

1 **Ontogeny of osmoregulation in the Pacific Blue Shrimp, *Litopenaeus stylirostris***
2 **(Decapoda, Penaeidae): deciphering the role of the Na⁺/K⁺-ATPase.**

3
4 Dominique Pham^{1*}, Guy Charmantier², Viviane Boulo^{1,2}, Nelly Wabete¹, Dominique
5 Ansquer¹, Clément Dauga¹, Evelyse Grousset², Yannick Labreuche^{3,4*}, Mireille
6 Charmantier-Daures²

7
8 ¹Lagon, Environnement et Aquaculture Durable, Ifremer, Boulouparis, Nouvelle-
9 Calédonie

10 ²Université de Montpellier, Adaptation Ecophysiologique et Ontogenèse, UMR 9190,
11 Marbec, UM, CNRS, IRD, Ifremer, Place Eugène Bataillon, CC 092, 34095
12 Montpellier, Cedex 05, France

13 ³Ifremer, Unite Physiologie Fonctionnelle des Organismes Marins, ZI de la Pointe du
14 Diable, CS 10070, F-29280 Plouzane, France

15 ⁴Sorbonne Universités, UPMC Paris 06, CNRS, UMR 8227, Integrative Biology of
16 Marine Models, Station Biologique de Roscoff, CS 90074, F-29688 Roscoff cedex,
17 France

18
19 These authors co-supervised the work

20
21
22
23
24
25
26 * Corresponding authors:

27 E-mail addresses: dominique.pham@ifremer.fr, telephone: (687) 35 25 83 ; fax: (687)
28 35 11 77; ylibreuche@sb-roscoff.fr, telephone: (33) 02 98 29 56 47 ; fax: (33) 02 98
29 29 23 24

30

31 **ABSTRACT**

32

33 The role of the main ion transporting enzyme Na⁺/K⁺-ATPase in osmoregulation
34 processes was investigated in *Litopenaeus stylirostris*. The development and
35 localization of the osmoregulation sites were studied during ontogenesis by
36 immunodetection of Na⁺K⁺-ATPase using monoclonal antibodies and transmission
37 electron microscopy (TEM). Osmoregulation sites were identified as the pleurae and
38 branchiostegites in the zoeae and mysis stages. In the subsequent post-metamorphic
39 stages the osmoregulatory function was mainly located in the epipodites and
40 branchiostegites and osmotic regulation was later detected in the gills. The presence of
41 ionocytes and microvilli in these tissues confirmed their role in ionic processes. The
42 complete open reading frame of the mRNA coding for the α -subunit of Na⁺K⁺-ATPase
43 was characterized in *L. stylirostris*. The resulting 3092-bp cDNA (LsNKA) encodes a
44 putative 1011-amino-acid protein with a predicted molecular mass of 112.3 kDa. The
45 inferred amino acid sequence revealed that the putative protein possesses the main
46 structural characteristics of the Na⁺K⁺-ATPase α -subunits.

47 Quantitative RT-PCR analyses indicated that LsNKA transcripts did not significantly
48 vary between the different developmental stages. The number of transcripts was about
49 2.5-fold higher in the epipodites and gills than in any other tissues tested in juveniles. A
50 reverse genetic approach was finally implemented to study the role of LsNKA in vivo.
51 Knock down of LsNKA expression by gene-specific dsRNA injection led to an increase
52 of shrimp mortality following an abrupt salinity change compared to control animals.
53 These data strongly suggest that LsNKA plays an important role in osmoregulation
54 when the shrimp are challenged by changing salinities.

55

56 Key words : penaeid, *Litopenaeus stylirostris*, ontogeny, osmoregulation, Na⁺/K⁺-
57 ATPase, RNA interference.

58

59

60 **1. Introduction**

61

62 In New Caledonia (South Pacific) the shrimp culture industry is based upon the Pacific
63 blue shrimp *Litopenaeus stylirostris*, an exogenous species imported in the early 1970s
64 from Latin Americas and which represents today the second largest export business
65 after the nickel industry. However, this production has been constantly decreasing in the
66 last 4 years, in part because of recurrent difficulties encountered by the hatcheries to
67 obtain nauplii and because of unexplained mortalities occurring during the larval
68 rearing period. A better understanding of the key physiological functions of
69 *L. stylirostris* in adapting to the surrounding environment is therefore needed to sustain
70 this industry.

71 The establishment of a species in a given habitat relies on the ability of each of its
72 developing stages to adapt to salinity (and to its abrupt changes) as it is one of the main
73 environmental parameters that wields a selective pressure on aquatic organisms. As
74 illustrated by the number of published studies, a large amount of information is now
75 available regarding the processes involved in osmoregulation of crustacean adult stages
76 (reviewed in Lucu and Towle, 2003; Charmantier et al., 2009; Henry et al. 2012). By
77 comparison, data regarding the changes in ionoregulatory mechanisms occurring
78 throughout the post-embryonic development remain relatively scarce. In crustaceans,
79 three alternative ontogenetic patterns have been proposed (Charmantier, 1998). The
80 first pattern corresponds to a weak osmoregulatory ability that does not predictably vary
81 with the progress of developmental stages; the second pattern is based on the
82 establishment of efficient osmoregulation in the first post-embryonic stages, comparable
83 to those occurring in adults. Finally, in the third proposed pattern, the early
84 postembryonic stages are osmoconformers or they slightly osmoregulate. Thereafter, a
85 shift occurs at the metamorphic larva-juvenile transition and the animals gain
86 osmoregulation capabilities which they keep into adulthood. Several penaeid shrimp
87 belong to the latter group, including the Kuruma shrimp *Penaeus japonicus*
88 (Charmantier, 1986; Charmantier et al., 1988) and the Pacific blue shrimp *L. stylirostris*
89 (Pham et al., 2012), which, as adults, are hyper-hypo-osmoregulators .

90 In a great variety of adult decapod crustacean species and other aquatic invertebrates,
91 osmoregulatory tissues in branchial cavity are restricted to the gills (Wheatly and
92 Henry, 1987; Flick and Haond, 2000; Lignot et al, 2005). Different studies conducted in
93 brachyuran crabs have pointed out that osmoregulatory structures are mainly located in

94 the posterior gills, whereas anterior gill lamellae generally possess thin respiratory
95 epithelia enabling diffusive gas exchange (Cieluch et al, 2004; Chung and Lin, 2006).
96 However other epithelia in the branchial chamber (branchiostegite, pleura, epipodites)
97 are involved in the osmoregulation processes in many species such as *P. japonicus*
98 (Bouaricha et al., 1994), *Crangon crangon* (Cieluch et al., 2005) or *Macrobrachium*
99 *amazonicum* (Boudour-Bouchecker et al., 2013) depending on the developmental stage.
100 As in adults, the occurrence of osmoregulation in young post-embryonic stages implies
101 the existence of an efficient ionic regulation (mainly of Na⁺ and Cl⁻ ions) based on
102 enzymes involved in ion transport. Among these enzymes, the Na⁺/K⁺-ATPase is
103 considered to play a central role (Péqueux, 1995; Charmantier, 1998; Charmantier et al.,
104 2001; Weihrauch et al., 2001; Castilho et al., 2001; Lucu and Towle, 2003; Charmantier
105 et al., 2009; McNamara and Faria, 2012, Henry et al., 2012). The native holoenzyme is
106 a tetramer composed of two catalytic units possessing the ATP binding site (the α-
107 subunits) of approximately 100 kDa and two β-subunits of 40 kDa thought to play a role
108 in binding the complex in the basolateral membrane. In addition, the Na⁺/K⁺-ATPase
109 has been shown in vertebrates to contain a third unit (γ-subunit) that modulates the
110 enzyme function according to the specific demands of a given tissue (Toyoshima et al.,
111 2011). However, this latter subunit has not yet been characterized in any crustacean
112 species. From a functional point of view, this sodium-pump enables ion transport
113 directly through the transfert of 3 Na⁺ ions from the cytosol to the hemolymph in
114 exchange of 2 K⁺ using energy derived from ATP hydrolysis and indirectly via the
115 establishment of ionic electrochemical gradients. This enzyme is therefore of particular
116 interest to investigate the ontogeny of osmoregulation and to ascertain functional and
117 morphological identification of ion transport.

118 The present study was aimed at investigating the development and localization of the
119 osmoregulation sites during the post-embryonic development of *L. stylirostris* through
120 the immunodetection of Na⁺/K⁺-ATPase activity using monoclonal antibodies. We also
121 characterized the gene encoding the sodium-pump and measured its expression during
122 post-embryonic development as well as in sub-adult animals using quantitative real-
123 time PCR. Finally, this gene was silenced by RNA interference to provide further
124 evidence for its *in vivo* role in the control of osmoregulation.

125

126 **2. Material and methods**

127

128 **2.1 Animals**

129 *L. stylirostris* shrimp from a selected line (SPR43, Weppe et al., 1992) were obtained
130 from the IFREMER facility (IFREMER, BP 2059, 98846 Noumea cedex, New
131 Caledonia). Nauplii bred by unilateral eyestalk ablated females (Pham et al., 2012) were
132 stocked at a density of 180 larvae.L⁻¹ and maintained under constant conditions (35 ppt,
133 29°C and 14:10 h day:night photoperiod). Microparticles and *Artemia* nauplii were
134 supplied *ad libitum*. From day 3, erythromycin (2 ppm) was added every other day to
135 the tank water until day 9. After this period, seawater exchanges were performed at a
136 50% to 100% daily ratio as long as the animals were kept in the hatchery (i.e. 10 days
137 after becoming postlarvae). After reaching PL4 stage (i.e. 10 days old postlarvae at
138 29°C), animals were transferred in 2m³ nursery ponds at a density of 20/liter and reared
139 till they reached PL7 stage or later stages, as confirmed by the rostral formula [5-0]
140 (Pham et al., 2012). After harvesting, animals were stocked at a density of 2-4/m² in
141 earthen grow-out pond and fed *ad libitum* for experimental purposes. Water renewal
142 rates were progressively increased from 5% to 15% a day for a duration of 4 months.

143

144 **2.2 Histology and immunofluorescence light microscopy**

145 Sections were prepared from stages Zoea 2 to PL9, and from 25g adults. For larvae and
146 postlarvae, whole animals were fixed in Bouin's fixative for 24 h, rinsed and kept in
147 70% ethanol until further use. For adults, gills were dissected and fixed. Samples were
148 washed and dehydrated in a graded ethanol series and embedded in Paraplast (Paraplast
149 Plus, Sigma, P3683). Sections of 4 µm were cut on a Leitz Wetzlar microtome,
150 collected on poly-L-lysine-coated slides, and stored at 37°C for 48 h. One series was
151 used for classic light microscopy with Masson's trichome method for tissue topography,
152 while the other series was used for immunohistology. The immunocytochemistry
153 procedure has been previously described (Cieluch et al., 2005). Immunolocalization of
154 Na⁺/K⁺-ATPase was done through immunofluorescence light microscopy using a rabbit
155 polyclonal antibody H300 raised against an internal region of Na⁺/K⁺-ATPase α1 of
156 human origin [Na⁺/K⁺-ATPase α (H-300); Santa Cruz Biotechnology]. This antiserum
157 has previously been shown to display positive and specific immunoreactivity toward the
158 Na⁺/K⁺-ATPase of several crustaceans (Boudour-Bouchecker et al., 2014).

159

160 **2.3 Transmission Electron Microscopy**

161 Transmission electron microscopy was done as previously described by Martinez et al.
162 (2005). Briefly, PL9 postlarvae were fixed for 24 h with 2.5% glutaraldehyde in a
163 mixture of freshwater and seawater adjusted to the hemolymph osmotic pressure.
164 Following rinsing, gills, epipodites and branchiostegites were excised and post-fixed
165 for 2 h at 4°C in osmic acid (OsO₄), washed in distilled water and dehydrated in a
166 graded ethanol series and propylene oxide, then embedded in Spurr's resin. Semithin
167 and ultrathin sections were cut on a Reichert OMU3 ultramicrotome. Semithin sections
168 were stained with toluidine blue, while ultrathin sections were contrasted with 2%
169 uranyl acetate in 70° ethanol and lead citrate. Observations were made on a JEOL 1200
170 EX2 transmission electron microscope at 70kV.

171

172 **2.4 RNA extractions and full-length cDNA cloning of *LsNKA***

173 Fresh tissues, including eyestalk (Ey), hepatopancreas (Hp), gills (Gi), epipodites (E),
174 pleopods (Pl), abdominal muscle (Mu), were collected from five shrimp (mean weight =
175 30 g) as parallel samples for subsequent RNA extractions. Larvae were collected from
176 the following stages: nauplius (Nii), zoea II (Z2), mysis II (M2), postlarvae I (P1).
177 Three pools were used as replicates. Tissues and larvae were kept in RNA *later* reagent
178 (Ambion) following sampling and stored at -20°C until further use. Total RNA was
179 extracted using RNeasy columns (Qiagen) according to the manufacturer's instructions.
180 RNA quantity, purity and integrity were verified spectrophotometrically (A_{260}/A_{280}) and
181 by electrophoresis on 1% agarose gels.

182 To isolate a cDNA of *LsNKA*, specific primers (A-120 and A-122, Table 1) were
183 designed on highly-conserved regions from *Penaeus monodon* and *Homarus*
184 *americanus* Na⁺/K⁺-ATPase α -subunit nucleotide sequences (accession numbers:
185 AY140650.1 and DQ399796.1, respectively) and from *L. vannamei* expressed sequence
186 tag (EST) sequences, available at www.marinegenomics.org (Marine Genomics
187 accession numbers: MGID368619 et MGID455880) (for a review, see Lucu and Towle,
188 2003; O'Leary et al., 2006). A PCR was subsequently performed to amplify a 1180-bp
189 amplicon from gill cDNA. After sequencing by GATC-Biotech (Konstanz, Germany) a
190 BlastX match of this fragment revealed high sequence homologies (>98% identities)
191 with the Na⁺/K⁺-ATPase α nucleotide sequence from *P. monodon* (accession
192 number: EF672699.1) and *Fenneropenaeus indicus* (accession number: HM003691.1).
193 Based on *P. monodon* and *F. indicus* nucleotide sequence alignment a new set of

194 primers (A-119 and A-132, Table 1) was designed in the 5'- and 3'-regions and used to
195 generate the full-length cDNA of *LsNKA*.

196

197 **2.5 Sequence data analysis and phylogenetic tree construction**

198 A list of known sequences of the members of NKA proteins was obtained from
199 GenBank and EMBL databases using the BLAST (Basic Local Alignment Search Tool)
200 program (Altschul et al., 1997). Sequence alignments were performed using
201 CLUSTAL O software (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The identification of
202 putative protein motifs was performed using the MotifScan softwares (Pfam HMMs
203 global models database) available at SIB (Swiss Institute of Bioinformatics;
204 <http://hits.isb-sib.ch>), the Protein motifs Search (<http://www.ebi.ac.uk/Tools/ppsearch/>)
205 and the TMPRED program available at EMBnet (European Molecular Biology
206 Network, http://www.ch.embnet.org/software/TMPRED_form.html). The theoretical
207 isoelectric point (pI) and molecular weight (Mw) were estimated by the ProtParam Tool
208 (<http://web.expasy.org/protparam/>). Trees were built using the MEGA software
209 (Molecular Evolutionary Genetics Analysis, version 5.0) applied to the Neighbor-
210 Joining and maximum likelihood methods (Felsenstein, 1985; Tamura et al., 2007).
211 Bootstrap values (%) of 1000 replicates were calculated for each node of the consensus
212 tree obtained.

213

214 **2.6 Preparation of dsRNA**

215 Double-stranded RNAs (dsRNA) were generated as previously described (Labreuche et
216 al., 2009). Briefly, the DNA template used for *in vitro* transcription was pCR4 vector
217 (Invitrogen) hosting a 298-bp portion of *LsNKA* cDNA. The formation of dsRNA was
218 monitored by determining the size shift in 1% agarose gel electrophoresis. Finally,
219 dsRNAs were diluted to a final concentration of 250 ng/μl in sterile saline solution
220 (10 mM Tris-HCl pH 7.5, 400 mM NaCl) and stored at -80°C.

221 In order to test the effectiveness of dsRNA injections, 240 Shrimp (1.0–1.5 g) were
222 brought to the laboratory for acclimation (salinity 27 ppt). After 5 days, the animals
223 were intramuscularly injected between the third and fourth abdominal segment with
224 either 10 μg (20 μl volume) of dsRNA or the same volume of injection buffer as a
225 control, and returned to two different culture tanks. Forty-eight hours after treatment,
226 gills were excised from 10 animals from each treatment and kept in RNA *later* reagent
227 (Ambion) and stored at -20°C for subsequent RNA extractions. At the same time, an

228 acute salinity exposure experiment was performed to investigate the effects of *LsNKA*
229 dsRNA injection on shrimp survival. To this end, dsRNA-injected and control shrimp
230 were exposed to salinities of 5, 27 and 40 ppt in 30 liter glass tanks. This experiment
231 was conducted in triplicates with 10 shrimp per tank. Mortality was recorded after 48
232 hours.

233

234 **2.7 Quantitative real-time PCR**

235 Quantitative real-time RT-PCR (qPCR) was performed on an ABI 7300 system as
236 previously described (Labreuche et al., 2010). Amplification efficiencies for all qPCR
237 primers were determined according to Pfaffl et al. (2002) and the specificity of the PCR
238 amplification was verified from the melting curve. Each run included the cDNA
239 control, negative controls (total RNA treated with DNase I), and blank controls (water).
240 The relative mRNA expression levels were determined using the two standard curve
241 methodology (QuantiTect® SYBR Green PCR Handbook). The elongation factor 1-
242 alpha gene (EF1, accession no. AY117542.1) was used as the internal reference
243 (normaliser) mRNA (de Lorgeril et al., 2008). Real-time PCR primer sequences are
244 indicated in Table 1.

245

246 **2.8 Statistical analysis**

247 All numerical data were expressed as the mean \pm standard error. One-way analysis of
248 variance (ANOVA) or the Kruskal-Wallis test was used for mRNA expression analysis.
249 These statistical analyses were performed with Statgraphics Plus 5.0 software.

250 **3. Results**

251

252 **3.1 Histology and Na⁺/K⁺-ATPase immunolocalization**

253 NKA distribution was visualized using immunohistochemistry (Fig. 1). The resulting data are
254 summarized in Table 2. A positive immunoreactivity for Na⁺/K⁺-ATPase could be observed
255 in zoea 2, restricted to the epithelium of the pleurae in the branchial cavity (Table 2 & Fig.
256 1A). At this larval stage, optical microscopy allowed the identification of well-developed
257 branchiostegites, which were observed without any immunolabelling, whereas epipodites and
258 gills appeared undifferentiated. In mysis 2, a positive immunolabeling was detected along the
259 inner epithelium of the branchiostegites. At this stage, simple evaginations of the body wall
260 formed early gill buds, free of fluorescence staining (Fig. 1B). At PL1, epipodites were
261 clearly observed, showing a strong immunofluorescence compared to the other positive
262 organs of the branchial cavity (i.e. the branchiostegites and pleurae) (Fig. 1C). At PL4, gill
263 filaments were easily observable but remained free of immunofluorescence (Fig. 1D); the
264 pleura epithelium was also immunonegative and remained without Na⁺/K⁺-ATPase detection
265 at the later stages. At PL9, positive immunoreactivity was intense in the epipodites and
266 branchiostegites (Fig. 1E,F); in contrast, gills were still free of immunofluorescence staining
267 whereas positive immunoreactivity in gills was observed in adults (Figure 2). Sections
268 without the primary antibody used as negative controls showed no specific staining (data not
269 shown).

270 As shown by optical microscopy, PL9 larvae possess all the potential osmoregulatory organs
271 in the branchial chamber (i.e gills, epipodites and branchiostegites) (Fig. 3A) and display an
272 adult hyper-hypo-osmoregulatory pattern (Pham et al, 2012). Given these facts, electron
273 microscopy was carried out at this stage only. The structure of gill lamellae appeared well
274 developed (Fig. 3B, C), constituted by a very thin epithelium, with scarce nuclei under the
275 cuticle, and a central connective tissue. Large hemolymph lacunae were observed between the
276 two tissues. Cells with the features of ionocytes were present in the epipodites and
277 branchiostegites. The epipodites (Fig. 3D,E) were composed of two identical facing epithelia
278 separated by a hemolymph lacuna maintained opened by pillar cells joining the two epithelia.
279 Both epithelia were formed by cells with numerous mitochondria associated with infoldings
280 of the basal membrane of the cell. Deep and abundant microvilli were located at the apical
281 part of the cell under the thin cuticle. Typical ionocytes were also present in the internal

282 epithelia of the branchiostegites (Fig. 3F,G) with pillar cells presenting apical microvilli as
283 well as deep and abundant infoldings associated with mitochondria.

284 **3.2 Full-length cDNA cloning of *LsNKA***

285 The entire cloned sequence of the *LsNKA* cDNA was characterized in this study and found to
286 contain 3092 bp, with a 3036-bp open reading frame (ORF) and a 5'untranslated region of
287 56 bp (deposited under the Genbank accession number: JN561324) (Fig. 4). A BlastN
288 analysis showed high-scoring matches (> 97%) to corresponding Na⁺/K⁺-ATPase α -subunit
289 cDNA sequences from other penaeids including *Penaeus monodon* (Genbank accession
290 number : EF672699.1), *Fenneropenaeus indicus* (HM012803.1) and *L. vannamei*
291 (AY140650.1). The deduced amino acid sequence encodes a putative 1011 amino-acid
292 polypeptide with a predicted molecular mass of 112.3 kDa. A putative Kozak consensus
293 sequence (5'-CAGCCATGG-3') was found around the likely start codon (indicated in bold)
294 (Kozak, 1991). Hydrophobicity analyses subsequently performed using the TMPred program
295 (http://www.ch.embnet.org/software/TMPRED_form.html) allowed the identification of 10
296 transmembrane domains, in agreement with the topology of Na⁺/K⁺-ATPase α -subunits from
297 other invertebrates such as the euryhaline shore crab *Pachygrapsus marmoratus* (Jayasundara
298 et al., 2007). In addition, a scan of the putative sequence against the PROSITE database
299 allowed the identification of an ATP binding site (V⁵⁰³MKGAPERIL⁵¹²) (Horisberger et al.,
300 1991) as well as a P-type ATPase phosphorylation site (D³⁶⁴KTGTLT³⁷⁰) (Fagan & Saier,
301 1994). Phylogenetic tree analyses revealed that the *L. stylirostris* Na⁺/K⁺-ATPase α -subunit
302 clustered with those from other invertebrate species (especially those characterized in other
303 marine invertebrates) (Fig. 5).

304

305 **3.3 Tissue expression of Na⁺/K⁺-ATPase α -subunit gene**

306 Quantitative real-time RT-PCR (qPCR) was subsequently performed to determine the relative
307 abundance of Na⁺/K⁺-ATPase α -subunit mRNA in different tissues of *L. stylirostris*. As
308 shown in Fig. 6, the Na⁺/K⁺-ATPase α -subunit transcripts were expressed in all tested tissues.
309 However, the Na⁺/K⁺-ATPase α -subunit mRNA abundance was about 2.5-fold higher in the
310 epipodites and gills than in any other tested tissues. No statistical difference in mRNA
311 transcript levels was observed within the different tissues that constitute the gills (i.e.
312 arthrobranchs and pleurobranchs) ($P > 0.05$).

313

314 **3.4 Expression of Na⁺/K⁺-ATPase α -subunit gene at various stages of development**

315 Whole shrimp at different stages of development (nauplius, zoea 2, mysis 2 and postlarvae 2)
316 were analyzed for the expression of the Na⁺/K⁺-ATPase α -subunit-encoding gene. The total
317 RNA sample from shrimp at each stage was tested by qRT-PCR and the relative expression
318 was calculated relatively to the mRNA level of the elongation factor 1- α gene. The expression
319 of this control gene has been demonstrated to remain statistically stable during larval
320 development (data not shown). Expression of mRNA was detected in all measured samples
321 (Fig. 7), with the highest levels at the nauplius and PL2 stages. However, no significant
322 difference was observed between the different developmental stages during this study
323 ($P > 0.05$).

324

325 **3.5 Functional study of the shrimp Na⁺/K⁺-ATPase α -subunit gene**

326 An experiment was conducted to explore the functional role of *LsNKA* in osmoregulation. In
327 the course of this experiment, we first checked that *LsNKA* message was successfully down-
328 regulated using sequence-specific dsRNA. As determined by qPCR (Fig. 8), intramuscular
329 injection of *LsNKA* sequence-specific dsRNA resulted in a statistically significant depletion
330 of cognate mRNA levels. Depletion was restricted to injection with dsRNA with no
331 suppression of *LsNKA* expression occurring in control animals. As summarized in Fig. 9, the
332 transfer of saline-injected control shrimp from 27 ppt to different salinity levels (5, 27 or
333 40 ppt) did not induce any significant increase in mortality ($P > 0.05$). Knock down of *LsNKA*
334 expression by dsRNA injection statistically affected the survival of animals transferred under
335 isosmotic conditions (i.e. 27 ppt) with a mortality of 18% after 48 hours (ANOVA, $P < 0.05$).
336 A transfer of shrimp injected with dsRNA from 27ppt to 5ppt and 40ppt increased the shrimp
337 susceptibility and led to a mortality of 57% and 38%, respectively which were significantly
338 higher than mortalities in control groups with 0% and 3%, respectively (ANOVA, $P < 0.05$).

339

340 4. Discussion

341

342 In species with a biphasic life cycle, such as *L. stylirostris*, *L. vannamei*, *P. japonicus* and *C.*
343 *crangon*, osmoregulatory capabilities are linked to the occurrence of specialized
344 osmoregulatory structures such as the pleura, branchiostegite, gills and epipodites (Bouaricha
345 et al., 1994; Charmantier et al., 1988; Cieluch et al., 2004; review in Charmantier et al., 2009;
346 Pham et al., 2012; Chong-Robles et al., 2014).

347 In zoeae, we identified the pleura as the unique site of Na⁺/K⁺-ATPase localization while the
348 involvement of the branchiostegites in ion transport occurred from the mysis stages only, as
349 indicated by the detection of Na⁺/K⁺-ATPase by immunostaining. We also reported here the
350 abundance of ionocytes on epipodites, a category of cells known to be specialized for active
351 ion transport (Taylor and Taylor, 1992), as well as a reduced involvement of the gills in the
352 osmoregulation processes in *L. stylirostris* postlarvae, as indicated by the lack of positive
353 staining in the gill filaments. These observations are supported by our data of TEM, no
354 differentiated epithelia being detected in the gills of PL9 post-larvae, while ionocytes were
355 found in branchiostegites and epipodites. In contrast, the positive immunostaining observed in
356 gills of juveniles suggests a late implication of this tissue in osmoregulatory functions. In
357 summary, our results evidenced a shift in the location of ion-transporting epithelia during
358 post-embryonic development of *L. stylirostris*: osmoregulation occurs in the pleura and
359 branchiostegites during the first larval stages (zoeae and mysis), while the osmoregulatory
360 function shifts in the subsequent stages from the pleura to the epipodites and is accompanied
361 by the acquisition of a hyper-hypo-osmoregulation pattern. Similar types of tissue
362 differentiation and shifts of function have also been described in the few other peneids in
363 which the development and localization of the osmoregulation sites have been investigated
364 such as *Penaeus aztecus* (Talbot et al., 1972) or *P. japonicus* (Bouaricha et al., 1994), as well
365 as in other decapod crustaceans, with some degree of variations in the chronology and
366 location of the osmoregulatory sites. For instance, in the European lobster *Homarus*
367 *gammarus*, the branchiostegites and epipodites have also been described as the main sites of
368 osmoregulation, the gills being free of immunostaining (Flik and Haond, 2000; Lignot and
369 Charmantier, 2001). In this species, the epipodites are already present in embryos and play an
370 effective role in osmoregulation while the branchiostegites are involved after metamorphosis
371 only. In the carid shrimp *C. crangon* and *M. amazonicum*, the branchiostegites participate to
372 ionic exchange as early as in zoeae stages (Cieluch et al., 2005; Boudour-Bouchecker et al.,
373 2013).

374 Our results show that the capacity to osmoregulate in *L. stylirostris*, progressively acquired
375 during the larval development, and associated with an increasing tolerance to salinity
376 variations (Pham et al., 2012), is linked to the progressive differentiation of different organs
377 in the branchial cavity where Na^+/K^+ -ATPase is detected. In postlarval stages, we found that
378 the gills are probably not involved in osmoregulation, in contrast with other species such as
379 the crayfish *Astacus leptodactylus* (Lignot et al., 2005) or brachyuran crabs like *Carcinus*
380 *maenas* (Cieluch et al., 2004), in which the posterior gills are involved in osmoregulation
381 while anterior gills are mainly devoted to respiratory functions.

382 To assess these findings, we attempted to characterize the gene encoding the sodium pump to
383 provide more sensitive information regarding the pattern of ontogenetic regulation. We cloned
384 and characterized a Na^+/K^+ -ATPase α -subunit homologue, *LsNKA*. The inferred amino acid
385 sequence of *LsNKA* cDNA revealed that the putative protein possesses the main structural
386 characteristics of the Na^+/K^+ -ATPase α -subunits, i.e. 10 membrane-spanning helices as well
387 as an ATP binding site and a P-type ATPase phosphorylation site, confirming the high
388 conservation of Na^+/K^+ -ATPase α -subunits between species. A putative signal peptide
389 cleavage site ($\text{G}^{95}\text{AILCF}^{100}$) was also identified within the first predicted transmembrane
390 region, as described in other marine invertebrates (Lucu and Towle, 2003). When subtracting
391 the amino acid residues located upstream of this putative cleavage site, the theoretical
392 molecular mass was determined at 101 kDa, a value consistent with the apparent molecular
393 weight estimated by Western blotting (Figure S1, supplementary data).

394 In vertebrates, the Na^+/K^+ -ATPase α -subunit has several isoforms differing in sensitivity to
395 proteases, cross-linking agents or electrophoretic mobility for instance. Thus far, four α -
396 subunit isoforms have been identified in mammals (Mobasher et al., 2000), while five and
397 nine Na^+/K^+ -ATPase α -isoforms have been characterized in the rainbow trout *Oncorhynchus*
398 *mykiss* and in the zebrafish *Danio rerio*, respectively (Rajarao et al., 2001; Richards et al.,
399 2003). In the brine shrimp *Artemia salina*, two isoforms have been found and reported to be
400 differentially expressed during early development (Peterson et al., 1978; Cortas et al., 1989).
401 The sea urchin *Hemicentrotus pulcherrimus* possesses also two α -subunit isoforms encoded
402 by a single gene and produced by differential exon splicing (Yamazaki et al., 1997). In the
403 present study, our experiments did not permit to identify additional isoforms or alternative
404 splicing products among the different osmoregulatory organs of *L. stylirostris*. Whether or not
405 α -subunit cDNA variants can be found in this species remains therefore to be elucidated.

406 We also investigated the expression pattern of *LsNKA* in *L. stylirostris* tissues. *LsNKA* mRNA
407 expressions were detected in all the studied tissues, as also reported in the shrimp

408 *Macrobrachium nipponense* (Wang et al., 2003) but also in all the metazoans studied so far.
409 The wide distribution of this enzyme may be linked to its known implication in other
410 physiological processes such as cellular volume regulation or calcium absorption during
411 ecdysis (Lucu and Towle, 2003).

412 As immunofluorescence had shown that branchial cavity organs are progressively involved in
413 osmoregulation during ontogenesis, we next investigated the mRNA expression pattern of
414 LsNKA. Our results demonstrate that this gene is constitutively expressed in all the
415 studied stages, starting in nauplius. Similar data were reported in the crayfish
416 *Astacus leptodactylus* demonstrating that the Na⁺/K⁺-ATPase α -subunit gene was
417 constitutively expressed in all the studied ontogenetic stages starting at the metanauplius stage
418 (Serrano et al., 2007). However in our study, its expression level did not significantly vary
419 afterwards, whereas our immunological results showed that the branchial cavity organs are
420 progressively involved in osmoregulation process. Several hypotheses may explain this result.
421 First, due to the size of the animals, mRNA expression was measured on whole larvae. And
422 NKA expression relative to antennal glands and gut, which are others osmoregulatory organs
423 in crustaceans (Mantel and Farmer, 1983) could have masked the differences detected in
424 branchial cavity tissues with immunocytochemistry. Considering also the ubiquitous
425 localization of this enzyme (including tissues not related to osmoregulation), we hypothesize
426 that this procedure may have partially reduced variations of LsNKA gene expression between
427 the different larval stages. Another explanation is that the sodium pump is considered to be a
428 “housekeeping” protein involved in different signaling pathways via interaction with other
429 proteins, e.g. in regulation of membrane trafficking and in the operation of cell junctions.
430 Therefore we also hypothesize that the osmoregulatory capacity of *L. stylirostris* at different
431 larval stages may not result from de novo synthesis of new enzyme (characterized by an
432 increase in the abundance of the corresponding mRNA) but from activation of pre-existing
433 enzyme. To date, information on the embryonic ontogeny of osmoregulation remains scarce
434 in decapod crustaceans. Further studies are thus needed to determine whether or not transcript
435 expression fits the Na⁺/K⁺-ATPase activity.

436 Finally to address the *in vivo* role of LsNKA in osmoregulation we developed a RNAi-based
437 approach. Since its first description in nematodes (Fire et al., 1998), this reverse genetics
438 approach has opened avenues for the study of candidate genes in most metazoan species,
439 including crustaceans. For example, several studies have contributed to solve gene functions
440 involved in immune response, molting, reproduction or glucose metabolism in shrimp
441 (reviewed in Robalino et al., 2011). We have previously shown that *in vivo* gene silencing

442 lasted for more than 4 days but less than eight, with maximum level of knockdown observed
443 after 48 hours (de la Vega et al., 2008; Labreuche et al., 2009). First, we have shown that
444 injection of *LsNKA* sequence-specific dsRNA into shrimp induced a significant depletion of
445 cognate mRNA levels. Then, we submitted the animals to an abrupt salinity change (from 27
446 ppt to 5 or 40 ppt). While the transfer of control (saline-injected) animals did not induce any
447 significant mortality, we showed that *LsNKA* silencing significantly increased the shrimp
448 susceptibility to salinity changes: after transfer to 40 or 5 ppt respectively, mortality was 2 to
449 3 times higher in the *LsNKA*-knockdown animals than in *LsNKA* dsRNA-injected group kept
450 at 27 ppt. These results demonstrated the biological implication of this gene in ionic
451 regulation process; they also indirectly confirmed the role of Na^+/K^+ -ATPase in different
452 signaling pathways since we observed mortalities in *LsNKA*-knockdown animals kept under
453 isosmotic conditions.

454

455 This study brings further information towards understanding the mechanisms behind the
456 osmoregulation processes that occur during the post-embryonic development of *L.*
457 *stylirostris*. It is well known now that osmoregulation in crustaceans relies on the coordinated
458 action of a suite of transport proteins and transport-related enzymes, including, among others,
459 the V-Type ATPase, carbonic anhydrase (reviewed in Henry et al, 2012). We are currently
460 developing efforts to identify and characterize other enzymes for future studies investigating
461 the molecular bases of the physiological and evolutionary responses of *L. stylirostris* shrimp
462 to osmoregulatory challenges in their estuarine environment. Such knowledge will greatly
463 participate to improve growth under aquaculture conditions and set up better acclimatizing
464 procedures in animals.

465

466 **Acknowledgments**

467 The authors are indebted to Dr Elizabeth Ficko-Blean for the critical review of this
468 manuscript and her help in editing the English language. This work was supported by the
469 DEDUCTION project funded by the Institut Français de Recherche pour l'Exploitation de la
470 Mer (IFREMER), the Provincial Institutions (Province Sud, Province Nord and Province des
471 Iles Loyauté) and by the Government of New Caledonia. Thanks are due to the zootechnical
472 staff of the experimental hatchery of Saint-Vincent (F. Broutoi, J.R. Mailliez, J.M. Peignon)
473 for their help in supplying *L. stylirostris* larvae and postlarvae. The authors also thank the
474 “Plate-forme de Recherche pour les Sciences du Vivant de Nouvelle-Calédonie (PFV-NC)”

475 for allowing access to the qPCR facility and the Electron Microscopy department in
476 Université de Montpellier 2. Any opinions, findings, and conclusions or recommendations
477 expressed in this material are those of the authors and do not necessarily reflect the views of
478 the supporting bodies mentioned herein.

479

480 **References**

481

482 Altschul, S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J.,
483 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search
484 programs. *Nucleic Acids Res.* 25 (17): 3389-402.

485

486 Bouaricha, N., Charmantier-Daures, M., Thuet, P., Trilles, J.-P. and Charmantier, G., 1994.
487 Ontogeny of Osmoregulatory Structures in the Shrimp *Penaeus japonicus* (Crustacea,
488 Decapoda). *The Biological Bulletin*, 186, n°1: 29-40.

489

490 Boudour-Bouchecker, N., Boulo, V., Lorin-Nebel, C., Elguero, C., Grousset, E., Anger, K.,
491 Charmantier-Daures, M., Charmantier, G., 2013. Adaptation to freshwater in the palaemonid
492 shrimp *Macrobrachium amazonicum*: comparative ontogeny of osmoregulatory organs. *Cell*
493 *Tissue Res.* 353: 87–98.

494

495 Boudour-Bouchecker, N., Boulo, V., Charmantier-Daures, M., Evelyse Grousset, E., Anger K.,
496 Charmantier, G., Lorin-Nebel, C., 2014. Differential distribution of V-type H⁺-ATPase and
497 Na⁺/K⁺-ATPase in the branchial chamber of the palaemonid shrimp *Macrobrachium*
498 *amazonicum*. *Cell Tissue Res.* 357, 195-206.

499

500 Castilho, P.C., Martins, I.A., and Bianchini, A., 2001. Gills Na⁺,K⁺-ATPase and
501 osmoregulation in estuarine crab *Chasmagnatus granulata* Dana, 1851 (Decapoda,
502 Grapsidae). *J. Exp. Biol. Ecol.* 256, 215-227.

503

504 Charmantier, G., 1986. Variation des capacités osmoregulatrices au cours du développement
505 post-embryonnaire de *Peaneus japonicus* Bate, 1888 (Crustacea, Decapoda). *C.R. Acad. Sci.*
506 *Paris.* 303, 217-222.

507

508 Charmantier, G., 1998. Ontogeny of osmoregulation in crustaceans: a review. *Invert. Reprod.*
509 *Develop.* 33, 177-190.

510

511 Charmantier, G., Charmantier-Daures, M., Bouaricha, N., Thuet, P., Aiken D. E, Trilles, J.-P.,
512 1988. Ontogeny of osmoregulation and salinity tolerance in two decapod crustaceans:
513 *Homarus americanus* and *Penaeus japonicus*. *Biol. Bull.* 175, No. 1, 102-110.

514

515 Charmantier, G., Haond, C., Lignot, J. H., Charmantier-Daures, M., 2001. Ecophysiological
516 adaptation to salinity throughout a life cycle: a review in homarid lobsters. *J. Exp. Biol.* 204,
517 967-977.

518

519 Charmantier, G., Charmantier-Daures, M., Towle, D., 2009. Osmotic and ionic regulation in
520 aquatic arthropods. In "Osmotic and ionic regulation. Cells and animals", D. H. Evans, Ed.,
521 CRC Press, New York, NY, Oxford, UK, 165-230.

522

523 Chong-Robles, J., Charmantier, G., Boulo, V., Lizarraga-Valdez, J., Enriquez-Paredes, L.M.,
524 Giffard-Mena, I., 2014. Osmoregulation pattern and salinity tolerance of the white shrimp
525 *Litopenaeus vannamei* (Boone, 1931) during post-embryonic development. *Aquaculture* 422–
526 423, 261–267.

527

528 Chung, K.-F., Lin, H.-C., 2006. Osmoregulation and Na,K-ATPase expression in
529 osmoregulatory organs of *Scylla paramamosain*. *Comp. Biochem. Physiol. Part A* 144, 48-
530 57.

531

532 Cieluch, U., Charmantier, G., Grousset, E., Charmantier-Daures, M., 2005. Osmoregulation,
533 immunolocalization of Na⁺/K⁺-ATPase, and ultrastructure of branchial epithelia in the
534 developing Brown Shrimp, *Crangon crangon* (Decapoda, Caridea). *Physiol. Biochem. Zoo.*
535 78(6), 1017-1025.

536

537 Cieluch, U., Anger, K., Aujoulat, F., Buchholz, F., Charmantier-Daures, M., Charmantier G.,
538 2004. Ontogeny of osmoregulatory structures and functions in the green crab *Carcinus*
539 *maenas* (Crustacea, Decapoda). *J. Exp. Biol.* 207, 325-336.

540

541 Cortas, N., Arnaout, M., Salon, J., Edelman, I.S., 1989. Isoforms of Na,K-ATPase in *Artemia*
542 *salina*: II. Tissue Distribution and Kinetic Characterization. *J. Membrane Biol.* 108: 187-195.

543

544 de la Vega, E., O'Leary, N.A., Shockey, J.E., Robalino, J., Payne, C., Browdy, C.L., Warr,
545 W.G., Gross, P.S., 2008. Antilipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a
546 broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and
547 fungal infection. *Mol. Immunol.* 45:1916–25.

548

549 De Lorgeril, J., Gueguen, Y., Goarant, C., Goyard, E., Mugnier, C., Fievet, J., Piquemal, D.,
550 Bachère, E., 2008. A relationship between antimicrobial peptide gene expression and capacity
551 of selected shrimp line to survive a *Vibrio* infection. *Mol. Immunol.* 45, 3438-3445.

552

553 Fagan, M.J., Saier, M.H. Jr., 1994. P-type ATPases of eukaryotes and bacteria: sequence
554 analyses and construction of phylogenetic trees. *J. Mol. Evol.* 38:57-99.

555

556 Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap.
557 *Evolution*, 39: 783-791.

558

559 Fire, A., Xu, S.Q., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent
560 and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*
561 Vol. 391, 806-811.

562

563 Flik, G., Haond, C., 2000. Na⁺ and Ca²⁺ pumps in the gills, epipodites and branchiostegites of
564 the European lobster *Homarus gammarus* : effects of dilute seawater. *J. Exp. Biol.* 203, 213-
565 220.

566

567 Henry, R.P., Lucu, C., Onken, H., Weihrauch, D., 2012. Multiple functions of the crustacean
568 gill : osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of
569 toxic metals. *Frontiers in Physiology.* 3 : 431.

570

571 Horisberger, J.D., Lemas, V., Kraehenbühl, J.P., Rossier, B.C., 1991. Structure-function
572 relationship of Na,K-ATPase. *Annual Review of Physiology*, 53: 565-84.

573

574 Jayasundara, N., Towle, D.W., Weihrauch, D., Spanings-Pierrot, C., 2007. Gill-specific
575 transcriptional regulation of Na⁺/K⁺-ATPase alpha-subunit in the euryhaline shore crab
576 *Pachygrapsus marmoratus* : sequence variants and promoter structure. *J. Exp. Biol.* 210 (Pt
577 12) : 2070-81.

578

579 Kozak, M., 1991. Structural features in eukaryotic mRNAs that modulate the initiation of
580 translation. *J. Biol. Chem.* 266: 19867-19870.

581

582 Labreuche, Y., O’Leary, N.A., de la Vega, E., Veloso, A., Gross, P.S., Chapman, R.W., 2009.
583 Lack of evidence for *Litopenaeus vannamei* Toll receptor (IToll) involvement in activation of
584 sequence-independent antiviral immunity in shrimp. *Dev. Comp. Immunol.* 33 : 806–10.
585

586 Labreuche, Y., Veloso, A., De La Vega, E., Gross, P.S., Chapman, R.W., Browdy, S.L., Warr,
587 G.W., 2010. Non-specific activation of antiviral immunity and induction of RNA interference
588 may engage the same pathway in the Pacific white leg shrimp *Litopenaeus vannamei*. *Dev.*
589 *Comp. Immunol.* 34: 1209–1218.
590

591 Lignot, J.-H., Charmantier, G., 2001. Immunolocalization of Na⁺,K⁺-ATPase in the branchial
592 cavity during the early development of the European lobster *Homarus gammarus* (Crustacea,
593 Decapoda). *J. Histochem. Cytochem.* 49, 1013-1023.
594

595 Lucu, C., Towle, D.W., 2003. Na⁺,K⁺-ATPase in the gills of aquatic crustacea. *J. Comp.*
596 *Biochem. Physiol.* 135 A, 195-214.
597

598 Lignot, J.-H., Nugroho Susanto, G., Charmantier-Daures, M., Charmantier, G., 2005.
599 Immunolocalization of Na⁺/K⁺-ATPase in the branchial cavity during the early development
600 of the crayfish *Astacus leptodactylus* (Crustacea, Decapoda). *Cell. Tissue Res.* 319, 331–339.
601

602 McNamara, J.C., Faria, S.C., 2012. Evolution of osmoregulatory patterns and gill ion
603 transport mechanisms in the decapod crustacea: a Review. *J. Comp. Physiol. B* 182, 997-
604 1014.
605

606 Mantel, L.H., Farmer, L.L., 1983. Osmotic and ionic regulation. In: Mantel, L. H. (Ed.), *The*
607 *Biology of Crustacea*, vol. 5. Academic Press, New York, pp. 53–161.
608

609 Martinez, A-S., Charmantier, G., Compère, P., Charmantier-Daures, M., 2005. Branchial
610 chamber tissues in two caridean shrimps: the epibenthic *Palaemon adspersus* and the deep-
611 sea hydrothermal *Rimicaris exoculata*. *Tissue Cell* 37: 153–165.
612

613 Mobasher, A., Avila, J., Cozar-Castellano, I., Brownleader, M. D., Trevan, M., Francis, M.
614 J., Lamb, J. F., Martin-Vasallo, P., 2000. Na⁺, K⁺-ATPase isozyme diversity; comparative

615 biochemistry and physiological implications of novel functional interactions. *Bioscience Rep.*
616 20, 51-91.

617

618 O’Leary, N. A., Trent, H. F., III, Robalino, J., Peck, M.E.T., McKillen, D.J., Gross, P.S.,
619 2006. Analysis of multiple tissue-specific cDNA libraries from the Pacific whiteleg shrimp,
620 *Litopenaeus vannamei*. *Integr. Comp. Biol.* 46, 931-939.

621

622 Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST(C))
623 for group-wise comparison and statistical analysis of relative expression results in real-time
624 PCR. *Nucleic Acid Res.* 30 (9), e36.

625

626 Péqueux, A., 1995. Osmotic regulation in crustaceans. *J. Crust. Biol.* 15 : 1–60.

627

628 Peterson, G.L., Edwing, R.D., Conte, F.P., 1978. Membrane differentiation and *de nova*
629 synthesis of the (Na⁺K⁺)-activated adenosine triphosphate during development of *Artemia*
630 *salina nauplii*. *Dev. Biol.* 67: 90-98.

631

632 Pham, D., Charmantier, G., Wabete, N., Boulo, V., Broutoi, F., Mailliez, J.-R., Peignon, J.-
633 M., Charmantier-Daures, M., 2012. Salinity tolerance, ontogeny of osmoregulation and
634 zootechnical improvement in the larval rearing of the Caledonian blue shrimp, *Litopenaeus*
635 *stylirostris* (Decapoda, Penaeidae). *Aquaculture* 362–363, 10–17.

636

637 Rajarao, S. J., Canfield, V. A., Mohideen, M. A., Yan, Y. L., Postlethwait, J. H., Cheng, K. C.,
638 Levenson, R., 2001. The repertoire of Na,KATPase α and β subunit genes expressed in the
639 zebrafish, *Danio rerio*. *Genome Res.* 11, 1211-1220.

640

641 Richards, J. G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na⁺/K⁺-ATPase α -
642 isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J.*
643 *Exp. Bio.* 206 : 4475 –4486.

644

645 Robalino, J., Chapman, R.W., de la Vega, E., O’Leary, N.A., Gorbach, D.M., Du, Z.-Q.,
646 Rothschild, M.F., Browdy, C.L., Warr, G.W., Labreuche, Y., 2011. Advances in genomics
647 and genetics of Penaeid Shrimp, in aquaculture biotechnology (eds G. L. Fletcher and M. L.
648 Rise), Wiley-Blackwell, Oxford, UK.

649

650 Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing
651 phylogenetic trees. *Mol. Biol. Evol.* 4 : 406-425.

652

653 Serrano, L., Towle, D.W., Charmantier, G., Spanings-Pierrot, C., 2007. Expression of
654 Na⁺/K⁺-ATPase α -subunit mRNA during embryonic development of the crayfish *Astacus*
655 *leptodactylus*. *Comp. Biochem. Physiol. Part D* 2, 126-134.

656

657 Talbot, P., Clark, W.H., Lawrence, A.L., 1972. Light and electron microscopic studies on
658 osmoregulatory tissue in the developing brown shrimp, *Penaeus aztecus*. *Tissue Cell* 4, 271–
659 286. Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary
660 Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24 : 1596-1599.

661

662 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA 4: molecular evolutionary genetics
663 analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.

664

665 Taylor, H.H., Taylor, E.W., 1992. Gills and lungs: The exchange of gases and ions. In:
666 Harrison, F.W., Humes, A.G. (Eds.), *Microscopic Anatomy of Invertebrates*. Vol 10. Decapod
667 Crustacea. Wiley-Liss, New York : 203–293.

668

669 Toyoshima, C., Kanai, R., Cornelius, F., 2011. First crystal structures of Na⁺,K⁺-ATPase:
670 new light on the oldest ion pump. *Structure*, 19(12):1732-8.

671

672 Wang, W.-N., Wang, A.-L., Wanga, D.-M., Wanga, L.-P., Liua, Y., Sun, R.-Y., 2003. Calcium,
673 phosphorus and adenylate levels and Na⁺–K⁺-ATPase activities of prawn, *Macrobrachium*
674 *nipponense*, during the moult cycle. *Comp. Biochem. Physiol. Part A* : 297–305.

675

676 Weihrauch, D., Ziegler, A., Siebers, D., Towle, D.W., 2001. Molecular characterization of V-
677 type H(+)-ATPase (β -subunit) in gills of euryhaline crabs and its physiological role in
678 osmoregulatory ion uptake. *J. Exp. Biol.* 204, 25-37.

679

680 Weppe, M., Bonami, J.R., Lightner, D.V., 1992. Demostracion de altas cualidades de la cepa
681 de *P. stylirostris* AQUACOP SPR 43 resistente al virus IHHN. Memorias Congreso
682 Ecuatoriano Acuicultura, pp 229–232.
683
684 Wheatly, M.G., Henry, R.P., 1987. Branchial and antennal gland Na^+/K^+ -dependent ATPase
685 and carbonic anhydrase activity during salinity acclimation of the euryhaline crayfish
686 *Pacifastacus leniusculus*. J. Exp. Biol. 133, 73–86.
687
688 Yamazaki, K., Okamura, C., Ihara, T. and Yasumasu, I., 1997. Two types of Na^+/K^+ -ATPase
689 alpha subunit gene transcript in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. Zool.
690 Sci. 14, 469-473.
691

692 **Figure captions**

693

694 **Figure 1.** Immunolocalization of Na⁺,K⁺-ATPase in organs of the branchial cavity in *L.*
695 *stylostris*. A: Zoea 2. Transverse section of cephalothorax. Positive immunostaining along
696 the pleura epithelium. B: Mysis 2. Transverse section of cephalothorax. Positive
697 immunostaining in the epithelia of the pleura and branchiostegite. C: PL1. Transverse section
698 of cephalothorax. Pleura, branchiostegite and the developing epipodites are
699 immunopositive. D: PL4. Transverse section of cephalothorax. Positive immunoreactivity
700 in epipodites and branchiostegite while gills are negative. E: PL9. Fully developed epipodites
701 are positive and gills are still negative. F: PL9. Strong immunopositive response in the pillar
702 cells of the branchiostegite.

703 BC : branchial cavity; Br : branchiostegite; GB : gill bud; Ep : epipodite; GF : gill filament;
704 He : Hepatopancreas; Pl : pleura. Bars = 100 μm.

705

706 **Figure 2.** Positive immunostaining of Na⁺,K⁺-ATPase in longitudinal section of gill
707 filaments in *L. stylostris* adults (25g). GF : gill filament. Bar = 50 μm.

708

709 **Figure 3.** Semi-thin section and ultrastructure of branchial organs from *L. stylostris* at PL9
710 stage (P25 at 29°C). A. Semi-thin section of the branchial cavity. B, C. Gill filament with its
711 thin epithelia. D,E. Two views of the epipodite cells with their extensive network of apical
712 microvilli. F. External part of the branchiostegite; note the thick cuticle and the deep
713 basolateral infoldings. G. Internal part of the branchiostegite with numerous microvillae under
714 a thin cuticle. Bars = 8μm (except A bar = 20 μm).

715 BI: basolateral infoldings; Br: Branchiostegite; CCT: central connective tissue; Cu: cuticle;
716 Ep: Epipodite; Epi: epithelia; Gf: gill filament; HL: hemolymph lacunae; Mi: mitochondrium;
717 Mv: microvilli; Nu: nucleus.

718

719 **Figure 4.** Complete nucleotide sequence and predicted amino acid sequence of the Na⁺/K⁺-
720 ATPase α-subunit cDNA amplified from *L. stylostris*. The P-type ATPase motif is indicated
721 by a yellow box, the likely ATPase binding site is symbolised by a solid red line. Predicted
722 transmembrane domains are indicated in a gray background. The start and stop codons are
723 indicated by bold lettering.

724

725 **Figure 5.** Phylogenetic analysis of amino acid sequences of Na⁺/K⁺-ATPase α-subunits from
726 organisms representing different taxa. Multiple alignments were created and curated in
727 MEGA 5.0. The tree was built using a Neighbor-joining method (Saitou & Nei, 1987) and is
728 drawn to scale, with branch lengths in the same units as those of the evolutionary distances
729 used to infer the tree. Percentages of bootstrapping branch corrections are shown beside the
730 branches. GenBank accession number are indicated between brackets.

731 .
732 **Figure 6.** Na⁺/K⁺-ATPase α-subunit mRNA transcript abundance in *L. stylirostris* shrimp
733 tissues. Expression values determined by quantitative real-time qPCR are presented as relative
734 abundance in relation to the elongation factor 1-alpha gene (EF1, accession no. AY117542.1).
735 Bars represent ± standard error of the mean. Different letters indicate statistically significant
736 differences among the results ($P < 0.05$).

737 Ey : eyestalk; Hp : hepatopancreas; AB : arthrobranchs; PLB : pleurobranchs; E : epipodites;
738 Pl: pleopods; Mu: abdominal muscle.

739
740 **Figure 7.** Na⁺/K⁺-ATPase α-subunit mRNA transcript abundance at different stages of the
741 larval development of *L. stylirostris* shrimp. Expression values determined by quantitative
742 real-time qPCR are presented as relative abundance in relation to EF1 gene. Bars
743 represent ± standard error of the mean. Different letters indicate statistically significant
744 differences among the results ($P < 0.05$).

745
746 **Figure 8.** *LsNKA* transcript abundance in shrimp gills following treatment with dsRNA.
747 Shrimp were injected on day 0 with saline (Control) or with 10 µg of *LsNKA* dsRNA. At 48 h
748 after this initial injection, 10 animals were randomly sampled in each group. Expression
749 values determined by quantitative real-time qPCR are presented as relative abundance in
750 relation to EF1 gene. Bars represent ± standard error of the mean.

751
752 **Figure 9.** Shrimp mortality following injection with *LsNKA* dsRNA and exposition to
753 different salinities (5, 27 or 40 ppt). Shrimps ($n = 240$) were injected intramuscularly with
754 either saline or *LsNKA* dsRNA. At 48 h after this initial injection, animals were transferred to
755 seawater tanks at different salinities (5, 27 or 40 ppt). This experiment was conducted in
756 triplicate with 10 shrimps per tank. One-way analysis of variance was used to detect the
757 differences in survival rates after data arc sin($\sqrt{\quad}$) transformation. Student's *t*-tests were used to

758 resolve differences among treatment means with $\alpha = 0.05$. Different lower-case letters
 759 indicate significant difference between treatments ($P < 0.05$).

760

761

762 **Tables**

763

764 **Table 1. List of primers used in this study**

765

| Primer name | Sequence (5' – 3') | |
|------------------------|--|---------------------------------|
| PCR primers | | |
| A-119 | TGT ATC CTC TGC TGC CGT CCT | |
| A-120 | ACC CCA CCC AAG CAG ACT C | |
| A-122 | TGT TGC GTG CAC GCC AGC CC | |
| A-132 | ATA GTA GGT CTC CAG TTC CAT C | |
| qRT-PCR primers | | |
| | target gene | |
| A-52 | EF1 | TGC TCA CAT TGC CTG CAA GT |
| A-53 | EF1 | CCT TAC CAG TAC GCC TGT CGA T |
| A-133 | Na ⁺ /K ⁺ ATPase | TCC CAT CCT GAA ACG TGA AGT AA |
| A-134 | Na ⁺ /K ⁺ ATPase | CTA CAC ACT TCA GCA GAG CAG CTT |

766

767

768 **Table 2** : Localization of the Na⁺/K⁺ATPase activity in the organs of the branchial cavity
 769 during the larval and postlarval development of *Litopenaeus stylirostris*. N : absence of
 770 organ; + : positive fluorescence immunolocalization; - : negative fluorescence
 771 immunolocalization.

772

| Stage | Pleura | Branchiostegite | Epipodite | Gills |
|---------|--------|-----------------|-----------|-------|
| Zoea 2 | + | - | N | N |
| Mysis 2 | + | + | N | N |
| PL1 | + | + | + | - |
| PL2 | - | + | + | - |
| PL4 | - | + | + | - |
| PL9 | - | + | + | - |

Fig 1

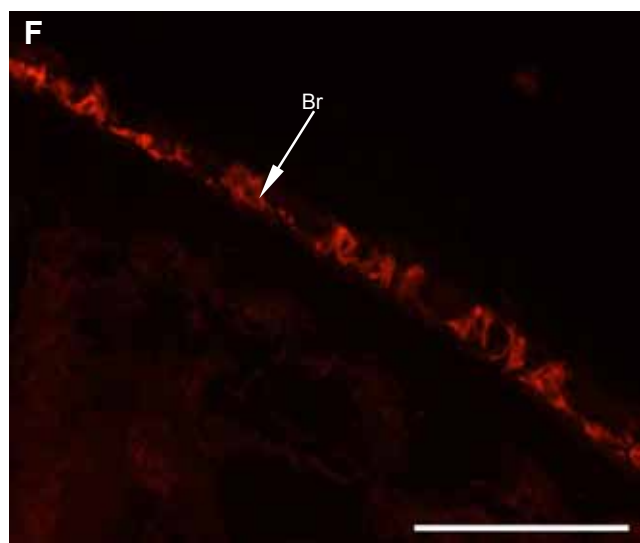
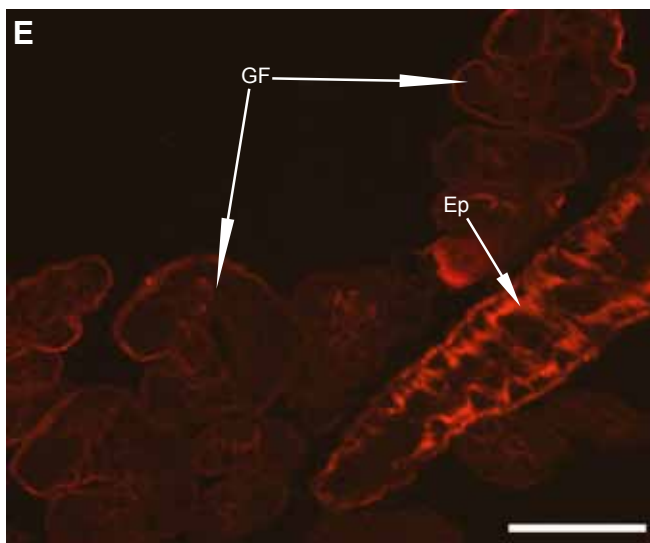
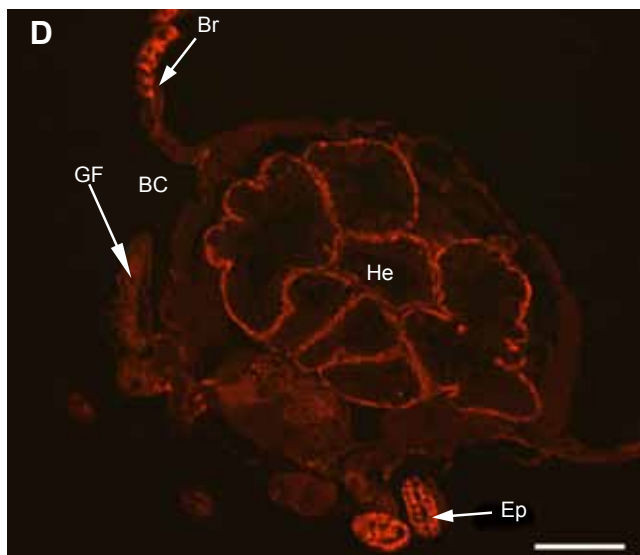
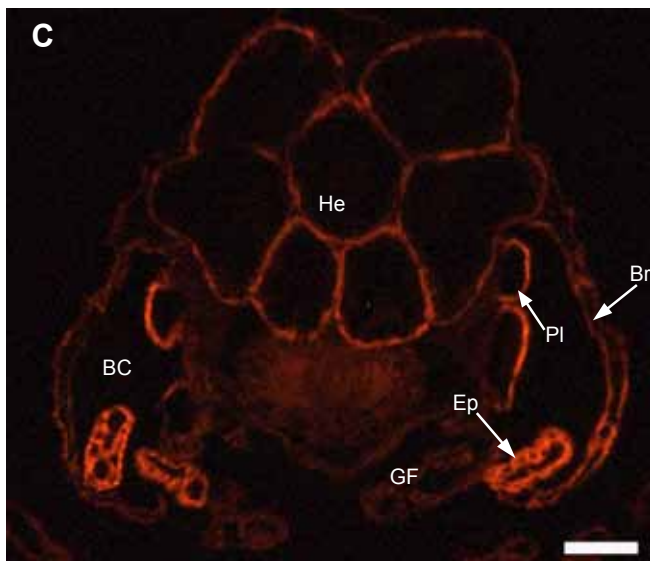
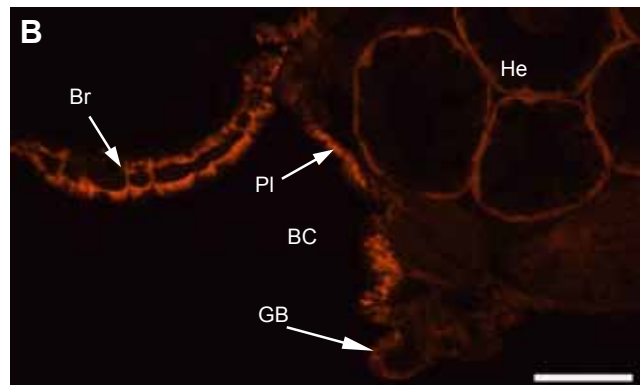
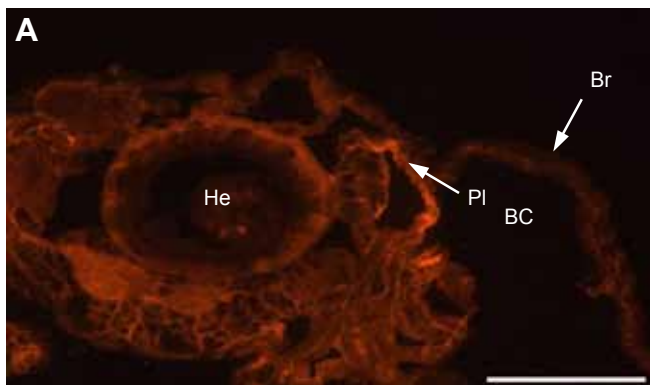


Fig. 2

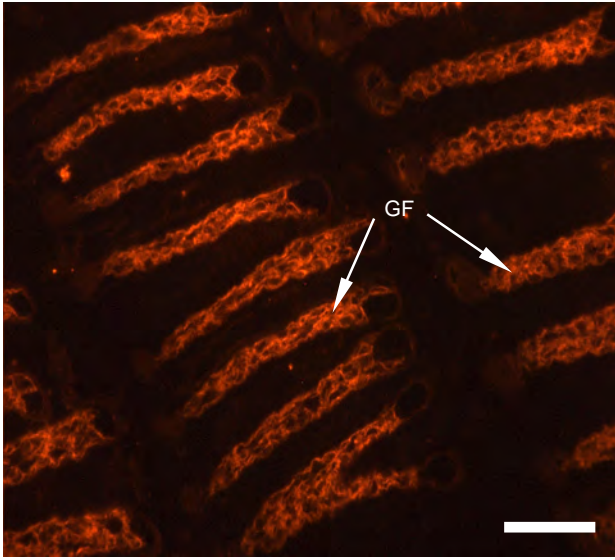


Fig 3

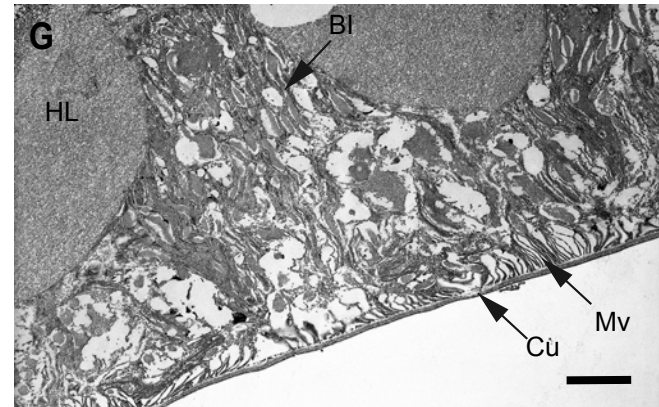
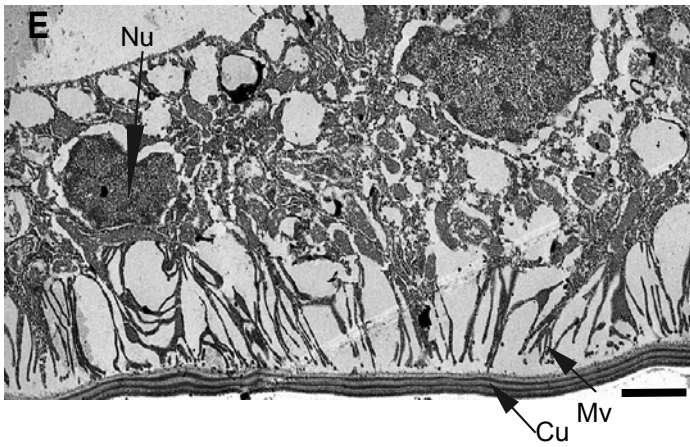
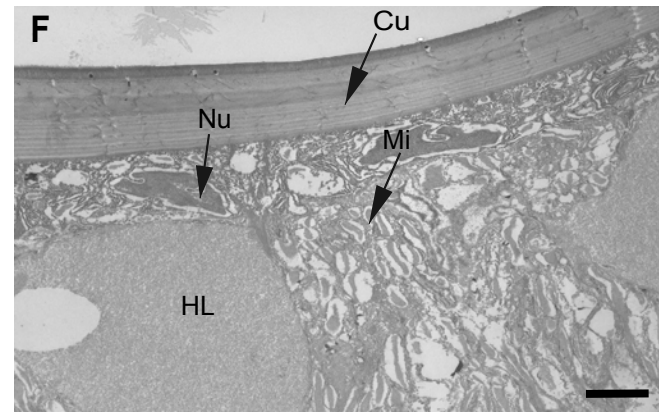
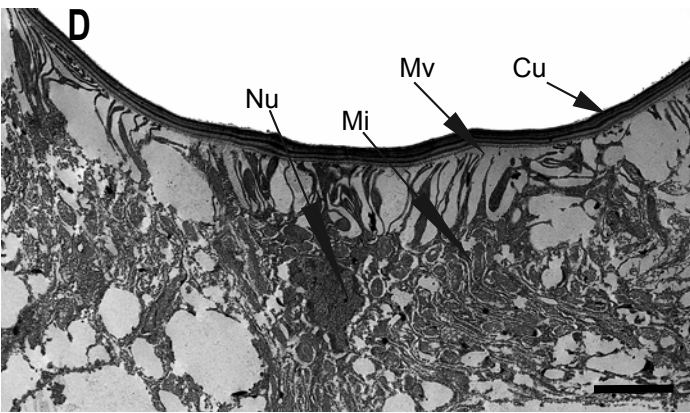
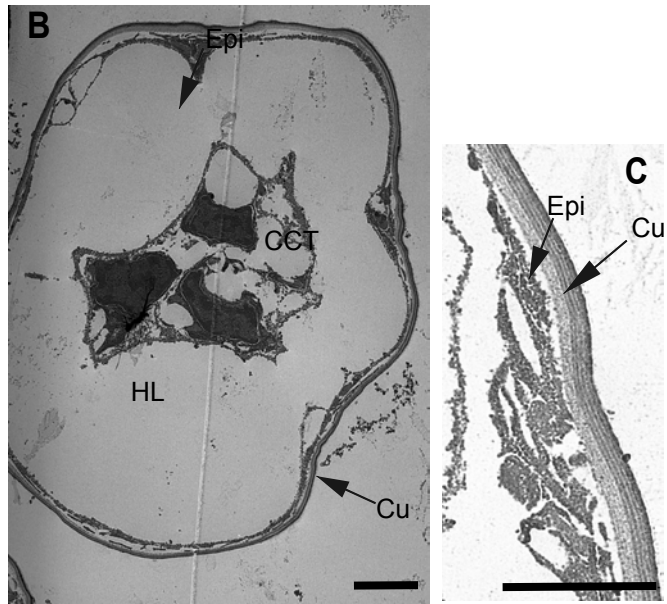
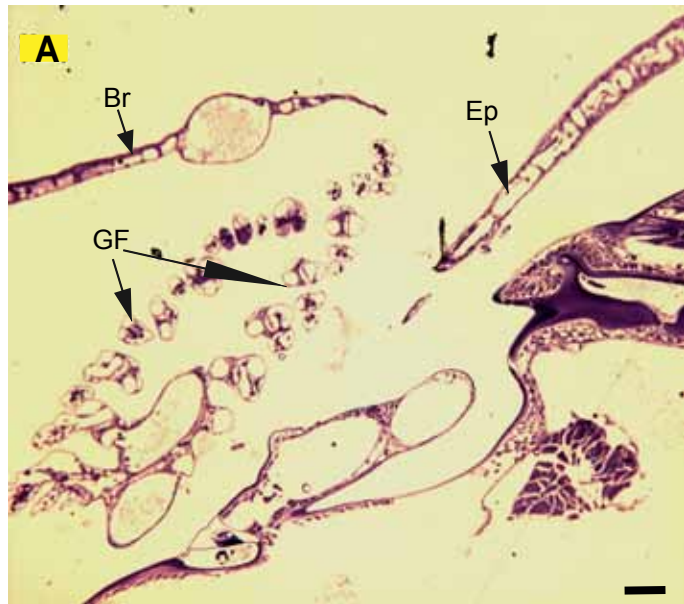


Figure 4

1 TGTaTCCTCTGCTGCCGTCCTCTGCTGAGCGCCGAGTGTGCGCTCTTCAGCAGCC**ATGG**CCGATTCTAAGAAAAAGCCC 80
1 Y P L L P S S C * A P S V A S S A A **M** A D S K K K P 17

81 CAGAAGGCTAAAGGGAAGAAGGGAGATAAGGATTTGAATGATCTGAAGCAGGAGTTGGAACCTGATGAGCACAAGGTCCC 160
18 Q K A K G K K G D K D L N D L K Q E L E L D E H K V P 44

161 AATTGAGGAACCTTTCAACGTCTCACTGTTAACCCAGACACAGGTCTATCACAAAGTGAGGCTAAGCGCCGTATTGAAC 240
45 I E E L F Q R L T V N P D T G L S Q S E A K R R I E R 71

241 GAGATGGGCCGAACGCTCTTACCCACCCAAAGCAGACTCCAGAATGGGTCAAGTTCGCAAAAACCTCTTCGGTGGTTTC 320
72 D G P N A L T P P K Q T P E W V K F C K **N L F G G E** 97

321 TCACTCTGCTGTGGATTGGCGCTATCCTCTGCTTCAATTGAGACAGCTGCAGAAGAGGAGCCCAACAA 400
98 **S L L L W I G A I L C F I A Y S I** E T A A E E E P N K 124

401 GGACAATTTGTACCTGGGCATTGTGCTCACAGCTGTCGTGATCATCACAGGCGTCTTCTCATATTACCAAGAAAGCAAGA 480
125 D N **L Y L G I V L T A V V I I T G V F S Y Y** Q E S K S 151

481 GCTCCCGTATTATGGAATCTTTCAAGAACATGGTCCCTCAGTATGCTATTGTTCTTCGAGATGGCGAGAAGCAGAATGTT 560
152 S R I M E S F K N M V P Q Y A I V L R D G E K Q N V 177

561 CAGGCTGAGGAACGTGCATAGGAGACATTTAGAGGTCAAGTTTGGTGATCGTATCCAGCTGATATCCGTGTCATCGA 640
178 Q A E E L C I G D I V E V K F G D R I P A D I R V I E 204

641 AAGCAGGGGCTTCAAGGTTGACAACCTTCCCTGACTGGAGAATCCGAACCCAGAGCCGATCACCCGAATACACTTCCG 720
205 S R G F K V D N S S L T G E S E P Q S R S P E Y T S E 231

721 AGAACCCCTTGAGACCAAGAACTGGCTTTCTTCCACCAATGCTGTCGAGGGTACTTGAAGGTATCGTTATCATG 800
232 N P L E T K N L A F F S T N A V E G T C K G I V I M 257

801 ATGGTGACAACACTGTGATGGGTGCTATTGCTGGTTGGCATCCGGATTGGAAACTGGTGAACCCCATGCCAAGGA 880
258 I G D N T V M G R I A G L A S G L E T G E T P I A K E 284

881 AATTACCCATTTCAATTCACATCATTACTGGTGTGGCTGTGTTCTTGGGTGTGACCTTCTTCGTTATTGCCCTTCATCCTTG 960
285 I T H **F I H I I T G V A V F L G V T F F V I A F I** L G 311

961 GGTACCATTGGTTGGATGCTGTTGTGTTCTCCTCATTGGTATCATTGTAGCCAATGTGCTGAGGGTCTGCTAGCCACTGTC 1040
312 Y H W L D A V **V F L I G I I V A N V P E G L L A T V** 337

1041 ACTGTGTGCTGACTCTTACTGCCAAGCGCATGGCTGCCAAGAAGTGCCTTGTAAGAAGTGGAGGCTGTGGAACCCCT 1120
338 **T V C L** T L T A K R M A A K N C L V K N L E A V E T L 364

1121 GGGTTCACCTCCACCATTGCTCTGATAAGACTGGTACCCTCACCCAGAATCGTATGACAGTAGCACATATGTGGTTCG 1200
365 G S T S T I C S **D K T G T L T** Q N R M T V A H M W F D 391

1201 ACAATACCATCATTGAAGCTGATACATCTGAAGATCAGTCTGGCTGCCAGTATGACAAGACCTCACAAGGCTGGAAGGCT 1280
392 N T I I E A D T S E D Q S G C Q Y D K T S Q G W K A 417

1281 CTGTCTAGAATTGCTGCCCTCTGTAACCGTCTGAATTCAGACTGGTATGGAAAACACTCCCATCCTGAAACGTGAAGT 1360
418 L S R I A A L C N R A E F K T G M E N T P I L K R E V 444

1361 AAACGGCGATGCTTCTGAAGCTGCTCTGCTGAAGTGTGTAGAATTGGCTGTGGTGATGTTAAGGGCTGGCGTGCACGCA 1440
445 N G D A S E A A L L K C V E L A V G D V K G W R A R N 471

1441 ACAAGAAGGTATGTGAAATTCCTTTCAACTCCACCAACAAGTACCAAGTATCCATCCACGAGACCGAGGATAAGAACGAC 1520
472 K K V C E I P F N S T N K Y Q V S I H E T E D K N D 497

1521 CCACGATACCTTGTGTGATGAAGGGAGCCCTGAGAGGATCCTGGAACGTTGCTCCACCATCTACATCAATGGAGAGGA 1600
498 P R Y L V V **M K G A P E R I L** E R C S T I Y I N G E E 524

1601 AAAGCCCTCGACGAAGAAATGAAGGAAGCTTCAACAATGCCTACCTTGAATTGGGCGGTCTGGAGAGCGTGTACTTG 1680
525 K A L D E E M K E A F N N A Y L E L G G L G E R V L G 551

1681 GTTCTGTGACTACATGCTGCCAAGTACCAAGTACCCTCTGGATAACCCCTTCGATGCTGATGCTGTGAACCTCCCTGTC 1760
552 F C D Y M L P T D K Y P L G Y P F D A D A V N F P V 577

1761 CATGGTCTGCGCTTCGTTGGTCTGATGTCCATGATTGATCCTCCTCGTGTGCTGTACCCGATGCTGTAGCAAAGTGCAG 1840
578 H G L R F V G L M S M I D P P R A A V P D A V A K C R 604

1841 ATCTGCTGGTATCAAGGTTATCATGGTTACTGGTGTGATCACCCATCACTGCCAAGGCTATTGCCAAGTCTGTAGGTATCA 1920
605 S A G I K V I M V T G D H P I T A K A I A K S V G I I 631

1921 TCTCTGAAGAAACGAGACTGTTGAGGACATTGCACAGAGGTTGAACATTCATCAAGGAGGTCGACCCCACTGAAGCA 2000
632 S E G N E T V E D I A Q R L N I P I K E V D P T E A 657

2001 AAGGCTGCTGTAGTTCACGGTCTGAACTTCGTGACATGACATCCGAGCAGTTGGATGATGTCTCCTCCACCACACTGA 2080
658 K A A V V H G S E L R D M T S E Q L D D V L L H H T E 684

2081 AATCGTGTTCGCCGTACCTCCCCACAACAAGCTGATCATTGTAGAAGGTTGCCAGCGTATGGGTGCCATTGTGGCTG 2160
685 I V F A R T S P Q Q K L I I V E G C Q R M G A I V A V 711

2161 TAACGGTGTGGTGTGAATGATTCTCCTGCTCTGAAGAAGGCTGATATTGGTGTGCTATGGGTATTGCTGGTCTGAT 2240
712 T G D G V N D S P A L K K A D I G V A M G I A G S D 737

2241 GTGTCCAAGCAAGCTGCTGACATGATTCTGTTGGACGACAACCTTTGCTTCCATTGTCACCGGTGTTGAAGAGGGCAGACT 2320
 738 V S K Q A A D M I L L D D N F A S I V T G V E E G R L 764

2321 TAtTTTCGACAACCTGAAGAAATCCATTGCTTACACCCTGACATCTAACATCCCTGAAATCTCTCCCTTCTGTTCTTCA 2400
 765 I F D N L K K S I A Y T L T S N I P E I S P F L F F M 791

2401 TGATTGCCTCAGTCCCACTTCTCTTGGAACTGTGACCATCCTCTGCATtGATCTGGGTACTGACATGGTGCCTGCCATT 2480
 792 I A S V P L P L G T V T I L C I D L G T D M V P A I 817

2481 TCCCTTGCCTATGAAGAAGCTGAGTCTGATATTATGAAGCGCCAGCCCCGAAACCCATTACCGACAAGCTTGTGAACGA 2560
 818 S L A Y E E A E S D I M K R Q P R N P F T D K L V N E 844

2561 GAGGCTCATCTCAATGGCCTATGGTCAGATTGGTATGATCCAGGCCCTGGCAGGATTCTTACCTATTTTCGTGATCATGG 2640
 845 R L I S M A Y G Q I G M I Q A L A G F F T Y F V I M A 871

2641 CTGAGAACGGCTTCTGCCACCCCATCTCTTGGTCTCCGTGAGCGCTGGGACAGTAAGGCCATCAACGATCTGGAGGAT 2720
 872 E N G F L P P H L F G L R E R W D S K A I N D L E D 897

2721 CACTATGGACAGGAATGGACCTTCCACGACCGTAAGATTCTTGAGTACACCTGCCACACTGCTTTCTTACCTCCATTGT 2800
 898 H Y G Q E W T F H D R K I L E Y T C H T A F F T S I V 924

2801 GATTGTGCAGTGGGCCGATTGATCATTTGCAAGACCCGCCGTAACCTCCATGTCCACCAGGGCATGAAGAACTGGGTGC 2880
 925 I V Q W A D L I I C K T R R N S I V H Q G M K N W V L 951

2881 TGAACCTTGGTCTCGTCTTTGAAACCACTTTGGCTGCCTTCTTCTTACACCCAGGCATGGACAAGGGTCTTCGCATG 2960
 952 N F G L V F E T T L A A F L S Y T P G M D K G L R M 977

2961 TACCCACTGAAGTTCTATTGGTGGCTGCCTGCTCTCCGTTCTCCATCCTTATCTTTCATCTACGATGAGATACGTCGCTT 3040
 978 Y P L K F Y W W L P A L P F S I L I F I Y D E I R R F 1004

3041 CATCTGCGAAGGAACCCCTGGTGGTGGATGGAACCTGGAGACCTACTAT**TAA** 3092
 1005 I L R R N P G G W M E L E T Y Y * 1020

Figure 5

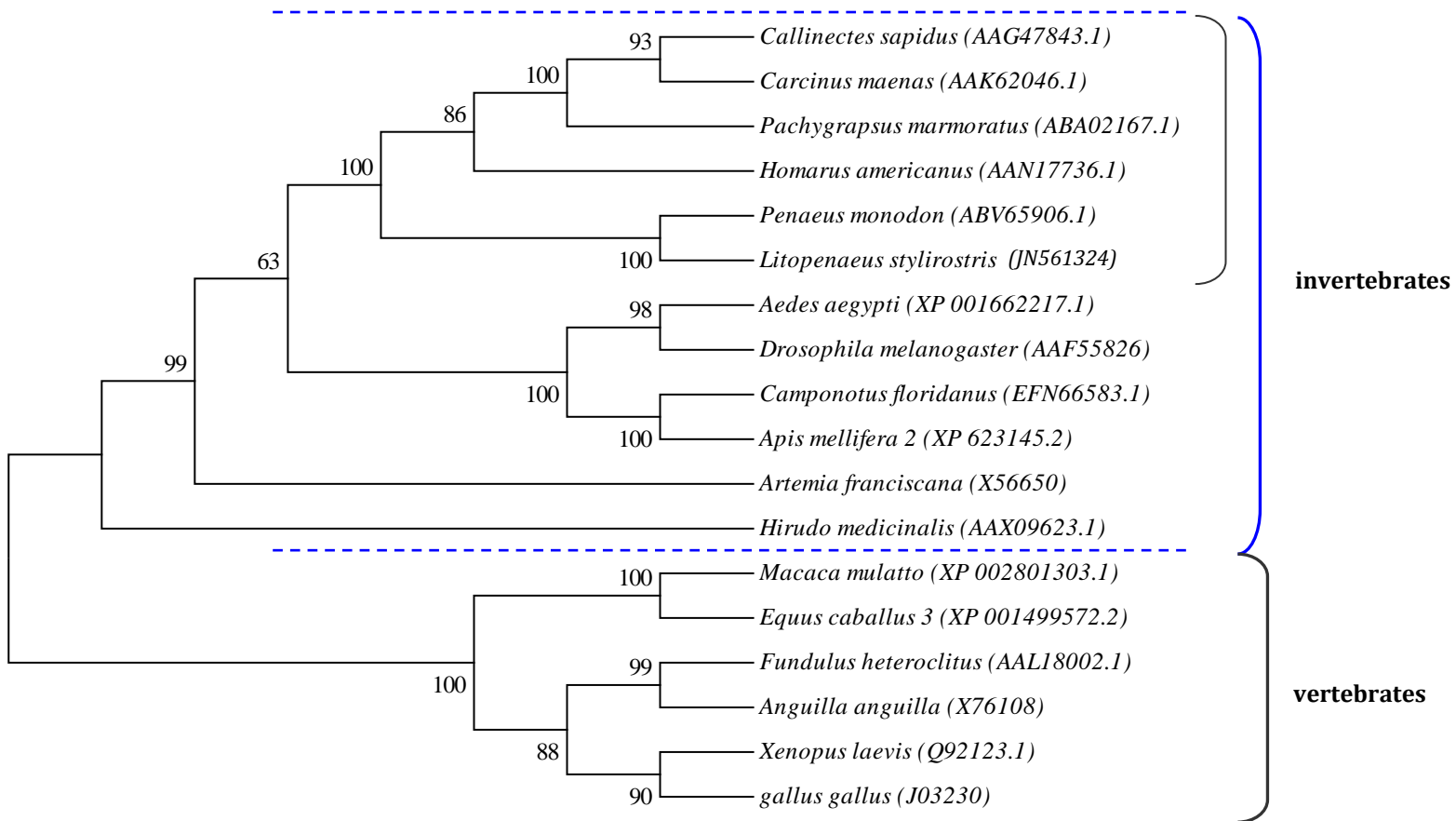


Figure 6

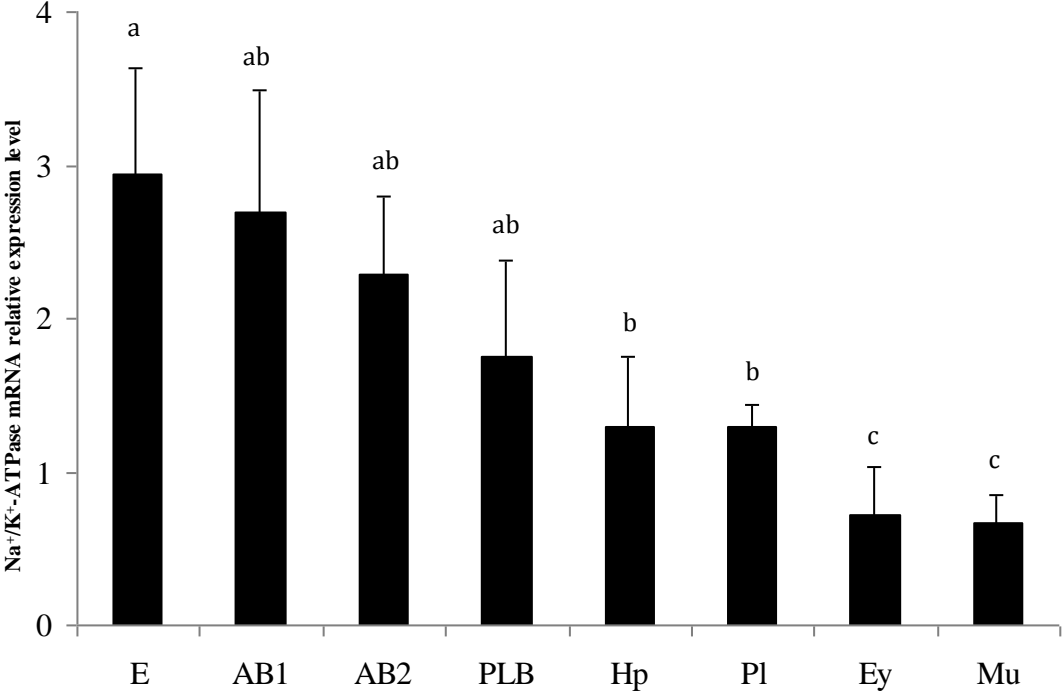


Figure 7

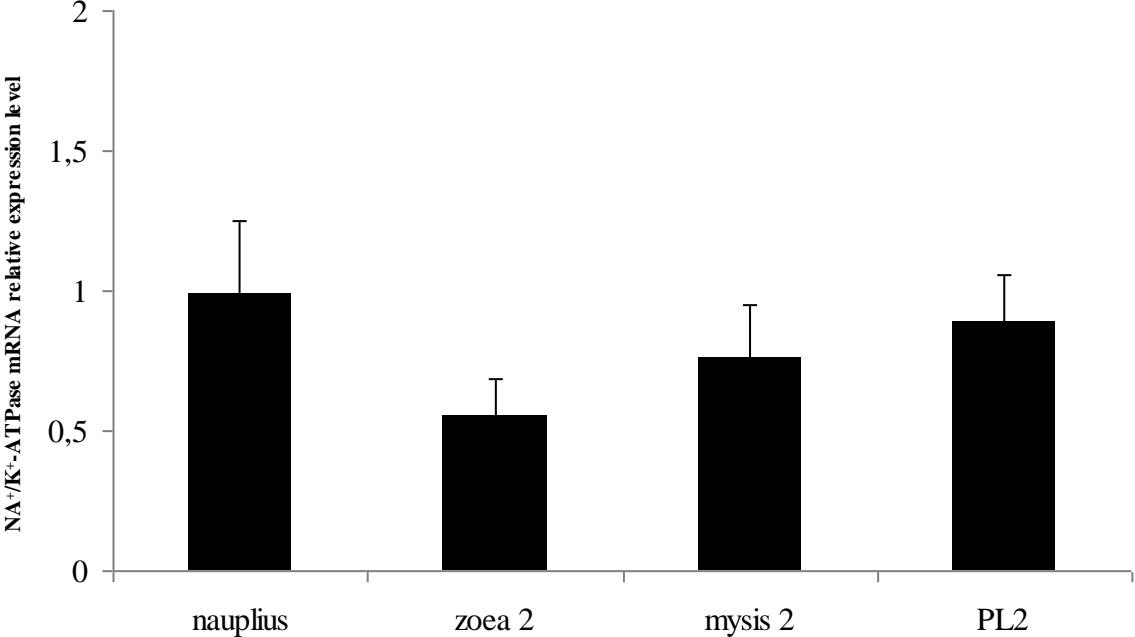


Figure 8

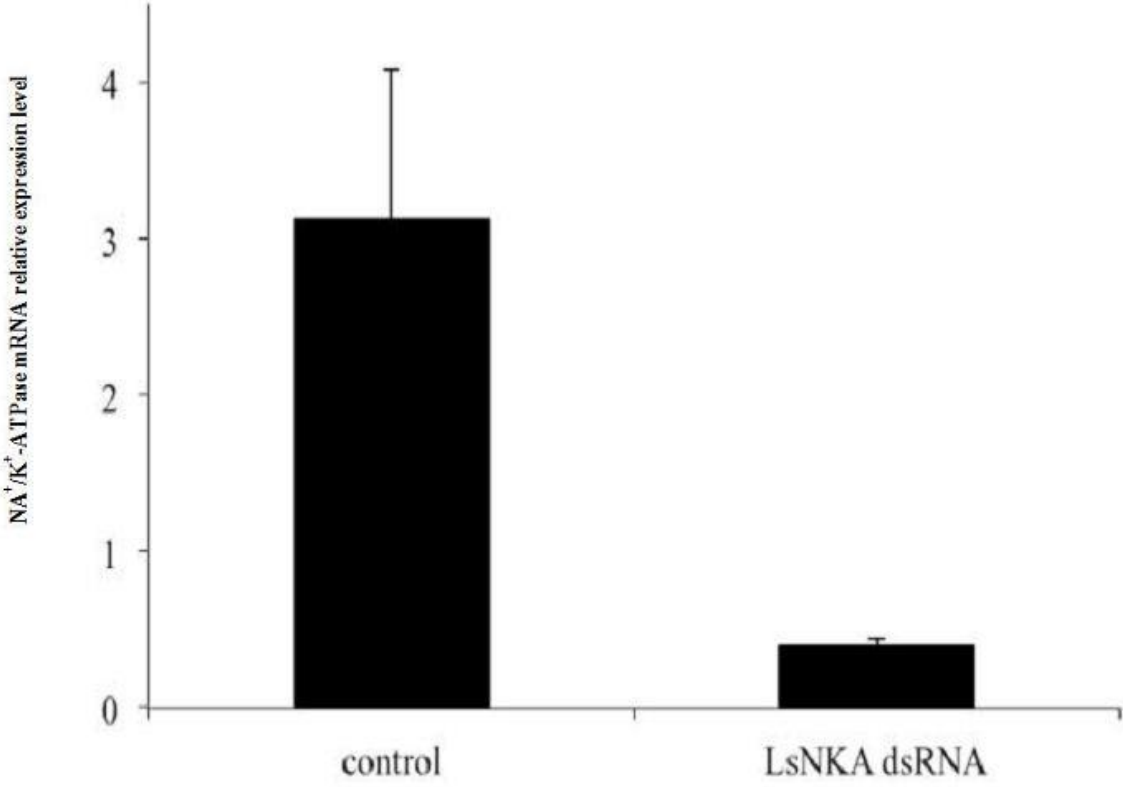
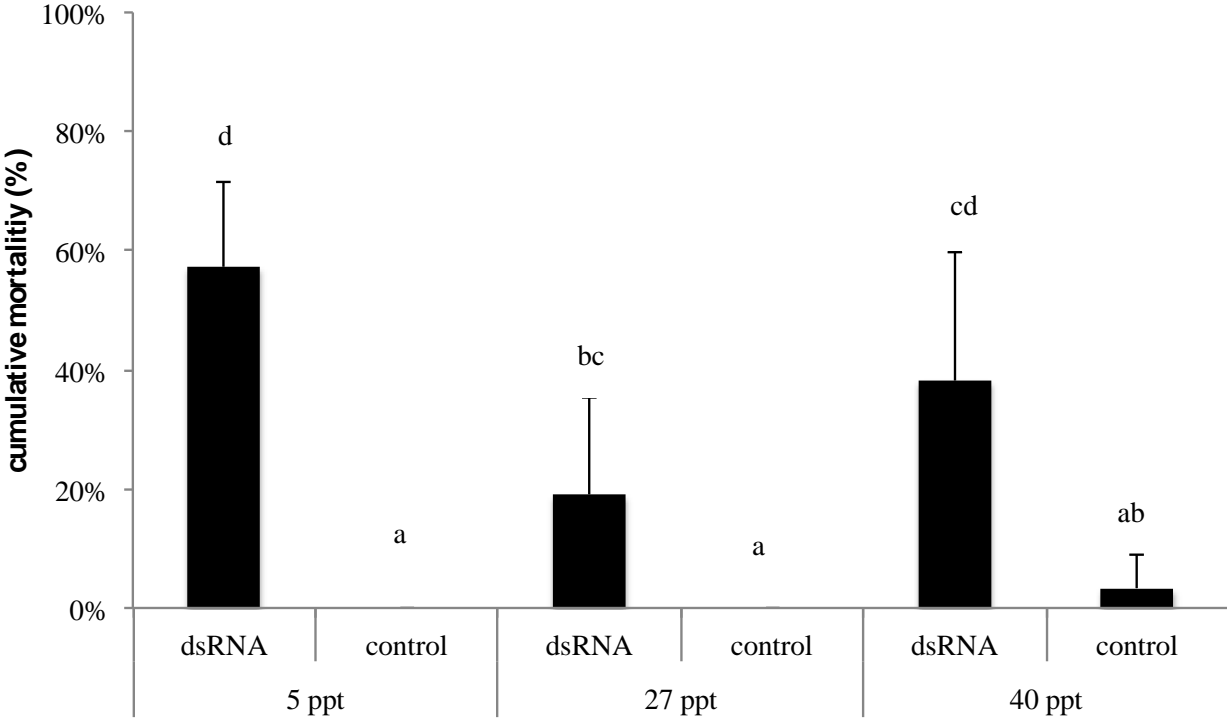


Figure 9



Supplementary data

Figure S1

Western Blot analysis of LsNKA in gills of *Litopenaeus stylirostris* cultured at 35 ppt. Lane 1 : sample 1, Lane 2 : sample 2

