Calcium handling in *Sparus auratus*: effects of water and dietary calcium levels on mineral composition, cortisol and PTHrP levels

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Summary

Juvenile gilthead sea bream (*Sparus auratus* L.; 10–40 g body mass) were acclimatized in the laboratory to full strength (34‰) or dilute (2.5‰) seawater and fed normal, calcium-sufficient or calcium-deficient diet for nine weeks. Mean growth rate, whole-body calcium and phosphorus content and accumulation rates were determined, as well as plasma levels of ionic and total calcium, cortisol and parathyroid hormone related protein (PTHrP; a hypercalcemic hormone in fish). When confronted with limited calcium access (low salinity and calcium-deficient diet), sea bream show growth arrest. Both plasma cortisol and PTHrP increase when calcium is limited in water or diet, and a positive relationship was found between plasma PTHrP and plasma ionic calcium ($R^2=0.29$, $N=18$, $P<0.05$).

Furthermore, a strong correlation was found between net calcium and phosphorus accumulation ($R^2=0.92$, $N=16$, $P<0.01$) and between body mass and whole-body calcium ($R^2=0.84$, $N=25$, $P<0.01$) and phosphorus ($R^2=0.88$, $N=24$, $P<0.01$) content. Phosphorus accumulation is strongly calcium dependent, as phosphorus accumulation decreases in parallel to calcium accumulation when the diet is calcium deficient but phosphorus sufficient. We conclude that PTHrP and cortisol are involved in the regulation of the hydromineral balance of these fish, with growth-related calcium accumulation as an important target.

Key words: calcium balance, PTHrP, cortisol, hypocalcemia, growth, phosphorus, *Sparus auratus*.

Introduction

In teleost fish, as in other vertebrates, calcium is of key importance for numerous physiological processes. The skeleton of vertebrates consists mainly of calcium phosphate and calcium carbonate. It serves an important role as it determines body shape, protective aspects (scales, bone plates) and as a buffer internal reservoir for calcium and phosphorus. In teleosts, ~99% of the whole-body calcium fraction is incorporated into bones and scales (Flik et al., 1986). Indeed, calcium is also of major importance for many other physiological processes, such as vision, muscle contraction, vitellogenesis, signal transduction, blood coagulation and membrane permeability (Riccardi, 1999).

In fish blood, calcium is either complexed (e.g. to citrate), protein bound or present as free ion. The free calcium fraction accounts for about half of the total calcium fraction and is the physiologically important fraction (Hanssen et al., 1991). Fish regulate their ionic plasma calcium level more strictly than their protein-bound calcium level, and this may relate to the fact that even minor disruptions in ionic calcium concentrations lead to severe stress and disturbance of calcium balance (Flik et al., 1995).

Unlike terrestrial vertebrates, which depend solely on the diet as their calcium source, fish live in an environment with a readily available source of calcium. Seawater has a calcium concentration of ~10 mmol l^{-1}, whereas the total plasma calcium concentration of marine fish ranges from 2 to 3 mmol l^{-1}; thus, marine fish live in a hypercalcic environment and face an inward gradient of Ca^{2+}. As calcium availability in the environment varies, fish have developed calcium regulatory systems that can react rapidly to changes in environmental calcium concentrations (Wendelaar Bonga and Pang, 1991; Bjornsson et al., 1999).

Endocrine control of calcium metabolism in fish is regulated by both hyper- and hypocalcemic hormones. Stanniocalcin (Lafeber et al., 1988; Wagner et al., 1998) acts as the major hypocalcemic (in fact anti-hypercalcemic, as it inhibits Ca^{2+} influx) hormone. Increased calcium levels in the medium induce hypercalcemic conditions and, by doing so, promote stanniocalcin release into the bloodstream, where it reduces the calcium influx in the gills and intestine. Prolactin (Kaneko and Hirano, 1993; Mancera et al., 1993; Flik et al., 1994) and PTHrP (parathyroid hormone related protein; Guerreiro et al., 2001) act as major hypercalcemic hormones. PTHrP is phylogenetically the predecessor of PTH, which appeared only after the water/land transition of vertebrates. Although recent reports indicate that fish express PTH (Danks

et al., 2003; Gensure et al., 2004), they also have PTHrP, which has a number of physiological functions, such as bone development, placental calcium transport and cellular growth and development (Martin et al., 1997). In sea bream (*Sparus auratus* L.), PTHrP has been detected in several tissues and plasma by radioimmunoassay using antisera raised against the human peptide (Danks et al., 1993; Devlin et al., 1996) and, more recently, the sea bream peptide (Rotllant et al., 2003). PTHrP has also been found in several other fish species (Ingleton and Danks, 1996; Danks et al., 1998; Trivett et al., 1999, 2001). In addition, hormones such as calcitonin (Wagner et al., 1997), growth hormone (Flik et al., 1993), vitamin D (Sundell et al., 1992) and cortisol (Flik and Perry, 1989) are also known to be involved in the calcium balance of fish.

Sea bream is a euryhaline marine teleost that is important for Mediterranean aquaculture. The intensive culture of this species leads to a high number of morphological malformations, which typically result in growth arrest, increased stress sensitivity and an increased incidence of disease outbreaks (Andrades et al., 1996; Carrillo et al., 2001). Improvement of our understanding of calcium regulation is of paramount importance in improving proper development and growth of this species in aquaculture settings.

We investigated calcium regulation after long-term exposure to limited calcium availability. The calcium balance of the fish was monitored through assessment of whole-body calcium and phosphorus content, plasma calcium levels and the relationship between calcium and phosphorus accumulation. In this context, we addressed hypercalcemic endocrine factors, *viz* PTHrP and cortisol, and investigated their relationship with calcium availability.

The experiments were achieved under controlled laboratory studies where sea bream were exposed to dilute seawater (hypocalcic values of 0.7 mmol l^{-1}) and/or a calcium-deficient diet for prolonged periods of time.

Materials and methods

Fish

Juvenile sea bream of approximately 1 g mass were obtained from a stock bred at a commercial fish farm (Viveiro Vilanova, Lda., V. N. Milfontes, Portugal). They were transported to the facilities at Radboud University Nijmegen, where they were held in an aerated flow-through system with 600-litre round tanks at a salinity of 34‰ and a temperature of 23°C. Water quality (pH, NO_2^-, NO_3^-, NH_4^+) was measured once a week and the salinity was checked daily. The photoperiod was 12 h:12 h and the fish stock was fed with commercial pellets (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total body mass per day.

Experimental set-up

To conduct the experiments, the required number of fish was randomly selected from the stock group and transferred to six identical 60-litre round tanks and left to acclimate. After one week, the salinity was lowered from control salinity (34‰; 10.5 mmol l^{-1} calcium) to test salinity (2.5‰; 0.7 mmol l^{-1} calcium) by continuous flow-through with demineralized water, and the diet was gradually changed from the control pellets (Trouvit) to the test pellets (Hope Farms, Woerden, The Netherlands). The calcium-deficient and -sufficient diets were identical in appearance (shape and colour). Although we observed temporary loss of appetite when switching from control to diet pellets, feeding was resumed to comparable levels after three days. This potential problem was addressed by keeping the control diet fish group on a low diet regime (0.5–1% food of the total mass) during the adaptation time to the new diet.

In the first experiment, five groups (A–E) of sea bream (start mass, 17.4±4.6 g; N=20 per group; protandrous fish; not sexually mature) were used. Group A is designated the control group (34‰, control diet). The following test groups were included: group B (34‰, calcium-sufficient diet), group C (34‰, calcium-deficient diet), group D (2.5‰, calcium-sufficient diet) and group E (2.5‰, calcium-deficient diet). The fish were exposed to experimental conditions for six weeks and were fasted for 24 h before sampling. After three weeks (t=1), all fish were weighed and 10 fish were euthanized with 2-phenoxyethanol (1:100; Sigma-Aldrich, St Louis, MO, USA), freeze-dried until constant mass was reached and subsequently dissolved in concentrated nitric acid (70%; 1 ml g^{-1} dry mass; Sigma-Aldrich) for mineral analyses. Vials were carefully capped to avoid evaporation of the digest and the samples were stored at 4°C. For the second sampling period [after six weeks (t=2)], this procedure was repeated with the remaining fish (N=10).

For the second experiment, the fish (N=24 per group) were exposed to experimental conditions for up to nine weeks; sampling took place after three (t=1), six (t=2) and nine (t=3) weeks. At each sampling time, eight fish were randomly selected, euthanized and weighed. Blood was taken from the caudal veins using 1 ml tuberculin syringes, rinsed with Na^+-heparin (Leo Pharma, Weesp, The Netherlands; 5000 U ml^{-1}) and diluted five times with demineralized water. Blood thus collected was centrifuged at 13 600 g for 10 min. Plasma was stored at –20°C.

Whole-body mineral concentrations

The nitric acid digests of fish were diluted 1000× with demineralized water, and whole-body calcium and phosphorus were measured by Inductively Coupled Plasma Atomic Emission Spectrophotometry (ICP-AES, Plasma IL200; Thermo Electron, MA, USA). Mineral concentrations (µmol l^{-1}) of the digests were assessed, and content calculated and expressed as µmol g^{-1} dry mass, based on digest total volume and fish dry mass.

In addition to calcium and phosphorus accumulation rates (µmol h^{-1}), the correlation between the net accumulation of calcium and phosphorus was also calculated. Also, the relationship between mass and whole-body calcium (µmol) was determined and the so-obtained formula of this
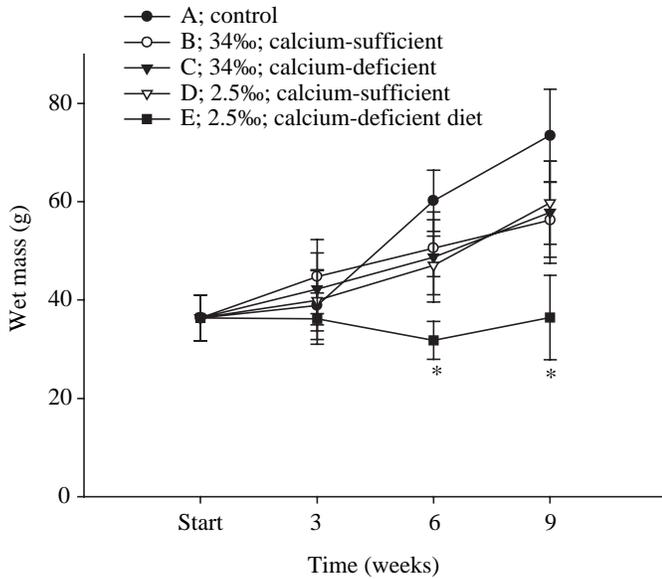
Fig. 1. Growth during the nine-weeks exposure to calcium-limited conditions. In the group exposed to limited calcium in both external calcium sources, diet and medium, growth arrest occurs. Values are means ± S.D. Asterisks represent significant difference from control group ($P<0.05$).

relationship was used to calculate the whole-body calcium levels of the second sampling group at $t=1$. Data of the measured whole-body calcium at $t=1$ and $t=2$ and the calculated data of the second group at $t=1$ were then pooled in full logarithmic plots of the relationship between mass and whole-body calcium at different calcium-limiting conditions. This was also done for the relationship between mass and whole-body phosphorus.

Plasma parameters

Plasma Ca^{2+} (μmol l^{-1}) concentration was measured with a Stat Profile pHOx plus analyser (Nova Biomedical, Waltham, MA, USA). Plasma osmolality was measured using a cryoscopic osmometer (Gonotec Osmosat 030, Berlin, Germany) and expressed in mOsmol kg^{-1}, and plasma total calcium was measured with a calcium kit (Roche, Mannheim, Germany). Plasma cortisol was measured by radioimmunoassay (RIA) as described by Arends et al. (1999), and plasma PTHrP was measured according to Rotllant et al. (2003).

Statistical analysis

All data were tested for significance by one-way analysis of variance (ANOVA), followed by either Dunn's multiple comparison post test (non-parametric) or the Bonferroni *t*-test (parametric), where appropriate. Significance was accepted when $P<0.05$. All values are expressed as means ± standard deviation (S.D.). Correlation regression between two groups was determined with power function. Because no variation was found in the results of the various parameters between three, six and nine weeks, the data for each parameter were pooled to one data set per group.

Results

Growth

No mortality occurred and all groups ate well during the experiments. Growth of the fish during the nine-weeks exposure to experimental conditions is shown in Fig. 1. Control fish increased in mass more than the test groups. No growth was observed in group E, which was exposed to both 2.5‰ salinity and a calcium-deficient diet.

Whole-body calcium and phosphorus content

Net calcium and phosphorus fluxes in μmol h^{-1} (Fig. 2A) and the correlation between calcium and phosphorus accumulation rates (Fig. 2B; $R^2=0.92$, $N=16$, $P<0.01$) demonstrate that net calcium and phosphorus accumulation follow the same pattern, although phosphorus availability was never limited under the experimental conditions. The highest accumulation was observed in the control group (group A); significantly lower net calcium and phosphorus accumulations
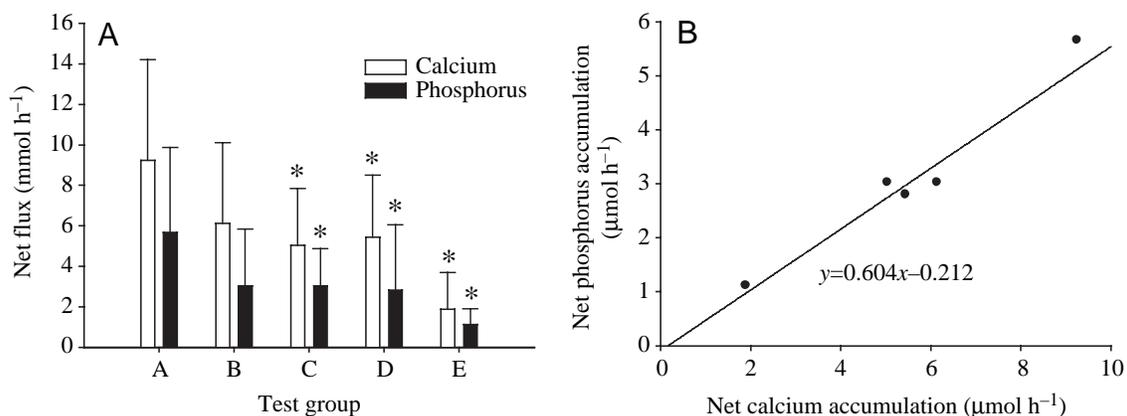

Fig. 2. (A) Net calcium and phosphorus accumulation (μmol h^{-1}) during three weeks under calcium-limited conditions and (B) the relationship between net calcium and net phosphorus accumulation ($R^2=0.92$, $N=16$, $P<0.01$). Values are means ± S.D. Asterisks represent significant difference from control group ($P<0.05$).
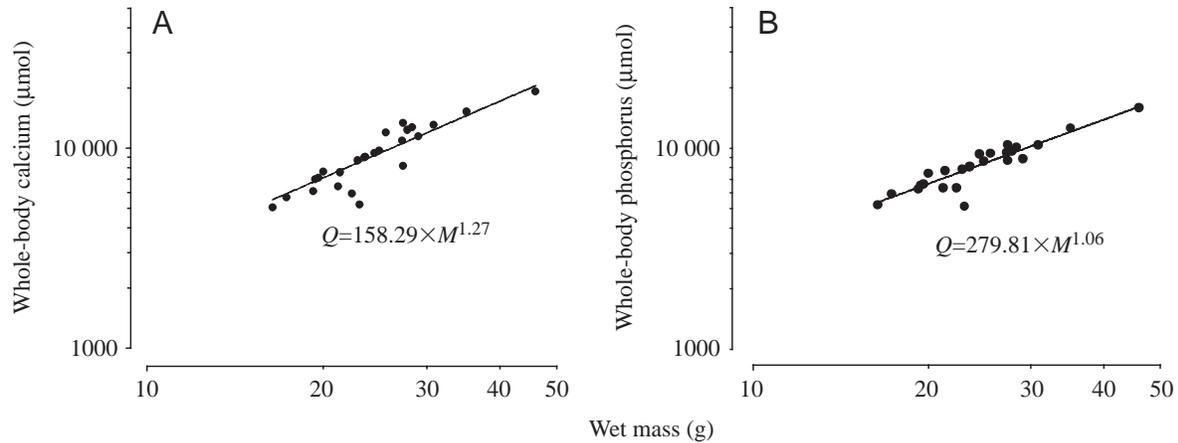
Fig. 3. The relationships between (A) wet mass and whole-body calcium content (R^2=0.84, N=25, P<0.01) and (B) wet mass and whole-body phosphorus content (R^2=0.88, N=24, P<0.01) for the control fish. For the test groups, similar correlations were found, although with lower regression slopes.

Table 1. *The calculated power functions for relationships between whole-body calcium and phosphorus and wet mass*

Group	Condition		Accumulation power function	
	Salinity (‰)	Diet	Calcium	Phosphorus
A	34	Control	Q=158.29×$M^{1.27}$	Q=279.81×$M^{1.06}$
B	34	Calcium sufficient	Q=393.17×$M^{1.04}$	Q=848.67×$M^{0.74}$
C	34	Calcium deficient	Q=645.47×$M^{0.85}$	Q=576.35×$M^{0.83}$
D	2.5	Calcium sufficient	Q=393.78×$M^{1.03}$	Q=480.26×$M^{0.91}$
E	2.5	Calcium deficient	Q=560.07×$M^{0.91}$	Q=570.22×$M^{0.83}$

The regression slopes for calcium and phosphorus are lower with limiting calcium availability.

were seen in the test groups where calcium was limited (groups C and D). Group E exhibited the lowest net calcium and phosphorus accumulation. The fish performed equally well on control and calcium-sufficient pellets (groups A and B).

A logarithmic plot of the relationship between whole-body calcium and body mass (M) shows a strong positive correlation (Fig. 3A; R^2=0.84, N=25, P<0.01). For the control fish (group A), the relationship is described by the power function Q=158.29×$M^{1.27}$ (Table 1), where the calculated slope of regression (1.27) reflects the rate of calcium accumulation (Q; μmol) in the fish (Flik et al., 1985, 1993). The test groups show lower power values (plots not shown), with comparable regressions in group B and group D. The two groups exposed to calcium-deficient diet (groups C and E) expressed the weakest slopes of regression. Overall, the power function decreased with lower calcium availability.

Similar power functions were made for the relationship between whole-body phosphorus and body mass (Fig. 3B; R^2=0.88, N=24, P<0.01). The regression slopes are comparable with the slopes that were found for the relationship between calcium and body mass, with the steepest slope in the control group (Q=279.81×$M^{1.06}$) and lower phosphorus–body mass regression slopes at calcium-limiting conditions (Table 1).

Plasma parameters

In contrast to plasma total calcium levels, which differ significantly in groups where calcium is limited in any way (groups C–E), plasma ionic calcium is strictly regulated except
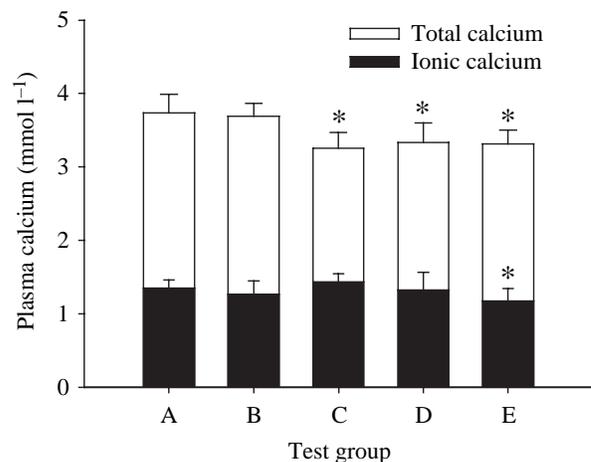

Fig. 4. Total and ionic plasma calcium levels show the more strict correlation in ionic calcium compared with total calcium. Values are means ± s.D. Asterisks represent significant difference from control group (P=<0.05).
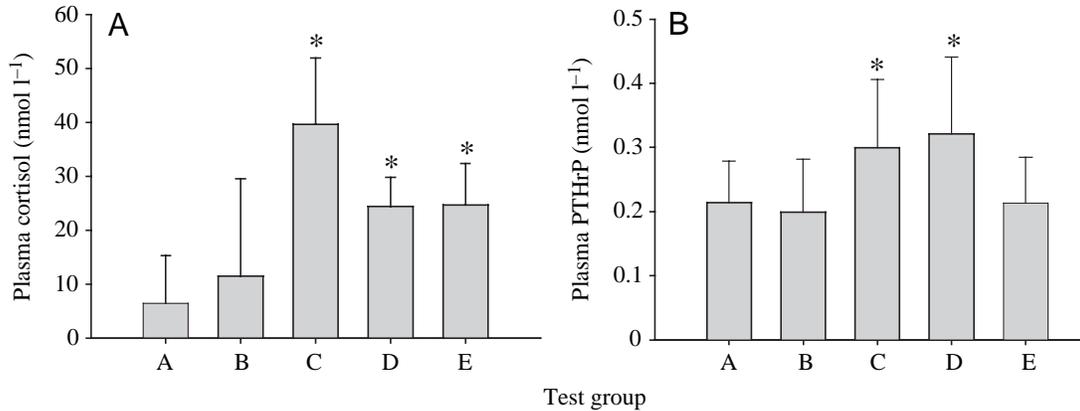
Fig. 5. (A) Plasma cortisol is shown to increase at calcium-limiting conditions. (B) Parathyroid hormone related protein (PTHrP) increases when calcium is limited in the diet or the medium. However, when both external calcium sources are limited, PTHrP shows no rise in plasma level. Asterisks represent significant difference ($P<0.05$) compared with the control group. Values are means ± S.D.

when fish are fed a calcium-deficient diet and exposed to calcium-limited water (Fig. 4). Under these conditions, plasma ionic calcium did in fact decline significantly. Plasma cortisol levels (Fig. 5A) are low in controls (6.51 ± 8.78 nmol l^{-1}) and significantly and chronically elevated in the test groups (up to 39.67 ± 12.34 nmol l^{-1}) where calcium access was limited and a decline in total calcium measured. Plasma PTHrP measurements show concentrations of 0.21 ± 0.06 nmol l^{-1} (Fig. 5B) for the control group and a significantly higher plasma PTHrP level of 0.30 ± 0.11 nmol l^{-1} and 0.32 ± 0.12 nmol l^{-1} in groups C and D, exposed to either a calcium-deficient diet or a low salinity, respectively. Group E, exposed to both 2.5‰ and a calcium-deficient diet, expressed a comparable PTHrP level as the control group.

For the control group, the positive correlation between plasma PTHrP and plasma ionic calcium is shown in Fig. 6. For PTHrP and total calcium, no such relationship was found
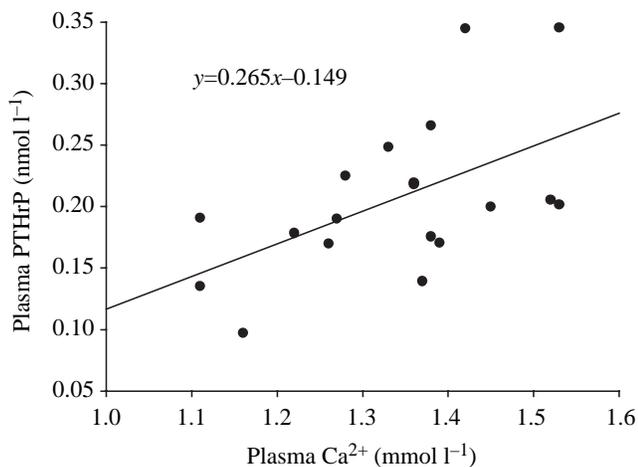
(plot not shown). Also, for the test groups, significant correlations were absent.

Discussion

This study provides new key observations on prolonged exposure to diluted seawater and/or a calcium-deficient diet in sea bream.

(1) When growth stops, sea bream still, or with priority, maintain their plasma calcium, and in particular the physiologically important free calcium fraction, at a concentration that ensures their survival for a prolonged period of time. Strong relationships were found between body mass and whole-body calcium and phosphorus for all groups tested, with decreasing slopes (decreasing whole-body calcium and phosphorus content) under decreasing calcium availability in water and diet.

(2) Net calcium and phosphorus accumulation rates decline when calcium is limited. A strong positive correlation was found between net calcium and phosphorus accumulation, although phosphorus was not limited in the experimental set-up.

(3) In control fish, a positive correlation was found between plasma PTHrP and ionic calcium concentrations.

(4) Plasma ionic calcium levels are strictly regulated whereas total plasma calcium levels show significant differences under calcium-limiting conditions. Interestingly, when hypocalcemia was observed, plasma cortisol and PTHrP levels were mildly increased, which we take as an indication for a hypercalcemic action or function of these hormones. The mild endocrine responses concur with an allostasis concept where these mild elevations would represent a normal allostatic load (McEwen and Wingfield, 2003).

Fig. 6. A positive relationship between plasma parathyroid hormone related protein (PTHrP) and ionic calcium ($R^2=0.29$, $N=18$, $P<0.05$) shows that PTHrP is involved in the strict control of plasma ionic calcium.

Whole-body calcium

With respect to the calcium balance, prolonged exposure to diluted seawater (2.5‰, which is a hypocalcemic medium) and a calcium-deficient diet results in growth arrest in sea bream.

This phenomenon has been described for several other teleost species (Flik et al., 1986; Morgan and Iwama, 1991; Woo and Kelly, 1995; Sampaio and Bianchini, 2002). Interestingly, the apparent growth arrest allows the fish to maintain plasma calcium balanced at a level that ensures their survival for prolonged times. Apparently, the calcium stores realised under control conditions have a significant buffer capacity. We calculate, for a 50 g sea bream, a total calcium content of 29.2 mmol under control conditions and of 16 mmol when water and diet are low in calcium. This indicates a 42% decrease in the total calcium pool. Such drastically lower calcium content may be possible only in aquatic vertebrates.

Plasma calcium

Ionic calcium levels are strictly regulated and fish are able to maintain these physiologically important free calcium levels when calcium availability is reduced in the diet and/or the medium. However, when calcium availability is strongly reduced in both of the external calcium sources (the diet and the medium), a slight but significant decrease in ionic calcium is observed. The strict control of ionic calcium means that the calcemic regulation system must be able to react swiftly on variable external calcium availability. A positive correlation between the hypercalcemic hormone PTHrP and ionic calcium is indeed found. This indicates that PTHrP is involved in the calcemic endocrine control of plasma calcium balance in fish. Total calcium is not as tightly regulated as ionic calcium by the calcemic control mechanisms, which means that larger variations in plasma total calcium concentration are found, indicating a change in binding protein level compared with the control group. Indeed, no positive relationship between plasma total calcium and plasma PTHrP is found here.

Calcium and phosphorus accumulation

The positive correlation found between body mass and whole-body calcium is not affected by severe and chronic decreases in external calcium availability. A similar relationship was found between body mass and whole-body phosphorus for all experimental conditions. This is remarkable, because the experimental conditions were focussed on calcium-limiting conditions, with phosphorus concentrations unaffected. Since the phosphorus concentration in seawater is very low, fish must depend on their diet for phosphorus, which they accumulate at the same rate as that for calcium (Roy and Lall, 2003). Yet, we have demonstrated that phosphorus accumulation is impeded under conditions of low calcium availability (Vielma and Lall, 1998; Chavez-Sanchez et al., 2000). Indeed, intestinal adsorption of phosphorus has been shown to be coupled to calcium adsorption in a variety of vertebrates (Mol et al., 1999). These studies mainly focus on the relationship between calcium and phosphorus in relation to availability in diet and or medium and subsequently growth. In the present study, we observed growth arrest under limiting calcium concentrations. Since most of the whole-body calcium and phosphorus is incorporated in bone and scales as calcium phosphate and calcium carbonate complexes, growth arrest due to calcium-limiting conditions apparently also leads to a subsequent decrease in net phosphorus influx.

PTHrP and cortisol

So far, only limited information is available on plasma PTHrP in sea bream. Danks et al. (1993) measured PTHrP in sea bream plasma and found 12.43 ± 1.48 pmol l^{-1}. Here, we present PTHrP values of 0.21 ± 0.06 to 0.32 ± 0.12 nmol l^{-1}. These values are in line with the values reported by Rotllant et al. (2003), where, using the same RIA as in this study, PTHrP values of 2.5 ± 0.29 ng ml^{-1} (0.61 ± 0.07 nmol l^{-1}) in 100–150 g fish were found. The lower values reported may well be caused by a lower immunoreactivity of the heterologous antisera with fish PTHrP, explained by different amino acids in the human N-terminal PTHrP sequence compared with fish consensus (discussed by Rotllant et al., 2003).

The plasma PTHrP levels in the two groups that were exposed to either a calcium-deficient diet or a diluted medium show a significant increase compared with the plasma PTHrP level of the control group. However, when calcium was limited in both diet and medium, plasma PTHrP level did not increase when compared with the control fish. A possible explanation for this is that the results show that, although decreased, growth is continuing in the groups in which the fish still had access to a natural calcium source, either in the diet or medium. For this growth, a positive net calcium accumulation is required (which may well be supported by a hypercalcemic action of PTHrP), which is supported by our results. On the other hand, in the fish in group E, growth arrest occurs during the experiment. The net calcium accumulation in this group was 4.5-fold lower compared with the control group and 2–3-fold compared with the other test groups. Under their apparent growth arrest, no net calcium influx for skeletal formation is required. Apparently, the calcemic endocrine system successfully controls blood plasma calcium levels to a level that ensures proper physiology and survival of the fish.

Cortisol values are approximately two times higher in the 2.5‰ group and 3–4 times higher in the calcium-deficient diet groups than in the control group. Although significantly higher, these values still do not exceed the basal level documented for this species, indicating that the fish were not stressed. Arends et al. (1999) measured basal cortisol levels of 25 nmol l^{-1} in sea bream. These values are in the same range as the basal levels in our experiment. It has been shown before that subtle differences in basal cortisol levels could account for changes in osmolarity, Na$^+$/K$^+$-ATPase activity and plasma calcium levels (Metz et al., 2003). Flik and Perry (1989) demonstrated increased cortisol secretion during hypocalcemic stress in freshwater rainbow trout, inducing the uptake of calcium ions from the water by regulating the Ca^{2+} pumps in the gills. Also, elevated plasma cortisol levels have been shown to play a role in hypo-osmotic adaptation. Mancera et al. (1994) showed increased cortisol levels in sea bream after transfer from 39‰ to brackish water of 7‰. The results reported here are corroborated by these early findings.

In the present study, we have demonstrated that sea bream can cope well with limited calcium availability in either diet or medium. The fish continued to grow, and upregulated hypercalcemic hormones, PTHrP and cortisol, allow the fish to maintain the physiologically important ionic calcium level constant.

In the case of limiting calcium availability in both external calcium sources, growth arrest occurs in sea bream, and whole-body calcium level can be so maintained at such a level that no large net calcium accumulation is needed for skeletal formation. The relatively small net calcium accumulation rate that is still achieved by the fish can thus be used to maintain plasma calcium balance in such a way that it ensures the survival of the fish for a prolonged period of time.

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