In vitro screening for estrogenic endocrine disrupting compounds using Mozambique tilapia and sea bass scales

Patrícia I. S. Pinto[a,#,*], M. Dulce Estêvão[a,b,#], Soraia Santos[a], André Andrade[a], Deborah M. Power[a,*]

[a] Centre of Marine Sciences (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[b] Escola Superior de Saúde, Universidade do Algarve, Av. Dr. Adelino da Palma Carlos, 8000-510 Faro, Portugal

[#] These authors contributed equally to the study

Running title: *In vitro* bioassay using fish scales

Ms. has 22 pages, 4 figures, 2 tables, 4 suppl. files (provided as separate file)

*Corresponding authors:
Dr. Patricia Isabel Silvestre Pinto
Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas
8005-139 Faro, Portugal
Tel: +351 289800900, ext. 7336; Fax: +351 289800051
ppinto@ualg.pt
Dr. Deborah M Power,
Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas
8005-139 Faro, Portugal
Tel: +351 289800900, ext. 7335; Fax: +351 289800051
dpower@ualg.pt

Abstract

A wide range of estrogenic endocrine disruptors (EDCs) are accumulating in the environment and may disrupt the physiology of aquatic organisms. The effects of EDCs on fish have mainly been assessed using reproductive endpoints and *in vivo* animal experiments. We used a simple non-invasive assay to evaluate the impact of estrogens and EDCs on sea bass (*Dicentrarchus labrax*) and tilapia (*Oreochromis mossambicus*) scales. These were exposed to estradiol (E2), two phytoestrogens and six anthropogenic estrogenic/anti-estrogenic EDCs and activities of enzymes related to mineralized tissue turnover (TRAP, tartrate-resistant acid phosphatase and ALP, alkaline phosphatase) were measured. Semi-quantitative RT-PCR detected the expression of both membrane and nuclear estrogen receptors in the scales of both species, confirming scales as a target for E2 and EDCs through different mechanisms. Changes in TRAP or ALP activities after 30 minutes and 24 h exposure were detected in sea bass and tilapia scales treated with E2 and three EDCs, although compound-, time- and dose-specific responses were observed for the two species. These results support again that the mineralized tissue turnover of fish is regulated by estrogens and reveals that the scales are a mineralized estrogen-responsive tissue that may be affected by some EDCs. The significance of these effects for whole animal physiology needs to be further explored. The *in vitro* fish scale bioassay is a promising non-invasive screening tool for E2 and EDCs effects, although the low sensitivity of TRAP/ALP quantification limits their utility and indicates that alternative endpoints are required.

Key words: alkaline phosphatase, bioassay, endocrine disrupting compounds, estradiol, fish scales, tartrate-resistant acid phosphatase

1. Introduction

The fish skeleton is composed of both an articulated endoskeleton and an exoskeleton formed by mineralized scales, which provide protection and support and are important for fish locomotion and for mineral homeostasis (Bereiter-Hahn and Zylberberg, 1993). Scales are calcified structures of dermal origin that cover part of the body of almost all bony fish and are maintained by continuous cycles of formation and resorption, mediated by osteoblasts (OSB) and osteoclasts (OSC) similar to those present in mammalian bone (Olson and Watabe, 1980; Sire and Arnulf, 2000). Scales have been proposed as a model to study several aspects of mammalian and fish bone physiology (e.g. de Vrieze et al., 2015; Pasqualetti et al., 2012; Suzuki et al., 2000). Since they are abundant, easy to sample and culture and rapidly regenerated, non-invasive assays have been developed to evaluate the impact of pollutants, drugs, mechanical loading, stress or hypercalcemia on mineral turnover (Aerts et al., 2015; Carnovali et al., 2016; Kitamura et al., 2010; Pinto et al., 2014; Suzuki et al., 2007). Measured endpoints have mainly been related to the enzymatic activity of markers for mineral turnover and include alkaline phosphatase (ALP, an osteoblast marker) and tartrate-resistant acid phosphatase (TRAP, an osteoclast marker) (Persson et al., 1995; Yoshikubo et al., 2005), as well as the expression of a limited number of genes.

Estrogenic endocrine disruptors (here defined as EDCs) include a wide range of natural and anthropogenic compounds that can disrupt (mimic or block) the normal functions of the estrogen system (reviewed in Pinto et al. 2014). They may have an impact on humans, ecosystems and wildlife and are particularly relevant in aquatic organisms like fish that may experience life-long exposures to these compounds in the environment or in aquaculture and may bioaccumulate them (Brander et al., 2013). Assessment of the effects of EDCs in fish has mainly used *in vivo* animal experiments and assessed the effects on reproductive parameters, while the impact on other physiological systems remain to be addressed (Pinto et al., 2014). Fish scales are now well established as a target tissue for estrogens, as they express both membrane and nuclear estrogen receptors (Pinto et al., 2009; Pinto et al., 2016) and 17β-estradiol (E2) has been shown to regulate scale mineral turnover in several fish species *in vitro* and *in vivo* (Persson et al., 1995; Persson et al., 1997; Suzuki et al., 2000; Suzuki et al., 2009). This also makes them potential targets for EDCs and the evaluation of EDC impacts on mineral turnover in fish scales can be used as an alternative endpoint to evaluate their overall adverse outcomes for fish.

In the present study, an *in vitro* scale bioassay was optimized for scales from a marine (European sea bass, *Dicentrarchus labrax*) and a freshwater fish (Mozambique tilapia, *Oreochromis mossambicus*), as species-specific estrogen or EDCs responsiveness and effects should be considered when interpreting EDC impact (Dang, 2010; Hutchinson et al., 2006; Miyagawa et al., 2014). The optimized

assays were used to assess the response to the natural estrogen, estradiol (E2), two phytoestrogens and six anthropogenic pollutants reported as estrogenic or anti-estrogenic EDCs. The endpoints used were the enzymatic activities of ALP and TRAP, as markers of mineral deposition or resorption, respectively. In addition, analysis of both nuclear and membrane expression allowed the responsiveness of scales to estrogenic compounds to be evaluated in both species.

2. Material and Methods

2.1. Scales sampling

Manipulation of animals was performed in compliance with international and national ethical 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. Scales were obtained from: 1) juvenile sea bass, *Dicentrarchus labrax* (1 year, 100-300 g, originated from local fish farms and maintained in 500 L flow-through seawater tanks in CCMAR's marine station, Ramalhete, at natural photoperiod (12:12 light-dark) and temperature (15-21ºC) for Spring; or from 2) adult male Mozambique tilapia, *Oreochromis mossambicus* (80-200 g, obtained from a stock raised from fertilized eggs at the University of Algarve, Portugal, and maintained in 150 L closed circuit freshwater aquaria at a water temperature of 24°C and 12:12 light-dark photoperiod). Before sampling, fish were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (MS-222, Sigma-Aldrich), washed with clean seawater and weighted. Individual scales were plucked with forceps from approximately the same position (first 2-3 rows below the dorsal fin and above the midline, see Figure 1) in all fish. Collected scales included both the mineralized elasmoid scale structure and part of the epidermis layer and superficial dermis, usually sampled with the scale (Sire and Akimenko, 2004; Pinto et al. 2017), done in a standardized way for all fish and treatments. Scales were randomly transferred to 6-well micro-well plates containing Modified Eagle´s Medium (MEM) with Hanks' Balanced Salts (Gibco) supplemented with an 1% penicillin–streptomycin mixture (Sigma-Aldrich) and were immediately used for *in vitro* assays. For analysis of gene expression, scales were sampled from the same area (Figure 1) from anesthetized sea bass and tilapia, placed in 500 µL RNA Later (ThermoFisher Scientific), incubated at 4 ºC overnight and then stored at -20ºC until RNA extraction.

2.2. Compounds of interest

Compounds tested in the present study included water-soluble estradiol (Sigma-Aldrich), the phytoestrogens genistein (Gen, Abcam) and daidzein (Dai, Abcam) and the anthropogenic pollutants

bisphenol A (BPA, Sigma), 4-tert-octylphenol (OP, Sigma), triphenyltin (TPT, Sigma), dibutyl phthalate (DBP, Sigma), bis-(2-ethylhexyl) phthalate (DEHP, Sigma) and benzyl butyl phthalate (BBP, Sigma). These compounds were chosen based on their structural similarities to estrogen (Supplementary File 1) and on previous reports referring to their estrogenic activities using other endpoints (see discussion).

2.3. Experimental Set up

Several variables were considered when optimizing the experimental conditions both for the sea bass and tilapia scale culture and for enzymatic activity quantification. The scale culture conditions were defined using E2 for the positive control test compound and initially various concentrations (10^{-5} to 10^{-10} M) and times of exposure (30 min and 1, 6 h) were used to test for short term responses on TRAP or ALP activities. Since no significant differences were observed in the E2 responses between these short-term incubation times (example in Supplementary File 2), the 30-minute exposure time was chosen for the EDC exposure assays as it allows the measurement of short term changes in enzymatic activities. In addition, a 24 h time of exposure was chosen to test for longer term responses.

Optimization of TRAP and ALP enzymatic quantification included varying the pH of the buffer (tested between 5.1-5.5 for TRAP and 9.3-9.7 for ALP), the substrate concentration (from 0.05 to 10 mM), incubation time (from 10 to 40 min) and temperature (from 20 to 30 ºC). No significant differences in enzymatic activities were observed for the different tested pHs and thus the already reported values used for other species (5.3 for TRAP and 9.5 for ALP) were chosen (Persson et al., 1995; Yoshikubo et al., 2005). The selected substrate concentration was 5 mM as it was a non-saturating value for both TRAP and ALP activities (Supplementary File 2). The assay temperature was set as 30ºC and incubation time at 15 and 30 minutes for TRAP and ALP, respectively, chosen to obtain absorbance values in an adequate range for the quantification of the enzymatic activities. Finally, the enzymatic assays were run in 2 ml tubes followed by centrifugation, to avoid interference in the absorbance measurements due to particles in suspension, associated with skin debris released from sampled scales, observed when microplates were used. The final protocol used for the scale assay is detailed below in topic 2.4.

2.4. In vitro *scale bioassay*

Four scales per fish (from 3 individual fish of each species) were analysed for each compound, concentration and incubation time. Scales were transferred to 24-well microplates with culture media (4 scales/well in 0.4 ml MEM), alone (control group) or supplemented with the tested compounds at different concentrations (10^{-10}, 10^{-8}, and 10^{-6} M), and then incubated for 30 minutes or 24 h at 21°C.

The activities of TRAP and ALP were determined in scales using a colorimetric assay that measures the amount of substrate p-nitrophenyl-phosphate (pNPP) converted into product p-nitrophenol (pNP) (Persson et al., 1995). After incubation of scales in culture media, TRAP and ALP enzymatic assays were performed in duplicate 2 ml tubes (2 scales/tube) with 400 µl of TRAP (0.1 M sodium acetate pH 5.3; 20 mM sodium potassium tartrate) or ALP (0.1 M Tris HCl pH 9.5; 1 mM $MgCl_2$; 0.1 mM ZnCl) assay buffer, respectively, containing 5 mM of substrate pNPP, and maintained at 30ºC under agitation for 15 (TRAP) or 30 (ALP) minutes. After this incubation, 200 µl of NaOH 2 M was added to stop the reaction and the tubes were centrifuged at 7000 x *g* for 5 minutes. 250 µl of the reaction mix from each tube were transferred in duplicate to an appropriate multi-well plate for spectrophotometry and the absorbance was measured at 405 nm. TRAP and ALP activities were expressed in nmol pNP/min/scale.

2.5. Semi-quantitative reverse transcriptase (RT)-PCR

Total RNA was extracted from frozen scales of sea bass and tilapia (n=3 fish per species, 15 scales per fish), collected as described in 2.1, from the same fish used for the *in vitro* assays. RNA was extracted using PureZol RNA isolation reagent (Bio-Rad) and following the manufacturer's instructions. Mechanical disruption was carried out using an Ultra Turrax homogenizer (IKA) equipped with a dispersing element S25N-8G-ST (recommended for fibrous tissues). Total RNA (6–8 µg) was treated with DNase (DNA-free kit, Ambion) and cDNA synthesis was 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 expression of the three nuclear estrogen receptor subtypes (*esr1, esr2a* and *esr2b*) and two membrane G-protein-coupled estrogen receptors (*gper* and *gperl*) was analysed by semi-quantitative RT-PCR using specific primers previously optimized for sea bass (Pinto et al., 2016) or designed for tilapia (see Supplementary File 3). Primers designed for tilapia were based on available sequences for *O. mossambicus* nuclear estrogen receptors (Esterhuyse et al., 2010) or for a membrane estrogen receptor predicted from the *O. niloticus* genome (Accession number **ENSONIT00000026534**, www.ensembl.org) and identified as *gperl* by similarity with the sea bass transcript. For tilapia *gper,* the same primers designed for sea bass were used due to their elevated sequence similarity (Supplementary File 3).

PCR reactions contained 2 µl of each individual cDNA (diluted 1:5), 300 nM of each specific primer and 1x Sso Fast EvaGreen Supermix (Bio-Rad) in a final reaction volume of 15 µl and were run for 40 cycles on a StepOnePlus qPCR thermocycler (Applied Biosystems, UK) using the cycling conditions recommended by the supplier and optimized primer annealing temperatures for each primer pair (Supplementary File 3). All PCR reactions had a single product melt curve and were run on

agarose gels (2%), isolated, and then sequenced to confirm amplicon identity and primer specificity. Amplification of the 18S ribosomal RNA sub-unit (18S) was carried out as previously described (Pinto et al., 2006) and used as the reference gene.

2.6. Statistics

Results are presented as the mean ± SEM (n = 3 fish for each treatment), using SigmaPlot v12 (SPSS Inc). Differences in enzymatic activity values were evaluated by two-way ANOVA in SigmaPlot, using compound concentration and time as factors. No interactions were detected between the two factors. Statistical differences within each factor, when significant ($p<0.05$), were assessed by Multiple Comparisons (Holm-Sidak test) versus control, defined as 30 min for the factor "time" and as the experimental control group at each time for the factor "compound concentration". Statistical differences were considered significant when p-values were < 0.05.

Results and Discussion

In this study we tested the utility of an *in vitro* scale bioassay to screen for the effects of estrogenic EDCs on the marine European sea bass and the freshwater Mozambique tilapia, important commercial fish species for fisheries and aquaculture and for which significant genomic information is available. Previous studies have reported the use of scale bioassays to evaluate the effects of a limited number of EDCs (reviewed in Pinto et al., 2014). In line with recent recommendations we decided to test for species-specific estrogen or EDC responsiveness and impacts (Dang, 2010; Hutchinson et al., 2006; Miyagawa et al., 2014), using an optimized assay and the scales from the marine sea bass and fresh water tilapia.

Using semi-quantitative RT-PCR analysis carried out in scales sampled from three fish per species (Figure 2), abundant expression of nuclear estrogen receptor subtypes *esr2a* and *esr2b* and the membrane receptor *gperl* was detected in both sea bass and Mozambique tilapia scales, while the nuclear receptor *esr1* and the membrane receptor *gper* were expressed at very low levels. The results support the reported expression of estrogen receptors in sea bass scales, evaluated by quantitative RT-PCR (Pinto et al., 2016), and revealed the nuclear and membrane bound receptor had a similar expression pattern in tilapia scales. We have previously shown in sea bass that both *esr2a* and *gperl* were up-regulated by E2 injection, and are thus good candidates to mediate estrogenic responses in this tissue (Pinto et al., 2016). The results of the present study indicate that tilapia scales are also responsive to E2 and EDCs, although they differed in sensitivity relative to the sea bass tissue. In this study we demonstrated both 30 min and 24 h responses of fish scales to E2 and estrogenic compounds, which may contribute to assess in this tissue the relative importance of membrane and nuclear

receptors, that are typically associated with rapid and longer term estrogenic responses (Pinto et al., 2014; Thomas et al., 2010), respectively.

The experimental conditions used in previous fish scale bioassays (e.g. Suzuki and Hattori, 2002; Suzuki and Hattori, 2003; Yachiguchi et al., 2014; Yoshikubo et al., 2005), including for example the duration of exposure, were modified in the present study to establish the optimal assay conditions for the two species studied. Several variables were tested using E2 exposure as the positive control and optimal conditions were selected based upon the amplitude of the TRAP and ALP activities. Different incubation times were selected for the *in vitro* incubation of scales with the tested EDCs to evaluate possible impacts that might be associated with direct regulation of the enzymatic activities (30 minutes) or involving changes in gene expression (24 h). A crucial aspect in the development of the reported assay that increased the robustness of our analysis relative to previous studies (e.g. Suzuki and Hattori, 2002; Suzuki et al., 2009; Yachiguchi et al., 2014) was that multiple measures were taken for each individual by increasing the number of scales used and also the number of biological replicates utilized in the assays.

The panel of tested compounds included two phytoestrogens and six anthropogenic EDCs (Supplementary File 1), previously shown to have estrogenic or anti-estrogenic effects or to bind estrogen receptors (references presented below for each compound). Tables 1 and 2 summarize the effects observed on TRAP and ALP activities in sea bass and tilapia scales, respectively. The responses obtained for the compounds, E2, Gen, DEHP and TPT, that had the most pronounced effects are illustrated as examples in Figures 3 (sea bass scales) and 4 (tilapia). A slight time-effect was observed for most assays with a reduction in enzymatic activities in control groups between 30 min and 24h; this could be probably related to depletion of nutrients during culture or other factors not explored, but comparison of compound effects were always carried out with the control at respective exposure times.

The natural estrogen, E2 induced a significant increase ($p<0.05$) in ALP enzymatic activity, 30 min after exposure of sea bass scales, at a concentration of both 10^{-6} M and 10^{-8} M while after a longer period (24 h) no effect was detected (Fig.3). TRAP activity was not significantly affected by E2 at the tested conditions. In contrast, in tilapia scales 10^{-6} and 10^{-8} M of E2 induced significant decreases in TRAP activity after 24 h ($p<0.05$; Fig.4) but no effects were observed on ALP activity. These results confirmed previous observations that E2 affects both TRAP (osteoclastic) and ALP (osteoblastic) activities in fish scales, *in vitro* and *in vivo* (Persson et al., 1997; Suzuki et al., 2009; Suzuki et al., 2000), indicating an overall stimulation of mineral turnover. The timing and the observed effects on

sea bass and tilapia differed from that described in goldfish, nibbler fish and wrasse, as in these species E2 increased both TRAP and ALP activities after 6 to 72 h of exposure *in vitro* (Suzuki et al., 2009; Suzuki et al., 2000), although shorter times were not assayed. However, in another study using female goldfish, E2 did not significantly affect ALP activity (Suzuki et al., 2000). No significant changes were observed in TRAP activity in sea bass of 100 g in the present study (Table 1), but in previous experiments performed with adult sea bass (400 g) significant decreases in TRAP activity were observed after 30 min incubation (Supplementary file 4). Overall, the results of our study indicate that scale responsiveness seems to be age-dependent and that E2 affects both TRAP and ALP activities in sea bass and tilapia, indicating a role for this hormone in the regulation of scale mineral turnover.

We propose that the way in which E2 regulates scale turnover is dependent on a number of factors, including the species, the environment they inhabit (different scale responses were found in the seawater sea bass and freshwater tilapia), the age, size or seasonal factors, the concentration of E2 and variations in calcium demand (Norberg et al., 1989; Pinto et al., 2014; Yoshikubo et al., 2005). Although the pattern of distribution of tilapia's estrogen receptors were similar to those in sea bass scales, in the present study we observed different responses to the tested compounds in tilapia scales, related both with the timing of the observed effect and the compounds that affected scale turnover markers. The species-specific differences could be related to modified receptor cellular context determining estrogenic responses and/or result from different functional characteristics of their estrogen receptors (Miyagawa et al., 2014; Pinto et al., 2014). Whether these differences could be related to some of the referred factors (e.g. different reproductive strategies, ages or environmental conditions) remains to be investigated.

This study also evaluated the effects of two phytoestrogens (Gen and Dai), which are plant derived compounds with estrogen-like activity. Fish may be exposed both in the wild, due to environmental pollution, or in aquaculture when they are fed plant-based feeds (Cos et al., 2003; Liu et al., 2010; Matsumoto et al., 2004). Gen and Dai have previously been shown to bind and activate fish and mammalian nuclear ERs, with apparently higher or similar affinity and transcriptional activation potency for ERβ than for ERα subtypes (e.g. An et al., 2001; Dang, 2010; Hawkins and Thomas, 2004; Sassi-Messai et al., 2009). Thus, an impact on fish scale estrogenic mechanisms is expected, due to the higher expression of *er*β*s vs er*α subtypes. However, in this study no significant effects on ALP or TRAP activities were detected in sea bass or tilapia after phytoestrogen treatment although a slight tendency for increasing enzymatic activities were observed for Gen in sea bass (Fig.3). We have previously reported a significant increase in both TRAP and ALP activities 24 h after injection of Gen in juvenile sea bass *in vivo.* Increases in circulating levels of E2 and vitellogenin (Vtg) and changes in

306 scale gene expression were also detected, including an up regulation of the *alp* gene 5 days' post-
307 injection (Pinto et al., 2016). Other estrogenic effects of phytoestrogens have been reported in fish,
308 mainly related to reproductive (e.g. Vtg synthesis) or growth and metabolism endpoints (Cleveland,
309 2014; Cleveland and Manor, 2015; Latonnelle et al., 2002; Sassi-Messai et al., 2009). Previous studies
310 also reported effects of these phytoestrogens on bone metabolism in mice (e.g. Dang and Lowik, 2004;
311 Piekarz and Ward, 2007). Our results combined with previously observed effects of Gen in sea bass *in*
312 *vivo* (Pinto et al., 2016) suggest that exposure to phytoestrogens may induce alterations in the activity
313 of enzymes associated with fish mineral homeostasis. However, while these endpoints appear to have
314 a low responsiveness to the *in vitro* treatment, the previously reported effects of Gen injection may
315 mainly result from indirect rather than direct actions on sea bass scales.
316
317 Several environmental pollutants of anthropogenic origin, previously shown to have estrogenic effects,
318 were also evaluated. They included bisphenol A, a compound widely used in a variety of plastics and
319 resins and previously shown to disrupt several physiological systems in fish and other organisms
320 (Canesi and Fabbri, 2015). BPA was also shown to bind and signal through nuclear ERs from mammals
321 to fish and through mammalian GPER (Canesi and Fabbri, 2015; Dang, 2010; Miyagawa et al., 2014;
322 Sanchez et al., 2016). BPA reduces TRAP and ALP activities in goldfish scales (Suzuki and Hattori,
323 2003), but had no significant effect on sea bass or tilapia scales in the present study.
324
325 Another widespread phenolic environmental contaminant tested was 4-tert-octylphenol (Sharma et al.,
326 2009; Ying et al., 2002). OP has previously been shown to bioaccumulate in juvenile rainbow trout
327 skin and bone (Ferreira-Leach and Hill, 2001) and estrogenic disruptive effects described in fish are
328 mainly related to reproductive endpoints such as Vtg production (Genovese et al., 2014). In mammals,
329 perinatal and postnatal OP exposure caused a reduction in bone growth width and ALP expression
330 (Kamei et al., 2008). In addition, OP can bind to fish ERα and β and it transactivates mammalian
331 nuclear ERα and β subtypes (Dang, 2010; Fu et al., 2007; Miyagawa et al., 2014). However, in the
332 present study no significant effects of OP on enzymatic activities of TRAP and ALP were observed in
333 sea bass and tilapia scales.
334
335 We tested the effects of an organotin biocide compound, triphenyltin, which is a persistent contaminant
336 of aquatic environments resulting from industrial or agricultural sources (reviewed in Yi et al., 2012).
337 TPT (10^{-6} M) significantly increased ($p<0.05$) ALP activity after 30 min exposure in tilapia (Fig.4),
338 while after 24 h it caused a significant increase ($p<0.05$) in sea bass ALP activity (Fig.3). Disruption
339 of ALP activity in fish scales had been previously reported for a related compound, tributyltin (TBT),

which caused a decrease after 6 and 18 h exposure in goldfish (Suzuki et al., 2006). In addition, both TPT and TBT disrupted bone metabolism in mouse cell lines (Watt and Schlezinger, 2015; Yonezawa et al., 2007). TPT bioaccumulates in fish and other aquatic organisms, inducing high toxicity, malformations and disrupting reproduction (Yi et al., 2012). TPT acts as an antagonist of E2 and blocks human ERβ transcriptional activity (Cho et al., 2012), although so far no interactions with GPERs or fish estrogen receptors have been described.

Finally, the effects of three phthalates (DBP, DEHP and BBP), that are persistent environmental pollutants and commonly used as plasticizers (Gao and Wen, 2016), were investigated on scale enzymatic activities. In sea bass, only DEHP (10^{-8} and 10^{-10} M) induced a significant increase in TRAP activity ($p<0.05$), at 30 min (Fig.3), while no effects were observed in ALP activity. In contrast, DEHP had no effect on tilapia scales (Fig.4) but a significant effect was observed for BBP (10^{-6} M), which decreased TRAP activity ($p<0.05$) after 24h. There are several studies indicating that exposure to phthalates in rodents causes fetal toxicity, evident as severe skeletal malformations and imbalance in bone homeostasis (reviewed in Agas et al., 2013). Here we report for the first time to our knowledge that two enzymes related to mineral homeostasis in fish bone may be impacted by phthalates. Although phthalates appear to be weak binders or activators of fish and mammalian ERs (Dang, 2010; Oehlmann et al., 2009), several estrogenic and anti-estrogenic effects have previously been detected, including on fish reproductive parameters (reviewed in Oehlmann et al., 2009). The molecular mechanisms of phthalate action may involve disruption of estrogen signalling pathways in fish scales through estrogen receptors or indirectly through other receptors (Wojtowicz et al., 2016).

In summary, the optimised *in vitro* scale bioassay was used to characterize estrogenic responses of sea bass and tilapia scales and to determine potential disruption of mineral turnover by pollutants not yet studied in mineralized tissues. The results indicated that exposure to exogenous estrogens through environmental pollution or through feeding in aquaculture (as is the case of phytoestrogens) can have an adverse impact not only on reproductive parameters but also on other systems. This *in vitro* fish scale assay is a promising non-invasive screening tool for E2 and EDC effects, complying with the 3Rs of animal welfare. However, technical limitations have to be overcome including the limited sensitivity of assays for TRAP and ALP activities. Alternative endpoints using non-invasive tools are under investigation. In terms of the mechanism of action of E2 or EDCs, both rapid (30 minutes) and longer-term (24 h) estrogenic responses were detected for the first time on the enzymatic activities associated with mineral turnover. These could be in part mediated through direct actions of these compounds on the membrane and nuclear estrogen receptors detected in scales of both species. Further

studies will be required to evaluate how disruption of mineralized tissues homeostasis contributes to the total outcome of exposure to these EDCs on fish health, which may be underestimated when analysing currently used endpoints.

Acknowledgments

This work was supported by the Foundation for Science and Technology of Portugal (FCT), through projects PTDC/AAG-GLO/4003/2012 and the infrastructure project CCMAR/Multi/04326/2013 and a fellowship to PP (SFRH/BPD/84033/2012). The authors acknowledge Dr Zelia Velez for technical contributions during assay optimization and Victor Mendes for technical assistance in some of the *in vitro* scale assays.

References

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., De Saeger, S., 2015. Scales tell a story on the stress history of fish. PLoS One 10, e0123411.

Agas, D., Sabbieti, M.G., Marchetti, L., 2013. Endocrine disruptors and bone metabolism. Arch. Toxicol. 87, 735-751.

An, J., Tzagarakis-Foster, C., Scharschmidt, T.C., Lomri, N., Leitman, D.C., 2001. Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J. Biol. Chem. 276, 17808-17814.

Bereiter-Hahn, J., Zylberberg, L., 1993. Regeneration of teleost fish scale. Comp. Biochem. Physiol. 105A, 625-641.

Brander, S.M., Connon, R.E., He, G., Hobbs, J.A., Smalling, K.L., Teh, S.J., White, J.W., Werner, I., Denison, M.S., Cherr, G.N., 2013. From 'omics to otoliths: responses of an estuarine fish to endocrine disrupting compounds across biological scales. PLoS One 8, e74251.

Canesi, L., Fabbri, E., 2015. Environmental effects of BPA: focus on aquatic species. Dose Response 13, 1-14.

Carnovali, M., Luzi, L., Banfi, G., Mariotti, M., 2016. Chronic hyperglycemia affects bone metabolism in adult zebrafish scale model. Endocrine 54, 808-817.

Cho, E.M., Lee, H.S., Moon, J.S., Kim, I.S., Sim, S., Ohta, A., 2012. Organotin compounds act as inhibitor of transcriptional activation with human estrogen receptor. J. Microbiol. Biotechnol. 22, 378-384.

Cleveland, B.M., 2014. *In vitro* and *in vivo* effects of phytoestrogens on protein turnover in rainbow trout (*Oncorhynchus mykiss*) white muscle. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 165, 9-16.

Cleveland, B.M., Manor, M.L., 2015. Effects of phytoestrogens on growth-related and lipogenic genes in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 170, 28-37.

Cos, P., De Bruyne, T., Apers, S., Vanden Berghe, D., Pieters, L., Vlietinck, A.J., 2003. Phytoestrogens: recent developments. Planta Med. 69, 589-599.

Dang, Z., 2010. Comparison of relative binding affinities to fish and mammalian estrogen receptors: the regulatory implications. Toxicol Lett 192, 298-315.

Dang, Z., Lowik, C.W., 2004. The balance between concurrent activation of ERs and PPARs determines daidzein-induced osteogenesis and adipogenesis. J. Bone Miner. Res. 19, 853-861.

de Vrieze, E., Zethof, J., Schulte-Merker, S., Flik, G., Metz, J.R., 2015. Identification of novel osteogenic compounds by an *ex-vivo* sp7:luciferase zebrafish scale assay. Bone 74, 106-113.
Esterhuyse, M.M., Helbing, C.C., van Wyk, J.H., 2010. Isolation and characterization of three estrogen receptor transcripts in *Oreochromis mossambicus* (Peters). J. Steroid Biochem. Mol. Biol. 119, 26-34.
Ferreira-Leach, A.M., Hill, E.M., 2001. Bioconcentration and distribution of 4-tert-octylphenol residues in tissues of the rainbow trout (*Oncorhynchus mykiss*). Mar. Environ. Res. 51, 75-89.
Fu, K.Y., Chen, C.Y., Chang, W.M., 2007. Application of a yeast estrogen screen in non-biomarker species *Varicorhinus barbatulus* fish with two estrogen receptor subtypes to assess xenoestrogens. Toxicol. In Vitro 21, 604-612.
Gao, D.W., Wen, Z.D., 2016. Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. Sci. Total Environ. 541, 986-1001.
Genovese, G., Regueira, M., Da Cuna, R.H., Ferreira, M.F., Varela, M.L., Lo Nostro, F.L., 2014. Nonmonotonic response of vitellogenin and estrogen receptor alpha gene expression after octylphenol exposure of *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquat. Toxicol. 156, 30-40.
Hawkins, M.B., Thomas, P., 2004. The unusual binding properties of the third distinct teleost estrogen receptor subtype ERβa are accompanied by highly conserved amino acid changes in the ligand binding domain. Endocrinology 145, 2968-2977.
Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R., 2006. Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. Environ. Health Perspect. 114 Suppl 1, 106-114.
Kamei, S., Miyawaki, J., Sakayama, K., Yamamoto, H., Masuno, H., 2008. Perinatal and postnatal exposure to 4-tert-octylphenol inhibits cortical bone growth in width at the diaphysis in female mice. Toxicology 252, 99-104.
Kitamura, K., Suzuki, N., Sato, Y., Nemoto, T., Ikegame, M., Shimizu, N., Kondo, T., Furusawa, Y., Wada, S., Hattori, A., 2010. Osteoblast activity in the goldfish scale responds sensitively to mechanical stress. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156, 357-363.
Latonnelle, K., Le Menn, F., Kaushik, S.J., Bennetau-Pelissero, C., 2002. Effects of dietary phytoestrogens *in vivo* and *in vitro* in rainbow trout and Siberian sturgeon: interests and limits of the *in vitro* studies of interspecies differences. Gen. Comp. Endocrinol. 126, 39-51.
Liu, Z.H., Kanjo, Y., Mizutani, S., 2010. A review of phytoestrogens: their occurrence and fate in the environment. Water Res. 44, 567-577.
Martins, R.S., Pinto, P.I., Guerreiro, P.M., Zanuy, S., Carrillo, M., Canario, A.V., 2014. Novel galanin receptors in teleost fish: identification, expression and regulation by sex steroids. Gen. Comp. Endocrinol. 205, 109-120.
Matsumoto, T., Kobayashi, M., Moriwaki, T., Kawai, S., Watabe, S., 2004. Survey of estrogenic activity in fish feed by yeast estrogen-screen assay. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 139, 147-152.
Miyagawa, S., Lange, A., Hirakawa, I., Tohyama, S., Ogino, Y., Mizutani, T., Kagami, Y., Kusano, T., Ihara, M., Tanaka, H., Tatarazako, N., Ohta, Y., Katsu, Y., Tyler, C.R., Iguchi, T., 2014. Differing species responsiveness of estrogenic contaminants in fish is conferred by the ligand binding domain of the estrogen receptor. Environ. Sci. Technol. 48, 5254-5263.
Norberg, B., Bjornsson, B.T., Brown, C.L., Wichardt, U.P., Deftos, L.J., Haux, C., 1989. Changes in plasma vitellogenin, sex steroids, calcitonin, and thyroid hormones related to sexual maturation in female brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. 75, 316-326.
Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 364, 2047-2062.

Olson, O.P., Watabe, N., 1980. Studies on formation and resorption of fish scales. IV. Ultrastructure of developing scales in newly hatched fry of the sheepshead minnow, *Cyprinodon variegatus* (Atheriniformes: Cyprinodontidae). Cell Tissue Res. 211, 303-316.

Pasqualetti, S., Banfi, G., Mariotti, M., 2012. The zebrafish scale as model to study the bone mineralization process. J. Mol. Histol. 43, 589-595.

Persson, P., Johannsson, S.H., Takagi, Y., Bjornsson, B.T., 1997. Estradiol-17β and nutritional status affect calcium balance, scale and bone resorption, and bone formation in rainbow trout, *Oncorhynchus mykiss.* J. Comp. Physiol., B: Biochem. Syst. Environ. Physiol. 167, 468-473.

Persson, P., Takagi, T., Björnsson, B.T., 1995. Tartrate resistant acid phosphatase as a marker for scale resorption in rainbow trout, *Oncorhynchus mykiss*: effects of estradiol-17β treatment and refeeding. Fish Physiol. Biochem. 14, 329 - 339.

Piekarz, A.V., Ward, W.E., 2007. Effect of neonatal exposure to genistein on bone metabolism in mice at adulthood. Pediatr Res. 61, 48-53.

Pinto, P.I., Estevao, M.D., Andrade, A., Santos, S., Power, D.M., 2016. Tissue responsiveness to estradiol and genistein in the sea bass liver and scale. J. Steroid Biochem. Mol. Biol. 158, 127-137.

Pinto, P.I., Estevao, M.D., Power, D.M., 2014. Effects of estrogens and estrogenic disrupting compounds on fish mineralized tissues. Mar Drugs 12, 4474-4494.

Pinto, P.I., Estevao, M.D., Redruello, B., Socorro, S.M., Canario, A.V., Power, D.M., 2009. Immunohistochemical detection of estrogen receptors in fish scales. Gen. Comp. Endocrinol. 160, 19-29.

Pinto, P.I., Passos, A.L., Martins, R.S., Power, D.M., Canario, A.V., 2006. Characterization of estrogen receptor βb in sea bream (*Sparus auratus*): phylogeny, ligand-binding, and comparative analysis of expression. Gen. Comp. Endocrinol. 145, 197-207.

Pinto, P.I., Thorne, M.A., Power, D.M., 2017. European sea bass (*Dicentrarchus labrax*) skin and scale transcriptomes. Marine Genomics, *In press* (Doi: 10.1016/j.margen.2017.05.002).

Sanchez, R.C., Gomez, R., Salazar, E.P., 2016. Bisphenol A Induces Migration through a GPER-, FAK-, Src-, and ERK2-Dependent Pathway in MDA-MB-231 Breast Cancer Cells. Chem. Res. Toxicol. 29, 285-295.

Sassi-Messai, S., Gibert, Y., Bernard, L., Nishio, S., Ferri Lagneau, K.F., Molina, J., Andersson-Lendahl, M., Benoit, G., Balaguer, P., Laudet, V., 2009. The phytoestrogen genistein affects zebrafish development through two different pathways. PLoS One 4, e4935.

Sharma, V.K., Anquandah, G.A., Yngard, R.A., Kim, H., Fekete, J., Bouzek, K., Ray, A.K., Golovko, D., 2009. Nonylphenol, octylphenol, and bisphenol-A in the aquatic environment: a review on occurrence, fate, and treatment. J. Environ. Sci. Health, Pt. A: Environ. Sci. Eng. Toxic Hazard. Subst. Control 44, 423-442.

Sire, J.-Y., Arnulf, I., 2000. Structure and development of the ctenial spines on the scales of a teleost fish, the cichlid *Cichlasoma nigrofasciatum*. Acta Zool. (Stockh.) 81, 139-158.

Suzuki, N., Hattori, A., 2002. Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. J. Pineal Res. 33, 253-258.

Suzuki, N., Hattori, A., 2003. Bisphenol A suppresses osteoclastic and osteoblastic activities in the cultured scales of goldfish. Life Sci. 73, 2237-2247.

Suzuki, N., Hayakawa, K., Kameda, T., Triba, A., Tang, N., Tabata, M.J., Takada, K., Wada, S., Omori, K., Srivastav, A.K., Mishima, H., Hattori, A., 2009. Monohydroxylated polycyclic aromatic hydrocarbons inhibit both osteoclastic and osteoblastic activities in teleost scales. Life Sci. 84, 482-488.

Suzuki, N., Kitamura, K., Nemoto, T., Shimizu, N., Wada, S., Kondo, T., Tabata, M.J., Sodeyama, F., Ijiri, K., Hattori, A., 2007. Effect of vibration on osteoblastic and osteoclastic activities: Analysis of bone metabolism using goldfish scale as a model for bone. Adv. Space Res. 40, 1711-1721.

Suzuki, N., Suzuki, T., Kurokawa, T., 2000. Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). Peptides 21, 115-124.

Suzuki, N., Tabata, M.J., Kambegawa, A., Srivastav, A.K., Shimada, A., Takeda, H., Kobayashi, M., Wada, S., Katsumata, T., Hattori, A., 2006. Tributyltin inhibits osteoblastic activity and disrupts calcium metabolism through an increase in plasma calcium and calcitonin levels in teleosts. Life Sci. 78, 2533-2541.

Thomas, P., Alyea, R., Pang, Y., Peyton, C., Dong, J., Berg, A.H., 2010. Conserved estrogen binding and signaling functions of the G protein-coupled estrogen receptor 1 (GPER) in mammals and fish. Steroids 75, 595-602.

Thomas, P., Dong, J., 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J. Steroid Biochem. Mol. Biol. 102, 175-179.

Watt, J., Schlezinger, J.J., 2015. Structurally-diverse, PPARgamma-activating environmental toxicants induce adipogenesis and suppress osteogenesis in bone marrow mesenchymal stromal cells. Toxicology 331, 66-77.

Wojtowicz, A.K., Szychowski, K.A., Wnuk, A., Kajta, M., 2016. Dibutyl Phthalate (DBP)-induced apoptosis and neurotoxicity are mediated via the Aryl Hydrocarbon Receptor (AhR) but not by Estrogen Receptor Alpha (ERα), Estrogen Receptor Beta (ERβ), or Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) in mouse cortical neurons. Neurotox. Res., 1-13.

Yachiguchi, K., Sekiguchi, T., Nakano, M., Hattori, A., Yamamoto, M., Kitamura, K., Maeda, M., Tabuchi, Y., Kondo, T., Kamauchi, H., Nakabayashi, H., Srivastav, A.K., Hayakawa, K., Sakamoto, T., Suzuki, N., 2014. Effects of inorganic mercury and methylmercury on osteoclasts and osteoblasts in the scales of the marine teleost as a model system of bone. Zool. Sci. 31, 330-337.

Yi, A.X., Leung, K.M., Lam, M.H., Lee, J.S., Giesy, J.P., 2012. Review of measured concentrations of triphenyltin compounds in marine ecosystems and meta-analysis of their risks to humans and the environment. Chemosphere 89, 1015-1025.

Ying, G.G., Williams, B., Kookana, R., 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates-a review. Environ. Int. 28, 215-226.

Yonezawa, T., Hasegawa, S., Ahn, J.Y., Cha, B.Y., Teruya, T., Hagiwara, H., Nagai, K., Woo, J.T., 2007. Tributyltin and triphenyltin inhibit osteoclast differentiation through a retinoic acid receptor-dependent signaling pathway. Biochem. Biophys. Res. Commun. 355, 10-15.

Yoshikubo, H., Suzuki, N., Takemura, K., Hoso, M., Yashima, S., Iwamuro, S., Takagi, Y., Tabata, M.J., Hattori, A., 2005. Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. Life Sci. 76, 2699-2709.

Legends

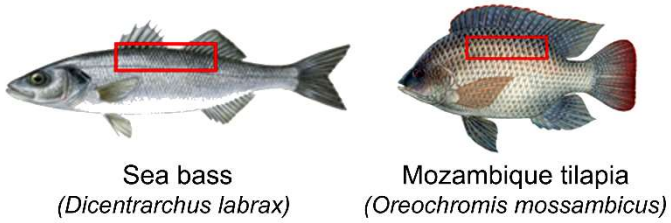
Figure 1. Area of the fish body selected for scale sampling. The sampled area is represented for both sea bass and Mozambique tilapia.

Figure 2. Expression of estrogen receptor transcripts in sea bass and tilapia scales. mRNA expression of nuclear (*esr1*, *esr2a* and *esr2b*) and membrane G protein-coupled (*gper* and *gperl*) estrogen receptors was determined by RT-PCR in scales of sea bass and tilapia (n=3 individuals per species, each presented in different columns). 18S ribosomal RNA was used as the reference gene.

Figure 3. Changes in TRAP/ALP activities in sea bass scales in response to selected compounds. Enzymatic activities are represented for scales exposed to 10^{-6}, 10^{-8} and 10^{-10} M of estradiol (E2), genistein (Gen), triphenyltin (TPT) and Bis-(2-ethylhexyl) phthalate (DEHP). * indicates statistical significance at $p<0.05$, determined by two-way ANOVA *versus* the control at the respective time.

Figure 4. Changes in TRAP/ALP activities in tilapia scales in response to selected compounds. Enzymatic activities are represented for scales exposed to 10^{-6}, 10^{-8} and 10^{-10} M of estradiol (E2), genistein (Gen), triphenyltin (TPT) and Bis-(2-ethylhexyl) phthalate (DEHP). * indicates statistical significance at $p<0.05$, determined by two-way ANOVA *versus* the control at the respective time.

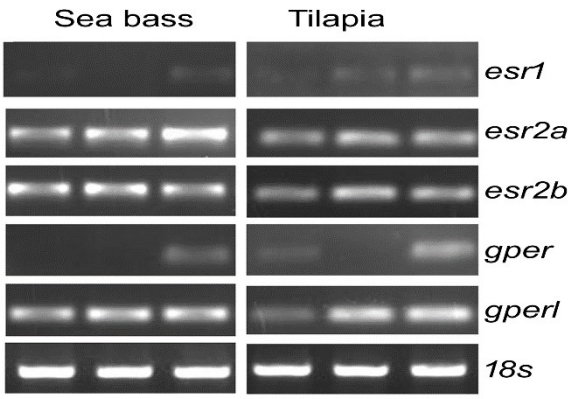
Figure 1



(This figure is intended to be reproduced in colour on the web and in black-and-white in print)

584 **Figure 2**

585

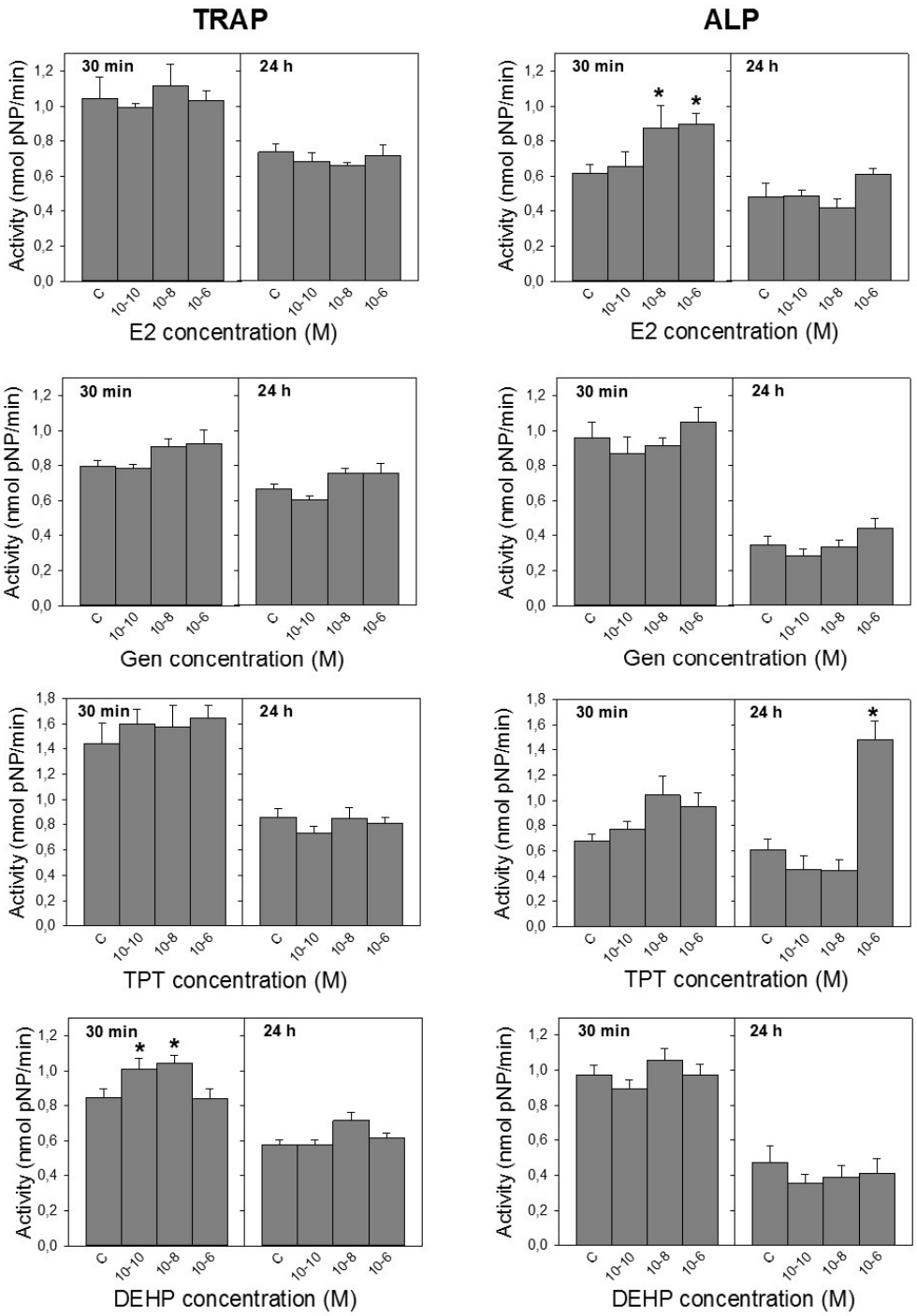


586

587 **Figure 3**

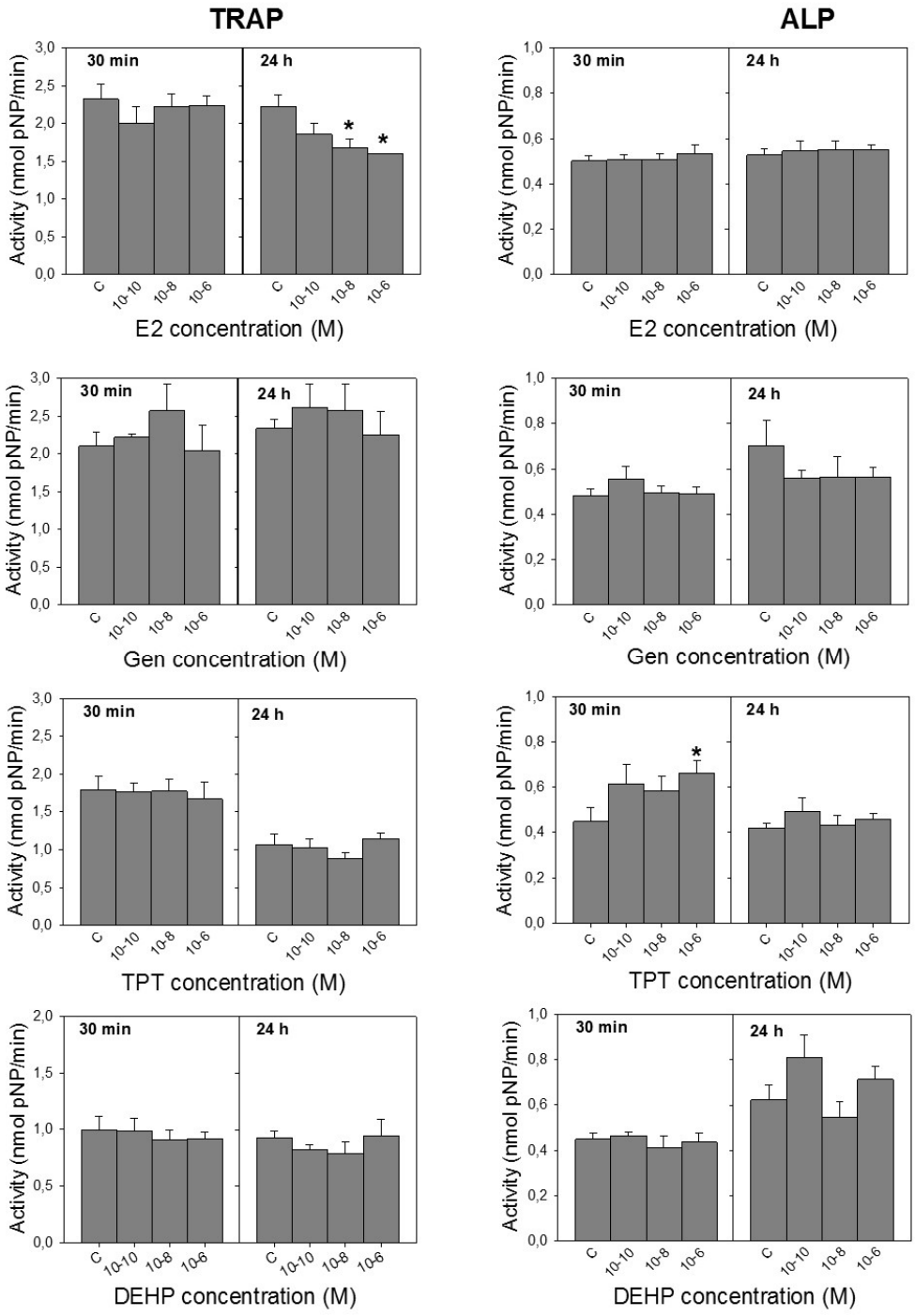
588

589



590

591 **Figure 4**



592

593

594

595

596 **Tables**

597 **Table 1.** Summary of the effects of *in vitro* incubations of scales from sea bass with E2 and tested
598 EDCs. Compounds were tested for 30 min or 24 h, at 10^{-6}, 10^{-8} or 10^{-10} M, and effects measured on
599 scale TRAP and ALP enzymatic activities. Arrows indicate significant increases (↑) or decreases (↓);
600 nc indicates that no change was observed. Results were considered statistically significants at $p<0.05$,
601 determined by two-way ANOVA *versus* the control group at each incubation time. Estradiol (E2),
602 genistein (Gen), daidzein (Dai), bisphenol A (BPA), 4-tert-octylphenol (OP), triphenyltin (TPT),
603 dibutyl phthalate (DBP), bis-(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP).
604
605

	TRAP		ALP	
Compounds	**30 min**	**24 h**	**30 min**	**24 h**
E2	nc	nc	↑ 10^{-6}; 10^{-8} M	nc
Gen	nc	nc	nc	nc
Dai	nc	nc	nc	nc
BPA	nc	nc	nc	nc
OP	nc	nc	nc	nc
TPT	nc	nc	nc	↑ 10^{-6} M
DBP	nc	nc	nc	nc
DEHP	↑10^{-8}; 10^{-10} M	nc	nc	nc
BBP	nc	nc	nc	nc

606
607

608 **Table 2.** Summary of the effects of *in vitro* incubations of scales from tilapia with E2 and tested EDCs.
609 Compounds were tested for 30 min or 24 h, at 10^{-6}, 10^{-8} or 10^{-10} M, and effects measured on scale
610 TRAP and ALP enzymatic activities. Arrows indicate significant increases (↑) or decreases (↓); nc
611 indicates that no change was observed. Results were considered statistically significants at $p<0.05$,
612 determined by two-way ANOVA *versus* the control group at each incubation time. For abbreviation
613 of compound names see Table 1.
614
615

	TRAP		ALP	
Compounds	**30 min**	**24 h**	**30 min**	**24 h**
E2	nc	↓ 10^{-6};10^{-8} M	nc	nc
Gen	nc	nc	nc	nc
Dai	nc	nc	nc	nc
BPA	nc	nc	nc	nc
OP	nc	nc	nc	nc
TPT	nc	nc	↑ 10^{-6} M	nc
DBP	nc	nc	nc	nc
DEHP	nc	nc	nc	nc
BBP	nc	nc	nc	↓ 10^{-6} M