Parathyroid hormone-related protein and calcium regulation in vitamin D-deficient sea bream (*Sparus auratus*)

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Abstract

Gilthead sea bream (*Sparus auratus* L.) were fed a vitamin D-deficient diet for 22 weeks. Growth rate, whole body mineral pools and calcium balance were determined. Plasma parathyroid hormone-related protein (PTHrP) and calcitriol levels were assessed. Expression of mRNA for *pthrp* and *pth1r* was quantified in gills and hypophysis. Fish on vitamin D-deficient diet (D- fish) showed reduced growth and lower calcium turnover (calcium influx, efflux and accumulation rates decreased) and unaltered plasma calcium levels. Plasma calcitriol levels became undetectable, PTHrP levels decreased in the D- fish. In controls, a significant increase in plasma PTHrP level over time was seen, i.e. it increased with body mass. Relationships were found between

plasma PTHrP and the whole body pools of calcium, phosphorus and magnesium, indicative of a role for PTHrP in bone development. Expression of *pthrp* and *pth1r* mRNA was down-regulated in the hypophysis of D- fish, whereas in gill tissue, *pthrp* and *pth1r* mRNA were up-regulated. We conclude that lower *pthrp* mRNA expression and plasma values in D- fish reflect lower turnover of PTHrP under conditions of hampered growth; up-regulation of *pthrp* mRNA in gills indicate compensatory paracrine activity of PTHrP during calcitriol deficiency to guarantee well-regulated branchial calcium uptake. This is the first report to document a relation between PTHrP and calcitriol in fish.

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Introduction

In teleost fish, as in higher vertebrates, calcium plays a key role in a variety of biochemical and physiological processes. Fish have access to and use the essentially infinite sources of calcium in the water (through branchial uptake mechanisms). Their skeleton and dermal scales, important for shape, armour, structure and muscle attachment, serve as an important internal reservoir for calcium and phosphorus. In teleosts, about 99% of the total calcium pool is incorporated into the skeleton and dermal scales in the form of calcium phosphates and to a lesser extent as calcium carbonate (Flik *et al.* 1986).

In fish blood, the total calcium concentration is 2–3 mmol/l, of which the physiologically important ionic fraction is about 1.25 mmol/l (Hanssen *et al.* 1991). The blood calcium level may vary among species and within species that are euryhaline, but is always strictly regulated at varying set points and in accordance with environmental calcium availability by hyper- and hypocalcaemic hormones. Of these hormones, stanniocalcin, produced by the corpuscles of Stannius, is the dominant hypocalcaemic (in fact anti-hypercalcaemic) hormone. Stanniocalcin secretion is stimulated by increased plasma calcium concentrations and inhibits

calcium influx from the environment through the gills and intestine (Verboost *et al.* 1993).

Parathyroid hormone-related protein (PTHrP) is an important hypercalcaemic factor in early vertebrates and is present in the cartilaginous sharks and rays (Ingleton *et al.* 1995, Trivett *et al.* 1999) and bony fishes (Danks *et al.* 1993, Ingleton 2002). Three different receptors for PTHrP have been identified in zebrafish (*Danio rerio*, Rubin & Jüppner 1999) and more recently, in sea bream (*Sparus auratus* L.; Rotllant *et al.* 2005), of which PTHrP-R1 is the most common.

In sea bream, PTHrP has been detected in the plasma and several tissues, using an RIA with antisera to the human peptide (Devlin *et al.* 1996) and more recently, with a homologous RIA (Rotllant *et al.* 2003). PTHrP is involved in a number of physiological functions in this fish, such as bone and scale development (Martin *et al.* 1997, Redruello *et al.* 2005), vitellogenesis (Guerreiro *et al.* 2002, Bevelander *et al.* 2006) and calcium regulation (Guerreiro *et al.* 2001, Abbink *et al.* 2004, 2006, Fuentes *et al.* 2006).

Vitamin D is not synthesized by fish through photochemical processes in the skin and therefore, the diet is assumed to be the key source of vitamin D. Teleosts have large stores of vitamin D in their liver, and this precursor can be

converted into hydroxylated metabolites (Graff *et al.* 1999). The active metabolite of vitamin D, $1,25(\text{OH})_2\text{D}_3$ or calcitriol, is a steroid hormone that exerts its effects through a high affinity vitamin D₃-receptor (DeLuca & Zierold 1998; our personal observations on salmon, trout and carp). Calcitriol plays a role in fish calcium metabolism by stimulation of intestinal calcium absorption (Swarup *et al.* 1991, Sundell *et al.* 1993) and is a key factor in bone formation (Haga *et al.* 2004); the effects of calcitriol can be considered hypercalcaemic in mammals and fish alike.

Sundell *et al.* (1992) demonstrated increased calcium absorption after calcitriol administration and localized calcitriol receptors in several calcium regulating tissues (gill, intestine) in Atlantic cod (*Gadus morhua* L.). Moreover, vitamin D and its metabolites, including calcitriol, have been found in plasma of various fish species (Takeuchi *et al.* 1991, Horvli *et al.* 1998).

In this study, we investigated the influence of feeding juvenile gilthead sea bream, a vitamin D-deficient diet, for prolonged time on the regulating role of PTHrP in maintaining the calcium balance. We hypothesized that denying fish vitamin D through a vitamin D-deficient diet would result in recruitment of hypercalcaemic PTHrP to maintain calcium balance and counteract the imminent threat of hypercalcaemic.

Materials and Methods

Set-up and timing of analyses

The experiments were carried out under controlled laboratory conditions and lasted for 5 months. Fish were weighed at the start of the experiment, after acceptance of the diet, i.e. 2 weeks later and then every 4 weeks, to follow growth performance. Fish were sampled at 4-week intervals to assess whole body mineral pools as of 6 weeks after the start of the experiments every 4 weeks; calcium balance (calcium fluxes) were determined at 18 weeks of the experiment when significant differences in growth were observed. Blood was collected as of 6 weeks into the experiment and used to prepare plasma for analyses of calcium (weeks 18 and 22), PTHrP (weeks 10, 14, 18 and 22) and calcitriol (weeks 18 and 22) levels; mRNA expression for *pthrp* and *pth1r* were assessed by real-time quantitative PCR (RQ-PCR; weeks 18 and 22). The small volume of plasma available per fish forced us to make this analysis schedule.

Fish

Juvenile gilthead sea bream (*S. auratus* L.) weighing around 0.5 g were obtained from a commercial fish farm (Viveiro Vilanova, Lda., VN Milfontes, Portugal) and flown to The Netherlands without mortality. The fish were kept in a round 1200-l tank with aerated flow-through, a constant salinity of 34‰ and a water temperature of $23 \pm 1^\circ\text{C}$. The water quality

was monitored continuously for nitrogenous waste products and pH. The fish were fed daily with commercial pellets (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total body mass. This ration allowed the fish to grow and did not lead to detectable nitrogenous waste build-up in the tanks. The treatment of the fish was in agreement with the Declaration of Helsinki and Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of the Radboud University Nijmegen.

Experimental set-up

The fish were kept in control tanks for 2 months post-transport. At the start of the experiment ($t=0$), 350 fish (3.91 ± 0.69 g) were randomly selected from stock and transferred to two 500-l tanks (175 fish in each tank) with identical water conditions as in the stock tank. One week after transfer, the diet was changed from commercial pellets (Trouvit) to the test pellets (Hope Farms, Woerden, The Netherlands). The two diets did not differ in energetic value; the phosphorus content of the diets was 5.8 g/kg, the calcium content 11.0 g/kg. The only difference between the diets was the vitamin D and D₃ contents of the sufficient diet, which were 1 IU/g.

Within a week, the fish accepted the new diet and showed normal appetite, i.e. the fish ate all the food provided ($t=2$). Indeed, the fish continued to grow during the change in diet (time period $t=0$ to $t=2$ weeks of the experiment; Fig. 1). The experimental fish were always fed first and the controls received an equivalent amount of food as taken by the experimental fish. The fish were given a vitamin D-sufficient diet (controls; D+ fish) or a vitamin D-deficient diet (D− fish). Information on the diet is available on request.

The experiment had five sampling points for various analyses, starting at 6 weeks into the experiment ($t=6$; 4 weeks after acceptance of the new diet) with 4-week intervals.

Figure 1 Growth of sea bream during 22 weeks of feeding a control or a vitamin D-deficient diet. The fish in both groups continued to grow throughout the experiment. As of 14 weeks of feeding the vitamin D-deficient diet, reduced body mass was observed in the experimental fish. Asterisks indicate significance of differences with controls at $P < 0.05$, $n = 175$ at $t=0$ down to $n = 50$ at $t=22$.

The day before sampling, the fish were not fed. To collect blood, fish were deeply anaesthetised with 2-phenoxyethanol (1:500; Sigma–Aldrich); the caudal vessels were punctured with a 23-G needle fitted to a tuberculine syringe, rinsed with sodium heparin (Leo Pharma, Weesp, The Netherlands; 5000 U/ml) to avoid blood clotting. Next, the fish were killed by spinal transection. Bone and muscle samples were taken to assess whole body mineral pools; plasma was analysed for calcium, PTHrP and calcitriol levels. Selected tissue sub-samples were taken to assess mRNA expression levels for *pthrp* and *pth1r*.

Drinking rate (DR) and calcium influx ($F_{in}^{Ca^{2+}}$) were evaluated, using ^{51}Cr -EDTA (25.75 GBq/mg; Perkin–Elmer, Boston, MA, USA) and $^{45}CaCl_2$ (0.55 GBq/mg; Perkin–Elmer) respectively, according to procedures that have been validated extensively (Flik *et al.* 1986), also for sea bream (Guerreiro *et al.* 2004).

Whole-body mineral content

The body mass of the fish was determined upon transfer from stock tank to test tank ($t=0$), after acclimatisation to the experimental diets ($t=2$, after 2 weeks) and at the subsequent five sampling points ($t=6$, after 6 weeks, etc.); the experiment lasted a total period of 22 weeks.

At every sampling point, 15 fish of each group were euthanized with 2-phenoxyethanol (1:500), were freeze-dried until dry (dry weight; DW) and dissolved in concentrated nitric acid (70%; 3 ml/g DW; Sigma–Aldrich) for whole body mineral analyses. The nitric acid digests were diluted 500× with demineralised water and analysed for calcium, phosphorus and magnesium, using inductively coupled plasma atomic emission spectrophotometry (ICP–AES, Plasma II200; Thermo Electron, MA, USA). From these analyses, whole body pools were calculated. Mineral content was expressed in $\mu\text{mol/g}$ DW, based on the fish DW and the total digest volume.

Plasma parameters

Blood was taken as indicated above from 15 fish of both groups at sampling points $t=6$ to $t=22$. The collected blood was centrifuged at 13.600 *g* for 10 min and the plasma so obtained was stored at -20°C . Plasma Ca^{2+} , Na^+ , K^+ , glucose, lactate (mmol/l) and pH were measured using a Stat Profile pHox plus analyser (Nova Biomedical, Waltham, MA, USA) and plasma total calcium (mmol/l) was measured

using ICP–AES. Plasma PTHrP level (nmol/l) was measured with a homologous RIA according to Rotlant *et al.* (2003) and the plasma calcitriol level (pmol/l) was measured according to Hoof van *et al.* (1993).

Drinking and calcium influx

After 18 weeks into the experiment ($t=18$), 20 fish from each group were randomly divided and placed into identical vessels, with 10 fish per vessel to determine the DR and the calcium influx according to earlier described procedures (Flik *et al.* 1985, Abbink *et al.* 2006).

pthrp and *pth1r* mRNA expression

At 18 ($t=18$) and 22 weeks ($t=22$) into the experiment, tissue samples from the gill and pituitary gland were taken from eight fish from the D+ and D– group. The small size of the fish and consequently of the pituitary gland did not allow us to take samples for RQ-PCR analysis at time points $t=6$, $t=10$ and $t=14$; mRNA expression was successfully analysed in individual fish on the subsets of samples taken at $t=18$ and $t=22$ weeks. RQ-PCR was used to quantitate mRNA expression levels for *pthrp* and *pth1r* in these tissue samples according to Hang *et al.* (2005), using the house-keeping gene β -actin as an endogenous control.

Statistical analysis

All data are expressed as means $\pm$ S.D. Differences among groups were assessed by ANOVA. Significance of differences was assessed by parametric (Student's *t*-test) or non-parametric (Mann–Whitney *U*-test) tests where appropriate and $P<0.05$ was taken as the fiducial limit.

Results

Calcium balance

No mortality was observed during the experiment; a lower body mass of the D– fish compared with the D+ fish occurred as of 14 weeks of feeding the deficient diet (Fig. 1). Plasma minerals, glucose, lactate and pH were unaffected in the vitamin D-deficient fish after 22 weeks of feeding the diet (Table 1). The growth related calcium accumulation rate (Fig. 2A) is lower in the D– fish ($0.29 \pm 0.29 \mu\text{mol/h}$) when compared with the D+ fish ($0.53 \pm 0.20 \mu\text{mol/h}$).

Table 1 Plasma minerals, glucose, lactate (in mmol/l) and pH in sea bream were unaffected in the vitamin D-deficient fish after 22 weeks of feeding the diet ($n=8$; values are given in mean $\pm$ S.D.)

	Total Ca	Ca^{2+}	Na^+	K^+	Glucose	Lactate	pH
D+ fish	2.3 ± 0.7	1.10 ± 0.21	159 ± 9	4.2 ± 0.5	7.7 ± 3.6	3.1 ± 1.1	7.66 ± 0.04
D– fish	2.2 ± 0.4	0.99 ± 0.15	155 ± 4	4.4 ± 0.4	5.2 ± 3.2	3.3 ± 2.4	7.64 ± 0.04

Figure 2 Analyses of calcium fluxes show reduced calcium turnover, with a decreased calcium influx, efflux and accumulation rate in the vitamin D-deficient fish (A); plasma total and ionic calcium levels did not differ between control and experimental fish (B).

Unidirectional calcium influx, $F_{\text{in}}^{\text{Ca}^{2+}}$, was $2.62 \pm 1.51 \mu\text{mol/h}$ in the D+ fish and had decreased to $1.58 \pm 1.14 \mu\text{mol/h}$ in the D- fish. The calcium efflux (calculated as the difference between calcium influx minus net accumulation rate) was $2.09 \mu\text{mol/h}$ in the D+ fish and $1.30 \mu\text{mol/h}$ in the D- fish. However, the ratio between calcium influx, efflux and accumulation remained constant and the plasma total and ionic calcium concentrations were not affected by the vitamin D-deficient diet (Fig. 2B).

Endocrinology

Feeding the fish a vitamin D-deficient diet decreased the calcitriol concentration to a level below the assay's detection limit ($<175 \text{ pmol/l}$) after 18 and 22 weeks of feeding the diet ($n=8$). In the controls, plasma calcitriol remained at a constant level throughout the experimental period ($228 \pm 35 \text{ pmol/l}$ after 18 weeks and $245 \pm 58 \text{ pmol/l}$ after 22 weeks; $n=8$). In addition, strongly decreased plasma PTHrP levels were found in the D- fish after 18 and 22 weeks of feeding the diet (Fig. 3; 18 weeks: D+ fish: $0.21 \pm 0.05 \text{ nmol/l}$, $n=15$; D- fish: $0.13 \pm 0.03 \text{ nmol/l}$, $n=15$, $P<0.001$ and 22 weeks: D+ fish: $0.19 \pm 0.04 \text{ nmol/l}$, $n=13$; D- fish: $0.13 \pm 0.04 \text{ nmol/l}$, $n=13$, $P<0.001$).

In the control fish, body weight and plasma PTHrP were positively correlated (Fig. 3; $R^2=0.14$; $n=42$; $P<0.01$), as were the whole body pools of the major bone minerals and PTHrP (calcium: Fig. 4A; $R^2=0.69$; $n=4$; $P<0.05$; phosphorus: Fig. 4B; $R^2=0.75$; $n=4$; $P<0.05$ and magnesium: Fig. 4C; $R^2=0.68$; $n=4$; $P<0.05$). In the D- fish, all these relationships were abolished. Relationships between body mass of the fish and whole body pools for calcium, phosphorus and magnesium were unaffected in the D- fish (data not shown).

Figure 3 Fish fed the vitamin D-deficient diet show lower plasma PTHrP levels compared with controls. A correlation between plasma PTHrP and body mass was found in the control fish ($R^2=0.14$, $n=42$, $P<0.05$). In the experimental fish, no such correlation could be established ($R^2=0.01$, $n=37$, $P>0.05$).

The mRNA levels for *pthrp* and *pth1r* in the pituitary gland and gills did not show any differences after 18 and 22 weeks of feeding the diet and the data were therefore pooled. In the pituitary gland, mRNA expression was down-regulated in the D- fish when compared with the control group (Fig. 5), whereas in gills, an up-regulation for *pthrp* and *pth1r* mRNA was found in the D- fish compared with controls.

Discussion

We hypothesized that fish on a vitamin D-deficient diet would recruit hypercalcemic PTHrP for calcium balance and counteract the imminent threat of hypocalcemia. This hypothesis was, to our surprise, only partly confirmed. Under long-term vitamin D constraint sea bream show a lower growth rate due to decreased calcium turnover (yet the fish keep their calcium balance) and decreased plasma PTHrP and calcitriol levels. The positive correlations between plasma PTHrP and bone minerals (calcium, phosphorus and magnesium) became less prominent. Expression of *pthrp* and *pth1r* mRNA was down-regulated in the pituitary gland and up-regulated in gill tissue.

As suggested earlier (Abbink *et al.* 2006), we have evidence for an independent branchial PTHrP regulatory system, acting in a paracrine fashion and apart from an endocrine pituitary source of PTHrP. The branchial chloride cell expresses PTHrP (Flanagan *et al.* 2000) is key in calcium uptake in fish (Flik *et al.* 1995), and thus appears to be fitted with a paracrine (and/or auto- and/or intracrine) calciotropic or cell proliferation control mechanism. Such actions of PTHrP are well recognised.

Calcium balance

The absence of vitamin D in the diet slowed down bone formation and growth rate, and thus reduced the need to

Figure 4 Correlations between plasma PTHrP and whole body calcium, phosphorus and magnesium pools in control (A: calcium; $R^2=0.69$, $N=4$, $P<0.05$; B: phosphorus; $R^2=0.75$, $N=4$, $P<0.05$; C: magnesium; $R^2=0.68$; $N=4$; $P<0.05$). In the experimental fish, these correlations have faded (A: calcium; $R^2=0.42$, $N=4$, $P>0.05$; B: phosphorus; $R^2=0.38$, $N=4$, $P>0.05$; C: magnesium; $R^2=0.31$; $N=4$; $P>0.05$).

incorporate calcium into the skeleton and dermal scales, processes that require calcitriol (Graff *et al.* 2002) and PTHrP (Redruello *et al.* 2005, Rotlant *et al.* 2005), and indeed the levels of both hormones had decreased in fish kept on a vitamin D-deficient diet. An earlier study by Taveekijakarn

Figure 5 Expression of *pthrp* and *pthr1* mRNA is down-regulated in the pituitary gland of the experimental fish compared with controls. In gills, mRNA expression levels for these genes are up-regulated in response to a vitamin D-deficient diet. Asterisks indicate significance at $P<0.05$; $n=8$.

et al. (1996) described impeded growth in response to vitamin D-deficient diet in amago salmon (*Oncorhynchus rohdaurus*). However, Graff *et al.* (2002) found no difference in growth rate in Atlantic salmon (*Salmo salar* L.) fed a low-level vitamin D diet (0.2 mg/kg) for 3 months. Indeed, the low vitamin D level present in the latter diet could still suffice to guarantee growth, considering the relatively mild effects seen in our study with deficient diet; it should be noted that we did not observe any effects in the early phase of feeding the diet.

The fish of both groups continued to grow and their whole body calcium content increased, while a strict and constant relation between the total calcium, phosphorus and magnesium pools and body weight was kept, indicating well-adapted mineral handling. Under stress conditions often enhanced calcium efflux and decreased uptake mark disturbances of calcium balance (Flik *et al.* 1985); no such phenomena were seen in our study. Reduced growth rate and decreased accumulation rate coincided with down-scaled calcium influx and efflux rate, i.e. the calcium turnover decreased but calcium balance was not disturbed. This peculiar adaptive response may be easily overlooked (little seems to change other than growth rate) but lower calcium turnover coincides with altered calciotropic activities (see next Section). The lower calcium turnover was not accompanied by a decrease in plasma calcium levels, which remained constant during the experiment. Since, minor deflections in plasma ionic calcium levels can lead to severe physiological disruptions, also in fish (Flik *et al.* 1995), plasma ionic calcium levels must be and are regulated within narrow limits. Injections of calcitriol lead to increased plasma calcium levels, in line with predicted hypercalcemic actions of calcitriol (Fenwick *et al.* 1984, Sundell *et al.* 1993). Clearly, in some of the experimental fish in this study, calcitriol levels became even undetectable and PTHrP levels remained constant. The unaffected plasma ionic calcium level then

would indicate PTHrP turnover had decreased, yet PTHrP activity remained sufficient to cope with the threat of hypocalcemic conditions in the vitamin D-deficient fish.

Endocrinology

Two remarkable and new observations were made when the plasma PTHrP levels in the fish were analysed. First, the vitamin D-deficient diet resulted in undetectable calcitriol levels and in highly significant lower plasma PTHrP levels after 18 and 22 weeks of feeding the diet, indicating that the vitamin D-deficient diet results in adaptive responses in the plasma PTHrP level, but only after 14–18 weeks of feeding the diet. A second remarkable finding is the small, but significant tendency for plasma PTHrP to increase over time, i.e. it increased with the increasing mass of the fish. In earlier studies, Rotllant *et al.* (2003) measured plasma PTHrP in sea bream and found 2.5 ± 0.29 ng/ml (0.61 ± 0.07 nmol/l) in 100–150 g sea bream and Abbink *et al.* (2004) measured somewhat lower values 0.21 ± 0.06 to 0.32 ± 0.12 nmol/l in smaller, 40–60 g juvenile sea bream. These levels are in line with the values found in the present study and establish once more a relation between the plasma PTHrP level and the increasing body mass of the fish. In accordance, Abbink *et al.* (2006) showed that in juvenile sea bream the plasma PTHrP level increases with the body mass, but plateaus with increasing mass of the fish; we suggested a role for PTHrP in skeletal physiology in particular in juvenile fish (where the bone compartment is relatively large) and thus a decreasing need for hypercalcemic control with increasing body mass. The strong correlations between plasma PTHrP and the whole body content of the main minerals in bone (calcium, phosphorus and magnesium) that were found in the present study further strengthen the assumption that PTHrP is involved in skeletal calcium physiology. Taking the above-mentioned aspects into account, we argue that plasma PTHrP levels reflect the need for regulation of the bone compartment, which is compartment/size dependent.

Involvement of PTHrP in skeleton and scale calcium physiology has been suggested earlier. Rotllant *et al.* (2005) established PTHrP involvement in calcium reabsorption from scales when the activity of tartrate-resistant acid phosphatase (a marker for osteoclastic activity in mammalian bone) was enhanced when cultured sea bream scales were treated with N-terminal (1–34)PTHrP. Redruello *et al.* (2005) measured abolished osteonectin mRNA expression in sea bream scales treated with different doses (10 and 1000 nmol/l) of (1–34)PTHrP. Osteonectin is a calcium-binding glycoprotein that stimulates the mineralization process following differentiation of the osteoblastic cell lineage (Esteveao *et al.* 2005).

The loss of correlations between plasma PTHrP and the whole body pools for calcium, phosphorus and magnesium in the D– fish point to a disturbance of the bone formation process induced by the vitamin D deficiency. The subsequent decrease in growth rate and calcium turnover could have

evoked the adaptive responses of the branchial and pituitary PTHrP systems, as presented in this study. Expression levels for *pthrp* and *pth1r* mRNA in sea bream have been described earlier by Flanagan *et al.* (2000) and Hang *et al.* (2005) and have established a widespread tissue distribution of PTHrP, mostly with a low expression level. The distribution pattern is suggestive of para-, auto- or intracrine functions of PTHrP. However, the presence of PTHrP in the pituitary gland and the (high) circulating levels (in fish indeed at concentrations of other endocrines, in the nmol/l range) point to a classic endocrine function for PTHrP, as suggested earlier by Danks *et al.* (1993) and Abbink *et al.* (2006).

Down-regulated expression levels for *pthrp* and *pth1r* in the pituitary gland suggest that the vitamin D-deficient diet with its consequences for calcium turnover may target and feedback at the pituitary gland level. This is reflected then by a decreased plasma PTHrP level and would point to a specific calcitriol feedback on the pituitary somatolactin cells that produce PTHrP (Ingleton *et al.* 1998, Abbink *et al.* 2006). Clearly, coinciding lower calcium turnover and lower pituitary PTHrP turnover are indicated by our data.

The up-regulation of branchial *pthrp* and *pth1r* mRNA expression correlates well with long-term adjustment of uptake mechanisms under lower calcium turnover, and could be an adaptive response to the decrease in circulating PTHrP. Binding of plasma PTHrP to its receptor in the gills (that comprise a very large volume in the fish), we speculate, may contribute significantly to clearance of PTHrP from the plasma and this could explain, at least partly, the lower PTHrP level in the plasma as observed after long-term feeding the vitamin D-deficient diet. The enhanced levels of receptor mRNA further add to this assumption. Moreover, the decline in pituitary gland *pthrp* mRNA expression will contribute to the lower plasma PTHrP levels in the D– fish. Our results on differential effects of vitamin D-deficiency towards PTHrP and PTH1R in pituitary gland and gills resemble similar findings in rats where vitamin D-deficiency increased PTHrP mRNA in keratinocytes and decreased in fibroblasts and kidney cells; the receptor mRNA increased in keratinocytes and kidney, but not in fibroblasts (Errazahi *et al.* 2004). Apparently, such differential links between the vitamin D system and the PTHrP system (Xie *et al.* 1996) are universal among vertebrates.

In line with an earlier study where juvenile sea bream were given limited access to calcium (Abbink *et al.* 2006), the present study shows similar responses from the branchial and pituitary gland PTHrP system and is a further indication that the auto- or paracrine branchial PTHrP system acts independently from the endocrine pituitary gland PTHrP system.

This study shows that vitamin D deficiency in fish decreases plasma calcitriol and this decrease triggers an adjustment of the pituitary and branchial PTHrP systems to counteract the imminent threat of hypocalcemia. This is the first study to

reveal such relation between these two hypercalcemic hormones in fish and we speculate that the bone formation, which is calcitriol dependent, is pivotal in this relation.

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