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COMMUNICATION

Application of a real-time PCR assay to detect BK potassium channel expression in samples from Atlantic salmon (*Salmo salar)* and Rainbow trout (*Oncorhynchus mykiss)* acclimated to freshwater[#]

Uso de PCR en tiempo real para la deteccción de la expresión del canal de potasio BK en muestras de salmón del Atlántico (*Salmo salar)* y trucha arcoíris (*Oncorhynchus mykiss)* aclimatadas a agua dulce

CA Loncoman[a], L Gutiérrez[a], P Strobel[b], P Alarcón[a], C Contreras[a], I Conejeros[a], FJ Morera[a*]

[a]Institute of Pharmacology and Morphophysiology, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

[b]Institute of Animal Science, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

RESUMEN

El salmón del Atlántico (*Salmo salar*) y la trucha arcoíris (*Oncorhynchus Mykiss*) son especies que se reproducen en agua dulce y durante la smoltificación deben aclimatarse para vivir en agua de mar, gracias a la secreción del exceso de NaCl al ambiente. La industria salmonera utiliza la medición de la actividad de la Na^+/K^+ ATPasa como marcador molecular para determinar el tiempo de transferencia de los smolts al mar. Sin embargo, la baja precisión de este marcador es un importante problema en la industria. Como el mecanismo molecular de secreción de NaCl en las branquias en peces aclimatados al mar tiene el requisito termodinámico de reciclar el K^+ fuera de las células por medio de canales de potasio, nosotros hipotetizamos que canales de potasio expresados en branquias podrían ser candidatos a potenciales nuevos marcadores para monitorear el proceso de smoltificación. En este trabajo se utilizó la técnica de PCR en tiempo real para medir la expresión de ARNm del canal de potasio BK. Se encontró expresión en branquias, riñón e intestino en animales aclimatados a agua dulce, así como también se encontró, de forma interesante, un aumento de la expresión del canal de potasio BK al aumentar la salinidad al día 7 específicamente en branquia. El producto amplificado por PCR en tiempo real es único y su secuencia posee un 98% de identidad con la porción del canal BK descrito en la base de datos del NCBI. Este ensayo de PCR en tiempo real podría ayudar al monitoreo de los cambios de expresión del canal de potasio BK durante la smoltificacion para estudiar si es posible usar este canal como marcador molecular en el futuro.

Palabras clave: canal de potasio BK, *Salmo salar,* PCR en tiempo real.

SUMMARY

Atlantic salmon (*Salmo salar)* and Rainbow trout *(Oncorhynchus mykiss)* are two fish species that spawn in fresh water (FW) and, during development, acclimate to seawater (SW) by secreting excess NaCl to the environment. The salmon industry measures Na^+/K^+ ATPase (NKA) activity as a molecular marker to determine the timing of smolt transfer from FW to SW. However, the lack of other accurate molecular markers of smoltification remains a major issue for the fish farming industry. The molecular mechanism of NaCl secretion in gills from SW-acclimated fish has a thermodynamic requirement to recycle K^+ out of the cell via potassium channels therefore we hypothesised that potassium channel expression in gills may be a suitable candidate to monitor the smoltification process. In support of this hypothesis, we observed increased expression of BK potassium channel mRNA in gills from *S. salar* under conditions of high salinity (1.2%) compared to animals in FW. In this work, we designed a real-time PCR analysis in order to quantify mRNA levels of BK potassium channels in *S. salar* organ samples. We found differences in mRNA expression among gills, kidney and intestine. We also found a unique real-time PCR product in *S. salar* gills through melting curve analysis, agarose gel electrophoresis and cDNA sequencing. This PCR product showed a 98% of identity with the BK channel portion recorded by the NCBI Database and was differentially expressed in gills, kidney and intestine. This real-time PCR assay may become an important tool to study BK potassium channels expressed in the gills of *S. salar* and its changes during smoltification as putative new candidate to monitor this process.

Key words: BK potassium channel, *Salmo salar*, real time PCR.

INTRODUCTION

Historically, research on smoltification has been linked to the aquaculture industry, in particular to the quality of smolts during transfer time from FW to SW (McCormick 2013). Increased tolerance to salinity is often measured by either increased survival or by lower plasma electrolytes and osmolality after direct transfer from FW to high salinity, also called "SW challenge" (Clarke 1996, McCormick 2013). The main disadvantage of salinity tolerance tests is that they fail to distinguish between SW tolerance and transient adaptation because some fishes may show increased survival but ultimately

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* fjmorera@uach.cl

fail to fully adapt to long-term SW exposure (Iremonger 2008).

The usage of molecular markers during smoltification is a current need in order to make this process more accurate, since many physiological changes occur in parallel during the parr-smolt transformation. The parr-smolt transformation tends to revert relatively fast in fishes that are not able to get into SW environments before the smoltification time (the "smolt window") (Stefansson *et al* 2008). In fact, in most instances the salmon industry uses a single molecular marker of smoltification by inhibiting the Na^+/K^+ ATPase (NKA) with ouabain. However, it has been demonstrated that the NKA in teleosts possesses three major subunits –α, β and γ– and also displays a wide repertoire of α and β-subunit isoforms that are differentially regulated during FW to SW adaptation (Gharbi *et al* 2005, Nilsen *et al* 2007). Additionally, the activity of this protein is often considered an indicator of smolt quality (McCormick 1993, Clarke *et al* 1996). 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 environments, that is, a thermodynamic requirement to recycle K^+ out via conductive pathways such as potassium channels (Degnan 1985, Evans *et al* 1999, Evans *et al* 2005). The molecular identity of these K^+ channels has not been defined in salt secreting Mitochondrion Rich (MR) cells of atlantic salmon, Salmo salar (S. salar) as well as rainbow trout, *Oncorhynchus mykiss* (*O. mykiss*). However, there are several candidate K^+ channels that may be involved in the function of MR cells in teleosts (Marshall and Grosell 2006). For instance, cDNA fragments for an inward-rectifying K^+ (Kir) channel have been identified and are strongly induced in eel gills under SW (Suzuki *et al* 1999). ROMK, another Kir channel variant, has also been recognised as a K^+-conductive candidate in Oreochromis mossambicus (Furukawa *et al* 2012). The high-conductance voltage and Ca^{2+}-activated K^+ channel (called BK for "Big K^+" or large (big)-conductance K^+ channel), whose expression was detected in gills from the teleost fish Porichthys notatus (Rohmann *et al* 2009), is another plausible candidate. The high conductance voltage and Ca^{2+}-activated K^+ channel is one of the most broadly expressed 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 (Latorre *et al* 2010, Lee and Cui 2010). BK channels have been implicated in a variety of physiological processes (Berkefeld *et al* 2010), which range from regulation of smooth muscle tone (Brayden and Nelson 1992) to modulation of hormone and neurotransmitter release (Waring and Turgeon 2009). Interestingly, BK channels are also involved in modulating K^+ transport in the mammalian kidney (Bailey *et al* 2006, Pulznick and Sansom 2006, Wang and Giebisch 2009, Holtzclaw *et al* 2010) and colon epithelium (Sorensen *et al* 2010).

In this work, we detect mRNA of a BK channel in brain and gill samples from *S. salar,* then we quantify BK mRNA expression in gills from *S. salar* under a condition of high salinity (1.2% NaCl) versus FW using real time PCR. Finally, we compare BK channel mRNA expression in osmoregulatory organs such as gills, intestine and kidney in *S. salar* and *O. mykiss* adapted to FW.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

S. salar and *O. mykiss* were kept in tanks from the biosecurity hatchery laboratory at the Institute of Animal Pathology, Universidad Austral de Chile (UACh). All tanks used for these experiments were isolated and supplied with recirculating FW or saline water for salinity experiments. For tissue sampling, animals were overdosed with anaesthesia (benzocaine) for 10 minutes at 185-250 mg/kg. Afterwards, fish were measured and weighed obtaining values from 20 to 25 centimeters and 120 to 125 grams respectively. Additionally, all fishes sampled had absence of parr marks and clinical evidence of disease. All experiments were performed according to the protocols approved by the Ethical Committee of the Universidad Austral de Chile. Samples of brain, second and third arch gill, last portion of the intestine, and the final section of the kidney were harvested,weighed (100-200 mg), and disrupted under liquid nitrogen for RNA isolation.

RNA ISOLATION AND CDNA SYNTHESIS

Total mRNA from tissue samples was isolated by adding of TRIzol® (Life Technologies™) per 100mg of tissue. The mixture was homogenised with an ultraturrex IKA® T10 and 200 µl of chloroform and 375 µl of isopropanol were added. Further mRNA purification was achieved by passing TRIzol extracted samples through RNA extraction kit (E.Z.N.A®) columns and eluting with 30 µl of diethyl pyrocarbonate (DEPC) treated water (Mo Bio lab Inc.). RNA concentrations were measured in a nanodrop spectrophotometer (Thermo Scientific) and stored at -80°C. cDNA was synthesised with 1 µg/mL of total RNA and M-MLV reverse transcriptase (RT) and oligo (dT) 15-mer primers (Promega). The RT protocol consisted in incubation for 5 min at 70 °C, 5 min at 4 °C, 1 h at 42 °C,and 95 °C for 5 min. cDNA samples were stored at -20 °C for further analysis.

REAL TIME PCR AND DNA ELECTROPHORESIS

The mRNA levels of BK α-subunit and Elongation Factor-1 (EF-1) as a housekeeping gene were assessed by real time PCR in a Mx3000P™ (Stratagene®) using

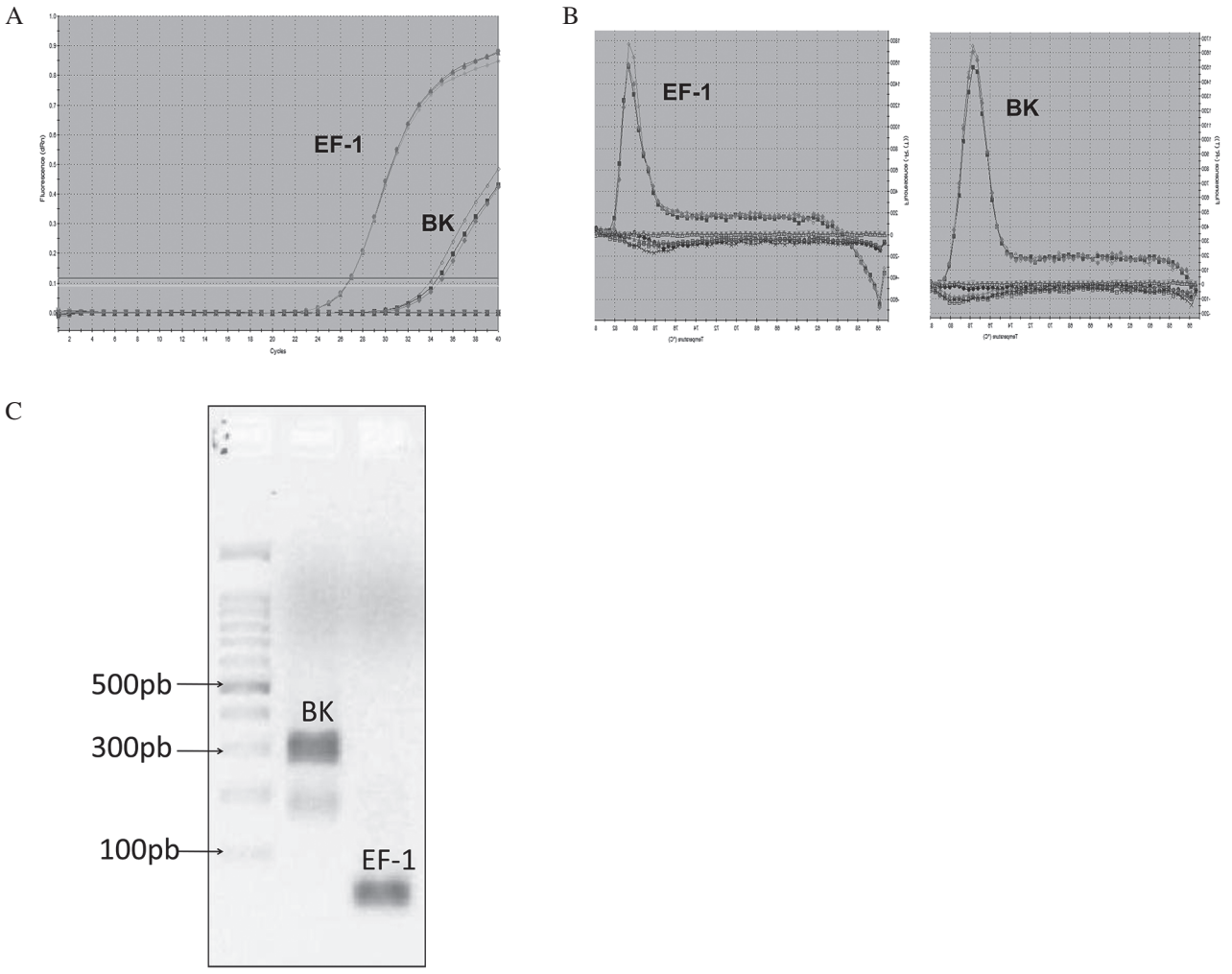

Figure 1. Detection of BK channel mRNA in brain from *Salmo salar* with real-time PCR. A) Amplification curves for EF-1 and BK primers in brain tissue from *Salmo salar.* B) Melting curves for EF-1 and BK products, respectively. C) Agarose gel electrophoresis of EF-1 and BK real time PCR products showing a product of 300 bp for BK, and less than 100bp for EF-1.

Detección del ARNm de canal BK en cerebro de *Salmo salar* mediante PCR en tiempo real. A) Curvas de amplificación obtenidas con los partidores de EF-1 y BK en cerebro de *Salmo salar.* B) Curvas de disociación de los productos de EF-1 y BK obtenidos. C) La electroforesis para los productos de EF-1 y BK amplificados por PCR en tiempo real muestra un tamaño de 300 pb para el producto de BK y de menos de 100 pb para EF-1, como era esperado.

the Maxima SYBR Green/ROX qPCR Master Mix 2X kit (Thermo Scientific). Primer sequences for BK and EF-1 detection were as follows:
BK forward, 5´TTCATGGTCTTCTTCATCCT3´;
BK reverse, 5´GAACAAGGCTTCCAGCTCAAG3´ (Rohmann *et al* 2009);
EF-1forward,5´CCCCTCCAGGACGTTTACAAA3´;
EF-1 reverse, 5´CACACGGCCCACAGGTACA3´(Olsvik *et al* 2005).

The thermal profile for real-time PCR amplification includes 1 cycle at 95 °C for 10 min, then 40 cycles of incubations at 95 °C for 15 sec followed by 60 °C for 45 sec, and one last cycle for melting curve analysis at 95 °C for 1 min, 55 °C for 30 sec and 95 °C for 30 sec.

Agarose gel electrophoresis was performed to check for unique real-time PCR products. Finally, unique PCR bands were isolated with QIAquick® (Quiagen®) and purified products were sent to Macrogen Inc. for sequencing. Sequence analyses and alignments were made using NCBI BLAST algorithm and the Clustal W2 multiple sequence alignment tool from The European Bioinformatics Institute.

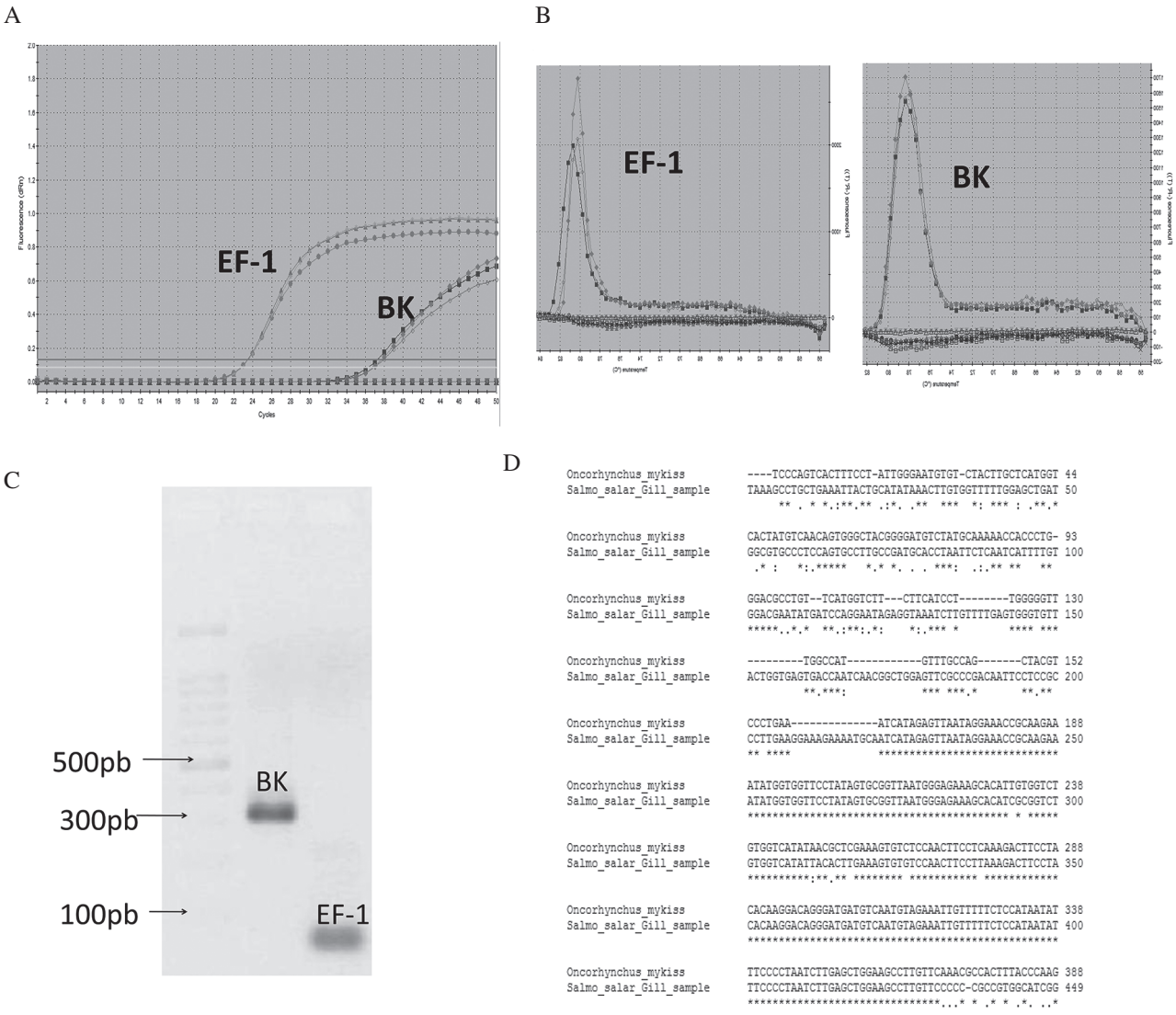

Figure 2. Detection of BK channel mRNA in gills from *Salmo salar* through real-time PCR and sequence alignments. A) Amplification curves for EF-1 and BK in tissue gills from *Salmo salar.* B) Melting curves for EF-1 and BK products, respectively. C) Agarose gel electrophoresis for BK and EF-1 real-time PCR products showing a 300bp band for BK and less than 100bp for EF-1. D) Alignment for *Oncorhynchus mykiss* sequence described by Rohman *et al* 2009, accession number FJ269021.1, and real-time product sequenced by Macrogen Inc. The product shows a 98% of identity in NCBI BLAST.

Detección del ARNm de canal BK en branquias de *Salmo salar* mediante PCR en tiempo real y alineamiento de las secuencias. A) Curvas de amplificación obtenidas con los partidores de EF-1 y BK en branquias de *Salmo salar.* B) Curvas de disociación de los productos de EF-1 y BK obtenidos. C) La electroforesis para los productos de EF-1 y BK amplificados por PCR en tiempo real muestra un tamaño de 300 pb para el producto de BK y de menos de 100 pb para EF-1, como era esperado. D) Alineamiento de la secuencia obtenida para el producto de PCR en tiempo real obtenido para el canal BK y la secuencia para el canal BK de *Oncorhynchus mykiss* descrita por Ronmann y cols. 2009 (Número de acceso FJ269021.1). El producto muestra un 98% de identidad de acuerdo con NCBI BLAST.

RESULTS AND DISCUSSION

DETECTION OF BK CHANNEL IN *S. SALAR* BY REAL-TIME PCR

First, we attempted to detect BK channel mRNA expression in brain tissue from *S. salar,* since these channels are highly expressed in the nervous system of metazoans, including teleost fish (Rohmann *et al* 2009). Indeed, we observed a putative BK channel mRNA in *S. salar* brain by real-time PCR. The amplification curves for this BK channel sequence and EF-1, as a housekeeping gene (Olsvik *et al* 2005), are shown in figure 1A. Melting curve analysis (figure 1B) suggests that a single PCR amplicon was produced. The expected size of the PCR product (300pb) was visualized by gel electrophoresis (figure 1C) and it was comparable to a previous product obtained from *O. mykiss* brain (Rohmann 2009). A secondary product of 150-170bp

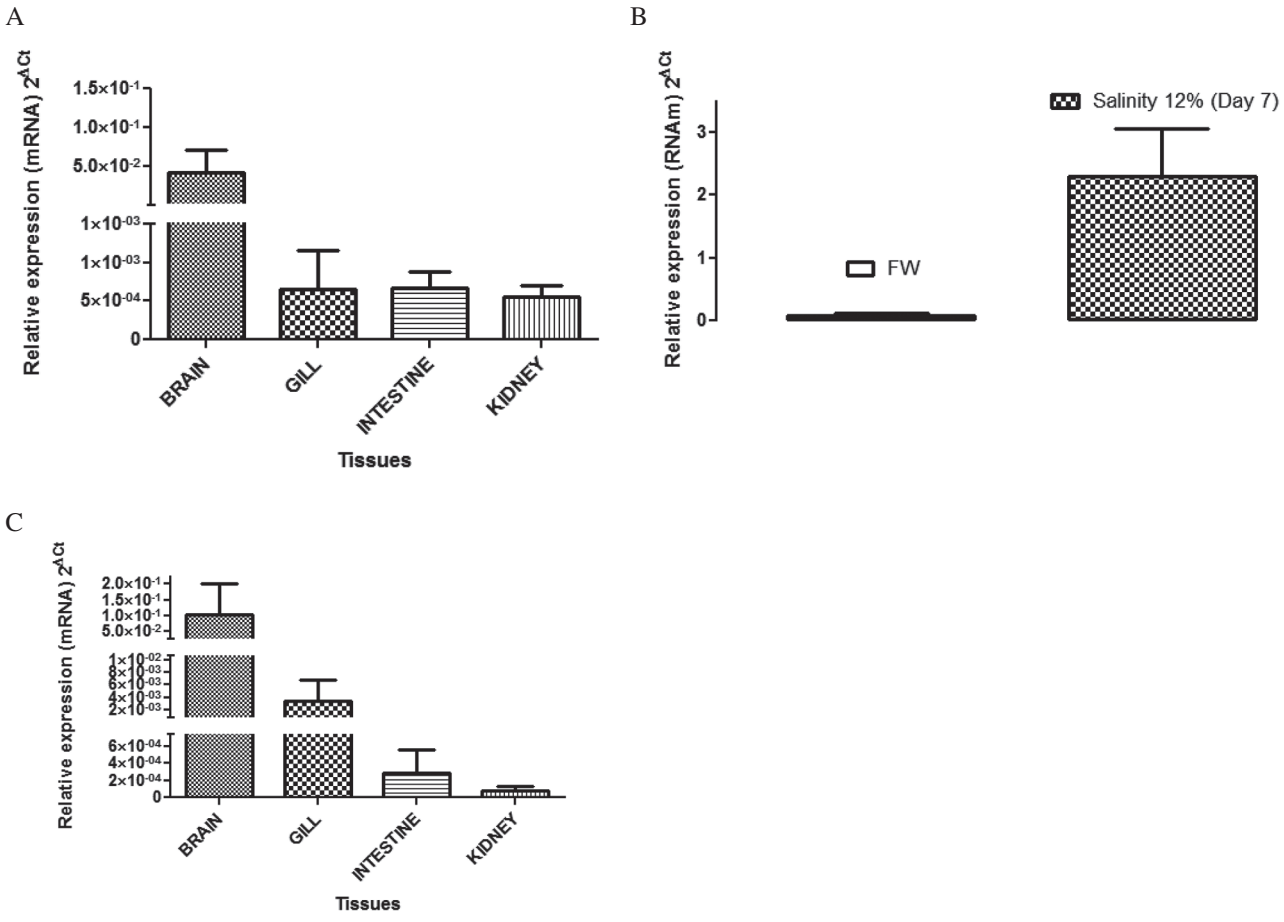

Figure 3. Relative mRNA expression of BK channel in osmoregulatory tissues from *Salmo salar* and *Oncorhynchus mykiss* and differential expression among fresh water and saline water. A) Relative expression of BK channel mRNA in brain and osmoregulatory organs from Atlantic salmon, *Salmo salar* FW (Freshwater)-acclimated. B) Increase of expression of BK channel transcripts (mRNA) in gills from *Salmo salar* at day 7 with 1.2% of salinity compared to expression in fish under FW. C) Relative expression of BK channel mRNA in brain and osmoregulatory organs from Rainbow trout, *Oncorhynchus mykiss,* freshwater (FW)-acclimated.

Expresión relativa del RNAm del canal BK en tejidos osmorreguladores de *Salmo salar* y *Oncorhynchus mykiss* y cambios de expresión entre agua dulce y salada. A) Expresión relativa del RNAm del canal BK en cerebro y tejidos osmorreguladores del Salmón del Atlántico *Salmo salar* aclimatados en agua dulce. B) Aumento de la expresión de los transcritos (ARNm) del canal BK en branquias de *Salmo salar* al día 7 de ser transferidos a agua con 1,2% de sal comparada con la de peces mantenidos en agua dulce. C) Expresión relativa del ARNm del canal BK en cerebro y tejidos osmorreguladores de trucha arcoiris *Oncorhynchus mykiss* aclimatadas en agua dulce.

also appears in the electrophoresis gel (figure 1C), which may correspond to an unrelated cDNA with lower sequence identity. Importantly, results obtained in other laboratories suggest that the RNA-arbitrarily-primed PCR (RAP-PCR) technique could be useful to distinguish between specific and non-specific products in species with limited genomic sequence information (Suzuki *et al* 1999). Our real-time PCR assay gave a similar result using cDNA from *S. salar* gills. Amplification and melting curves are shown in figure 2A and figure 2B, respectively. A single PCR amplicon of 300pb for a putative BK channel was produced (figure 2C). The identity of this PCR product was confirmed by DNA sequencing and sequence alignment with a reported BK channel fragment from *O. mykiss* (A. number FJ269021.1) (figure 2D). To our knowledge, this is the first experimental detection of BK channel expression in tissues from *Salmo salar*. mRNAs encoding BK channels were found in other tissues of FW-acclimated fish as well (figure 3). Among osmorregulatory tissues, gills from FW-acclimated *S. salar* (figure 3A) showed the highest BK channel mRNA expression. A similar expression pattern was found in FW-acclimated *O.mykiss* (figure 3C). Moreover, experiments performed in *S.salar* under a condition of salinity (1.2% NaCl) at day 7 compared to fishes with no salinity (day 0) showed an increase in BK channel expression in gills (figure 3B).

Real-time PCR analyses for monitoring mRNA expression profiles during smoltification have already been used with different NKA alpha subunit isoforms (Nielsen *et al* 2007). Our results suggest that it is plausible to use more

than one molecular marker to more accurately determine smoltification, however, more research is needed in order to develop robust and effective tests that are also practical on fish farming sites. In Chilean smoltification facilities, experience has shown that it is essential to use more than one marker to accurately predict the optimal timing for smolt transfer. Low quality smolts cause significant growth delays and high mortality rates at the sea stage. In Chile, during 2009 and 2010, 29% of smolts transferred to SW suffered growth retardation, and an alarming 34% of the smolt production had to be eliminated. Thus, the lack of accurate molecular markers remains a major issue for the industry and warrants further research on the physiology of smoltification and the techniques used to determine the crucial time window for transferring fishes from hatcheries to the sea.

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REFERENCES

Bailey MA, A Cantone, Q Yan, GG MacGregor, Q Leng, JB Amorim, T Wang, SC Hebert, G Giebisch, G Malnic. 2006. Maxi-K channels contribute to urinary potassium excretion in the ROMK-deficient mouse model of Type II Bartter's syndrome and in adaptation to a high-K+ diet. *Kidney Int* 70, 51-59.

Berkefeld H, B Fakler, U Schulte. 2010. Ca2+-activated K+ channels: from protein complexes to function. *Physiol Rev* 90, 1437-1459.

Brayden JE, MT Nelson. 1992. Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256, 532-535.

Clarke WC, RL Saunders, SD McCormick. 1996. Smolt Production. In: Pennel W, Barton BA (eds). *Principles of Salmonid Culture.* CRC Press, Elsevier, Amsterdam, The Netherlands, Pp 517-567.

Degnan KJ, KJ Karnaky, JA Zadunaisky. 1977. Active chloride transport in the *in vitro* opercular skin of a teleost (*Fundulus heteroclitus*), a gill-like epithelium rich in chloride cells. *J Physiol* 271,155-191.

Degnan KJ. 1985. The role of K+ and Cl- conductances in chloride secretion by the opercular epithelium. *J Exp Zool* 236, 19-25

Evans, DH, P Piermarini, WTW Potts. 1999. Ionic transport in the fish gill. *J Exp Zool* 283, 641-652.

Evans DH, PM Piermarini, KP Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev* 85, 97-177.

Furukawa F, S Watanabe, S Kimura, T Kaneko. 2012. Potassium excretion through ROMK potassium channel expressed in gill mitochondrion-rich cells of Mozambique tilapia. *Am J Physiol Regul Integr Comp Physiol* 302, R568-576.

Hoffmann EK, T Schettino, WS Marshall. 2007. The role of volume-sensitive ion transport systems in regulation of epithelial transport. *Comp Biochem Physiol A Mol Integr Physiol* 148, 29-43.

Holtzclaw JD, PR Grimm, SC Sansom. 2010. Intercalated cell BK-alpha/beta4 channels modulate sodium and potassium handling during potassium adaptation. *J Am Soc Nephrol* 21, 634-645.

Iremonger GS. 2008 Smoltification and Growth Retardation in New Zealand King Salmon *Oncorhynchus tshawytscha* (Walbaum). *Master Thesis*, University of Canterbury, Christchurch, New Zealand.

Karnaky KJ, KJ Degnan, JA Zadunaisky. 1977. Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. *Science* 195, 203-205.

Latorre R, FJ Morera, C Zaelzer. 2010. Allosteric interactions and the modular nature of the voltage- and Ca2+-activated (BK) channel. *J Physiol* 588, 3141-3148.

Lee US, J Cui. 2010. BK channel activation: structural and functional insights. *Trends Neurosci* 33, 415-423.

Marshall WS. 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *J Exp Zool* 293, 264-283.

Marshall WS, M Grosell. 2006. Ion Transport, Osmoregulation, and Acid-Base Balance. In: Evans, DH and JB Claiborne (eds). *The Physiology of Fishes*. 3rd ed. CRC Press, Elsevier, Boca Raton, Fl, USA, Pp177-230.

McCormick SD. 1993. Methods for non-lethal gill biopsy and measurement of Na^+/K^+-ATPase activity. *Can J Fish Aquatic Sci* 50, 656-658.

McCormick SD. 2013. Smolt physiology and endocrinology In: McCormick SD, Farrel AP, Brauner CJ (eds). *Fish Physiology: Euryhaline Fishes.* Academic Press, London, UK, Pp 199-251.

Nilsen TO, LOE Ebbesson, SS Madsen, SD McCormick, E Andersson, BT Björnsson, P Prunet, SO Stefansson. 2007. Differential expression of gill Na+, K+- ATPase α- and β-subunits, Na+, K+,2Cl− cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. *J Exp Biol* 210, 2885-2896.

Olsvik PA, KK Lie, AE Jordal, TO Nilsen, I Hordvik. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Mol Biol* 17, 21-30.

Pluznick JL, SC Sansom. 2006. BK channels in the kidney: role in K(+) secretion and localization of molecular components. *Am J Physiol Renal Physiol* 291, F517-529.

Potts WTW. 1984. Transepithelial potentials in fish gills. In: Hoar WS and DJ Randall (eds) *Fish physiology*, Academic Press, Orlando, FL, USA, Pp 105-128.

Rohmann KN, DL Deitcher, AH Bass. 2009. Calcium-activated potassium (BK) channels are encoded by duplicate slo1 genes in teleost fishes. *Mol Biol Evol* 26, 1509-1521.

Sorensen MV, JE Matos, HA Praetorius, J Leipziger. 2010. Colonic potassium handling. *Pflugers Arch* 459, 645-656.

Stefansson SO, BTh Björnsson, LOE Ebbesson, SD McCormick. 2008. Smoltification. In: Finn RN, Kapoor BG (eds). *Fish Larval Physiology.* Science Publishers, Inc. Enfield (NH) & IBH Publishing Co. Pvt. Ltd., New Delhi, India, Pp 639-681.

Suzuki Y, M Itakura, M Kashiwagi, N Nakamura, T Matsuki, H Sakuta, N Naito, K Takano, T Fujita, S Hirose. 1999. Identification by differential display of a hypertonicity-inducible inward rectifier potassium channel highly expressed in chloride cells. *J Biol Chem* 274, 11376-11382,

Tipsmark CK, DA Baltzegar, O Ozden, BJ Grubb, RJ Borski. 2008. Salinity regulates claudin mRNA and protein expression in the teleost gill. *Am J Physiol Regul Integr Comp Physiol* 294, 1004-1014.

Wang WH, G Giebisch. 2009. Regulation of potassium (K) handling in the renal collecting duct. *Pflugers Arch* 458,157-168.

Waring DW, JL Turgeon. 2009. Ca2+-activated K+ channels in gonadotropin-releasing hormone-stimulated mouse gonadotrophs. *Endocrinology* 150, 2264-2272.

Witters H, P Berckmans, C Vangenechten. 1996. Immunolocalization of Na+, K+- ATPase in the gill epithelium of rainbow trout, *Oncorhynchus mykiss*. *Cell Tissue Res* 283, 461-468.