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## RESEARCH ARTICLE

# Prolactin regulates luminal bicarbonate secretion in the intestine of the sea bream (*Sparus aurata* L.)

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### SUMMARY

**The pituitary hormone prolactin is a pleiotropic endocrine factor that plays a major role in the regulation of ion balance in fish, with demonstrated actions mainly in the gills and kidney. The role of prolactin in intestinal ion transport remains little studied. In marine fish, which have high drinking rates, epithelial bicarbonate secretion in the intestine produces luminal carbonate aggregates believed to play a key role in water and ion homeostasis. The present study was designed to establish the putative role of prolactin in the regulation of intestinal bicarbonate secretion in a marine fish. Basolateral addition of prolactin to the anterior intestine of sea bream mounted in Ussing chambers caused a rapid (<20 min) decrease of bicarbonate secretion measured by pH-stat. A clear inhibitory dose–response curve was obtained, with a maximal inhibition of 60–65% of basal bicarbonate secretion. The threshold concentration of prolactin for a significant effect on bicarbonate secretion was 10 ng ml$^{-1}$, which is comparable with putative plasma levels in seawater fish. The effect of prolactin on apical bicarbonate secretion was independent of the generation route for bicarbonate, as shown in a preparation devoid of basolateral $HCO_3^-$/$CO_2$ buffer. Specific inhibitors of JAK2 (AG-490, 50 μmol l$^{-1}$), PI3K (LY-294002, 75 μmol l$^{-1}$) or MEK (U-012610, 10 μmol l$^{-1}$) caused a 50–70% reduction in the effect of prolactin on bicarbonate secretion, and demonstrated the involvement of prolactin receptors. In addition to rapid effects, prolactin has actions at the genomic level. Incubation of intestinal explants of anterior intestine of the sea bream *in vitro* for 3 h demonstrated a specific effect of prolactin on the expression of the Slc4a4A $Na^+$–$HCO_3^-$ co-transporter, but not on the Slc26a6A or Slc26a3B $Cl^-$/$HCO_3^-$ exchanger. We propose a new role for prolactin in the regulation of bicarbonate secretion, an essential function for ion/water homeostasis in the intestine of marine fish.**

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Key words: prolactin, intestine, epithelial transport, bicarbonate secretion, sea bream, JAK2, MEK, PI3K, SLC4A4, SLC26A6, SLC26A3.

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### INTRODUCTION

The blood of marine teleosts has an osmolality of 300–370 mOsm kg$^{-1}$, while seawater has an osmolality of 1000 mOsm kg$^{-1}$ $H_2O$. This difference in osmolality causes a continuous diffusive water loss by the gills in marine fish that accompanies the branchial process of ion secretion which removes excess plasma salts (Evans et al., 2005). Consequently, water replacement becomes of the highest importance to sustain body ionic regulation. Thus, marine teleosts are required to drink substantial amounts of seawater (Fuentes and Eddy, 1997), which once ingested undergoes an initial desalting step in the oesophagus by selective absorption of NaCl (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983) to facilitate water absorption in the intestine.

An important physiological process related to intestinal fluid processing, and ultimately to water absorption, in the intestine of marine teleosts is the production of divalent carbonate aggregates within the intestinal lumen, which in addition to intestinal processing are a relevant part of the carbon cycling in the ocean (Wilson et al., 2009). The formation of carbonate aggregates, which also takes place in unfed fish, has at least two requirements: high calcium availability and high pH to drive the precipitation. The substrates calcium and/or magnesium are in high concentration in the intestinal lumen (i.e. ingested seawater) and, in addition, epithelial bicarbonate secretion creates alkaline conditions (Faggio et al., 2011; Fuentes et al., 2010b; Grosell, 2011; Kurita et al., 2008; Walsh et al., 1991; Wilson and Grosell, 2003; Wilson et al., 2009; Wilson et al., 2002). Thus, secreted bicarbonate immobilizes divalent ions in the form of carbonate aggregates, reduces fluid osmolality, and ultimately favours osmotic water absorption. The mechanisms responsible for bicarbonate secretion are not completely understood, although the involvement of some transporters has been established. On the basolateral membrane the $Na^+$–$HCO_3^-$ co-transporter (NBC), which belongs to the SLC4 gene family, leads to accumulation of cellular $HCO_3^-$ (Kurita et al., 2008; Taylor et al., 2010). In the apical membrane, $Cl^-$/$HCO_3^-$ anion exchangers belonging to the sulphate permease family, SLC26, secrete $HCO_3^-$ concomitantly with $Cl^-$ absorption (Ando and Subramanyan, 1990; Grosell and Genz, 2006; Kurita et al., 2008). In the absence of a contribution from the transcellular route, which is mediated by the action of basolateral SLC4, a significant amount of the apical alkaline secretion still takes place (Fuentes et al., 2010b; Taylor et al., 2010) and is probably produced by the hydration of $CO_2$ in the enterocyte driven by

carbonic anhydrase (Grosell et al., 2007). Bicarbonate transporters required for apical secretion are mostly non-electrogenic and rely on the electrogenic action of basolateral $Na^+/K^+$-ATPase (Grosell and Genz, 2006). The $Na^+/K^+$-ATPase extrudes $3\,Na^+$ in exchange for $2\,K^+$ and establishes a strong cytosolic negative potential and low intracellular concentration of $Na^+$ (Skou, 1990; Skou and Esmann, 1992).

Bicarbonate secretion for luminal aggregate formation is specific to marine fish (Grosell, 2011) and does not occur in freshwater fish. In addition, $Na^+/K^+$-ATPase activity and gene expression are higher in the intestine of seawater fish than in freshwater fish (Cutler et al., 2000; Fuentes et al., 1997; Jensen et al., 1998; Seidelin and Madsen, 1999).

The pituitary hormone prolactin is a pleiotropic endocrine factor involved in the regulation of water and electrolyte balance in most classes of vertebrates, including teleost fish (Bole-Feysot et al., 1998). The importance of this hormone in fish osmoregulation was first demonstrated in 1950s by studies on the euryhaline teleost *Fundulus heteroclitus* (Pickford and Phillips, 1959) and the osmoregulatory actions of prolactin in fish have been extensively reviewed (e.g. Manzon, 2002). The studies showed that fish were unable to survive in fresh water if subjected to hypophysectomy, while replacement therapy with prolactin allowed survival by limiting haemodilution. The target for prolactin action was later identified as the $Na^+/K^+$-ATPase (Pickford et al., 1970). The regulatory action of prolactin on gill $Na^+/K^+$-ATPase has since been established in a series of freshwater and euryhaline species such as *Salmo salar* (Tipsmark and Madsen, 2009), *Salmo trutta* (Seidelin and Madsen, 1999), *Oreochomis mossambicus* (Tipsmark et al., 2011), *Dicentrarchus labrax* (Varsamos et al., 2006) and *Sparus aurata* (Mancera et al., 2002).

We have recently demonstrated that intestinal bicarbonate secretion in marine fish is an endocrine-regulated process (Fuentes et al., 2010b) with inhibitory actions of the parathyroid hormone-related protein (PTHrP) and stimulatory action of stanniocalcin (STC). Prolactin receptors have been identified in the sea bream and the intestine is amongst the tissues with the highest expression of the receptor (Santos et al., 2001), even in seawater fish, making it a likely target for prolactin. Taking into consideration the likely importance of $Na^+/K^+$-ATPase for the electrogenic potential required for bicarbonate secretion, together with the established regulatory action of prolactin in gill $Na^+/K^+$-ATPase, we hypothesized that prolactin is involved in the regulation of bicarbonate secretion in marine fish intestine. The present study was designed to test this hypothesis using an *in vitro* model, the intestinal epithelium of the sea bream (*S. aurata*).

## MATERIALS AND METHODS

### Peptides and chemicals

Ovine prolactin (NIADDK-oPRL-21) was obtained from the National Institutes of Health (Bethesda, MD, USA) and has previously been shown to be bioactive in the sea bream (Mancera et al., 2002). All chemicals were of the highest grade and were obtained from Sigma-Aldrich (Madrid, Spain) unless stated otherwise.

### Animals

Sea bream juveniles (*S. aurata*) were obtained from commercial sources (CUPIMAR SA, Cadiz, Spain) and stocked at the Ramalhete Experimental Marine Station (CCMAR, University of Algarve). Fish were held in 1000 l seawater tanks (density <5 kg fish/tank), with flowing seawater (salinity 36 p.p.t., temperature 18–24°C), under natural photoperiod and were fed once a day (10:00 h) with 2% body mass commercial dry pellets (Provimi, Alverca, Portugal). All fish were fasted for 48 h before experimental manipulations. For tissue collection, fish (200–300 g body mass) were anaesthetized with 2-phenoxyethanol ($1\,ml\,l^{-1}$ water, Sigma-Aldrich) and killed by decapitation.

All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under licence of Group-1 from the Direcção-Geral de Veterinária, Ministerio da Agricultura, do Dessevolvimento Rural e das Pescas (Portugal).

### Intestinal bicarbonate secretion

A section of anterior intestine (3–5 cm caudal from the pyloric caeca) was dissected out and washed in chilled saline. The intestinal portions were defatted, cleaned with fresh saline, and opened longitudinally to produce a flat sheet that was then mounted on a tissue holder (P2413, $0.71\,cm^2$, Physiological Instruments, San Diego, CA, USA) and positioned between two half-chambers (P2400 Ussing chamber, Physiological Instruments) containing 1.5 ml of physiological saline. Basolateral and apical saline were prepared as previously described (Fuentes et al., 2006; Fuentes et al., 2010b) to simulate *in vivo*-like conditions and had the following composition: basolateral saline: $160\,mmol\,l^{-1}$ NaCl, $1\,mmol\,l^{-1}$ $MgSO_4$, $2\,mmol\,l^{-1}$ $NaH_2PO_4$, $1.5\,mmol\,l^{-1}$ $CaCl_2$, $5\,mmol\,l^{-1}$ $NaHCO_3$, $3\,mmol\,l^{-1}$ KCl, $5.5\,mmol\,l^{-1}$ glucose, $5\,mmol\,l^{-1}$ Hepes, pH 7.800, gassed with 0.3% $CO_2$ + 99.7% $O_2$; apical saline: $88\,mmol\,l^{-1}$ NaCl, $9.5\,mmol\,l^{-1}$ $MgCl_2$, $3\,mmol\,l^{-1}$ KCl, $7.5\,mmol\,l^{-1}$ $CaCl_2$, $126.5\,mmol\,l^{-1}$ $MgSO_4$ and $1\,mmol\,l^{-1}$ $Na_2HPO_4$, gassed with 100% $O_2$. The pH of apical saline was maintained constant at 7.800 by pH-stat. For experiments in which $NaHCO_3$ was omitted from the basolateral saline it was replaced by Hepes-Na in an equivalent concentration ($160\,mmol\,l^{-1}$ NaCl, $1\,mmol\,l^{-1}$ $MgSO_4$, $2\,mmol\,l^{-1}$ $NaH_2PO_4$, $1.5\,mmol\,l^{-1}$ $CaCl_2$, $5\,mmol\,l^{-1}$ Hepes-Na, $3\,mmol\,l^{-1}$ KCl, $5.5\,mmol\,l^{-1}$ glucose and $5\,mmol\,l^{-1}$ Hepes, pH 7.800) and continuously mixed and oxygenated by gently gassing with 100% $O_2$. The temperature was maintained at 21–22°C throughout the experiments.

All bioelectrical variables were monitored by means of Ag/AgCl electrodes (with tip asymmetry <1 mV) connected to either side of the Ussing chamber with 3 mm-bore agar bridges ($1\,mol\,l^{-1}$ KCl in 3% agar). Voltage (mV) was monitored by clamping the epithelia to $0\,\mu A\,cm^{-2}$ (VCC600 Physiologic Instruments). Epithelial resistance (tissue resistance, $R_t$, $\Omega\,cm^2$) or tissue conductance ($G_t$, $mS\,cm^{-2}$) were manually calculated by Ohm's law using the voltage deflection induced by a $10\,\mu A$ pulse of 3 s every minute. Bioelectrical parameters for each tissue were recorded after the tissue achieved a steady state, usually between 30 and 40 min of mounting in chambers. To allow pulsing for $R_t$ calculations during the titration, the VCC600 was grounded to the pH-stat assembly.

Bicarbonate secretion was measured by means of a pH-stat assembly consisting of an ABU80 microburette, a TT80 titration controller and a PHM84 meter (Radiometer, Copenhagen, Denmark) attached to a mini pH electrode (Hanna Instruments, Smithfield, RI, USA). Luminal saline was kept at a constant pH of 7.800 throughout the experiment by addition of acid titrant ($2.5\,mmol\,l^{-1}$ HCl). Bicarbonate secretion ($nmol\,h^{-1}\,cm^{-2}$) was calculated from the volume of titrant added, the concentration of the titrant and surface area ($cm^2$).

Experiments were only performed with a tissue sample if the voltage and bicarbonate secretion were stable over 1 h. Once a steady

state was achieved, bicarbonate secretion was monitored for 30 min to serve as the control. Hormones or pharmacological compounds were added to the Ussing chambers and the tissue response monitored over 1 h.

### Short-circuit current measurement

The anterior intestine was collected, isolated and mounted as previously described (Fuentes et al., 2010b) on a tissue holder (P2413, 0.71 $cm^2$, Physiological Instruments). The tissue was positioned between two half-chambers (P2400 Ussing chamber, Physiological Instruments) containing 2 ml of the serosal physiological saline (160 mmol $l^{-1}$ NaCl, 1 mmol $l^{-1}$ $MgSO_4$, 2 mmol $l^{-1}$ $NaH_2PO_4$, 1.5 mmol $l^{-1}$ $CaCl_2$, 5 mmol $l^{-1}$ $NaHCO_3$, 3 mmol $l^{-1}$ KCl, 5.5 mmol $l^{-1}$ glucose and 5 mmol $l^{-1}$ Hepes, pH 7.800). During the experiments the tissue was gassed with 0.3% $CO_2$ + 99.7% $O_2$ and the temperature was maintained at 21–22°C. All bioelectrical variables were monitored by means of Ag/AgCl electrodes (with tip asymmetry <1 mV) connected to either side of the Ussing chamber with 3 mm bore agar bridges (1 mol $l^{-1}$ KCl in 3% agar). Short-circuit current ($I_{sc}$, μA $cm^{-2}$) was monitored by clamping of epithelia to 0 mV. Voltage clamping and current injections were performed by means of a DVC-1000 voltage clamp amplifier (WPI, Sarasota, FL, USA) or a VCC MC2 voltage/current clamp (Physiologic Instruments). Bioelectrical parameters for each tissue were recorded after the tissue achieved a steady state, usually between 30 and 40 min after mounting in the chambers; prolactin (1000 ng $ml^{-1}$) was added to the basolateral side and $I_{sc}$ was monitored for the next 40 min.

### Identification of prolactin intracellular signalling pathways

The anterior intestine of sea bream was mounted on a tissue holder (P2413, 0.71 $cm^2$, Physiological Instruments) and positioned between two half-chambers (P2400 Ussing chamber, Physiological Instruments) with 1.5 ml of basolateral saline and 1.5 ml of apical saline. The temperature was maintained at 21–22°C. After an initial control period of 30 min, inhibitor was added to the basolateral side of the Ussing chamber for 40 min. The Janus kinase-2 (JAK2) inhibitor AG490 (tyrphostin, Sigma) was used at a concentration 50 μmol $l^{-1}$, mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor U0126 (Sigma) at concentration 10 μmol $l^{-1}$ and the phosphoinositide 3-kinase (PI3K) inhibitor LY-294002 at a concentration 75 μmol $l^{-1}$. After pre-incubation of the tissue with inhibitors, prolactin (1000 ng $ml^{-1}$) was added to the basolateral side and the secretion of bicarbonate was monitored for an additional 40 min period.

### Quantification of transporter gene expression

To establish the effects of prolactin on selected $HCO_3^-$ transporter expression, 3 h *ex vivo* cultures were used. The expression of the basolateral $Na^+$–$HCO_3^-$ co-transporter (Slc4a4) and the apical $Cl^-/HCO_3^-$ exchangers Slc26a6 and the Slc26a3 was analysed in response to different levels of prolactin in the culture medium (0, 1, 10, 100 and 1000 ng $ml^{-1}$). Fish were captured and decapitated as above, the abdominal cavity was exposed and the intestinal tract collected into oxygenated saline: 160 mmol $l^{-1}$ NaCl, 1 mmol $l^{-1}$ $MgSO_4$, 2 mmol $l^{-1}$ $NaH_2PO_4$, 1.5 mmol $l^{-1}$ $CaCl_2$, 5 mmol $l^{-1}$ $NaHCO_3$, 3 mmol $l^{-1}$ KCl, 5.5 mmol $l^{-1}$ glucose, 5 mmol $l^{-1}$ Hepes and 10 μl $ml^{-1}$ antibiotics (penicillin 10.000 IU $ml^{-1}$ + streptomycin 10.000 μg $ml^{-1}$, Sigma-Aldrich; pH 7.800; gassed with 0.3% $CO_2$ + 99.7% $O_2$). The anterior region of the sea bream intestine samples was flushed out with saline, cut open and flattened. Ten longitudinal sections of a similar size (20–30 mg) from the anterior intestine of the same individual were collected. Tissue explants (in duplicate) were transferred to individual wells of a sterile 24-well plate, suspended in 2 ml saline in an incubation chamber with a controlled atmosphere (0.3% $CO_2$ + 99.7% $O_2$). Samples from the same fish were exposed to different concentrations of prolactin (0, 1, 10, 100 and 1000 ng $ml^{-1}$) and incubated for 3 h. After incubation, samples were placed in RNA later (Sigma-Aldrich) and stored for 1 week at 4°C until use.

Total RNA was extracted with E.Z.N.A. total RNA isolation kit I (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions, and the quantity and quality assessed (Nanodrop 1000, Thermo Scientific, Barrington, IL, USA). Prior to cDNA synthesis, RNA was treated with DNase using a DNA-free kit (Ambion, Life Technologies, Paisley, UK) following the supplier's instructions. Reverse transcription of RNA into cDNA was carried out using RevertAid first strand cDNA synthesis kit (Fermentas, Thermo Scientific) following the manufacturer's instructions, with 500 ng of total RNA in a final reaction volume of 20 μl.

For primer design, ion transporter sequences from sea bream were identified in the EST collection database in the National Center of Biotechnology (NCBI, http://blast.ncbi.nlm.nih.gov/) using BLASTn queries of known fish sequences. Identity confirmation of sea bream transporter sequences isolated was achieved after tBLASTx (Altschul et al., 1997) analysis and maximum parsimony (MP) phylogenetic analysis. The MP tree with sea bream clustering was obtained using the 'Close-Neighbor-Interchange' algorithm in MEGA5 (Tamura et al., 2011) with alignments generated by CLUSTALW (see supplementary material Figs S1–S3 for details). Clustering of sea bream sequences into paralogue groups follows the notation described previously for the euryhaline pufferfish (Chang et al., 2012; Kurita et al., 2008). Specific primers were designed using Primer3 (http://frodo.wi.mit.edu/) running under the EBioX (http://www.ebioinformatics.org/) interface for Macintosh. Table 1 shows primer sequences, amplicon sizes and NCBI accession numbers of the ion transporter sequences analysed.

Real-time qPCR amplifications were performed in duplicate with 75 ng cDNA, 350 nmol $l^{-1}$ of forward and reverse primers and the reporter dye SsoFast EvaGreen Supermix (BioRad, Hercules, CA, USA) using the iCycler iQ (BioRad). The amplification protocol

Table 1. Primers for qPCR

| Gene | Sequence (5′ to 3′) | Amplicon (bp) | Accession no. |
|---|---|---|---|
| *Slc26A3* | Forward ATCTCGGCTCTGAAGGGACT | | |
| | Reverse GAGCATTTCTGTCCCTGCTC | 162 | AM973894 |
| *Slc26A6* | Forward GCGGGACTGTTCAGCGGAGG | | |
| | Reverse TGCGAACACGCCTGAACGGCA | 176 | FM155691.1 |
| *Slc4A4* | Forward ACCTTCATGCCACCGCAGGG | | |
| | Reverse CGCCGCCGCCGATAACTCTT | 128 | FM157528.1 |
| *18S* | Forward AACCAGACAAATCGCTCCAC | | |
| | Reverse CCTGCGGCTTAATTTGACTC | 139 | AY993930 |

was as follows: denaturing and enzyme activation step at 95°C for 2 min, followed by 35 cycles of 95°C for 5 s and 63°C for 10 s. At the end of amplification, a temperature-dependent dissociation step was carried out at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s.

To estimate amplification efficiencies, a standard curve was generated for each primer pair from 10-fold serial dilutions (from 100 to $1\times10^{-4}$ pg) of a pool of first-strand cDNA template from all samples. Standard curves represented the cycle threshold value as a function of the logarithm of the number of copies generated, and was defined arbitrarily as one copy for the most diluted standard. All calibration curves exhibited correlation coefficients >0.99, and the corresponding real-time PCR efficiencies were >99%. Normalization of expression for each target gene was performed using 18S ribosomal protein expression.

### Statistics

Results are shown as means ± s.e.m. unless otherwise stated. After assessment of normality and homogeneity of variances, differences between groups were established using Student's *t*-test, one-way analysis of variance (ANOVA) or repeated measures ANOVA (RM ANOVA) followed by the *post hoc* Bonferroni test. All statistical analyses were performed using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego, CA, USA). Groups were considered significantly different at $P<0.05$, unless stated otherwise.

## RESULTS

### Effect of prolactin on bicarbonate secretion and $I_{sc}$

The basolateral addition of prolactin (1000 ng ml$^{-1}$) to a preparation of the anterior intestine of sea bream resulted in a significant decrease ($P<0.05$, one-way RM ANOVA) of bicarbonate secretion within 20 min and reached a minimum within 40 min (Fig. 1A). The effects of prolactin on bicarbonate secretion were achieved without modification of epithelial selectivity, as shown by unchanged values of $R_t$ (Fig. 1B). Removal of prolactin from the basolateral saline and washing of the preparation with prolactin-free saline did not restore bicarbonate secretion to basal values, at least during the 1 h of measurement (Fig. 1C). Basolateral addition of prolactin (1000 ng ml$^{-1}$) did not evoke changes in $I_{sc}$ (Fig. 2).

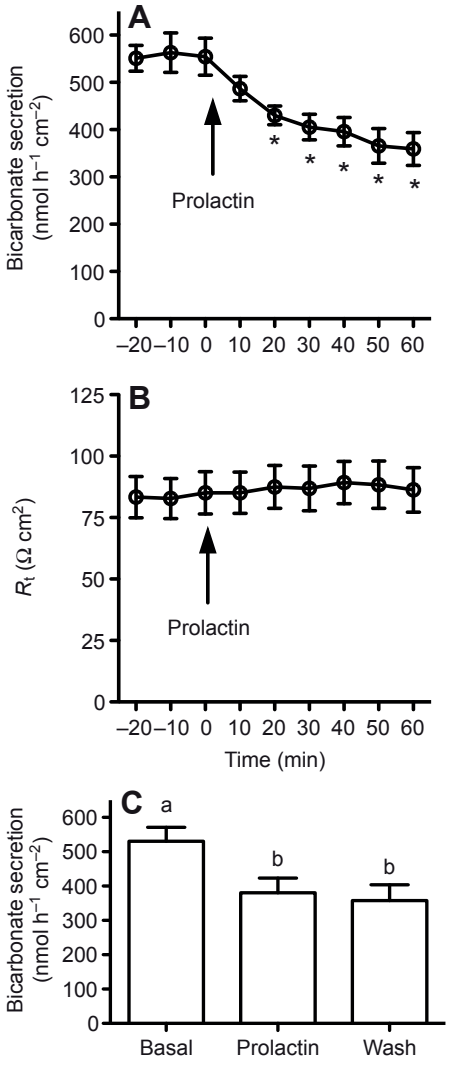


Fig. 1. (A) Bicarbonate secretion as measured by pH-stat and (B) tissue resistance ($R_t$) in the anterior intestine of sea bream in response to ovine prolactin (1000 ng ml$^{-1}$) applied to the basolateral side in the Ussing chambers. (C) Effect of removal of prolactin from the basolateral saline and replacement with prolactin-free basolateral saline (wash) on bicarbonate secretion. Data are means ± s.e.m. ($N=7$). Asterisks represent significant differences from control periods [$P<0.05$, one-way repeated measures (RM) ANOVA].

### Dose effect of prolactin on bicarbonate secretion

To establish whether the effect of prolactin on bicarbonate secretion in the anterior intestine of sea bream followed a typical dose–response curve, increasing doses of prolactin were added to Ussing preparations of the anterior intestine at 45 min intervals (Fig. 3). Prolactin applied between 1 and 1000 ng ml$^{-1}$ evoked a dose-dependent reduction of bicarbonate secretion in the anterior intestine of sea bream (Fig. 3). The lowest dose at which a statistically significant effect of prolactin on bicarbonate secretion was detected was 10 ng ml$^{-1}$ ($P<0.05$, one-way RM ANOVA) and higher doses induced a further decrease in intestinal bicarbonate secretion. For clarity, the data are also presented as dose–response inhibition of bicarbonate secretion by prolactin; maximal inhibitory effects reached 60–65% of basal bicarbonate secretion and were obtained with doses of prolactin between 100 and 1000 ng ml$^{-1}$ (Fig. 3B).

### Inhibition of basolateral $Na^+/K^+$-ATPase over-rides prolactin effects

Fig. 4 compares the inhibitory effects of prolactin (1000 ng ml$^{-1}$), ouabain (1 mmol l$^{-1}$) and a combination of both on bicarbonate secretion in the anterior intestine of sea bream. With the addition of basolateral prolactin alone to anterior intestine preparations of sea bream a plateau was reached from *ca*. 40 min onwards and showed stable inhibition of 50% bicarbonate secretion. Addition of 1 mmol l$^{-1}$ of the $Na^+/K^+$-ATPase inhibitor ouabain, alone or in combination with basolateral prolactin induced a continuous inhibition of intestinal bicarbonate secretion that reached *ca*. 70% at the end of the experimental period (60 min) and was significantly greater than the effect of prolactin alone ($P<0.05$, one-way ANOVA; Fig. 4).

### Prolactin inhibitory effect on bicarbonate secretion is cellular and transcellular

The response of bicarbonate secretion to basolateral prolactin was tested in the presence or absence of basolateral $HCO_3^-/CO_2$ (Fig. 5A,B). In both cases the application of prolactin (1000 ng ml$^{-1}$) had an inhibitory effect on bicarbonate secretion ($P<0.05$, Student's *t*-test) and the magnitude of inhibition was similar in the two experiments (66–68% in relation to basal secretion). However, the

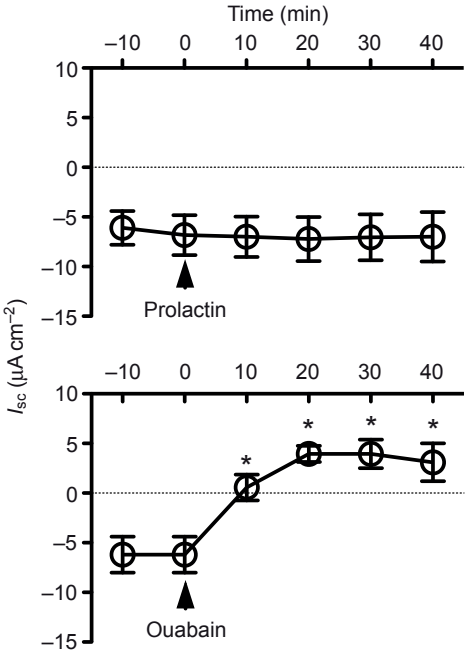


Fig. 2. Short-circuit current ($I_{sc}$) in the anterior intestine of sea bream in response to ovine prolactin (1000 ng ml$^{-1}$) or ouabain (1 mmol l$^{-1}$) applied to the basolateral side in the Ussing chambers. Data are means ± s.e.m. ($N$=5). Asterisks represent significant differences from basal values ($P$<0.05, one-way RM ANOVA).

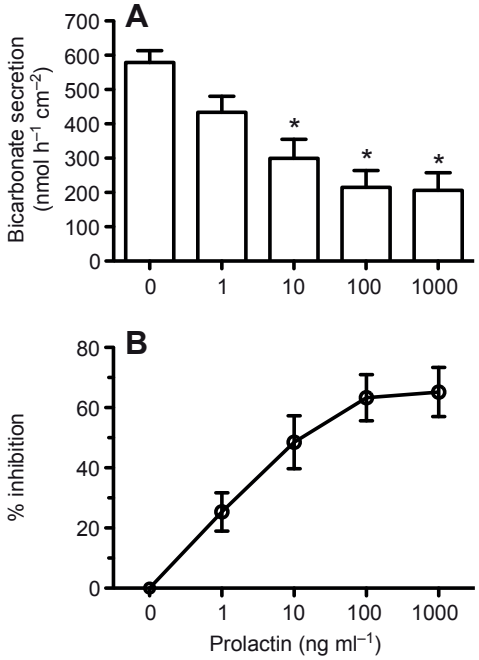


Fig. 3. (A) Bicarbonate secretion as measured by pH-stat in the anterior intestine of sea bream in response to increasing concentrations of ovine prolactin (0, 1, 10, 100 and 1000 ng ml$^{-1}$) applied to the basolateral side in the Ussing chambers. (B) Inhibitory effect of each dose relative to basal values (% inhibition). Data are means ± s.e.m. ($N$=4). Asterisks represent significant differences from basal values ($P$<0.05, one-way RM ANOVA).

absolute effect of prolactin was significantly higher in the presence than in the absence of basolateral $HCO_3^-/CO_2$ ($P$<0.05, Student's $t$-test; Fig. 5C). The inhibitory effect of prolactin on bicarbonate secretion was proportional to the transcellular/endogenous generation of bicarbonate for apical secretion.

### Prolactin effect on bicarbonate secretion is mediated by prolactin receptors

Several inhibitors of intracellular signalling cascades were used to determine the specific intracellular signalling pathways modified when prolactin activates its receptor in the sea bream intestine to bring about its effect on bicarbonate secretion. In the presence of a JAK2 pathway inhibitor (AG490, 50 μmol l$^{-1}$) the inhibitory effects of prolactin (1000 ng ml$^{-1}$) on bicarbonate secretion were significantly reduced (by 60%; $P$<0.05, Student's $t$-test). Interestingly, 75 μmol l$^{-1}$ LY294002, a specific PI3K inhibitor and 10 μmol l$^{-1}$ U0126, a specific MEK inhibitor, produced a similar significant reduction ($P$<0.05, Student's $t$-test) in the effect of prolactin on bicarbonate secretion in the anterior intestine of sea bream (Fig. 6). These results indicate that JAK2, PI3K and MEK intracellular signalling mediate the effect of prolactin on bicarbonate secretion in the sea bream intestine.

### Sea bream $HCO_3^-$ transporter clustering with fish paralogues

MP phylogenetic analysis of sea bream translated sequences (supplementary material Figs S1–S3) shows that the sea bream Slc26a6 anion exchanger corresponds to piscine paralogue A; sea bream Slc26a3 corresponds to piscine paralogue B; and sea bream Slc4a4 corresponds to piscine paralogue A. The classification follows the notation described previously for the euryhaline pufferfish (Chang et al., 2012; Kurita et al., 2008).

### $HCO_3^-$ transporter gene expression in response to prolactin

Prolactin at 10–1000 ng ml$^{-1}$ caused a statistically significant reduction in *Slc4a4* gene expression compared with untreated anterior intestine (Fig. 7A; $P$<0.05, one-way RM ANOVA). No significant effects were observed in the expression of apical *Slc26a3* and *Slc26a6* in response to prolactin (Fig. 7B,C).

## DISCUSSION

The present study establishes a role for prolactin in the regulation of bicarbonate secretion in the intestine of marine fish. The lines of evidence provided are as follows: bicarbonate secretion in the intestine of the sea bream is sensitive to physiological levels (ng) of prolactin, the effect of prolactin follows a typical dose–response curve and the effect of prolactin on bicarbonate secretion is reduced by blockade of prolactin intracellular signalling *via* JAK2, MEK or PI3K.

In the marine sea bream, prolactin receptor transcripts and protein are highly abundant in the intestine (Santos et al., 2001), indicating that this is a likely target for prolactin although its function at this site has never been described. In the present study, we report the inhibitory action of prolactin on intestinal bicarbonate secretion in the sea bream. The action is rapid, as measured by pH-stat in Ussing chambers, and reaches a plateau within 40 min. It also takes place without alteration of tissue barrier function, expressed electrically by $R_t$. Removal of prolactin from the basolateral chamber did not restore bicarbonate secretion to values obtained before to prolactin treatment (1000 ng ml$^{-1}$) within 3.5 h, the time period for maintenance of vital conditions in *in vitro* sea bream intestine preparations (Fuentes et al., 2006), and values remained stable until the end of the experiments. The rapid time frame of the response to prolactin is indicative that non-genomic pathways are responsible for this inhibitory action.

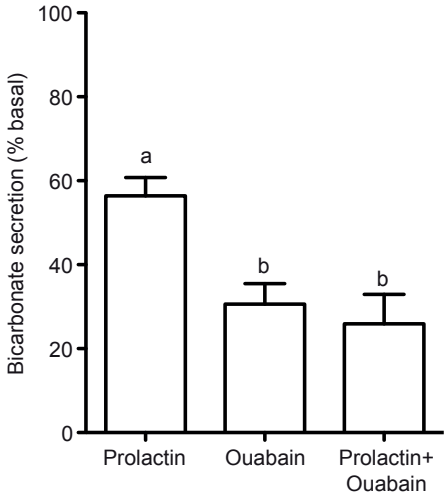


Fig. 4. Bicarbonate secretion as measured by pH-stat in the anterior intestine of sea bream. Results are shown as a percentage of control values after 60 min basolateral exposure to ovine prolactin alone (1000 ng ml$^{-1}$), ouabain alone (1 mmol l$^{-1}$) or a combination of ovine prolactin and ouabain (1000 ng ml$^{-1}$ + 1 mmol l$^{-1}$, respectively). Data are means ± s.e.m. ($N$=5–7). Different letters denote significantly different groups ($P$<0.05, one-way ANOVA).

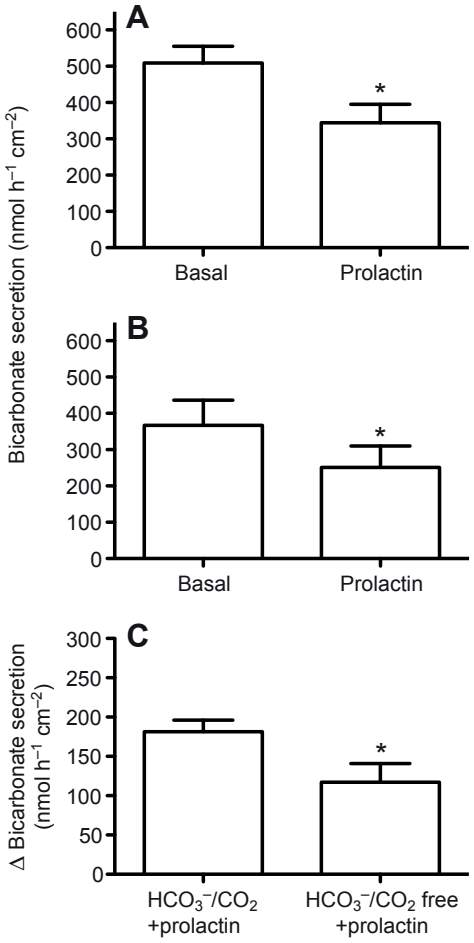


Fig. 5. Bicarbonate secretion as measured by pH-stat in the anterior intestine of sea bream in response to ovine prolactin (1000 ng ml$^{-1}$) in the presence (A) or absence (B) of basolateral $HCO_3^-/CO_2$. (C) Prolactin-dependent bicarbonate secretion with and without basolateral $HCO_3^-/CO_2$. Data are means ± s.e.m. ($N$=7). Asterisks represent significant differences between treatments ($P$<0.05, Student's $t$-test).

The concentration of prolactin in fish plasma in both freshwater and seawater is in the low ng ml$^{-1}$ range. In *Oreochromis mossambicus* and *Oreochromis niloticus* the range of plasma prolactin is 5–20 ng ml$^{-1}$ during short-term (under 24 h) transfers between salinities (Breves et al., 2010) and in seawater-adapted *O. mossambicus*is it is 3.4 ng ml$^{-1}$ (Takahashi et al., 2007). In freshwater cannulated brown trout (*Salmo trutta*), plasma prolactin levels are in the 0–30 ng ml$^{-1}$ range (Waring et al., 1996) and are 10–15 ng ml$^{-1}$ in freshwater rainbow trout (*Salmo gairdneri*) and 3–5 ng ml$^{-1}$ in seawater-adapted trout (Prunet et al., 1985). Circulating plasma prolactin levels in the sea bream have not been reported. However, based on the secretion pattern and pituitary content (Brinca et al., 2003; Fuentes et al., 2010a) the levels of prolactin in sea bream plasma are likely to be within the range described for other species. The bicarbonate secretion responses of the anterior intestine to basolateral prolactin revealed a significant threshold effect at the physiological level of 10 ng ml$^{-1}$, with inhibition of bicarbonate secretion of around 40% (Fig. 3B). An inhibitory plateau in excess of 60% was reached at levels ≥100 ng ml$^{-1}$ prolactin. This result reveals the likely importance of prolactin in the regulation of bicarbonate secretion *in vivo*. Even the lower plasma concentrations of prolactin are expected to have a direct regulatory effect on bicarbonate secretion and an indirect effect on the availability of water for intestinal absorption, with an end result of increased plasma osmolality.

Luminal secretion of bicarbonate in the enterocyte of marine fish is the result of transcellular transport and endogenous generation of $HCO_3^-$ from $CO_2$. The presence of a basolateral $Na^+/HCO_3^-$ co-transporter (Slc4a4) and apical $Cl^-/HCO_3^-$ exchangers (Kurita et al., 2008; Taylor et al., 2010) suggests that bicarbonate could be routed at a transcellular level. However, in the absence of basolateral bicarbonate, a significant amount of alkaline secretion still takes place (Fuentes et al., 2010b; Taylor et al., 2010), probably through hydration of the $CO_2$ in the intestinal epithelial cell by the action of carbonic anhydrase (Grosell et al., 2007). The values of intestinal bicarbonate secretion obtained in this study in the sea bream by pH-stat methods are in keeping with the range previously described in the same species using the double titration method (Fuentes et al., 2010b). In the conditions used for most of the experiments in this study (0.3% $CO_2$–5 mmol l$^{-1}$ $HCO_3^-$ in the basolateral saline) only about 30% of bicarbonate secretion is likely to be driven *via* transcellular pathways, while the remainder is probably (Grosell et al., 2007) produced by hydration of $CO_2$ in the enterocyte (shown in $CO_2/HCO_3^-$-free basolateral saline). Under these conditions, it seems likely that the inhibitory effects of prolactin on luminal bicarbonate secretion in the sea bream intestine are mediated by both transcellular and cellular mechanisms. This suggestion is supported by the significant effects of prolactin on bicarbonate secretion in the Ussing chamber, in both the presence and the absence of basolateral $HCO_3^-/CO_2$ (Fig. 5).

The mechanisms involved in bicarbonate secretion regulated by prolactin in the intestine of the sea bream have not been definitely established. The link between prolactin and $Na^+/K^+$-ATPase (Mancera et al., 2002; Tipsmark et al., 2011) was tested using the specific $Na^+/K^+$-ATPase inhibitor ouabain and resulted in a 70% inhibition of total bicarbonate secretion. In the anterior intestine of the Gulf toadfish, ouabain has a similar effect (Grosell and Genz,

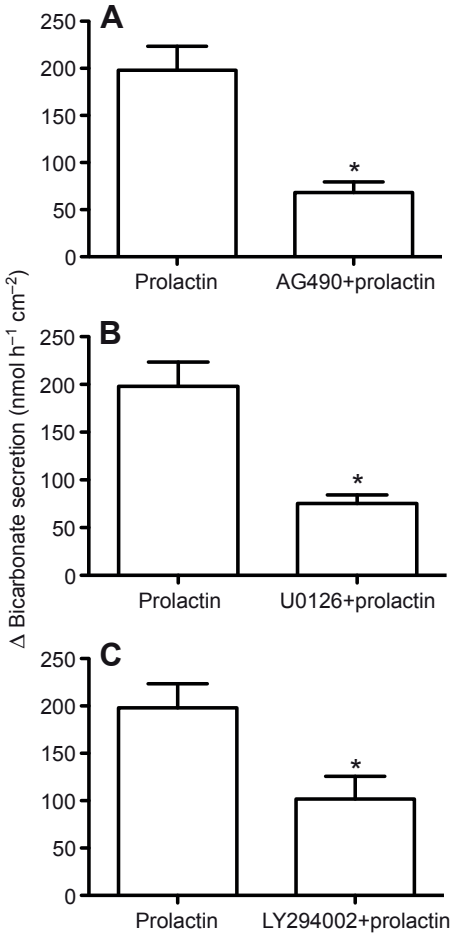


Fig. 6. Prolactin-dependent bicarbonate secretion as measured by pH-stat in the anterior intestine of sea bream in response to ovine prolactin (1000 ng ml$^{-1}$) alone (left) or after the addition (right) of (A) a JAK2 inhibitor (AG490, 50 μmol l$^{-1}$), (B) a MEK inhibitor (U0126, 10 μmol l$^{-1}$) or (C) a PI3K inhibitor (LY294002, 75 μmol l$^{-1}$). Data are means ± s.e.m. (*N*=5). Asterisks represent a significant decrease of prolactin effect in the presence of inhibitors (*P*<0.05, Student's *t*-test).

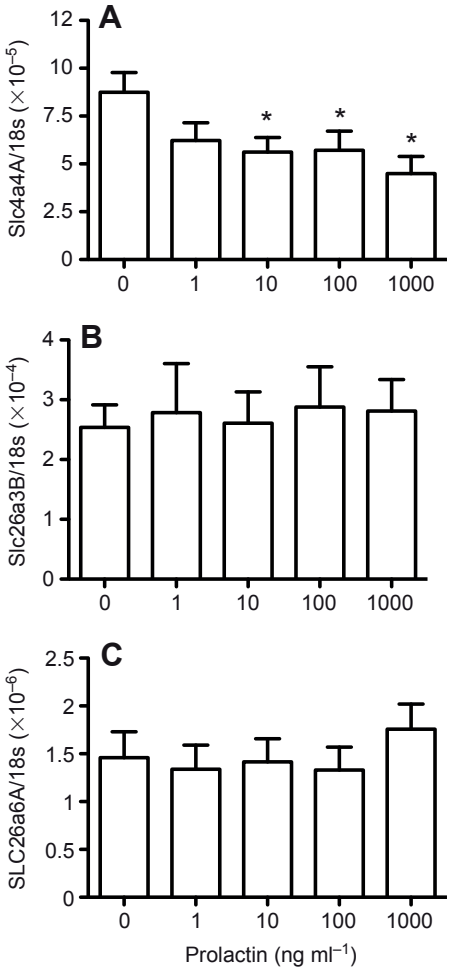


Fig. 7. (A) *Slc4a4A*, (B) *Slc26a3B* and (C) *Slc26a6A* gene expression relative to *18S* as measured by qPCR in anterior intestine of sea bream after *in vitro* culture for 3 h in the absence (0) or presence of prolactin (1, 10, 100 and 1000 ng ml$^{-1}$). Data are means ± s.e.m. (*N*=6). Asterisks indicate significant differences from control (*P*<0.05, one-way RM ANOVA).

2006). The effect of ouabain was explained by the reduction of the basolateral $Na^+$ gradient, which is essential to drive $H^+$ extrusion *via* a sodium–proton exchanger (NHE) required for apical bicarbonate secretion (Grosell and Genz, 2006). The inhibitory effect of ouabain would account for the reduction in apical bicarbonate secretion derived from transcellular and cellular $HCO_3^-$, although this possibility was not tested in the present study. In the anterior intestine of the sea bream, the inhibition of apical bicarbonate secretion in response to ouabain was significantly greater than the effect of prolactin alone. Furthermore, prolactin added in combination with ouabain failed to induce additional inhibition of apical bicarbonate secretion. Taken together, these results indicate that ouabain probably offsets the effect of prolactin on the $Na^+/K^+$-ATPase. However, the reduction by ouabain of the basolateral $Na^+$ gradient required for apical bicarbonate secretion would mask the effects of prolactin on additional mechanisms. Interestingly, prolactin and ouabain have different effects in voltage-clamped preparations of anterior intestine in Ussing chambers. While no effect was observed in response to prolactin, ouabain totally abolished the absorptive current (Fig. 2). This result may indicate that the effects of prolactin are mediated *via* a non-electrogenic transporter. A recent study in the Gulf toadfish (Taylor et al., 2010) proposed the basolateral $Na^+$–$HCO_3^-$ co-transporter Slc4a4 to be a limiting step for apical bicarbonate secretion in the marine fish intestine. It is tempting to speculate that prolactin has a regulatory action on basolateral Slc4a4, as indicated by the genomic effect of prolactin on the expression of *Slc4a4* gene but not on the expression of the apical anion transporters genes *Slc26a6* and *Slc26a3* (Fig. 7). However, the inhibitory effect of prolactin on bicarbonate secretion in preparations devoid of basolateral $HCO_3^-/CO_2$ points to an effect of prolactin on other important mechanisms.

In higher vertebrates, prolactin receptors signal *via* specific intracellular cascades involving the JAK2 pathway, PI3K and/or MEK, depending on the tissues analysed (al-Sakkaf et al., 1997; Jantarajit et al., 2007; Piccoletti et al., 1994; Yamauchi et al., 1998). In fish, despite the evidence showing physiological actions of prolactin in osmoregulation, little effort has been devoted to the study of prolactin intracellular cascades. In our *in vitro* model of anterior intestine, prolactin effects on bicarbonate secretion were significantly reduced by the action of the JAK2, PI3K and MEK inhibitors. This indicates that the effects of prolactin on bicarbonate secretion are specifically mediated by prolactin

receptors. In Caco-2 cell line monolayers and in rat duodenum, prolactin-stimulated transepithelial calcium movements are mediated by the actions of PI3K, but not by MEK or JAK2 (Jantarajit et al., 2007), while in the mammary gland cell line HC11, JAK2 mediates the actions of prolactin in $Cl^-$ transport (Selvaraj et al., 2000). Although further studies are required, it appears that the action of prolactin on bicarbonate secretion in the anterior intestine of the sea bream is the result of signalling through the three intracellular pathways. Whether this biological action of prolactin takes place *via* the previously characterized sea bream prolactin receptor (Santos et al., 2001) or whether multiple receptors are involved requires further investigation. More than a single functional receptor has been characterized in some fish, including the Mozambique tilapia (Fiol et al., 2009), the Nile tilapia and the black sea bream (Huang et al., 2007). In the last two, functional prolactin receptors sensitive to $ng\,ml^{-1}$ prolactin in gene promoter assays are expressed in the intestine and could be responsible for the actions of prolactin on intestinal bicarbonate secretion observed in this study.

In a previous study (Fuentes et al., 2010b) it was demonstrated that the calcitropic hormones PTHrP and STC had a significant role in the regulation of bicarbonate secretion and that this opposed the effects of both factors on net calcium transport in the intestine of the sea bream, thus modulating intestinal aggregate formation and water availability for absorption. The present study has established that prolactin has an osmoregulatory function in the intestine of marine fish compatible with its known actions on adaptation to freshwater. Sustained intestinal bicarbonate secretion is a special feature of the intestine of marine fish directed at enabling water absorption, and the actions of prolactin downregulate this process. The effects are rapid, compatible with plasma circulating levels of prolactin in freshwater and seawater fish, and in the light of the involvement of JAK2, MEK or PI3K intracellular pathways are likely to be mediated by prolactin receptors. In addition to its rapid effects, prolactin regulates the expression of intestinal *Slc4a4*, proposed to be the rate-limiting step (Taylor et al., 2010) for apical bicarbonate secretion in the marine fish intestine. Based on evidence from the present study, a new functional role for prolactin is proposed; namely, regulation of bicarbonate secretion in the intestine of marine fish, which is an essential function in ion/water homeostasis. It will be important to establish whether other endocrine factors directly regulate intestinal bicarbonate secretion and whether calcium sensing (e.g. calcitropic factors) or osmosensing (e.g. prolactin) is the predominant signal for regulation of this process.

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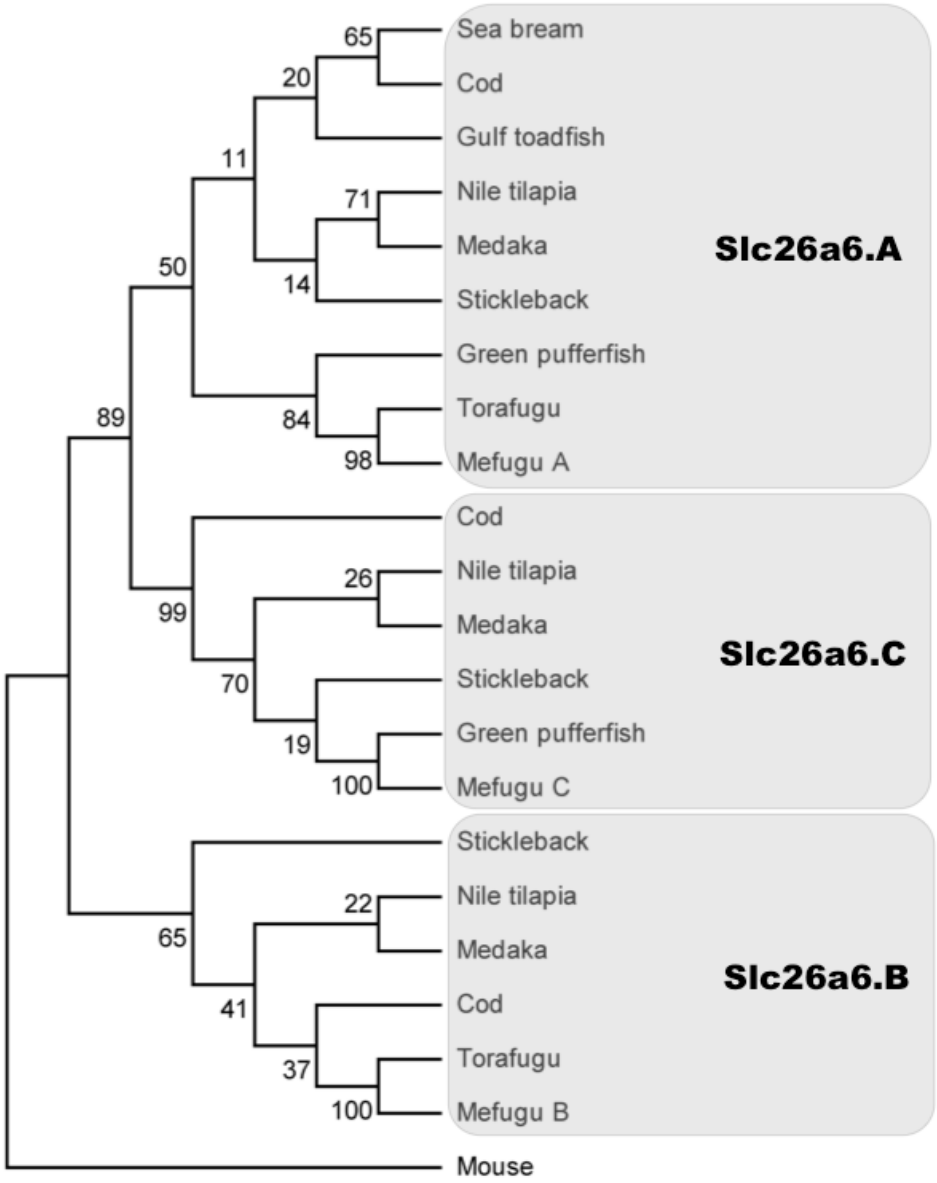


**Fig. S1.** Slc16a6 evolutionary history inferred using the maximum parsimony (MP) method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa. The MP tree was obtained using the Close-Neighbor-Interchange algorithm in MEGA5 using alignments generated by CLUSTALW. GenBank and Ensembl accession numbers of amino acid sequences are as follows. Branch Slc26a6.A: sea bream (*Sparus aurata*, this study translated FM155691.1), cod (*Gadus morhua*, ENSGMOP00000018827), Gulf toadfish (*Opsanus beta*, ABQ01444.1), Nile tilapia (*Oreochromis niloticus*, ENSONIP00000015691), medaka (*Orizyas latipes*, ENSORLP00000011505), stickleback (*Gasterosteus aculeatus*, ENSGACP00000002987), green pufferfish (*Tetraodon nigroviridis*, ENSTNIP00000008886), torafugu (*Takifugu rubripes*, ENSTRUP00000007745), mefugu A (*Takifugu obscurus*, BAE75796.1). Branch Slc26a6.B: stickleback (*Gasterosteus aculeatus*, ENSGACP00000007629), Nile tilapia (*Oreochromis niloticus*, ENSONIP00000002151), medaka (*Orizyas latipes*, ENSORLP00000003731), cod (*Gadus morhua*, ENSGMOP00000002315), torafugu (*Takifugu rubripes*, ENSTRUP00000007000), mefugu B (*Takifugu obscurus*, BAE75797.1|). Branch Slc26a6.C: cod (*Gadus morhua*, ENSGMOP00000010092), Nile tilapia (*Oreochromis niloticus*, ENSONIP00000002151), medaka (*Orizyas latipes*, ENSORLP00000002390), stickleback (*Gasterosteus aculeatus*, ENSGACP00000001974), green pufferfish (*Tetraodon nigroviridis*, ENSTRUP00000038102), mefugu C (*Takifugu obscurus*, BAE75798.1).

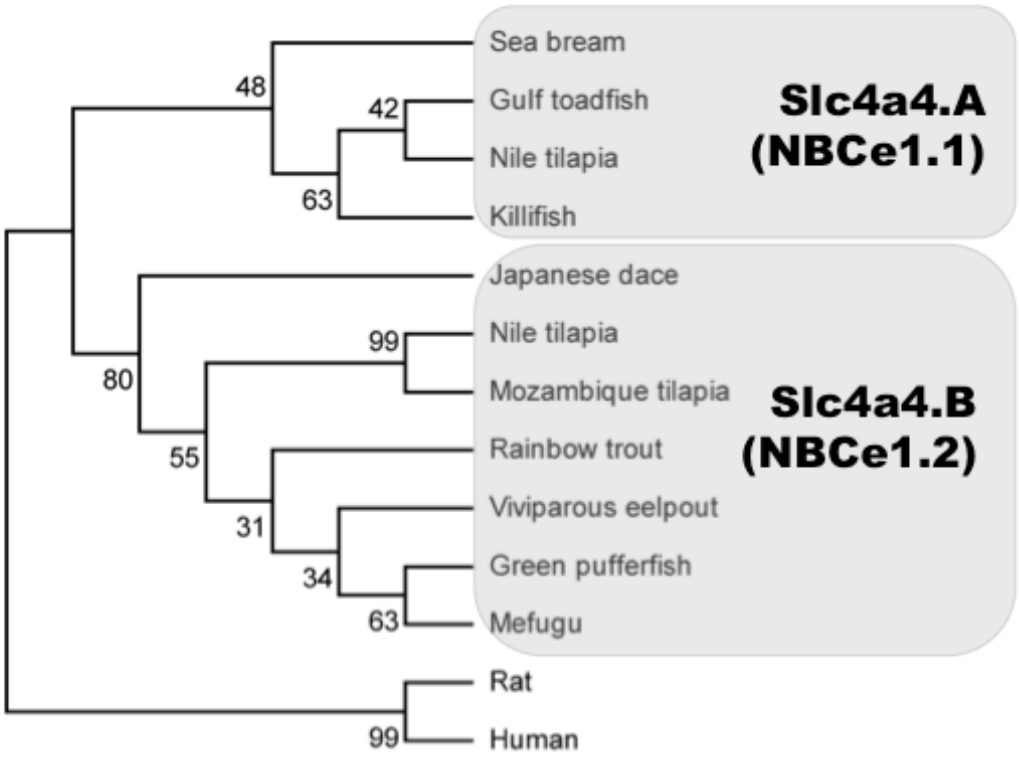


**Fig. S2.** Slc4a4 (NBCe1) evolutionary history inferred using the Maximum Parsimony (MP) method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa. The MP tree was obtained using the Close-Neighbor-Interchange algorithm in MEGA5 using alignments generated by CLUSTALW. GenBank and Ensembl accession numbers of amino acid sequences are as follows. Branch Slc4a4.A (NBCe1.1): sea bream (*Sparus aurata*, this study translated FM157528.1), Gulf toadfish (*Opsanus beta*, ACK37865.1), Nile tilapia (*Oreochromis niloticus*, XP_003444524.1), killifish (*Fundulus heteroclitus*, ACU44673.1). Branch Slc4a4.B (NBCe1.2): Japanese dace (*Tribolodon hakonensis*, BAB83084.1), Nile tilapia (*Oreochromis niloticus*, XP_003449699.1), Mozambique tilapia (*Oreochromis mossambicus*, BAJ49842.1), rainbow trout (*Oncorhynchus mykiss*, NP_001117797.1), viviparous eelpout (*Zoarces viviparous*, ACB46956.1), green pufferfish (*Tetraodon nigroviridis*, CAAE01014533.1), mefugu C (*Takifugu obscurus*, BAG15993.1).

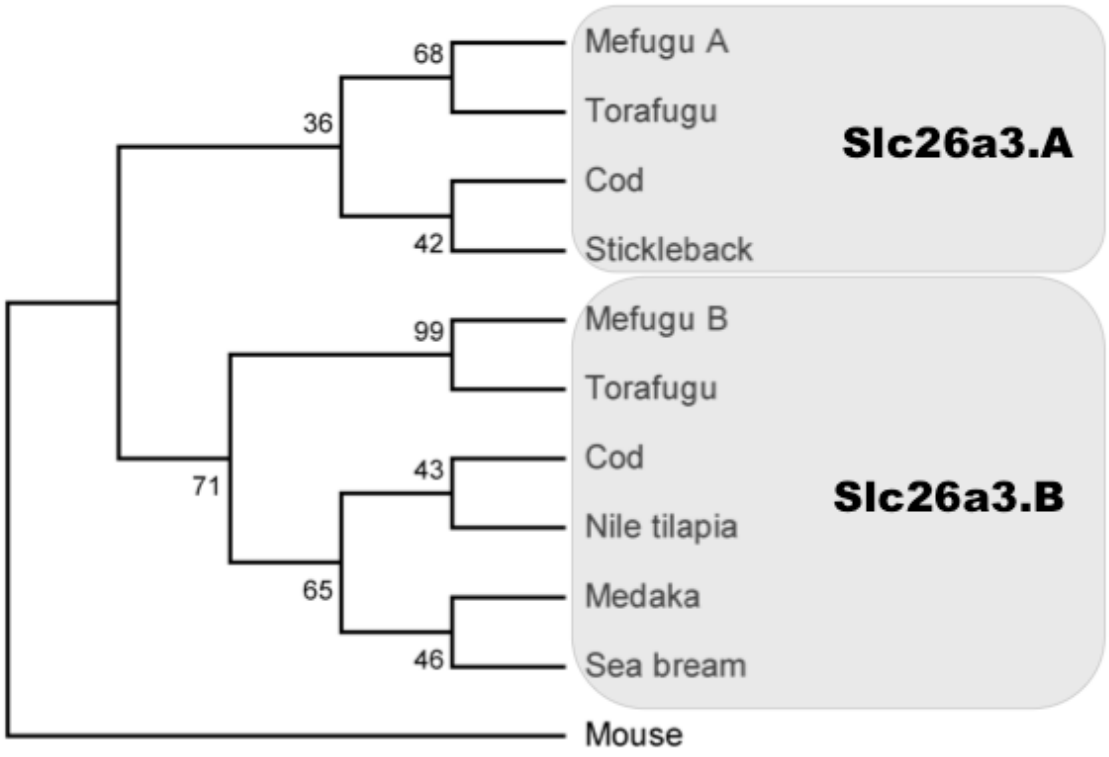


**Fig. S3.** Slc26a3 evolutionary history inferred using the Maximum Parsimony (MP) method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa. The MP tree was obtained using the Close-Neighbor-Interchange algorithm in MEGA5 using alignments generated by CLUSTALW. GenBank and Ensembl accession numbers of amino acid sequences are as follows. Branch Slc26a3.A: mefugu A (*Takifugu obscurus*, BAE75796.1), torafugu (*Takifugu rubripes*, ENSTRUP00000015967), cod (*Gadus morhua*, ENSGMOP00000014051), stickleback (*Gasterosteus aculeatus*, ENSGACP00000025627). Branch Slc26a3.B: mefugu B (*Takifugu obscurus*, BAE75794.1), torafugu (*Takifugu rubripes*, ENSTRUP00000014138), cod (*Gadus morhua*, ENSGMOP00000014075), Nile tilapia (*Oreochromis niloticus*, ENSONIP00000015007), medaka (*Orizyas latipes*, ENSORLP00000015168), sea bream (*Sparus aurata*, this study translated AM973894).