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Endocrine regulation of carbonate precipitate formation in marine fish intestine by Stanniocalcin and PTHrP

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Running head title: Hormones and intestinal precipitates

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SUMMARY

In marine fish, high epithelial bicarbonate secretion by the intestine generates luminal carbonate precipitates of divalent cations that play a key role in water and ion homeostasis. *In vitro* studies highlight the involvement of the calciotropic hormones PTHrP (Parathyroid hormone related protein) and stanniocalcin (STC) in the regulation of epithelial bicarbonate transport. The present study tests the hypothesis that calciotropic hormones have a regulatory role in carbonate precipitate formation *in vivo*. To test this hypothesis sea bream (*Sparus aurata*) juveniles received single intraperitoneal injections of piscine PTHrP(1-34), the PTH/PTHrP receptor antagonist PTHrP(7-34), purified sea bream STC or were passively immunized with polyclonal rabbit antisera raised against sea bream STC (STCAb). Endocrine effects on the expression of the basolateral sodium bicarbonate co-transporter (Slc4a4.A), the apical anion exchangers (Slc26a6.A and Slc26a3.B) and the V-type proton pump beta subunit (Atp6v1b) in the anterior intestine were evaluated. In keeping with their calciotropic nature the hypocalcemic factors PTHrP(7-34) and STC up-regulated gene expression of all transporters. In contrast, the hypercalcemic factor PTHrP(1-34) and STC antibodies down-regulated transporters involved in the bicarbonate secretion cascade. Changes in intestine luminal precipitate contents provoked by calcaemic endocrine factors validated these results. 24 hours post-injection either PTHrP(1-34) or immunization with STCAb reduced the content of carbonate precipitates in the intestine of the sea bream. In contrast, the PTH/PTHrP receptor antagonist PTHrP(7-34) increased not only the precipitated fraction, but also the concentration of HCO_3^- equivalents in the intestinal fluid. These results confirm the hypothesis that calciotropic hormones have a regulatory role in carbonate precipitate formation *in vivo* in the intestine of marine fish. Furthermore, they illustrate for the first time in fish the counter-acting effect of PTHrP and STC and reveal an unexpected contribution of calcemic factors to acid-base balance.

Key words: intestinal physiology, calcium regulation, bicarbonate secretion, ion transporter, endocrine regulation, calciotropic hormones

INTRODUCTION

Marine teleosts sustain an ionic equilibrium with seawater to keep their plasma osmolality within narrow limits. To achieve this, the gills remove excess salts from the body (Evans et al., 2005), in a secretion process that indirectly causes water loss. Therefore, water replacement by drinking becomes essential to sustain ion regulation (Fuentes and Eddy, 1997a). In addition to regulation of the amount of water ingested by endocrine and environmental factors (Fuentes et al., 1996; Fuentes and Eddy, 1997a; Fuentes and Eddy, 1997b; Guerreiro et al., 2004; Guerreiro et al., 2001), the processing of imbibed fluid has a major impact in fish ion regulation. Ingested water is first processed in the esophagus where it undergoes selective absorption of NaCl (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983), which lowers fluid osmolality, and is believed to enhance water absorption in the intestine.

Essential to intestinal fluid processing, and thus intestinal water absorption, is the production of luminal divalent carbonate precipitates in marine teleosts (Grosell, 2006; Grosell, 2011; Kurita et al., 2008; Walsh et al., 1991; Wilson et al., 2002). Luminal precipitate formation requires high concentrations of divalent ions and a chemically favorable high pH. The high divalent ion content required for precipitate formation is supplied by the high calcium content of imbibed seawater, that reaches the intestine in a concentration range that varies between 3-15 mM depending on the species (Fuentes et al., 2006; Genz et al., 2011; Grosell et al., 2001; Wilson et al., 2002). The alkaline pH of the luminal fluid that provides a favorable chemical environment for carbonate precipitation results from high rates of epithelial HCO_3^- secretion by the enterocytes (Grosell, 2006; Grosell, 2011)

Mechanisms for epithelial HCO_3^- secretion are dependent on luminal Cl^- and involve apical Cl^-/HCO_3^- exchangers (Ando and Subramanyan, 1990; Grosell et al., 2005; Wilson et al., 1996), in particular, members of the SLC26 family, that secrete HCO_3^- and absorb Cl^- (Grosell and Genz, 2006; Grosell et al., 2009b; Kurita et al., 2008). In addition to apical mechanisms, a basolateral Na^+–HCO_3^- co-transporter (NBC), which belongs to the SLC4 gene family, leads to accumulation of cellular HCO_3^- to fuel apical secretion (Kurita et al., 2008; Taylor et al., 2010). Furthermore, a Bafilomycin-sensitive H^+ pump participates in the regulation of apical HCO_3^- secretion in seawater adapted rainbow trout and Gulf toadfish (Grosell et al., 2009a; Guffey et al., 2011) and is also

important for regional specialization of fluid processing in the sea bream intestine (Gregorio et al., 2013) .

Calcium as well as HCO_3^- immobilization in the luminal carbonate precipitates, results in reduced fluid osmolality and favors water absorption. Therefore, HCO_3^- secretion in marine fish intestine drives not only luminal precipitate formation, but also water absorption (Grosell, 2011). In keeping with this suggestion, a recent study in the Gulf toadfish demonstrated a role for the HCO_3^-–sensing soluble adenylyl cyclase (sAC) in intestinal water absorption (Tresguerres et al., 2010), a process also occurring in the sea bream intestine (Carvalho et al., 2012).

The importance of fish intestinal carbonate precipitate production in the ocean carbon cycle has recently been highlighted (Wilson et al., 2009) and brings a new dimension to the ecophysiology of fish and their environment. The endocrine factors that regulate drinking, fluid processing, epithelial HCO_3^- secretion and precipitate production require more detailed study. *In vitro* studies have revealed that the calciotropic hormones STC (stanniocalcin) and PTHrP (Fuentes et al., 2010) regulate not only calcium transport, but also the epithelial rate of HCO_3^- secretion in sea bream. Prolactin (Ferlazzo et al., 2012) and both trans-membrane and soluble adenylyl cyclases participate in the regulation of intestinal epithelial HCO_3^- secretion (Carvalho et al., 2012) and hypercalcemic PTHrP regulates drinking rates in vivo in sea bream larvae (Guerreiro et al., 2001) and intestinal calcium transport *in vitro* (Fuentes et al., 2006). Taken together the results from *in vitro* studies support a role for the calciotropic hormones, STC and PTHrP, in processing of intestinal fluid in marine fish. Therefore, the objectives of the present study were to establish the relative importance of the calciotropic hormones PTHrP and STC, i) in the regulation of the main molecular mechanisms SLC26a6, SLC26a3, SLC4a4 and Atp6v1b involved in epithelial HCO_3^- secretion in the intestine of the sea bream, and ii) establish their physiological relevance in intestinal precipitate formation *in vivo*.

RESULTS

Western blot

Western blot analysis of sea bream serum with an antibody generated against purified sea bream STC identified a single immunoreactive band (Figure 1). The apparent size of

the immunoreactive band under denaturing reducing conditions is 3 kDa smaller that the purified STC (26kDa). This apparent disparity in size is likely related to the removal of the signal peptides of 30 amino acids in the N-terminus of stanniocalcin as previously described for other fish species (Amemiya et al., 2006; Amemiya et al., 2002). Pre-absorption of the sea bream anti-STC sera with a protein extract of isolated Stannius corpuscles ablated the immunoreactive band obtained in western blots of serum and purified STC and confirms antisera specificity.

Immunohistochemistry

STC immunoreaction (Figure 2) was restricted to the corpuscles of Stannius and no immunoreaction was detected in the adjacent kidney (Figure 2A, D). Moreover, the immunoreaction was restricted to the cell cytoplasm (Figure 2E). No immunoreaction was obtained when antisera pre-absorbed with extracts of the corpuscles of Stannius was used (Figure 2C, F).

Plasma calcium

Intra-peritoneal injections of PTHrP(1-34) alone, STC antisera alone or a combination of both treatments induced comparable significant increases in circulating plasma calcium in sea bream juveniles 24 hours post-injection (Figure 3A).

Intra-peritoneal injections of the PTHrP receptor antagonist PTHrP(7-34) alone or STC alone resulted in absolute plasma calcium levels lower than those of the controls, but the effect was not significant (Figure 3B). However, intra-peritoneal injection of PTHrP(7-34) and STC combined as a single treatment significantly reduced plasma calcium in sea bream juveniles (Figure 3B).

qPCR

Intra-peritoneal injections of PTHrP(1-34) alone, STC antisera alone or a combination of both treatments induced comparable significant decreases in the expression of all genes analyzed i.e. SLC4a4, Atp6v1b, SLC26a3 and SLC26a6 in the anterior intestine of sea bream juveniles 24 hours post-injection (Figure 4).

Intra-peritoneal injections of the PTHrP receptor antagonist PTHrP(7-34) alone or in combination with STC resulted in a significant increase in the expression of SLC4a4, Atp6v1b, SLC26a3 and SLC26a6 in the anterior intestine of sea bream juveniles 24

hours post-injection (Figure 5). However, no significant effects were achieved in response to STC alone, although the general trend was an increase in the expression of all transporters (Figure 5).

Intestinal luminal fluid and precipitate analysis

HCO_3^- concentration in the intestinal fluid of control fish averaged 45 mEquiv l^{-1} (Figure 6) and the total carbonate in the precipitate expressed as the average of $CaCO_3$+$MgCO_3$ was in the order of 350 nEquiv/gram of body mass in control fish. Intra-peritoneal injections of PTHrP(1-34) alone resulted in a trend for lower levels of HCO_3^- in the intestinal fluid and a significant decrease of total carbonate content in the intestinal precipitate fraction (Figure 6). In contrast, intra-peritoneal injections of the PTHrP receptor antagonist PTHrP(7-34) resulted in a significant increase in HCO_3^- in the intestinal fluid and in total carbonate content of the intestinal precipitate (Figure 6). Additionally, intra-peritoneal injection of STC antibodies significantly decreased HCO_3^- in the intestinal fluid and total carbonate content of the intestinal precipitate. A STC treatment alone was omitted as insufficient purified native sea bream STC was available to treat sea bream juveniles of 20-25 gram of body mass.

DISCUSSION

The present study establishes *in vivo* effects of the calciotropic hormones, PTHrP and STC, on the mechanisms of carbonate precipitation in the sea bream intestine. This claim is based on the fact that the hypercalcemic or hypocalcemic factors: 1) regulate in a reproducible manner key molecular mechanisms involved in the epithelial HCO_3^- secretion cascade; 2) the regulatory effect is predictable; and 3) they modify luminal intestinal bicarbonate/carbonate contents both in the intestinal fluid and the precipitate fraction.

In a previous study (Fuentes et al., 2010) the *in vitro* actions of PTHrP(1-34) and STC in the regulation of epithelial HCO_3^-secretion in the intestine of the sea bream was revealed. To extend the *in vitro* results to an *in vivo* situation both PTHrP(1-34) and the receptor antagonist PTHrP(7-34), which has an established bioactivity in sea bream *in vivo* (Fuentes et al., 2007a), was utilized. The effect of the receptor antagonist PTHrP(7-34) on epithelial HCO_3^-secretion *in vivo* indicates the effects of PTHrP(1-34) on epithelial HCO_3^-secretion in the intestine is through one of the 3 previously

characterized teleost PTH receptors. The STC receptor(s) sequence(s) remain unidentified, which consequently precludes the design of receptor–specific antagonists. However, a previous study in tilapia (Fenwick and Flik, 1995) revealed that passive immunization with antibodies against fish STC is a useful tool to uncover the physiological actions of STC in fish calcium physiology. For this reason specific polyclonal antibodies raised against the purified native sea bream STC (Fuentes et al., 2010) that recognize STC in western blotting (Figure 1) and IHC (Figure 2) were used in the present study. A significant characteristic of this antibody is its ability to modify plasma calcium levels in the expected direction (increase) within 24 hours of administration as a single intraperitoneal injection to sea bream juveniles (Figure 3). This effect is similar to the action of PTHrP(1-34) when administered alone or in combination with STC antibodies. Together, these results support the idea that manipulation of plasma calcium can be achieved by administering PTHrP to potentiate hypercalcaemia, or by inhibition of the hypocalcemic-blocking agent by administration of STC antibodies. The notion that PTHrP and STC have counter-effects on plasma calcium regulation was consolidated, by the demonstration that the PTH/PTHrP receptor antagonist PTHrP(7-34), or purified sea bream STC administered alone failed to significantly decrease plasma calcium. In contrast, co-administration of both PTHrP(7-34) and STC significantly decreased plasma calcium levels *in vivo*.

The PTHrP/STC endocrine regulatory system(s) provide alternative mechanisms for the control of plasma calcium levels, and likely regulates other putative downstream actions of PTHrP and STC in fish. In this context, both PTHrP and STC have opposite effects in the regulation of HCO_3^- secretion in the intestine of marine fish *in vitro* (Fuentes et al., 2010). In this intestinal model, the process of apical HCO_3^- secretion relies on luminal Cl^-, suggesting the involvement of Cl^-/HCO_3^- exchangers (Grosell et al., 2009b; Grosell et al., 2005; Wilson et al., 1996). Amongst the members of the SLC26 family, SLC26a6 and SCL26a3, were targeted in the present study as they exhibit Cl^-/HCO_3^- exchange activity (Mount and Romero, 2004). For example, the mefugu SLC26a6 shows high capacity of HCO_3^- transport when expressed in *Xenopus* oocytes (Kurita et al., 2008), and its expression is salinity dependent in both mefugu (Kurita et al., 2008) and in the sea bream (Gregorio et al., 2013). There is a limited body of literature describing the endocrine control of anion exchangers in vertebrates and the present study contributes

to this by demonstrating SLC26a6 and SCL26a3 expression is regulated by different combinations of hypercalcemic or hypocalcemic factors in the anterior intestine of the sea bream. Thus, hypercalcemic factors (eg. PTHrP(1-34) and passive immunization with STC antisera) down-regulate the expression of both transporters. Further studies will be required to establish why STC administration failed to modify SLC26a6 and SCL26a3 expression but it may be related to the dose of STC given or the already high levels of STC detected in fish plasma (Mayer-Gostan et al., 1992).

A proton pump is present in the marine fish intestine (Gregorio et al., 2013; Guffey et al., 2011) and is probably associated with intestinal fluid processing via modulation of epithelial HCO_3^- secretion. In the intestine of the Gulf toadfish, the proton pump constitutes a functional metabolon with the SLC26a6 anion exchanger, that drives intestinal Cl^- uptake against a steep electrochemical gradient (Grosell et al., 2009b) and enables epithelial competence for HCO_3^- secretion. In light of this idea, it was unsurprising that the regulation of expression of *Atp6v1b* (proton pump beta subunit) in the anterior intestine of the sea bream paralleled the response to endocrine regulation described for the SLC26a6 (Figures 4 and 5).

In fish intestine, SLC4a4 is located in the basolateral membrane of the enterocyte and is highly expressed in seawater fish (Kurita et al., 2008). SLC4a4 is a high capacity HCO_3^- supply that drives transcellular secretion at the apical membrane of the enterocyte and is suggested to act as a limiting step of HCO_3^- secretion in the intestine of marine fish (Chang et al., 2012; Taylor et al., 2010). The capacity of calcitropic factors to modulate the expression of SLC4a4 in the anterior intestine of the sea bream (Figures 4 and 5) provides evidence that reinforces the relationship between epithelial calcium movements and HCO_3^- secretion in the intestine of marine fish. However, the present study reveals that the antagonistic action of PTHrP and STC goes beyond calcium regulation and that they also affect key mechanisms of the intestinal HCO_3^- secretion cascade. In particular the calcitropic hormones had a similar effect on the transcript abundance of SLC26a3, SLC26a6, SLC4a4 and *Atp6v1b* transporters in the sea bream intestine and this contrasts with the *ex-vivo* effect of prolactin (Ferlazzo et al., 2012) where only SLC4a4 transcript abundance was modified in the intestine. The divergence between PTHrP, STC and PRL action may arise from the experimental approach (*in vivo vs ex-vivo*) or may simply reflect the physiological roles and selectivity of these

hormones.

The intestinal molecular response to hypercalcemic/hypocalcemic treatment established an important feature; the molecular mechanisms targeted by both calcitropic hormones in the formation of carbonate precipitates are common. In light of this result the relative importance, if any, of such regulation in normal physiology could only be accessed *in vivo*. The intestinal fluid and the precipitate fraction were treated as different compartments, since the fluid acts as an intermediate step between epithelial HCO_3^- secretion and the final precipitation in the form of intestinal carbonate precipitates. The precipitated fraction represented the values as ∑carbonate to account for magnesium and calcium content of the precipitates (Perry et al., 2011; Woosley et al., 2012). The endocrine effects observed (Figure 6) were parallel in both fractions and the results obtained clearly demonstrate the endocrine regulation of intestinal carbonate precipitation. Hypercalcemic factors reduced the amount of carbonate precipitates e.g PTHrP(1-34) treatment reduced precipitated carbonates to about 50%, while immunization with STC antibodies reduced precipitates by about 65%. In contrast, hypocalcemic factors increased precipitate output: the PTH/PTHrP receptor antagonist PTHrP(7-34) induced about a 3 fold increase in the precipitate fraction present in the intestinal lumen.

Fish intestinal carbonate precipitate production has a significant (Wilson et al., 2009) impact in the ocean carbon cycle. Consequently, the characterization of the endogenous endocrine response to the predicted ocean acidification will be essential to model and establish the contribution of fish in a changing environment. The present study demonstrates the predictable effect of the calciotropic hormones STC and PTHrP on the regulation of intestinal mechanisms related to epithelial HCO_3^- secretion, and exposes the end result on precipitate formation. Assuming that intestinal water absorption and epithelial HCO_3^- are functionally associated (Carvalho et al., 2012; Grosell, 2011; Tresguerres et al., 2010) the endocrine regulation of epithelial HCO_3^-secretion may have a central role in this process. The endogenous modulation of carbonate precipitation in the intestine highlights a novel physiological role for calcium-regulating factors and likely impacts the general osmoregulatory process in marine fish, and may have a global impact in the ocean carbon cycle.

METHODS AND TECHNIQUES

Peptides and chemicals

In vivo and *in vitro* PTHrP(1-34) and PTHrP(7-34) bioactivity has been previously reported in fish (Canario et al., 2006; Fuentes et al., 2006; Fuentes et al., 2007a; Fuentes et al., 2007b; Guerreiro et al., 2001; Rotllant et al., 2005). Stanniocalcin (STC) was purified from the sea bream Stannius corpuscles and bioactivity has been confirmed using *in vitro* Ussing type chambers (Fuentes et al., 2010). All chemicals were of the highest grade and obtained from Sigma-Aldrich (Madrid, Spain) unless stated otherwise.

Animals

Sea bream (*Sparus aurata*) juveniles were obtained from commercial sources (CUPIMAR SA, Cádiz, Spain) and maintained in open-seawater circuits under natural conditions of water temperature (18– 20°C), photoperiod, and salinity (37 ppt) at a density <5 kg/m^3. For maintenance, fish were fed twice daily to a final ration of 2% of the body weight, with a commercial sea bream diet (Trow España S.A.; Cojóbar, Burgos, Spain). All fish were fasted for 24 hours (h) before experimental manipulations. 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 a Group-1 license from the Direcção-Geral de Veterinária, Ministerio da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

STC antibody validation

Antibodies were commercially produced in rabbits (Genemed Synthesis, Sigma-Aldrich, Madrid, Spain) with a triple boost of 1 mg purified native sea bream STC (Fuentes et al., 2010). All antibody procedures were performed with the neat antiserum and no purification attempt was made. For tissue and serum collection sea bream (n=4, body mass 250 g) were anesthetized (2-phenoxyethanol 1:10,000; Sigma-Aldrich, Madrid, Spain) and blood was obtained by puncture of the caudal peduncle, left to clot on ice and serum obtained by centrifugation (10,000 rpm for 5 min) and stored at -20ºC for later use. Corpuscles of Stannius were dissected out after decapitation of sea bream, and either fixed in Bouin or flash-frozen in liquid-N_2 and stored at -20ºC for later use.

SDS-PAGE and Western blot: corpuscles of Stannius were homogenized in extraction buffer (240 mM Tris; 48% glycerol; 13.5% sodium dodecylsulfate (SDS) and total protein in the extract quantified (Nanodrop 1000, Thermo Scientific, Waltham, MA, USA). Serum samples (2 µl/lane) and purified sea bream STC (800 ng/lane) were fractioned by SDS-PAGE on 15% polyacrylamide gels under reducing conditions at 100 V and blotted onto enhanced chemiluminescence (ECL) membranes (Hybond-P PVDF ECL, Amersham Biosciences) in a vertical tank transfer system (Mighty Small Hoefer, Amersham Pharmacia) for 60 min at 250 mA. The membranes were incubated with blocking solution [3% (wt/vol) bovine serum albumin (BSA); 0,1% (vol/vol) Tween 20 in Tris-buffered saline (TBS)] overnight at 4ºC. Subsequently, membranes were washed in TBS-T and incubated for 2 h at room temperature with constant agitation with either the diluted STC primary antisera (1:50,000 in TBS-T) or antisera pre-absorbed with an extract of Stannius corpuscles. Antibody pre-absorption was performed overnight at 4ºC with an antibody dilution of 1:1,000 in TBS-T in the presence of an extract of sea bream Stannius corpuscles (see above) with total crude protein 1μg/ml. After incubation with primary antibodies the excess antisera was removed, and the membranes were washed several times in TBS-T and incubated with a secondary antibody (anti-rabbit IgG-peroxidase conjugate 1:35,000, Sigma-Aldrich, Madrid, Spain) for 1 h at room temperature. Excess secondary antibody was removed and the membranes were washed several times in TBS-T and incubated with streptavidin-horseradish peroxidase conjugate (GE Healthcare, Amersham) diluted 1:50,000 in TBS-T at room temperature for 1 h. Excess streptavidin was removed; membranes washed several times in TBS-T and developed using the ECL Prime Western blotting reagents Kit (GE Healthcare, Amersham) following the manufacturer's protocol.

Immunohistochemistry (IHC): After fixation with Bouin, kidney tissue containing the Stannius corpuscles were rinsed in PBS, dehydrated, embedded in wax, and serial sagittal sections (5 µm thickness) prepared and mounted onto poly-L-lysine coated slides for subsequent IHC. Prior to antibody incubation, sections were washed with a solution of PBS (1X), (0.1%) Triton X-100 and 10% sheep serum (Sigma-Aldrich, Madrid, Spain) for 1 h at room temperature. Slides were incubated overnight at 4ºC with rabbit polyclonal STC antibody (1:2,500 dilution). After washing with PBS (1X,

0.1% Triton X-100) the sections were incubated overnight at 4ºC with a secondary antibody [goat anti-rabbit IgG H-R Hyligth-488 (1:400 dilution, AnaSpec, Fremont, CA, USA)] and then washed in PBS (1X, 0.1%Triton X-100), and mounted with VectaShield (Vector Labs, Burlingame, CA, USA). Fluorescent microscopy images were captured using a Zeiss Z2 Axioscope fluorescent microscope coupled to a Zeiss HR camera. Preliminary testing was conducted to optimize anti-STC dilution. None of the negative controls (normal host sera, omission of primary antibody) gave a signal.

Plasma calcium and intestinal transporter expression

Unfed sea bream (n = 80, body mass 2.5 - 3.5 g) were anaesthetized in 2-phenoxyethanol (1:10,000; Sigma-Aldrich, Madrid, Spain) and weighed to the nearest 0.1 g.

In a first experiment fish received a single intraperitoneal injections of, pre-immune rabbit antiserum (5 $\mu l.g^{-1}$) PTHrP 1–34 (0.5 $\mu g.g^{-1}$), rabbit STCAb (5 $\mu l.g^{-1}$) and a combination of PTHrP 1–34 (0.5 $\mu g.g^{-1}$) + STCAb (5 $\mu l.g^{-1}$). In a second experiment fish were injected with 0.9% NaCl (5 $\mu l.g^{-1}$), PTHrP 7–34 (0.5 $\mu g.g^{-1}$), purified sea bream STC (5 $\mu g.g^{-1}$), or a combination of PTHrP 7–34 (0.5 $\mu g.g^{-1}$) + purified sea bream STC (sbSTC, 5 $\mu g.g^{-1}$). Final injection volumes were of 5 $\mu l.g^{-1}$ in all treatments. Dosages for PTHrP 1-34 and PTHrP 7-34 (receptor antagonist) used in the present study were based on those previously used in sea bream juveniles (Fuentes et al., 2007a). After injection fish were transferred to 60 L tanks with flowing seawater for recovery. Twenty-four hours after injection fish were anesthetized with a terminal dose of 2-phenoxyethanol (1:1,000) and blood samples were collected into heparinized glass capillaries from the caudal peduncle after severing the tail. Plasma was obtained by centrifugation of whole blood (10,000 rpm for 5 min), and stored at -20°C for later analysis.

After decapitation of fish samples from the anterior intestine, which corresponds to a section 1-1.5 cm in length caudal to the point of insertion of pyloric caeca, were collected from individual fish and incubated overnight in RNA*Later* (Ambion, Austin, TX, USA) at 4ºC and stored at -20ºC until utilized for RNA extraction (within 2 weeks). Plasma calcium levels were determined in duplicate using a commercial calcium kit (Spinreact, Girona, Spain) with a Benchmark Microplate Reader (Bio-Rad, USA).

Total RNA was extracted from samples of the anterior intestine with a Total RNA Kit I (E.Z.N.A, Omega Bio-tek, Norcross, GA, USA) and the quantity and quality of RNA assessed (Nanodrop 1000, Thermo Scientific, Waltham, MA, USA). Prior to cDNA synthesis RNA was treated with DNase using a DNA-free Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Reverse transcription of RNA into cDNA was carried out using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific) with 500 ng of total RNA in a reaction volume of 20 µl. Primers and reaction conditions followed those previously described (Ferlazzo et al., 2012; Gregorio et al., 2013). Table 1 shows primer sequences and predicted amplicon sizes. Real-time qPCR amplifications were performed in duplicate in a final volume of 10 µl with 5 µl SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) as the reporter dye, 20 ng cDNA, and 0.5 pM/µl of each forward and reverse primers. Amplifications were performed in 96-well plates using the *One-step Plus* sequence detection system (Applied Biosystems, Foster City, CA, USA) with the following protocol: denaturation and enzyme activation step at 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 10 sec. After the amplification phase, a temperature-determining dissociation step was carried out at 65°C for 15 s, and 95°C for 15 s. For normalization of cDNA loading, all samples were run in parallel using 18S ribosomal RNA (*18S*). To estimate efficiencies, a standard curve was generated for each primer pair from 10-fold serial dilutions (from 10 to 0.001 ng) from a cDNA pool that included all samples. Standard curves represented the cycle threshold value as a function of the logarithm of the number of copies generated, defined arbitrarily as one copy for the most diluted standard. All calibration curves exhibited correlation coefficients R^2>0.98, and the corresponding real-time PCR efficiencies were >99%.

Endocrine effects on intestinal contents

The experiment was designed to test the effect of PTHrP (1-34), PTHrP (7-34), and the polyclonal sea bream antibody STCAb in the luminal fluid contents of the sea bream. STC was not tested as insufficient quantities were available.

Sea bream juveniles (n=36, body mass 20-25 g) were anaesthetized in 2-phenoxyethanol (1:10,000) weighed to the nearest 0.1 g and received a single intraperitoneal injection of 0.9% NaCl (5ul.g^{-1}, Control), PTHrP 1–34 (0.5 µg.g^{-1}),

PTHrP 7–34 (0.5 μg.g^{-1}) or STCAb (5 μl.g^{-1}). Individual control groups (0.9% NaCl injected) were run in parallel with each hormone treatment. After injection fish were transferred for recovery to 150-liter tanks supplied with well-aerated running seawater. Twenty-four hours after treatment, fish were anesthetized with 2-phenoxyethanol (1:1000) and decapitated. The intestinal fluid of individual fish was collected from the excised intestinal tract clamped (from pyloric caeca to anal sphincter) with two mosquito forceps, and emptied into pre-weighed vials and centrifuged (12,000g, 5 min) to separate fluid from precipitate.

The intestinal fluid was transferred to pre-weighted vials and volume measured to the nearest 0.1 μl (0.1 mg assuming a density of 1). Intestinal fluid titrable alkalinity (HCO_3^-+ CO_3^{2-}) was manually measured using the double titration method and a combination semi-micro pH electrode (HI1330B, Hanna Instruments) attached to a pH-meter (PHM84, Radiometer Copenhagen) as follows: 50 μl of the intestinal fluid samples were diluted in 10 ml NaCl (40 mM), gassed with CO_2 free gas for 30 min to remove CO_2 and titrated to pH 3.8 with 10 mM HCl, an additional gassing period of 20 min was applied to remove any remaining CO_2 and the sample was back titrated to its original pH with 10 mM NaOH. The volume difference between added acid and base in both titrations and titrant molarities were used to calculate total HCO_3^- equivalents (mEquiv/l). Intestinal precipitates were re-suspended in 400 μl of triple distilled water, homogenized with a glass in glass homogenizer and a 100 μl aliquot was double titrated as described for the intestinal fluid and normalized by fish mass and expressed as nEq/g.

Statistics

All results are shown as mean ± standard error of the mean (Mean ± SEM). After assessing homogeneity of variance and normality statistical analysis of the data was carried out using as appropriate Student t-test or one-way analysis of variance followed by the post hoc Bonferroni test to establish differences from control groups. All statistical analyses were performed using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego, CA, USA). Groups were considered significantly different for $p < 0.05$.

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603 **Table 1. Primers for qPCR**

Gene	Primer	Sequence (5' to 3`)	Tm (ºC)	Size (bp)
Slc26a3.B	F R	ATCTCGGCTCTGAAGGGACT GAGCATTTCTGTCCCTGCTC	60	162
Slc26a6.A	F R	GCGGGACTGTTCAGCGGAGG TGCGAACACGCCTGAACGGCA	60	176
Slc4a4.A	F R	ACCTTCATGCCACCGCAGGG CGCCGCCGCCGATAACTCTT	60	128
Atp6v1b	F R	TGTCTGCCTTTTCCTGAACC TGGGGATCTGGGTGATAGAG	60	180
18S	F R	AACCAGACAAATCGCTCCAC CCTGCGGCTTAATTTGACTC	60	139

604

FIGURE LEGENDS

Figure 1. Left: Protein profile of a Coomassie blue-stained acrylamide gel (left) of sea bream serum (Ser) and purified sea bream stanniocalcin (STC). **Center**: Western blot (WB) using antisera (1:50,000) raised against purified sea bream stanniocalcin revealing a single immunoreactive band in the serum. **Right**: Decreased signal in WB using pre-absorbed antisera (WB pre-absorbed) with an extract of sea bream Stannius corpuscles (see MATERIALS AND METHODS for further details).

Figure 2. Immuno-localization of stanniocalcin in serial sections of sea bream kidney and Stannius corpuscles (see MATERIALS AND METHODS for further details). Merge (A, D), Green/488 (B, E) and bright field (C, F). Note that positive STC signal is limited to the Stannius corpuscles and appears with highest intensity in the center of the glands. Scale bars: A, B and C 100 μm; D, E and F 10 μm. Black arrowhead in C indicates the position of a Stannius Corpuscle.

Figure 3. Plasma calcium levels ($mmol.L^{-1}$) in sea bream juveniles in response to a single intra-peritoneal injection of: **A**) Rabbit pre-immune serum (C, Control), PTHrP(1–34), rabbit anti-sea bream stanniocalcin antiserum (STC-Ab) or a combination of PTHrP(1–34)+STC-Ab. **B**) saline (C, Control), PTHrP 7–34, purified sea bream stanniocalcin (STC) or a combination of PTHrP 7–34+STC. Each column represents the means ±SEM (n=6-8). Asterisks indicate significant differences ($P < 0.05$) from corresponding control groups.

Figure 4. Relative expression (arbitrary units, gene of interest/18s) of *Slc4a4*, beta subunit of the V-type proton pump (*Atp6v1b*), *Slc26a3* and Slc26a6 in the anterior intestine of sea bream juveniles in response to a single intra-peritoneal injection of rabbit pre-immune serum (C, Control), PTHrP(1–34), rabbit anti-sea bream stanniocalcin antiserum (STC-Ab) or a combination of PTHrP(1–34)+STC-Ab. Each column represents the mean ± SEM (n=7-8). Asterisks indicate significant differences ($P < 0.05$) from the corresponding control groups.

636 **Figure 5.** Relative expression (arbitrary units, gene of interest/18s) of *Slc4a4*, beta
637 subunit of the V-type proton pump (*Atp6v1b*), *Slc26a3* and Slc26a6 in the anterior
638 intestine of sea bream juveniles in response to a single intra-peritoneal injection of
639 saline (C, Control), PTHrP 7–34, purified sea bream stanniocalcin (STC) or a
640 combination of PTHrP 7–34+STC. Each column represent means ± SEM (*n*=6-8).
641 Asterisks indicate significant differences ($P < 0.05$) from the corresponding control
642 groups.
643
644 **Figure 6**. Intestinal fluid total HCO_3^- (mEquiv/L; left axis) and intestinal precipitate ∑
645 carbonates ($CaCO_3$ + $MgCO_3$, nEquiv/g body mass; right axis) in response to a single
646 intra-peritoneal injection of saline (Control), PTHrP(1–34) or rabbit anti-sea bream
647 stanniocalcin antiserum (STC-Ab). Each column represents the mean ± SEM (n=5-7).
648 Asterisks indicate significant differences ($P < 0.05$) from corresponding control groups.
649

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628 bream serum (Ser) and purified sea bream stanniocalcin (STC). **Center**: Western blot
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640

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652 pre-immune serum (C, Control), PTHrP(1–34), rabbit anti-sea bream stanniocalcin
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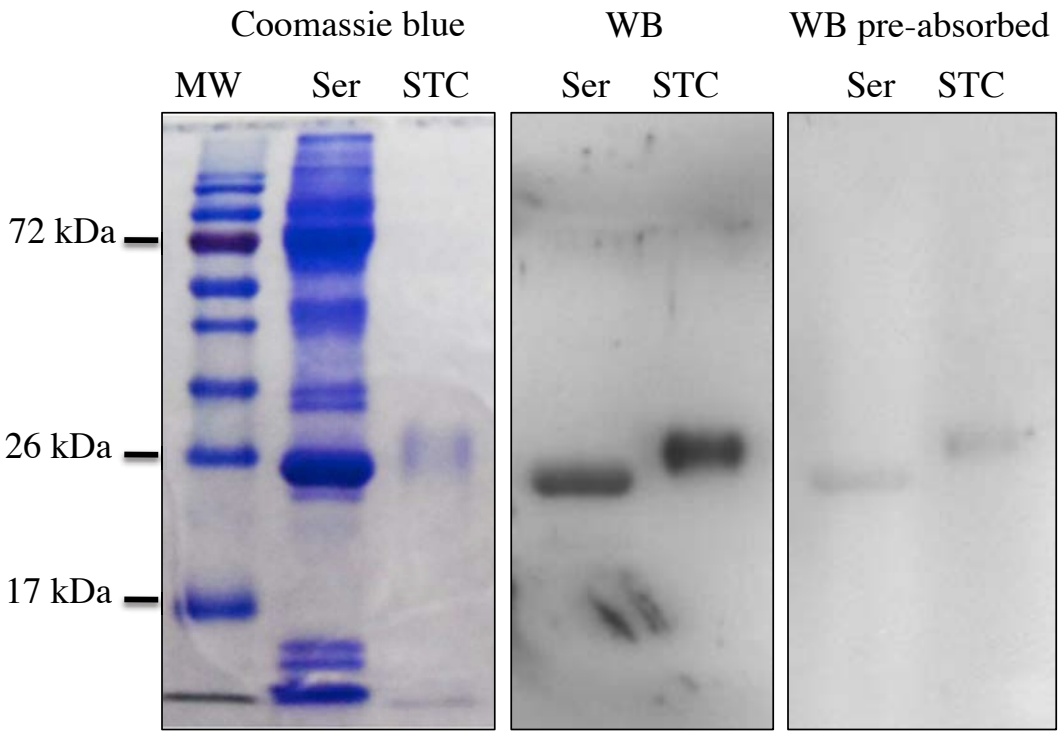
669

670 **Figure 1. Gregorio *et al.***



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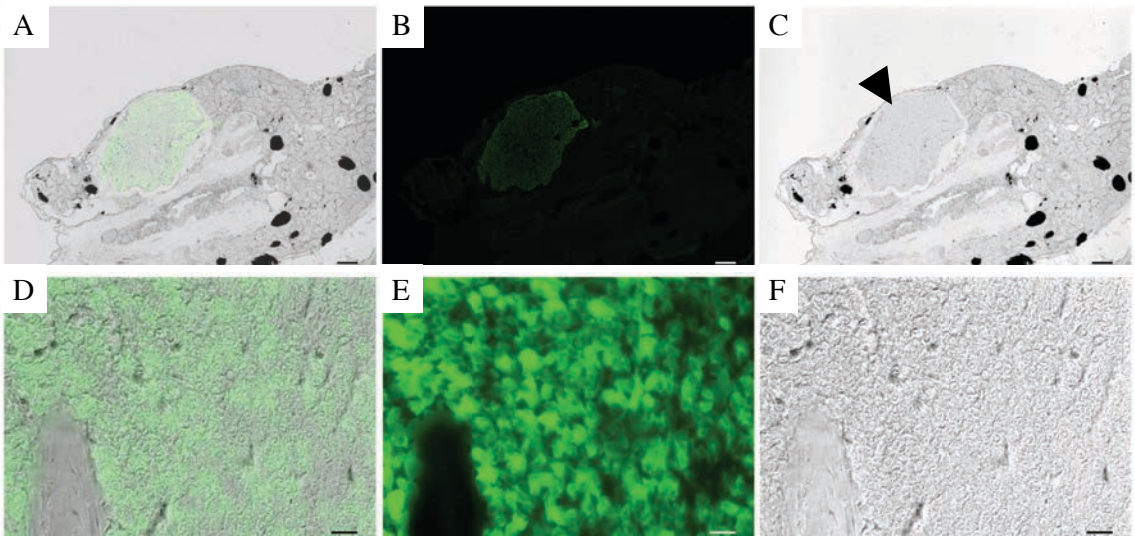
672 **Figure 2. Gregorio *et al.***



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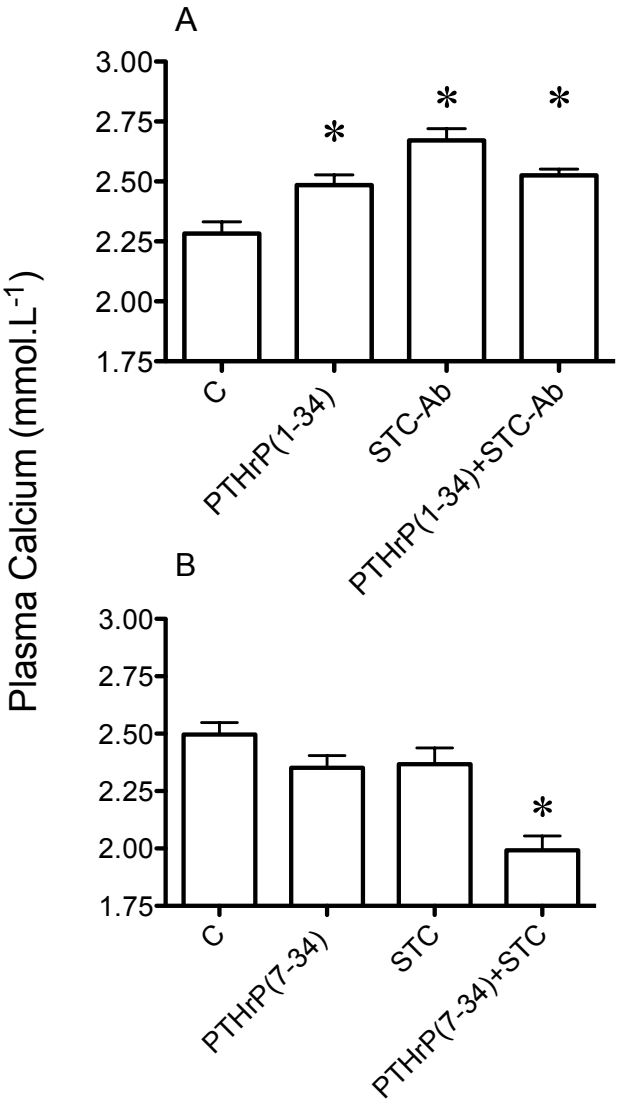
675 **Figure 3. Gregorio *et al.***



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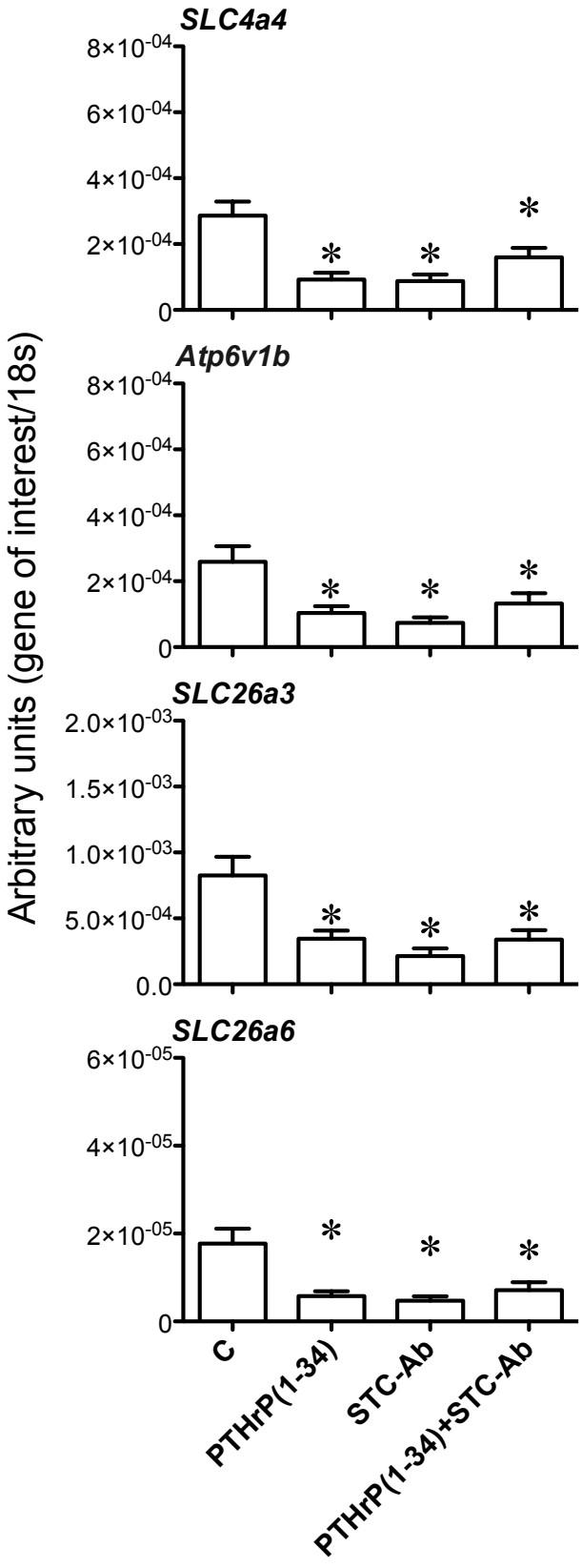

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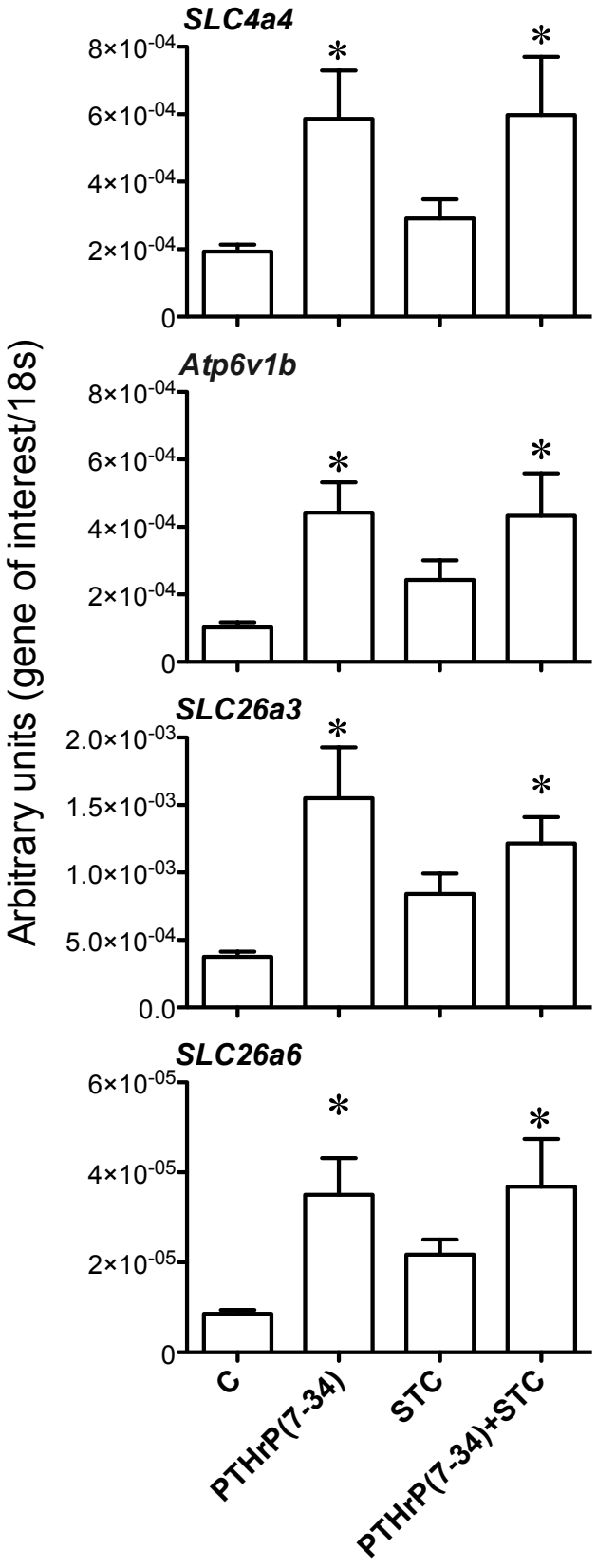
681 **Figure 5. Gregorio *et al.***



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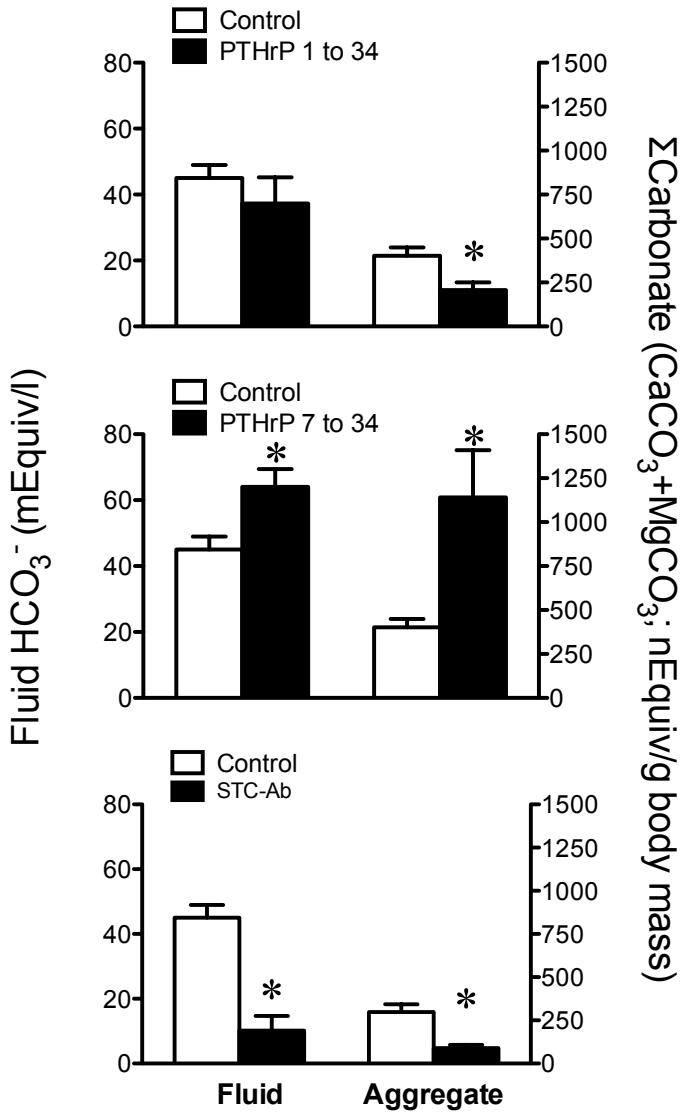
684 **Figure 6. Gregorio *et al.***



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