Tissue responsiveness to estradiol and genistein in the sea bass liver and scale

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Abstract

As in mammals, estrogens in fish are essential for reproduction but also important regulators of mineral homeostasis. Fish scales are a non-conventional target tissue responsive to estradiol and constitute a good model to study mineralized tissues effects and mechanisms of action of estrogenic compounds, including phytoestrogens. The responsiveness to estradiol and the phytoestrogen genistein, was compared between the scales and the liver, a classical estrogenic target, in sea bass (*Dicentrarchus labrax*). Injection with estradiol and genistein significantly increased circulating vitellogenin (for both compounds) and mineral levels (estradiol only) and genistein also significantly increased scale enzymatic activities suggesting it increased mineral turnover. The repertoire, abundance and estrogenic regulation of nuclear estrogen receptors (ESR1, 2a and 2b) and membrane G-protein receptors (GPER and GPER-like) were different between liver and scales, which presumably explains the tissue-specific changes detected in estrogen-responsive gene expression. In scales changes in gene expression mainly consisted of small rapid increases, while in liver strong, sustained increases/decreases in gene expression occurred. Similar but not overlapping gene expression changes were observed in response to both estradiol and genistein. This study demonstrates for the first time the expression of membrane estrogen receptors in scales and that estrogens and phytoestrogens, to which fish may be exposed in the wild or in aquaculture, both affect liver and mineralized tissues in a tissue-specific manner.

Keywords: estrogenic compounds, estrogen responsive genes, fish scales, G protein-coupled estrogen receptor, nuclear estrogen receptors, phytoestrogens.

Abbreviations:

ALP, alkaline phosphatase; E_2, estradiol; ESRs, nuclear estrogen receptors; ESR1, estrogen receptor 1; ESR2a, estrogen receptor 2a; ESR2b, estrogen receptor 2b; Gen, genistein; GPER, G-protein-coupled estrogen receptor; GPER-like, G-protein-coupled estrogen receptor-like; OSB, osteoblasts; OSC, osteoclasts; pNP, *para*-nitrophenol; pNPP, *para*-nitrophenyl-phosphate; PTHrP, parathyroid hormone-related protein; TRAP, tartarate-resistant acid phosphatase; Vtg, vitellogenin; For gene name abbreviation see table 2.

1. Introduction

Estrogens and in particular estradiol (E_2), are best known for their role in reproduction but they also regulate other processes such as mineral homeostasis. In fish liver and gonads, E_2 functions are well established and are associated with increased hepatic vitellogenin (Vtg) production for yolk accumulation in oocytes and with the control of sexual differentiation and function (Nelson et al. 2013). E_2 also has a hypercalcaemic role during vitellogenesis in female fish, a period of increased calcium (Ca) demand, which appears to involve both increased Ca influx from the environment and Ca mobilization from mineralized tissues, including the scales (Persson et al. 1994; Persson et al. 1995; Guerreiro 2002).

The scales are mineralized appendages that function as a mechanical barrier but also contribute to the effectiveness of the skin as an innate immune barrier and are an essential mineral reservoir in fish (Pinto et al. 2014). In common with mammalian bone, the scales are maintained by cycles of tissue formation and resorption mediated respectively by osteoblasts (OSB) and osteoclasts (OSC), while osteocytes have only been identified in some bony tissues of salmonid and cyprinid fish (Meunier et al. 1992). Given the crucial importance of Ca and P (phosphorus) in normal physiology, the homeostasis of these ions in fish is under tight control by several hormones including stanniocalcin, parathyroid hormone-related protein (PTHrP), vitamin D and E_2 (Guerreiro et al. 2007), but their precise functions and mechanisms of action remain to be fully described.

The majority of E_2 actions are mediated by nuclear receptors that act as ligand activated transcription factors. In fish scales and other mineralized tissues three estrogen receptor (ESR) subtypes have been detected (reviewed by Pinto et al. 2014; Nelson et al. 2013; Pinto et al. 2006a): the ESR1 form (also named ERα) and two duplicate ESR2 forms, ESR2a (also named ERβa, ERβ1 or ERβ) and ESR2b (also named ERβb, ERβ2 or ERγ). Evidences of their expression indicates that the actions of E_2 on fish mineralized tissues is most likely direct, particularly in the scales, and explains how E_2 regulates

the Ca deposition and mobilization cycles in this tissue (e.g. Persson et al. 1995; Yoshikubo et al. 2005). However, the way in which E_2 modulates the responsiveness of fish scales remains to be detailed. For example, the existence in scales of the recently identified membrane-associated estrogen receptors (e.g. the G protein-coupled estrogen receptor, GPER, Thomas et al. 2010; Prossnitz et al. 2011) that may mediate alternative rapid responses to estrogens remains to be investigated as does the existence of other indirect actions. Furthermore, the effect on fish scales of estrogenic compounds increasingly present in the environment and in the diet is unknown (Pinto et al. 2014).

Phytoestrogens are plant-derived polyphenolic non-steroid compounds with chemical structures similar to E_2 which have been demonstrated to have estrogen-like activity (Cos et al. 2003; Liu et al. 2010) and to bind to blood steroid-binding proteins and nuclear estrogen receptors, from fish to mammals (Latonnelle et al. 2002; Liu et al. 2010) having the potential to disrupt the endocrine system by competing with endogenous molecules. In mammals phytoestrogens are proposed to have protective effects against osteoporosis, like E_2, but also have the potential to affect development, metabolism, fertility and the reproductive system (Szkudelska et al. 2007; Cederroth et al. 2012).

An increased demand in recent decades for high quality protein alternatives for inclusion in fish feeds has accompanied the global expansion of the aquaculture industry. Plant ingredients, mainly soybean meal, are increasingly being used in fish feeds (Dersjant-Li 2002) but they are particularly rich in phytoestrogens with genistein being one of the main isoflavones present (Matsumoto et al. 2004; Kobayashi et al. 2006). Exposure of fish to phytoestrogens can also occur through contaminated waste and surface waters (Liu et al. 2010). Fish phytoestrogen exposure causes reproductive effects such as increases in Vtg synthesis (Pelissero et al. 1991; Latonnelle et al. 2002) and effects on growth and metabolism (Cleveland 2014; Cleveland et al. 2015), but their potential impact on mineralized tissues in fish has not been established.

The present work compared for the first time in a marine teleost, the Atlantic sea bass (*Dicentrarchus labrax*), the estrogen and phytoestrogen responsiveness of a classical estrogen target, the liver, with

the scales, a non-conventional target tissue that can be accessed in a non-invasive manner. The estrogenic effects of E_2 and genistein (Gen) on mineral homeostasis were evaluated by measuring plasma Ca and P levels and alkaline phosphatase (ALP, an OSB marker) and tartrate-resistant acid phosphatase (TRAP, an OSB marker) activities in the scales. The tissue responsiveness and probable modes of action of E_2 and Gen in scales and liver was established by measuring membrane and nuclear estrogen receptors and some putative responsive genes and abundance of plasma Vtg was used as an indicator of liver induction. Putative responsive genes analyzed included previously identified E_2 responsive genes related to Ca metabolism (Lehane et al. 1999; Bevelander et al. 2011) and also some of the responsive genes identified in a previous global differential gene expression study between liver and testis (Pinto et al. 2006c).

2. Methods

2.1. Animals

Manipulation of animals was performed in compliance with international and national ethics guidelines for animal care and experimentation (Guidelines of the European Union Council, 86/609/EU). The work was carried out under a "Group-I" license from the Portuguese Government Central Veterinary service to the Centre of Marine Sciences, CCMAR-CIMAR and conducted by a certified investigator (DMP). Immature sea bass were obtained from local fish farms and maintained at Ramalhete Marine Station (CCMAR, Faro, Portugal) in 500 L flow-through seawater tanks at natural temperature and photoperiod for winter and fed with commercial dry pellets at 1% body weight/day.

2.2. Treatments and sampling

Three weeks before the experiments to characterize *in vivo* estrogenic responses of sea bass scales and liver (Table 1), immature sea bass (n=10 per experimental group) were randomly distributed between tanks and left to acclimatize. Treatments consisted of a single intraperitoneal (i.p.) injection with

coconut oil alone (control) or containing 5 mg/kg of the test compound, E_2 (Sigma-Aldrich, Madrid, Spain) or genistein (Gen, AbCam). At the end of the exposure period (1 or 5 days), fish were anesthetized with 2-phenoxyethanol (Sigma-Aldrich, diluted 1:5,000 in seawater), washed with clean seawater and were then measured and weighted. Blood samples were collected from the caudal vein with heparinized 1ml syringes (1000 U/ml ammonium heparin, Sigma-Aldrich) and fish were sacrificed by decapitation. Plasma was collected by centrifugation of whole blood (10,000 rpm for 5 min) and stored at -20 ºC. Sampled tissues included the liver and individual scales that were collected from approximately the same position (below the dorsal fin) in all fish. Sampled tissues were immediately frozen in liquid nitrogen and stored at -80 ºC.

Table 1
Experimental conditions and parameters analyzed in the *in vivo* experiment carried out

Experimental conditions	
Date	January 2014
Tank volume	90L
Temperature	18ºC
Fishes per tank	10
Fish weight (g)	59.4±0.7
Fish SL (cm)	18.25 ±0.43
Exposure route	Intraperitoneal injection
E_2 doses	5 mg/Kg
Gen doses	5 mg/Kg
Exposure duration	24 h and 5 days
Samples	Blood, liver, scales, skin-scale
Measured parameters	
E_2 and cortisol plasma levels	Yes
Ca/P plasma levels	Yes
Vtg plasma levels	Yes
Ca/P scale content	Yes
ALP/TRAP enzymatic activity	Yes
Gene expression	Yes

2.3. Hormone and mineral plasma levels

Estradiol and cortisol levels were quantified in individual heat denatured plasma samples, analyzed in duplicate by radioimmunoassay (RIA) using specific antiserum against E_2 (Guerreiro et al. 2002) and cortisol (Rotllant et al. 2005a). Free hormones were separated from total bound hormones using

dextran-coated charcoal. No cross-reactivity of the E_2 antiserum was detected in relation to Gen, when serial dilutions of Gen up to 5000 pg/ml were tested. Total plasma Ca and P were measured in duplicate 2.5 µl plasma samples from individual fish using o-cresolphtalein and phosphomolybdate colorimetric assays, respectively (Spinreact 1001060 and 1001150, Barcelona, Spain).

2.4. Vitellogenin relative plasma levels

Plasma Vtg was detected by SDS-PAGE as previously described (Guerreiro et al.2002). In brief, 10 µl of plasma diluted 1:10 in Tris buffer, pH 7.8 were mixed with an equal volume of sample buffer, boiled for 5 min, centrifuged (60 s, 1700x*g*) and fractionated on an 8 % polyacrylamide gel. Molecular weight markers (Bio-Rad Laboratories, USA) were run on all gels. Proteins were detected using Coomassie blue staining, scanned and digital images captured with an Alpha Imager System (Alpha Innotech). The intensity of the band corresponding to Vtg, (approx 180 KDa, Ibarz et al. 2013) was quantified using Image J v.1.48 software. The total protein concentration (mg/ml plasma) was measured in each plasma sample diluted 1:75 using the Bradford method (Bio-Rad Protein Assay Kit) with bovine serum albumin (BSA, Sigma-Aldrich) as a standard. The total plasma protein loaded in each sample was then calculated and used to normalize intensity values for the Vtg bands, which were expressed as band volume (relative pixels/mm^2)/µg total protein.

2.5. Scale mineral contents

Pools of approximately 35 scales from each experimental group were weighed and incinerated at 550ºC for 14 hours. Scale ashes were weighted, dissolved in nitric acid (150 µl/2.5 mg) for 24 h at room temperature and then neutralized by adding an equal volume of 2 M sodium hydroxide. Ca and P contents in scale ashes were determined in duplicate using the colorimetric assays described above (section 2.3) and results are expressed as µmol/mg ash.

2.6. TRAP / ALP enzymatic activities

Scales were transferred to individual wells in a 96-well microplate containing 100 µl of buffer (20 mM tartrate in 0.1 M sodium acetate pH 5.3) for TRAP analysis or 100 µl of buffer (100 mM Tris-HCl pH

9.5; 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) for ALP analysis. For the TRAP or ALP activity determination, 2 mM *para*-nitrophenyl-phosphate (pNPP) was added to each well, assays were incubated at 24 °C for 30 min under agitation, the reaction was stopped by adding 100 µl of 2 M NaOH, and the absorbance measured at 405 nm using a microplate reader (Benchmark, Biorad). The amount of pNPP converted into *para*-nitrophenol (pNP) was determined using an pNP standard curve. After the enzyme assays the scales were rinsed in deionized water, dried at 50 ºC overnight, weighed and the TRAP and ALP activity expressed in nmol pNP/min/mg scale (dry weight).

2.7. Transcript expression quantification

Total RNA was extracted from frozen tissues using an automated Maxwell 16 Instrument and a Maxwell 16 SEV total RNA purification kit (Promega, UK), after mechanical disruption using an Ultra Turrax homogenizer (IKA, Germany) equipped with a dispersing element S25N-8G for liver (soft tissue) and S25N-8G-ST for scales (fibrous tissue). Total RNA (1.5 - 4 µg for scales, 8 µg for liver) was treated with DNase (DNA-free kit, Ambion, UK) and cDNA synthesis carried out in 20 µl reactions containing 500 ng of DNase-treated RNA and 200 ng of random hexamers as previously described (Martins et al. 2014). Transcript levels of nuclear and membrane estrogen receptors, OSC and OSB markers (*trap* and *alp*, respectively) and selected estrogen-responsive genes (see Table 2) were measured by quantitative real time RT-PCR (qPCR) using the relative standard curve method and the EvaGreen chemistry.

Preparation of qPCR reactions and thermocycle conditions were as previously described (Martins et al. 2014) except that reactions were run on a StepOnePlus qPCR thermocycler (Applied Biosystems, UK). Duplicate 15 µl reactions containing 2 µl of each individual cDNA (diluted 1:5, n=10/experimental group), 300nM of each specific primer and 1x Sso Fast EvaGreen Supermix (Bio-Rad) were run for 40 cycles using the cycling conditions recommended by the supplier and optimized primer annealing temperatures (Table 2). All qPCR reactions had a single peak melt curve and primer specificity was confirmed by sequencing the amplicons. No amplification products were obtained

when reverse transcriptase was omitted from the cDNA syntheses reactions (-RT control), confirming the absence of genomic DNA contamination. Standard curves prepared from serial dilutions of quantified amplicons for each gene were included in all qPCR plates to permit product quantification and for determination of efficiency, which ranged between 91-105% with $R^2 > 0.99$.

Table 2
List of the amplified genes and the primers used for analysis of gene expression by quantitative RT-PCR.

	Gene name and abbreviation	**Fw/Rv [a]**	**Primer sequence (5'-3')**	**Ta [b]**	**bp [c]**
Nuclear estrogen receptors	*Estrogen receptor 1 (esr1)*	Fw	AAACCACCTCAACACCCGTCTACAG	62	173
		Rv	GCACACGGCACAGAAACGCATA		
	Estrogen receptor 2a (esr2a)	Fw	TGTCATCGGGCGGGAAGG	60	188
		Rv	GCTCTTACGGCGGTTCTTGTCT		
	Estrogen receptor 2b (esr2b)	Fw	CGCAACCTCCGTCTCACCTG	60	158
		Rv	GGGCACCTCTAAACTCTGAAATGG		
Membrane estrogen receptors	*G protein-coupled estrogen receptor (gper)*	Fw	GCCACCCTTCTCCCTTTCACC	62	157
		Rv	TTCGCCCAATCAGAGAGTAGCAT		
	GPER-like (gperl)	Fw	ACAGCAGCGTCTTCTTCTTAACC	60	122
		Rv	AGATGAGGACACCCAGATAAGGCAG		
Genes related to Ca metabolism	*Tartrate-resistant acid phosphatase (trap)*	Fw	GTATCCATCTTAATTGCTGCCATCC	58	200
		Rv	CCCATCTGCTCTGCTACTTTGC		
	Alkaline phosphatase (alp)	Fw	ACGCAGGCAAGTCGGTAGG	58	121
		Rv	GCTTCAGGTGGCATCTCATTGTC		
Known estrogen-responsive genes	*Vitellogenin 2 (vtg2)*	Fw	TGCTCTGCTGTCTGGTTTCG	60	150
		Rv	GTGGCTTGGAACTCAATAGATGC		
	Choriogenin L (chgl)	Fw	AACATCGACTGCCTTTGCCATC	60	130
		Rv	GCCGCCAGACCCACTTCC		
	Transferrin (trf)	Fw	ACACTGCTGGACTGAACAACTACGA	60	146
		Rv	GGATTTCTTCCCGCTGAGGT		
Known estrogen-responsive genes related to Ca metabolism	*Osteonectin (osn)*	Fw	AAGAAGGGCAAAGTGTGTGAGG	60	151
		Rv	TGGCAAAGAAGTGGCAAGAGG		
	Parathyroid hormone-related protein a (pthrpa)	Fw	TTTGATGGTCCTGCTTTCCTTTCC	63	86
		Rv	ACGGTGAGAGTGGGTTTGATGAAG		
	Type 1 PTH/PTHrP receptor (pth1r)	Fw	TGTTGTGCTCTACTCTGGATCGG	61	78
		Rv	GCTTCGGTGATGGACTTGAGG		
	Type 3 PTH/PTHrP receptor (pth3r)	Fw	AGATGGCACTGGTTAAAGAAGG	58	152
		Rv	ATCACACTGGCGGTAGGCTC		
Reference genes	*18S ribosomal RNA (18s)*	Fw	TGACGGAAGGGCACCACCAG	60	158
		Rv	AATCGCTCCACCAACTAAGAACGG		
	β-Actin (bact)	Fw	TCAAGGAGAAACTGTGCTA	58	173
		Rv	CATACCGAGGAAGGAAGG		
	EF1α Elongation factor 1-alpha (ef1a)	Fw	GACACAGAGACTTCATCAAG	58	114
		Rv	GTCCGTTCTTAGAGATACCA		

[a] Forward (Fw) or reverse (Rv) primers; [b] Optimized annealing temperature used for each pair of primers.

[c] Amplicon size in base pairs (bp).

Candidate reference genes tested included 18S ribosomal RNA sub-unit (18S), beta actin (β-actin) and elongation factor 1α (EF1α). The combinations showing the least variation between cDNA samples and between experimental treatments (18S and EF1α) were chosen to normalize gene expression data. Copy number of target or reference genes were calculated as described in (Pinto et al. 2013) and normalized by dividing calculated gene copy number by the geometric mean of the two reference genes. Relative expression levels were calculated for each individual as log2 of the fold change compared to the control (mean expression in the control group at the same sampling point).

2.8.Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM). Differences between groups at each sampling point were analyzed by one-way analysis of variance (one-way ANOVA, SigmaStat v.3.50, Systat Software, USA) on log2 transformed data, followed by the Tukey test. In some cases when ANOVA did not detect significant differences, pair-wise comparisons were evaluated for significance using a t-test. A Pearson correlation analysis was performed to test the relationships between log2 gene expression levels. The alpha level set at 0.05 was divided by the number of comparisons (0.05/n-1, n = number of genes compared) and only p values lower than the corrected value were considered significant (Bonferroni correction).

3. Results

The response of the scales and liver to E_2 and Gen was characterized one and five days after injection *in vivo*. The responsiveness of animals to the treatment was determined by measuring plasma estradiol, cortisol, Ca, P and Vtg followed by gene expression analyses.

3.1. Plasma levels

A significant ($p < 0.001$) increase in plasma E_2 levels was detected at 1 and 5 days after E_2 injection, while Gen only caused a significant increase in plasma E_2 1 day after injection (Fig. 1). The basal mean values of plasma cortisol were approximately 400 ng/ml and no significant correlation was found with the responsive parameters analyzed.

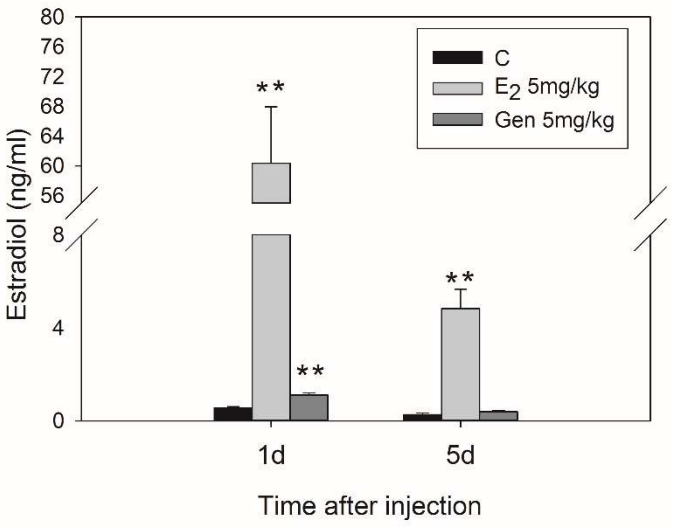

Figure 1. Circulating plasma levels of estradiol.
Results are expressed in ng/ml estradiol (E_2) in the plasma of control (C), E_2 or genistein (Gen) injected sea bass, as mean ± SEM (n = 10). ** indicates significant differences between the experimental group and the control at each sampling point ($p < 0.001$, one-way ANOVA).

Plasma calcium and phosphorus significantly ($p < 0.001$) increased 5 days after E_2 injection but no significant changes were observed 1 day after injection. Gen had no effect on plasma Ca and P levels at any time point (Fig. 2A and B). Due to technical limitations related to the amount of material available for analysis, scale mineral content was only analyzed in one sample (pool) per experimental group and Ca and P levels were not significantly modified by E_2 and Gen treatment at 1 and 5 days (data not shown). Finally, both E_2 and Gen induced a significant ($p < 0.001$) increase in plasma Vtg levels 1 day after injection, but after 5 days Vtg was only significantly ($p < 0.001$) increased in the E_2 group (Fig. 2 C).

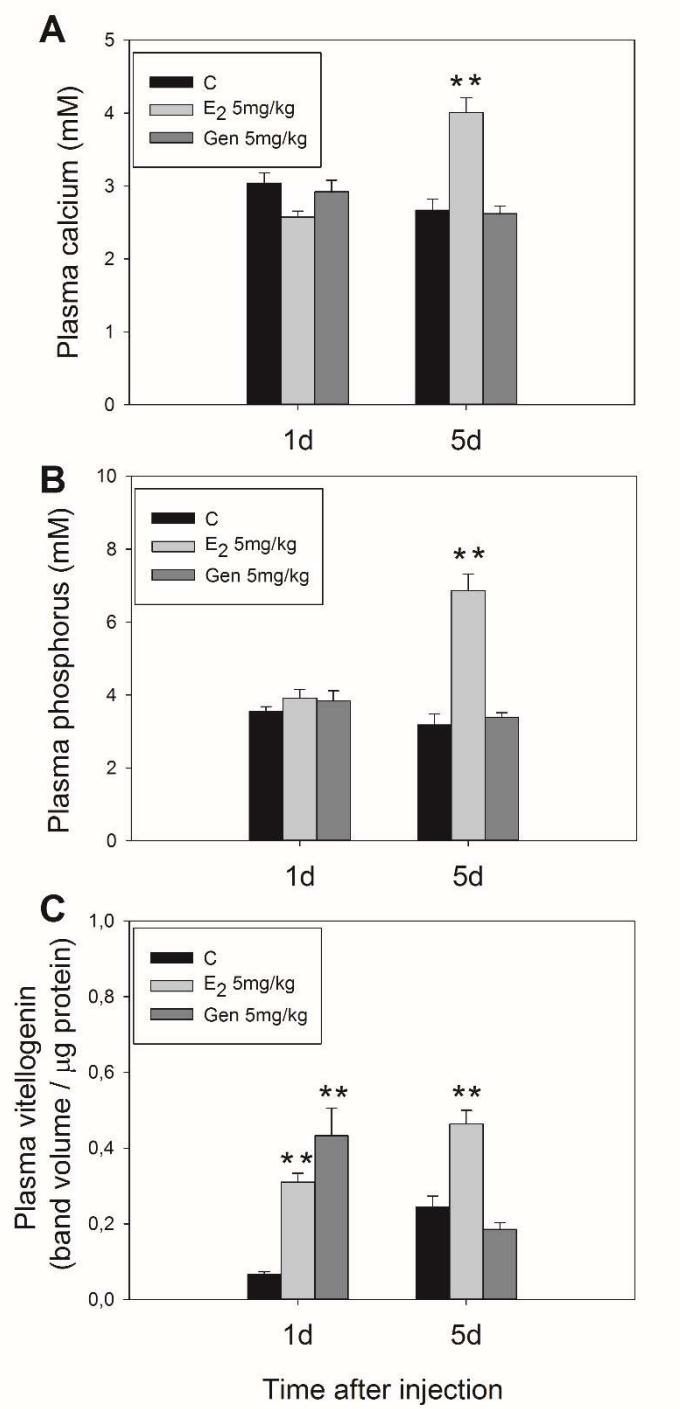

Figure 2. Total plasma calcium, phosphorus and vitellogenin.
Calcium (A) and phosphorus (B) were measured using a colorimetric assay (n = 10) and vitellogenin (C) was estimated by image analysis of SDS-PAGE bands (n = 9), in control (C), estradiol (E_2) or genistein (Gen) injected sea bass. Results are expressed as mean ± SEM and ** indicate significant differences between the experimental group and the control at each sampling point ($p < 0.001$, one-way ANOVA).

3.2. TRAP and ALP activity and transcript abundance in scales

E_2 did not significantly modify TRAP or ALP activity at 1 or 5 days after E_2 exposure although high individual variation was observed. Gen induced a significant ($p < 0.05$) increase in both TRAP and ALP activities 1 day after injection while at 5 days no significant effect was detected (Fig. 3 A). *Trap* and *alp* transcripts in scales were not significantly modified by E_2 or Gen after 1 day (Fig. 3 B). However, transcript abundance of *trap* and *alp* was positively correlated with the expression of other genes significantly up-regulated by E_2 and Gen in scales (see below, Fig. 5), such as *gperl*, *osn* and *chgl* ($p < 0.006$, correlation coefficient > 0.5, data not shown). A positive correlation ($p < 0.0002$ and correlation coefficient 0.65) was also observed between *trap* and *alp* gene expression in scales. Longer exposure (5 days) to both E_2 and Gen induced a significant up-regulation in *alp* mRNA levels but not in *trap* in scales (Fig. 3 B).

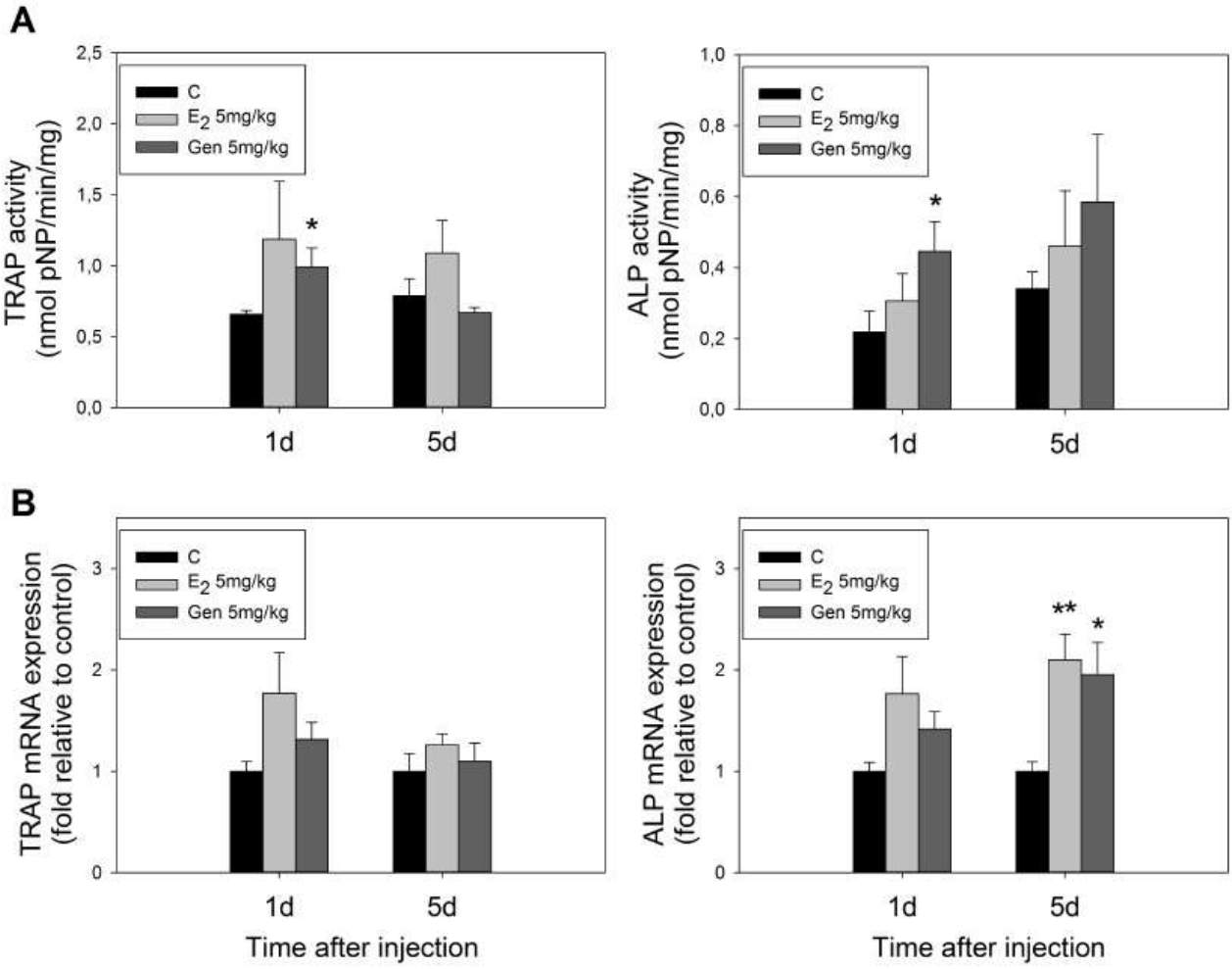

Figure 3. Scale enzyme activity and gene expression of tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP).

Enzyme activities (A) and q-PCR gene expression profiles (B, expressed as the fold change relative to the control group) from fish sampled at 1 and 5 days after treatment with estradiol (E_2) and genistein (Gen) are presented as mean ± SEM (n = 7 for enzymatic activities and n=10 for q-PCR). * or ** indicate significant differences compared to the control ($p < 0.05$ or $p < 0.001$, respectively, t-student).

3.3. Transcript abundance of estrogen receptors in scales and liver

To characterize the estrogen receptor repertoire, relative mRNA expression of nuclear (*esr1*, *esr2a* and *esr2b*) and membrane (*gper* and *gperl*) estrogen receptors was analyzed by qPCR in scales and liver from control fish. Results revealed similar levels of abundance between *esr2a*, *esr2b* and *gperl* in scales while *esr1* and *gper* were undetectable. In liver high expression levels of *esr2b*, moderate levels of *esr1*, *esr2a* and *gperl* and very low levels of *gper* were observed (Fig. 4). While *esr2a* and *gperl* were expressed at similar levels between the two tissues, the transcript abundance of *esr1*, *esr2b* and *gper* were all significantly ($p < 0.001$) higher in liver than in scales.

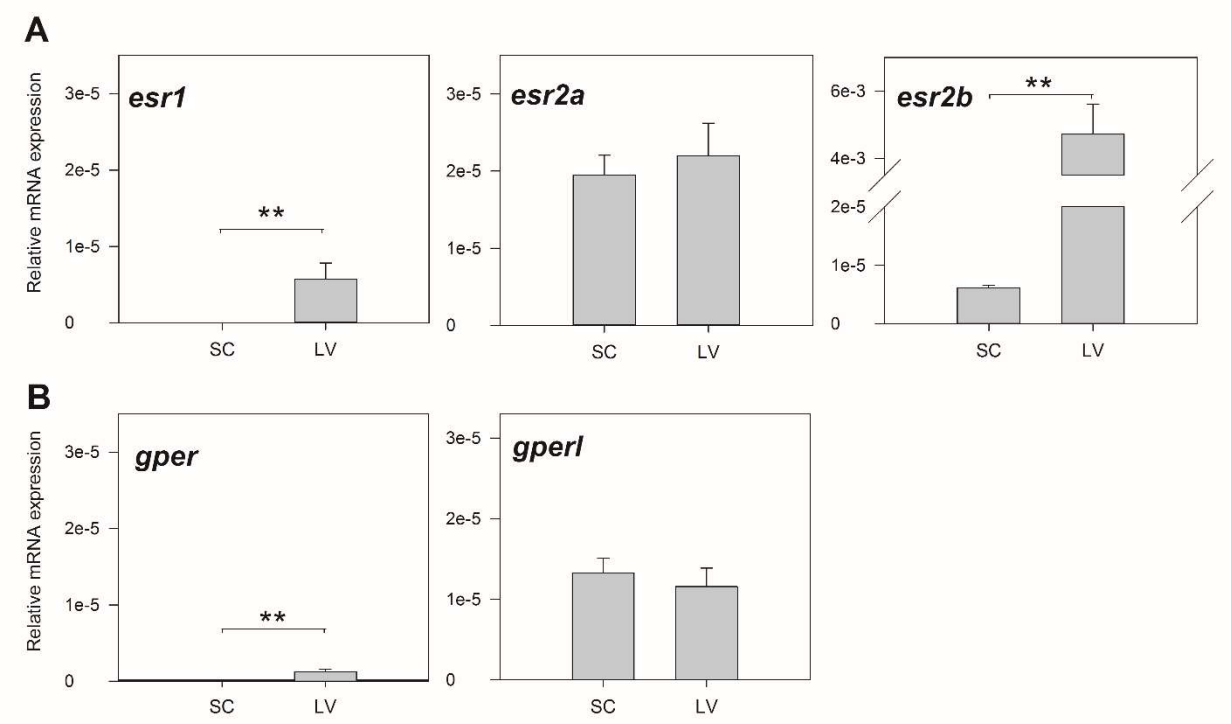

Figure 4. Relative gene expression of nuclear (A) and membrane (B) estrogen receptors in scales (SC) and liver (LV) of control fish.
Expression levels detected by qPCR were normalized by dividing detected copy numbers for the target gene by the geometric mean of the two reference genes and expressed as mean ± SEM (n = 10). Expression levels did not significantly differed between fish sampled 1 and 5 days after injection and are presented for fish sampled after 1 day. ** indicate significant differences compared to the control ($p < 0.001$, one-way ANOVA).

3.4. Transcript regulation of estrogen receptors and responsive genes in scales

Other estrogen-responsive genes previously identified in the liver (Table 2) such as vitellogenin (*vtg2*), choriogenin l (*chgl*) and transferrin (*trf*) were analyzed to evaluate regulation by E_2 and Gen in both scales and liver. In addition to *trap* and *alp*, other genes related to Ca metabolism were also included in the analysis such as osteonectin (*osn*), parathyroid hormone-related protein a (*pthrpa*) and type 1 and 3 PTH/PTHrP receptors (*pth1r* and *pth3r*). The expression levels of *esr1*, *gper*, *pth3r* and *pthrpa* in scales were too low for accurate quantification and they were not included in the analysis, while for the other genes the results of fold change in response to E_2 and Gen are presented in Figs 5 and 6. In scales 1 day after the *in vivo* treatment E_2 and Gen induced a significant up-regulation of *esr2a*, *gperl*, *vtg2*, *chgl*, *osn* and *pth1r* ($p < 0.05$ or $p<0.001$, see Fig. 5) but *esr2b* and *trf* transcript abundance was not modified. After a longer exposure period (5 days), *esr2a* and *trf* were significantly up-regulated ($p < 0.001$ and $p < 0.05$, respectively) in response to E_2. *Pth1r* was significantly up-regulated by both E_2 ($p < 0.001$) and Gen ($p < 0.05$). All other transcripts returned to basal levels with the exception of *chgl* which was significantly ($p < 0.05$) down-regulated by E_2.

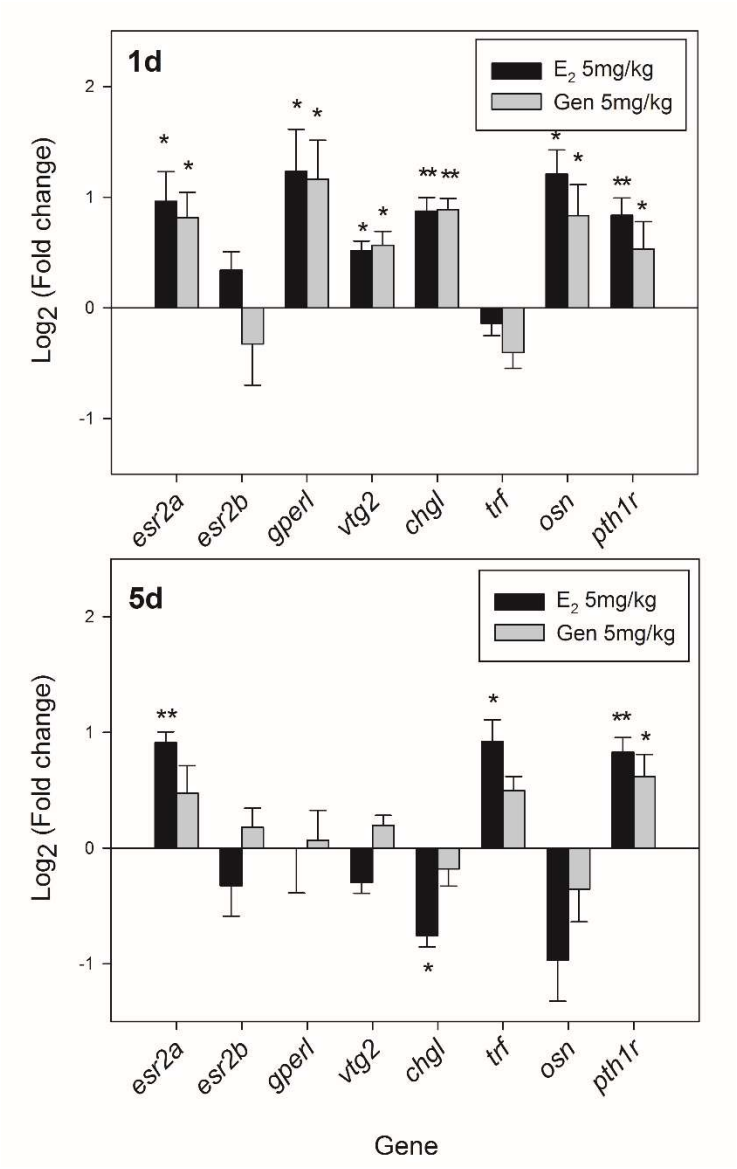

Figure 5. Gene expression profiles in sea bass scales.
Results are expressed as mean ± SEM (n = 10) of log2 of the fold change (quantified by qPCR) of the estradiol (E_2) and genistein (Gen) treated samples compared to the mean of the control group at the respective sampling times (1 and 5 days, d). See table 2 for gene name abbreviations. * or ** indicate significant differences compared to the control ($p < 0.05$ or $p < 0.001$, respectively, one-way ANOVA).

3.5. Transcript regulation of estrogen receptors and responsive genes in liver

In liver, *esr1*, *vtg2* and *chgl* were significantly and highly up-regulated after 1 and 5 days exposure to E_2 and Gen (Fig. 6; $p < 0.001$ except for *esr1* in response to Gen). At the shorter exposure period (1 day), E_2 induced a significant down-regulation of *esr2a* ($p < 0.001$) and *gper* and *pth1r* ($p < 0.05$) while Gen caused a significant ($p < 0.001$) down-regulation of *pth3r*. The expression of *pthrpa* was

too low for quantification and was excluded from the analysis. Five days after injection, genes in the liver that were significantly down-regulated by E_2 included *esr2a*, *esr2b*, *gperl,osn* and *pth3r*, ($p < 0.001$) and *gper* and *trf* ($p < 0.05$). Gen only significantly decreased transcript abundance of *esr2a* ($p < 0.05$) and *osn* ($p < 0.001$) 5 days after treatment.

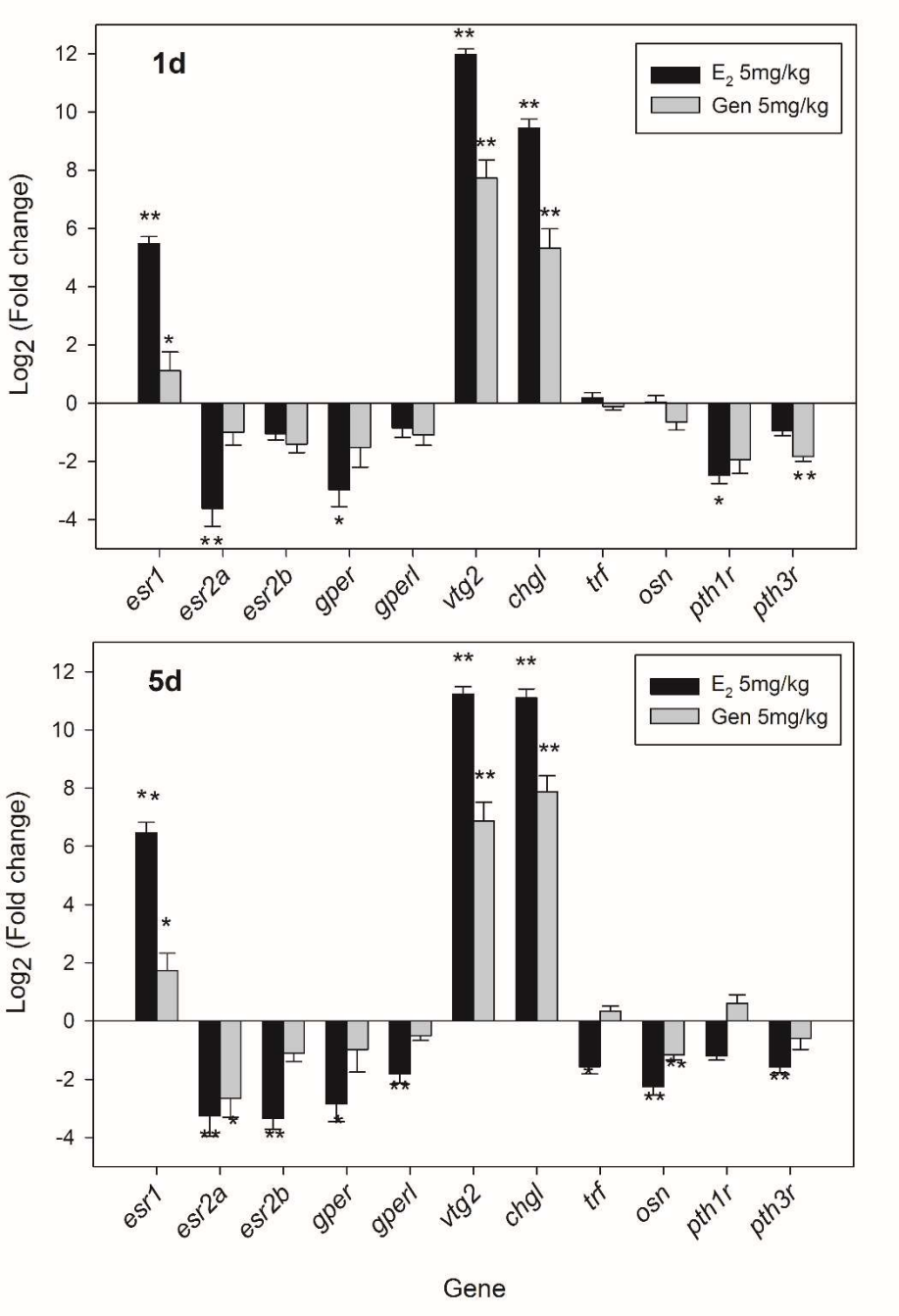

Figure 6. Gene expression profiles in sea bass liver.
Results are expressed as mean ± SEM (n = 10) of log2 of the fold change (quantified by qPCR) of the estradiol (E_2) and genistein (Gen) treated samples compared to the mean of the control group at the respective sampling times (1 and 5 days, d). See table 2 for gene name abbreviations. * or ** indicate significant differences compared to the control ($p < 0.05$ or $p < 0.001$, respectively, one-way ANOVA).

Figure 7 summarizes basal transcript expression and the response to E_2 and Gen of the liver and scales. Analysis of the global change in gene expression revealed that in liver the response to E_2 and Gen was prolonged and that strong up-regulation occurred for only a few of the genes analyzed (*esr1*, *vtg2*, *chgl*) while in scales the response was short term (1 day) with more responsive genes which were less strongly up-regulated (Figs. 5, 6 and 7).

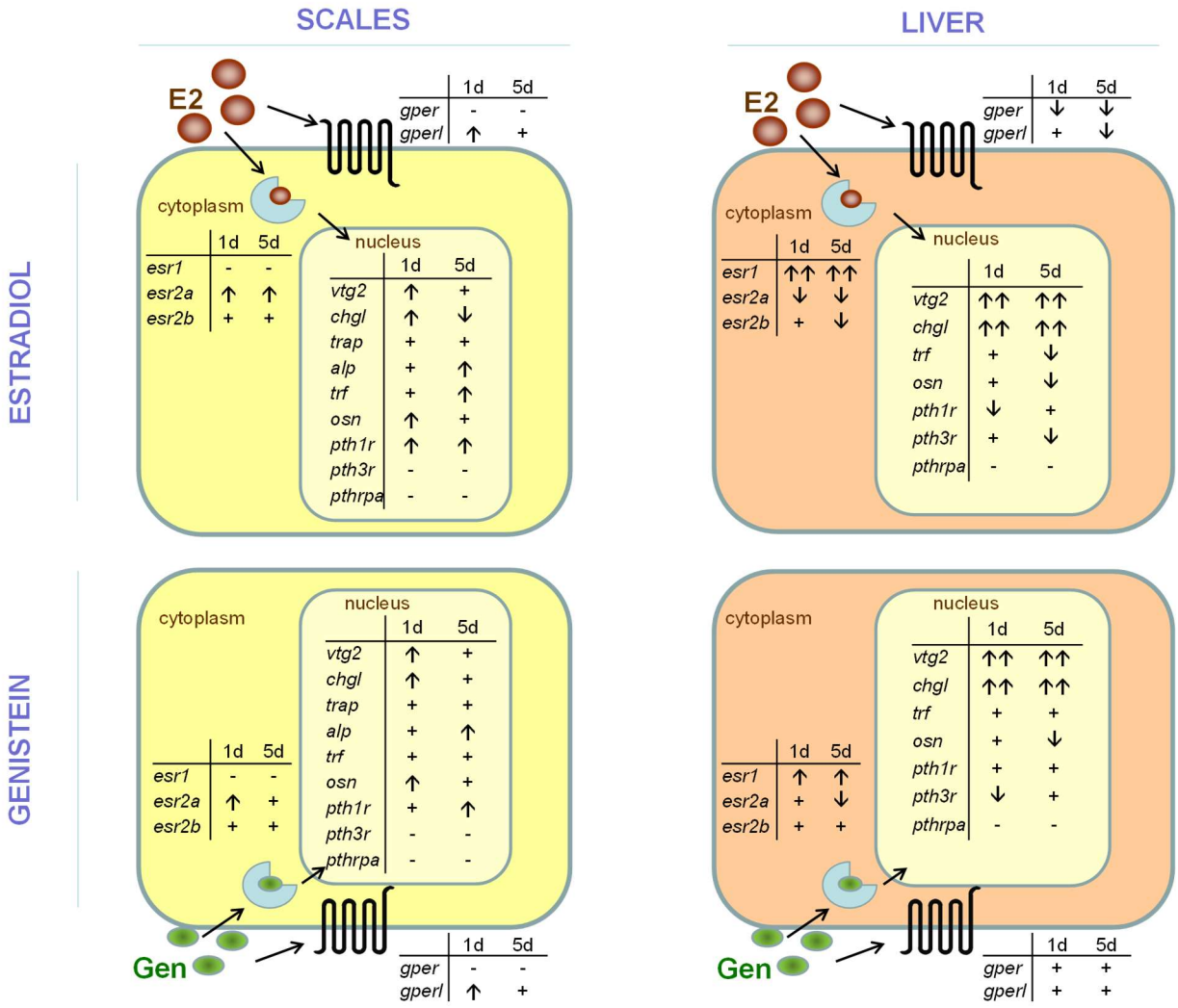

Figure 7. Simplified model of the gene expression changes identified in response to estradiol (E_2) and genistein (Gen) in scales or in liver.
In the model the genes represented in the membrane are the membrane receptors *gper* and *gperl*, in the cytoplasm the nuclear receptors *esr1*, *esr2a* and *esr2b*, and in the nucleus the target genes *vtg2*, *chgl*, *trf*, *osn*, *pth1r, pth3r* and *pthrpa* (for both tissues) and also *trap* and *alp* (only in the scales). For each analyzed gene, "+" indicates that expression was detected but not significantly changed, "-" indicates

non-detectable or very low expression (for *esr1, gper, pth3r* and *pthrpa* in scales and *pthrpa* in liver) and arrows pointing up and down indicate statistically significant up- or down-regulation by E_2 or Gen, after 1 or 5 days treatment (1d or 5d in the title of each table).

4. Discussion

Estradiol has previously been shown to participate in the regulation of calcium metabolism in several fish species by mobilizing Ca from the scales (Guerreiro et al. 2007; Pinto et al. 2014). Other estrogenic compounds, such as phytoestrogens, have been shown to affect calcium homeostasis in mammals (Sirotkin et al. 2014) but their effects on fish mineralized tissues are unknown. In this study, E_2 and Gen effects were compared between liver and the mineralized scales using immature sea bass, as they have negligible levels of endogenous sex steroids. E_2 and Gen doses and the timing of sampling were chosen based on previous studies demonstrating physiological responses to both compounds in fish (Persson et al. 1997; Pinto et al. 2006b; Ibarz et al. 2013; Cleveland 2014).

The treatment regime with E_2 was effective as indicated by the high levels of E_2 measured in the plasma of treated compared to control fish 1 day after treatment. In the Gen treated group, the significantly increased E_2 plasma levels suggest it increased E_2 synthesis (since the E_2 RIA did not cross-react with Gen), possibly by increasing testosterone synthesis and/or by increasing aromatization. Previous reports have shown Gen regulation of aromatase activity both in mammals and fish and binding of Gen to aromatase and to sex hormone-binding globulin (Pelissero et al. 1996; Rusin et al. 2011), which may affect E_2 bioavailability and synthesis. Thus, effects of Gen observed in the present study may result from a direct effect via receptors expressed in scales and liver, but indirect effects may also occur as a consequence of its actions on other tissues (e.g. liver or HPG axis) or via the detected increase in circulating E_2 levels. It will be interesting to evaluate if the increase in plasma E_2 resulted from a direct effect of Gen on aromatase activity or on fish aromatase genes expression in sea bass tissues.

In periods of increased calcium demand, such as vitellogenesis in female teleosts, the level of total Ca in plasma is known to correlate with E_2 and Vtg plasma levels and in males and immature fish exposed to exogenous E_2 treatments plasma Ca and Vtg levels are also increased (Guerreiro et al. 2002; Pinto et al. 2006b; Bevelander et al. 2011; Falahatkar et al. 2014). In this study E_2 induced an increase in plasma Ca and P levels 5 days after its injection in sea bass and increased Vtg levels after only 1 day and this increase was still evident 5 days after treatment (Fig. 2). The present results and those of previous studies indicate that Vtg plasma levels may be a faster endpoint of estrogenic exposure than plasma minerals (Guerreiro et al. 2002; Pinto et al. 2006b; Ibarz et al. 2013). The observed increase in circulating Ca could be due to higher whole body calcium influx (Guerreiro et al. 2002) or to calcium mobilization from mineralized tissues (Mugiya et al. 1977; Carragher et al. 1991; Persson et al. 1995; Armour et al. 1997). The increased Vtg levels detected in plasma in response to E_2 exposure were expected since E_2-induced secretion of Vtg from the liver of teleost fish and other oviparous animals is a typical and well characterized response (Arukwe et al. 2003).

In contrast to E_2, Gen induced an increase in Vtg after 1 day but not 5 days and did not affect Ca and P plasma levels (Fig. 2). It will be interesting to establish if exposure to Gen directly induces Vtg synthesis by the liver or if this increase was due to the Gen-induced increase in E_2 circulating levels 1 day after treatment, as mentioned above.

The effects of E_2 and Gen on scale turnover was assessed by determining the activity of OSB and OSC by measuring their enzymatic markers ALP and TRAP, respectively. A significant increase in ALP and TRAP in response to Gen occurred after one day (Fig. 3), but high individual variability in response to E_2 meant that no significant change in enzyme activity was measurable. Several reports have previously described an increase in TRAP and ALP activities in scales of several marine and freshwater fish species in response to E_2 (Pinto et al. 2014) and our results suggest that Gen may have a similar action on scale turnover. This is further supported by the significant up-regulation of *alp* transcript expression after 5 days in the scales of E_2 and Gen treated sea bass. Furthermore, after 1 day

E_2 and Gen treatment did not significantly affect *trap* expression but its expression was highly correlated with other up-regulated genes. Overall, the results suggest an increase in both OSB and OSC activities as a consequence of both E_2 and Gen treatments which may be indicative of increased scale turnover and the results support the notion that calcium mobilization and deposition may be coupled processes (Khosla 2010). The increase in *osn* and *pth1r* expression by E_2 and Gen in scales (Fig. 5) reinforces this hypothesis as these genes are associated with calcium deposition and mobilization, respectively, and both genes have previously been shown to be estrogen-responsive (Lehane et al. 1999; Rotllant et al. 2005b; Delany et al. 2009; Bevelander et al. 2011).

Analyzing the results obtained for gene expression (Figs. 5, 6 and 7) it is noticeable that the responsiveness of scales to E_2 and Gen was higher 1 day compared to 5 days after treatment and that most genes were up-regulated. In contrast, the liver appeared to have a similar response at the two time points considered in the study (especially the well described strong up-regulation of *esr1*, *vtg2* and *chgl* (Pinto et al. 2006b; Nelson et al. 2013)), which may reflect both slow and rapid mechanisms of action in response to E_2 and Gen. The rapid responses observed in fish scales resembles what has been described in mammalian bone, a tissue in which both classical direct actions mediated by nuclear ESRs localized in OSB, OSC and osteocytes occur, along with alternative estrogenic modes of action (reviewed by Spelsberg et al. 1999; Centrella et al. 2012; Pinto et al. 2014). These include non-nuclear estrogen actions such as rapid responses mediated by membrane receptors GPERs or membrane sub-populations of nuclear ESRs (Centrella et al. 2012). In this study, we demonstrated for the first time in fish scales the expression of membrane estrogen receptors, namely two *gper* genes which appear to be teleost fish duplicates of mammalian *gper1* (unpublished results). These are good candidates for mediating the rapid effects observed in this tissue after one day of treatment, namely the increase in TRAP and ALP activities in response to Gen (Fig. 2) and the up-regulation of estrogen receptors and responsive genes (Figs. 5 and 7). Expression of GPERs by scales also provide a feasible explanation

for rapid estrogenic effects previously observed in fish scales, e.g. an increase in TRAP activity in sea bream scales after 1h exposure to E_2 (Rotllant et al. 2005b).

In mammalian bone, in addition to the multiple cellular mechanisms of estrogen action there is also cross-talk between cell types and between the signaling of E_2 with other hormones and factors such as PTHrP, cytokines and growth factors (Khosla 2010; Centrella et al. 2012). In the present study, the expression of the PTH/PTHrP receptor *pth1r* and its up-regulation by E_2 and Gen in scales supports the interaction between the two systems. This is in agreement with our previous studies indicating that the hypercalcemic effects of E_2 in fish are in part mediated by PTHrP (Fuentes et al. 2007), which appears to act via *pth1r* expressed in scales to control calcium mobilization (Rotllant et al. 2005b). We also detected *pth3r* and *pthrpa* expression although at very low levels, which leaves open the possibility of local paracrine actions for *pthrpa* in fish scales. Other E_2-responsive genes analyzed included *trf*, which had opposing responses in scales and liver (Figs. 5 and 6). Previous tissue-specific regulation of *trf* by E_2 was demonstrated in the testis and liver of sea bream, while in the skin transferrin protein levels were also regulated by E_2 and/or scale regeneration (Ibarz et al. 2013). The E_2 induced up-regulation of *trf* in scales in the present study may reflect an innate immune response of skin and scales since the protein product is proposed to be protective against bacteria (Garcia-Fernandez et al. 2011; Ibarz et al. 2013). Although *vtg2* and *chgl* are considered to be typical liver transcripts, their expression and regulation by E_2 in scales (Fig. 5) are in agreement with previous reports of extra-hepatic expression including skin (e.g. Wang et al. 2005; Pinto et al. 2006c; Kim et al. 2015) and their possible function in fish scales remains to be investigated.

Overall, this study reveals that scales and liver responded differently to E_2 and Gen as shown by the differences in the timing and magnitude of the gene expression changes in response to the treatments (Fig. 7). Tissue-specific effects of several estrogen-responsive genes have previously been reported between fish liver and testis (Pinto et al. 2006c). Tissue-specific responses may be due to different cellular contexts in terms of the abundance and type of estrogen receptors in each tissue as well as the

presence of other molecular determinants of estrogen cellular actions, namely co-regulators or other transcription factors, and the cross-talk between different cell types and signaling systems (Pinto et al. 2014). Indeed, differences were found between the basal levels of expression and the estrogenic regulation of nuclear and membrane receptors between scales and liver of immature fish. In scales *esr1* and *gper* had negligible expression, *esr2b* was moderately expressed and *esr2a* and *gperl* were expressed and rapidly up-regulated by E_2 and Gen (Figs. 4, 5 and 7), suggesting these may be the main receptors mediating estrogenic effects in this tissue. Based on these results we hypothesize that the estrogen membrane receptor, *gperl*, may be associated with rapid responses in fish scales, particularly because its expression was up-regulated after 1 but not 5 days of estrogenic treatments. The nuclear receptor *esr2a* has previously been reported to be up-regulated in response to E_2 in sea bream skin (Ibarz et al. 2013) and in the present study it was also significantly increased 1 and 5 days after treatment, a time span compatible with the classical genomic actions of E_2.

In sea bass liver, nuclear estrogen receptors appear to be preponderant (Figs. 4, 6 and 7) and *esr1* was highly inducible by E_2, while the *esr2s* had a high basal expression but as previously described were down-regulated by E_2 (Pinto et al. 2006b). In contrast, the membrane receptors *gper* and *gperl* in liver were relatively low abundance and down-regulated by E_2, suggesting that in liver in contrast to scales the responsiveness to E_2 may be mainly via classical estrogenic mechanisms mediated by nuclear estrogen receptors. This is in agreement with recent evidence supporting the idea that estrogenic control of vitellogenesis in fish liver is mediated by intracellular and not membrane estrogen receptors (Nagler et al. 2010; Nelson et al. 2013). The evidence from the present study of strong up-regulation of *era* and down-regulation of both *esr2s* suggests a major role for ESR1 compared to ESR2s, which is in agreement with the paradigm that ESR1 up-regulation sensitizes the liver for Vtg induction by E_2 as reviewed in (Nelson et al. 2013).

Comparing Gen and E_2 responses it was noticeable that the two compounds induced similar effects on TRAP and ALP activities and on scale and liver gene expression (Fig. 7). This is in agreement with

the proposed anti-osteoporotic effects of phytoestrogens that are agonists of E_2 in mammalian bone (Sirotkin et al. 2014) and also in fish where they induce Vtg synthesis, affect growth and liver gene expression (Latonnelle et al. 2002; Cleveland 2014; Cleveland et al. 2015). Gen binds fish and mammalian ESRs (Latonnelle et al. 2002; Liu et al. 2010) and mammalian GPER (Thomas et al. 2006), and although the binding and/or activation of fish GPERs by Gen remains to be characterized, some of the observed Gen effects in the present study suggest a direct action on ESRs and GPERs expressed in sea bass scales and liver. However, more in depth studies are needed to detail the mechanisms involved, as well as the likely impact of phytoestrogen exposure on development, growth, survival and health of both wild and aquaculture fish as well as possible source dependent effects (eg. comparison of ingestion in feeds with exposure through aquatic contamination (Dersjant-Li 2002; Liu et al. 2010)).

5. Conclusions

We have clearly demonstrated that estrogens and phytoestrogens affect both liver and scales in fish, causing: 1) an increase in scale enzymatic activities indicative of higher mineral turnover, 2) increases in plasma mineral levels (only E_2) and hepatic Vtg production, and 3) significant changes in gene expression in liver and scales.

In fish scales the expression of plasma membrane estrogen receptors (GPERs) was demonstrated for the first time (in addition to the expression of nuclear ESR2 sub-types) and we hypothesize that they may mediate the rapid effects observed for E_2 and Gen in fish scales. We demonstrated that scales and liver express different ESRs/GPERs that are differentially regulated by E_2 and Gen, suggesting direct actions of estrogenic compounds on these tissues, although there appears to be tissue-specific consequences for gene expression (with different genes, magnitude and timing) and therefore likely different mechanisms of action. In scales E_2 and Gen caused small rapid increases in gene expression and in liver strong, sustained increases in “classical target genes” (*esr1*, *vtg2* and *chgl*) or sustained

down-regulation of the other genes. The increase in circulating E_2 caused by Gen also highlights potential indirect effects of this phytoestrogen.

Overall the study provides evidence for different responsiveness and impacts of estrogens and phytoestrogens on a classical E_2 responsive tissue (the liver) and a poorly characterized, but important mineralized tissue (the scales) and establishes the basis for future studies to detail the exact mechanisms involved and the possible consequences that exposure to estrogens and phytoestrogens in the environment or in food may have in whole animal physiology.

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