pattern was observed. Total osmolality and the concentrations
358 of all three ions were tightly regulated across the salinities tested.

359

360 2.2.3. Gene expression profiles of putative osmoregulatory genes during ontogeny

361



362

363 Figure 6. Expression levels (dCt) for two isoforms putatively identified as Na^+/K^+-
364 ATPase alpha subunit in four embryonic stages corresponding with <16-cells (<24
365 hpf), pre-dorsal organ (pre-DO, <48 h), dorsal organ present (DO, 9 dpf), gills
366 present (Gill, 18 dpf) and adults (*n* = 2-3 pools of 50 embryos per developmental
367 stage, and n= 3 pools of 10 adults). Transcripts are labelled ATPase alpha long
368 (transcript length= 4398 bp) and ATPase alpha short (transcript length= 4317 bp) for
369 convenience.

Both Na^+/K^+-ATPase transcripts were expressed throughout development. The expression of the longer transcript differed significantly between stages (ANOVA, $F_{4,8}$=32.39, P<0.001), showing greater expression at <16 cells, followed by a significant downregulation in dorsal organ embryos (approximately 30% downregulation compared to the <16-cell stage) before increasing again in embryos presenting gills and in adults to levels similar to those of the <16-cell embryos (1.37 and 2.49-fold increase respectively compared to <16-cell embryos). The expression of the short transcript increased with ontogeny (ANOVA, $F_{4,8}$=15.41, P<0.001) being lowest in the early stages (<16 cells and pre-DO) and highest at the late stages following a 4.67, 13.68 and 23.59-fold increase in dorsal organ embryos, gill embryos and adults respectively in relation to the <16-cell stage (Figure 6). The expression ratio between the two transcripts was reasonably stable throughout ontogeny with the long transcript having higher expression levels (ΔCt long - ΔCt short= 1.66-2.08, except for dorsal organ embryos, where expression levels were most similar 1.13).

3. Discussion

3.1. Adult osmo- and iono-regulatory pattern

Adult *Gammarus chevreuxi* exhibited a pronounced hyper-hypo-osmoregulatory pattern. The only other gammarideans for which this pattern is recorded is the high shore *Orchestia gammarellus* (Moore and Francis, 1985; Morritt, 1988) and individuals of a brackishwater population of the predominantly marine amphipod

395 *Gammarus oceanicus* (Normant et al., 2005). However, in *G. oceanicus* where
396 population differentiation is well attested (Croker and Gable, 1977), individuals from
397 more oceanic populations show a hyper-iso- osmoregulatory pattern (Aarset and
398 Zachariassen, 1988; Brodie and Halcrow, 1978) in common with the adult stage of
399 many marine or estuarine gammaridean amphipod species (Dorgelo, 1974; Kiko et
400 al., 2009; Werntz, 1963). The strong osmoregulatory capacity of adult *G. chevreuxi*,
401 is matched by an equally strong pattern of haemolymph ion regulation including
402 sodium, which is a major component of effective osmoregulation (Lignot and
403 Charmantier, 2015).

404

405 3.2. Ontogeny of osmoregulation and the dorsal organ

406 The adult pattern of hyper-hypo-osmoregulation appeared in embryonic *G. chevreuxi*
407 well before the ontogeny of the gills (14 dpf), the presumed primary site of ion
408 exchange in crustaceans (Freire et al., 2008; Henry et al., 2012), and the pattern did
409 not change thereafter. This points to an osmoregulatory role for extrabranchial
410 structures prior to gill ontogeny. Appearing at 6 dpf the oval shaped, silver-stained
411 area on the egg surface, which has a darker outer ring surrounding it, corresponds
412 with the position of the DO. This is consistent with the DO ultrastructural description
413 of Meschenmoser (1989). The most dense staining occurs where the DO connects
414 to the vitelline membrane in *G. chevreuxi* (this study) and other peracarids (Bregazzi,
415 1973; Meschenmoser, 1989), and is likely to be indicative of active ion pumping.
416 While the DO may well be implicated in osmoregulation, the adult pattern of hyper-
417 hypo-regulation in *G. chevreuxi* was present before the DO becomes visible (6dpf).
418 However, before 6dpf, both hyper- and hypo-regulatory ability were not as strong as
419 they were post-DO formation. The results of silver staining and the correlation

420 between improved osmoregulation and the appearance of the DO, support the idea
421 that, for *G. chevreuxi*, and in many other amphipod and isopod crustaceans, the DO
422 is involved in osmotic and ionic regulation (Bregazzi, 1973; Martin and Laverack,
423 1992; Meschenmoser, 1989; Morritt and Spicer, 1995; Strömberg, 1972; Vlasblom
424 and Bolier, 1971; Wright and O'Donnell, 2010).

425 At 14 dpf, the DO began to degenerate in *G. chevreuxi* and at the same time the gills
426 became visible and showed weak silver staining. The centre of the DO stained
427 (unlike the vitelline membrane) in all developmental stages, up until the point where
428 it had totally degenerated, suggesting it remained functional until very late in
429 embryonic development. Interestingly, like *G. chevreuxi,* the coxal gills of late stage
430 *G. duebeni* embryos also showed little stain retention. Morritt and Spicer (1995)
431 suggested therefore, that the coxal gills may only provide major osmoregulatory
432 function post-hatching in this species, as it is only then the gills stain darkly.
433 However, the lower staining intensity could equally be indicative of a decrease in
434 chorion permeability.

435

436 3.3. Gene expression and osmoregulation

437 Na^+/K^+-ATPases are ubiquitous, highly conserved transport proteins consisting of
438 alpha and beta subunits. The alpha subunit is the catalytic unit, and the beta subunit
439 facilitates insertion in the membrane (Skou, 1990). In adult crustaceans, Na^+/K^+-
440 ATPase activity is largely responsible for epithelial movement of monovalent ions
441 across specialized ion-regulatory cells and tissues, mostly located in the gills (Leone
442 et al., 2017; Lignot and Charmantier, 2015). Its activity is associated with
443 osmoregulation in euryhaline crustaceans, with significant increases in activity

444 demonstrable during both hyper- and hypo-osmoregulation (reviewed by Lucu and
445 Towle, 2003). Long-term increases in activity are likely a result of enzyme activation
446 and *de novo* protein synthesis *via* enhanced transcription (Havird et al., 2013). An
447 increase in the activity of Na^+/K^+ -ATPase during embryonic development has been
448 recorded in a wide range of decapod species (Felder et al., 1986; Ituarte et al., 2008;
449 Taylor and Seneviratna, 2005; Wilder et al., 2001). While gill Na^+/K^+ -ATPase activity
450 has been measured in adult amphipods (Brooks and Lloyd Mills, 2006), nothing is
451 known of how enzymatic activity, or its associated gene expression, changes during
452 embryonic development in these peracarids.

453

454 In *G. chevreuxi*, we found evidence of regulation during ontogeny of two different
455 transcripts encoding the alpha subunit. More than one copy of the Na^+/K^+ ATPase
456 alpha gene is reported in crustaceans including the waterflea *Daphnia pulex* (Macias
457 et al., 1991), the brine shrimp *Artemia franciscana* (Baxter-Lowe et al., 1989) and the
458 barnacle *Balanus improvisus* (Lind et al., 2013), which is divided into two classes;
459 Na^+/K^+-ATPase 1 and 2. In addition, some crustacean species present different
460 isoforms of the alpha 1 variant, representing different splice variants. This has been
461 well characterised in the barnacle, *B. improvisus,* where the long and short forms
462 differ by 27 amino acids at the N-terminus. Analysis of the transcripts identified in *G.*
463 *chevreuxi*, suggests that these mRNAs represent a long and short slice variant
464 differing only by 81 bp. Alignment of these mRNA sequences to the long and short
465 Na^+/K^+ ATPase alpha 1 splice variants identified in the barnacle *B. improvisus*, the
466 shrimp *Penaeus monodon* and the crab *Pachygrapus marmoratus* (alignment given
467 in Lind et al., (2013)), revealed similarity between the two main variants for each
468 individual including or excluding the 27 amino acids. This suggests that the *G.*

469 *chevreuxi* transcripts identified herein belong to the Na^+/K^+ ATPase alpha 1 class,
470 and are homologous products of alternative splicing of a 27 amino acid exon (Figure
471 S1). While the functional importance of this exon in the encoded proteins is not
472 known, we have observed differences in expression patterns between the two
473 homologs. Expression of both transcripts was detected in all three developmental
474 stages examined. This supports the idea that ion transporting activity may occur
475 before the formation of osmoregulatory organs, potentially through Na^+/K^+-ATPase-
476 rich ionocytes. Such activity may be required to ensure the maintenance of osmotic
477 balance during hydration. Overall, the expression of both transcripts was greatest in
478 the late stages, embryos with gills and adults, which is consistent with the increased
479 activity levels observed during ontogeny in other crustaceans (Felder et al., 1986;
480 Ituarte et al., 2008; Taylor and Seneviratna, 2005; Wilder et al., 2001). However,
481 while the expression in one of the transcripts increased with ontogeny, the other
482 showed complex patterns, with reduced expression after the appearance of the
483 dorsal organ, compared to all other stages, suggesting different stage specific splice
484 variants may play a role during ontogeny. Differences in expression of different
485 splice variants of the alpha subunit between life stages have been described in the
486 barnacle *Amphibalanus* (as *Balanus) improvisus*, with dominance of a longer mRNA
487 over the short variant in cyprids, whereas in the adult, the short isoform was clearly
488 dominant (Lind et al., 2013). Interestingly, the long mRNA form is up-regulated in
489 relation to the short form during low salinity conditions, indicating the long protein
490 might have a more prominent functional role in maintaining haemolymph
491 hyperosmotic to the surrounding water under low salinity (Lind et al., 2013). The
492 expression ratio between the two transcripts remain constant throughout ontogeny,
493 except in dorsal organ embryos, where expression of both transcripts was most

494 similar. Differences in the ratio between two different isoforms during development
495 have been previously observed in the brine shrimp *Artemia salina* (Salon et al.,
496 1989), but the functional significance of these changes is not known and warrants
497 further investigation. It should be acknowledged, that, while the ontogenic changes in
498 expression of these two transcripts is interesting, they only represent a minor
499 component of the osmoregulatory genes in *G. chevreuxi.* Further gene expression
500 profiling across developmental stages and salinities is required to fully characterise
501 the molecular mechanisms underpinning the development of osmoregulation in this
502 species.

503

504 3.4. Comparison with other amphipod species

505 The ontogeny of osmoregulation in the three gammaridean species that have been
506 investigated to date all fit "Pattern 2" of Charmantier and Charmantier-Daures
507 (2001). Here, the 'adult' pattern is established around hatching, and adults are
508 euryhaline and can live in environments where salinity is high, low or variable.

509 While the ontogeny and development of osmoregulatory function in embryonic *G.*
510 *chevreuxi* was similar to that of embryonic *O. gammarellus* (Morritt and Spicer,
511 1999, 1996c), it differed from embryonic *G. duebeni* (Morritt and Spicer, 1995). A
512 hyper-hypo-regulatory pattern was already established in the earliest embryonic
513 (stage 2/3) examined of *O. gammarellus* and persisted through to hatching, although
514 it disappeared and developed again in hatchlings (Morritt and Spicer, 1999).
515 Paradoxically, the embryonic pattern was stronger than that of the adult (Morritt and
516 Spicer, 1996b) even though embryos are retained within the brood pouch where
517 there is tight maternal control of osmotic pressure of the exosomatic water within

518 (Morritt and Spicer, 1996a). This species can be subject to salinity extremes over a
519 number of different timescales (Moore and Francis, 1985).

520

521 *G. duebeni,* although more aquatic than *O. gammarellus*, also lives in habitats
522 subjected to large salinity variations but shows a slightly different osmoregulatory
523 pattern. Adult and early embryonic (stage 2/3) *G. duebeni* are hyper-iso-
524 osmoregulators. However, in medium and late embryos (stages 5-7) regulation was
525 hyper-hypo-osmotic, before reverting to hyper-isosmotic in hatchlings (Morritt and
526 Spicer, 1995). The significance of this transient ability to hyper-hypo-regulate is not
527 known, although Morritt and Spicer (1995) suggested it may be associated with the
528 appearance of the coxal gills, the putative primary osmoregulatory organs in
529 juveniles and adults, and with the concomitant disappearance of the DO. However,
530 while in *G. duebeni* the DO begins to degenerate around stage 5 and has
531 disappeared by hatching, in *G. chevreuxi* the DO remains visible right up until
532 hatching.

533 To conclude, the ontogeny of osmoregulatory function in the gammaridean
534 amphipod *G. chevreuxi* is consistent with the incomplete adult hypothesis proposed
535 by Adolph (1968) and Spicer and Gaston (1999), However the ontogenies of that
536 same function in the closely-related *G. duebeni* and in the talitrid amphipod
537 *Orchestia gammarellus* are more consistent with the physiological competency
538 hypothesis.

539

540

541 **Figure captions**

542

543 Figure 1. Light microscopy images of *Gammarus chevreuxi* embryos developed in
544 the marsupium of females and removed for examination at A) 2-cells, B) <16 cells,
545 C) 6 dpf, D) 9 dpf, E) 14 dpf and F) 18 dpf. DO and G indicate embryonic dorsal
546 organ and gills respectively. Scale bar = 200 µm.

547

548 Figure 2. Scanning electron microscopy images of dechorionated *Gammarus*
549 *chevreuxi* embryos fixed at 14 dpf indicating, A) the position of the embryonic dorsal
550 organ, B) the dorsal organ, C) the association between the dorsal organ and the
551 chorion, and D) the position of the gills. DO and G indicate embryonic dorsal organ
552 and gills respectively. Scale bars (10-100 µm) and magnifications (x230-1500) are
553 shown for each image.

554

555 Figure 3. Light microscopy images of *Gammarus chevreuxi* embryos developed in
556 the female marsupium. Embryos were stained with $AgNO_3$ at A) 6 dpf, B) 9 dpf, C)
557 14 dpf, and D) 18 dpf. Arrows with accompanying letter indicate silver stained areas,
558 corresponding with the locations of the embryonic dorsal organ (DO) and gills (G).
559 Scale bar = 200µm.

560

561 Figure 4. Osmolality of *Gammarus chevreuxi* homogenate of embryos removed from
562 the mother at three different stages of development (i.e. pre-dorsal organ (pre-DO,
563 dark grey), dorsal organ present (DO, dark blue), and gills present (Gill, light blue))
564 and subsequently exposed to salinities of 2, 15 and 33 for 24 h *in vitro*. Points
565 represent mean concentrations and standard deviations of three biological replicates

566 per treatment, each consisting of pools of 25 embryos. Broken line represents the
567 isosmotic line.
568
569 Figure 5. Osmotic and ionic regulation in adult *Gammarus chevreuxi* acclimated to
570 four salinities (S= 2, 10, 25 and 35) for seven days. Haemolymph (A) osmolality (B)
571 sodium (Na^{+}) concentration, (C) calcium (Ca^{2+}) concentration and (D) magnesium
572 (Mg^{2+}) concentration. Points represent mean concentrations and standard errors of
573 pooled biological replicates from the respective treatments (n= 12, 15, 15 and 15 for
574 salinities 2, 10, 25 and 35 respectively (A); n= 40, 44, 40 and 19 for salinities of 2,
575 10, 25 and 35 respectively (B-D)). Broken lines represent isosmotic lines.
576
577 Figure 6. Expression levels (dCt) for two isoforms putatively identified as Na^{+}/K^{+}-
578 ATPase alpha subunit in four embryonic stages corresponding with <16-cells (<24
579 hpf), pre-dorsal organ (pre-DO, <48 h), dorsal organ present (DO, 9 dpf), gills
580 present (Gill, 18 dpf) and adults (*n* = 2-3 pools of 50 embryos per developmental
581 stage, and n= 3 pools of 10 adults). Transcripts are labelled ATPase alpha long
582 (transcript length= 4398 bp) and ATPase alpha short (transcript length= 4317 bp) for
583 convenience.
584

585 **Acknowledgements**

586 We thank Andy Fisher, Glenn Harper and Marie Palmer for technical support with
587 ICO-OES, SEM microscopy and animal husbandry respectively. We also thank Dr
588 Luke Holmes for help with graphical outputs. The RNA-seq work was funded by a
589 NERC NBAF Pilot Scheme Grant (NBAF622) to JIS and MT. RNA sequencing and

590 transcriptome assembly was carried out at the NBAF GenePool genomics facility,

591 University of Edinburgh.

592

593

594 **References**

595

596 Aarset, A.V., Zachariassen, K.E., 1988. Low temperature tolerance and osmotic
597 regulation in the amphipod *Gammarus oceanicus* from Spitsbergen waters.
598 Polar Res. 6, 35–41. https://doi.org/10.1111/j.1751-8369.1988.tb00579.x

599 Adolph, E.F., 1968. Origins of physiological regulations. Academic Press Ltd.,
600 London, UK. https://doi.org/10.1016/C2013-0-12479-8

601 Anger, K., 2003. Salinity as a key parameter in the larval biology of decapod
602 crustaceans. Invertebr. Reprod. Dev. 43, 29–45.
603 https://doi.org/10.1080/07924259.2003.9652520

604 Augusto, A., Greene, L.J., Laure, H.J., McNamara, J.C., 2009. The ontogeny of
605 isosmotic intracellular regulation in the diadromous, freshwater palaemonid
606 shrimps, *Macrobrachium amazonicum* and *M. olfersi* (Decapoda). J. Crustac.
607 Biol. 27, 626–634. https://doi.org/10.1651/s-2796.1

608 Baxter-Lowe, L.A., Guo, J.Z., Bergstrom, E.E., Hokin, L.E., 1989. Molecular cloning
609 of the Na,K-ATPase alpha-subunit in developing brine shrimp and sequence
610 comparison with higher organisms. FEBS Lett. 257, 181–187.
611 https://doi.org/10.1016/0014-5793(89)81816-2

612 Beadle, L.C., 1957. Comparative Physiology: Osmotic and ionic regulation in aquatic
613 animals. Annu. Rev. Physiol. 19, 329–358.
614 https://doi.org/10.1146/annurev.ph.19.030157.001553

615 Bolt, S.R., 1983. Haemolymph concentration and apparent permeability in varying
616 salinity conditions of *Gammarus duebeni*, *Chaetogammarus marinus* and
617 *Gammarus locusta*. J. Exp. Biol. 107, 129 LP – 140.

618 Bouaricha, N., Charmantier-Daures, M., Thuet, P., Trilles, J.P., Charmantier, G.,
619 1994. Ontogeny of osmoregulatory structures in the shrimp *Penaeus japonicus*
620 (Crustacea, Decapoda). Biol. Bull. 186, 29–40. https://doi.org/10.2307/1542034

621 Bregazzi, P.K., 1973. Embryological development in *Tryphosella kergueleni* (Miers)
622 and *Cheirimedon femoratus* (Pfeffer) (Crustacea: Amphipoda). B. Antarct. Surv.
623 Bull 63–74.

624 Brodie, D.A., Halcrow, K., 1978. Hemolymph regulation to hyposaline and
625 hypersaline conditions in *Gammarus oceanicus* (Crustacea: Amphipoda).
626 Experientia 34, 1297–1298. https://doi.org/10.1007/BF01981429

627 Brooks, S.J., Lloyd Mills, C., 2006. Gill Na^+, K^+-ATPase in a series of hyper-
628 regulating gammarid amphipods: enzyme characterisation and the effects of
629 salinity acclimation. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 144, 24–
630 32. https://doi.org/10.1016/j.cbpa.2006.01.023

631 Brown, A.C., Terwilliger, N.B., 2007. Developmental changes in ionic and osmotic
632 regulation in the Dungeness Crab, *Cancer magister*. Biol. Bull. 182, 270–277.
633 https://doi.org/10.2307/1542121

634 Burggren, W., 2018. Developmental phenotypic plasticity helps bridge stochastic
635 weather events associated with climate change. J. Exp. Biol. 221, jeb161984.
636 https://doi.org/10.1242/jeb.161984

637 Burggren, W., Warburton, S., 2005. Comparative developmental physiology:an
638 interdisciplinary convergence. Annu. Rev. Physiol. 67, 203–223.
639 https://doi.org/10.1146/annurev.physiol.67.040403.104223

640 Burggren, W.W., 2005. Developing animals flout prominent assumptions of
641 ecological physiology. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 141,
642 430–439. https://doi.org/10.1016/j.cbpb.2005.03.010

643 Charmantier, G., 1998. Ontogeny of osmoregulation in crustaceans: A review.
644 Invertebr. Reprod. Dev. 33, 177–190.
645 https://doi.org/10.1080/07924259.1998.9652630

646 Charmantier, G., Aiken, D.E., 1987. Intermediate larval and postlarval stages of
647 *Homarus americanus* H. Milne Edwards, 1837 (Crustacea: Decapoda). J.
648 Crustac. Biol. 7, 525–535. https://doi.org/10.2307/1548301

649 Charmantier, G., Charmantier-Daures, M., 2001. Ontogeny of osmoregulation in
650 crustaceans: The embryonic phase. Am. Zool. 41, 1078–1089.
651 https://doi.org/10.1093/icb/41.5.1078

652 Charmantier, G., Charmantier-Daures, M., 1994. Ontogeny of osmoregulation and
653 salinity tolerance in the isopod crustacean *Sphaeroma serratum*. Mar. Ecol.
654 Prog. Ser. 114, 93–102. https://doi.org/10.3354/meps114093

655 Charmantier, G., Charmantier-Daures, M., Towle, D.E., 2008. Osmotic and ionic
656 regulation in aquatic arthropods, in: Evans, D.H. (Ed.), Osmotic and Ionic
657 Regulation: Cells and Animals. CRC Press, London, pp. 165–230.
658 https://doi.org/10.1201/9780849380525

659 Cieluch, U., Charmantier, G., Grousset, E., Charmantier-Daures, M., Anger, K.,
660 2005. Osmoregulation, immunolocalization of Na^+/K^+-ATPase, and ultrastructure
661 of branchial epithelia in the developing brown shrimp, *Crangon crangon*
662 (Decapoda, Caridea). Physiol. Biochem. Zool. 78, 1017–1025.
663 https://doi.org/10.1086/432856

664 Collins, M., Tills, O., Spicer, J.I., Truebano, M., 2017. *De novo* transcriptome
665 assembly of the amphipod *Gammarus chevreuxi* exposed to chronic hypoxia.
666 Mar. Genomics. https://doi.org/10.1016/j.margen.2017.01.006

667 Conte, F.P., 1984. Structure and function of the crustacean larval salt gland, in:

668 Bourne, G.H., Danielli, J.F., Jeon, K.W.B.T. (Eds.), Membranes. Academic
669 Press, pp. 45–106. https://doi.org/https://doi.org/10.1016/S0074-7696(08)61314-
670 5

671 Croker, R.A., Gable, M.F., 1977. Geographic variation in Western Atlantic
672 populations of *Gammarus Oceanicus* Segerstrale (Amphipoda). Crustaceana
673 32.

674 Dahl, E., 1978. The amphipod functional model and its bearing upon systematics
675 and phylogeny. Zool. Scr. 6, 221–228. https://doi.org/10.1111/j.1463-
676 6409.1978.tb00773.x

677 Dall, W., 1970. Osmoregulation in the lobster *Homarus americanus*. J. Fish. Res.
678 Board Canada 27, 1123–1130. https://doi.org/10.1139/f70-128

679 Dorgelo, J., 1974. Comparative ecophysiology of gammarids (Crustacea:
680 Amphipoda) from marine, brackish and fresh-water habitats, exposed to the
681 influence of salinity-temperature combinations. Hydrobiol. Bull. 8, 90–108.
682 https://doi.org/10.1007/BF02254909

683 Felder, J.M., Felder, D.L., Hand, S.C., 1986. Ontogeny of osmoregulation in the
684 estuarine ghost shrimp *Callianassa jamaicense* var. *louisianensis* Schmitt
685 (Decapoda, Thalassinidea). J. Exp. Mar. Bio. Ecol. 99, 91–105.
686 https://doi.org/https://doi.org/10.1016/0022-0981(86)90230-3

687 Freire, C.A., Onken, H., McNamara, J.C., 2008. A structure-function analysis of ion
688 transport in crustacean gills and excretory organs. Comp. Biochem. Physiol. A.
689 Mol. Integr. Physiol. 151, 272–304. https://doi.org/10.1016/j.cbpa.2007.05.008

690 Gilles, R., 1975. Mechanisms of ion and osmoregulation, in: O. Kinne (Ed.), Marine
691 Ecology. Wiley-Interscience, New York, pp. 259–347.

692 Havird, J.C., Henry, R.P., Wilson, A.E., 2013. Altered expression of Na^+/K^+-ATPase
693 and other osmoregulatory genes in the gills of euryhaline animals in response to
694 salinity transfer: A meta-analysis of 59 quantitative PCR studies over 10 years.
695 Comp. Biochem. Physiol. - Part D Genomics Proteomics 8, 131–140.
696 https://doi.org/10.1016/j.cbd.2013.01.003

697 Henry, R.P., Lucu, C., Onken, H., Weihrauch, D., 2012. Multiple functions of the
698 crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion,
699 and bioaccumulation of toxic metals. Front. Physiol. 3, 431.
700 https://doi.org/10.3389/fphys.2012.00431

701 Hornung, E., 2011. Evolutionary adaptation of oniscidean isopods to terrestrial life:
702 Structure, physiology and behavior. Terr. Arthropod Rev. 4, 95–130.
703 https://doi.org/10.1163/187498311X576262

704 Ituarte, R.B., López Mañanes, A.A., Spivak, E.D., Anger, K., 2008. Activity of Na^+,K^+-
705 ATPase in a “freshwater shrimp”, *Palaemonetes argentinus* (Caridea,
706 Palaemonidae): Ontogenetic and salinity-induced changes. Aquat. Biol. 3, 283–
707 290. https://doi.org/10.3354/ab00089

708 Kiko, R., Werner, I., Wittmann, A., 2009. Osmotic and ionic regulation in response to

709 salinity variations and cold resistance in the Arctic under-ice amphipod
710 *Apherusa glacialis*. Polar Biol. 32, 393–398. https://doi.org/10.1007/s00300-008-
711 0531-z

712 Krogh, A., 1939. Osmotic regulation in aquatic animals, in: Osmotic Regulation in
713 Aquatic Animals. Cambridge at the University Press, Cambridge.

714 Leone, F.A., Lucena, M.N., Garçon, D.P., Pinto, M.R., McNamara, J.C., 2017. Gill
715 ion transport ATPases and ammonia excretion in aquatic crustaceans, in:
716 Weihrauch, D., O'Donnell, M. (Eds.), Acid-Base Balance and Nitrogen Excretion
717 in Invertebrates. Springer International Publishing, Cham, pp. 61–107.
718 https://doi.org/10.1007/978-3-319-39617-0_3

719 Lignot, J.H., Charmantier, G., 2015. Osmoregulation and excretion, in: Watling, L.,
720 Thiel, M. (Eds.), The Natural History of Crustacea. pp. 249–285.

721 Lind, U., Alm Rosenblad, M., Wrange, A.-L., Sundell, K.S., Jonsson, P.R., André, C.,
722 Havenhand, J., Blomberg, A., 2013. Molecular characterization of the α-subunit
723 of Na^{+}/K^{+} ATPase from the euryhaline barnacle *Balanus improvisus* reveals
724 multiple genes and differential expression of alternative splice variants. PLoS
725 One 8, e77069–e77069. https://doi.org/10.1371/journal.pone.0077069

726 Lockwood, A.P., 1962. The osmoregulation of crustacea. Biol. Rev. 37, 257–303.
727 https://doi.org/10.1111/j.1469-185X.1962.tb01613.x

728 Lucu, C., Towle, D.W., 2003. $Na^{+}+K^{+}$-ATPase in gills of aquatic crustacea. Comp.
729 Biochem. Physiol. A. Mol. Integr. Physiol. 135, 195–214.

730 Macias, M.T., Palmero, I., Sastre, L., 1991. Cloning of a cDNA encoding an *Artemia*
731 *franciscana* Na/K ATPase alpha-subunit. Gene 105, 197–204.
732 https://doi.org/10.1016/0378-1119(91)90151-z

733 Martin, J.W., Laverack, M.S., 1992. On the distribution of the crustacean dorsal
734 organ. Acta Zool. 73, 357–368. https://doi.org/10.1111/j.1463-
735 6395.1992.tb01108.x

736 Mclusky, D.S., Heard, V.E.J., 1971. Some effects of salinity on the Mysid *Praunus*
737 *Flexuosus*. J. Mar. Biol. Assoc. United Kingdom 51, 709–715.
738 https://doi.org/10.1017/S0025315400015083

739 Meschenmoser, M., 1989. Ultrastructure of the embryonic dorsal organ of *Orchestia*
740 *cavimana* (crustacea, amphipoda); with a note on localization of chloride and on
741 the change in calcium-deposition before the embryonic moult. Tissue Cell 21,
742 431–442. https://doi.org/https://doi.org/10.1016/0040-8166(89)90056-6

743 Moore, P.G., Francis, C.H., 1985. On the water relations and osmoregulation of the
744 Beach-Hopper *Orchestia gammarellus* (Pallas) (Crustacea: amphipoda). J. Exp.
745 Mar. Bio. Ecol. 94, 131–150. https://doi.org/10.1016/0022-0981(85)90054-1

746 Morritt, D., 1988. Osmoregulation in littoral and terrestrial talitroidean amphipods
747 (Crustacea) from Britain. J. Exp. Mar. Bio. Ecol. 123, 77–94.
748 https://doi.org/10.1016/0022-0981(88)90110-4

749 Morritt, D., Spicer, J.I., 1999. Developmental ecophysiology of the beachflea

750 *Orchestia gammarellus* (Pallas) (Crustacea: Amphipoda: Talitridae) III.
751 Physiological competency as a possible explanation for timing of hatchling
752 release. J. Exp. Mar. Bio. Ecol. 232, 275–283. https://doi.org/10.1016/S0022-
753 0981(98)00116-6

754 Morritt, D., Spicer, J.I., 1996a. Developmental ecophysiology of the beachflea
755 *Orchestia gammarellus* (Pallas) (Crustacea: Amphipoda). I. Female control of
756 the embryonic environment. J. Exp. Mar. Bio. Ecol. 207, 191–203.
757 https://doi.org/https://doi.org/10.1016/S0022-0981(96)02634-2

758 Morritt, D., Spicer, J.I., 1996b. The culture of eggs and embryos of amphipod
759 crustaceans: Implications for brood pouch physiology. J. Mar. Biol. Assoc.
760 United Kingdom 76, 361–376. https://doi.org/10.1017/s0025315400030605

761 Morritt, D., Spicer, J.I., 1996c. Developmental ecophysiology of the beachflea
762 *Orchestia gammarellus* (Pallas) (Crustacea: Amphipoda) II. Embryonic
763 osmoregulation. J. Exp. Mar. Bio. Ecol. 207, 205–216.
764 https://doi.org/https://doi.org/10.1016/S0022-0981(96)02635-4

765 Morritt, D., Spicer, J.I., 1995. Changes in the pattern of osmoregulation in the
766 brackish water amphipod *Gammarus duebeni* Lilljeborg (crustacea) during
767 embryonic development. J. Exp. Zool. 273, 271–281.
768 https://doi.org/10.1002/jez.1402730402

769 Mueller, C.A., Eme, J., Burggren, W.W., Roghair, R.D., Rundle, S.D., 2015.
770 Challenges and opportunities in developmental integrative physiology. Comp.
771 Biochem. Physiol. A. Mol. Integr. Physiol. 184, 113–124.
772 https://doi.org/10.1016/j.cbpa.2015.02.013

773 Normant, M., Kubicka, M., Lapucki, T., Czarnowski, W., Michalowska, M., 2005.
774 Osmotic and ionic haemolymph concentrations in the Baltic Sea amphipod
775 *Gammarus oceanicus* in relation to water salinity. Comp. Biochem. Physiol. A.
776 Mol. Integr. Physiol. 141, 94–99. https://doi.org/10.1016/j.cbpb.2005.04.007

777 Potts, W.T.W., Parry, G., 1964. Osmotic and ionic regulation in animals. Oxford
778 (London, New York & Paris): Pergamon Press.

779 Rivera-Ingraham, G.A., Lignot, J.-H., 2017. Osmoregulation, bioenergetics and
780 oxidative stress in coastal marine invertebrates: raising the questions for future
781 research. J. Exp. Biol. 220, 1749–1760. https://doi.org/10.1242/jeb.135624

782 Robertson, G., Schein, J., Chiu, R., Corbett, R., Field, M., Jackman, S.D., Mungall,
783 K., Lee, S., Okada, H.M., Qian, J.Q., Griffith, M., Raymond, A., Thiessen, N.,
784 Cezard, T., Butterfield, Y.S., Newsome, R., Chan, S.K., She, R., Varhol, R.,
785 Kamoh, B., Prabhu, A.L., Tam, A., Zhao, Y., Moore, R.A., Hirst, M., Marra, M.A.,
786 Jones, S.J.M., Hoodless, P.A., Birol, I., 2010. De novo assembly and analysis of
787 RNA-seq data. Nat. Methods 7. https://doi.org/10.1038/nmeth.1517

788 Salon, J., Cortas, N., Edelman, I.S., 1989. Isoforms of Na,K-ATPase in *Artemia*
789 *salina*: I. Detection by FITC binding and time course. J. Membr. Biol. 108, 177–
790 186. https://doi.org/10.1007/BF01871028

791 Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25

792 years of image analysis. Nat. Methods 9, 671–675.
793 https://doi.org/10.1038/nmeth.2089

794 Schoffeniels, E., 1970. Osmoregulation in aquatic arthropods. Chem. Zool. 5, 255–
795 286.

796 Seneviratna, D., 2006. Ontogeny of osmoregulation in embryos of intertidal crabs
797 (*Hemigrapsus sexdentatus* and *H. crenulatus*, Grapsidae, Brachyura): putative
798 involvement of the embryonic dorsal organ. J. Exp. Biol. 209, 1487–1501.
799 https://doi.org/10.1242/jeb.02167

800 Sexton, E.W., 1928. On the rearing and breeding of *Gammarus* in laboratory
801 conditions. J. Mar. Biol. Assoc. United Kingdom 15, 33–55.
802 https://doi.org/10.1017/S0025315400055521

803 Skou, J.C., 1990. The energy coupled exchange of Na^+ for K^+ across the cell
804 membrane: The Na+, K^+-pump. FEBS Lett. 268, 314–324.
805 https://doi.org/https://doi.org/10.1016/0014-5793(90)81278-V

806 Spicer, J.I., Gaston, K.J., 1999. Physiological diversity and its ecological
807 implications. Blackwell Science Ltd, Oxford.

808 Spicer, J.I., Tills, O., Truebano, M., Rundle, S.D., 2018. Developmental Plasticity and
809 Heterokairy, in: Development and Environment. Springer International
810 Publishing, Cham, pp. 73–96. https://doi.org/10.1007/978-3-319-75935-7_4

811 Strömberg, J.-O., 1972. *Cyathura Polita* (Crustacea, Isopoda), some embryological
812 notes. Bull. Mar. Sci. 22, 463–482.

813 Susanto, G.N., Charmantier, G., 2002. Ontogeny of ssmoregulation in the crayfish
814 *Astacus leptodactylus*. Physiol. Biochem. Zool. 73, 169–176.
815 https://doi.org/10.1086/316736

816 Taylor, H.H., Seneviratna, D., 2005. Ontogeny of salinity tolerance and hyper-
817 osmoregulation by embryos of the intertidal crabs *Hemigrapsus edwardsii* and
818 *Hemigrapsus crenulatus* (Decapoda, Grapsidae): Survival of acute hyposaline
819 exposure. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 140, 495–505.
820 https://doi.org/10.1016/j.cbpb.2005.03.005

821 Thabet, R., Ayadi, H., Koken, M., Leignel, V., 2017. Homeostatic responses of
822 crustaceans to salinity changes. Hydrobiologia 799, 1–20.
823 https://doi.org/10.1007/s10750-017-3232-1

824 Truebano, M., Tills, O., Spicer, J.I., 2013. Embryonic transcriptome of the
825 brackishwater amphipod *Gammarus chevreuxi*. Mar. Genomics 28, 5–6.
826 https://doi.org/10.1016/j.margen.2016.02.002

827 Vlasblom, A.G., Bolier, G., 1971. Tolerance of embryos of *Marinogammarus marinus*
828 and *Orchestia gammarella* (Amphipoda) to lowered salinities. Netherlands J.
829 Sea Res. 5, 334–341. https://doi.org/10.1016/0077-7579(71)90016-0

830 Werntz, H.O., 1963. Osmotic regulation in marine and fresh-water Gammarids
831 (Amphipoda). Biol. Bull. 124, 225–239. https://doi.org/10.2307/1539498

832 Wilder, M.N., Huong, D.T.T., Okuno, A., Atmomarsono, M., Yang, W.-J., 2001.
833 Ouabain-sensitive Na/K-ATPase activity increases during embryogenesis in the
834 giant freshwater prawn *Macrobrachium rosenbergii*. Fish. Sci. 67, 182–184.
835 https://doi.org/10.1046/j.1444-2906.2001.00219.x

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

839

840

841