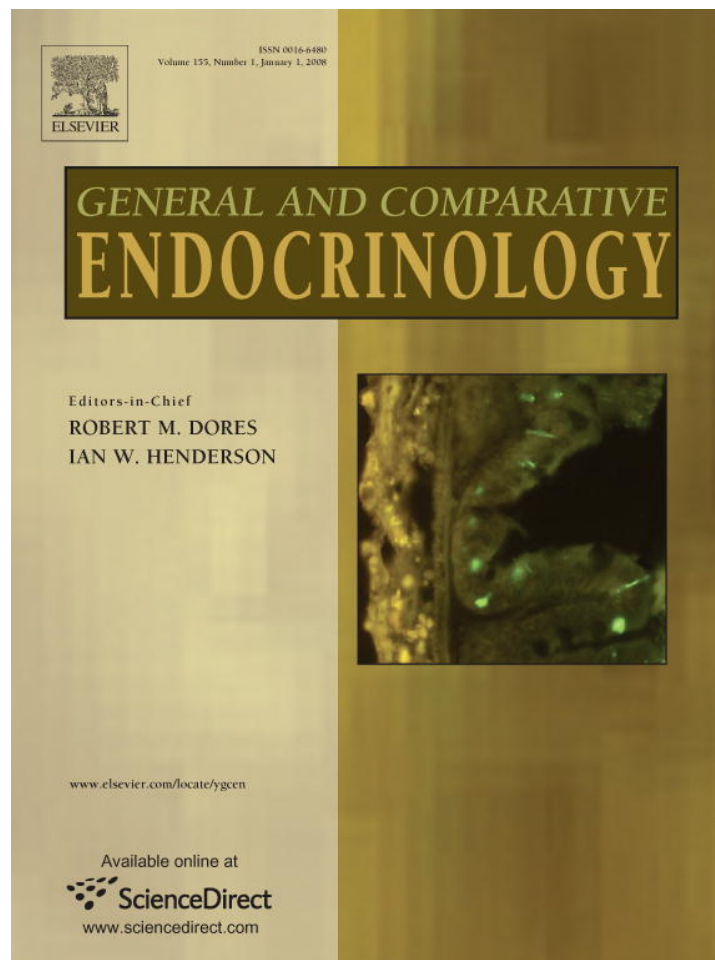


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Melatonin synthesis under calcium constraint in gilthead sea bream (*Sparus auratus* L.)

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Abstract

Brain or blood plasma melatonin was analysed as a measure for pineal melatonin production in sea bream. Access to calcium was limited by diluting the seawater to 2.5‰ and removing calcium from the diet or by prolonged feeding of vitamin D-deficient diet. Interactions/relations between melatonin and calcium balance and the hypercalcemic endocrines PTHrP and calcitriol were assessed. Restricting calcium availability in both water and diet had no effect on plasma melatonin, but when calcium was low in the water or absent from food, increased and decreased plasma melatonin was observed, respectively. Fish on a vitamin D-deficient diet (D– fish) showed decreased plasma calcitriol levels and remained normocalcemic. Decreased brain melatonin was found at all sampling times (10–22 weeks) in the D– fish compared to the controls. A positive correlation between plasma Ca^{2+} and plasma melatonin was found ($R^2 = 0.19$; $N = 41$; $P < 0.01$) and brain melatonin was negatively correlated with plasma PTHrP ($R^2 = 0.78$; $N = 4$; $P < 0.05$). The positive correlation between plasma levels of melatonin and Ca^{2+} provides evidence that melatonin synthesis is influenced by plasma Ca^{2+} . The decreased melatonin production in the D– fish points to direct or indirect involvement of calcitriol in melatonin synthesis by the pineal organ in teleosts. The hypercalcemic factors PTHrP and calcitriol appeared to be negatively correlated with melatonin and this substantiates an involvement of melatonin in modulating the endocrine response to cope with hypocalcemia. It further points to the importance of Ca^{2+} in melatonin physiology.

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Keywords: Melatonin; Hypocalcemia; PTHrP; Calcitriol; Sea bream; Diet

1. Introduction

Melatonin (*N*-acetyl-5methoxytryptamine) is a product of tryptophan metabolism in the pineal gland and retina in all classes of vertebrates. Melatonin synthesis shows a circadian rhythm in vertebrates, including fishes, with synthesis increased during darkness and decreased during the light period (Ekström and Meissl, 1997). The rhythm of melatonin biosynthesis results from variations in activity of arylalkylamine *N*-acetyltransferase, the light-sensitive, key enzyme in melatonin production (Liu and Borjigin,

2005). The pineal gland does not store melatonin and therefore, levels of melatonin assayed in plasma or brain extracts directly reflect synthetic activity of the pineal gland (Kulczykowska, 2002).

The past decades have provided a plethora of data on physiological parameters that are linked to melatonin activity (Davis, 1997; Dubocovich and Markowska, 2005). Melatonin activity is pivotal in circadian as well as circannual biorhythms (Meissl and Brandstätter, 1992; Reiter, 1993; Vera et al., 2006). In Atlantic salmon, *Salmo salar*, melatonin was shown to be involved in early development and control of the timing of parr-smolt transformation (Porter et al., 1998). Melatonin *per se* decreases tartrate-resistant acid phosphatase and alkaline

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phosphatase activities in cultured goldfish (*Carassius auratus*) scales and counteracts stimulatory effects of estradiol on these enzymes (Suzuki and Hattori, 2002). These phosphatases are the markers of choice for osteoclastic and osteoblastic activity and it thus follows that melatonin influences calcium physiology of scales (and bone) in fish (Fjellidal et al., 2004).

Many aspects of pinealocyte activity are under some control of (plasma) calcium activity and therefore, pineal function often relates directly or indirectly to calcium metabolism (Morton and Reiter, 1991). Indeed, in two fishes, viz. rainbow trout (*Oncorhynchus mykiss*) and summer flounder (*Paralichthys dentatus*), melatonin synthesis capacity appears to be positively correlated to plasma free calcium levels (Kroeber et al., 2000; Gozdowska et al., 2003). This relation between plasma calcium and melatonin activity warranted the research presented here. We reasoned that an analysis of plasma calcium levels and of calcitropic hormone activities would shed better light on the relation between calcemic conditions and melatonin activity, considering the strict calcemic control in fish (as in all vertebrates).

Fish have essentially unlimited access to calcium in their environment (water and diet; external calcium sources); in addition, their skeleton and dermal scales represent internal calcium sources (Flik et al., 1986). Physiological processes, such as vitellogenesis, that demand sumptuous amounts of calcium or variations in environmental calcium availability (e.g., migration into soft water), require a swift calcemic endocrine system to keep plasma calcium balanced. Plasma Ca^{2+} is the physiologically important fraction in (fish) blood (Hanssen et al., 1991) and this fraction in particular is regulated within narrow limits, as even minor deviation of set point may evoke (severe) stress (Flik et al., 1995).

Calcium regulation in fishes involves the antihypercalcemic stanniocalcin (the hormone inhibits calcium influx from the water via the gills and by doing so exerts hypocalcemic effects; Verbost et al., 1993). It has long been thought that fish lack typical hypercalcemic endocrine factors, as antihypercalcemic control by stanniocalcin seemed to suffice in explaining calcemic control (Wagner et al., 1998). Indeed, only very recently the genes for parathyroid hormone (PTH), which is the dominant hypercalcemic factor for terrestrial vertebrates, were found in fish (Danks et al., 2003). However, earlier, fish were shown to express genes for parathyroid hormone related protein (PTHrP; Power et al., 2000; Flanagan et al., 2000; Canario et al., 2006). PTHrP behaves in fish as a hypercalcemic hormone and appears key in fish calcium physiology (Ingleton et al., 2002; Trivett et al., 2001). Recently, we established a strict relationship between PTHrP levels in plasma and plasma Ca^{2+} in juvenile sea bream (Abbink et al., 2006). PTHrP is involved in both the regulation of calcium uptake from the environment (Guerreiro et al., 2001) and regulates calcium resorption from scales (Rotllant et al.,

2005). In addition to PTHrP, calcitriol ($1.25[\text{OH}]_2\text{D}_3$) exerts hypercalcemic effects in fish; it is the active metabolite of vitamin D that plays an important role in bone formation (Haga et al., 2004) and it stimulates intestinal calcium absorption (Swarup et al., 1991). Sundell et al. (1993) demonstrated calcitriol receptors in several calcium regulating tissues (gill, intestine, kidney) in Atlantic cod (*Gadus morhua*) and demonstrated increased calcium absorption after calcitriol administration, in line with hypercalcemic function. We reasoned that feeding our fish a vitamin D-deficient diet for prolonged times should compromise their calcium physiology and thus we analysed such fish in this study.

Juvenile sea bream were limited for at least 3 weeks in their calcium access by feeding a calcium deficient diet, decreasing water calcium content, or both. The water calcium content was decreased by dilution of the seawater (34–2.5‰ salinity) and by doing so, the water calcium concentration decreased from 10 to 0.7 mmol l^{-1} (Abbink et al., 2004).

Indeed, compared to untreated control fish in seawater, all experimental groups in these experiments show slightly elevated cortisol levels, although we discussed that these rises were very mild and considered still within the limits of values for non-stress situations (Abbink et al., 2004). We realise ourselves that even mild elevations of cortisol may affect neuroendocrine regulatory systems including the melatonin system (Larson et al., 2004). However, as will be shown in this paper the melatonin response to the treatments given does not parallel the earlier published cortisol responses.

In a second series of experiments, fish were fed a vitamin D-deficient diet for up to 22 weeks (Abbink et al., in press) and compared to controls that were fed a vitamin D-sufficient diet. The rationale behind these two experiments was to limit calcium availability, either directly (via water and diet) or indirectly (via vitamin D-deficiency) to impose an imminent hypocalcemia and activate hypercalcemic endocrines (PTHrP). The fish limited in their access to calcium in water and diet became hypocalcemic (for the Ca^{2+} fraction). The fish kept on the vitamin D-deficient diet remained normocalcemic, but calcium turnover decreased, indicated by decreased branchial in- and efflux of calcium and a lower calcium accumulation rate. Unexpectedly, in both experiments, plasma PTHrP levels remained constant or even decreased, while *pthrp* and *pth1r* (the main PTHrP receptor; Rubin and Jüppner, 1999) mRNA levels were down-regulated in the pituitary gland, results interpreted to indicate lower turnover of PTHrP.

Thus we followed these studies by further exploring the relationship between melatonin production and calcium regulation. In the present study, we analysed the brain or blood plasma melatonin concentration of these fish and their controls to assess interactions/relations between melatonin and calcium balance and the hypercalcemic endocrines PTHrP and calcitriol.

2. Materials and methods

2.1. Fish

Juvenile gilthead sea bream (*Sparus auratus*) were obtained from a commercial fish farm (Viveiro Vilanova, Lda., V.N. Milfontes, Portugal) and kept in a round 1500-L tank with an aerated flow-through system and full strength sea water (34‰ salinity; 10.5 mmol⁻¹ calcium) at 23 °C and a photoperiod of 12 h light/12 h dark. The fish were fed a ration of 2% of the total body mass daily of commercial fish pellets (Trouvit, Trouw, Putten, The Netherlands). At the time of the experiments (spring–summer), the fish weighed between 10 and 40 g body mass. The experimental setup and sampling procedures were described recently (Abbink et al., 2004; in press). In short, for the first series of experiments, four groups of fish were used. The control group A, exposed to full strength seawater (SW) and fed a control diet (Ca+ diet). Three experimental groups: group B; exposed to dilute sea water of 2.5‰ salinity (DSW), group C: fed a calcium deficient diet (Ca– diet), and group D: exposed to DSW and fed a Ca– diet). This experiment lasted for up to 3 weeks.

In the second series of experiments, fish kept in full strength seawater were fed a vitamin D-deficient (D– diet) or control diet (D+ diet) for up to 22 weeks and sampled every 4 weeks ($N = 7–8$). Upon completion of the experiments, the fish were quickly and deeply anaesthetised in 0.1% v/v 2-phenoxyethanol (Sigma–Aldrich, St. Louis, MO, USA) and after blood had been taken from the caudal vessels by puncture with a 24-G needle fitted to a tuberculin syringe, the fish were killed by spinal transection and the brain was promptly dissected. Animal handling followed the approved university guidelines. Plasma PTHrP level (nmol⁻¹) was measured with a homologous radioimmunoassay according to Rotllant et al. (2003) and plasma calcitriol (pmol⁻¹) was measured according to Van Hoof et al. (1993).

2.2. Melatonin

The brains of the fish from the vitamin D experiment were snap-frozen in liquid nitrogen and stored at –70 °C. Sonification of the brains was performed in 0.05 mol⁻¹ phosphate buffer containing 0.01% thimerosal (Sigma–Aldrich). After centrifugation of the brain homogenate at 15,000g for 20 min, supernatant was collected and assayed for melatonin and total protein as reference. Protein was determined by the Lowry method with Peterson's modification (Peterson, 1977), using a total protein kit (Sigma–Aldrich); bovine serum albumin (BSA) was used as a reference.

Melatonin concentration in plasma and brain samples was quantified by radioimmunoassay (RIA), using a total melatonin kit (IBL, Hamburg, Germany) with a certified extraction procedure. Solid phase extraction of melatonin from all samples (100 µl) was carried out on an Octadecyl C₁₈ Speedisk Column, 10 µm (J.T. Baker, Phillipsburg, NJ, USA). Samples were eluted with methanol according to a procedure previously described for melatonin extraction from fish plasma (Kulczykowska and Iuvone, 1998). After extraction, samples were dried and then resuspended in Dulbecco's phosphate-buffered saline containing 0.01% thimerosal and

assayed by RIA. Samples were counted in a Wallac Wizard γ -counter (Wallac, Turku, Finland). The detection limit was 3.0 pgml⁻¹ in plasma and 3.5 pgml⁻¹ in brain extract. The intra- and inter-assay coefficients of variation for plasma melatonin were 8.0% and 15.0%, respectively. The intra- and inter-assay coefficients of variation for brain melatonin were 8.4% and 14.7%, respectively. Two different serum or brain samples and controls (available from IBL–Hamburg kit) were measured in 10 replicates to determine intra-assay precision in the same assay. The inter-assay precision was determined by analysis of two different serum or brain samples and controls (available from IBL–Hamburg kit), in triplicate in three independent assays. The RIA data were validated by HPLC assay (Kulczykowska and Iuvone, 1998): randomly selected samples of brain and plasma were assayed for melatonin by both HPLC and RIA. The results obtained by either method were identical.

2.3. Statistics

Data are presented as means \pm standard deviation (s.d.). For statistical analysis of the data, analysis of variance (ANOVA and two-way ANOVA) was used to assess differences among groups and Tukey's test was applied as post-hoc test, where appropriate. To determine relationships, regression and weighted non-linear regression analyses were performed; Pearson's correlation coefficient and y -intercept were determined where appropriate. Significance of differences was accepted when $P < 0.05$.

3. Results

In fish that were restricted in their calcium access (Table 1), the total calcium level was reduced when calcium was limited in the diet (group C), whereas exposure to DSW (group D) resulted in decreased Na⁺, K⁺, total calcium and osmolality. Hypocalcemia (defined as decreased plasma Ca²⁺) was only seen when calcium was restricted in both water and diet (group B).

Fig. 1 shows plasma melatonin after 3 weeks calcium restriction. Exposure to both DSW and a Ca– diet had no effect on plasma melatonin ($P > 0.05$). Feeding the fish (held in normal sea water) a Ca– diet decreased plasma melatonin ($F = 12.223$; $P < 0.0001$; post hoc: $P < 0.05$), whereas exposure to DSW (and fed a normal diet) resulted in an increase of plasma melatonin compared to the controls ($F = 12.223$; $P < 0.001$; post hoc: $P < 0.001$).

In the D– fish, a strongly decreased brain melatonin was found at all sampling times compared to the controls (Fig. 2; $F = 97.3$; $P < 0.001$). The lower brain melatonin in the D– fish was established at the first sampling point, viz. after 10 weeks on the diet and was consistent throughout the subsequent experimental period. In addition, a

Table 1
Mineral analysis of plasma of sea bream fed a calcium deficient diet (Ca–) while kept in dilute seawater (DSW), fed the Ca– diet in normal seawater or kept in DSW fed a normal diet

Condition	Na ⁺	K ⁺	Ca total	Ca ²⁺	Osmolality
Control	175 \pm 12	5.4 \pm 0.9	3.7 \pm 0.3	1.30 \pm 0.17	381 \pm 17
Ca– diet and DSW	161 \pm 8*	5.5 \pm 1.4	3.3 \pm 0.4*	1.15 \pm 0.14*	358 \pm 23*
Ca– diet	172 \pm 7	5.2 \pm 0.6	3.3 \pm 0.5*	1.35 \pm 0.09	373 \pm 24
DSW	161 \pm 10*	5.1 \pm 1.1	3.3 \pm 0.4*	1.32 \pm 0.24	360 \pm 28*

Values are in mmol⁻¹, osmolality is expressed in mOsmol kg⁻¹. Asterisks (*) represent significant difference from the control group ($P < 0.05$), $N = 8$ per group.

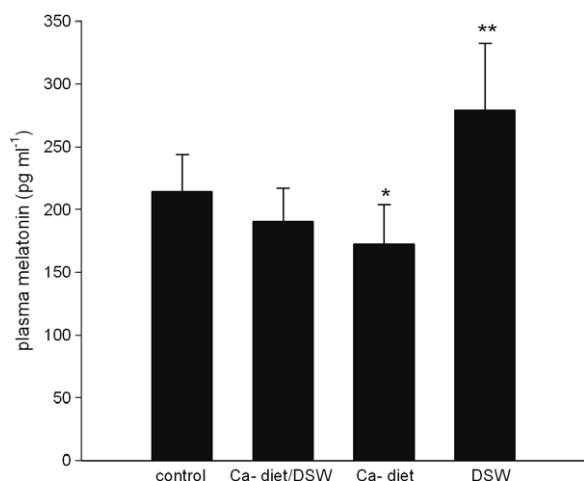


Fig. 1. Plasma melatonin levels after 3 weeks under conditions of limited calcium access. Plasma melatonin was not affected in fish kept on a Ca-deficient diet and in diluted seawater (DSW). Fish exposed to the Ca-deficient diet showed a decrease in plasma melatonin, whereas plasma melatonin was increased in fish kept in DSW. Asterisks (*) represent statistical different from the control group (* $P < 0.05$ and ** $P < 0.01$).

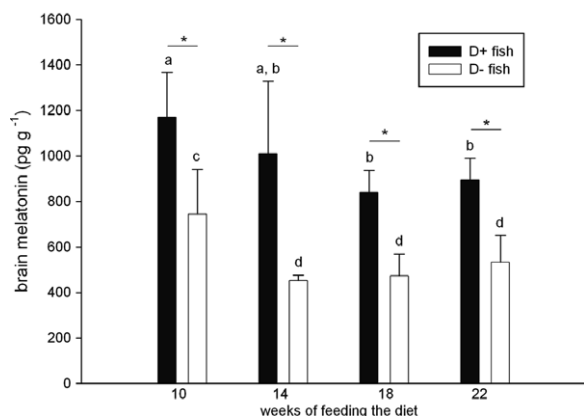


Fig. 2. In fish fed a vitamin D-deficient diet, melatonin synthesis in the brain is significantly lower than in controls at all four time points. The reduction in melatonin was consistent and had already been established at the first sampling point. Asterisks (*) represent significant difference from accompanying control group ($P < 0.001$). The decrease in melatonin synthesis over time for the two groups is indicated by a,b for the D+ fish and c,d for the test fish ($P < 0.05$).

decrease in brain melatonin was observed in time ($F = 9.54$; $P < 0.01$).

A positive correlation between plasma Ca^{2+} and plasma melatonin was found (Fig. 3; $R^2 = 0.19$; $N = 41$; $P < 0.01$).

Brain melatonin is negatively correlated with plasma PTHrP (Fig. 4; $R^2 = 0.78$; $N = 4$; $P < 0.05$) and this relationship was not affected by feeding the fish a vitamin D-deficient diet ($R^2 = 0.90$; $N = 4$; $P < 0.05$), although plasma melatonin and PTHrP levels were lower in the latter group (D+ fish: $y = -6171 \pm 1503 x + 1363 \pm 196$; D- fish: $y = -5663 \pm 1717 x + 1992 \pm 309$. $P = 0.96$ for the slopes of the regression lines and $P < 0.05$ for the y-intercept).

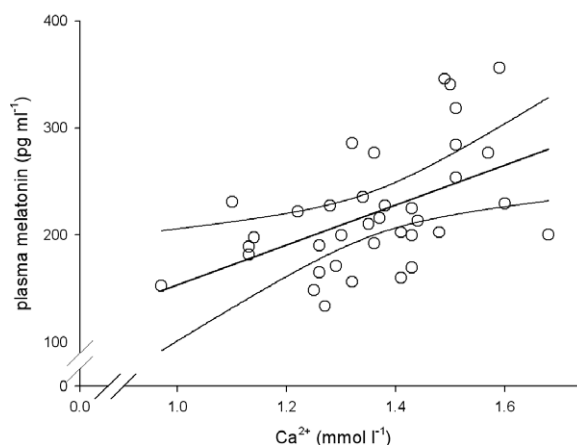


Fig. 3. Plasma melatonin correlates positively to plasma Ca^{2+} (pooled data from all fish analysed for melatonin); $R^2 = 0.19$; $N = 41$; $P < 0.01$. Confidence intervals (95%) are included in thinner lines.

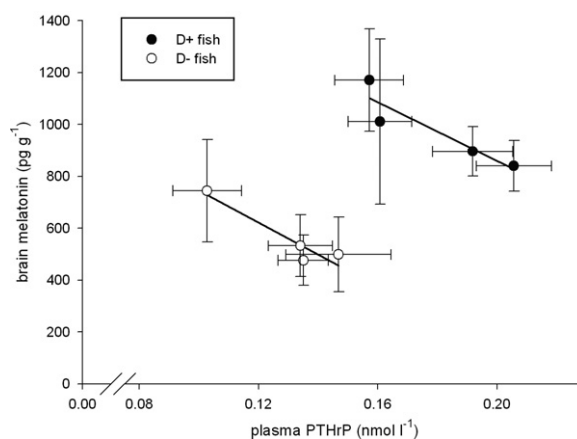


Fig. 4. Brain melatonin (production) correlates negatively to plasma PTHrP. Feeding fish a vitamin D-deficient diet does not affect this correlation, but levels of PTHrP and melatonin are decreased in concert; $R^2 = 0.78$; $N = 4$; $P < 0.05$ for the controls and $R^2 = 0.90$; $N = 4$; $P < 0.05$ for the test fish. N values represent group averages for each sampling point.

4. Discussion

In the evaluation of changes in melatonin activity *in vivo* a plethora of considerations comes to mind. Melatonin controls the rhythmic adaptations to daily and seasonal cycles in fish (Bolliet et al., 1997). A variety of physiological and environmental conditions interferes with melatonin synthesis: reproduction (Mayer et al., 1997), osmoregulatory demands (Kulczykowska 2002), photoperiod and water temperature (Garcia-Allegue et al., 2001) all affect melatonin production.

Sea bream is a protandrous fish and, being juvenile, the fish used in the present study were all sexual immature; the water temperature (23 °C) and the photoperiod (12 h light/12 h dark) were kept constant and the experiments were completed in the same season. We are therefore convinced that such factors were not confounders in our experiments

and that the results obtained relate mainly to calcium handling and physiology. One could argue that the effects seen in fish exposed to diluted seawater relate to altered osmoregulation or a variety of metabolic alterations which cause alterations in downstream endocrine events as a result of calcium depletion. Indeed, these faculties cannot be excluded as indicated by significant, albeit mild changes in plasma cortisol (Abbink et al., 2004) and osmolarity (this paper); yet, it should be kept in mind that diluted seawater also means a dilution of external calcium (from 10 to 0.7 mmol l⁻¹), from hypercalcemic to hypocalcemic conditions.

The positive relation between plasma levels of melatonin and Ca²⁺ provides further evidence that melatonin synthesis is influenced by plasma Ca²⁺ (plasma melatonin and brain melatonin reflect the synthesis capacity of the pineal gland; Kulczykowska 2002). Earlier studies (Kroeber et al., 2000; Gozdowska et al., 2003) indeed confirm the relation between plasma Ca²⁺ and the capacity of (night) melatonin production; Begay et al. (1994) observed increased melatonin synthesis in response to an increased plasma Ca²⁺ level in rainbow trout and Meissl et al. (1996) found inhibited melatonin production in a hypocalcemic/low calcium medium in cultured trout pinealocytes.

Fish on a vitamin D-deficient diet (D– fish) showed decreased plasma calcitriol levels and remained normocalcemic. Growth rate was reduced, which translated in lower net calcium accumulation rate, that was confirmed by decreased branchial calcium in- and efflux (Abbink et al., in press). Feeding the fish a D– diet and the subsequent decreased calcitriol level had no visible effect on plasma Ca²⁺, although a decreased calcium turnover was observed (Abbink et al., in press). The decrease in melatonin over time that was observed relates to the time of the year at which the experiments were conducted (spring–summer). Sokolowska et al. (2004) showed that melatonin levels are high in early spring (March) and decrease towards the summer (July–August).

The strongly decreased melatonin production in the D– fish points to direct or indirect involvement of calcitriol in melatonin synthesis by the pineal organ in teleosts. To the best of our knowledge, there are no reports of interactions between melatonin and calcitriol in fish and reports in mammals are scarce. An interplay between melatonin and calcitriol was shown by Bizzarri et al. (2003): vitamin D (calcitriol?) enhances the synthesis of the transforming growth factor TGF-β₁, which is the most relevant negative growth regulator in breast cancer cells. Melatonin was found to increase the sensitivity of the tumor cells to vitamin D (calcitriol), thereby increasing the release of TGF-β₁ and inhibiting tumor cell growth.

The decreased melatonin synthesis in the fish fed a D– diet is in accordance with the reduced melatonin production observed in the fish fed a Ca– diet, and this suggests diet-specific effects on melatonin synthesis under calcium constraint. Melatonin produced in the intestine is the most important source of extra-pineal melatonin. The melatonin level in the intestinal tract is not subject to

any (daily) rhythmic changes in fish (Bubenik and Pang, 1997), which indicates that the influence of plasma melatonin on intestinal melatonin physiology increases in darkness, when pineal melatonin production is up-regulated.

Rubio et al. (2004) showed that increased plasma melatonin in European sea bass (*Dicentrarchus labrax* L.), realised through orally administration in gelatin capsules, significantly reduced food intake, suggesting melatonin involvement in the process of feeding and digestion. In the present study, the indirectly (D– diet) or directly (Ca– diet) and dietary-induced calcium restraint and the subsequent calcemic endocrine action to maintain calcium balance could well have interfered with (intestinal) melatonin physiology, limiting the production of the hormone. This conclusion needs further experimentation for confirmation.

The increased melatonin production in the fish exposed to DSW is in accordance with previous studies. Kleszczyńska et al. (2006) measured plasma melatonin in sea bream adapted to different salinities and found the highest plasma melatonin in fish that were exposed to the lowest salinity. An important factor in adaptation to hypo-osmotic and hypocalcemic conditions in euryhaline fishes is prolactin (PRL; Flik et al., 1994), a hypercalcemic hormone in fish that is well-known for its key role in the control of low salinity adaptation. Falcon et al. (2003) showed that melatonin reduced PRL secretion in cultured rainbow trout pituitary gland cells and provided the first evidence that melatonin modulates the secretion of PRL in teleosts. Clearly, our results indicate a positive correlation between a (presumably) enhanced PRL activity in DSW and observed enhanced melatonin production. This *in vivo* result does not corroborate the observation by Falcon et al. (2003) and suggests multivariable control; the increase in PRL in response to DSW exposure might overrule the inhibition of a PRL cell response to melatonin as observed *in vitro*.

We here argue that PTHrP is involved in the regulation of melatonin synthesis. The negative correlation between melatonin production and plasma PTHrP presented in this study is indicative of a relationship between the two factors. In accordance, the reduction of melatonin production in response to a decrease in vitamin D (calcitriol) availability (this study) points to a relationship between melatonin synthesis and hypercalcemic endocrines (PTHrP and calcitriol). Whatever the effect, this highlights the importance of calcium in melatonin physiology, although further research is needed to investigate the role of melatonin in modulating hypercalcemic factors under calcium constraint.

This study provides new observations on the relation between melatonin production and calcium metabolism in sea bream exposed to indirect or direct calcium constraint. Limited calcium availability in the water increased melatonin production, whereas indirectly (D– diet) or directly (Ca– diet) and dietary-induced calcium restraint decreased melatonin production. These opposite effects were abolished under calcium constraint in both diet and

water. The hypercalcemic factors PTHrP and calcitriol appear to be correlated with melatonin, which we take as a clear indication of involvement of melatonin in modulating the endocrine response to cope with hypocalcemia and further points to the importance of Ca^{2+} in melatonin physiology.

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