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1 **Osmoregulatory performance and immunolocalization of Na⁺/K⁺-ATPase in the**
2 **branchiopod *Artemia salina* from the Sebkhah of Sidi El Hani (Tunisia)**

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6 Imene SELLAMI ^{1,2}, Guy CHARMANTIER ¹, Hachem B. NACEUR ², Adnane KACEM ²,
7 Catherine LORIN-NEBEL ^{1*}

8
9 ¹ Univ. Montpellier, UMR MARBEC (CNRS, IFREMER, IRD, UM), France

10
11 ² LR14ES06 Bioressources, Integrative Biology and Valorization, Higher Institute of
12 Biotechnology of Monastir, University of Monastir, Avenue Tahar Haddad, BP 74, 5000
13 Monastir, Tunisia

14
15 * Corresponding author : Catherine Lorin-Nebel, catherine.lorin@umontpellier.fr
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20 **Abstract**

21 *Artemia salina* is an extremophile species that tolerates a wide range of salinity, especially
22 hypertonic media considered lethal for the majority of other aquatic species. In this study, *A.*
23 *salina* cysts were hatched in the laboratory and nauplii were acclimated at three different
24 salinities (60, 139 and 212 ppt). Once in the adult phase, their hemolymph osmolality was
25 measured. The animals were strong hypo-osmoregulators in the entire range of tested
26 salinities, with up to 10 fold lower hemolymph osmolalities than their surrounding
27 environment. Immunostaining of Na⁺/K⁺-ATPase was done on sections and on whole body
28 mounts of adults in order to localize the ionocytes in different organs. An intense Na⁺/K⁺-
29 ATPase immunostaining throughout the cells was observed in the epithelium of the ten pairs
30 of metepipodites. A positive immunoreactivity for Na⁺/K⁺-ATPase was also detected in the
31 maxillary glands, in the epithelium of the efferent tubule and of the excretory canal, as well as
32 in the anterior digestive tract. This study confirms the strong hypo-osmotic capacity of this
33 species and affords an overview of the different organs involved in osmoregulation in *A.*
34 *salina* adults.

35
36
37 **Keywords:** *Artemia salina*, hypo-osmoregulation, Na⁺/K⁺-ATPase, immunolocalization,
38 metepipodites, maxillary glands
39
40

41 1. Introduction

42

43 *Artemia salina* (Linnaeus, 1758) is part of a complex of euryhaline species of
44 branchiopod crustaceans qualified as an animal extremophile with an exceptionally wide
45 salinity tolerance among animals, and particularly an ability to survive and thrive in
46 hypertonic media, a "forbidden environment" from which most metazoans are excluded as
47 stated by Eads (2004) (Abatzopoulos et al., 2002; Browne and Bowen, 1991; Clegg and
48 Trotman, 2002). These animals have attracted the attention of scientists over decades and
49 have been the subject of extensive studies in order to understand the mechanisms allowing
50 them to tolerate ionic and osmotic stresses imposed by their environment.

51 These remarkable crustaceans are able to withstand ionic contents and salinities that, in
52 their upper values, are lethal for the majority of other aquatic species, ranging from 9 ppt
53 (Brisset et al., 1982) to 340 ppt (Gajardo and Beardmore, 2012; Post and Youssef, 1977) and
54 up to crystallizing brine close to 600 ppt (Croghan, 1958a, b). *Artemia salina* is a highly
55 potent hypo-osmotic regulator in media more concentrated than one third of seawater (salinity
56 of about 11 ppt corresponding to an osmolality of 300 mOsm.kg⁻¹). For instance, early studies
57 had shown that its hemolymph osmolality is maintained between 400 and 800 mOsm.kg⁻¹ for
58 salinities ranging from 34 to 340 ppt (1000 to 10 000 mOsm.kg⁻¹) (Croghan, 1958b).

59 Such levels of hypo-osmoregulation suppose the existence of powerful ion excretion
60 abilities in order to compensate the passive invasion of ions from the concentrated external
61 medium. Moreover, animals have to limit water loss by osmosis, but water uptake
62 mechanisms are not well known in crustacean, notably in those high-salinity environments. In
63 larvae, Conte (1984) has extensively described the mechanisms of osmoregulation in the
64 nauplii of *A. salina* that use a special salt-secreting gland, also called salt gland or neck or
65 nuchal organ, to excrete salts that penetrate by diffusion. Similarly, Russler and Mangos
66 (1978) have shown that this organ is the main route for sodium excretion.

67 In adult *A. salina*, different studies have shown a similarity between their mechanisms
68 of osmoregulation and those proposed for marine teleosts and hypo-regulating crustaceans
69 (Croghan, 1958b, c, d; Copeland, 1967; Smith, 1969a, b). Brine shrimps have well-developed
70 active mechanisms for absorbing NaCl from the gut lumen to the hemolymph. Hence, water
71 follows passively to compensate water lost by osmosis to the external concentrated medium
72 (Croghan, 1958d). Also in adults, sodium and chloride, which enter the body through
73 diffusion given their high concentration in the external medium, are excreted to the medium
74 by specialized organs acting as gills, the metepipodites. The ten pairs of flattened, leaf-like

75 metepipodites borne by the phyllopods have been described as the sites of active outward
76 transport of ions (Copeland, 1967; Croghan, 1958c).

77 In particular, a detailed study of the metepipodites of *A. salina* was conducted by
78 Copeland (1967). Using silver nitrate, he confirmed that the metepipodites were the site of ion
79 (chloride) exchanges, a finding reported earlier by Croghan (1958c) and later confirmed by
80 other authors (Holliday et al., 1990). Copeland's ultrastructural exploration revealed the
81 presence and association of two types of cells in the metepipodites, the "light" and "dark
82 cells". The latter are columnar cells with projected stellate flanges; they extend from the
83 apical cuticle to the basal membrane lining the hemolymph, i.e. they face the external medium
84 as well as the hemolymph, and they present deep interdigitations with the light cells. Stacks of
85 mitochondria, called "mitochondrial pumps" by Copeland, are located in the dark cells in
86 close association with their membrane, particularly in their projections. From these
87 observations, Copeland concluded that the metepipodites represent "a highly specialized
88 tissue for the secretion of salt". Given the location of the dark cells, their ultrastructural
89 features and particularly the abundance of mitochondria in their cytoplasm, "it would appear
90 likely that the dark cell is responsible" for the "release of salt to the external environment"
91 (Copeland, 1967). This hypothesis was confirmed by later experimental findings. In fact, the
92 crude homogenates of metepipodites showed a very high specific enzyme activity of Na^+/K^+ -
93 ATPase, which increased proportionally with the salinity of the external medium (Holliday et
94 al., 1990). The same author found that the digestive tract and maxillary glands also had a high
95 Na^+/K^+ -ATPase content, which suggests that these organs are also involved in ion transports
96 and probably for a part in osmoregulatory processes. Membrane-bound Na^+/K^+ -ATPase was
97 partially purified from *A. salina* nauplii (Morohashi and Kawamura, 1984) and the α isoform
98 of the enzyme was isolated in salt glands and intestine of nauplii (Cortas et al., 1989). Also in
99 naupliar larvae, Na^+/K^+ -ATPase α and β isoforms were immunolocalized in the basal
100 membranes of the salt gland cells (Sun et al., 1991) and the mRNA expression of their $\alpha 1$ and
101 $\alpha 2$ isoforms was quantified in the same organs (Conte, 2008; Escalante et al., 1995; Sun et al.,
102 1992). While the localization of the enzyme is known in the larval salt gland, to our
103 knowledge surprisingly few reports are available regarding the localization of Na^+/K^+ -ATPase
104 in adult *Artemia*. Recently, an illustration of the presence of Na^+/K^+ -ATPase in a
105 metepipodite of *A. franciscana* has been made available in a preliminary report (Drenth,
106 2017).

107 The present study aims to confirm the ability of *A. salina* to osmoregulate at very high
108 salinities, and to determine the localization of Na⁺/K⁺-ATPase in the metepipodites, maxillary
109 glands and digestive tract of adults, in order to improve the understanding of osmoregulatory
110 mechanisms allowing a Tunisian *Artemia salina* population (from the hyper-saline lagoon
111 Sebkhah of Sidi El Hani) to withstand high salinities.

112

113 **2. Materials and methods**

114

115 *2.1. Cysts sampling*

116

117 *Artemia* cysts were collected in spring 2017 from the banks of the hypersaline Sebkhah
118 of Sidi El Hani where the salinity exceeded 280 ppt.

119 The Sebkhah of Sidi El Hani is a NW–SE lengthened depression in the Sahel area (eastern
120 Tunisia) (Fig. 1). The Sidi El Hani discharge area is approximately 370 km² and its average
121 water depth is 0.4 m, and 0.8 m at some locations (Fig. 1). The seasonal temperature
122 fluctuates between 2 and 13 °C in winter and between 33 and 39 °C in summer (Ali et al.,
123 2013). The seasonal average of salinity oscillates between a minimum of 180 ppt in winter
124 registered after rainfall and up to 320 ppt in summer. The ionic composition of Sidi El Hani is
125 mostly based on Na⁺ (115 gL⁻¹), Cl⁻ (191 gL⁻¹), Mg²⁺ (8 gL⁻¹) and SO₄²⁻ (13 gL⁻¹). The
126 presence of *Artemia* in the site was first reported by (Gauthier, 1928).

127

128 *2.2. Culture experiments*

129

130 Upon collection, cysts were mixed with salt collected in the Sidi El Hani area, for
131 conservation. Once in the laboratory, cysts of *Artemia salina* were cleaned, separated and
132 stored according to the protocol of Sorgeloos *et al.* (1986). Cysts were incubated over 48 hrs
133 in 1 liter of filtered seawater at a salinity of 35 ppt and a temperature of 25 °C, under constant
134 illumination (2000 lux) and continuous aeration to keep them in suspension (Lavens and
135 Sorgeloos, 1996). Following hatching, the nauplii were transferred to 3 containers of 5L filled
136 with water at different salinities, 60, 139 and 212 ppt. These media had been prepared from a
137 mixture of seawater sterilized in an autoclave and raw salt (harvested at the Sebkhah of Sidi El
138 Hani). In each container, the salinity of the water was checked using a salinometer (Lovibond
139 SensoDirect con110), and the osmolality was measured with an Advanced Instruments 3300
140 micro-osmometer in later experiments. The containers were placed at a temperature of 25-27

141 °C and a photoperiod of 16 hrs L / 8 hrs D. *Artemia* nauplii density was adjusted to about 50
142 individuals per L. These larvae were fed twice a week by adding 100 ml of Chlorophyceae
143 *Dunaliella salina* culture at an approximate density of 100,000 cells.ml⁻¹ (the salinity of the
144 microalga culture was the same as that used for the culture of nauplii). Water was renewed
145 once a week to ensure the elimination of *Artemia salina* waste and agglomerated microalgae.
146 Once the adult phase was reached, some animals were fixated for histology, others were
147 transported over 24 hrs in sealed containers to the Marbec laboratory (Montpellier, France)
148 for further experiments.

149

150 2.3. Measure of hemolymph osmolality

151

152 Hemolymph osmolality was measured in 8-12 adult *A. salina* per condition. According
153 to previous results on different crustacean species (Charmantier, 1998), the animals were
154 exposed directly to the experimental media for 48 hrs in covered beakers filled with water at
155 three different salinities maintained at 24 °C, aerated and covered with parafilm to prevent
156 evaporation. The salinities of the media, 60, 139 and 212 ppt, corresponded to osmolalities of
157 1737, 4064 and 6192 mOsm.kg⁻¹, according to measures made on an Advanced Instrument
158 Model 3300 micro-osmometer. Prior to sampling, the specimens were carefully rinsed with
159 deionized water, dried on filter paper and quickly immersed into mineral oil to prevent
160 evaporation and desiccation. Remaining adherent water was removed using a hand-made
161 glass micropipette. A second micropipette was then inserted dorsally into the heart to obtain
162 hemolymph samples, which were then immediately measured with reference to a 300
163 mOsm.kg⁻¹ standard solution on a Kalber-Clifton nano-osmometer (Clifton Technical Physics,
164 Hartford, NY, USA) requiring about 30 nl.

165

166 2.4. Histology and immunolocalization of Na⁺/K⁺-ATPase

167

168 In order to localize the ionocytes, and to visualize and semi-quantify Na⁺/K⁺-ATPase,
169 we performed histology, immunofluorescence staining, whole-mounts and microscopic
170 observations. About twenty adult brine shrimp from each salinity condition were fixed for 24
171 hrs by immersion in Bouin's fixative. Once rinsed in several baths of 70 % ethanol, samples
172 were dehydrated in a graded ethanol series and finally embedded in Paraplast (Sigma).
173 Longitudinal and transverse sections of 4 µm were cut on a Leitz Wetzlar microtome,
174 collected on poly-L-lysine coated glass slides and stored at 37 °C for 48 hrs. One series of

175 slides was stained using the Masson's Trichrome staining protocol and observed under a Leica
176 Diaplan microscope. The other series was used for *in situ* immunolocalization of Na⁺/K⁺-
177 ATPase. Slides were dewaxed (butanol and LMR), hydrated through a descending series of
178 ethanol baths (from 100 % to 50 %) and rinsed in phosphate-buffered saline (PBS; 137 mM
179 NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4, Sigma). The slides were then immersed
180 for 10 min into 0.02 % Tween 20, 150 mM NaCl dissolved in PBS, pH 7.3. After blocking in
181 5 % skimmed milk (SM) in PBS at 37 °C for 20 min, the slides were rinsed twice with PBS.
182 Primary labelling was carried out overnight at 4 °C in a wet chamber with the primary
183 monoclonal antibody (α 5) mouse anti-Na⁺/K⁺-ATPase (Hybridoma Bank, University of Iowa)
184 diluted in 0.5% SM-PBS at 10 μ g.mL⁻¹. Using a procedure similar to that of this study, this
185 antibody was shown to specifically react with Na⁺/K⁺-ATPase in several crustacean species,
186 such as *Porcellio scaber* (Ziegler, 1997), *Homarus gammarus* (Lignot et al., 1999), *Astacus*
187 *leptodactylus* (Lignot et al., 2004), *Carcinus maenas* (Cieluch et al., 2004), *Crangon crangon*
188 (Cieluch et al., 2005), *Eriocheir sinensis* (Cieluch et al., 2007), *Macrobrachium amazonicum*
189 (Boudour-Bouchecker et al., 2014) and *Eurytemora affinis* (Gerber et al., 2016). Once rinsed
190 three times in PBS to remove unbound primary antibody, the sections were incubated for 1 hr
191 with a secondary antibody (donkey anti-mouse Alexa Fluor® 488 (Invitrogen, Life
192 Technologies) at 10 μ g.mL⁻¹ in SM-PBS) and rinsed again three times in PBS. Nuclei of
193 some slides were counterstained using DAPI at 1 μ g/ml for 6 min, followed by three washes in
194 PBS.

195 Several protocols of preparation and immunostaining of whole mounts of *A. salina*
196 have been tried. A limitation of antibody penetration was encountered probably due to the
197 cuticle barrier. EDTA, ultrasonic treatment from 5 s to 2 min, and Triton X-100 were tested in
198 different combinations. Positive staining was obtained using ultrasonic pulses at 35 KHz from
199 10 s to 20 s as indicated thereafter. Even in the best preparations, only some metepipodites of
200 the animals showed positive staining. Following pretreatment with ultrasonic pulses (from 10
201 s to 20 s) to enhance probe accessibility, whole mounts of *A. salina* adults were pre-incubated
202 in 5 % skimmed milk (SM) in PBS and 0.3 % Triton X-100 (PBS-TX) for 2 hrs at room
203 temperature. After blocking, the animals were rinsed several times with PBS. Primary
204 labelling was carried out overnight at 4 °C in a wet chamber with the primary monoclonal
205 antibody (α 5) mouse anti-Na⁺/K⁺-ATPase (Hybridoma Bank, University of Iowa) diluted at
206 10 μ g.mL⁻¹ in 0.5 % SM-PBS. Following three rinses in PBS, the specimens were incubated
207 for 3 hrs with a secondary antibody (donkey anti-mouse Alexa Fluor® 488 (Invitrogen, Life

208 Technologies) at $10 \mu\text{g.mL}^{-1}$) and rinsed again in PBS. Nuclei of some mounts were
209 counterstained using DAPI at $1 \mu\text{g.mL}^{-1}$ for 6 min, followed by three washes in PBS.

210 Sections and whole mount preparations were then mounted in an anti-bleaching
211 mounting medium (Immunohistomount, Santa Cruz Biotechnology) and observed with a
212 Leica Diaplan microscope equipped with a special filter for fluorescence (450-490 nm) and
213 coupled to a Leica DC300F digital camera and FW4000 software. Observations and
214 photography of selected slides were conducted using a Leica TCS SP2 confocal microscope
215 of the MRI platform, Montpellier University.

216

217 *2.5. Quantification of the Na^+/K^+ -ATPase (NKA) immunostaining intensity in* 218 *phyllopods*

219

220 To determine the relative importance of different phyllopods in ion transport in each
221 salinity condition, we compared the relative fluorescence intensity of immunostaining in
222 metepipodites of *Artemia* adults. Several photographs were taken in 5 individuals for each
223 studied salinity using a constant exposure time. Longitudinal sections were used to visualize
224 the different metepipodites and their Na^+/K^+ -ATPase content within an animal.

225 The photographs were analyzed using the public domain ImageJ software (version 1.49, v),
226 according to a protocol successfully used in other crustaceans (Boudour-Bouchecker et al.,
227 2013; Gerber et al., 2016; Issartel et al., 2010) and in fish (Ouattara et al., 2009; Riou et al.,
228 2012; Sucré et al., 2012). The intensity of immunostaining, i.e. the staining intensity of the
229 immunolabelled area, was measured by quantifying the pixel intensity of the immunolabeled
230 area using ImageJ software. These measurements were performed using the ImageJ software's
231 "Average Gray Value" parameter, which determines the sum of the gray values of all pixels in
232 the selected area relative to the number of pixels, expressed in calibrated units (optical
233 density). In a second step, the ImageJ software was used to quantify the difference in average
234 pixel intensity of fluorescence between different salinities among metepipodites within the
235 same adults.

236

237 *2.6. Statistics*

238 Statistical analyses (hemolymph osmolality and fluorescence intensity) were performed
239 using Graphpad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA).
240 Normality and homogeneity of variance were respectively checked using D'Agostino-Pearson
241 test and Barlett's test. For data fitting homogeneity of variance requirement, a one-way

242 ANOVA regarding or regardless salinity as the main factor was performed; critical
243 differences between groups were appraised using the Tukey's multiple comparisons test. For
244 data not fitting homogeneity of variance and data due to the small sample size, a non-
245 parametric Kruskal-Wallis test followed by a multiple comparisons Dunn's test was used.
246 Data are presented as means \pm SD, and the level of statistical significance was set at $p < 0.05$.

247

248

249 **3. Results**

250

251 The main results of this study are summarized in Fig. 1S.

252

253 *3.1. Hemolymph osmolality*

254

255 *Artemia* adults were extremely strong hypo-osmoregulators. Their hemolymph
256 osmolality, which ranged between 406 ± 21 and 608 ± 16 mOsm.Kg⁻¹ in our experiment, was
257 significantly different according to salinity (Fig. 2; one-way ANOVA, $p < 0.001$). It is worth
258 noting that hemolymph osmolalities differed by only 202 mOsm.Kg⁻¹ between the lower and
259 higher tested salinities that themselves differed by more than 4450 mOsm.Kg⁻¹.

260

261 *3.2. Morphological description*

262

263 The morphology of *Artemia* adults is illustrated in Fig. 3. Eleven pairs of phyllopods
264 (Ph1-11) were observed. The ten pairs of flattened leaf-like metepipodites are attached at the
265 middle part of the ten phyllopods, (Me1-10). The eleventh pair of phyllopods is sexually
266 dimorphic between the sexes and does not bear a metepipodite (Fig. 3A, 4A, B, C, 5A, B).
267 Each metepipodite is a flattened leaf-like oval structure (Fig. 3A, C, 5G), with average
268 minimum and maximum dimensions of 225 ± 50 μ m and 486 ± 84 μ m. Metepipodites 1 and 2
269 are smaller (Fig. 3A).

270 Internally, we observed a longitudinal digestive tract, and a pair of maxillary glands
271 located on each side of the digestive tract in the anterior part of the body (Fig. 6).

272

273 *3.3. Histology and immunolocalization of Na⁺/K⁺-ATPase (NKA)*

274

275 Metepipodites are flat structures formed of two identical facing monolayered epithelia
276 covered by a thin cuticle, limiting a hemolymph lacuna (Fig. 5). A hemolymphatic space at
277 the base of each metepipodite allows a communication with the phyllopod (Fig. 5C, D). NKA
278 localization was visualized using immunohistochemical analysis of longitudinal and
279 transverse sections. An intense immunostaining throughout most of the cells was observed in
280 the epithelium of the ten pairs of metepipodites (Figs 3B, C, 4C, 5). The immunopositive cells
281 were large ($57\pm 3 \mu\text{m}$ cellules) and contained a voluminous nucleus with average diameter of
282 $10\pm 1 \mu\text{m}$ (Fig. 5F, F'). Immunostaining intensity appears lower in some cells (Figs 3C, 5G).

283

284 The semi-quantification of fluorescence intensity of metepipodites showed no
285 significant difference between the different metepipodites and among the analyzed salinities
286 and an overall high variability was observed between animals (Fig. 2S).

287

288 A positive immunoreactivity for Na^+/K^+ -ATPase was detected in the maxillary glands,
289 where a strong immunostaining was observed in the epithelium of the efferent tubule and in
290 the excretory canal (Fig. 6C-H). The proximal coelomic sac was not immunostained (Fig.
291 6C). The anterior digestive tract revealed a positive immunolabelling in the basal cell part as
292 shown in Fig. 6D-F.

293 Negative control sections without the primary antibody did not show immunolabelling
294 in any organ or tissue (not shown).

295

296

297 **4. Discussion**

298

299 Brine shrimps, *Artemia* species, are usually considered as extremophiles especially for
300 their ability to face challenging salinities (up to 10-fold that of standard seawater) considered
301 lethal for the majority of other aquatic species (Gajardo and Beardmore, 2012). Such
302 environmental pressures demand an ability to regulate hemolymph osmolytes according to the
303 external medium via powerful osmoregulatory mechanisms (Campbell et al., 2012;
304 Charmantier et al., 2009; Freire et al., 2003). Blood osmolality change is often used as an
305 indicator for osmoregulatory acclimation and tolerance to salinity stress (Varsamos et al.,
306 2005). This study has confirmed the hypo-osmotic capacity of *Artemia* adults that can
307 maintain their hemolymph osmolality up to 10 fold lower than their surrounding environment
308 (for example hemolymph osmolality of $608 \pm 16 \text{ mOsm.Kg}^{-1}$ in a medium at $6192 \text{ mOsm.Kg}^{-1}$

309 ¹, 212 ppt). Due to these exceptional physiological abilities, brine shrimps are considered the
310 most powerful hypo-osmoregulators among aquatic metazoans, with the highest tolerance to
311 salinity (Cole and Brown, 1967). Extensive similar studies have concluded that the
312 hemolymph osmotic pressure was markedly hypotonic even in the most concentrated media
313 (Medwedewa, 1927; Plattner, 1955). Holliday *et al.* (1990) stated that *A. salina* is a weak
314 hyporegulator in 50 % SW and an increasingly strong hyporegulator in 100 %, 200 % and 400
315 % SW. Different studies have also shown a similarity between the mechanisms of
316 osmoregulation in *Artemia* adults and those proposed for marine teleosts and hypo-regulating
317 crustaceans (Croghan, 1958b, c, d; Copeland, 1967; Thuet *et al.*, 1968; Smith, 1969a, b).
318 Croghan (1958b, c, d), in an extensive study of osmoregulation in brine shrimps, has shown
319 that they have well-developed active mechanisms for absorbing NaCl from the gut lumen to
320 the hemolymph, resulting in a passive influx of water to hemolymph that compensates water
321 lost by osmosis to the external concentrated medium (Croghan, 1958d). Sodium and chloride,
322 which also enter the body through diffusion given their high concentration in the external
323 medium, are excreted to the medium by specialized organs.

324
325 Na⁺/K⁺-ATPase is known as the major driving force that ensures ion exchanges (Cieluch *et*
326 *al.*, 2004; Geering, 2008; Lignot *et al.*, 1999; Lignot and Charmantier, 2001; Lucu and Towle,
327 2003; Thabet *et al.*, 2016; Thuet *et al.*, 1988) as well as generating an electrical gradient
328 fuelling other transporters (Campbell *et al.*, 2012; Esbaugh *et al.*, 2019; Pivovarov *et al.*,
329 2019; Sáez *et al.*, 2009). Its abundance in an organ suggests an involvement in ion transport.
330 The present study affords an overview of the different organs involved in osmoregulation in
331 *A. salina* adults. Immunofluorescence staining of Na⁺/K⁺-ATPase was used in order to
332 localize ionocytes in these organs.

333 Previous studies first addressed osmoregulation in embryos and during early post-
334 embryonic development. Several extensive studies have reported that active ion excretion
335 mediated by Na⁺/K⁺-ATPase is carried out by the naupliar salt gland (or dorsal / neck / nuchal
336 organ) that develops in late pre-naupliar embryonic stages; this organ becomes apparently
337 functional shortly before hatching. Later in development, thoracic epipodites take over ion
338 transport according to the stage of development (Conte, 1984; Conte *et al.*, 1977, 1972;
339 Mitchell and Crews, 2002; Peterson *et al.*, 1978; Russler and Mangos, 1978; Thuet, 1982).
340 Working in naupliar larvae, Sun *et al.* (1991) reported that Na⁺/K⁺-ATPase α and β subunits
341 were immunolocalized in the basal membranes of the salt gland cells. The mRNA expression

342 of $\alpha 1$ and $\alpha 2$ paralogs was quantified in the same organs (Conte, 2008; Escalante et al., 1995;
343 Sun et al., 1991).

344 In the present study, an intense Na^+/K^+ -ATPase immunostaining throughout most of the
345 cells was observed in the epithelium of the ten pairs of metepipodites. Our results confirm
346 those found in earlier works that attributed active outward transport of ions to metepipodites
347 in adults (Copeland, 1967; Croghan, 1958c; Holliday et al., 1990). In a major study using
348 electron microscopy, Copeland (1967) concluded that the metepipodites represent “a highly
349 specialized tissue for the secretion of salt and a special cell type found in these structures (the
350 'dark cell'), rich in mitochondria, is thought to be responsible for this ion transport”. The
351 numerous immunostained cells that we observed in metepipodites therefore correspond to the
352 “dark cells” as described by Copeland (1967). As we detected no difference in
353 immunostaining intensity among metepipodites, this suggests a similar cellular Na^+/K^+ -
354 ATPase content among different metepipodites and an involvement of all metepipodites in
355 active transport. Later experimental findings conducted on crude homogenates of
356 metepipodites revealed a very high specific enzyme activity of Na^+/K^+ -ATPase, which
357 increased proportionally with the salinity of the external medium (Holliday et al., 1990). A
358 slight but not significant increase in immunostaining intensity has been observed between 60
359 and 139 ppt and could indicate a slightly increased Na^+/K^+ -ATPase content within the cells
360 lining phyllopods at 139 ppt. Recently, a preliminary report of Drenth (2017) confirmed the
361 presence of Na^+/K^+ -ATPase in a metepipodite of *A. franciscana* and the salt extrusion occurs
362 in a mitochondrial rich, membraneous cell layer in the metepipodites of the brine shrimp. The
363 same investigator suggested that brine shrimp upregulate only the $\alpha 2$ form of Na^+/K^+ -ATPase
364 in response to increasing salinity, while the $\alpha 1$ form remains relatively unchanged.

365 A positive immunoreactivity for Na^+/K^+ -ATPase was also detected in the anterior digestive
366 tract and in the maxillary glands. In these paired glands, Na^+/K^+ -ATPase immunostaining was
367 observed in the epithelium of the efferent tubule and of the excretory canal, while the
368 coelomic sac remained unstained. Our results confirmed that these organs are also involved in
369 ion transport and probably for a part in osmoregulatory processes. In the same context, Tyson
370 (1969) had shown that the efferent duct of the maxillary gland of *A. salina* presents
371 ultrastructural features typical of transporting epithelia. Holliday *et al.* (1990) measured a
372 high Na^+/K^+ -ATPase activity in the digestive tract. Studying the physiology of the gut in *A.*
373 *salina* and its implication in osmoregulation, Croghan (1958c) found that the concentration of
374 both sodium and chloride ions in the gut fluids was always lower than that in the hemolymph,
375 pointing to an active uptake of NaCl across the gut epithelium, which in turn controls water

376 balance and prevents dehydration in hypertonic media. Indeed, Drenth (2017) confirmed the
 377 basolateral localization of Na⁺/K⁺-ATPase in the gut of *A. franciscana* adults, where ions and
 378 water are taken in from the environment into the hemolymph (Russler and Mangos, 1978).

379

380 **5. Conclusion**

381

382 In this work, we have focused on the hypo-osmoregulatory capacity of adult *A. salina*
 383 and particularly on their main organs involved in active ion transport. We have confirmed that
 384 brine shrimps are powerful hypo-osmoregulators as they keep their hemolymph osmolality
 385 strongly lower than even the most concentrated media. Immunofluorescence and whole
 386 mounts staining of Na⁺/K⁺-ATPase have been used to illustrate the histological and cellular
 387 structure of metepipodites related to osmoregulation; despite their major function, their
 388 functional histology had surprisingly not been re-addressed since the major works of the
 389 1960's. We have also confirmed the involvement in osmoregulation of the maxillary glands
 390 and of the anterior part of the digestive tract.

391

392

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 396 help in confocal microscopy.

397

398 **List of References**

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576 **Legends of Figures**

577

578 Abbreviations. ADT: anterior digestive tract; Cs: coelomic sac; E: eye; EC: excretory canal; Me:
579 metepipodite; MG: maxillary gland; N: nucleus; Ph: phyllopod; PI: posterior intestine; T: tubule.

580

581 **Fig. 1.** Geographical location of the Sebkhha of Sidi El Hani (Tunisia). Google Earth Pro V. 7.3.2.5776.

582

583 **Fig. 2.** Adult *Artemia salina*. Osmotic pressure of the hemolymph of animals maintained at three
584 different salinities (60 ppt: 1737 mOsm.Kg⁻¹, 139 ppt: 4064 mOsm.Kg⁻¹, 212 ppt: 6192 mOsm.Kg⁻¹,
585 N=8-12). The dotted line is the isosmotic line. Different letters indicate significant differences
586 (p<0.001).

587

588 **Fig. 3.** Adult *Artemia salina*. Morphology and localization of metepipodites (Me) and maxillary
589 glands (MG). (A) Dorsal view showing the eleven pairs of phyllopods (Ph) and the ten pairs of
590 metepipodites. (B), (C) Whole-mounts and immunolocalization of Na⁺/K⁺-ATPase; (B) Anterior view
591 with immunostaining in the two maxillary glands and in one metepipodite 1; (C) Median view with
592 several phyllopods and immunostained metepipodites. Scale bars: (A) 500 µm; (B), (C) 200 µm.

593

594 **Fig. 4.** Adult *Artemia salina*. Localization of metepipodites (Me) on phyllopods (Ph). (A), (B)
595 Longitudinal vertical and horizontal histological sections. (C) Longitudinal horizontal section:

596 immunolocalization of Na⁺/K⁺-ATPase in metepipodites 1-9 attached to phyllopods. Scale bars: (A),
 597 (B) 500 μm, (C) 40 μm.

598

599 **Fig. 5.** Adult *Artemia salina*. Histological structure and immunolocalization of Na⁺/K⁺-ATPase (NKA)
 600 and DNA staining of nuclei on phyllopods and metepipodites sections. (A), (B), (F), (F') Merged
 601 staining of Na⁺/K⁺-ATPase (green) and DNA (blue). (C), (D), (E) Phyllopods bearing metepipodites
 602 with NKA immunostaining (D, E) or Trichrome Masson's staining (C). (G) Immunolocalization of
 603 NKA on metepipodites in horizontal (Me4) and transverse (Me5, 6) sections. Scale bars: (A), (B) 40
 604 μm; (C), (D), (E), (F), (G) 20 μm; (F') 5 μm.

605

606 **Fig. 6.** Adult *Artemia salina*. Histological structure, whole-mounts and immunolocalization of Na⁺/K⁺-
 607 ATPase (NKA) in the anterior digestive tract (ADT), in the maxillary gland (MG), and in
 608 metepipodite (Me). (A), (B) Transverse histological sections of the anterior digestive tract and of the
 609 maxillary gland. (C), (D), (E) NKA localization in transverse sections of the anterior digestive tract
 610 and of the maxillary gland. (F) Horizontal section of the anterior digestive tract and of the two
 611 maxillary glands. (G), (H) Whole mounts showing NKA localization in the maxillary gland and
 612 metepipodite 1. Scale bars: (A), (B), (C), (D), (F) 20 μm; (E) 40 μm; (G) 200 μm; (H) 50 μm.

613

614 **Fig. 1S.** Present results (in red) and previous knowledge on osmoregulation in the branchiopod
 615 *Artemia* at different development stages involving different osmoregulatory organs.

616

617 **Fig. 2S.** Adult *Artemia salina*. Relative immunostaining intensity for Na⁺ /K⁺-ATPase (NKA) in the
 618 different metepipodites (1-10) and its difference according to different salinities. (A) Comparison
 619 between the metepipodites (1-10) regardless of salinity (N=5-6). (B) Comparison between the studied
 620 salinities regardless of the location of metepipodite (N=3-12).

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Fig. 1.

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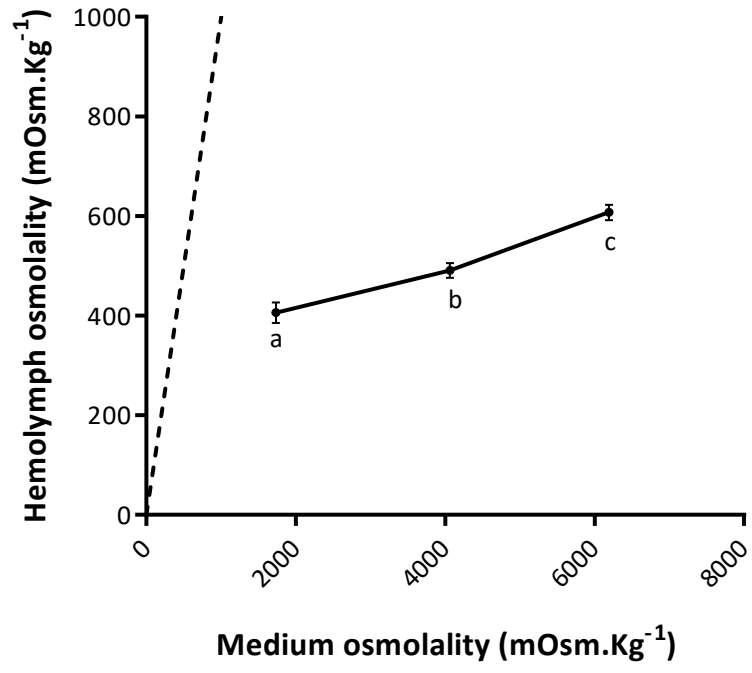
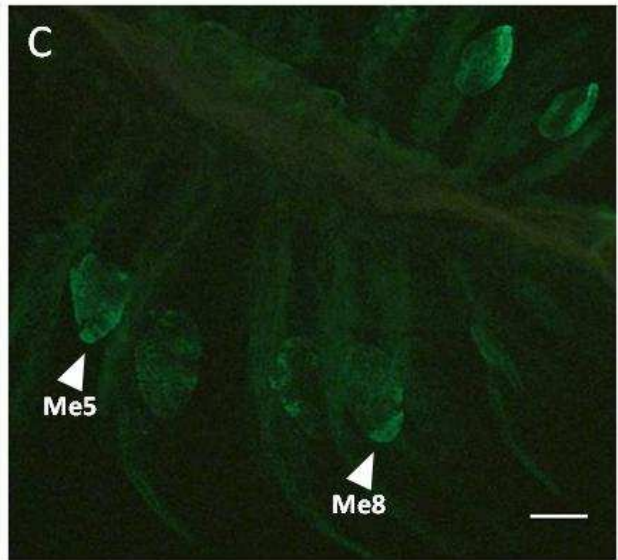
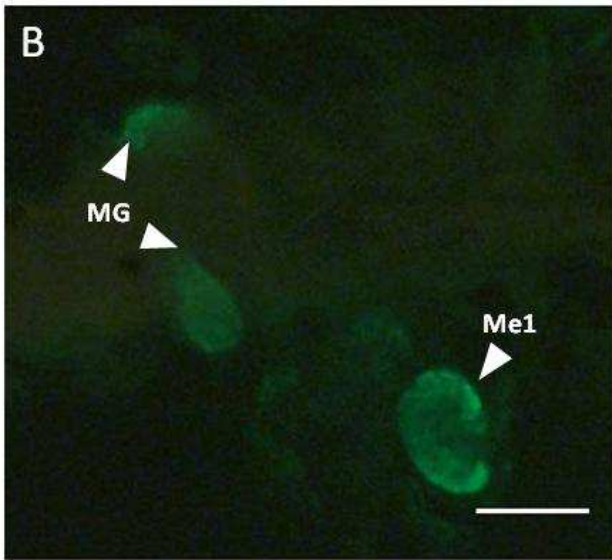
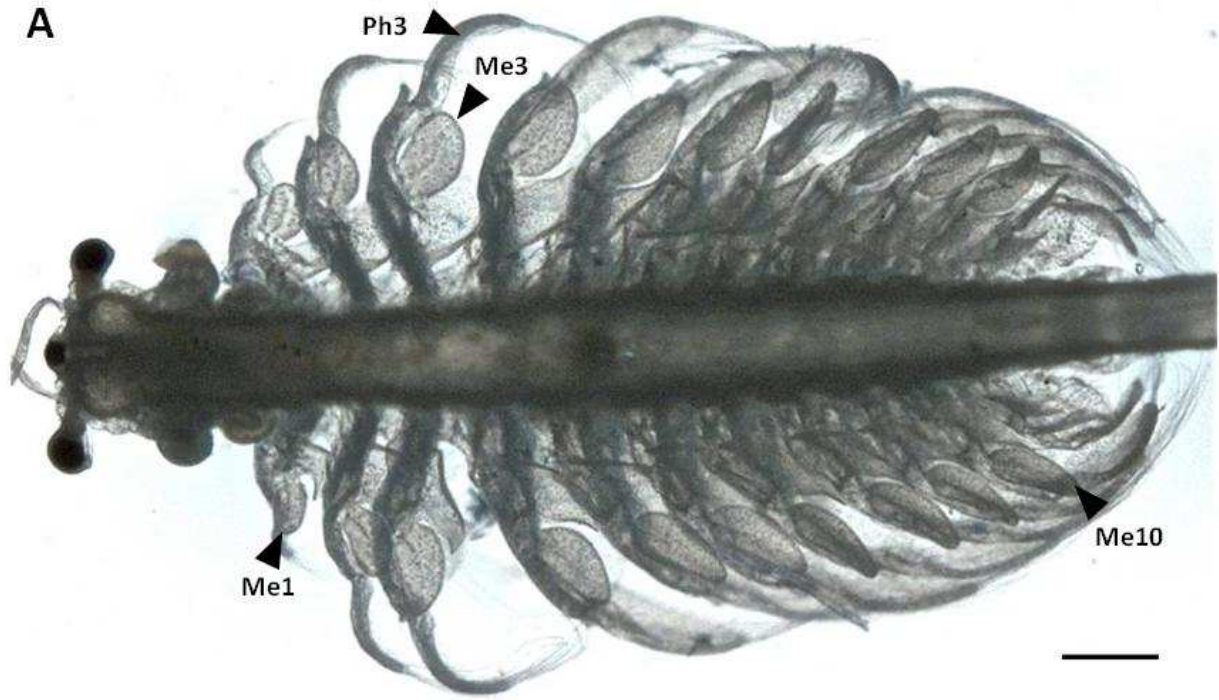


Fig. 2.

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Fig. 3.

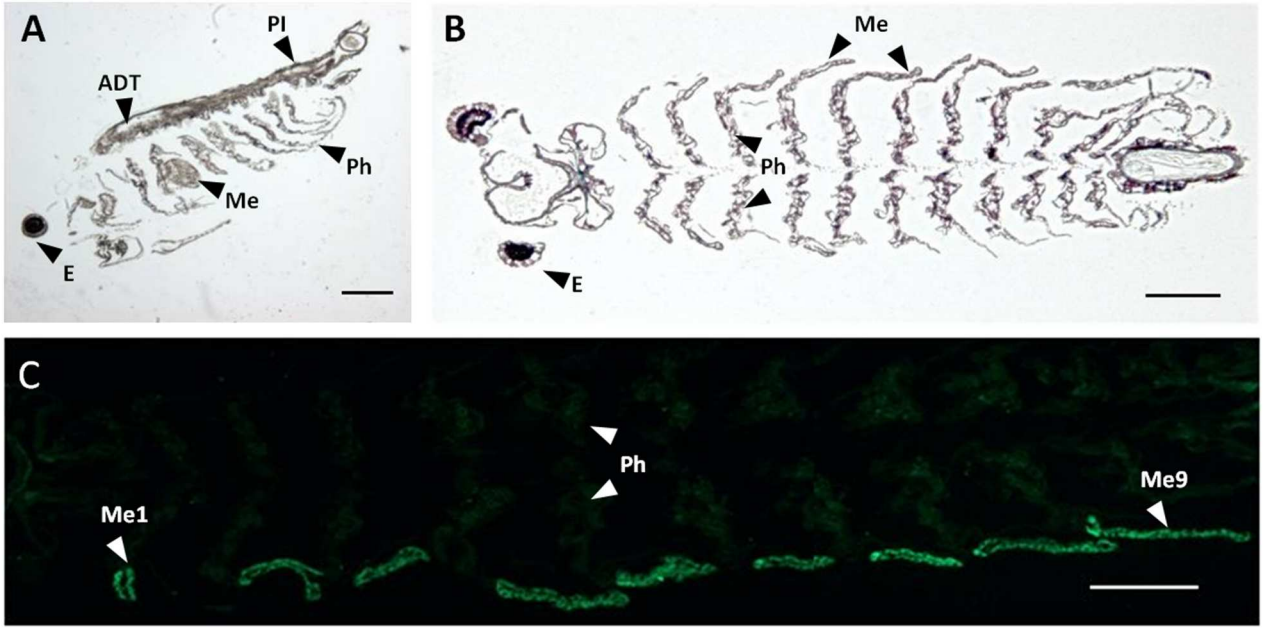


Fig. 4.

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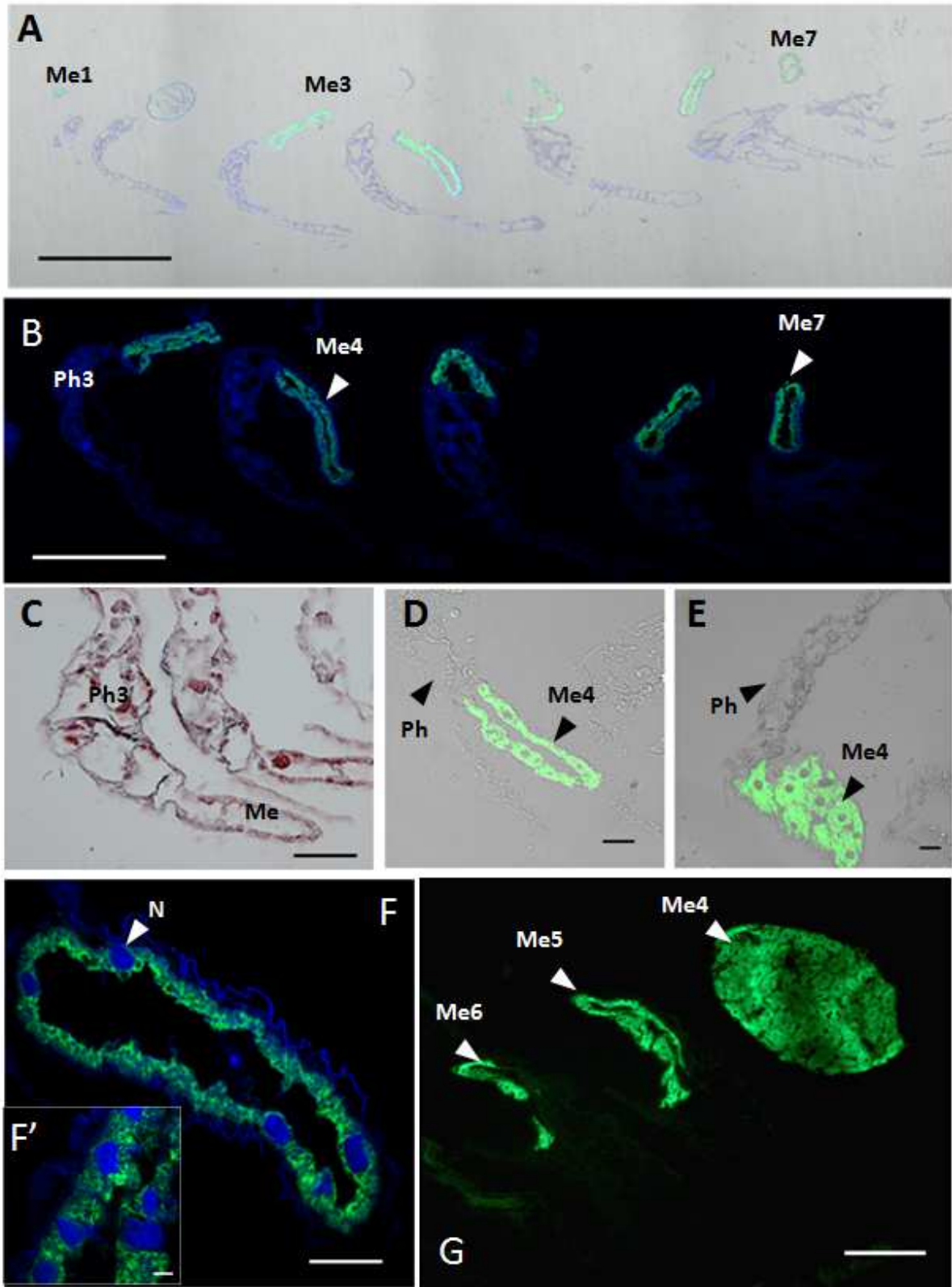


Fig. 5.

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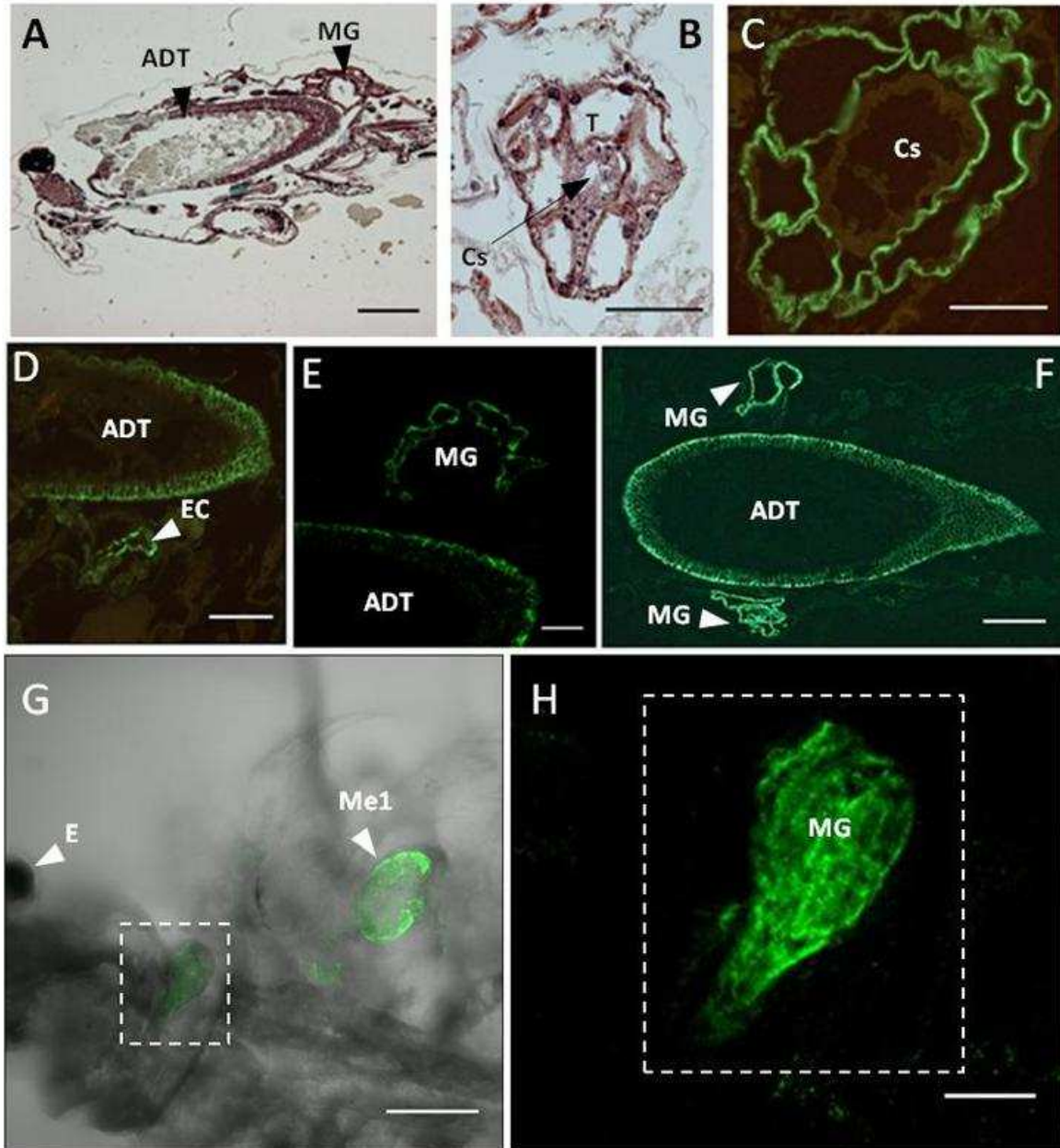


Fig. 6.

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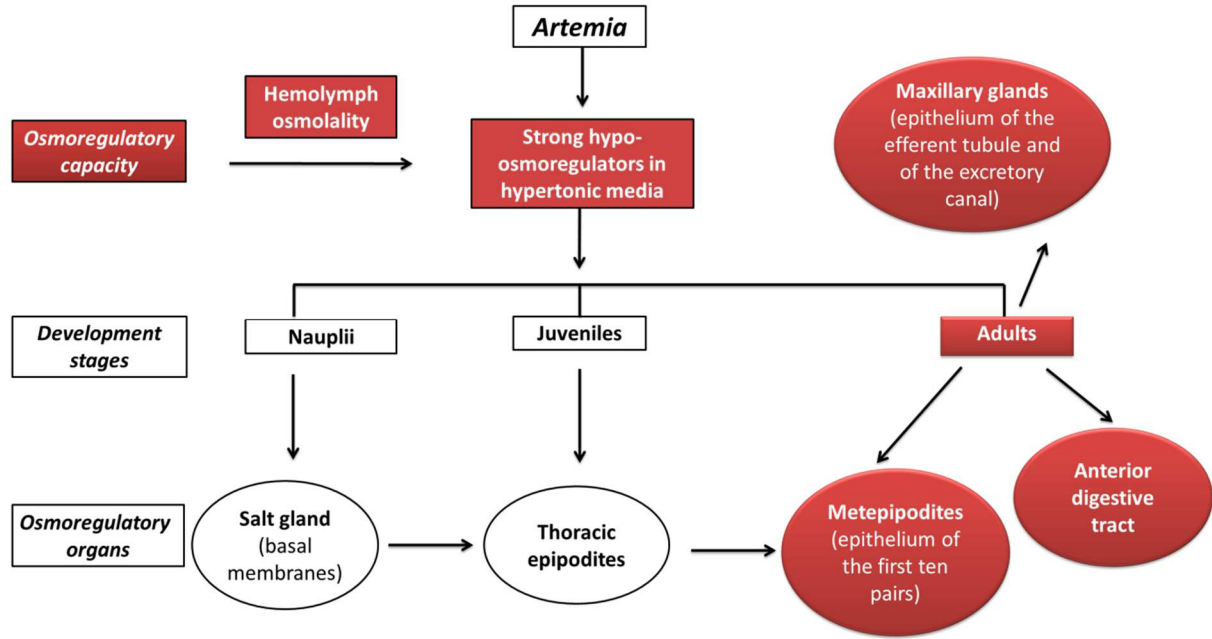
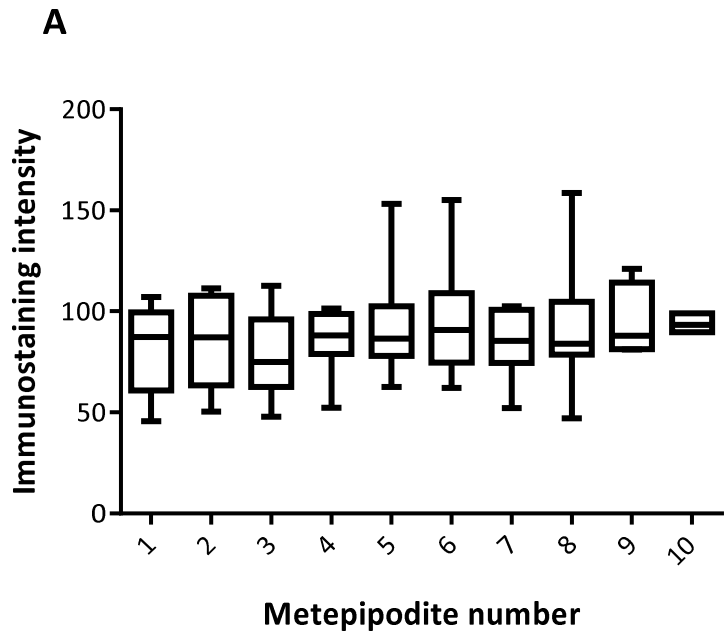


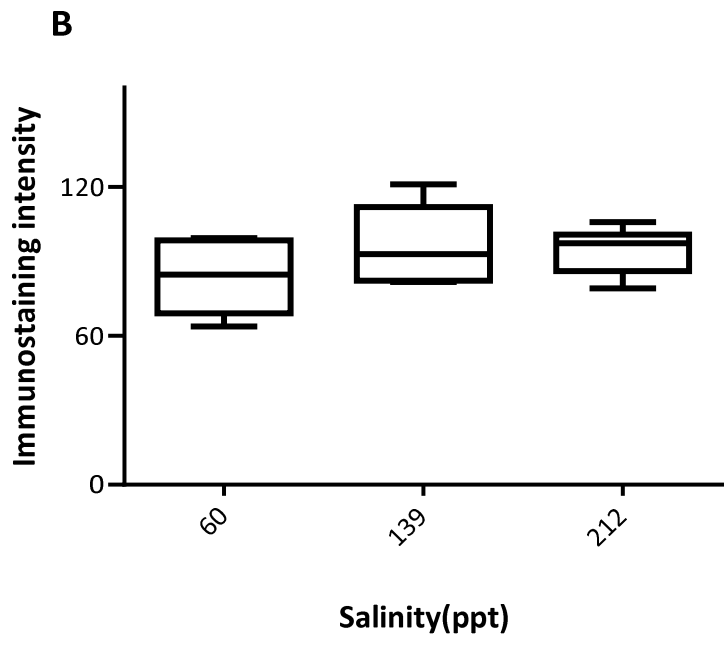
Fig. 1S.

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Fig. 2S.