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Short communication

BK potassium channel mRNA level changes in gills of Atlantic salmon after brackish water transfer

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ABSTRACT

BK potassium channels are implicated in a variety of physiological processes and they have been characterized in many species from *Drosophila* to humans although detailed studies about their properties and functions in the osmoregulation of teleosts are still lacking. In this study, we have cloned 911 bp corresponding to a partial *Salmo salar* BK channel coding sequence with high level of homology to BK channel sequences from other teleosts. Also, we have followed the BK gene expression pattern in gills of Atlantic salmon during brackish water (BW) acclimation. Interestingly, transfer from freshwater to BW induced an upregulation in mRNA BK channel expression in gills 7 days after transfer to BW, followed by a decrease in the expression. These results are the first describing mRNA changes of BK channel in gills of Atlantic salmon during BW acclimation, and they could suggest a possible role for BK channel during this adaptive response. Additionally, our results could position BK potassium channels expressed in the gills of *Salmo salar* as new candidates to be a marker of smoltification process, which may complement, and possibly outperform NKA activity measurement, the current gold standard of the Chilean industry.

1. Introduction

Atlantic salmon (*Salmo salar* Linnaeus 1758) begin their life cycle in freshwater (FW), where they are hyperosmotic with respect to the external medium. In this situation, osmotic pressure favors the entry of water into the body and the loss of salt by diffusion across the gill. To compensate these water and ions passive flow, the fish excretes water as diluted urine and obtains salts from food and water by active uptake through both gill and intestine epithelium. As the Atlantic salmon moves downstream into seawater (SW), the osmotic gradient is reversed and internal fluids reach approximately one-third of the SW osmolarity, hence they become hyposmotic relative to the external medium. As compensatory mechanisms, salmonids drink seawater constantly, their urine production is high in salt concentration and they reduce their water loss and actively secrete salts across the gill's epithelium through specialized cells called ionocytes, mitochondria-rich (MR) cells or chloride cells (McCormick, 2013;Stefansson et al., 2008).

The fine-tuning of the ion-transporting machinery in the gill epithelia is a key osmoregulatory event during the process that prepares salmonids for downstream migration and entrance into SW, which is known as parr-smolt transformation or smoltification (McCormick, 2013). Ion transport is primarily carried out by MR cells (Marshall and Grosell, 2006), and requires rearrangement and changes in the expression of Na^+, K^+-ATPase (NKA), Na-K-Cl cotransporter-1 (NKCC1), and other critical ion transport proteins at the cell surface. In the most of euryhaline teleosts, a strong upregulation of gill NKA and NKCC1 are associated with SW acclimation (Hiroi et al., 2008;Hiroi and McCormick, 2007, 2012).

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Sodium chloride (NaCl) secretion by teleost gills is accomplished via secondary active transport of $Cl^-$and passive transport of Na^+. The driving force for active transport is provided by the NKA, which maintains intracellular Na^+at low levels and intracellular K^+at high levels (Marshall, 2002). However, this mechanism of NaCl secretion needs an additional condition to work under SW: a thermodynamic requirement to recycle K^+out via conductive pathways (potassium channels). The molecular identity of this K^+conductance is still unknown in salmonids but there are several K^+channels that may be involved in the function of MR cells in other teleosts (Furukawa et al., 2012;Loncoman et al., 2015;Rohmann et al., 2009;Suzuki et al., 1999). We hypothesized that the high conductance voltage- and Ca^{2+}-activated K^+(BK) channel could be one of the members of the potassium channel family involved in these function in the gills of salmonids as they are involved in modulating K^+transport in osmorregulatory organs of other species (Sorensen et al., 2010;Wang and Giebisch, 2009;Welling, 2016).

BK channels are one of the most broadly expressed potassium channels in metazoans. The name “big K” stems from its single-channel conductance that can be as large as 250 pS under symmetrical 100 mM K^+solutions (Contreras et al., 2013;Latorre et al., 2016). BK channels are homotetramers of the pore-forming α-subunit, encoded by the gene *Slo1* (*Kncma1*) (Lee and Cui, 2010), and they are members of the voltage-dependent potassium (Kv) channel superfamily (Hoshi et al., 2013;Latorre et al., 2010;Yang et al., 2015). BK channels are implicated in a variety of physiological processes in different species (Latorre et al., 2016;Morera et al., 2015), ranging from regulation of smooth muscle tone (Hu and Zhang, 2012;Wu and Marx, 2010) to modulation of hormone and neurotransmitter release (Braun et al., 2008;Orio et al., 2002). Interestingly, BK channels are also involved in modulating K^+transport in mammalian kidney (Holtzclaw et al., 2010;Sausbier et al., 2006;Welling, 2016) and colon epithelium (Sorensen et al., 2010).

BK channels have been characterized in many species from *Drosophila* to humans but detailed studies about their properties and functions in teleosts remain unknown. An exception is the early work that identified BK currents as one of the major outward currents in hair cells of teleost fish (Steinacker and Romero, 1991;Sugihara and Furukawa, 1989). Recently, the genomic organization of *Slo1* in the zebrafish*Danio rerio*has been reported (Rohmann et al., 2009). BK channel transcripts have also been detected in the intestinal epithelium of the European eel*Anguilla anguilla* (Lionetto et al., 2008, 2010), in the nervous system of different teleost fish, and in gills from different fish like: the midshipman fish*Porichthys notatus* (Rohmann et al., 2009), the Mozambique tilapia*Oreochromis mossambicus* (Furukawa et al., 2012) and, more recently, in gills of rainbow trout (*Onchorynchus mykiss*) and Atlantic salmon (*Salmo salar*) by our research team (Loncoman et al., 2015). Previously we have shown that BK channels were expressed in gills of rainbow trout and Atlantic salmon acclimated to FW by using a real time PCR assay (Loncoman et al., 2015). Now, we aim to further study the expression of mRNA BK channels in gills from Atlantic salmon and to explore if low levels of saline water can regulate BK channel expression.

2. Materials and methods

2.1. Experimental animals and sampling

Atlantic salmons were maintained in tanks supplied with recirculating FW at ambient temperature (around 12 °C) in the Fish Vivarium at the Laboratorio Húmedo de Alta Bioseguridad, Institute of Animal Pathology, Faculty of Veterinary Sciences of the Universidad Austral de Chile. Fish were fed ad libitum with commercial pellets. Animal care, sampling and euthanasia was conducted according to the principles and procedures approved by the Institutional Animal Care and Use Committee of the Universidad Austral de Chile and the protocols established in the Laboratorio Húmedo de Alta Bioseguridad. For RNA purification, second and third arch gills, brain, last portion of the intestine as well as the final section of the kidney were collected, weighted (100–200 mg), and disrupted using liquid nitrogen to finally perform RNA purification using TRIzol (Life Technologies) and RNA extraction kit (E.Z.N.A) according to instructions from the manufacturer.

2.2. Freshwater to brackish water transfer time-course experiment

To investigate changes of BK channel mRNA after saline water transfer, a time-course experiment was conducted and mRNA BK channel expression was evaluated in the intestine, kidney and gills over time after BW transfer. A total of forty-eight Atlantic salmon FW-acclimated were transfer into four circular tanks with an animal density not higher than 10 Kg/m^3per tank. Twenty-four animals were allocated to two tanks named as control group (FW-control), which was supplied with FW during the entire experiment. Other twenty-four specimens were allocated to two tanks named as BW group, being supplied with BW consisting in 1.2 PSU (using commercial salt) during the entire experiment. Sampling was carried out at 0 (immediately after transfer/undisturbed controls), 7, 14 and 21 days after the animals were placed in the tanks. Each day, six animals from the FW tanks along with six animals from the BW tanks were randomly selected and sampled. In addition to measuring the changes of BK channel mRNA, gill NKA activity was measured as it has been previously shown that these changes are useful to monitor the progress of saline acclimation (Hiroi and McCormick, 2007;Nilsen et al., 2007).

2.3. Analyses

2.3.1. Cloning and sequencing

Heterologous primers (see Supplemental Table 1, BK sequencing) were designed following mRNA (cDNA) alignments from different teleost species in order to unveil differences of cDNA sequence of BK channel in gills from *Salmo salar*. Partial cDNA sequences were obtained by performing Sanger sequencing of BK channel mRNA from Atlantic salmon's gills (Macrogen, KOREA). Chromatograms were handled and aligned using Geneious 8.1.9 software (Kearse et al., 2012). The sequence cloned was uploaded to genebank (accession number: KY472824.1).

2.3.2. Nucleotide alignment

Nucleotide alignment for the sequence obtained from the BK channel in*Salmo salar* (accession number KY472824.1) and other BK channel coding sequences (CDS) from *Salmo salar*, *Anguilla anguilla, Oreochromis mossambicus, Ornithorhynchus mykiss* and *Danio rerio*. Accession numbers are: XM_014215742, EU267177, AB669174, FJ269021 and XM_017358787, respectively. The alignement was performed using Clustal W algorythm within Geneious 8.1.9 software. BK *Salmo salar* gill nucleotide sequence (XM_014215742) was used as a reference sequence.

2.3.3. Phylogenetic analysis

The phylogenetic tree was obtained through a study of maximum parsimony using the sequenced BK channel. The analysis included 19 nucleotide sequences for BK channels: *Salmo salar* (KY472824.1), *Anguilla anguilla* (EU267177.1), *Chelonia mydas* (XM_007067519.1), *Danio rerio* (FJ269019.1), *Oncorhynchus mykiss* (FJ269021.1), *Mus musculus* (NM_001253378.1),*Orcinus orca* (XM_004273065.2), *Oreochromis mossambicus* (AB669174.1), *Xenopus laevis* (NM_001085690.1), *Xenopus tropicalis* (XM_012966946.2), *Homo sapiens* (NM_001322835.1), *Pan troglodytes* (XM_016918715.1), *Gorilla gorilla gorilla* (XM_019035069.1), *Chlorocebus sabaeus* (XM_007962901.1), *Trachemys scripta* (AF086646.1), *Chrysemys picta bellii* (XM_005300578.2), *Alligator sinensis* (XM_006016306.1), *Nipponia*

nippon (XM_009468101.1) and *Falco cherrug* (XM_005433518.2). Codon positions were included; first, second, third and non-coding. All positions containing gaps and missing data were eliminated. A total of 544 positions were used in the final dataset. Evolutionary analyzes were carried out with MEGA6 software. The consistency index is 0.627315 (0.583979), the retention rate is 0.764275 (0.764275), and the composite index is 0.479441 (0.446321) for all sites and parsimony informative sites (in parentheses).

2.3.4. RNA isolation and cDNA synthesis

Total RNA was purified using a protocol previously described (Loncoman et al., 2015). Total RNA was measured using Nanodrop (Thermo Fisher Scientific) according to instructions from the manufacturer. 1 μg of total RNA was normalized to synthetized cDNA using M-MLV reverse transcriptase (Promega cat#M1701) following the instructions from the manufacturer.

2.3.5. Real-time PCR experiments

Gene expression was assessed by RT-qPCR using a Mx3000P(R) qPCR thermal cycler (Stratagene). Relative expression of mRNA BK channels (Loncoman et al., 2015) along with the elongation factor as housekeeping (ELF1α) gene (Olsvik et al., 2005) were measured on samples obtained at each time point. Primers targeting each gene of interest are described in Supplemental Table 1. SYBR Green Universal Master mix Assay (Applied Biosystems Inc.) was used following the instructions from the manufacturer. The thermal profile used for all primers was as follows: Initial denaturation at 95 °C, 40 cycles of 30 s at 95 °C and annealing/extension at 60 °C for 60 s. Eventually, melting curve analysis was used to differentiate PCR products. Further information about qPCR protocols can be found inLoncoman et al. (2015).

2.3.6. Gill Na^+,K^+-ATPase (NKA) activity

Gill NKA activity was determined using the micro-assay method of McCormick (1993). Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as μmol ADP/mg protein/h at 25 °C. Total protein was measured in undiluted samples in triplicate (Pierce BCA Protein kit # 23225). Both assays were run on Microplate Reader MultiscanGo (Thermo Scientific) using ScanIT 3.2 software.

2.3.7. Statistics

Statistical analyses were carried out using Statistica 7.0 software. Prior to the statistical analysis, all data were tested for normality, independence and homogeneity of variance among the different groups using Kolmogorov–Smirnov, Shapiro-Wilk and Levene tests and a Hartley F-max test. Two-way ANOVA was used to test differences in gene expression between the groups of fish and for possible interactions between condition and time. A post-hoc Tukey-test was used to identify significantly different groups. Differences were considered significant at $P \leq 0.05$.

3. Results and discussion

3.1. Sequencing of BK channels cDNA in gills from Salmo salar and phylogenetic analysis

In the present study, we successfully identified 911 bp sequence length corresponding to a partial *Salmo salar* BK channel cDNA sequence with a high level of homology to publicly available BK channel sequences from other teleost fish (Fig. 1A). This sequence presents a high percentage of homology (~94%) with a putative *Salmo salar* BK sequence (GeneBank accession number: XM_014215742) (see Fig. 1A). Additionally, we performed a phylogenetic analysis using a total of 544 positions of our *Salmo salar* BK channel sequence and they were analyzed with other 18 nucleotide sequences for BK channels from mammalian, birds, reptiles, amphibian and fish. A phylogenetic tree was obtained through a study of maximum parsimony and the *Salmo salar* BK channel sequence was sorted out inside the group of fishes, being very close to the *Oncorhynchus mykiss* BK channel sequence (Fig. 1B).

3.2. Effects of BW challenge on BK channel mRNA expression in gills

To investigate the effect of salinity challenge over BK channel mRNA expression in gills from Atlantic salmon, mRNA of BK channel as gene of interest along with ELF1α as housekeeping gene were measured at 0 (immediately after transfer/undisturbed controls), 7, 14 and 21 days in FW-control and BW groups after they were transferred to FW or BW, respectively. Additionally, as it has been described that NKA activity (Fig. 2), as well as relative expression of NKAα1a and NKAα1b subunits, change over time during salinity challenge (Nilsen et al., 2007); we used these measurements as positive controls to compare our BK changes results. Relative expression of BK channel rises at day 7 in the BW group (Fig. 2), which correlates with an upregulation of mRNA relative expression of NKAα1b and a downregulation of NKAα1a (Data not shown) as previously described byNilsen et al. (2007), indicating that NKAα1a is the most abundant isoform in FW, whereas NKA α1b predominates in SW (McCormick et al., 2013). Therefore, as both BK and NKAα1b upregulate at day 7, and this upregulation precedes an increment in the NKA activity at day 14 (Fig. 2), we hypothesized that these two changes at day 7 could be used as an early molecular marker for smoltification. One-week time frame from day 7 to 14 after SW acclimation could bring major issues or benefits in terms of management for the salmon industry. However, smoltfication is a far more complex phenomenon and we have just included salinity in our current experiment, which is, of course, part of smoltification, but further studies during smoltification are required in order to draw wider conclusions.

3.3. Expression profile of BK channel mRNA after brackish water challenge in other tissues

To further investigate the effect of salinity challenge in BK channel mRNA expression in other tissues, samples of brain, last portion of the intestine as well as the final section of the kidney were obtained during the time-course transfer experiment described previously. In FW, expression of BK channel was found at similar levels in most of analyzed organs (Fig. 3). In the brain, the expression of BK channel was upregulated after 14 days in the BW group and it kept rising up to day 21 (Fig. 3A). In the intestine, the BK channel expression was upregulated after 7 days of in the BW group (Fig. 3B) and it kept rising up to day 14. After day 14, the BK expression deceased until it reached basal level 21 days after transfer (Fig. 3B). The only exception to this pattern was the brain, where the BK channel expression was kept in high levels until the end of the experiment (Fig. 3A). As known, the brain is not an osmoregulatory organ but constantly senses osmolarity. Additionally, it has been studied that the brain undergoes many changes during smoltification which have an impact at physiological development level (McCormick, 2013).

The molecular identities of potassium channels involved in the salt secretion mechanism on salmonids gills in seawater environment are still poorly understood, but there are several K^+channels that may be involved in the function of MR cells in other teleosts. One plausible candidate is the inward-rectifying K^+channel (eKir), highly expressed in gills of the seawater-acclimated Japanese eel (Suzuki et al., 1999). Here we have tested the BK channel, whose expression was detected in gills from the teleost fish *Porichthys notatus*, rainbow trout and Atlantic salmon (Loncoman et al., 2015;Rohmann et al., 2009). Recently, an inward-rectifying K+ channel (Kir1.1 or ROMK for Renal Outer Medullary K+ channel) and a BK channel have been detected in MR cells from Mozambique tilapia (Furukawa et al., 2012).Furukawa et al.

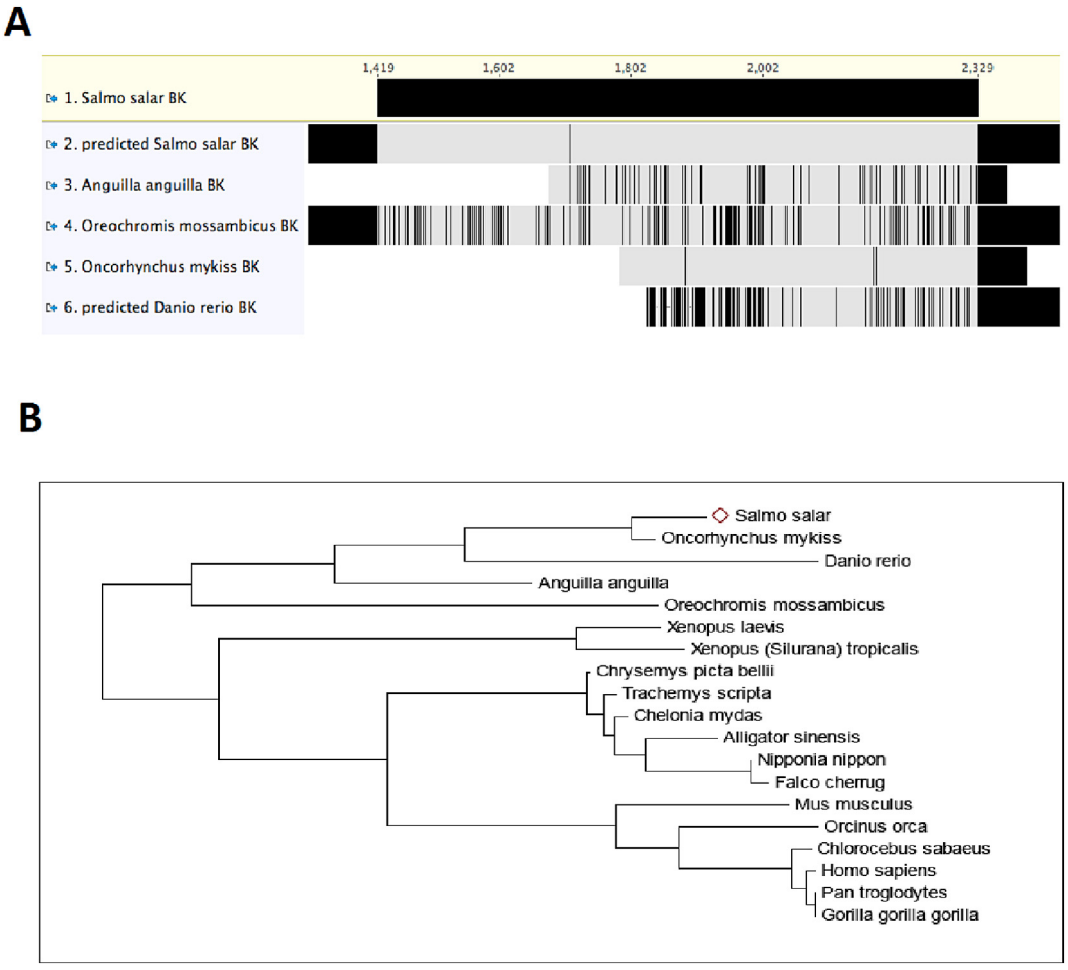

Fig. 1. Sequence multiple alignment and phylogenetic tree for the sequence obtained from the BK channel in *Salmo salar*. (A) Nucleotide alignment for the sequence obtained from the BK channel in *Salmo salar* and other BK channel coding sequences (CDS) from *Salmo salar*, *Anguilla anguilla, Oreochromis mossambicus, Ornithorhynchus mykiss* and *Danio rerio*. Accession numbers are: XM_014215742, EU267177, AB669174, FJ269021 and XM_017358787, respectively. As summary, these 6 sequences have 87.2% pairwise identity. (B) The phylogenetic tree was obtained through a study of maximum parsimony sequence cloned BK channel. The analysis included 19 nucleotide sequences.

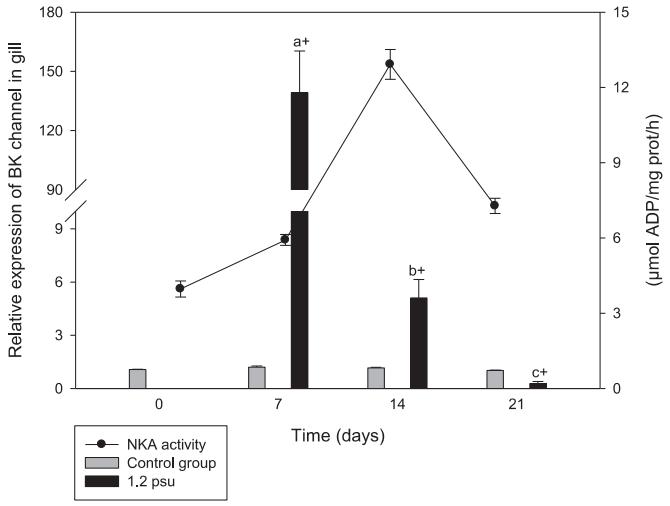

Fig. 2. Changes in BK channel expression in *Salmo salar* gills under 1.2 psu of salinity. Time course of changes in BK channel mRNA expression in fish under 1.2 psu salinity (black bar) and freshwater (control group, grey bar). Measuring of the activity of NKA enzyme in fish in 1.2 psu salinity (•) is also displayed. Values are expressed as the mean ± SEM (n = 6). Different letters indicate significant differences between sampling days. Symbol (+) indicate significant differences between control and treated groups (two-way ANOVA, post-hoc Tukey's test, $P < 0.05$).

(2012)studied some potassium channels and transporter involved in K^+excretion in the gills from tilapia. cDNA sequences of ROMK channel, BK channel, K^+-Cl^-cotransporters (KCC1, KCC2, and KCC4) were identified in tilapia as the candidate molecules that are involved in K^+handling. Among the cloned candidate molecules, only ROMK showed marked upregulation of mRNA levels in response to high external K^+concentration. They also showed that BK channel expression is downregurated in saltwater (Furukawa et al., 2012).

Our present results are the first to describe mRNA changes of BK channel in gills from Atlantic salmon during BW acclimation, and they suggest a possible role for BK channel during this adaptive osmoregulatory response. However, the quest for understanding the function of different potassium channels in gills from salmonids is still ongoing. For instance, experimental evidence from cellular biology field shows that β1 subunit of NKA interacts with the cytoplasmic C-terminal region of BK channels, which results in regulating steady-state levels of BK channel on the cell surface (Jha and Dryer, 2009). Therefore, it would be interesting to test whether that interaction is responsible for the up- and down-regulation we described here. In perspective, this could be an important endeavor to provide additional molecular markers for the smoltification process in salmon aquaculture.

Chilean salmon industry uses the enzymatic activity of the Na^+/K^+ATPase (NKA), whose activity increases during the transition from parr to smolt, as a single molecular marker to determine the timing to smolt transfer to seawater. Any improvement in the methodology to determine the smoltification window with more accuracy and the

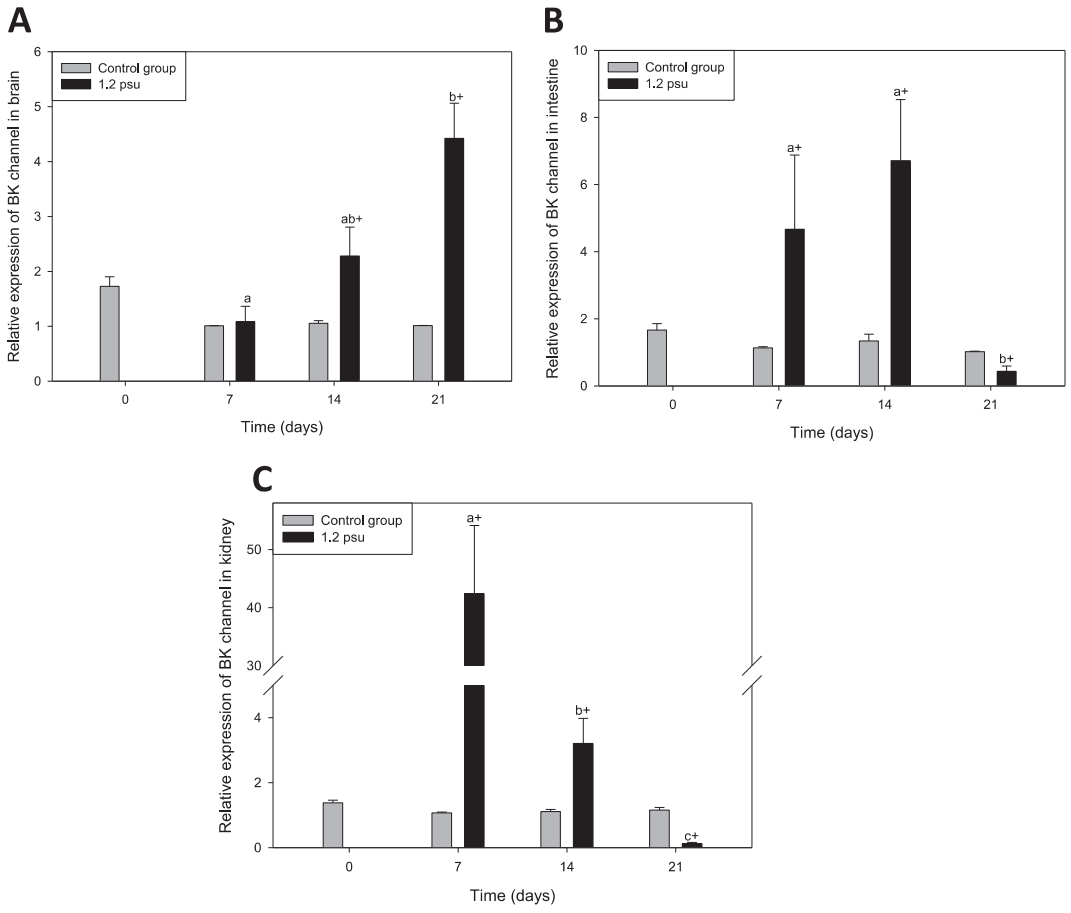

Fig. 3. Changes in BK channel expression in different *Salmo salar* tissues under 1.2 psu of salinity. Relative expression of BK channel in brain (A), intestine (B) and kidney (C) under 1.2 psu of salinity (black bar) or freshwater (control condition, grey bar) in many tissues. Values are expressed as the mean ± SEM (n = 6). Different letters indicate significant differences between sampling days. Symbol (+) indicate significant differences between control and treated groups (two-way ANOVA, post-hoc Tukey's test, $P < 0.05$).

seeding time of fish in seawater will have a major impact in salmon aquaculture production. It is of great interest to test whether changes in BK channel expression may be a potential new complementary marker to determine more precisely the timing to seawater transfer of Atlantic salmon at a relatively low cost.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.03.032.

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