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Effect of salinity and temperature on the expression of genes involved in branchial ion transport processes in European sea bass

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Abstract

The responses of European sea bass to temperature increase and salinity decrease were investigated measuring mRNA expression levels of main genes involved in ion transport. Juvenile fish were pre-acclimated to seawater (SW) at 18 °C (temperate) or 24 °C (warm) for two weeks and then transferred for two weeks to either fresh water (FW) or SW at the respective temperature. Unlike temperate conditions, there is no change in Na^+/K^+-ATPase α1a (*nka α1a*) and Na^+/H^+ exchanger 3 (*nhe3*) mRNA expression following FW transfer in warm conditions. This is linked to the high expression of these genes in warm SW compared to temperate SW. Na^+/Cl^--cotransporter (*ncc2a*) expression however is increased following FW transfer in temperate and warm conditions. Main transporters involved in ion excretion ($Na^+/K^+/2Cl^-$-1 cotransporter, *nkcc1* and cystic fibrosis transmembrane conductance regulator, *cftr*) as well as nitrogen excretion (Rh-glycoproteins, *rhcg1* and *rhbg*) and acid-base regulation (V-H^+-ATPase, *vha-a* and *b*) are highly expressed in SW warm conditions *vs* FW warm. Overall, our results suggest a higher activation of ion transport processes in warm conditions and more strikingly in SW. This is linked to a strong interplay between diverse ion transporters in order to coordinate physiological responses at the gill level.

Keywords: *Dicentrarchus labrax,* ion transporter, gill, mRNA expression, thermal acclimation, salinity

1. Introduction

Temperature is considered a main factor affecting organism distribution, life-history traits and biological processes (Crockett and Londraville, 2006; Hutchison and Maness, 1979; Schulte, 2011). The effects of temperature on ion-regulatory mechanisms and other physiological processes have been shown in several fish species (Burton, 1986; Chou et al., 2008; Gibbons et al., 2018; Kreiss et al., 2015; Metz et al., 2003; Michael et al., 2016a; Morrison et al., 2006; Vargas-Chacoff et al., 2018) including the European sea bass *Dicentrarchus labrax* (Claireaux and Lagardère, 1999; Masroor et al., 2018). Ion homeostasis is mainly relying on ion pumps such as the branchial Na^+/K^+-ATPase (NKA) and V-H^+-type ATPase (VHA), both creating an electrochemical gradient for active ion transport across gill basolateral and apical membranes (Evans, 2008; Evans et al., 2005, 1999; Hwang and Lin, 2014; Vasić et al., 2008). It has been reported that active ion transporters represent a higher thermal sensitivity than carrier-mediated diffusive transporters (Moyes and Ballantyne, 2011) which could lead to imbalances between active and passive ion transport. Ion transport alteration at the gills due to high temperatures can also occur due to changes in membrane integrity and fluidity that could affect the proper insertion and function of ion transporters (Moyes and Ballantyne, 2011). As Na^+ and Cl^- are thought to be taken up by different uptake pathways, respectively in exchange for H^+ and HCO_3^-, imbalance in Na^+/Cl^- ratio likely affect acid-base status (Goss et al., 1998; Jensen et al., 1998). The fish gill is a plastic organ involved in gas exchange, ion regulation, acid-base balance and nitrogen excretion and significantly contributes to physiological homeostasis in changing environments (Evans et al., 2005). In response to temperature and/or salinity changes, fish gills are subject to significant morphological remodeling (Gibbons et al., 2018; Metz et al., 2003; Tzaneva and Perry, 2010) and changes in ionocyte density and distribution (Metz et al., 2003;

63 Mitrovic and Perry, 2009). We have previously shown that gill morphological parameters
64 differed in sea bass that have been pre-acclimated at two different temperatures, 24 °C and 18 °C
65 in seawater (SW) and fresh water (FW) with particularly a lower ionocyte density in warm FW
66 (Masroor et al., 2018). Plasma Na^+ levels were decreased in warm (24 °C) compared to
67 temperate conditions (18 °C) at both salinities. On the contrary, plasma Cl^- levels were higher in
68 FW warm compared to temperate conditions and no effect of temperature was reported in SW.
69 These results may point out an effect of increased temperatures on enhanced FW Cl^- uptake and
70 decreased Na^+ uptake (or enhanced Na^+ loss) at the gill (Hwang and Lee, 2007) and/or kidney
71 levels (Hickman and Trump, 1969). Membrane transporters and channels, including Na^+/H^+
72 exchangers (NHE2/3), VHA, ammonia transporters (Rhesus (Rh) glycoproteins, mainly RHBG
73 and RHCG1), NKA and Na^+/Cl^- cotransporters (NCC2) contribute directly or indirectly to ion
74 homeostasis (Hwang and Lin, 2014). Basolateral $Na^+/K^+/2Cl^-$-1 (NKCC1) and apical cystic
75 fibrosis transmembrane conductance regulator (CFTR) are involved in ion excretion in SW
76 (Hwang and Lin, 2014). The mechanisms for ion uptake in FW have been reviewed in several
77 reports and involve a multitude of transporters working together to take up Na^+ and/or Cl^- ions in
78 exchange of different counterions, mainly H^+ and HCO_3^- (Dymowska et al., 2012; Hsu et al.,
79 2014; Hwang, 2009). Recently, Blondeau-Bidet et al. (2019) have shown that in sea bass *D.*
80 *labrax* maintained in FW for two weeks, gill NHE3 and NCC2 have been detected in different
81 ionocyte subtypes at the apical cell part (NHE3- and NCC2-type cell), coupled to basolateral
82 NKA in order to absorb Na^+ or Na^+, Cl^- (Hwang et al., 2011; Kumai and Perry, 2012; Tang and
83 Lee, 2011). ClC-3 is a volume-activated chloride channel involved in transepithelial Cl^- transport
84 (Bossus et al., 2013; Tang and Lee, 2011) and cell volume regulation (Duan et al., 1999, 1997;
85 Hermoso et al., 2002; Wang et al., 2000). Changes in expression of these and other transporters

86 have been reported in numerous species following salinity transfers, but the effect of increased or
87 decreased temperature on the expression of these genes in different salinity conditions is less
88 known (Chou et al., 2008; Gibbons et al., 2018; Hu et al., 2016; Kyprianou et al., 2010; Logan
89 and Buckley, 2015; Logan and Somero, 2011; Michael et al., 2016a; Morrison et al., 2006).
90 Several paralogs exist in fish for *nka α1* with potentially different functions and expression
91 patterns depending on the considered species (Hwang and Lee, 2007). Two paralogs have been
92 identified in sea bass, *nka α1a* and *nka α1b,* with *nka α1a* being the most expressed paralog in
93 sea bass gills whatever the considered salinity (Blondeau-Bidet et al., 2016). Temperature
94 generally increases NKA transcript expression and protein activity but the response highly
95 depends on the species and even the population (Michael et al., 2016a; Morrison et al., 2006).
96 Michael et al. (2016a) have reported population-specific differences regarding mRNA expression
97 levels of *nka* in cod *Gadus morhua* populations maintained at the same temperature but
98 originating from a different thermal niche. The European sea bass *Dicentrarchus labrax*
99 (Linnaeus 1758) is an important aquaculture species along the Mediterranean and Atlantic
100 coasts. It is considered as a highly eurythermal (tolerates temperatures from 4 to 35 °C) and
101 euryhaline species (tolerating from FW up to 70 ppt) (Barnabé, 1989; Dülger et al., 2012;
102 Madeira et al., 2013). In the wild, sea bass adults and juveniles are frequently exposed to
103 fluctuations of environmental parameters, notably during their stay in lagoons and estuaries
104 (Dufour et al., 2009; Newton and Mudge, 2003). In these habitats, temperatures can reach values
105 over 25 °C in the summer months. The Mediterranean basin is considered as one of the 'Hot-
106 spots' by Giorgi (2006) and in the context of climate change, temperatures will continue to rise
107 in the future. Temperature acclimation and preference have been studied in sea bass by
108 investigating oxygen consumption (Dalla Via et al., 1998), fish distribution (Trancart et al.,

109 2016), food intake (Dülger et al., 2012; Person-Le Ruyet et al., 2004) and swimming speed
110 (Claireaux et al., 2006). *D. labrax* optimal growth rate was reported at 25 °C (Person-Le Ruyet et
111 al., 2004) which is close to the warm temperature analyzed in our study.

112 In this study, we analyzed branchial ion regulatory mechanisms at the transcript level with a
113 particular focus on selected genes involved in osmoregulation, acid-base regulation and ammonia
114 excretion. We compared mRNA levels of *nka α1a* (*atp1a1a*), *nka α1b* (*atp1a1b*), *cftr*, *nkcc1*
115 (*slc12a2*), *nhe3* (*slc9a3*), *ncc2a* (*slc12a3-like*), *clcn3* (*clc-3*), *vha-a* (*atp6v1a*) and *vha-b*
116 (*atp6v1b2*), *rhbg* and *rhcg1* in gills from fish acclimated at two different temperatures (18 °C
117 and 24 °C) and transferred from SW to FW.

118

119 **2. Material and Methods**

120 **2.1. Experimental conditions**

121 Experimental conditions have been previously described in Masroor et al. (2018). Briefly,
122 juvenile sea bass *D. labrax* originating from a Western Mediterranean population were obtained
123 from the Ifremer Station at Palavas-les-Flots (Herault, France). Fish were brought to the
124 Montpellier University at the age of 14 months and maintained for one week in 3,500 L tanks
125 containing natural seawater (SW) form the Mediterranean Sea at 38 ppt and 18 °C, under a 12 h
126 light/12 h dark photoperiod. Fish were transferred to 200 L tanks (14 fish/tank, density of 6-7
127 kg/m3, two replicates per conditions) to be acclimated either at 18 °C or 24 °C (with a
128 temperature increase of 0.2 °C/h). After two weeks of temperature acclimation, fish were
129 transferred directly either to dechlorinated tap water (fresh water, FW), or to SW (7 fish/tank,
130 two replicates for each conditions) and maintained in this salinity two weeks before sampling.

131 Ionic composition (in mEq.L^{-1}) of the FW was Na^+ (0.12), K^+ (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29),
132 Cl^- (0.98), NO_3^- (0.06) SO_4^{2-} (0.61). Water was aerated and mechanically/biologically filtered
133 (Eheim System, Germany). Temperature, salinity, oxygen and nitrogen levels were checked
134 daily. A quarter of the water volume was changed every two days. Fish were fed *ad libitum* twice
135 a week with fish granules (Aphymar feed, Meze, Herault, France) until 2 days before sampling.
136 At the end of the experiment, fish were anesthetized in a solution of phenoxy-2-ethanol (240
137 ppm) prior to tissue collection. The fish used for the experiment had a length of 20.77±1.32 cm
138 (mean ± SD) and average weight was 86.87±20.23 g. Four groups were compared: SW at 18 °C
139 (temperate SW), SW at 24 °C (warm SW), FW at 18 °C (temperate FW) and FW at 24 °C (warm
140 FW). These experiments respected the guidelines of the European Union (directive 86/609) and
141 of the French law (decree 87/848) regulating animal experimentation.

142 **2.2. Blood parameters, protein and cellular measurements**

143 Following anesthesia, blood was sampled using a 1-ml syringe coated with heparin (Li-heparin,
144 Sigma-Aldrich, France). Plasma was obtained following centrifugation of 8 min at 10,000g at 4
145 °C and frozen at -20°C until analysis. For pH measurements, plasma was thawed on ice then
146 measured at 20 °C using a minimum of 15 µl of plasma with an InLab Ultra-Micro ISM pH
147 probe coupled to a SevenMulti pH meter (Mettler Toledo, Ohio, USA) calibrated with National
148 Bureau of Standards (NBS) buffers. It must be noted that samples were frozen and thawed twice
149 before performing plasma pH measurements. Analysis of plasma Na^+ and Cl^-, gill Na^+/K^+-
150 ATPase activities and gill ionocyte densities (in filaments and lamellae) have been performed on
151 the same fishes and methods are described in Masroor et al. (2018).

152 **2.3. RNA extraction and reverse transcription**

153 Gill tissues were collected from gills of SW- and FW-exposed sea bass in temperate and warm
154 conditions. The epithelium of the first gill arch was scraped with a sterile scalpel, immerged in
155 Trizol® reagent and flash frozen in liquid nitrogen. Tissues were then stored at -80 °C until
156 analysis. Total RNA was extracted using Trizol® reagent according to the manufacturer's
157 instructions. RNA quantity and purity were assessed by measuring the A260/A280 ratio using
158 the NanoDrop® ND-1000 V3300 spectrometer (Nanodrop Technology Inc., Wilmington,
159 Delaware, USA). RNA quality was checked using Agilent bioanalyzer (Agilent) using
160 electrophoretic trace method. One microgram of the total RNA was treated with DNase I
161 amplification grade (Invitrogen™, Life Technologies). Reverse transcription was performed
162 using 200 U M-MLV reverse transcriptase (Invitrogen™) and first strand of complementary
163 DNA (cDNA) was generated using 12.5 ng/µl of random primers (Invitrogen™), dNTPs (10
164 mM) and 40 U of RNase OUT (Invitrogen™), following manufacturer's instruction.

165 **2.4. Quantification at the transcript level**

166 Specific primers (forward and reverse) for different transporters are listed in Table 1.
167 Quantitative real-time PCR analyses (qRT-PCR) were performed using the LightCycler® 480
168 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany) with 2X
169 LightCycler-FastStart DNA Master SYBER-Green I™ Mix (Roche), forward and reverse
170 primers (at a final concentration of 0.5 µM) and cDNA. The qRT-PCR conditions were:
171 denaturation at 95 °C for 10 min, followed by 45 cycles of repeated amplification (95 °C, 10 s),
172 hybridization (60 °C, 10 s) and elongation (72 °C, 10 s), and a final step at 40 °C for 30 s. A
173 melting curve program was performed to control the amplification specificity, and the
174 amplification products were sequenced. *Ef1α* (encoding elongation factor 1α) was used as
175 reference gene as in previous studies performed on salinity challenged sea bass (Blondeau-Bidet

176 et al., 2016; Lorin-Nebel et al., 2006) and as recommended by Mitter et al. (2009). Ultra-pure
177 water was used as a no-template control in the qRT-PCR. Efficiencies were determined and
178 given in Table 1. The relative expression ratio of each target gene was calculated using the ΔCt
179 method with the formula: Efficiency $(E)^{-\Delta Ct}$ and the efficiency of each primer pair.

180 **2.5. Statistical analysis**

181 Statistical analyses were performed using Graphpad Prism (version 6, GraphPad Software
182 Incorporated, La Jolla, CA, 268 USA). Outliers were identified using the ROUT method based
183 on the False Discovery Rate (with a Q set at 1%). Normality and homogeneity of variance were
184 respectively checked using D'Agostino-Pearson test and Bartlett test. For nka *a1a, cftr, clc-3,*
185 *rhcg1*, *nkcc1*, *nhe3, ncc2a* and *vha-a,* data were square root transformed to fit homogeneity of
186 the variance assumption. Two-way factorial analysis of variance with temperature and salinity as
187 the main factors was performed. Critical differences between groups were appraised using the
188 Fisher's least-square difference test. Linear correlations were determined using the Spearman
189 correlation (Two-tailed). Data are presented as box and whisker plots showing median, minimum
190 and maximum values. Level of statistical significance was set at $p<0.05$. Linear correlation
191 analysis was carried out with data from relative mRNA expression levels from this study and
192 physiological parameters (plasma Na^+ and Cl^- levels, plasma Na^+/Cl^- ratio) recently reported in
193 Masroor et al. (2018) obtained in the same fish (Table 3).

194

195 **3. Results**

196 **3.1. Quantitative gene expression**

197 For *nka α1a* (*atp1a1a)* and *nka α1b* (*atp1a1b)*, there was a significant salinity effect (Table 2,
198 two-way ANOVA, $p<0.01$). Temperature and interaction between both parameters (salinity and
199 temperature) exerted a significant effect only on *nka α1a* expression (Table 2, two-way ANOVA,
200 $p<0.01$). In temperate conditions (18 °C), fish challenged to FW exhibited a 2.2 fold significantly
201 higher expression of *nka α1a* compared to SW controls (Fig. 1A, Table 4). In warm conditions
202 (24 °C), *nka α1a* expression was not significantly different between salinities (Fig. 1A).
203 Moreover, a significant higher *nka α1a* expression was measured in SW warm compared to SW
204 temperate.

205 On the other hand, *nka α1b* expression did not change in temperate conditions between SW and
206 FW (Fig. 1B). Conversely, a significant higher *nka α1b* expression was recorded in warm
207 conditions in SW *vs* FW- exposed fish (Fig. 1B).

208 For *cftr* and *nkcc1* (*slc12a2)*, there was a significant salinity (*cftr*, $p<0.0001$; *nkcc1*, $p<0.001$) and
209 temperature effect (*cftr*, $p<0.0001$; *nkcc1*, $p<0.05$) but the interaction between temperature and
210 salinity did not exert an effect on both of these genes (Table 2, two-way ANOVA). *Cftr*
211 expression was significantly lower in FW than in SW at both temperatures (Fig. 1C). In both
212 salinities, fish acclimated to 24 °C had a significantly higher expression of *cftr* than at 18 °C.
213 Regarding *nkcc1*, fish challenged to FW had a significantly lower expression than SW fish, at
214 both tested temperatures. *Nkcc1* expression did not change significantly between both tested
215 temperatures (Fig. 1D) in the FW- and SW-exposed groups.

216 For *ncc2a* (*slc12a3-like)* and *nhe3* (*slc9a3)*, there was a significant salinity (*ncc2a*, $p<0.0001$;
217 *nhe3*, $p<0.01$) and temperature effect ($p<0.01$). Interaction between both parameters did not exert
218 a significant effect (Table 2, two-way ANOVA). In temperate conditions, fish exhibited a

219 significantly higher expression of *nhe3* in FW compared to SW. In warm conditions however, no
220 differences were observed in the expression level of *nhe3* between both salinities (Table 4). In
221 SW warm conditions, fish showed a significantly higher expression of *nhe3* compared to fish
222 acclimated to SW temperate conditions (Fig. 1E). In temperate and warm conditions, *ncc2a*
223 expression was significantly higher in FW compared to SW (Fig. 1F). In SW, *ncc2a* expression
224 was higher in warm compared to temperate conditions (Fig. 1F, Table 4).

225 For *clc-3* (*clcn3),* the temperature ($p<0.0001$) was the only factor exerting a significant effect
226 (Table 2, two-way ANOVA). *Clc-3* expression was 2 and 1.6 fold higher at 24 °C compared to
227 18 °C in SW and FW, respectively (Fig. 1G).

228 Regarding expression of *vha-a* (*atp6v1a*), there was a significant salinity ($p<0.01$) and
229 temperature effect ($p<0.0001$) but the interaction between temperature and salinity was not
230 significant (Table 2, two-way ANOVA). In the case of *vha-b* (*atp6v1b2*), the temperature
231 ($p<0.05$) is the only factor exerting a significant effect (Table 2, two-way ANOVA). In
232 temperate conditions, no differences were observed between salinities. In warm conditions
233 however, significantly lower *vha-a* expression was detected in FW compared to SW. *Vha-a*
234 expression was 2 and 1.6 fold higher at 24 °C compared to 18 °C in SW and FW respectively
235 (Fig. 1H). *Vha-b* expression was significantly higher in fish challenged to 24 °C in SW,
236 compared to all other conditions (Fig. 1I).

237 For *rhbg*, there is no effect of neither salinity nor temperature (Table 2, two-way ANOVA, Fig.
238 1J) and no difference in *rhbg* expression was observed between all analyzed conditions. In case
239 of *rhcg1,* there was a significant salinity ($p<0.05$) and temperature effect ($p<0.0001$) but the
240 interaction between temperature and salinity did not exert an effect on *rhcg1* expression (Table

241 2, two-way ANOVA). No difference in *rhcg1* expression was observed between salinities in
242 temperate and warm conditions. Mean *rhcg1* expression was significantly increased by 4 and 3.3
243 fold at 24 °C compared to 18 °C in SW and FW, respectively (Fig. 1K). In warm conditions and
244 notably in SW at 24 °C, a high variability was observed.

245 **3.2 Plasma pH**

246 Mean plasma pH are shown in Fig. 1S. There was a significant salinity ($p<0.05$) and temperature
247 ($p<0.0001$) effect as well as a strong interaction between the two parameters ($p<0.001$) (Table 2,
248 two-way ANOVA). A significant 5% increase from 8.25 ± 0.23 to 8.66 ± 0.13 (mean ± SD) in
249 plasma pH was observed in temperate conditions following FW transfer (Table 4). No significant
250 differences were observed following FW transfer in warm conditions (24 °C) (pH=8.13 ± 0.27 in
251 SW and 8.19 ± 0.18 (mean ± SD) in FW).

252

253 **3.3. Correlations**

254 mRNA levels of transporters that are involved in ion secretion (*cftr* and *nkcc1*) and of key
255 pumps, *nka α1b* and *vha-a*, showed a positive correlation with plasma Cl^- level and negative
256 correlation with Na^+/Cl^- ratio, as shown in Table 3. mRNA levels of transporters involved in ion
257 uptake, *nhe3* and *ncc2a,* showed a negative correlation to plasma Cl^- and Na^+ level. mRNA
258 levels of *nka α1b*, *clc-3* and *vha-a* and *b* are negatively correlated to Na^+/Cl^- ratio. *Rhcg1* was
259 negatively correlated to plasma Na^+ levels. *Nka α1a* and *rhbg* were not correlated to the analyzed
260 blood parameters.

261

262 **4. Discussion**

263 European sea bass raised in SW were acclimated to two environmental relevant temperatures, 18
264 °C and 24 °C, in order to investigate the molecular mechanisms underlying acclimation to warm
265 temperature followed by a salinity decrease. The results showed that increased temperature
266 affected the expression of several genes involved in osmoregulation, acid-base balance and
267 ammonia excretion in gills, at both tested salinities.

268 In this study, we confirm previous results with higher branchial *nka α1a* mRNA levels in FW
269 compared to SW in temperate conditions (Blondeau-Bidet et al., 2016; Jensen et al., 1998). In
270 warm conditions however, *nka α1a* expression was similar between both salinities, which
271 partially explain changes observed previously at the protein activity level (Masroor et al., 2018).
272 NKA activity was increased by 1.72 fold in warm conditions following FW transfer (Table 4) but
273 was overall lower than in temperate conditions (Masroor et al., 2018). Despite *nka α1a* has been
274 identified as a main *nka α1* paralog in sea bass osmoregulatory tissues (Blondeau-Bidet et al.,
275 2016), other paralogous genes encoding for NKA (*nka α2, β,* …) may also change in warm
276 conditions and contribute to overall branchial ion transport capacities (Vargas-Chacoff et al.,
277 2018), but further analyses remain to be done to support this hypothesis in European sea bass.
278 Branchial *nka α1b* mRNA expression seems to be different only between salinities in warm
279 conditions with higher expression in SW than in FW. This tendency has already been shown in
280 long-term (2.5 years) acclimated European sea bass to FW *vs* SW controls (Blondeau-Bidet et
281 al., 2016). We did not observe a switch from *nka α1b* to *nka α1a* following FW transfer, a
282 phenomenon previously reported in salmons (McCormick et al., 2009). Recently, Vargas-
283 Chacoff et al. (2018) showed that temperature affect NKAα1b levels in salmon smolts
284 challenged from 14 °C to 24 °C in SW. In sea bass, it seems that significant differences in *nka*

285 *α1b* mRNA expression between SW and FW are observed only under specific circumstances as
286 long-term acclimation to extreme salinities (Blondeau-Bidet et al., 2016) or increased
287 temperatures (this study). It is known in the literature that *nka* expression is modulated by
288 temperature change (Michael et al., 2016a; Mitrovic and Perry, 2009; Nilsen et al., 2007) as it
289 seems to be the case in our study in SW notably. Posttranscriptional processes as differential
290 NKA phosphorylation might also be worth investigating in warm *vs* temperate conditions
291 (Féraille et al., 1999).

292

293 **4.1. Ion excretion mechanisms**

294 In SW, NKA generates the driving force for ion excretion involving basolateral NKCC1 and
295 apical CFTR (Evans et al., 2005). *Nkcc1* and *cftr* mRNA levels are higher in SW compared to
296 FW when temperature factor is the same, as previously shown in sea bass maintained in
297 temperate conditions (Bodinier et al., 2009; Lorin-Nebel et al., 2006) and numerous other teleost
298 species (Hiroi et al., 2005; Inokuchi et al., 2017; McCormick et al., 2003; Nilsen et al., 2007).
299 Contrary to *nkcc1*, whose expression is strongly affected by salinity only and to a much lesser
300 extent by temperature, there seems to be an additive effect of high salinity and increased
301 temperature on *cftr* expression. Thus, fish exposed to both, high salinity and high temperature
302 (SW, 24° C) have greater *cftr* expression levels than fish exposed to SW without temperature
303 increase. Bodinier et al. (2009) has shown the presence of apical CFTR in gill ionocytes of sea
304 bass maintained in temperate SW. Previous studies have shown no changes in ionocyte density
305 between SW conditions comparing both temperatures (Masroor et al., 2018). The high *cftr*
306 expression observed in warm SW thus suggests an enhanced *cftr* expression in those cells

307 without necessarily an increased number of ionocytes. CFTR might also be expressed in
308 pavement cells in warm conditions, as it is the case in killifish *F. heteroclitus*, or it could be sub-
309 apically localized as shown in this same species (Marshall et al., 2002). Our results show that
310 CFTR seems to be a key player in contributing to the maintenance of Cl^- balance in warm SW-
311 acclimated fish as it has been suggested in temperate conditions (Evans et al., 2005; Bodinier et
312 al., 2009), however further studies are necessary notably by analyzing protein CFTR levels in
313 warm-acclimated sea bass.

314

315 **4.2. Ion uptake mechanisms**

316 Sea bass gills display remarkable plasticity when it comes to adjusting ion transport in response
317 to salinity changes (Masroor et al., 2018). The switch from hypo- to hyper-osmoregulation is
318 achieved by the activation of ion transporters and channels that are involved in ion uptake
319 (Blondeau-Bidet et al., 2019; Hwang et al., 2011; L'Honoré et al., 2019). Other than NKA, key
320 transporters involved in branchial Cl^- and/or Na^+ uptake, such as *ncc2a* and *nhe3*, are negatively
321 correlated to plasma Na^+ and Cl^- levels, as expected, and higher expressed in temperate FW *vs*
322 SW conditions. These transporters are apically localized in sea bass FW-type ionocytes and are
323 thus essential for transepithelial Cl^- and/or Na^+ uptake (Blondeau-Bidet et al., 2019; Inokuchi et
324 al., 2017). Few studies have investigated the interactive effects of temperature and salinity on
325 these transporters. In a recent study on stickleback *Gasterosteus aculeatus* from a marine
326 ecotype, the combination of low salinity and low temperature (4 °C and 0.3 ppt) had no
327 interactive effect on the expression of *nhe3*, contrary to *nka*, where an additive effect was
328 observed (Gibbons et al., 2018). In European sea bass, no additive effect was observed for both

329 of these genes and similar patterns of expression were observed for *nhe3* and *nka α1a* (Fig. 1A,
330 E). Both transporters showed an increased expression in warm SW conditions *vs* temperate SW
331 but no change in expression was observed between salinities in warm conditions (Table 4). No
332 apical NHE3 expression has been observed so far in temperate SW conditions (Blondeau-Bidet
333 et al., 2019) and protein localization should be investigated further, notably in SW warm
334 conditions. *Ncc2a* is upregulated upon warm temperature acclimation, in SW notably. Contrary
335 to *nhe3* and *nka α1a*, there is a significant difference in *ncc2a* expression between salinities in
336 warm conditions and an average mRNA fold change of 1.6 following FW transfer (Table 4).
337 This suggests a differentiation of *ncc2a* expressing cells to take up Cl^- and Na^+. In previous
338 studies we have observed a 883-fold and 10- fold increase of lamellar ionocytes following FW
339 challenge in temperate and warm conditions respectively (Table 4) (Masroor et al., 2018).
340 Lamellar ionocytes are essentially NHE3-type cells whereas NCC2-type cells have been
341 essentially detected on gill filaments (Blondeau-Bidet et al., 2019). This lower density in
342 lamellar ionocytes (NHE3-type) might partially be linked to the lack of *nhe3* increase in warm
343 FW conditions. It is likely that the proportion of NCC- *vs* NHE3-type cells is different in warm
344 FW conditions, but this needs to be investigated further at protein level.

345 In pufferfish *Tetraodon nigroviridis* gills, *clc-3* mRNA expression did not change between FW
346 and SW groups (Tang et al., 2010) as we have also shown in this study in sea bass maintained at
347 18 °C. This differs from previous results in sea bass, where *clc-3* mRNA expression was lower in
348 FW than in SW whereas protein levels seemed to be higher in FW (Bossus et al., 2013; Tang et
349 al., 2010). In tilapia, *clc-3* mRNA expression was higher in deionized water in comparison to
350 FW and SW (Tang et al., 2010) and several studies suggest an involvement of ClC-3 in
351 basolateral Cl^- uptake in FW (Bossus et al., 2013; Tang and Lee, 2011, 2007). In this study, we

352 showed that *clc-3* expression is strongly affected by temperature but not by salinity. The high
353 *clc-3* expression in warm conditions might be linked to an overall increased ion transport and
354 increased need to regulate cell volume (Duan et al., 1997, 1999; Hermoso et al., 2002).
355 Interestingly, in our previous study, ionocyte cell area was significantly lower in FW warm
356 compared to the three other conditions (Masroor et al., 2018). These same fish also displayed
357 higher plasma chloride levels than in FW temperate conditions (Masroor et al., 2018) suggesting
358 that chloride uptake mechanisms are efficient. No difference in ionocyte cell volume was
359 observed in SW conditions between the two temperature regimes which suggest an efficient
360 ionocyte cell volume regulation in warm SW, maybe partially achieved by the increased ClC-3
361 expression as well as other cell volume-regulating proteins as aquaporins (Madsen, 2012) that
362 have not been analyzed in this study.

363

364 **4.3. Effect of temperature on acid-base regulation**

365 It is well known in marine fish that acid-base homeostasis is mainly regulated at the gill and
366 kidney levels (Heuer and Grosell, 2014) and in larvae at the integument level (Burggren and
367 Bautista, 2019). The link between ionic regulation and acid-base balance with regard to low pH
368 has been investigated in several species (Dymowska et al., 2012; Kwong et al., 2014). Plasma pH
369 levels observed in this study were high compared to the literature. In Shrivastava et al. (2019),
370 sea bass blood pH ranged from around 7.8 at 32 ppt to 7.6 at 2.5 ppt following a three-week
371 salinity challenge. The high levels we measured might be linked to two successive
372 freezing/thawing processes. Absolute values are therefore not compared, however, all samples
373 have been treated similarly and it seems relevant to compare pH changes among treatments. As

374 for the increased plasma Na^+/Cl^- ratio measured previously (Masroor et al., 208), a significantly
375 higher pH was recorded following FW transfer in temperate conditions clearly indicating a
376 metabolic alkalosis. An increased plasma pH following FW transfer might indicate that sea bass
377 are not fully acclimated to FW after 2 weeks as previously suggested (Jensen et al., 1998).
378 Moreover, L'Honoré et al. (2019) have shown differential FW tolerance in sea bass from the
379 West Mediterranean lineage. In 8-month-old fish, some animals were intolerant to FW and
380 displayed increased plasma Na^+/Cl^- ratios indicating a metabolic alkalosis as shown in this study.
381 It has to be noted that fish from this study are 14 months-old and do not show any other sign of
382 FW intolerance. At the gill level, acid secretion is thought to be coupled to Na^+ uptake either
383 through a Na^+/H^+ exchanger (NHE3 or NHE2) or VHA. In SW, the model with NHEs is more
384 likely given the favorable Na^+ gradient for Na^+/H^+ exchangers. The increased mRNA expression
385 of *nhe3* as well as *vha-a* and *vha-b* in SW warm conditions could be a response to the lower
386 blood Na^+/Cl^- ratio previously shown (Masroor et al., 2018). A low blood Na^+/Cl^- ratio is
387 generally an indicator of metabolic acidosis triggering acid excretion mechanisms (Heuer and
388 Grosell, 2014; Michael et al., 2016b). In 24 °C acclimated sea bass, there is no metabolic
389 acidosis as plasma pH levels are not different from temperate conditions. An overexpression of
390 *nhe3* together with carbonic anhydrase 2 (*ca2*) and Na^+/H^+ exchanger (*nbc1*) has been reported
391 in Osorezan dace *Tribolodon hakonensis* gills when fish were challenged to acidic waters
392 compared to fish maintained in neutral waters (Hirata et al., 2003). In this latter species, apical
393 NHE3 clearly participates to acid excretion as well as in other species studied (Hiroi et al., 2008;
394 Inokuchi et al., 2008; Ivanis et al., 2008). In zebrafish maintained in acidic FW, *vha-a* mRNA
395 expression as well as the density of VHA-enriched ionocyte subtype (called HR cells) were
396 increased following 7 days of acid exposure (Chang et al., 2009). *Vha-a* is negatively correlated

397 to the Na^+/Cl^- ratio (r= -0.6471, p<0.0001) and may be involved in proton excretion in sea bass
398 gills, however the subcellular localization of VHA is not yet clear in this species. Apical
399 (Sullivan et al., 1995; Yan et al., 2007) as well as basolateral (Catches et al., 2006; Malakpour
400 Kolbadinezhad et al., 2018; Uchiyama et al., 2012) localization of VHA has been reported in
401 fish. Basolateral VHA, by pumping protons out of the cell to the blood, would generate a
402 favorable electrochemical gradient for apical Cl^-/HCO_3^- exchange and could thus participate to
403 HCO_3^- excretion and chloride uptake (Piermarini and Evans, 2001) whereas apical localization
404 would rather be involved in acid secretion and might be rather functionally linked to a
405 basolateral anion exchanger (AE1) secreting Cl^- in exchange of HCO_3^- (Liu et al., 2016).
406 Interestingly, a high correlation is observed between *vha-a* and blood chloride levels (r= 0.903,
407 p<0.001) but further analyses are needed to fully understand if there is a functional link between
408 VHA and chloride levels.

409

410 **4.4. Effect of temperature on nitrogen excretion**

411 In a previous study on sea bass challenged to FW temperate conditions, *rhcg1* was significantly
412 upregulated compared to SW temperate but *rhbg* did not change between SW and FW conditions
413 (Blondeau-Bidet et al., 2019). This suggests a different handling for nitrogen excretion when
414 comparing different salinity regimes (Frick and Wright, 2002). In our study, we can observe a
415 slightly but not significantly increased *rhcg1* expression in FW compared to SW. More
416 strikingly, a significant upregulation of *rhcg1* was observed in warm temperature-challenged fish
417 at both salinities. In gill ionocytes, RHCG1 might operate in concert with NHE3 and VHA at the
418 apical cell part as shown in other species, but this remains to be analyzed further in sea bass

419 (Heuer and Grosell, 2014; Nawata et al., 2010). In this study, temperature did not affect *rhbg*
420 expression. In longjaw mudsucker *Gillichthys mirabilis* maintained in SW, warm temperatures
421 (28 °C) seemed to enhance the expression of different transport related genes including branchial
422 *rhbg* and *vha* compared to lower temperature groups (9 °C and 19 °C) and suggested an
423 increased ammonia excretion in warm conditions (Logan and Somero, 2010). Nawata et al.
424 (2010) showed that in seawater maintained pufferfish (*Takifugu rubripes*) exposed to ammonia,
425 gill *rhcg1*, *vha, nkcc1, nka* and *nhe3* were upregulated, which suggests a tight cooperation
426 between different ion transporters expressed in ionocytes under high ammonia. The involvement
427 of Rhesus proteins (Rh) in ammonia transport processes remains to be clarified in sea bass gills
428 as well as the functional link with other ion transporters, as VHA and NHE3 (Nawata et al.,
429 2007, 2010). However, our data strongly point to a role of *rhcg1* in ammonia excretion in warm
430 temperatures, probably linked to increased metabolism, as shown previously in the same sea bass
431 lineage challenged to increased temperatures (Claireaux and Lagardère, 1999). In another study,
432 Person-le-Ruyet et al. (2004) have shown in the same species a 3 times increased mean daily
433 ammonia excretion rate at 25 °C compared to 13 °C which is in accordance with our data.
434

435 **5. Conclusions**

436 In this study focusing on the mechanisms involved in salinity acclimation at two different
437 temperatures, we showed that branchial transcript levels of most analyzed transporters were
438 significantly affected by warm temperatures. We showed a more striking effect of temperature
439 on gene expression patterns in SW warm conditions compared to FW warm. Increased *rhcg1*
440 mRNA expression points to a potential up-regulation of ammonia excretion as a response to
441 enhanced metabolism in warm conditions. Data also support an activation of chloride secretion

442 in warm sea water contributing to the maintenance of the chloride balance. Moreover, an
443 increased expression in proteins involved in acid excretion (*nhe3* and *vha-a, and vha-b*) points to
444 an activation of acid secretion pathways to maintain plasma pH constant. Following FW transfer,
445 a differential response at both temperatures was observed regarding the expression of
446 transporters involved in Na^+ *vs* Cl^- uptake. These results as well as data obtained on blood
447 parameters (Masroor et al., 2018) indicate physiological as well as molecular modifications at
448 the gill level following a two-week acclimation at 24 °C.

449

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454

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Figure legend

Fig. 1. Box and whisker plot showing the median, minimum and maximum mRNA expression level of *nka α1a* (*atp1a1a*) (A), *nka α1b* (*atp1a1b*) (B), *cftr* (C), *nkcc1* (*slc12a2*) (D), *nhe3* (*slc9a3*) (E), *ncc2a* (*slc12a3-like*) (F), *clc-3 (clcn3)* (G), *vha-a* (*atp6v1a*) (H), *vha-b* (*atp6v1a*) (I), *rhbg* (J) and *rhcg1* (K) in gills of sea bass exposed to FW and SW at 18 °C (temperate) and 24 °C (warm). mRNA levels were normalized to *ef1α*. Different letters indicate significant differences between conditions (two-way ANOVA followed by a Fisher Least Significant Difference (LSD) post hoc test $p<0.05$, N= 8-13). FW: fresh water; SW: seawater.

Fig. 1S. Mean plasma pH in sea bass exposed to FW and SW at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant differences between conditions (two-way ANOVA followed by a Fisher Least Significant Difference (LSD) post hoc test $p<0.05$, N= 8-13).

Table 1: Sequences and efficiencies of the primers used for qRT-PCR. F: forward primer; R: reverse primer; Sequence ID: identification number from sea bass genome or GenBank identification number.

Sequence ID	Target gene	Primer name	Sequence (from 5′ to 3′)	Amplicon size	Efficiency	Reference
KP400258	*atp1a1a*	NKAα1a F NKAα1a R	CCTCAGATGGCAAGGAGAAG CCCTGCTGAGATCGGTTCC	146	1.89	(Blondeau-Bidet et al., 2016)
KP400259	*atp1a1b*	NKA α1b F NKA α1b R	AGCAGGGCATGAAGAACAAG CCTGGGCTGCGTCTGAGG	204	1.99	(Blondeau-Bidet et al., 2016)
DQ501276	*Cftr*	CFTR F CFTR R	GACTGATGCGTTCGGTAG CCTCAATGACATCTCCTTC	215	1.917	(Bodinier et al., 2009)
DLAgn_00080120	*slc12a2*	NKCC1 F NKCC1 R	TCAGCTCACAGTTCAAGGCC TTGTGGAGTCCATAGCGGC	102	2.08	(Lorin-Nebel et al., 2006)
JN998891	*clcn3*	ClC-3 F ClC-3 R	CAAGTACAGCAAGAACGAGGC ACAGCGTCTTGAGAGGGAAG	146	2.069	(Bossus et al., 2013)
DLAgn_00204050	*slc9a3*	NHE3 F NHE3 R	GGATACCTCGCCTACCTGAC AAGAGGAGGGTGAGGAGGAT	251	1.98	(Blondeau-Bidet et al., 2019)
DLAgn_00038210	*slc12a3-like*	NCC2a F NCC2a R	ATGATGAGCCTCTTCGAGCC ACAGAAGGTGATGAGAGCAGC	278	1.94	
DLAgn_00076370	*atp6v1a*	VHA-A F VHA-A R	GGCAGTCACATCACAGGAGG CCAGCTCCATCACCACATCG	154	1.98	
DLAgn_00018050	*atp6v1b2*	VHA-B F VHA-B R	TTGCCATAGTCTTCGCAGCC CTTCTCGCACTGGTAGGC	194	1.90	
DLAgn_00222650	*Rhbg*	RHBG F RHBG R	CCTCATGGTGACCCGAATCC TATGTGGACAGAGTGCAGGC	218	1.97	
DLAgn_00166370	*rhcg1*	RHCG1 F RHCG1 R	TCAGGGAATTGTGTGACCGC CCCAGCGTGGACTTGATTCT	118	2.01	
AJ866727	*ef1α*	EF1-F EF1-R	GGCTGGTATCTCTAAGAACG CCTCCAGCATGTTGTCTCC	239	2.09	(Lorin-Nebel et al., 2006)

Table 2: Two-way ANOVA results of gill gene expression data and plasma pH with salinity and temperature as the main factors. ns: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 per condition.

Genes	Interaction	Salinity	Temperature
nka α1a (*atp1a1a*)	**	**	**
nka α1b (*atp1a1b*)	Ns	**	ns
cftr	Ns	****	****
nkcc1 (*slc12a2*)	Ns	***	*
nhe3 (*slc9a3*)	Ns	**	**
ncc2a (*slc12a3-like*)	Ns	****	**
clc-3 (*clcn3*)	Ns	ns	****
vha-a (*atp6v1a*)	Ns	**	****
vha-b (*atp6v1b2*)	Ns	ns	*
Rhbg	Ns	ns	ns
rhcg1	Ns	*	****
pH	***	*	****

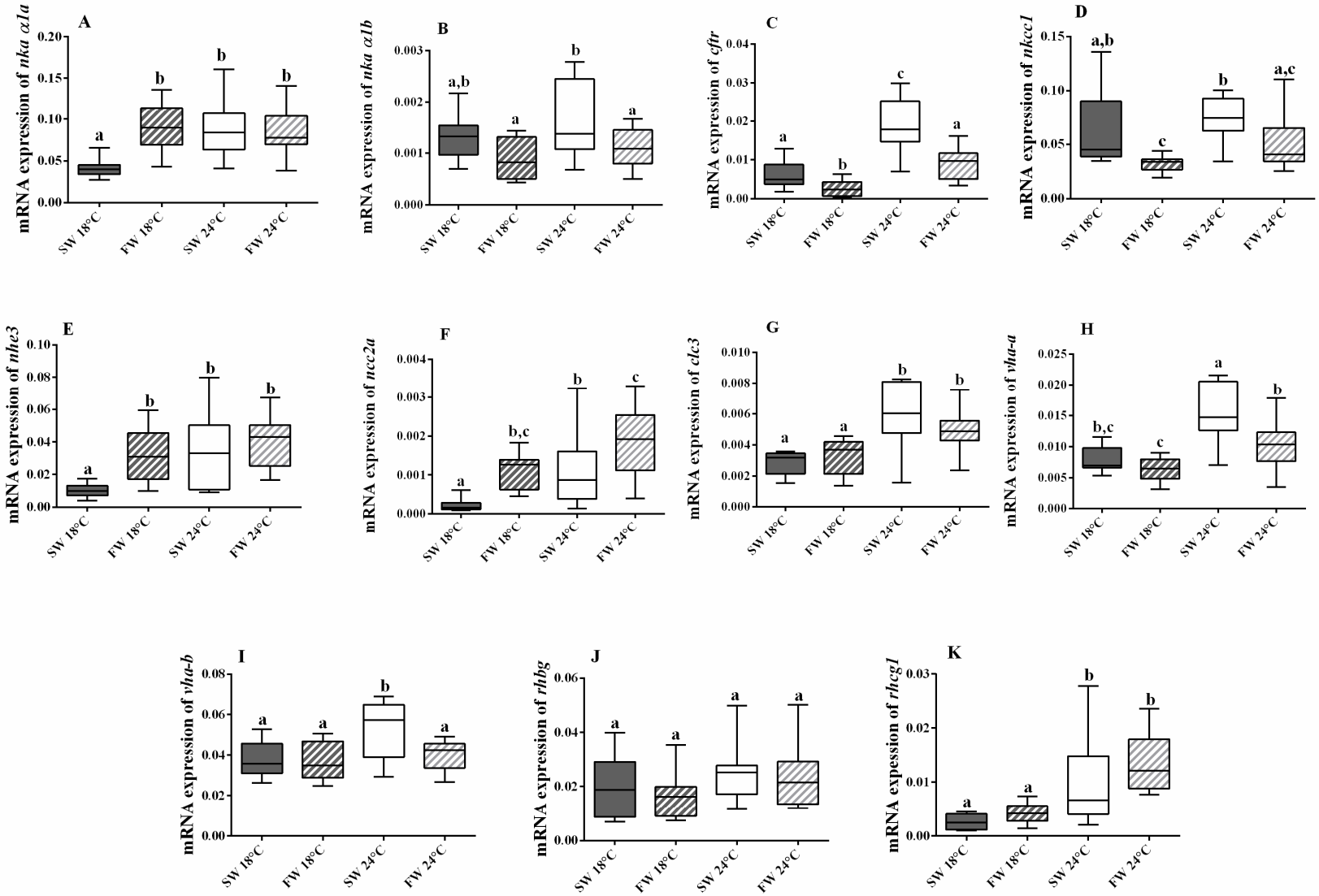
Table 3: Spearman correlation for gill gene expression *vs* plasma Cl^-, Na^+ and Na^+/Cl^- ratio (these data have been obtained from the same fish obtained in Masroor et al. (2018)). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 per condition.

Genes	Cl^- level ($mmol.L^{-1}$)	Na^+ level ($mmol.L^{-1}$)	Na^+/Cl^- ratio
nka α1a (*atp1a1.a*)	-0.2798	-0.2858	-0.02371
nka α1b (*atp1a1.b*)	0.3303*	-0.01329	-0.3031*
Cftr	0.5442***	0.07930	-0.3796*
nkcc1 (*slc12a2*)	0.4615**	-0.05339	-0.3365*
nhe3 (*slc9a3*)	-0.3059*	-0.3437*	0.005587
ncc2a (*slc12a3-like*)	-0.3913**	-0.4037**	-0.02552
clc-3 (*clcn3*)	0.1989	-0.2980	-0.4097**
vha-a (*atp6v1a*)	0.4903***	-0.2927	-0.6471****
vha-b (*atp6v1b2*)	0.2822	-0.2027	-0.3201*
rhbg	0.1040	-0.09353	-0.07771
rhcg1	-0.1198	-0.3655*	-0.1780

Table 4 : Fold changes for mean relative mRNA levels regarding genes involved in ion uptake (*nka α1a*, *ncc2a* and *nhe3*), Na^+/K^+-ATPase activity, ionocyte density at filament and lamellar level (data have been obtained from the same fish obtained in Masroor et al. (2018)) and plasma pH changes (indicated in % of change of mean pH values). Comparisons are done in sea bass following freshwater (FW) transfer at temperate (18 °C) and warm (24 °C) conditions.

	Gene name	Relative mRNA levels	NKA activity (µmol Pi. mg^{-1} protein. h^{-1}	Filament ionocytes (number/300 µm)	Lamellar ionocytes (number/300 µm)	Plasma pH
FW transfer at 18 °C	*nka α1a* (*atp1a1a*)	2.23	1.70	1.70	883.62	+5.00
	nhe3	3.19				
	ncc2a	5.20				
FW transfer at 24 °C	*nka α1a* (*atp1a1a*)	0.98	1.68	1.29	9.70	-0.83
	nhe3	1.26				
	ncc2a	1.61				

Fig. 1


A
mRNA expression of nka α1a
B
mRNA expression of nka α1b
C
mRNA expression of cftr
D
mRNA expression of nkcc1
E
mRNA expression of nhe3
F
mRNA expression of ncc2a
G
mRNA expression of clc3
H
mRNA expression of vha-a
I
mRNA expression of vha-b
J
mRNA expression of rhbg
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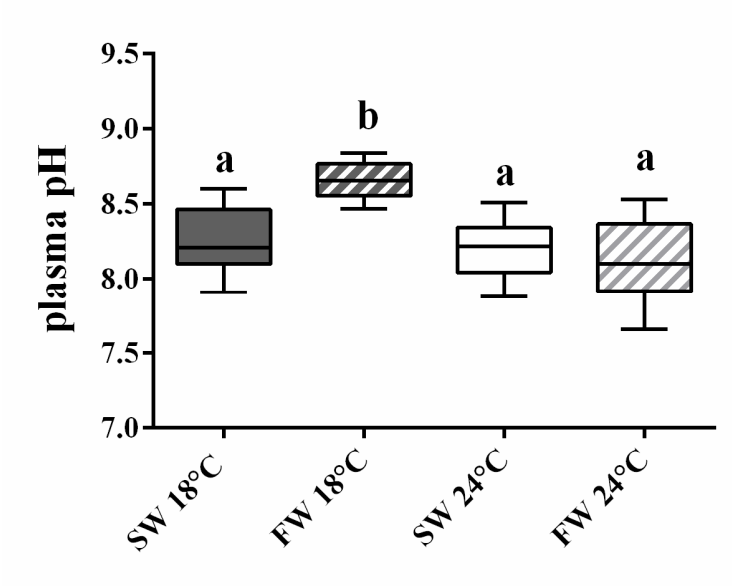
Fig. 1S