Regulation of calcium balance in the sturgeon *Acipenser naccarii*: a role for PTHrP

Juan Fuentes, Christophe Haond, Pedro M. Guerreiro, Nádia Silva, Deborah M. Power, and Adelino V. M. Canário

Centro de Ciências do Mar, CIMAR-Laboratório Associado, Universidade do Algarve, Campus de Gambelas, Faro, Portugal

Submitted 22 March 2007; accepted in final form 8 May 2007

Fuentes J, Haond C, Guerreiro PM, Silva N, Power DM, Canário AV. Regulation of calcium balance in the sturgeon *Acipenser naccarii*: a role for PTHrP. Am J Physiol Regul Integr Comp Physiol 293: R884–R893, 2007. First published May 9, 2007; doi:10.1152/ajpregu.00203.2007.—Calcium regulation in sturgeon is of special interest because they are a representative of the ancient fishes possessing mainly cartilaginous skeletons and a supposedly low calcium demand. The present study aimed to characterize the effect of a chronic absence of dietary calcium and the effect of parathyroid hormone-related protein (PTHrPA) (1-34) (7) on calcium balance in juvenile sturgeon (*Acipenser naccarii*). At rest, sturgeon juveniles are in net positive calcium balance, since whole body calcium uptake is significantly higher than efflux and calcium accumulates in the body. To study the importance of dietary calcium, the sturgeon were kept on a calcium-free diet for 8 wk. This manipulation impaired growth as measured by failure to gain weight or increase in length and indicates that dietary calcium is important for growth in sturgeon. An increased whole body calcium uptake partially compensated dietary calcium deficiency and was associated with increased gill chloride cell number in lamellae and filaments in parallel with increased gill Na⁺K⁺-ATPase activity. In addition, a single injection of piscine PTHrP(1-34) significantly increased whole body calcium uptake and decreased whole body calcium efflux. Administration of PTHrP significantly increased circulating plasma calcium 4–24 h postinjection. The increase in net calcium transport and increased plasma levels of calcium is consistent with the actions of a hypercalcemic factor. It would appear that the sturgeon rely on calcium for growth and tightly regulate calcium transport. The action in calcium balance is consistent with PTHrP acting as a hypercalcemic factor in sturgeon.

chloride cells; fish; growth

Unlike terrestrial vertebrates that rely on dietary calcium as a source of calcium, fish have access to a vast calcium pool in water even in the case of calcium-poor fresh water (15). The working model for teleost fish calcium balance entails the cooperation and cross-compensation (16, 19) of the gills and intestine to make enough calcium available for body functions. In freshwater teleosts, the gills are assumed to be the primary entry point for nondietary calcium (12) and in both freshwater and seawater species, calcium uptake from the water by the gill is mediated by the chloride cells. This cell type is a specialized ion-transporting cell characterized by the presence of numerous mitochondria and a high expression of the Na⁺K⁺-ATPase (12, 35, 37). This cell type contains all the machinery for transcellular calcium movement, such as the Na⁺/Ca²⁺ exchanger and the Na⁺-K⁺-ATPase (16, 19, 29). Extrabranchial chloride cells present in the fish skin may also play a role in calcium uptake (30, 32). In seawater, fish have high drinking rates, and the intestine becomes an additional route for calcium uptake from the water (19, 21).

In recent years, there have been significant advances in the understanding of the endocrine control of calcium balance in teleost fish. Although stanniocalcin secreted from the corpuscles of Stannius is hypocalcemic (or antihypercalcemic factor) (46), there is strong evidence that parathyroid hormone-related protein (PTHrP) has strong short-term hypercalcemic actions on calcium balance (7, 21, 24). A variety of other endocrine factors described as having long-term calcitropic actions in fish include prolactin (18), vitamin D (42), estrogen (22, 34), and cortisol (17).

A recent review (24) provides an up-to-date account of the structure and physiology of PTHrP in Teleosts. PTHrP, in particular the PTHrPA(1-34) form has been shown to have a role in calcium regulation in teleost, in particular in sea bream either at whole body level (24) and in in vitro studies in the intestine (21). The mature peptides of the PTHrPA in teleosts such as the puffer fish, (*Takifugu rubripes*), sea bream (*Sparus auratus*), and European flounder (*Platichthys flesus*) are very similar, and identity is over 90% in the first 34 amino acids (24). In contrast, in cartilaginous fish, the PTHrP mRNA has not been isolated. However, PTHrP and PTHrP receptor immunoreactivity have been identified using human antiserum in elasmobranchs (3, 26, 43, 44).

The physiological and endocrine model for calcium regulation in fish has been developed from studies on teleosts. Yet, considering the evolutionary history of fishes and the many adaptations to different environments, it is unlikely that any single model is representative of the diversity of fish species. The sturgeons (chondrosteans) offer alternative models to study the evolution of calcium regulation, as they are representatives of an ancient branch of the fish phylogenetic tree (10). Calcium regulation in chondrosteans is of particular interest because they have a cartilaginous skeleton, which may imply a reduction in calcium needs and thus representing a different model of calcium homeostasis.

No account on the calcium balance of sturgeon exists in the literature, and the potential function of PTHrP has not been studied in this group of fish. Thus, the present study aimed to characterize the mechanisms by which calcium balance is attained in the sturgeon (*Acipenser naccarii*) and to examine the potential action of piscine PTHrPA(1-34) (7) on calcium balance in this species.

Address for reprint requests and other correspondence: J. Fuentes, Centro de Ciências do Mar, CIMAR-Laboratório Associado, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal (e-mail: jfuentes@ualg.pt).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Fish

Immature juvenile sturgeon (Acipenser naccarii, Bonaparte 1836) were obtained from commercial dealers and transported to Ramalhete Marine Station (University of Algarve, Faro, Portugal). Fish (43 ± 2 g body mass; 25 ± 0.45 cm fork length) were maintained in 60-liter glass freshwater aquaria (four fish per tank) in a closed circuit with a biological filter at 27°C for 2 wk before experiments. Sea bream (Sparus auratus, 26 ± 1 g body mass) were obtained from a stock raised at the University of Algarve and maintained in 60-liter glass freshwater aquaria (four fish per tank) in a closed circuit with a biological filter at 27°C for 2 wk before death. All fish were fed daily at a rate of 2% of body weight on commercial pellets (Durafsoja, Provimi, Faro, Portugal). Food was withheld 24 h prior to, and on the day of experiments and sampling. All experimental work was carried out in compliance with national regulations for the use of laboratory animals.

Comparative Analysis of Plasma and Whole Body Calcium

For comparison of whole body calcium and plasma parameters between teleosts and chondrosteans, tilapia (n = 14; 7 males and 7 females), sea bream juveniles (n = 18; immature), and sturgeon (n = 10; immature) were used. Fish were killed with an overdose of anesthetic with 2-phenoxyethanol (1:1,000 vol/vol, Sigma-Aldrich, Madrid, Spain), weighed (to the nearest gram) and measured (to the nearest millimeter). Before blood collection, the puncture area was dried with tissue paper to avoid contamination of the samples with water. A 0.3-ml blood sample was collected by caudal puncture into 1-ml heparinized syringes (ammonium heparin, 30 units/ml; Sigma-Aldrich), and plasma was separated by centrifugation of whole blood (10,000 rpm for 3 min.), aliquoted into 1.5-ml vials, snap-frozen, and stored in liquid nitrogen until analysis. The plasma parameters measured were PTHrP, total calcium, and the filtered plasma calcium fraction.

Determination of Whole Body 45Ca Fluxes in Sturgeon

Whole body calcium uptake. For determinations of whole body calcium uptake, three fish per tank in duplicate (six fish per treatment) were transferred to freshwater flush tanks (1.5 l/fish) set up in closed circuit with aeration. After a 30-min recovery period, the tank water was spiked with 37 kBq/l 45CaCl2 (specific activity 14.8 MBq/nmol; NEN, Life Sciences Products, Boston, MA) and allowed to mix. The tank water was replaced with tracer-free water. The 45Ca activity in the fish carcass (i.e., the whole intestine excluded), WCa, is the 45Ca specific activity in the water (cpm/nmol), t is the duration of exposure (h), and w is fish wet weight (g). Whole body calcium influx rate is expressed as nanomoles per hour per gram.

Whole body calcium efflux. Fish were loaded with 45Ca by intraperitoneal injection of 37 kBq/45CaCl2 dissolved in 300 μl 0.9% NaCl and returned to holding tanks for 36 h for recovery and to achieve a constant specific activity of the readily available calcium pool in the whole fish. For determinations of whole body calcium efflux, fish were obtained from experiment 1, or control and PTHrP injected from experiment 2 (see below) were lightly anesthetized (2-phenoxyethanol, 1:20,000 vol/vol) and transferred to 0.5 liter freshwater flush tanks (fish to tank ratio 1:10) with aeration. Five-milliliter water samples were taken after 30 min and every 20 min thereafter for 2 h. Fish were then killed with an overdose of anesthetic (1:250); fish were then weighed, and a blood sample was collected and plasma was separated. Water and plasma samples (200 μl) were measured for 45Ca radioactive decay as previously described. Calcium efflux rate (ER) was calculated as follows: ER = (Aw·Ct)/(At·W), where Aw is the final specific activity in water, Ct is the total plasma calcium (in millimoles per liter), At is the specific activity of 45Ca in the plasma, t is time (h), and W is fish wet weight (g). Results are expressed as nanomoles per hour per gram. Calculations of calcium efflux from individual fish were taken to show initial velocity if individual timed collections compiled with linearity (R² > 0.98), to rule out back flux.

Experiment 1: Effect of Dietary Calcium on Growth and Calcium Balance

After the acclimation period, 20 juvenile sturgeons were randomly allocated in four 65-liter tanks (5 fish/tank; 2 tanks per treatment), and the diet was gradually (over 4 days) changed from the control pellets (calcium-sufficient) to the test pellets (calcium-deficient; provided by Prof. G. Flik, Radboud University Nijmegen and previously tested in the sea bass (1)). Fish were fed twice daily to a final ration of exactly 1.5% of the total body mass per day. The calcium-deficient and -sufficient diets were identical in appearance (shape and color), and no reduction in feeding was observed after switching to test diet pellets, and all food offered was consumed by the animals. The fish were kept on the experimental regime for 8 wk and were fasted for 24 h before each sampling. The sturgeon were sampled immediately before (time 0) and 2, 4, 6, and 8 wk after the start of the experiments.

At each sampling, the sturgeon were weighed (to the nearest gram) and measured (to the nearest millimeter) under anesthesia. A blood sample (0.3 ml) was collected and processed as described above. Fish were returned to their holding tanks and resumed normal feeding the day after sampling. Food rations were adjusted after each sampling using the recorded total wet weight of fish in the tanks and maintained constant until the next sampling. No mortalities occurred during the experiment. The condition factor (K) was calculated using the equation: K = (W/L³)×1,000, where W is body weight (g) and L is standard length (cm). At the end of experiments (week 8), both control fish and those maintained on a calcium-deficient diet were subject to the same experimental procedure. All fish in one tank (n = 5) were for 10 min until reduced to a paste. A subsample was transferred to preweighed glass vials and digested with nitric acid (1:1 weight:volume) at 30°C for 1 wk. After digestion, samples were neutralized with equal volumes of 2 M NaOH and transferred to scintillation vials (2 ml per vial to count the whole subsample). All samples, including tank water and digested carcasses, were bleached with 2 ml 35% hydrogen peroxide (Fluka; Sigma-Aldrich) to prevent color quenching, incubated overnight at room temperature, and then counted for 15 min in a Beckman LS6000IC scintillation counter after the addition of 20 ml scintillation cocktail (OptiPhase HiSafe II, Wallac; Amersham Pharmacia Biotech, Piscataway, NJ).

Whole body calcium uptake (CU) was calculated according to the following equation: CU = (FC/WCa)(t·w), where FC is the total 45Ca activity in the fish carcass (i.e., the whole intestine excluded), WCa is the 45Ca specific activity in the water (cpm/nmol), t is the duration of exposure (h), and w is fish wet weight (g). Whole body calcium influx rate is expressed as nanomoles per hour per gram.

Whole body calcium efflux. Fish were loaded with 45Ca by intraperitoneal injection of 37 kBq/l 45CaCl2 dissolved in 300 μl 0.9% NaCl and returned to holding tanks for 36 h for recovery and to achieve a constant specific activity of the readily available calcium pool in the whole fish. For determinations of whole body calcium efflux, fish from experiment 1, or control and PTHrP injected from experiment 2 (see below) were lightly anesthetized (2-phenoxyethanol, 1:20,000 vol/vol) and transferred to 0.5 liter freshwater flush tanks (fish to tank ratio 1:10) with aeration. Five-milliliter water samples were taken after 30 min and every 20 min thereafter for 2 h. Fish were then killed with an overdose of anesthetic (1:250); fish were then weighed, and a blood sample was collected and plasma was separated. Water and plasma samples (200 μl) were measured for 45Ca radioactive decay as previously described. Calcium efflux rate (ER) was calculated as follows: ER = (Aw·Ct)/(At·W), where Aw is the final specific activity in water, Ct is the total plasma calcium (in millimoles per liter), At is the specific activity of 45Ca in the plasma, t is time (h), and W is fish wet weight (g). Results are expressed as nanomoles per hour per gram. Calculations of calcium efflux from individual fish were taken to show initial velocity if individual timed collections compiled with linearity (R² > 0.98), to rule out back flux.
Clathrate and three (from each duplicate tank) were transferred to flux boxes (see above). The remaining fish were anesthetized to record weight and length and for a final collection of plasma as previously described. A biopsy of the left gill filaments was collected from individual fish in 100 μl of ice-cold sucrose-EDTA-imidazole buffer (SEI; or 150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) in 0.5-ml vials, snap-frozen and stored in liquid nitrogen or fixed in Bouin’s fixative and used for quantification of branchial chloride cell numbers (see Chloride Cell Detection and Quantification).

**Experiment 2: Effect of Piscine PTHrP(1-34) on Calcium Balance**

After the acclimation period of 2 wk, groups of four sturgeon juveniles per tank (two tanks per treatment) were anesthetized, weighed to the nearest gram, and injected intraperitoneally with saline (0.9% NaCl, control fish) or freshly prepared piscine (1-34) Takifugu rubripes PTHrPA (7, 23, 24) to give final doses of 0, 0.9% NaCl, control fish) or freshly prepared piscine (1-34) Takifugu rubripes PTHrPA (7, 23, 24) to give final doses of 0, 10 ng/g or 50 ng/g (0.9% NaCl, control fish) or freshly prepared piscine (1-34) Takifugu rubripes PTHrPA (7, 23, 24) to give final doses of 0, 10, or 50 ng PTHrP(1-34)/g wet weight, transferred to freshwater flux tanks, and processed as described above. Experiments were performed twice as to give a final n = 8 per treatment.

For determinations of whole body calcium uptake, control and PTHrP-injected fish (n = 6 per treatment) were slightly anesthetized (2-phenoxethanol, 1:20,000 vol/vol), injected to give final doses of 0, 10, or 50 ng PTHrP(1-34)/g wet weight, transferred to freshwater flux tanks, and processed as described above.

**Blood Plasma Calcium and PTHrP**

Calcium was measured in triplicate by colorimetric assay (Sigma-Aldrich procedure no. 587). The plasma total calcium fraction was measured by ultraviolet light of plasma by centrifugation (12,000 g, 10 min at 4°C) through a 10-kDa cutoff filter (Millipore) followed by colorimetric assay (Sigma-Aldrich procedure no. 587). Values for calcium are shown as millimoles per liter for plasma or milligram per gram for whole body measurements. Plasma levels of PTHrP were measured by radioimmunoassay (RIA) after assessing parallelism of values of PTHrP are shown as nanograms per milliliter. Measurement of PTHrP was carried out on dewatered sections following the general methodology indicated above with the following differences: endogenous peroxidase inactivation with 0.35% H2O2 for 20 min in 100% methanol; incubation for 1 h with goat-anti-mouse IgG H + L, 1 h with the M/PAP. Sections were mounted in DePex (Merck, Boston, MA) and analyzed with a microscope (Leica DM 2000) equipped with a digital camera (Leica DFX 400).

In the sectioned tissue, two different regions of the gill filament were considered for chloride cell distribution/density analysis according to the results of the whole mount HIC: region 1, the afferent side of the filament devoid of lamellae; and region 2, the median part of the filament-bearing lamellae. For each fish, five images were recorded from each of the defined regions of the filament. In region 1, the length of the epithelium lining the filament was measured and immunopositive cells counted. In region 2, the length of the epithelium lining the lamellae (4 to 6 lamellae per fish) was measured, and the immunopositive cells in this epithelium were counted. In addition, the length of the interlamellar filament epithelium was measured and the immunopositive cells in this epithelium were counted. The density of immunopositive cells was expressed as the number of chloride cells per linear millimeter of lining epithelium. The data obtained from the different images were averaged for each fish, and eight fish per group (one control group and one calcium-deficient diet group) were used. Three sets of data were obtained for each sample, the density of chloride cells in the afferent filament epithelium (AFE), the interlamellar filament epithelium (IFE), and the lamellar epithelium (LE). All measurements were performed using the open source software Image J (2).

**Detection and Identification of Calcified Tissue**

A preliminary analysis to assess the presence of calcified tissue was conducted. Sturgeon snout, vertebrae, and ganoid scales were fixed for histology in sublimated Bouin-Hollande and embedded in paraffin (Histosec, Merck). Serial sections (5 μm) were mounted on poly-L-lysine-coated glass slides. Sections were stained with Masson’s Trichrome to distinguish mineralized tissue and nonmineralized connective tissue (11). Briefly, to stain nuclei, sections were immersed in Mayer’s hematoxylin, followed by staining of mineralized tissue by an acid fuchsin/xylidine Ponceau solution. Differentiation was performed with phosphomolybdic acid followed by staining of the non-mineralized tissue with fast green. Alcian blue staining of mucopolysaccharides in connective tissue allowed the identification of cartilage and was carried out with dewatered and rehydrated sections by immersion for 30 min in Alcian blue (1% Alcian blue in 3% acetic
acid); sections were then counterstained with Harris hematoxylin. Vertebrae and ganoid scales were fixed in ethanol and the presence of calcium detected with alizarin red S whole-mount staining, using an adaptation of a previously described method (13). Stained sections and whole mount preparations were analyzed using a microscope (Olympus BH2) and a stereomicroscope (Olympus SZ-60) coupled to a digital camera (Olympus DP11) linked to a computer for digital image analysis.

Statistics

Values are shown as means ± SE, unless otherwise stated. After assessing normality and homogeneity of variances, differences between groups were established as appropriate by Student’s t-test, or one-way ANOVA followed when necessary by the a posteriori Bonferroni multicomparison test to identify groups different from controls. For chloride cell distribution/proliferation data were analyzed using a two-way ANOVA considering treatment (food calcium level) and histological location (lamellae, interlamellae, and filament epithelia) as main factors, followed by the Bonferroni multicomparison test to identify significantly different groups. Differences between groups were considered significant at P < 0.05.

RESULTS

Basal Plasma and Body Calcium

Table 1 shows the summary of comparative values of whole body calcium and plasma calcium parameters in juvenile sea bream, tilapia, and sturgeon. Plasma calcium in sturgeon juveniles ranged between 1.2 and 1.3 mM, roughly half that of teleost fish. While the ratio of filtered (i.e., ionic) to total calcium in the sea bream and tilapia ranged between 0.4 and 0.8, in sturgeon it was 0.99, which means that virtually all of the calcium circulating in sturgeon plasma is ionic.

Whole body 45Ca fluxes in juvenile sturgeon indicate that there is a net calcium uptake of 12.6 nmol·h⁻¹·g⁻¹ (Fig. 1). The overall positive net influx is a consequence of the high whole body calcium uptake and the significantly lower calcium efflux (P < 0.05, Student’s t-test).

Whole body calcium in sturgeon juveniles (5.67 ± 0.19 mg/g) was slightly lower than that of the sea bream (6.36 ± 0.14 mg/g) and tilapia (14) (12 mg/g). The high body calcium content and the significant net calcium uptake observed in sturgeon juveniles raise questions about the potential localization of this calcium. The distribution of calcium in the endoskeleton compared with the exoskeleton of sturgeon is in agreement with the generally accepted absence of calcification of the endoskeleton (Fig. 2), as exemplified by the vertebrae (Fig. 2E). In contrast, the sturgeon ganoid scales contain abundant calcium (Fig. 2, C and D) as does the snout area (Figs. 2, A and B), indicating that the exoskeleton most likely accounts for the high values of whole body calcium detected.

Dilutions of sturgeon blood plasma show good cross-reaction and parallelism to the standard curve in the PTHrP RIA and measurements of blood plasma in normal untreated juvenile sturgeon were 0.7 ± 0.07 ng/ml (n = 10), roughly one-third of the plasma levels measured in the normal untreated juvenile sea bream and tilapia (Table 1).

Table 1. Whole body and plasma parameters in sturgeon, tilapia, and sea bream

<table>
<thead>
<tr>
<th></th>
<th>Sturgeon</th>
<th>Tilapia</th>
<th>Sea Bream</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body calcium, mg/g</td>
<td>5.67±0.19</td>
<td>12*</td>
<td>6.36±0.14</td>
</tr>
<tr>
<td>Plasma total calcium, mmol/l</td>
<td>1.32±0.02</td>
<td>5.68±1.21 (female)</td>
<td>2.56±0.08</td>
</tr>
<tr>
<td>Plasma free calcium, mmol/l</td>
<td>1.30±0.03</td>
<td>1.96±0.05</td>
<td>1.65±0.04</td>
</tr>
<tr>
<td>Ratio free/total calcium</td>
<td>0.99±0.02</td>
<td>0.40±0.08 (female)</td>
<td>0.64±0.01</td>
</tr>
<tr>
<td>Plasma PTHrP, ng/ml</td>
<td>0.71±0.07</td>
<td>2.11±0.11</td>
<td>2.43±0.08</td>
</tr>
</tbody>
</table>

Results are shown as means ± SE; n = 10 for sturgeon, n = 18 for sea bream, n = 7 for tilapia female, and n = 7 for tilapia male. Tilapia results not separated by sex were not significantly different (Student’s t-test) and were pooled. PTHrP, parathyroid hormone-related protein. *Data for male tilapia from Ref. 14.

Effect of Calcium-Deficient Diet on Calcium Balance

The effect of feeding calcium-deficient and calcium-sufficient diets on body mass, length, and condition factor (K) in sturgeon juveniles is shown in Fig. 3, A–C. In both treatments, there was a positive mass gain during the 8 wk, although lack of dietary calcium significantly impairs growth in weight (Fig. 3A, F = 8.92, P = 0.024) and length (Fig. 3B, F = 27.2, P < 0.001). The value of K in fish maintained on a calcium-deficient diet was consistently and significantly lower than in the calcium-sufficient control group throughout the experimental period (Fig. 3C, P < 0.05, one-way ANOVA).

Plasma levels of calcium in response to feeding with calcium-deficient and calcium-sufficient diets in sturgeon juveniles are shown in Fig. 4A. Fish fed the calcium-deficient diet had higher plasma levels of calcium at all sampling points, with the exception of the data point on week 6. After 8 wk of treatment, plasma calcium in fish fed with the calcium-sufficient diet ranged from 1.3 to 1.45 mM, while fish fed the on calcium-deficient diet ranged from 2.1 to 2.2 mM.

The effect of dietary calcium on whole body calcium uptake is shown in Fig. 4B. In fish fed with calcium-sufficient diets, calcium uptake averaged 12.4 ± 0.8 nmol·h⁻¹·g⁻¹, and an increase of whole body calcium uptake of 28% was observed in the calcium-deficient diet group (P < 0.05, Student’s t-test).

Gill Na⁺-K⁺-ATPase activity

The effect of dietary calcium on gill Na⁺-K⁺-ATPase activity is shown in Fig. 4C. In the calcium-sufficient diet group, the activity was 3.4 ± 0.3 nmol·h⁻¹·mg protein⁻¹ and in-
creased 40% in the calcium-deficient diet group (\(P < 0.05\), Student’s \(t\)-test).

Numerous putative chloride cells, immunopositive for \(\text{Na}^+\)-\(\text{K}^+\)-ATPase, were observed in the sturgeon gill filaments (Fig. 6A). Chloride cells were mainly distributed in the epithelium lining the blood afferent (afferent or efferent refer to blood flow hereafter) side of the filament (AFE), the epithelium lining the lamellae (LE), and the epithelium lining the interlamellar spaces (IFE, Fig. 5D). Few or no chloride cells were observed on the efferent side of the filament, either on the filament epithelium or the lamellar epithelium close to this edge of the filament (Fig. 5, A–D). In the calcium-sufficient diet group, the density of chloride cells was significantly different in the three distinct areas (AFE, IFE and LE). The highest density of chloride cells was observed on the IFE and was approximately one-half in the AFE, whereas the LE had the lowest chloride cell density. In the calcium-deficient diet group, the distribution of chloride cells was similar to that of the control group, but the density of chloride cells increased significantly (Fig. 5E). There was no significant interaction between the main factors (location in epithelia vs. food calcium level; two-way ANOVA \(F = 2.05, P = 0.14\)), indicating that the effect of dietary calcium is homogeneous in the epithelial areas under study. Overall, there was a 20% increase of chloride cell density in the three distinct epithelia of the gill filament (Fig. 5E).

**Effect of PTHrP on Calcium Balance**

Alterations of circulating levels of PTHrP in response to the injection of piscine PTHrP(1-34) are shown in Fig. 6A. There were no differences between control and PTHrP-treated groups at 4 h postinjection. However, by 24 h, circulating PTHrP levels were significantly higher in fish injected with 10 and 50 ng/g PTHrP(1-34) (\(P < 0.05\), one-way ANOVA).

PTHrP(1-34) induced a significant increase in circulating plasma calcium levels (Fig. 6A). This increase was effective as early as 4 h after injection with 50 ng/g and becomes significantly higher than controls at 24 h at doses of 10 and 50 ng/g 24 h after the injection of PTHrP (Fig. 6B). In addition, the ratio of filtered to total plasma calcium shows a significant decrease of 10% at doses of 10 and 50 ng/g 24 h after the injection of PTHrP (Fig. 6C).

PTHrP(1-34) had two main actions related to whole body calcium balance. It evoked a significant reduction of calcium efflux at 50 ng/g and not at a dose of 10 ng/g (Fig. 7A) and resulted in an increase in calcium uptake at both doses tested with no evident dose-response (Fig. 7B). The combination of both effects resulted in a substantial increase in the already
inside positive net calcium uptake in both PTHrP-treated groups.

DISCUSSION

The present study demonstrates that despite the absence of a calcified endoskeleton, calcium is required for normal growth in Acipenser naccarii, and the gill plays an important role in calcium uptake from the water. Furthermore, PTHrP remains to be identified in chondrosteans; its administration had a hypercalcemic effect, suggesting that the PTH/PTHrP family of peptides may be important for calcium balance in this group.

Calcium Balance and Calcium Dietary Deficiency in Sturgeon

The lack of a calcified endoskeleton in sturgeon suggests smaller calcium requirements for calcification and matrix formation. However, although whole body calcium content of sturgeon A. naccarii is slightly lower than that of sea bream, S. auratus or tilapia, O. mossambicus a substantial amount of calcium is present in the body (Table 1). In normal conditions, in freshwater, sturgeon are in positive net calcium balance, and uptake is around 10-fold higher than calcium lost to the water. The accumulation of calcium in the body is in keeping with the high net calcium uptake described for this species 12.6 nmol·h⁻¹·g⁻¹, which is in the same range as freshwater teleosts such as tilapia 19.8 nmol·h⁻¹·g⁻¹ (14) or even sea-
water fish such as the sea bream 18 nmol·h⁻¹·g⁻¹ (22). The identification of all areas in the body in which calcium accumulates still requires a detailed study, but the histology results suggest significant amounts of calcium are deposited in the exoskeleton, that is, the ganoid scale system and cranium.

Total calcium circulates at much lower levels in _A. nacarii_ and Actipenseridae (38) compared with sea bream or tilapia (see Table 1 for comparison). We show also that, strikingly, in the sturgeon _A. nacarii_ nearly all (99%) of the calcium circulates in the plasma unbound to protein (i.e., in ionic form), which is significantly higher than in sea bream (around 65%) or tilapia (between 45 and 75% depending on sex, Table 1). This feature is, as far as we know, unique to this group of vertebrates. Thus, the high calcium uptake observed in our study in sturgeon, together with a low efflux and the limited amount of calcium circulating in the plasma reinforces the idea of calcium accumulation in the exoskeleton.

In teleosts, the lack of dietary calcium combined with reduced water calcium availability results in reduced or even arrested (with limiting water calcium) growth, as recently shown for the sea bream (1). Although lack of dietary calcium impaired growth in _A. nacarii_, the fish were still able to maintain positive growth. Thus, if calcium is important to maintain positive growth, compensatory mechanisms to increase calcium uptake may have been activated. The cross-regulation of intestinal (dietary) and branchial calcium uptake was first demonstrated in goldfish (25), where increased branchial calcium uptake was confirmed in the absence of dietary calcium. Furthermore, it has been recently shown that increased dietary calcium results in lower gill uptake in rainbow trout (5). Indeed, the measurements of whole body calcium uptake in juveniles of _A. nacarii_ show that lack of dietary calcium results in increased epithelial calcium uptake (Fig. 4B). Thus sturgeon is regulating calcium, and because the potential sources of calcium can be the surrounding water and the diet, the available routes of calcium entry into the body are the gill and the intestine. The low freshwater calcium content (0.7 mM) and the expected low drinking rate for all juveniles of freshwater fish (20), such as the sturgeon used in this study, suggest the intestine may have only a minor role in the compensatory response to lack of dietary calcium, and the gill is the likely target of increased epithelial calcium uptake.

In fish, the branchial epithelium contains specialized ion-transporting cells, the chloride cells (also called mitochondrion-rich cells), which are believed to be the main site of active ion transport (12, 35, 37) and nearly all of the calcium uptake in the gills of _A. naccarii_, an immunostaining procedure widely used in fish to specifically visualize the chloride cells. In control fish, the labeling procedure revealed the presence of numerous chloride cells located mostly on the afferent region of the gill epithelium. The afferent and efferent regions of the filament (Af and Ef) are mostly located on the epithelium lining the afferent region of the filament (Af) and on the region of the lamellae (Lam) close to the afferent region of the filament (Ef) (A and B). The efferent region of the filament (Ef) (A and B) is devoid of CCs. C: transverse section through the afferent region of the filament. On both sides of the filament, the afferent filament epithelium (AFE) hosts numerous CCs. D: transverse section through the filament showing lamellae close to the afferent region of the filament. The lamellar epithelium (LE) and the interlamellar filament epithelium (IFE) display numerous CCs. The AFE, the IFE, and the LE illustrated in _B_ and _C_ were chosen for CC quantification. Scale bars: _A_, 500 μm; _B_, 200 μm; _C_ and _D_, 50 μm. E: chloride cell (CC) density in different regions of the gill epithelium in juvenile sturgeon fed on calcium-sufficient and calcium-deficient diet for a period of 8 wk. Results are shown as means ± SE (n = 8 per treatment). *Significant (Students t-test _P_ < 0.05) differences between treatments in discrete gill regions.
On the filament epithelium covering the trailing edge and the interlamellar spaces but also on the lamellae. Chloride cells have already been described in the gill of *A. naccarii* using transmission electron microscopy (8) or the zinc iodide-osmium staining procedure (9, 33). According to the authors, in freshwater acclimated fish, the chloride cells are present in the lamellar and interlamellar epithelia, but the filament epithelium was not studied (8, 33) or did not show chloride cells (9). Chloride cells were also observed all over the filament epithelium in the sturgeon *Acipenser oxyrinchus* (4).

A whole mounted staining of gill filament was also performed after immunostaining for the Na\(^+\)/K\(^+\)-ATPase. This technique clearly illustrated the distribution of chloride cells and particularly showed that the presence of lamellar chloride cells is a characteristic feature of the *A. naccarii* in normal conditions and confirms our observations made on paraffin sections.

In our study, we observed an increase in chloride cell density of ~20% in the calcium-deficient diet group compared with controls. This increase in chloride cell numbers in the gill correlates with the increase of gill Na\(^+\)/K\(^+\)-ATPase activity. The values of gill Na\(^+\)/K\(^+\)-ATPase activity measured in *A. naccarii* are similar to values published for other freshwater species or euryhaline species adapted to freshwater such as stingray (36), rainbow trout (28), or killifish (40). Both the increase in chloride cell numbers and the increase in the activity of the Na\(^+\)/K\(^+\)-ATPase in response to deficient dietary calcium suggests the activation of compensatory mechanisms leading to an increase in calcium uptake from the water (18), in which apical epithelial calcium channels in gill nonchloride cells could also be involved (41). However, the impaired growth of the sturgeon on reduced calcium diets indicates that branchial epithelial transport only partially compensates for dietary deficiency.

**PTHrP and Calcium Regulation in Sturgeon**

Similar to what has been observed in teleosts (7, 21, 23), PTHrP(1-34) strongly modified calcium balance in the sturgeon *A. naccarii*. The action of PTHrP(1-34) was achieved through two processes: 1) stimulation of calcium uptake and 2) inhibition of calcium efflux from the fish to water. Both effects are consistent with its action as a hypercalcemic factor and are similar to PTHrP effects in sea bream larvae (23). Because in our study, the sturgeon were in freshwater where low drinking rates are expected, most of the effect of PTHrP(1-
34) was likely to have been through the gills. Considering the apparent similarity in calcium handling between sturgeon and teleosts, it is likely that basolateral calcium movement in the gill is mediated by both the Na\(^+/\)Ca\(^{2+}\) exchanger and Ca\(^{2+}\)-ATPase. Both transport systems respond to prolactin treatment in teleosts (16) and could be likely targets for PTHrP. This is supported by the fact that in the parathyroidectomized rat kidney, PTH is able to restore the levels of protein expression of Na\(^+/\)Ca\(^{2+}\) exchanger (NCX1, basolateral) and the epithelial calcium channel (transient receptor potential cation channel subfamily V member 5, apical) (45). This relationship may also hold true in fish, and it is possible that one or more members of the PTH/PTHrP family is involved in the regulation of transcellular calcium transport in the epithelium.

The hypercalcemic action of PTHrP in sturgeon is reflected by the direct increase in total plasma calcium in response to single injections. After hormonal treatment, the plasma ionic fraction remains unchanged and the fraction of calcium bound to protein is significantly increased. This fact suggests that the regulation of plasma calcium in chondrostean fish is very tight, keeping constant the concentration of ionic calcium in circulation. This observation suggests that calcium binding proteins can act as temporary stores keeping ionic calcium constant and that PTHrP may be involved in this process. Although the nature and identity of the calcium-binding proteins is unknown, in adult sturgeon total calcium increases in parallel with vitellogenin (27).

In conclusion, the present study demonstrates that sturgeon tightly regulate calcium balance and that PTHrP is a likely candidate in the endocrine regulation of calcium in this group of fish. The bioactivity of PTHrP(1-34) in sturgeon, further supports the suggestion for a generalized importance of this peptide family in vertebrates and fish, in particular (7). The targets and mechanisms by which PTHrP exerts its hypercalcemic require scrutiny.

ACKNOWLEDGMENTS

The authors thank J. Reis, M. Viegas, and A. Silva (Ramalhete Marine Station, Faro, Portugal) for fish care. The α5 monoclonal antibody developed by D. M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA.

GRANTS

This research has been carried out with the financial support of the Commission of the European Union, Quality of Life and Management of Living Resources specific Research Technology and Development program (Q5RS-Q5RS-2001-02904) and by European Social Fund and national funds under Portuguese National Science Foundation (FCT) project POCTI/CTV/48946/2002. P. Guerreiro and C. Haond were in receipt of FCT fellowships SFRH/BPD/9464/02 and SFRH/BPD/11527/02, respectively.

REFERENCES

26. Ingleton PM, Hazon N, Ho PM, Martin TJ, Danks JA. Immunodetection of parathyroid hormone-related protein in plasma and tissues of an