Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The version of the following full text has not yet been defined or was untraceable and may differ from the publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/35553

Please be advised that this information was generated on 2017-12-06 and may be subject to change.

PTHrP Potentiating Estradiol-induced Vitellogenesis in Sea Bream (*Sparus auratus,* L.)

Gideon S. Bevelander[1], Xiaoming Hang[2,3], Wout Abbink[1], Tom Spanings[1], Adelino V. M. Canario[4] and Gert Flik[1,*]

[1]*Department of Animal Physiology, Institute for Neuroscience, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands,* [2]*Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom,* [3]*Institute of Environmental Systems Biology, Dalian Maritime University, Linghai Road, Dalian, 116026_P.R. China and* [4]*Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal*

*Author for correspondence (e-mail: g.flik@science.ru.nl)

Nitro PDF Trial
www.nitropdf.com

Short title:

Vitellogenesis in *Sparus auratus*

Key words: PTHrP, Vitellogenesis, Estradiol, Sparus auratus, Calcium, Hepatocytes

Abstract

In fish, vitellogenin is an important nutritional precursor protein produced solely in the liver and released into the blood where it binds calcium. In the gilthead sea bream (*Sparus auratus*) 17β-Estradiol (E_2) plays an important role in the synthesis of vitellogenin, but also the pituitary hormones prolactin (PRL) and growth hormone (GH) can stimulate vitellogenin induction in fish. Considering the emerging involvement of PTHrP in fish calcium metabolism and the importance of calcium regulation in reproduction, we investigated the possible role of PTHrP in vitellogenesis. E_2-naive and E_2-primed sea bream hepatocytes were used in an in-vitro primary hepatocyte culture and stimulated with a recombinant sea bream PTHrP (sbPTHrP) to establish the contribution of sbPTHrP alone or in combination with E_2 to the regulation of hepatic vitellogenin synthesis. Hepatocytes stimulated solely with sbPTHrP were not affected in their vitellogenesis. However, in hepatocytes stimulated with E_2 in combination with sbPTHrP a higher vitellogenin production was seen than with E_2 alone. It is concluded that sbPTHrP has a potentiating effect on estradiol stimulation of vitellogenin production by sea bream hepatocytes. The sea bream provides a unique model where vitellogenesis regulation can be studied on E_2-naïve liver cells, both *in vivo* and *in vitro*.

Nitro PDF Trial
www.nitropdf.com

Introduction

In fish, and other oviparous species, vitellogenin is an important nutritional precursor protein produced solely in the liver and released into the blood where it binds calcium. Circulating vitellogenin is transported to the ovary and taken up by developing oocytes to give rise to two major yolk proteins: lipovitellin and phosvitin. Lipovitellin supplies the necessary amino acids and lipids for the developing embryo, while phosvitin delivers the minerals important for skeletal development (Polzonetti-Magni et al., 2004). In the gilthead sea bream (*Sparus auratus*) 17β-Estradiol (E_2) plays an important role in the synthesis of vitellogenin (Guerreiro et al., 2002; Mosconi et al., 2002). Several studies have demonstrated that not only E_2 but also the pituitary hormones prolactin (PRL; Kwon and Mugiya, 1994) and growth hormone (GH; Mosconi et al., 2002; Peyon et al., 1996) stimulate vitellogenesis in fish. In Japanese eel, *Anguilla japonica*, a multihormonal control by GH and/or PRL as well as E_2 is essential for proper vitellogenin synthesis (Kwon and Mugiya, 1994). In female European silver eel (*Anguilla anguilla*) GH potentiates the effect of E_2 on cultured hepatocytes (Mosconi et al., 2002; Peyon et al., 1996). In sea bream the liver responds directly to stimulation with recombinant sea bream GH and a homologous pituitary homogenate as well as E_2 stimulation (Mosconi et al., 2002; Peyon et al., 1996).

When E_2 induces vitellogenesis also plasma total calcium levels rise as vitellogenin binds calcium and the vitellogenin concentration in plasma becomes very significant. Importantly, in primary rainbow trout hepatocyte cultures E_2 induced vitellogenesis, and this induction is dependent on extracellular calcium (Yeo and Mugiya, 1997). The hypercalcemic effects of E_2 result from calcium mobilization from bone (scales), enhanced intestinal absorption and kidney reabsorption (Persson, 1997). In sea bream

Nitro PDF Trial
www.nitropdf.com

however, E_2 does not induce bone resorption in scales and the high plasma calcium levels cannot be explained by renal and intestinal calcium reabsorption.

A 13-fold increase of plasma hypercalcemic parathyroid hormone related protein (PTHrP) levels was shown in sea bream treated with E_2-implants; the rise in PTHrP precedes the rise in plasma calcium levels, and this establishes that PTHrP probably mediates the hypercalcemic effects of E_2; PTHrP is thought to stimulate calcium uptake from the water via the gills in this species (Guerreiro, 2002).

PTHrP was first discovered in the late 80's as a hypercalcemic factor originating from certain cancers of the head, neck, breast, lung and kidney, causing the syndrome Humoral Hypercalcemia of Malignancy (HHM). PTHrP is an important factor in various tissues in normal physiology with roles in calcium transport, cell proliferation, relaxation of vascular tissue and has specific roles during early development (Philbrick et al., 1996; Wysolmerski and Stewart, 1998).

In fish, PTHrPs and several PTH/PTHrP receptors have been characterized and cloned (Danks et al., 1993; Devlin et al., 1996; Flanagan et al., 2000; Gensure et al., 2005; Gensure et al., 2004; Ingleton, 2002; Power et al., 2000; Rotllant et al., 2003; Rubin and Juppner, 1999). High to moderate levels of immunoreactive PTHrP have been detected in various tissues such as the pituitary gland, liver, head kidney, oesophagus, gill, intestine, skin and muscle. High PTHrP levels have been measured in pituitary extracts and plasma of sea bream and flounder, which indeed supports that PTHrP in teleosts can act as an endocrine factor (Rotllant, Worthington et al. 2003).

Nitro PDF Trial
www.nitropdf.com

Few physiological studies have been conducted on the effect of PTHrP in defined tissues. Piscine PTHrP (1-34) was shown to down-regulate mRNA expression of osteonectin in sea bream scales (Anjos et al., 2005) and in the same species it was found that PTHrP (1-34) stimulates cortisol production by interrenal cells (Rotllant et al., 2005). PTHrP stimulates whole body calcium influx and reduces calcium efflux in whole sea bream larvae (Guerreiro et al., 2001). Considering the emerging involvement of PTHrP in fish calcium metabolism and the pivotal importance of calcium regulation in ovarian maturation, we set out to study the possible involvement of PTHrP in hepatic vitellogenesis; recombinant, full length PTHrP (1-125) was used (Anjos et al., 2005).

Nitro PDF Trial
www.nitropdf.com

Material and Methods

Fish

Juvenile sea bream of approximately 1g mass were obtained from a stock bred at a commercial fish farm (Viveiro Vilanova, Lda., V. N. Milfontes, Portugal). They were transported by air to the facilities at the Radboud University Nijmegen, where they were held in 600-litre round tanks with an aerated flow-through system; water salinity was 34‰ and the water temperature was 22°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 commercial pellets (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total estimated body mass per day. Fish used for experiments were juvenile sea bream of approximately 90 g mass.

Hepatocyte Isolation

Hepatocytes were isolated according to Mommsen et al. (1994), with minor modifications. After anaesthesia of the fish with 2-phenoxyethanol (0.1%v/v), the liver was perfused via the heart with Ca^{2+}-free hepatocyte buffer (HB; 175 mM NaCl, 5.4 KCl, 0.81mM $MgSO_4$, 0.33mM Na_2HPO_4, 0.44mM KH_2PO_4, 5mM $NaHCO_3$, pH 7.63) containing 1mM EGTA for 10-15 minutes at room temperature.

After clearing the blood from the liver, the liver was excised from the abdominal cavity and transferred onto a sterile petri dish, cut into smaller fragments with a razor blade and the tissue was treated with HB containing collagenase at a concentration of 0.3 mg/ml for about 30-45 minutes (depending on liver mass) to dissociate the cells. The softened liver fragments were further minced with a razor blade and the homogenate was filtered twice, through a 200 and a 50 μm sterile nylon mesh screen. The cell suspension was transferred into a sterile tube (Greiner, 50ml) and subsequently centrifuged 3 times at 80g for 4 minutes at 10°C. Cell pellet washes of HB containing 1.5 mM $CaCl_2$ (HB-Ca^{2+}) and a 50/50 mixture of L-15 medium (L-15, Gibco) (containing antibiotics/antimycotics (Gibco), 5 mM $NaHCO_3$ and 10mM NaCL) and HB-Ca^{2+} were conducted in between centrifugations.

After the last centrifugation, the cell pellet was resuspended in a small volume of L-15 medium; cell yield was assessed by determining the number of cells using a counting chamber and cell viability monitored by trypan blue exclusion. Only cell preparations with more than 90% viability were used for experiments. Isolated hepatocytes were plated in 24-well culture plates (Greiner) at a density of $1x10^6$/ml in a volume of 1 ml. Cells were incubated at 22°C (the temperature at which the fish are kept) at saturated humidity.

Nitro PDF Trial
www.nitropdf.com

Effect of E_2 and PTHrP on vitellogenin synthesis

Blood parameters after E_2 priming

Sea bream is a protandrous fish and therefore in initial experiments the (male) fish were estrogenized to induce liver vitellogenesis. Fish received a peritoneal implant of coconut butter containing 0, 5 or 10mg/kg body weight (BW) E_2 and were left for 8 days. After estrogenization fish were euthanized with 2-phenoxyethanol (0.1%v/v; Sigma-Alderich, St Louis, MO, USA) and fish blood was taken from the caudal vessels with a 1ml tuberculin syringe, rinsed with Na^+-heparin (Leo Pharma, Weesp, The Netherlands; 5000 U ml^{-1}). Collected blood was centrifuged at 13 600 g for 10 min. Plasma was stored at –20°C for subsequent analysis of total calcium, estradiol and vitellogenin (VTG) levels (see below).

In-vivo and in-vitro priming

To establish the effect of in-vivo priming, fish received a single injection of 10mg/kg BW E_2 and hepatocytes were isolated 8 days later. Next, the in-vitro hepatocyte culture was divided into two groups for the first 48hrs. One group of cells was treated with E_2 (10^{-6}M) and one group was treated with vehicle.

After the first 48hrs of in-vitro priming the E_2 treated group was further divided into 2 groups: one was further treated with just E_2 and one group exposed to a combination of E_2 and 10^{-8}M sbPTHrP. The vehicle, E_2 and the E_2 plus sbPTHrP groups were cultured for 2 x 24 hrs, media was collected from each well and used to assess vitellogenin release with an enzyme-linked immunosorbent assay (ELISA; see below). As an extra control, E_2-naïve hepatocytes were cultured for 4 days, treated with vehicle and the medium analysed.

Nitro PDF Trial
www.nitropdf.com

As the higher dose of 10mg/kg made the liver cells highly active and refractory to further manipulation, fish were then primed with a lower dose, 5mg/kg BW E_2. Hepatocytes were isolated 8 days later and liver material from up to 4 fish was pooled. The hepatocyte culture was continued for 4 days and the cells were treated with E_2 (10^{-6}M) and/or sbPTHrP (10^{-8}M). The medium was collected and changed every day from each culture plate well and analysed with the vitellogenin ELISA (see below).

In-vitro priming

The last series of experiments was run on tissues of control, i.e. E_2-naïve (no E_2 exogenously administered) fish. Hepatocytes were isolated and cultured for 6 days treated with E_2 (10^{-6}M) and/or sbPTHrP (10^{-8}M). The hepatocyte culture medium was sampled and changed every other day (after one day of culture VTG levels were below detection) to measure production of vitellogenin using the ELISA (see below).

Nitro PDF Trial
www.nitropdf.com

Chemicals

17β-Estradiol (E_2) was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). E_2 was dissolved in ethanol and the final concentration of ethanol in the culture medium never exceeded 0.02%. The recombinant parathyroid hormone related protein (sbPTHrP 1-125) used in this study was obtained from Manchester University where the sbPTHrP was produced and purified (Anjos et al., 2005).

Plasma analyses

Plasma E_2-levels were analysed by an ELISA method (IBL, Hamburg). The lower sensitivity limit of the E_2-ELISA was 10 pg/ml. Plasma total calcium was measured with a calcium kit (Roche, Mannheim, Germany). Plasma vitellogenin was measured using a vitellogenin ELISA (see below).

Vitellogenin assay

Vitellogenin concentration in the medium was measured by a standard ELISA protocol in 96-well plates using purified sea bream vitellogenin as standard, a polyclonal rabbit anti-sea bream vitellogenin antiserum (raised by Dr Per-Erik Olsson, Orebro University, Sweden) as primary anti-antibody and a secondary HRP-conjugated goat-anti rabbit antibody (Nordic Labs, Tilburg, The Netherlands). Briefly, the standard curve and sample dilutions were prepared in coating buffer (0.1 M Na_2CO_3, pH 9.6); 100µl of each was added to each well and incubated overnight. The wells were washed 3 times with PBS-Tween (phosphate buffered saline, 0.05% Tween-20, pH 7.4) and incubated for 1 h at RT with PBS-Tween containing 0.5% dry-milk to block non-specific binding. The wells were washed 3 times in PBS-Tween followed by incubation with primary antibody (diluted 1:3000) for 1h at RT. After washing with PBS-Tween the secondary antibody incubation (diluted 1:3000) was performed for 1 h at RT. The wells were washed for 3 times with PBS-Tween and 200µl of OPD-H_2O_2 substrate buffer (McIlvaine Buffer containing 0.05% o-phenylenediamine and 0.375% H_2O_2) was added to each well. The reaction was stopped with 4 M H_2SO_4 after 10 minutes and the plates were read using a microplate reader (Bio-Rad microplate reader model 680). The lower detection limit of the VTG-ELISA was 1 ng/ml. The intra-assay coefficient of variation was 4.6% (n=6) and inter-assay coefficient variation was 11.9% (n=6).

Nitro PDF Trial
www.nitropdf.com

Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM) or as mean values ± standard deviation (SD), depending on the number of experiments run. Analysis of variance (ANOVA) was used to assess differences between groups, followed by the Bonferroni test (SPSS Windows version 13.0, Chicago, IL). Significance was accepted when $P<0.05$.

Nitro PDF Trial
www.nitropdf.com

Results

Blood parameters after E_2 priming

In plasma of naïve juvenile sea bream E_2-levels were extremely low (0.02 µM ± .005; Table 1). E_2 implants significantly increased estradiol levels ($P<0.05$; Table 1) and doubling the E_2 dose led to a doubling of plasma E_2-levels. The significant degree of hypercalcemia ($P<0.05$; Table 1) essentially plateaued at the 5mg/kg dose. Both concentrations of E_2 have a significant ($P<0.05$; Table 1) effect on plasma vitellogenin release compared to control, however VTG levels did not differ between the two treatments.

In-vivo and in-vitro priming

In-vivo primed with 10mg/kg BW E_2

On day 3 of culture, addition of E_2 alone or combined with sbPTHrP did not affect the VTG-production compared to vehicle treated liver cells, which resulted from priming the fish (Fig.1). Vehicle-treated liver cells produced significant amounts of VTG compared to vehicle-treated liver cells of E_2-naïve sea bream ($P<0.05$). VTG production had declined on day 4 of culture for the vehicle treated group to 100ng/ml, being significantly elevated compared to VTG levels of E_2-naïve liver cells. The combined addition of E_2 and sbPTHrP further stimulated VTG production compared to day 3 ($P<0.05$). Day 4 shows significant differences between VTG levels in control, E_2 and the combination of E_2 and sbPTHrP treated hepatocytes. With VTG production in E_2 treated cells being significantly elevated compared to control ($P<0.05$) and the combination of E_2 and sbPTHrP is significantly elevated compared to control and E_2 treated hepatocytes ($P<0.05$; Fig.1).

Nitro PDF Trial
www.nitropdf.com

In-vivo primed with 5mg/kg BW E_2

We then set out for an experiment where fish were primed with 5mg/kg E_2.

VTG production declined during the first three days of culture independently of in-vivo or in-vitro treatment (Fig.2). While control and sbPTHrP group dropped below the 50 ng/ml, the E_2 group as well as the group co-treated with E_2 and sbPTHrP stayed significantly elevated compared to control and sbPTHrP treated cells ($P<0.05$; Fig.2.). On day 3 a significant difference of VTG levels was detected between E_2 treated liver cells and the liver cells treated with E_2 and sbPTHrP ($P<0.05$). On day 4 all experimental groups show a significant rise in VTG levels compared to control ($P<0.001$) and significantly differ from each other ($P<0.001$; Fig. 2). Noteworthy is the sudden significant rise of VTG levels from the sbPTHrP treated hepatocytes compared to control on day 4 of culture.

In-vitro priming

The effect of E_2 and/or sbPTHrP on the isolated hepatocytes of unprimed fish is shown in Fig.3. For all experimental groups almost undetectable levels of vitellogenin were measured in the first 2-4 days (< 5 ng/ml; results not shown). In control as well as sbPTHrP (10^{-8}M) treated liver cells the vitellogenin concentration remained very low (< 15 ng/ml) up to 6 days. Vitellogenin levels rose significantly ($P<0.05$) when treated with E_2 (10^{-6}M) after 6 days *in vitro*. When hepatocytes were co-treated with E_2 and sbPTHrP a significantly ($P<0.05$) higher amount of vitellogenin was produced on day 6 than with E_2 only (Fig.3)

Nitro PDF Trial
www.nitropdf.com

Discussion

This study is the first to show that PTHrP is directly involved in fish vitellogenesis. Two important observations have to be taken into regard from this study. First, the recombinant sea bream PTHrP proved to have potentiating effects on E_2 induced VTG production and second the protandrous gilthead sea bream provides us with E_2-naïve liver cells. This makes this fish a good model for in vitro hepatocyte bioassays for the study of vitellogenesis as hepatocytes can be primed *in vivo* or *in vitro* without prior 'substantial experience' to E_2.

To perform this research a primary culture was set up for sea bream hepatocytes. Research has been conducted on sea bream liver before, but only *in vivo* or *in vitro* using liver fragments of a certain size (Carnevali et al., 2005; Mosconi et al., 2002), not on isolated cells. Isolated hepatocytes in our hands remained viable for up to at least 8 days (trypan blue exclusion) and remained E_2 sensitive, which is in accordance with other studies on this and other species (Flouriot et al., 1993; Islinger et al., 1999; Kim and Takemura, 2003; Kim et al., 2003; Kwon and Mugiya, 1994; Peyon et al., 1996; Riley et al., 2004; Tollefsen et al., 2003; Yeo and Mugiya, 1997).

E_2 is known to upregulate its own receptor, preceding the upregulation of vitellogenin mRNA (Flouriot et al., 1997) and this can be an explanation for the observed result in control (unprimed) juvenile sea bream where, after 2-4 days, no response is measured in either of the groups, only after 6 days the response can be seen clearly (Fig.3). The vitellogenic response observed after 6 days corresponds with similar kinetics in the study of Peyon et al. (Peyon et al., 1996).

In the cells of E_2-naïve fish no direct effect of sbPTHrP on vitellogenesis was observed, but importantly sbPTHrP alone did stimulate VTG production in hepatocytes of fish primed with 5mg/kg BW E_2; only on day 4 of subsequent culture the direct effect of sbPTHrP was observed (Fig.2). We take this as a 'memory effect'

Nitro PDF Trial
www.nitropdf.com

induced by the E_2 priming (Peyon et al., 1993). Indeed, plasma E_2 levels were strongly elevated after both priming treatments and suggest that such high E_2 levels *in vivo* could have lasting and dose-dependent effects on the hepatocytes in culture, as can be seen with the high dosage priming (10 mg/kg E_2; Fig. 1) where no difference was shown between vehicle and experimental treated cells. Accordingly, in controls VTG production finally decreased after 3 days of culture, whereas with a lower E_2 priming dose the decline of VTG sets in earlier (Fig.2). The potentiating effect of sbPTHrP in the 'lower dose primed' fish became significant as of day 4 of culture.

For a long time E_2 was considered to be the main trigger in the process of hepatic vitellogenesis, it is now known that this process is under a multihormonal regulation in which factors such as pituitary hormones (GH, PRL) and steroid hormones, e.g. progesterone and cortisol play a role (Polzonetti-Magni et al., 2004). In a study of Mosconi and co-workers hepatocytes were treated, (besides E_2 and GH), with a pituitary homogenate inducing VTG production (Mosconi et al., 2002), such a homogenate may well contain significant amounts of PTHrP (Ingleton, 2002; Rotllant et al., 2003). This study demonstrates PTHrP as one of the factors involved in the process of hepatic vitellogenesis, with known expression of PTHrP and the PTHrP-receptor in sea bream liver (Hang et al., 2005) and the pituitary as the main source suggested (Rotllant et al., 2003). Although the mechanism for the potentiating effect of PTHrP on the E_2 induced hepatocytes has not been studied in more detail here, it is known that extracellular calcium is an important factor during vitellogenesis (Yeo and Mugiya, 1997). E_2 significantly stimulates the secretion of PTHrP into the bloodstream before the rise in plasma calcium levels and this suggests that PTHrP is a good candidate for the modulation of the hypercalcemic action of E_2 as it elevates plasma PTHrP that has hypercalcemic actions (Guerreiro, 2002).

Nitro PDF Trial
www.nitropdf.com

This hypothesis of a hypercalcemic action of PTHrP and possibly even acting as the main calcium up-regulator is supported by studies performed on sea bream larvae where PTHrP was able to stimulate whole body calcium influx and reduce calcium efflux (Guerreiro et al., 2001), and elevated levels of PTHrP were measured when calcium is limited in water or in diet (Abbink et al., 2004).

However, other possible mediators of the calciotropic action of E_2 may be factors such as GH and PRL (Flik et al., 1994; Takagi et al., 1992).

For long, fish were thought to lack parathyroid hormone (PTH), but certainly fish including sea bream express PTHrP. As PTH and PTHrP signal through the same receptor (Gensure et al., 2005) it was thought that the PTHrP gene was the ancestral gene to PTH (Blair et al., 2002). Recently PTH genes and their products were demonstrated in zebrafish and the puffer fish (Canario et al., 2006; Gensure et al., 2004) and has opened new fields of research. PTH induces calcium influx in rat hepatocytes (Klin et al., 1994) and the puffer fish PTH-like ligand induces calcium whole body influx in sea bream larvae (Canario et al., 2006). In both cases the pathway, via which the PTH action is realised, involves the G protein-adenylate cyclase-cAMP system. Indeed, also PTHrP can act through this pathway (Canario et al., 2006; Gensure et al., 2005) via shared receptors. Via the second messenger pathway another possible mechanism may be responsible for the potentiating action of PTHrP. Protein kinases can enhance the E_2 dependent estradiol-receptor (ER) transcription activity. E_2, cAMP and certain growth factors, for instance insulin-like growth factor-I, have been shown to mediate regulatory effects of certain genes via the ER (Cho and Katzenellenbogen, 1993).

IGF-I has been shown to stimulate vitellogenin synthesis in the frog and it was recently suggested that the GH effect on synthesis of vitellogenin is at least partly mediated through its action on IGF-I synthesis (Carnevali et al., 2005). It has been shown that

Nitro PDF Trial
www.nitropdf.com

PTHrP and PTH can induce elevations of IGF-I in humans (Horwitz et al., 2005) and in cultures of 21-day fetal rat calvaria (Canalis et al., 1990) . Moreover it is speculated that during fracture healing of bone PTHrP influences IGF-1 expression and both enhance the ossification in an autocrine/paracrine fashion (Okazaki et al., 2003). To elucidate the pathways in which PTHrP works in the process of hepatic vitellogenesis requires further study.

In conclusion, we have established a new role for PTHrP showing a potentiating effect on hepatocyte vitellogenesis when induced with E_2. This study with other studies on the effect of PTHrP on corticosteroidogenic activity (Rotllant et al., 2005), downregulation of osteonectin mRNA in scales (Anjos et al., 2005) and the ability to stimulate whole body calcium influx and reduce calcium efflux in whole sea bream larvae show that PTHrP in fish (Guerreiro et al., 2001), as in normal human physiology, is probably a pleiotropic hormone working on different levels in different tissues.

Nitro PDF Trial
www.nitropdf.com

Acknowledgements

This research has been carried out with financial support from the Commission of the European Union, Quality of Life Management of Living resources specific RTD programme (Q5RS-2001-020904).

Abbink, W., Bevelander, G.S., Rotllant, J., Canario, A.V., Flik, G., 2004. Calcium handling in Sparus auratus: effects of water and dietary calcium levels on mineral composition, cortisol and PTHrP levels. J Exp Biol 207, 4077-4084.

Anjos, L., Rotllant, J., Guerreiro, P.M., Hang, X., Canario, A.V., Balment, R., Power, D.M., 2005. Production and characterisation of gilthead sea bream (Sparus auratus) recombinant parathyroid hormone related protein. Gen Comp Endocrinol 143, 57-65.

Blair, H.C., Zaidi, M., Schlesinger, P.H., 2002. Mechanisms balancing skeletal matrix synthesis and degradation. Biochem J 364, 329-341.

Canalis, E., McCarthy, T.L., Centrella, M., 1990. Differential effects of continuous and transient treatment with parathyroid hormone related peptide (PTHrp) on bone collagen synthesis. Endocrinology 126, 1806-1812.

Canario, A.V., Rotllant, J., Fuentes, J., Guerreiro, P.M., Rita Teodosio, H., Power, D.M., Clark, M.S., 2006. Novel bioactive parathyroid hormone and related peptides in teleost fish. FEBS Lett 580, 291-299.

Carnevali, O., Cardinali, M., Maradonna, F., Parisi, M., Olivotto, I., Polzonetti-Magni, A.M., Mosconi, G., Funkenstein, B., 2005. Hormonal regulation of hepatic IGF-I and IGF-II gene expression in the marine teleost Sparus aurata. Mol Reprod Dev 71, 12-18.

Cho, H., Katzenellenbogen, B.S., 1993. Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. Mol Endocrinol 7, 441-452.

Danks, J.A., Devlin, A.J., Ho, P.M., Diefenbach-Jagger, H., Power, D.M., Canario, A., Martin, T.J., Ingleton, P.M., 1993. Parathyroid hormone-related protein is a factor in normal fish pituitary. Gen Comp Endocrinol 92, 201-212.

Devlin, A.J., Danks, J.A., Faulkner, M.K., Power, D.M., Canario, A.V.M., Martin, T.J., Ingleton, P.M., 1996. Immunochemical Detection of Parathyroid Hormone-Related Protein in the Saccus Vasculosus of a Teleost Fish. General and Comparative Endocrinology 101, 83- 90.

Flanagan, J.A., Power, D.M., L.A. Bendell, L.A., 2000. Cloning of the cDNA for Sea Bream (Sparus aurata) Parathyroid Hormone-Related Protein. General and Comparative Endocrinology 118, 373- 382.

Flik, G., Rentier-Delrue, F., Wendelaar Bonga, S.E., 1994. Calcitropic effects of recombinant prolactins in Oreochromis mossambicus. Am J Physiol 266, R1302-1308.

Flouriot, G., Pakdel, F., Ducouret, B., Ledrean, Y., Valotaire, Y., 1997. Differential regulation of two genes implicated in fish reproduction: vitellogenin and estrogen receptor genes. Mol Reprod Dev 48, 317-323.

Flouriot, G., Vaillant, C., Salbert, G., Pelissero, C., Guiraud, J.M., Valotaire, Y., 1993. Monolayer and aggregate cultures of rainbow trout hepatocytes: long-term and stable liver-specific expression in aggregates. J Cell Sci 105 (Pt 2), 407-416.

Nitro PDF Trial
www.nitropdf.com

Gensure, R.C., Gardella, T.J., Juppner, H., 2005. Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. Biochem Biophys Res Commun 328, 666-678.

Gensure, R.C., Ponugoti, B., Gunes, Y., Papasani, M.R., Lanske, B., Bastepe, M., Rubin, D.A., Juppner, H., 2004. Identification and characterization of two parathyroid hormone-like molecules in zebrafish. Endocrinology 145, 1634-1639.

Guerreiro, P.M., 2002. Calcium Balance in the Larval and Juvenile Gilthead Sea Bream, Sparus aurata. Phd Thesis.

Guerreiro, P.M., Fuentes, J., Canario, A.V., Power, D.M., 2002. Calcium balance in sea bream (Sparus aurata): the effect of oestradiol-17beta. J Endocrinol 173, 377-385.

Guerreiro, P.M., Fuentes, J., Power, D.M., Ingleton, P.M., Flik, G., Canario, A.V.M., 2001. Parathyroid hormone-related protein: a calcium regulatory factor in sea bream (*Sparus aurata* L.) larvae. Am. J. Regulatory Integrative Comp. Physiol. 281, R855-R860.

Hang, X.M., Power, D., Flik, G., Balment, R.J., 2005. Measurement of PTHrP, PTHR1, and CaSR Expression Levels in Tissues of Sea Bream (Sparus aurata) Using Quantitative PCR. Ann N Y Acad Sci 1040, 340-344.

Horwitz, M.J., Tedesco, M.B., Sereika, S.M., Syed, M.A., Garcia-Ocana, A., Bisello, A., Hollis, B.W., Rosen, C.J., Wysolmerski, J.J., Dann, P., Gundberg, C., Stewart, A.F., 2005. Continuous PTH and PTHrP Infusion Causes Suppression of Bone Formation and Discordant Effects on 1,25(OH)(2)Vitamin D. J Bone Miner Res 20, 1792-1803.

Ingleton, P.M., 2002. Parathyroid hormone-related protein in lower vertebrates. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 132, 87-95.

Islinger, M., Pawlowski, S., Hollert, H., Volkl, A., Braunbeck, T., 1999. Measurement of vitellogenin mRNA expression in primary cultures of rainbow trout hepatocytes in a non-radioactive dot blot/RNAse protection-assay. Science of the Total Environment 233, 109-122.

Kim, B.H., Takemura, A., 2003. Culture conditions affect induction of vitellogenin synthesis by estradiol-17 beta in primary cultures of tilapia hepatocytes. Comp Biochem Physiol B Biochem Mol Biol 135, 231-239.

Kim, B.H., Takemura, A., Kim, S.J., Lee, Y.D., 2003. Vitellogenin synthesis via androgens in primary cultures of tilapia hepatocytes. Gen Comp Endocrinol 132, 248-255.

Klin, M., Smogorzewski, M., Khilnani, H., Michnowska, M., Massry, S.G., 1994. Mechanisms of PTH-induced rise in cytosolic calcium in adult rat hepatocytes. Am J Physiol 267, G754-763.

Kwon, H.C., Mugiya, Y., 1994. Involvement of growth hormone and prolactin in the induction of vitellogenin synthesis in primary hepatocyte culture in the eel, Anguilla japonica. Gen Comp Endocrinol 93, 51-60.

Nitro PDF Trial
www.nitropdf.com

Mommsen, T.P., Moon, T.W., P.J. Walsh, P.J., 1994. Hepatocytes: isolation, maintenance and utilization. Biochemistry and molecular biology of fishes 3, 355-373.

Mosconi, G., Carnevali, O., Habibi, H.R., Sanyal, R., Polzonetti-Magni, A.M., 2002. Hormonal mechanisms regulating hepatic vitellogenin synthesis in the gilthead sea bream, Sparus aurata. American Journal of Physiology-Cell Physiology 283, C673-C678.

Okazaki, K., Jingushi, S., Ikenoue, T., Urabe, K., Sakai, H., Iwamoto, Y., 2003. Expression of parathyroid hormone-related peptide and insulin-like growth factor I during rat fracture healing. J Orthop Res 21, 511-520.

Persson, P., 1997. Estradiol-17beta and nutritional status affect calcium balance, scale and bone resorption, and bone formation in rainbow trout Oncorhynchus mukiss. J Comp Physiol B 167, 468-473.

Peyon, P., Baloche, S., Burzawa-Gerard, E., 1993. Synthesis of vitellogenin by eel (Anguilla anguilla L.) hepatocytes in primary culture: requirement of 17 beta-estradiol-priming. Gen Comp Endocrinol 91, 318-329.

Peyon, P., Baloche, S., Burzawa-Gerard, E., 1996. Potentiating effect of growth hormone on vitellogenin synthesis induced by 17 beta-estradiol in primary culture of female silver eel (Anguilla anguilla L.) hepatocytes. Gen Comp Endocrinol 102, 263-273.

Philbrick, W.M., Wysolmerski, J.J., Galbraith, S., Holt, E., Orloff, J.J., Yang, K.H., Vasavada, R.C., Weir, E.C., Broadus, A.E., Stewart, A.F., 1996. Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol Rev 76, 127-173.

Polzonetti-Magni, A.M., Mosconi, G., Soverchia, L., Kikuyama, S., Carnevali, O., 2004. Multihormonal control of vitellogenesis in lower vertebrates. Int Rev Cytol 239, 1-46.

Power, D.M., Ingleton, P.M., Flanagan, J.A., Canario, A.V.M., Danks, J., Elgar, G., Clark, M.S., 2000. Genomic structure and expression of parathyroid hormone-related protein gene (PTHrP) in a teleost, *Fugu rubripes*. Gene 250, 67- 76.

Riley, L.G., Hirano, T., Grau, E.G., 2004. Estradiol-17beta and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-I production in primary hepatocytes of the tilapia Oreochromis mossambicus. Comp Biochem Physiol C Toxicol Pharmacol 138, 177-186.

Rotllant, J., Guerreiro, P.M., Anjos, L., Redruello, B., Canario, A.V., Power, D.M., 2005. Stimulation of cortisol release by the N terminus of teleost parathyroid hormone-related protein in interrenal cells in vitro. Endocrinology 146, 71-76.

Rotllant, J., Worthington, G.P., Fuentes, J., Guerreiro, P.M., Teitsma, C.A., Ingleton, P.M., Balment, R.J., Canario, A.V., Power, D.M., 2003. Determination of tissue and plasma concentrations of PTHrP in fish: development and validation of a radioimmunoassay using a teleost 1-34 N-terminal peptide. Gen Comp Endocrinol 133, 146-153.

Nitro PDF Trial
www.nitropdf.com

Rubin, D.A., Juppner, H., 1999. Zebrafish express the common parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) and a novel receptor (PTH3R) that is preferentially activated by mammalian and fugufish parathyroid hormone-related peptide. J Biol Chem 274, 28185-28190.

Takagi, Y., Moriyama, S., Hirano, T., Yamada, J., 1992. Effects of growth hormones on bone formation and resorption in rainbow trout (Oncorhynchus mykiss), as examined by histomorphometry of the pharyngeal bone. Gen Comp Endocrinol 86, 90-95.

Tollefsen, K.E., Mathisen, R., Stenersen, J., 2003. Induction of vitellogenin synthesis in an Atlantic salmon (Salmo salar) hepatocyte culture: a sensitive in vitro bioassay for the oestrogenic and anti-oestrogenic activity of chemicals. Biomarkers 8, 394-407.

Wysolmerski, J.J., Stewart, A.F., 1998. The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. Annu Rev Physiol 60, 431-460.

Yeo, I.K., Mugiya, Y., 1997. Effects of extracellular calcium concentrations and calcium antagonists on vitellogenin induction by estradiol-17 beta in primary hepatocyte culture in the rainbow trout Oncorhynchus mykiss. Gen Comp Endocrinol 105, 294-301.

Nitro PDF Trial
www.nitropdf.com

Legends

Fig. 1. Vitellogenin release, after in-vivo priming (10mg/kg BW E_2). Hepatocytes treated with either vehicle, E_2 or a combination of E_2 and sbPTHrP. Values are expressed as means ± SEM (N= 4). On Day 3 no significant differences occurred between the groups. On Day 4 E_2 and the combination group differ significantly from control and each other. On both days VTG levels were significantly higher then VTG levels of cultured hepatocytes treated with vehicle from E_2-naïve fish. Letters denote significance at $P<0.05$.

Fig. 2. In-vitro vitellogenin synthesis, after lower dose priming of E_2 (5mg/kg BW), during 4 days of E_2 and/or sbPTHrP treatments. After 4 days a potentiating effect can be seen of the group treated with E_2 (10^{-6} M) and sbPTHrP (10^{-8} M). Values are expressed as means ± SD (n= 6). Letters denote significance per day of culture. On day 2 and 3 letters show significance at $P<0.05$ and on day 4 letters denote significant difference at $P<0.001$.

Fig. 3. Effect of E_2 and/or sbPTHrP on the production of vitellogenin in control (unprimed) hepatocytes in primary culture. Comparison of the vitellogenin synthesis in control vs. sbPTHrP, E_2 and E_2 + sbPTHrP on the last day of culture. Values are expressed as means ± SEM (N=6). Bars with different letters denote significant difference ($P< 0.05$).

Nitro PDF Trial
www.nitropdf.com

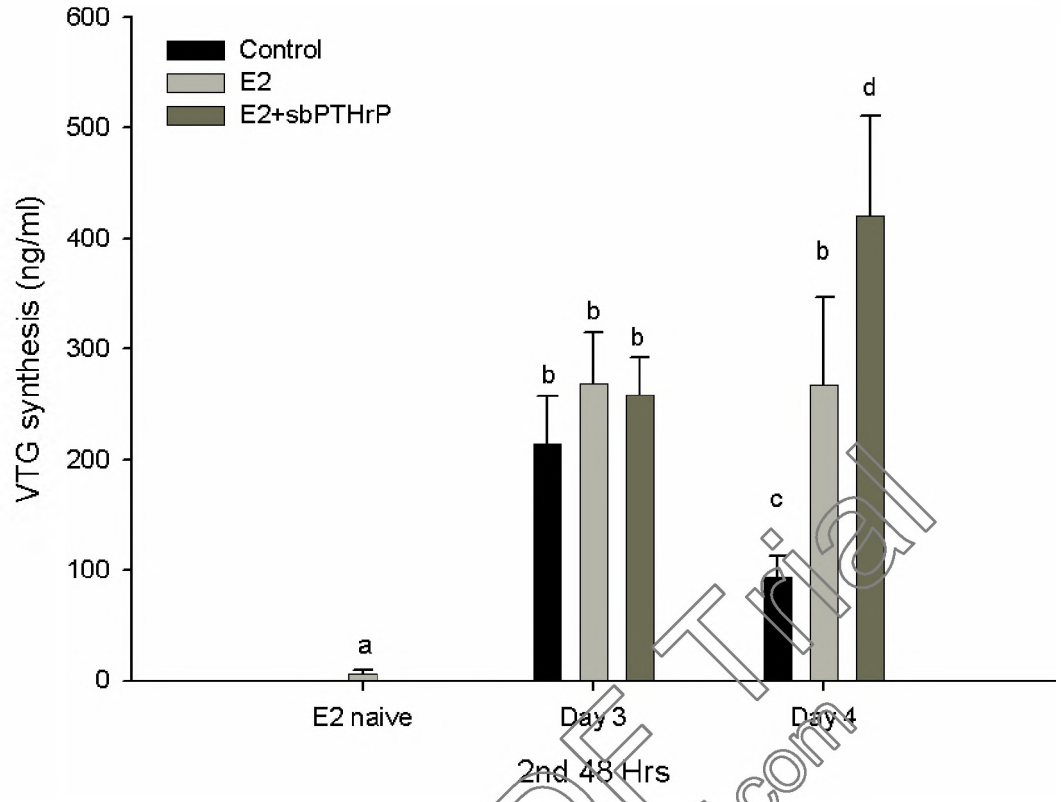
Tables

Table 1.

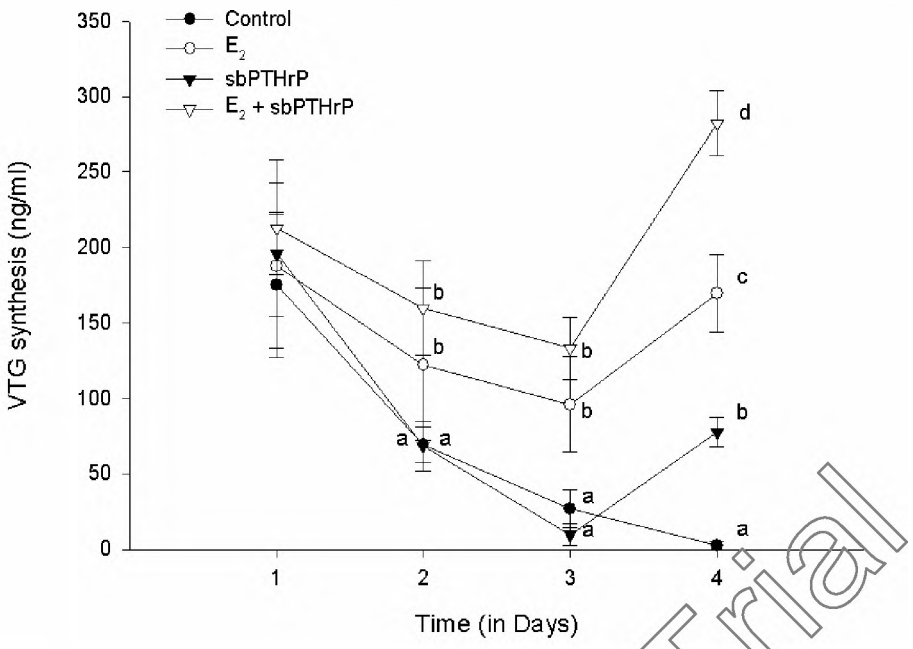
Plasma levels	*Control* $_{0mg}$	*E_2-injected* $_{5mg}$	*E_2-injected* $_{10mg}$
Estradiol (μM)	$0.02 \pm .005^a$	2.04 ± 1.33^b	4.53 ± 0.27^c
Total Calcium(mM)	1.85 ± 0.28^a	17.10 ± 2.4^b	20.16 ± 3.0^c
VTG (ng/ml)	5.29 ± 2.18^a	770.91 ± 38.67^b	804.02 ± 47.78^b

Table 1. Plasma levels of Estradiol, Total Calcium and Vitellogenin in sea bream injected with 0 (Control), 5mg or 10mg/kg BW 17β-Estradiol (E_2-injected) coconut butter implants. Values are the mean ± SD, N=10 for Control or N=8 for E_2-injected. Letters in the same row denote significant difference ($P<0.05$).

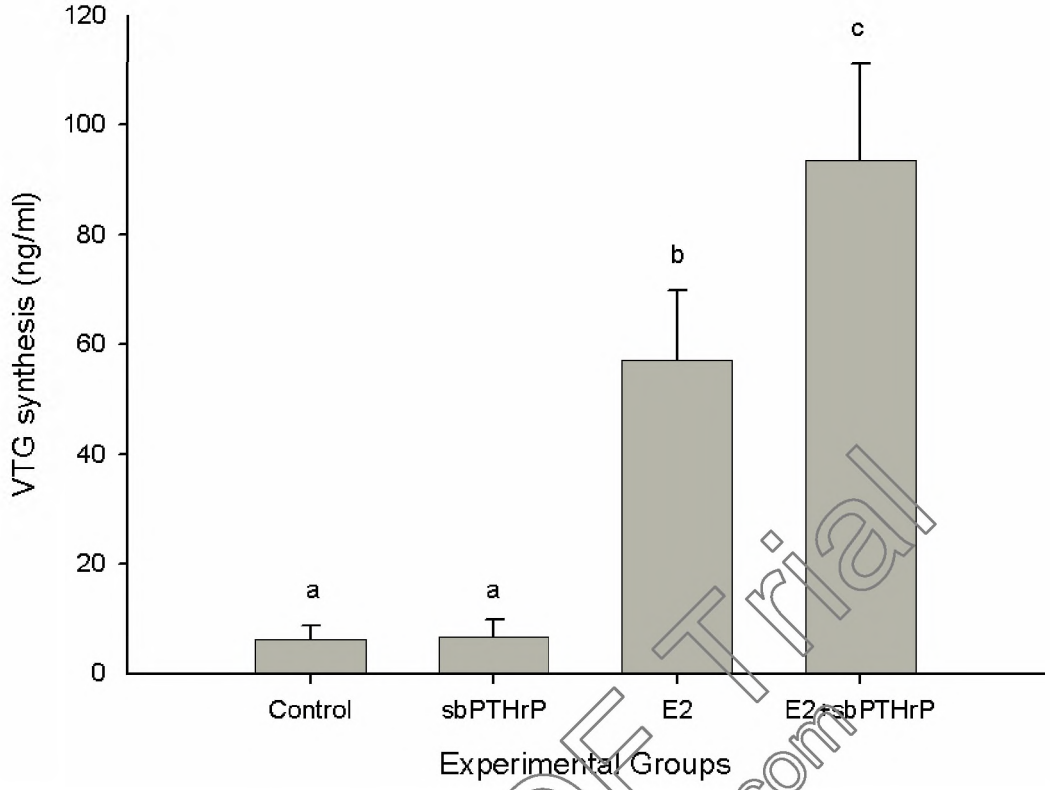
Nitro PDF Trial
www.nitropdf.com



Nitro PDF Trial
www.nitropdf.com



Nitro PDF Trial
www.nitropdf.com



Nitro PDF Trial
www.nitropdf.com