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

Masroor Waliullah [1], Farcy Emilie [1], Blondeau-Bidet Eva [3], Venn Alexander [2], Tambutté Eric [2], Lorin-Nebel Catherine [1, *]

[1] Univ Montpellier, CNRS, IFREMER, IRD, UM, MARBEC, France
[2] Centre Scientifique de Monaco, Marine Biology Department, 8 Quai Antoine 1er, 98000, Monaco

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.

Highlights

► The expression of various branchial ion transporters is increased at 24 °C. ► The induction of ion transport processes is less striking in fresh water. ► In warm seawater, CFTR seems to be a key player in the regulation of Cl^- balance. ► Transporters regulating H^+ and nitrogen excretion are highly expressed at 24 °C.

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;

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

have been reported in numerous species following salinity transfers, but the effect of increased or decreased temperature on the expression of these genes in different salinity conditions is less known (Chou et al., 2008; Gibbons et al., 2018; Hu et al., 2016; Kyprianou et al., 2010; Logan and Buckley, 2015; Logan and Somero, 2011; Michael et al., 2016a; Morrison et al., 2006).

Several paralogs exist in fish for *nka α1* with potentially different functions and expression patterns depending on the considered species (Hwang and Lee, 2007). Two paralogs have been identified in sea bass, *nka α1a* and *nka α1b*, with *nka α1a* being the most expressed paralog in sea bass gills whatever the considered salinity (Blondeau-Bidet et al., 2016). Temperature generally increases NKA transcript expression and protein activity but the response highly depends on the species and even the population (Michael et al., 2016a; Morrison et al., 2006). Michael et al. (2016a) have reported population-specific differences regarding mRNA expression levels of *nka* in cod *Gadus morhua* populations maintained at the same temperature but originating from a different thermal niche. The European sea bass *Dicentrarchus labrax* (Linnaeus 1758) is an important aquaculture species along the Mediterranean and Atlantic coasts. It is considered as a highly eurythermal (tolerates temperatures from 4 to 35 °C) and euryhaline species (tolerating from FW up to 70 ppt) (Barnabé, 1989; Dülger et al., 2012; Madeira et al., 2013). In the wild, sea bass adults and juveniles are frequently exposed to fluctuations of environmental parameters, notably during their stay in lagoons and estuaries (Dufour et al., 2009; Newton and Mudge, 2003). In these habitats, temperatures can reach values over 25 °C in the summer months. The Mediterranean basin is considered as one of the 'Hot-spots' by Giorgi (2006) and in the context of climate change, temperatures will continue to rise in the future. Temperature acclimation and preference have been studied in sea bass by investigating oxygen consumption (Dalla Via et al., 1998), fish distribution (Trancart et al.,

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

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

2. Material and Methods

2.1. Experimental conditions

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

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

2.2. Blood parameters, protein and cellular measurements

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

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

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

2.5. Statistical analysis

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

3. Results

3.1. Quantitative gene expression

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

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

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

For *ncc2a* (*slc12a3-like)* and *nhe3* (*slc9a3)*, there was a significant salinity (*ncc2a*, $p<0.0001$; *nhe3*, $p<0.01$) and temperature effect ($p<0.01$). Interaction between both parameters did not exert 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

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

3.2 Plasma pH

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

3.3. Correlations

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

4. Discussion

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

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

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

4.2. Ion uptake mechanisms

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

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

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

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

4.3. Effect of temperature on acid-base regulation

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

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

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

4.4. Effect of temperature on nitrogen excretion

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

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

5. Conclusions

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

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

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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	***	*	****

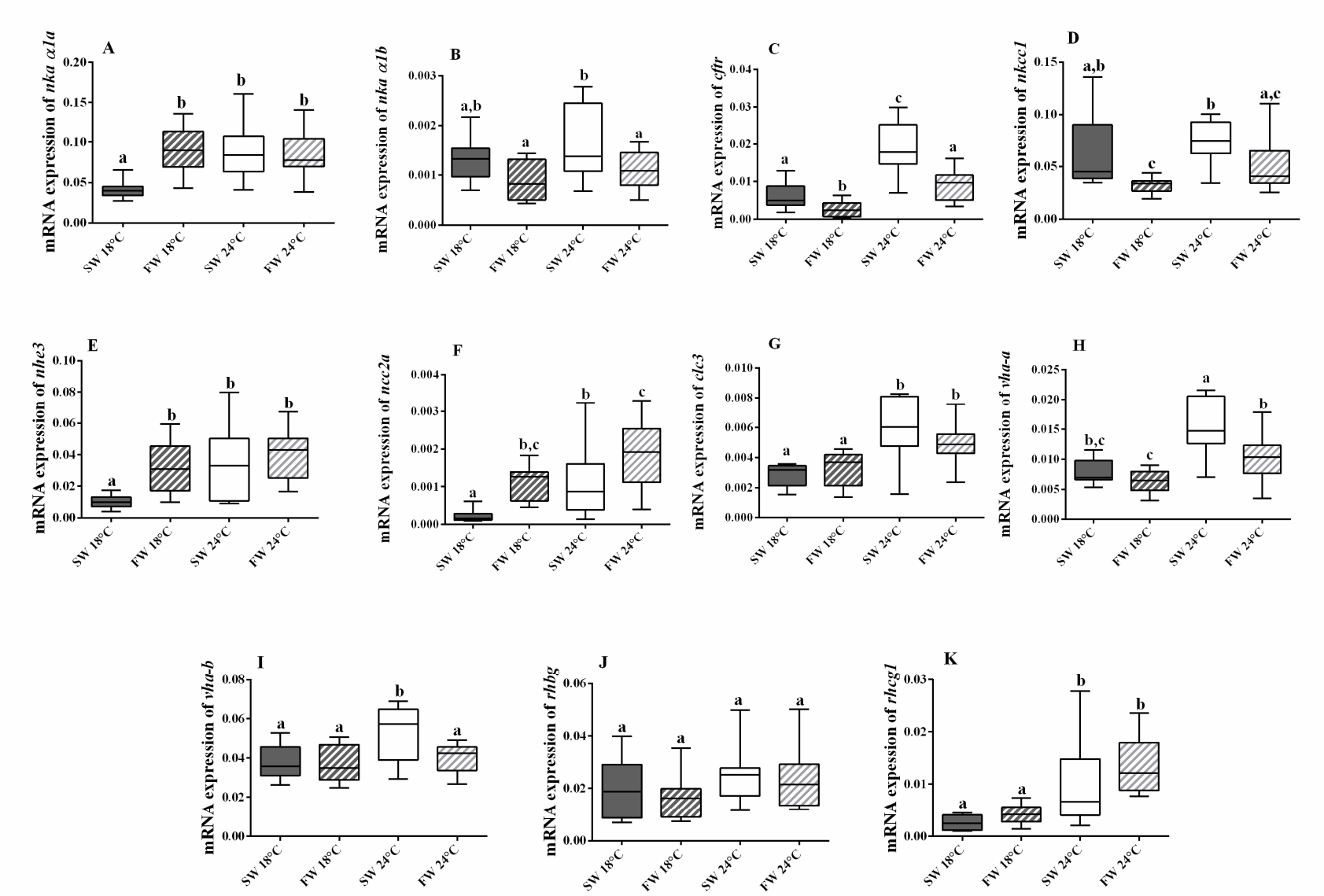
Table 3: Spearman correlation for gill gene expression *vs* plasma Cl^-, Na^+ and Na^+/Cl^- ratio (these data have been obtained from the same fish obtained in Masroor et al. (2018)). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 per condition.

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
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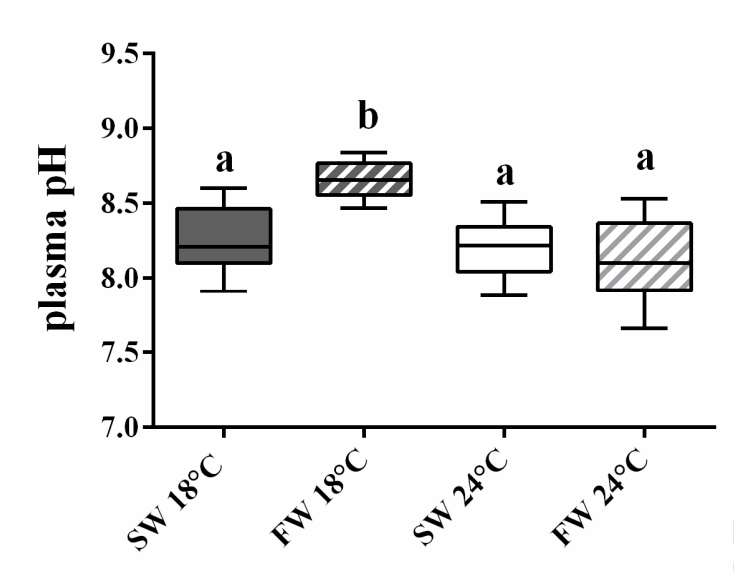
K
mRNA expression of rhcg1
SW 18°C
FW 18°C
SW 24°C
FW 24°C

Fig. 1S



Highlights:

- The expression of various branchial ion transporters is increased at 24 °C;
- The induction of ion transport processes is less striking in fresh water;
- In warm seawater, CFTR seems to be a key player in the regulation of Cl^- balance;
- Transporters regulating H^+ and nitrogen excretion are highly expressed at 24°C.