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Thibaut L'Honore, Emilie Farcy, Eva Blondeau-Bidet, Catherine Lorin-Nebel. Inter-individual variability in freshwater tolerance is related to transcript level differences in gill and posterior kidney of European sea bass. Gene, 2020, 741, pp.144547. 10.1016/j.gene.2020.144547 . hal-03411033

HAL Id: hal-03411033
https://hal.umontpellier.fr/hal-03411033v1

Submitted on 20 May 2022

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Version of Record: https://www.sciencedirect.com/science/article/pii/S037811192030216X
Manuscript_a5ce0c22d9ed2a4c533c1b4347093e2d

Inter-individual variability in freshwater tolerance is related to transcript level differences in gill and posterior kidney of European sea bass

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Key words

Intraspecific variation, osmoregulation, freshwater acclimation, ion transporters, SLC26A6, NKCC, PRLR

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Introduction

In transitional habitat such as lagoons, fish have to deal with a wide range of changing environmental parameters and therefore a high phenotypic plasticity is beneficial to cope with fluctuating environments. Salinity can rapidly drop in these habitats through freshwater (FW) supplies by rainfalls or rivers. Low-salinity environments can lead to differential distributions of marine species according to their acclimation capacity (Pierce et al., 2012; Wong et al., 1999). Differential habitat distribution has also been observed between individuals within the same species as in stickleback *Gasterosteus aculateus* and in mummichog, *Fundulus heteroclitus*, showing a differential capacity to regulate ions in FW (Scott et al., 2004; McCairns and Bernatchez, 2009). To maintain hydromineral balance in FW environments, fish have to minimise diffusive ion losses and compensate them by actively (re)absorbing ions at the gill and posterior kidney levels (Hickman and Trump, 1969; Dantzler, 1996). The European sea bass, *Dicentrarchus labrax*, is known to undertake seasonal migrations in transitional habitats where salinity fluctuates (Kelley, 1988; Barnabé, 1989; Waldman, 1995). Previous studies highlighted intraspecific differences in FW tolerance in this species at different ages (Giffard-Mena et al., 2008; L'Honoré et al., 2019; Nebel et al., 2005) with about 30% mortality following FW exposure. Fish that did not tolerate FW could survive when they were rechallenged to SW conditions and no intraspecific variation was observed in SW (Giffard-Mena et al., 2008; L'Honoré et al., 2019). In *F. heteroclitus,* Scott et al. (2004) concluded that the divergence in osmoregulatory capacities may result in different capacities to absorb and reabsorb Na^+ and Cl^- at gill and/or kidney levels. In Nebel et al. (2005), it has been suggested that the kidney might be responsible for the osmoregulatory failure detected in FW intolerant fish, linked to a low renal Na^+-K^+-ATPase (NKA) activity and a lower kidney tubular density. In L'Honoré et al., (2019), authors highlighted that intraspecific variation in FW tolerance of European sea bass is supported by strong differences in *nka α1a* expression in the posterior kidney while no difference was measured at the gill level. Authors also showed differences in corticosteroid receptors mRNA levels (*gr1, gr2* and *mr*) with lower expression levels in FW intolerant fish compared to FW tolerant. This suggests an impaired hormonal and stress regulation between both FW-tolerance phenotypes. Pituitary prolactin (PRL) is a key hormone involved in FW acclimation by promoting the maintenance of the hydromineral balance (Hirano 1986; Manzon, 2002; Sakamoto and McCormick, 2006; Breves et al., 2014; Bossus et al., 2017). PRL interacts with PRL receptor (PRLR) to regulate Na^+ efflux, water permeability and the differentiation of ionocytes expressing Na^+/Cl^- cotransporter (NCC) as shown in tilapia and zebrafish (Breves et al., 2013, 2010; Dharmamba et al., 1967; Dharmamba and Maetz, 1972). Prolactin receptors are cell surface receptors known to be expressed in osmoregulatory organs of

many teleost species (Manzon, 2002). As for many fish species like *Takifugu rubripes*, two paralogs of *prlr* were identified in *D. labrax* genome called *prlra* and *prlrb* (Lee et al., 2006; Tine et al., 2014). It remains to be determined if both *prlr* paralogs display the same expression pattern according to salinity and if mRNA levels differ between FW-tolerance phenotypes in European sea bass.

In fish, blood pH levels are salinity-dependent with lower blood pH at low salinity than in SW as it was demonstrated in European sea bass by Shrivastava et al. (2019). In 8 month-old European sea bass, FW intolerance was characterised by a lower blood osmolality associated with an elevated Na^+/Cl^- ratio, indicating a metabolic alkalosis (L'Honoré et al., 2019). To regulate a high blood pH, fish have to excrete bases as HCO_3^-, mainly associated with Cl^- uptake (Maetz and García Romeu, 1964; Goss and Wood, 1990, 1991; Tresguerres et al., 2006). The apical anion transporters SLC26A6, involved in Cl^-/HCO_3^- and Cl^-/oxalate exchanges, are known to be widespread among species in osmoregulatory organs including the posterior kidney (Mount and Romero, 2004; Sardella and Brauner, 2007; Xie et al., 2013; Knauf et al., 2018) and gills (Perry et al., 2009; Boyle et al., 2015) and could be potential entry routes for HCO_3^- in intolerant European sea bass to FW. In the gulf toadfish for example, *slc26a6* was highly expressed in kidney (Grosell et al., 2009) to reabsorb Cl^- from the lumen to the blood. Among three different SLC26 anion transporters in zebrafish (SLC26A6, SLC26A3 and SLC26A4, called *za6*, *za3* and *za4*), *za6* seemed to be the most expressed in gills and was overexpressed when fish were transferred to water with low Cl^- or $NaHCO_3$ (Perry et al., 2009). Moreover, gene knockdown of *slc26a6c* resulted in a reduction in Cl^- uptake in zebrafish larvae confirming the major role of this transporter in Cl^- uptake (Bayaa et al., 2009; Perry et al., 2009). Guh et al. (2015) localised SLC26 genes apically in gill ionocytes, called SLC26 cells, but to our knowledge nothing is known about SLC26 localisation in the fish kidney. In *D. labrax*, the only record about SLC26A6 (most probably SLC26A6c) is very recent and shows a high mRNA expression in the anterior intestine to promote Cl^- transepithelial transport to the blood and HCO_3^- excretion (Alves et al., 2019).

The basolateral Na^+/K^+-ATPase (NKA) (Hwang et al., 2011; Kumai and Perry, 2012) is a key player in Na^+ and Cl^- uptake and its importance in FW acclimation has been demonstrated in many fish species (Hiroi et al., 2008; Inokuchi et al., 2008; Watanabe et al., 2008; Hsu et al., 2014; Bollinger et al., 2016). *Nka α1a* transcriptional expression in the posterior kidney was previously shown to be related to FW tolerance in European sea bass (L'Honoré et al., 2019). One other key cotransporter known for Na^+ and Cl^- uptake in fish is the NCC-2A or NCC-like (SLC12A10 or SLC12A10.2), that plays a crucial role in FW acclimation in many fish species including European sea bass (Inokuchi et al., 2008; Wang et al., 2009; Hwang et al., 2011; Blondeau-Bidet et

al., 2019). SLC12A10 is localised apically in FW-type branchial ionocytes (*i.e.* NCC-type cells) in several species (Hiroi et al., 2008; Inokuchi et al., 2008; Guh et al., 2015; Blondeau-Bidet et al., 2019). It is highly expressed in FW compared to SW fish gills (Hiroi et al., 2008; Inokuchi et al., 2008; Wang et al., 2009; Blondeau-Bidet et al., 2019). In posterior kidney, *slc12a10.1* paralog has been shown to be highly expressed in zebrafish and in Mozambique tilapia gills compared to other organs (Hiroi et al., 2008; Wang et al., 2009). The Na^+/H^+ exchanger-3 (NHE3) is also localised in the apical membrane of another ionocyte subtype at the gill level (Inokuchi et al., 2008; Watanabe et al., 2008; Hwang et al., 2011; Blondeau-Bidet et al., 2019) and functionally coupled to several other ion transporters facilitating Na^+ uptake (Dymowska et al., 2015). Anion exchanger 1 (AE1) is mainly known to play a role in bicarbonate transport to the blood and Cl^- secretion at the gill and kidney (mammal medullary collecting duct cells) levels (Barone et al., 2004, Lee et al. 2011). AE1 is thought to be functionally linked to apical V-H^+-ATPase (VHA) in order to complete acid secretion. At the gill level, AE1 is localised basolaterally in zebrafish HR cells (Lee et al., 2011). Its role was investigated in medaka *Oryzias latipes* and in zebrafish gills (Lee et al., 2011; Hsu et al., 2014; Liu et al., 2016). In both species, the two analysed paralogs (*ae1a* and *ae1b*) were expressed in gills with *ae1b* being over-expressed in a low-Na^+ environment. It has also been suggested that AE1 is involved in Cl^- absorption and HCO_3^- secretion (Evans et al., 2005; Hwang and Lee, 2007; Hwang and Perry, 2010) but its localisation in basolateral membranes of ionocytes in pufferfish *Tetraodon nigroviridis* and in milkfish *Chanos chanos* seems not in favour with this hypothesis (Tang and Lee, 2007; Tang et al., 2011). In European sea bass, no data is available on VHA and AE1 localization. VHA mRNA expression and protein activity measurements have shown the presence of VHA in *D. labrax* gills in FW media (Sinha et al., 2015; Blondeau-Bidet et al., 2019).

Regarding $Na^+/K^+/2Cl^-$ cotransporters (NKCC), three paralogs have been described in teleosts: basolateral NKCC1a mainly expressed in the gills and NKCC1b, both dedicated to NaCl secretion, and the apical NKCC2 mainly expressed in the kidney and intestine and attributed to NaCl reabsorption (Haas and Forbush III, 2000; Teranishi et al., 2013). NKCC1 paralogs are expressed in SW-type ionocytes in numerous teleost species including the European sea bass (Lorin-Nebel et al., 2006; Inokuchi et al., 2008; Buhariwalla et al., 2012; Breves et al., 2014). NKCC2 was detected in tilapia and in *D. labrax* intestine (Hiroi et al., 2008; Alves et al., 2019), but there is no data available on its localisation in the gills and in the posterior kidney of *D. labrax*.

European sea bass exhibiting a FW intolerant phenotype face a severe hydromineral imbalance and we will analyse if this failure originates from a failure in ion uptake at the kidney and/or gill levels. The involvement of the gill in differential ion uptake capacity of *D. labrax* will be analysed by comparing the expression profile of

the main ion transporters in tolerant and intolerant fish to FW. Little information is available about ion transporter expression at the kidney level. This is a first tentative to identify key renal ion transporters in FW kidney and to detect intraspecific differences in the transcriptional profile between tolerant and intolerant fish to FW.

Materials and methods

1. Tissue sampling

European sea bass juveniles (N=350) were reared at Ifremer Station at Palavas-les-flots (Hérault, France) in recirculating SW (osmolality: 1208 mOsm kg^{-1}, Na^+: 515 mmol L^{-1}, Cl^-: 737 mmol L^{-1}) under a 12/12 hours light/dark photoperiod at 20°C. At the age of 8 months (13.59 ± 0.12 cm, 32.19 ± 2.62 g), 300 fish were then transferred to brackish water (BW; osmolality: 475 mOsm kg^{-1}) for 24h and then transferred to dechlorinated tap FW (osmolality: 8 mOsm kg^{-1}, Na^+: 2 mmol L^{-1}, Cl^-: 3.5 mmol L^{-1}) for two weeks. The 50 remaining fish were transferred from SW to SW as controls. Pellet food (Le Gouessant, France) was proposed to fish daily, but the fish did not feed during the two weeks of FW challenges.

Fish were sampled two weeks following FW challenge. FW intolerant fish were identified according to their behavior *e.g.* an atypical swimming (lower velocity sometimes associated with a darker color, isolation from the shoal) as described previously (Nebel et al., 2005; L'Honoré et al., 2019). Fish that did not show any of these traits were considered as FW tolerant fish. In this study we compared three groups: FW tolerant fish (FW_t, N=15), FW intolerant fish (FW_i, N=15), and SW control fish (SW, N=13). After the 2-week FW challenge, fish were killed by a lethal dose of benzocaine (at 100 ppm) and the first left gill arch and the posterior kidney (last posterior third of the excretory kidney) were excised and immerged into RNAlater (Qiagen, Mississauga, ON, Canada) for 24h at 4°C and then stored at -80°C for further analyses.

The experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS permit no. 9045-201701068219555).

2. Gene expression

2.1 RNA extraction and complementary DNA (cDNA) synthesis

Tissues were thawed on ice in lysis buffer using the total RNA extraction kit (Nucleospin® RNA, Macherey-Nagel, Germany) before performing the extraction. Quantity and purity (A260/280 ratio) of extracted RNA were verified using a spectrophotometer (NanoDrop™ One/OneC Spectrophotometer, Thermo Scientific, Waltham, MA, USA). One microgram of RNA was used to generate the complementary DNA (cDNA) using the qScript™ cDNA SuperMix (Quanta Biosciences™) providing all necessary components for first-strand synthesis: buffer, oligo(dT) primers, random primers and qScript reverse transcriptase.

2.2 Quantification of gene expression levels

384-wells plates were filled with an Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA). Each well contained a mix composed by 0.75 µL of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Manheim, Germany), 0.037 µL of each primer (forward and reverse primers at 0.2 µM final concentration), 0.21 µL of ultrapure water and 0.5 µL of cDNA. The dilution of the samples was determined according to the standard curves generated for each primer pair. Quantitative real-time PCR (qRT-PCR) was performed using a LightCycler®480 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany), with the conditions described in Blondeau-Bidet et al., 2019. All the primers used are listed in Table 1. For each organ and gene, efficiency was determined (ranged from 1.8 to 2.1) and was used for gene expression quantification (see below) (Table 1). PCR products were sequenced by Sanger sequencing in order to validate the identity of the amplified sequences in comparison with the sea bass genome. Elongation factor 1 α (*ef1α*) was chosen as a reference gene according to previous studies performed on European sea bass challenged to FW (Nebel et al. 2005; Mitter et al. 2009; Blondeau-Bidet et al. 2016, L'Honoré et al., 2019). Relative expression of the target gene was performed using the delta delta Ct method (Pfaffl, 2001) with the SW condition as the reference condition. Ultra-pure water was used as a negative control template, and each sample was analysed in duplicate.

3. Phylogenetic analysis and protein comparisons

Phylogenetic analyses of *slc26a6* and *nkcc (slc12a1 and slc12a2)* have been performed (Figs 1, 2). Nucleotide sequences from different species were obtained at Ensembl or NCBI (Tables 2, 3). *D. labrax* sequences were obtained from the European sea bass genome project (Tine et al., 2014). Multiple nucleotide alignments were performed with MUSCLE V3.8.31 (Edgar, 2004) and ambiguous regions were removed with Gblocks V0.91b

(Talavera and Castresana, 2007). The phylogenetic trees were inferred using the maximum likelihood (ML) method implemented in the PhyML program (v3.1/3.0 aLRT) (Guindon and Gascuel, 2003). Best model of evolution was selected using Modelgenerator V.85 (Keane et al., 2006) following the corrected Akaike Information Criterion (with four discrete gamma categories) and used to construct a phylogenetic tree. Bayesian posterior probabilities were computed with MrBayes 3.2.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Two different runs with four incrementally heated simultaneous Monte Carlo Markov chains were conducted over one million generations, applying respective substitution models determined for each partition in PartitionFinder2 (Lanfear et al., 2017). Trees were sampled every 100 generations to produce 10,000 trees. In order to estimate posterior probabilities, 25% of the trees were discarded as a burn-in stage, observing when average standard deviation of split frequency (ASDSF) values dropped below 0.01. Tree were generated and robustness of the phylogeny assumption was evaluated by bootstrapping procedure from 1000 data set replicates and with posterior probabilities (PP). The phylogenetic tree of *slc26a6* was rooted with the *slc26a6* sequences of two reptilians, the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and the painted turtle (*Chrysemys picta bellii*). The phylogenetic tree *of nkcc (slc12a1 and slc12a2)* was rooted with the *ncc3* sequences of the tropical frog (*Xenopus tropicalis*) and the lizard (*Anolis carolinensis*) (Hartmann et al., 2013).

Amino acid identities (in %) were performed with MUSCLE V3.8.31 (Edgar, 2004) implemented in Geneious® 9.1.8 software (Biomatters).

4. Statistical analysis

Analyses were performed using GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA). When parametric conditions were verified, one-way analyses of variances were performed followed by a Tukey's post-hoc test. In the case of non-parametric conditions, a Kruskal-Wallis test was performed followed by a Dunn's post-hoc test. Data are represented as box and whisker plots (from first quartile to third quartile) showing median, minimum and maximum values. Statistical differences were accepted from $P < 0.05$.

Results

Among the 300 fish challenged in FW, 28% of them were detected and characterised as FW intolerant fish and 70% as FW tolerant fish.

1. Phylogenetic analysis of European sea bass *slc26a6* and *nkcc* paralogous genes

193 Three paralogs of *slc26a6* have been identified in the European sea bass genome (Tine et al., 2014). The three
194 paralogs are called in this study *slc26a6 a*, *slc26a6 b* and *slc26a6 c* according to their phylogenetic position
195 (Fig. 1) computed by maximum likehood analysis against sequences of selected teleost fish (Table 2).
196 Comparatively, three paralogs were identified in *O. niloticus*, *O. latipes*, *G. aculeatus*, *F. heteroclitus* and in *D.*
197 *rerio* whereas two paralogs were identified in *T. nigroviridis* (a and c clades). The European sea bass *slc26a6*
198 paralogous genes obtained in this study strongly grouped with other teleosts *slc26a6* paralogs. *D. labrax*
199 *slc26a6c* is grouped with *slc26a6c* of *O. niloticus*, *O. latipes*, *T. nigroviridis* and shared 86%, 80% and 56%
200 identity in amino acid sequences respectively. European sea bass SLC26A6c only shares 49% and 54% amino
201 acid identity with its SLC26A6b and SLC26A6a. *Slc26a6b* of *D. labrax* is grouped with *O. niloticus slc26a6b*,
202 sharing 80% identity in amino acid sequences but only 53% with European sea bass SLC26A6a. European sea
203 bass *slc26a6b* is strongly grouped with *O. latipes* and *T. nigroviridis*, sharing 71% and 67% identity in amino
204 acid sequences respectively.

205 Two *slc12a1* (*slc12a1a* and *slc12a1b*) and one *slc12a2* were identified in the European sea bass genome that
206 correspond to NKCC1a, NKCC1b and NKCC2 according to their phylogenetical position (Fig. 2), computed by
207 maximum likehood analysis against sequences of selected teleost fish (Table 3). Three *nkcc* genes were also
208 found in *O. mossambicus*, *O. latipes*, *T. rubripes*, *G. aculeatus* and *G. morhua* while two were found in *T.*
209 *nigroviridis* (1b and 2 clades). European sea bass *nkcc* paralogs strongly grouped with teleost *nkcc* sequences.
210 European sea bass *nkcc1a* and *nkcc1b* are grouped with *O. latipes nkcc1a* and *nkcc1b* and share 90.2% and
211 91.9% identity in amino acid sequences respectively. *D. labrax* NKCC1a and NKCC1b only share 77.8%
212 identity between each other. European sea bass *nkcc2* is strongly grouped with *T. nigroviridis* and shares 82.5%
213 amino acid identity whereas it only shares 57.1 and 55.6% identity with NKCC1a and NKCC1b of European sea
214 bass amino acid sequences.

215 2. Relative gene expression in osmoregulatory tissues

216 Expression of selected genes was quantified in the gills and in the posterior kidney (Figs 3, 4, 5). Analysed
217 genes were: *ae1a* (*slc4a1-1*), *ae1b* (*slc4a1-2*), *nhe3* (*slc9a3*), *nkcc1a* (*slc12a2*), *nkcc1b (slc12a2-like), nkcc2*
218 *(slc12a1)*, *vha-b (atp6v1b), ncc1 (slc12a3), ncc-2a (slc12a3-like), slc26a6a, slc26a6b, slc26a6c, prlra* and
219 *prlrb.* Among them, *ae1b, slc26a6a, slc26a6b* and *nkcc2* expression levels have not been quantified in the gills.
220 *slc12a2-like* (*nkcc1b*) expression in the gill was below the quantification limit and is therefore not shown in the

221 results. *ae1a* and *nhe3* expression levels have not been quantified in the posterior kidney because their
222 expression was below the quantification limit.

223 2.1 Gill gene expression

224 *ae1a* was not differentially expressed between SW and both FW groups (FW_t and FW_i, Kruskal-Wallis test, P =
225 0.13, Fig. 3A). *nkcc1a* expression was significantly lower in both FW phenotypes (FW_t and FW_i) compared to
226 SW (Dunn's test, $P < 0.0001$ and $P = 0.0142$ respectively, Fig. 3B), but no significant differences were
227 measured between FW phenotypes.

228 *nhe3* relative expression showed no significant differences between SW, FW_t and FW_i, (Kruskal-Wallis test, P =
229 0.1081, Fig. 3C) but a higher variability was observed in FW conditions. *vha-b* expression levels were
230 significantly higher in SW than in both FW phenotypes (FW_t and FW_i), but no differences were measured
231 between FW_t and FW_i (Dunn's test, $P < 0.0001$, $P = 0.0007$ and $P = 0.9215$ respectively, Fig. 3D). No
232 significant differences have been measured for *ncc1* expression levels between the three groups (Kruskal-Wallis
233 test, $P = 0.0626$, Fig. 3E), but there is more variability in FW_i compared to SW and FW_t, with an almost
234 significant difference between FW_t and FW_i (Dunn's test, $P = 0.0676$). *ncc-2a* expression was significantly
235 higher in FW_t than in SW (Dunn's test, $P = 0.0170$, Fig. 3F), but no differences were measured between SW and
236 FW_i as between FW_t and FW_i (Dunn's test, $P = 0.0562$ and $P > 0.9999$ respectively). Among *slc26a6* paralogs,
237 only *slc26a6c* expression has been detected in the gills. FW_t exhibited a significantly lower expression of
238 *slc26a6c* compared to both SW and FW_i (Dunn's test, $P = 0.0309$ and $P = 0.0367$, Fig. 3G) whereas no
239 difference was observed between SW and FW_i (Dunn's test, $P > 0.9999$).

240 Prolactin receptor *prlra* relative expression was significantly higher in FW_i compared to SW fish (Dunn's test P
241 = 0.0001, Fig. 5A). No difference in *prlra* expression was recorded between SW and FW_t or between FW_i and
242 FW_t (Dunn's test, $P = 0.1324$ and $P = 0.0652$ respectively) but a high variability is observed in FW_i. *Prlrb*
243 expression was lower in FW groups compared to SW, but only significant between FW_t and SW fish (Dunn's
244 test, $P = 0.0045$, Fig. 5B). No difference was observed between FW_i and FW_t.

245 2.2 Posterior kidney gene expression

246 No significant differences were measured in *ae1b* expression levels between SW and FW_t. However a
247 significantly lower expression was measured in FW_i compared to SW but not to FW_t (Dunn's test, $P = 0.0261$
248 and $P = 0.8787$, Fig. 4A).

No significant differences were measured in *ncc1* and *nkcc1a* expression levels between the three groups (Kruskal-Wallis test, $P = 0.1016$ and $P = 0.2475$ respectively, Fig. 4B, C). Concerning *nkcc1b*, no differences were measured between SW and FW_t (Dunn's test, $P = 0.5448$, Fig. 4D) but FW_i expression levels were significantly lower than SW and almost significantly lower than FW_t (Dunn's test, $P = 0.0043$ and $P = 0.0529$ respectively). *nkcc2* expression was not significantly different between SW and FW_t or between FW_t and FW_i, but it was significantly lower in FW_i than in SW (Dunn's test, $P = 0.2269$, $P = 0.8044$ and $P = 0.0096$ respectively, Fig. 4E). In FW_t, among *slc12a* cotransporters analysed, *ncc1* is the most expressed paralog in the posterior kidney followed by the 5-fold less expressed *nkcc2* and the 25- and 50-fold less expressed *nkcc1a* and *nkcc1b* (not shown).

Concerning *slc26a6* paralogs, *slc26a6a* exhibited a significant lower relative expression in FW_t compared to SW (Dunn's test, $P = 0.0044$, Fig. 4F). In FW_i, *slc26a6a* expression was not significantly different from FW_t, whereas for *slc26a6b* and *slc26a6c,* FW_t were at significant higher levels than FW_i (Dunn's test, $P = 0.0141$ and Tukey's test, $P = 0.0018$, Fig. 4F, G, H) but not significantly different from SW. Overall, in FW_t the three paralogs were similarly expressed (not shown).

prlra expression was significantly higher in FW_t than in SW (Tukey test, $P < 0.0001$, Fig. 5C) whereas *prlrb* expression levels did not differ between SW and FW_t (Tukey test, $P = 0.8217$, Fig. 5D). A significantly lower expression of both *prlr* paralogs (*prlra*, *prlrb*) was measured in FW_i compared to FW_t (Tukey test, $P = 0.0139$ and $P = 0.0472$ respectively, Fig. 5C, D).

Discussion

The comparative analysis of key genes and proteins involved in the maintenance of hydromineral balance in fish exhibiting different capacities to tolerate FW is a powerful tool to investigate intraspecific variation in FW tolerance in euryhaline species. In FW, fish have to minimise ion loss and compensate through active ion uptake occurring at interfaces with their surroundings. Most studies investigating FW osmoregulation focused at the gill level in adults or at integument level in larvae (mostly zebrafish), showing that FW tolerance relies on the expression of key genes involved in hyper-osmoregulatory mechanisms (Evans and Clairborne, 2009). Less data are available on the involvement and importance of the kidney in ion re-uptake. In this study, we highlight the importance of taking into account not only the gill but also the posterior kidney in order to fully understand the mechanisms of ion and acid-base regulation.

1. Ion uptake capacities are not altered in gills of FW intolerant sea bass

Regarding gill ion uptake, slightly higher *nka α1a* expression levels and NKA protein activity were previously measured in FW_i compared to FW_t in European sea bass (Nebel et al., 2005; L'Honoré et al., 2019). This could indicate a compensatory response to the blood ion imbalance. In fact, several studies on *F. heteroclitus* or *D. labrax* suggested that a higher Na^+/Cl^- ratio may indicate a more alkaline state (Scott, 2004; Scott et al., 2004a; L'Honoré et al., 2019). In *D. labrax,* the FW_i phenotype was in this physiological condition (L'Honoré et al., 2019) due to a high Na^+/Cl^- ratio. Regarding diverse transporters, we measured no difference in *ae1a* (*slc4a1a*) relative expression between SW and FW phenotypes in gills but a higher variability in FW_i (Fig. 3A). AE1b has been demonstrated as the dominant paralog in medaka gills and it was more expressed in FW media than in saline waters from 10 to 30 ‰ (Liu et al., 2016). This seems not to be the case in European sea bass, as we could only quantify *ae1a*, which was not differently expressed between salinities. In medaka gills, an opposite response was measured regarding the two AE1 paralogs, AE1a and AE1b, when comparing SW and FW-acclimated fish suggesting different functions according to the salinity. Using inhibitors and the scanning ion-selective electrode technique (SIET), the role of AE1 seemed to be attributed to acid and Cl^- secretion in seawater-acclimated medaka larvae (Liu et al., 2016). In European sea bass, the physiological role of AE1 paralogs still needs to be investigated. Moreover, a different expression pattern is observed between *ae1a* and *vha-b*, which is not consistent with a functional link between apical VHA and basolateral AE1a as shown in medaka. The slightly higher *ae1a* expression in FW_i compared to FW_t is therefore probably not linked to an increased acid secretion by VHA. A 3-fold lower expression of *nkcc1a* was measured in FW compared to SW acclimated fish gills (Fig. 3B), as it has previously been shown in this species (Lorin-Nebel et al. 2006; Blondeau-Bidet et al., 2019) and many other teleosts (Hiroi et al., 2008; Inokuchi et al., 2008 Bollinger et al., 2016). This is consistent with the role played by basolateral NKCC1 in ion secretion. According to Inokuchi et al. (2017) and Blondeau-Bidet et al. (2019), FW transfer in Japanese and European sea bass might be followed by a migration towards gill lamellae of seawater-type ionocytes differentiating into FW-type ionocytes (NHE3-type cells) involved in Na^+ uptake. The observed decrease of *nkcc1a* expression in FW_i suggests a successful functional shift from SW to FW-type ionocytes. *nhe3* seems also slightly more expressed in both FW phenotypes compared to SW but unlike previous studies (Blondeau-Bidet et al., 2019), there is no significant difference between salinities. Interestingly, the variability of expression levels for *nhe3* is higher in FW_i compared to the other conditions, which could indicate differential responses within this group.

We measured a higher expression of *ncc-2a* in FW acclimated European sea bass than in SW (Fig. 3F) as it was already demonstrated in this species (Blondeau-Bidet et al., 2019). This reinforces the hypothesis of the differentiation of *ncc-2a* expressing ionocytes in FW, as it was observed in many other fish species such as medaka and tilapia, exhibiting an higher relative expression of *ncc* (homologous to European sea bass *ncc-2a*) in FW than in SW conditions (Hiroi et al., 2008; Inokuchi et al., 2008 Bollinger et al., 2016). FW_i fish also increase slightly but not significantly their *ncc-2a* expression suggesting that at least some individuals successfully differentiate NCC-type cells in gill filaments as shown previously in this species (Blondeau-Bidet et al., 2019). Interestingly, expression levels of *ncc1* were slightly higher and extremely variable in FW_i compared to the other groups (Fig. 3E), which was surprising as *ncc1* expression in the gills is generally low (Inokuchi et al., 2017). A compensatory role of *ncc1* in Na^+, Cl^- uptake in intolerant fish gills could be hypothesised as fish gills are known to have multiple ion uptake pathways, that compensate each other in case of stressful and ion-poor environments (Hwang et al., 2011; Hwang and Lee, 2007).

Slc26a6c expression is significantly higher in SW and FW_i compared to FW_t (Fig. 3G). In SW, where fish do not need to take up Cl^-, the high *slc26a6c* expression is surprising and might be linked to other functions as an increased need to excrete HCO_3^-. In fact, studies dealing with *slc26a6c* in fish mostly focus on FW environments and/or FW species (Bayaa et al., 2009; Perry et al., 2009). In this study we show that this gene might also be of importance in SW conditions. The high expression in FW_i indicates that these fish may compensate their low blood Cl^- levels through an increased Cl^- absorption and also activate the secretion of HCO_3^- to restore blood pH levels (L'Honoré et al., 2019). In zebrafish, a higher expression level of *za6c* was observed in the gills in hypo-osmotic conditions which is consistent with an involvement of this ion exchanger in chloride uptake in apical membranes (Bayaa et al., 2009; Perry et al., 2009). By displaying enhanced expression levels of Cl^- uptake genes such as *ncc1* and *slc26a6c,* FW_i sea bass seemed to activate Cl^- absorption at the branchial level to compensate low Cl^- circulating levels measured previously (L'Honoré et al., 2019).

2. FW intolerant sea bass differentially regulate chloride reabsorption by the kidney

In FW_i posterior kidney, lower expression of *nka α1a* were previously measured compared to FW_t, associated with lower Cl^- blood levels possibly due to a renal ion leakage (L'Honoré et al., 2019). In this study, we have measured a lower expression of two other transporters involved in acid-base and hydromineral regulations (*ae1b* and *slc26a6c*) in FW_i compared to FW_t suggesting that FW_i are actually less efficient to compensate ion loss at the kidney level. Contrary to zebrafish in which *ae1a* is the predominant paralog of AE1 in the kidney, in *D.*

labrax only *ae1b* could be detected (Lee et al., 2011). Expression of *ae1b* was low in FW_i compared to the other conditions. Its basolateral localisation has been demonstrated in goldfish (Fehsenfeld and Wood, 2018), suggesting a role in acid-base regulation. FW_i may decrease the expression of this transporter in order to limit excessive HCO_3^- levels in the blood as these fish already experience blood alkalosis (L'Honoré et al., 2019). In *D. labrax* kidney, expression levels of *ncc1* (*slc12a3*) are higher than other *slc12a* cotransporters, which suggest that NCC1 is predominant in *D. labrax* kidney for ion uptake. It is slightly more expressed in FW_t compared to SW fish and is highly variable in FW_i. *ncc1* (*slc12a3*) has been demonstrated to be also highly expressed in FW in Japanese sea bass kidney compared to SW (Inokuchi et al., 2017). In mefugu *Takifugu obscurus*, *ncc* (homologous to European sea bass *ncc1*) is localised apically and highly expressed in collecting duct of FW-acclimated fish, with a putative role in Na^+, Cl^- reabsorption (Kato et al., 2010). In killifish *F. heteroclitus*, rainbow trout *Oncorhynchus mykiss* and torafugu *Takifugu rubripes*, NKCC2 was localised apically and a role in Na^+, Cl^- reabsorption was suggested (Katoh et al., 2008; Kato et al., 2010). Compared to *ncc1*, *nkcc2* was about 6.5 times less expressed in European sea bass kidney in FW_t and it rather seems downregulated in FW media *vs* SW. Its role in ion uptake seems therefore secondary. FW_i sea bass exhibited the lowest expression levels of *nkcc2* and are thus certainly not using this ion uptake pathway to compensate ion loss. The basolateral *nkcc1a* and *nkcc1b* are respectively 15.8 and 27 times less expressed than *ncc1* in FW_t which is consistent with the role of NKCC1 paralogs in ion excretion rather than ion absorption. Whereas *nkcc1a* expression did not change between the different treatments and phenotypes, *nkcc1b* showed significantly lower expression in FW_i compared to the other conditions. The physiological significance of this transcriptional down-regulation needs to be further explored.

Among the three SLC26A6 paralogous genes investigated in the posterior kidney of European sea bass, *slc26a6a* exhibited significant lower expression in FW_t compared to SW: *slc26a6b* and *slc26a6c* expressions were not significantly modified between SW and FW_t, but they were lower in FW_i. A higher *slc26a6c* expression in low-Cl^- FW compared to normal FW has already been demonstrated in zebrafish gills and kidney with a suggested role in HCO_3^- excretion associated with a Cl^- (re)absorption (Bayaa et al., 2009). SLC26A6 family can exchange various anions with Cl^- such as sulfate (SO_4^{2-}), oxalate ($[COO^-]_2$) and bicarbonate (HCO_3^-) (Mount and Romero, 2004). The role of SLC26A6a in SO_4^{2-}/Cl^- exchange in mefugu proximal tubules has been already highlighted. According to Kato et al. (2009), SLC26A6a is acting as the main apical SO_4^{2-}/Cl^- exchanger and its expression is enhanced under SW conditions. Our results are concordant with this study since both FW sea bass phenotypes (FW_t and FW_i) exhibited lower *slc26a6a* expression levels than in SW. Less is known

about the role of SLC26A6b in the kidney. SLC26A6a and SLC26A6b have been shown to be both implicated in HCO_3^- secretion by the intestine (Xie et al., 2002) but their role in SO_4^{2-} or/and HCO_3^- secretion needs to be further investigated in the kidney of European sea bass and other teleost species.

3. Prolactin receptor transcript levels differ according to FW tolerance capacities

Contrary to mammals where only one *prlr* gene exists, teleost can have two distinct paralogs due to genome duplication events (Ocampo Daza and Larhammar, 2018). The relationship between PRLR and gene expression patterns of osmoregulatory-related genes has not yet been described in European sea bass and needs more attention. Gill *prlr* paralog expression levels seem to depend on circulating PRL levels as shown in Seale et al. (2012) for *prlra* and on extracellular osmolality as shown for *prlrb* in Fiol et al. (2009). Pituitary PRL acts as one of the major endocrine signals for FW acclimation in euryhaline teleosts (Manzon, 2002). Lower *prlra* expression levels in SW compared to FW have been already shown in Mozambique tilapia (Breves et al., 2010; Breves et al., 2011), whereas no differences where observed concerning branchial *prlrb* relative expression between salinities. In our study, *prlr* paralogs expression patterns are not concordant with those described in tilapia gills. Even if *prlra* seems to be slightly more expressed in FW_t than in SW, *prlrb* is significantly overexpressed in SW compared to FW_t. We have previously shown that FW_i exhibited decreased blood osmolality levels (L'Honoré et al., 2019), which could be one of the trigger to activate branchial *prlr* expression. In fact, we measured slightly but not significantly higher mRNA levels of both *prlr* paralogs in FW_i compared to FW_t. In zebrafish and Nile tilapia, PRL and PRLR positively regulate the expression of *ncc* in gill filament cultures *in vitro* (Breves et al., 2013, 2014), which suggests that NCC-type cells might express PRLR. In our study, we also highlighted higher branchial *ncc1* relative expression in FW_i compared to FW_t, suggesting a similar regulation of *ncc1* expression levels by *prlr*. Moreover, slightly higher *nka α1a* expression levels and increased gill NKA activity were previously measured in FW_i compared to FW_t (Nebel et al., 2005; L'Honoré et al., 2019), potentially linked to an increased number of ionocytes to compensate low blood osmolality and low circulating chloride levels.

In the posterior kidney, *prlra* is the predominantly expressed paralog (10-fold higher expressed than *prlrb*) whereas in the gills, *prlrb* was 10-fold more expressed than *prlra*. No differences in *prlra* relative expression have been reported following salinity change in Seale et al. (2012) but a lower relative expression of *prlrb* was shown in FW compared to SW (Fiol et al., 2009; Breves et al., 2011). It seems different in European sea bass posterior kidney where significantly higher expression levels of *prlra* are measured in FW_t compared to SW.

Moreover, no differences were observed for *prlrb* between SW and FW_t. Interestingly, both *prlra* and *prlrb* were less expressed in FW_i compared to FW_t, highlighting that FW_i and FW_t do not exhibit the same regulation mechanisms in response to the FW challenge. We showed a differential expression of prolactin receptors in FW between FW_t and FW_i, with a higher, though not significant, over-expression of branchial prolactin receptors in FW_i and a significantly lower expression of both renal prolactin receptors. These results suggest that FW intolerance in European sea bass might be due to an altered PRL-associated endocrine response in FW_i.

Conclusion

This study is the first to highlight that freshwater intolerance in European sea bass is linked with lower mRNA expression of *slc26a6* genes involved in Cl^- uptake in the posterior kidney. Regarding prolactin receptors, we showed a differential endocrine control in FW between the tolerant and intolerant European sea bass associated to the incapacity to maintain blood hydromineral balance in FW_i sea bass. Facing strong rainfalls during their migration to transitional habitats like lagoons, European sea bass may undergo strong salinity decreases triggering mortality in the freshwater intolerant phenotype. Thus, freshwater intolerance could affect some European sea bass in their migrations by preventing them to access transitional environments. It remains to be characterised how freshwater intolerance in *D. labrax* affects seasonal migratory behaviour in European sea bass.

Acknowledgments

We would like to thank Philippe Clair from the qPCR CEMEB platform for his help, and technicians of Ifremer Palavas-les-flots for the maintenance of the fish. This research was partially granted from CeMEB (Centre Méditerranéen Environnement et Biodiversité) labex project SALSA, granted to C. Lorin-Nebel & C. Cosseau.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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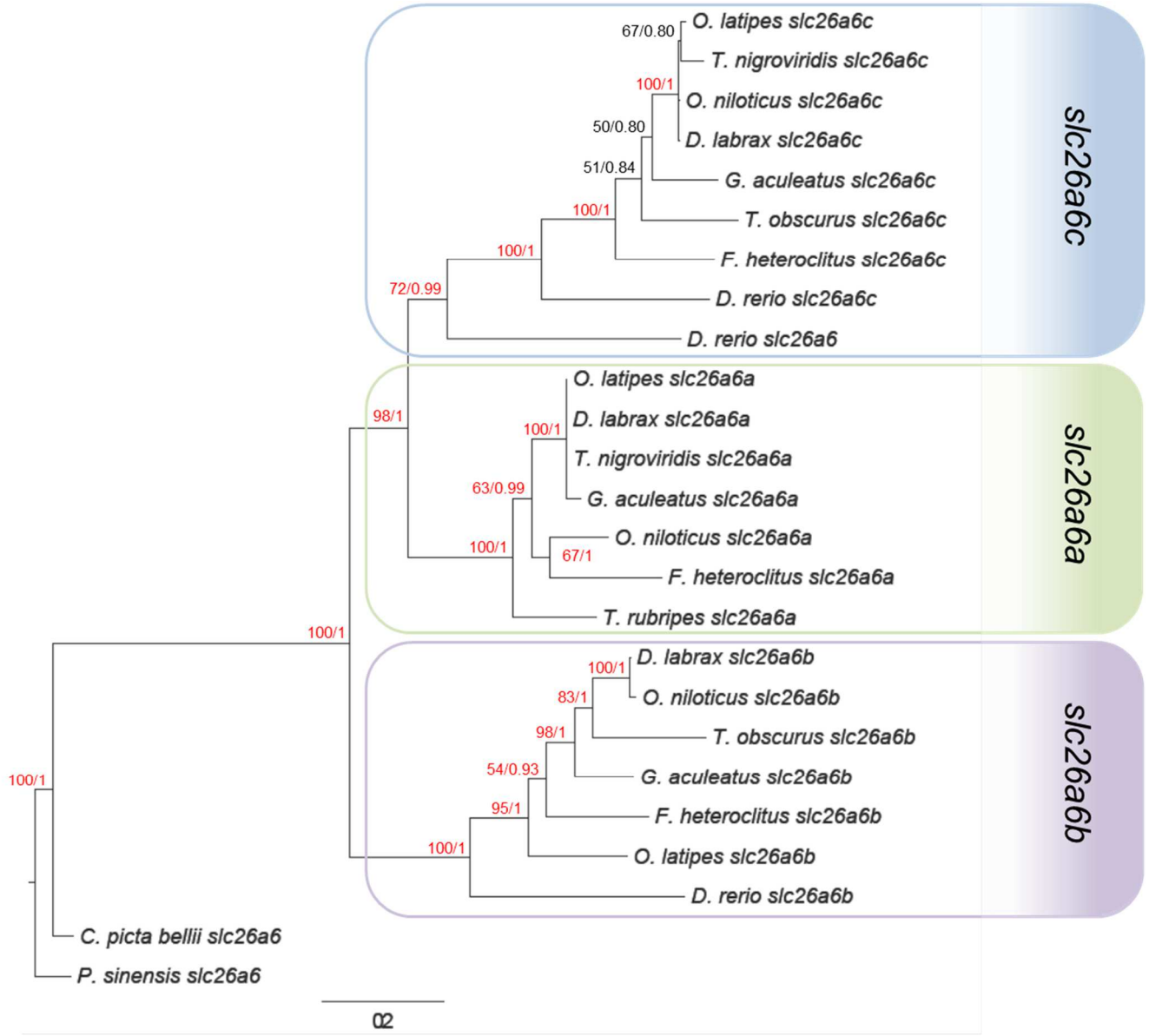

Fig. 1 Bayesian tree of *slc26a6*. A ML tree had identical topology for all nodes. Bootstrap values (in %) from the ML are shown before the Bayesian PP, confident nodes are indicated in red. Branch lengths represent the degree of divergence, with the scale bar indicating the distance representing 0.2 substitutions per position. The blue frame mainly comprises sequences of teleost *slc26a6c*. The green frame mainly comprises sequences of teleost *slc26a6a* and purple frame comprises sequences of teleost *slc26a6b*.

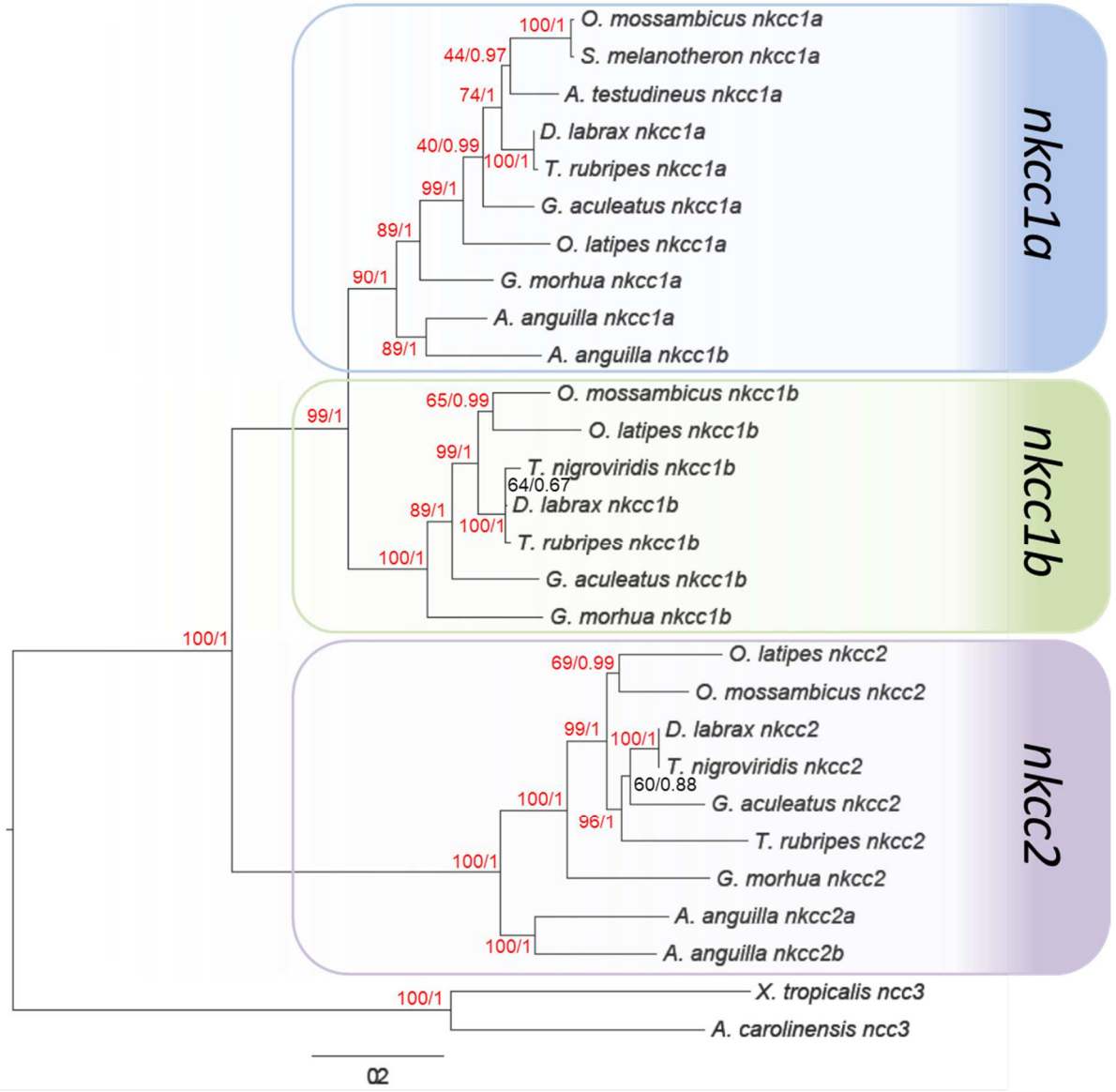

Fig. 2 Bayesian tree of *nkcc1a*, *nkcc1b* and *nkcc2* (*slc12a1a*, *slc12a1b* and *slc12a2*). A ML tree had identical topology for all nodes. Bootstrap values (in %) from the ML are shown before the Bayesian PP, confident nodes are indicated in red. Branch lengths represent the degree of divergence, with the scale bar indicating the distance representing 0.2 substitutions per position. The blue frame mainly comprises sequences of teleost *nkcc1a*. The green frame mainly comprises sequences of teleost *nkcc1b* and purple frame comprises sequences of teleost *nkcc2*.

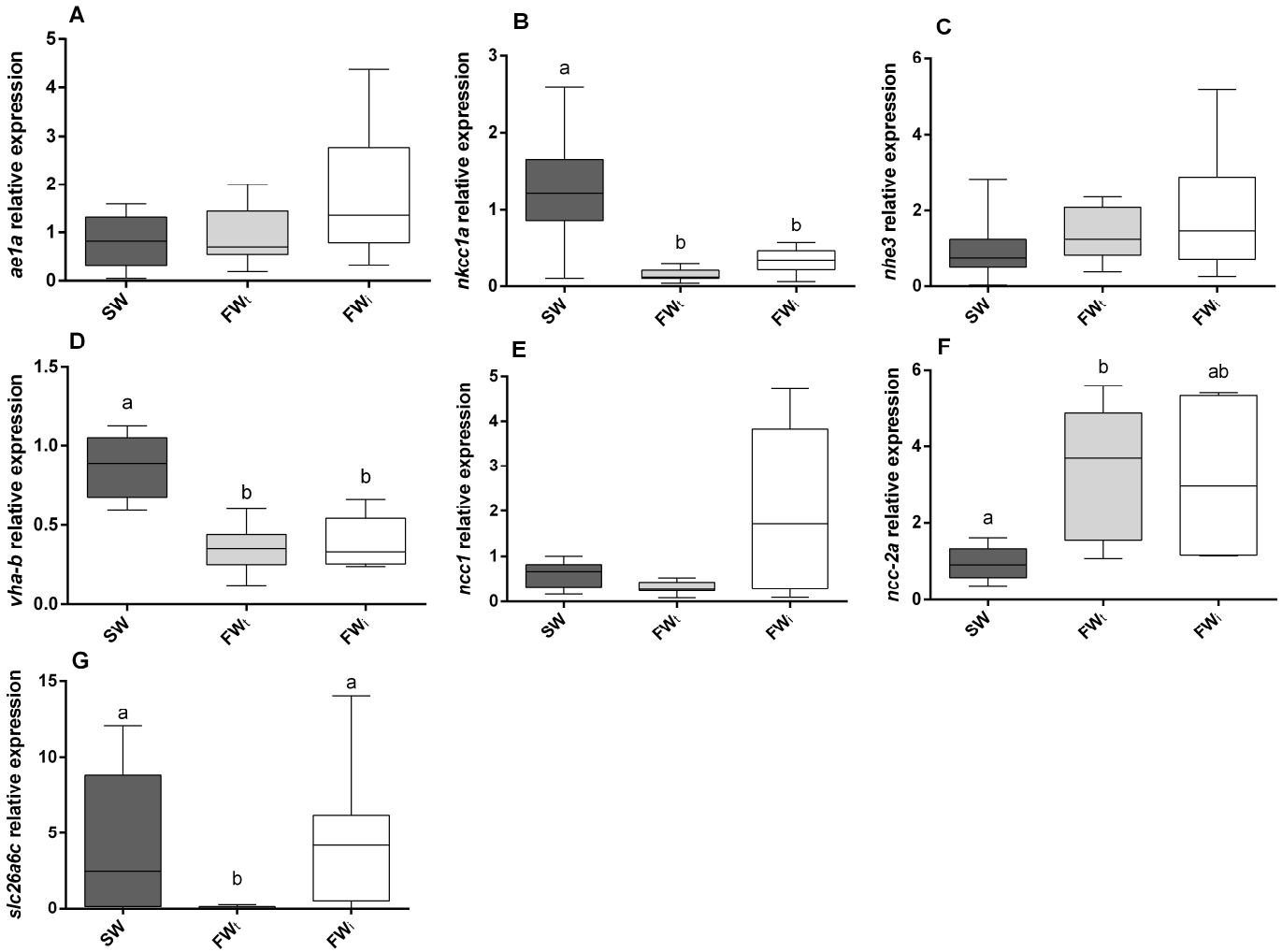

Fig. 3 Relative expression in gills of (A) *ae1a / slc4a1-1*, (B) *nkcc1a / slc12a2-1* , (C) *nhe3*, (D) *vha-b*, (E) *ncc1,* (F) *ncc-2a* and (G) *slc26a6c* in SW and after two weeks in FW. The expression has been normalised according to the expression of the elongation factor *ef1α*. Different letters denote significant differences between groups (one-way Anova followed by Tukey's test or Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=6-16). SW: control fish in SW, FW_i: intolerant fish to FW, FW_t: tolerant fish to FW.

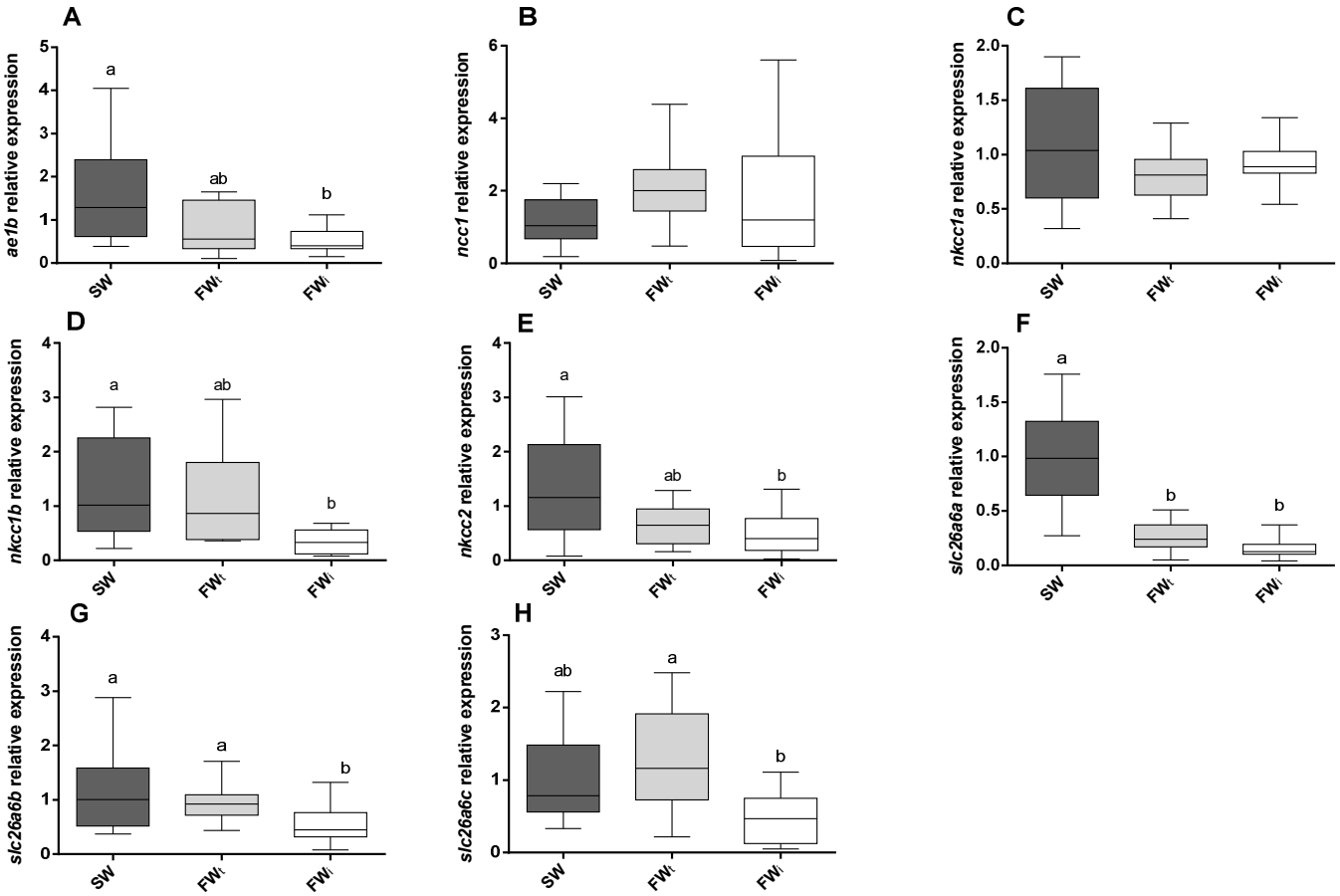

Fig. 4 Relative expression in posterior kidney of (A) *ae1 b / slc4a1-2*, (B) *ncc1 (slc12a3),* (C) *nkcc1a / slc12a2*, (D) *nkcc1b / slc12a2-like,* (E) *nkcc2 (slc12a1),* (F) *slc26a6a,* (G) *slc26a6b* and (H) *slc26a6c* in SW and after two weeks in FW. The expression has been normalised according to the expression of the elongation factor *ef1α*. Different letters denote significant differences between groups (one-way Anova followed by Tukey's test of Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=6-16). SW: control fish in SW, FW_i: intolerant fish to FW, FW_t: tolerant fish to FW.

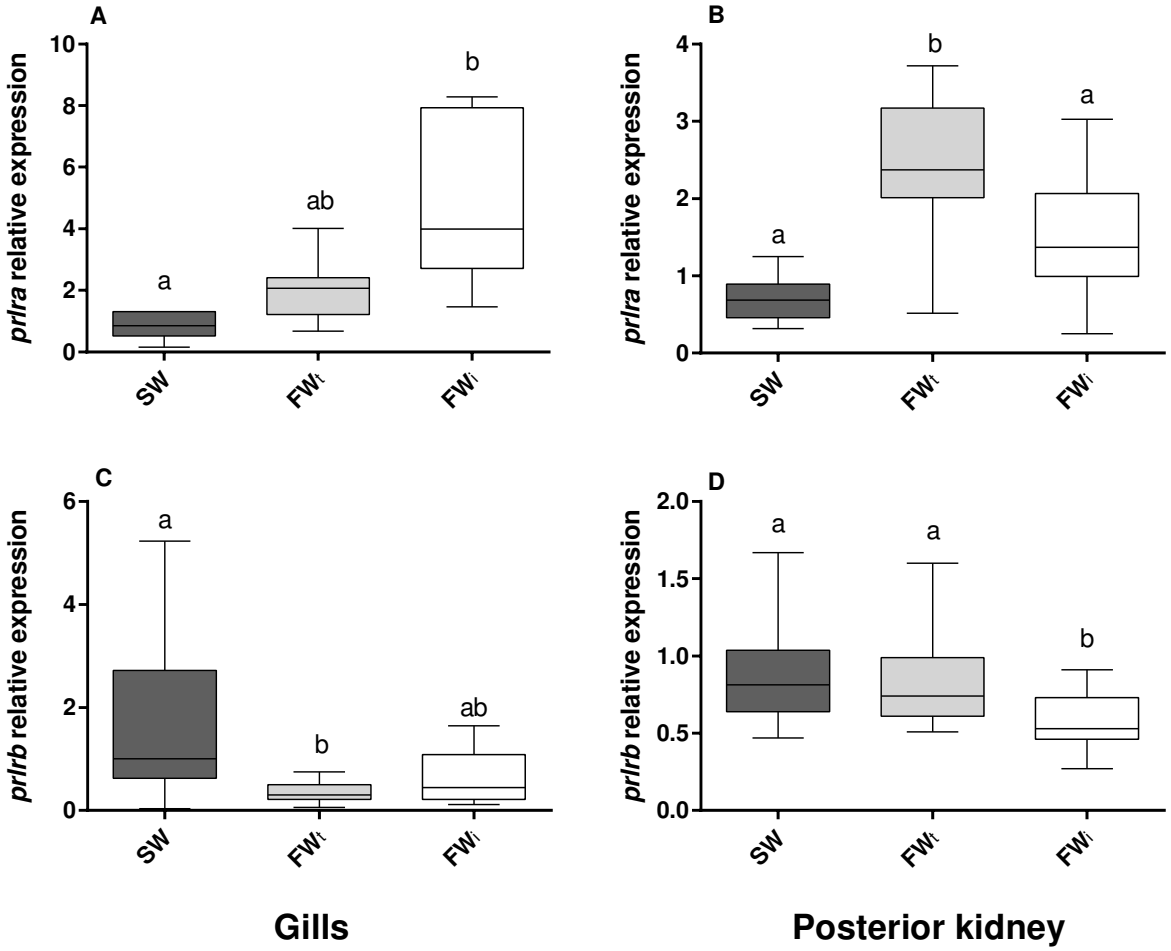

Fig. 5 Relative expression in the gills and in the posterior kidney of *prlr a* (A-C) and *prlr b* (B-D) in SW and after two weeks in FW. The expression has been normalised according to the expression of the elongation factor *ef1α*. Different letters denote significant differences between groups (one-way Anova followed by Tukey's test or Kruskal-Wallis test followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=6-16). SW: control fish in SW, FW_i: intolerant fish to FW, FW_t: tolerant fish to FW.

Table 1 Primer sequences used for the gene expression analysis

Target gene	Primer name	Sequences ID	Sequence (from 5' to 3')	Efficiency	Reference
ef1α	EF1α F	AJ866727.1	GGCTGGTATCTCTAAGAACG	1.9 (gills)	Nebel et al., 2005
	EF1α R		CCTCCAGCATGTTGTCTCC	1.9 (posterior kidney)	
nkcc1a	NKCC1A F	DLAgn_00123120	AGTTGGCAGTAAGGAGGTGG	2.1 (gills)	Blondeau-Bidet et al., 2019
	NKCC1A R		TCAGACTCAGAGGAGACTTGG	1.9 (posterior kidney)	
nkcc1b	NKCC1B F	DLAgn_00080120	TCAGCTCACAGTTCAAGGCC		This study
	NKCC1B R		TTGTGGAGTCCATAGCGGC	2.1 (posterior kidney)	
nkcc2	NKCC2 F	DLA_LG5_005810	GACACTGTGGAGGACGATGG		This study
	NKCC2 R		AGCATGCATCTCACCAGGAC	2.0 (posterior kidney)	
nhe3	NHE3 F	DLAgn_00204050	GGATACCTCGCCTACCTGAC	1.9 (gills)	Blondeau-Bidet et al., 2019
	NHE3 R		AAGAGGAGGGTGAGGAGGAT	1.9 (posterior kidney)	
ncc1	NCC1 F	DLAgn_00172790	TGACGTACTTGATCGCTGCC	1.9 (gills)	This study
	NCC1 R		AGTTGGTGATGGAGGCATGG	2,0 (posterior kidney)	
ncc2-a	NCC2-A F	DLAgn_00038210	ATGATGAGCCTCTTCGAGCC	2.1 (gills)	Blondeau-Bidet et al., 2019

	NCC2-A R		ACAGAAGGTGATGAGAGCAGC	2.0 (posterior kidney)	
slc26a6a	26A6-A F	DLAgn_00129890	TCATGTGTGTGTCTCCCAGC		This study
	26A6-A R		GAGAGTGCTACCAGGCTGAC	2.0 (posterior kidney)	
slc26a6b	26A6-B F	DLAgn_00133440	ATACCTGTGGAGCTGCTTGG		This study
	26A6-B R		TCAGGGCAAAGATTCGTCCC	1.9 (posterior kidney)	
slc26a6c	26A6-C F	DLAgn_00221070	GAAACGGACACAGAGGAGGG	1.9 (gills)	This study
	26A6-C R		GTCTGGTGTTCGAGGAGTGG	2.0 (posterior kidney)	
ae1a	AE1-A F	DLAgn_00101870	TCTGAAGGAATCGGTGGTGC	1.9 (gills)	This study
	AE1-A R		CTCGTTCTGGATCTCGGTGG		
ae1b	AE1-B F	DLAgn_00193420	TGAACAAGGGTGAGATCCGC		This study
	AE1-B R		ACAAAGCGAATAGGGACGGG	2.0 (posterior kidney)	
prlr a	PRLR-A F	DLA_LG19_005350	GGGACAGAGGCAGAAGACAT	2.0 (gills)	This study
	PRLR-A R		GAGGTAGGAGGATGTGGAGC	2.0 (posterior kidney)	
prlr b	PRLR-B F	DLA_LG20_006210	GAATGACAATGGGCCTCTGC	2.0 (gills)	This study
	PRLR-B R		GTTCCGTTGCTTCTGGGTTT	2.0 (posterior kidney)	

Table 2 Cl^-/HCO_3^- exchanger (slc26a6) nucleotide sequences used in the phylogenetic analysis

Nucleotide sequence ID	Fish species	Gene
XM_024104973.1	*Chrysemys picta bellii*	*slc26a6*
DLAgn_00129890	*Dicentrarchus labrax*	*slc26a6 a*
DLAgn_00133440	*Dicentrarchus labrax*	*slc26a6 b*
DLAgn_00221070	*Dicentrarchus labrax*	*slc26a6 c*
XM_680900.6	*Danio rerio*	*slc26a6*
XM_001344207.7	*Danio rerio*	*slc26a6 b*
FJ170818.1	*Danio rerio*	*slc26a6 c*
XM_012876467.2	*Fundulus heteroclitus*	*slc26a6 a*
XM_021318616.1	*Fundulus heteroclitus*	*slc26a6 b*
XM_012872750.2	*Fundulus heteroclitus*	*slc26a6 c*
ENSGACP00000002987	*Gasterosteus aculeatus*	*slc26a6*
ENSGACT00000007648.1	*Gasterosteus aculeatus*	*slc26a6 b*
ENSGACT00000001978.1	*Gasterosteus aculeatus*	*slc26a6 c*

ENSORLP00000011507	*Oryzia latipes*	*slc26a6 a*
XM_011476835.3	*Oryzia latipes*	*slc26a6 b*
ENSORLP00000002390	*Oryzia latipes*	*slc26a6 c*
ENSONIT00000015705.1	*Oreochromis niloticus*	*slc26a6 a*
ENSONIT00000002151	*Oreochromis niloticus*	*slc26a6 b*
ENSONIP00000018142	*Oreochromis niloticus*	*slc26a6 c*
XM_014577432.2	*Pelodiscus sinensis*	*slc26a6*
CAF95115.1	*Tetraodon nigroviridis*	*slc26a6 a*
CAG06912.1	*Tetraodon nigroviridis*	*slc26a6 c*
AB200328.1	*Takifugu rubripes*	*slc26a6 a*
AB200329.1	*Takifugu rubripes*	*slc26a6 b*
AB200330.1	*Takifugu rubripes*	*slc26a6 c*

Table 3 NKCC 1, 2 (*slc12a1/slc12a2*) nucleotide sequences used in the phylogenetic analysis

Nucleotide sequence ID	Fish species	Gene
JN180944.1	*Anabas testudineus*	*nkcc1a*
AJ486858.1	*Anguilla anguilla*	*nkcc1a*
AJ486859.1	*Anguilla anguilla*	*nkcc1b*
AJ564602.1	*Anguilla anguilla*	*nkcc2a*
AJ564603.1	*Anguilla anguilla*	*nkcc2b*
XM_003223867.1	*Anolis carolinensis*	*ncc3*
DLAgn_00080120	*Dicentrarchus labrax*	*nkcc1a*
DLAgn_00123120	*Dicentrarchus labrax*	*nkcc1b*
DLA_LG5_005810	*Dicentrarchus labrax*	*nkcc2*
ENSGMOT00000004750.1	*Gadus morhua*	*nkcc1a*
ENSGMOT00000001772.1	*Gadus morhua*	*nkcc1b*
ENSGMOT00000009346.1	*Gadus morhua*	*nkcc2b*
ENSGACT00000024304.1	*Gasterosteus aculeatus*	*nkcc1a*
ENSGACT00000019494.1	*Gasterosteus aculeatus*	*nkcc1b*

ENSGACT00000022179.1	*Gasterosteus aculeatus*	*nkcc2*
AY513737	*Oreochromis mossambicus*	*nkcc1a*
AY513738	*Oreochromis mossambicus*	*nkcc1b*
AY513739.1	*Oreochromis mossambicus*	*nkcc2*
ENSORLT00000024013.2	*Oryzias latipes*	*nkcc1a*
ENSORLT00000021359.2	*Oryzias latipes*	*nkcc1b*
ENSORLT00000038800.1	*Oryzias latipes*	*nkcc2*
GU066877.1	*Sarotherodon melanotheron*	*nkcc1*
ENSTRUP00000004453	*Takifugu rubripes*	*nkcc1a*
ENSTRUP00000027260	*Takifugu rubripes*	*nkcc1b*
ENSTRUT00000043584.2	*Takifugu rubripes*	*nkcc2*
CAG09669.1	*Tetraodon nigroviridis*	*nkcc1b*
CAF99849.1	*Tetraodon nigroviridis*	*nkcc2*
XM_002934280.4	*Xenopus tropicalis*	*ncc3*