Author's version (accepted manuscript):

Genistein and estradiol have common and specific impacts on the sea bass (*Dicentrarchus labrax*) skin-scale barrier

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ABSTRACT

Teleost fish scales play important roles in animal protection and homeostasis. They can be targeted by endogenous estrogens and by environmental estrogenic endocrine disruptors. The phytoestrogen genistein is ubiquitous in the environment and in aquaculture feeds and is a disruptor of estrogenic processes in vertebrates. To test genistein disrupting actions in teleost fish we used a minimally invasive approach by analysing scales plucked from the skin of sea bass (*Dicentrarchus labrax*). Genistein transactivated all three fish nuclear estrogen receptors and was most potent with the Esr2, had the highest efficacy with Esr1, but reached, in all cases, transactivation levels lower than those of estradiol. RNA-seq revealed 254 responsive genes in the sea bass scales transcriptome with an FDR<0.05 and more than 2-fold change in expression, 1 or 5 days after acute exposure to estradiol or to genistein. 65 genes were specifically responsive to estradiol and 106 by genistein while 83 genes were responsive to both compounds. Estradiol specifically regulated genes of protein/matrix turnover and genistein affected sterol biosynthesis and regeneration, while innate immune responses were affected by both compounds. This comprehensive study revealed the impact on the fish scale transcriptome of estradiol and genistein, providing a solid background to further develop fish scales as a practical screening tool for endocrine disrupting chemicals in teleosts.

Keywords: estradiol; estrogen-responsive genes; fish scale; genistein; transcriptome

Abbreviations: E₂, estradiol; Esr1, estrogen receptor 1; Esr2a, estrogen receptor 2a; Esr2b, estrogen receptor 2b; Gen, genistein; Gper, G-protein-coupled estrogen receptor; For gene name abbreviation see Supplementary Table S2.

1. Introduction

Teleost fish scales are specialized mineralized appendages that contribute to the effectiveness of fish skin as a mechanical, osmotic and innate immune barrier. The scales are also a dynamic mineral reservoir in teleost fish and like mammalian bone are mobilized in periods of increased calcium demand such as during reproduction, partly as a result of the hypercalcaemic action of estradiol (E_2) on the scales [1-3]. E_2 can also regulate mineral deposition in scales and has a positive effect on skin-scale regeneration [4, 5].

Estrogens can have multiple modes of action that include regulation of 1) responsive-gene transcription, classically associated with nuclear estrogen receptors (Esrs), 2) rapid non-genomic effects mainly mediated by G-protein coupled estrogen receptors (Gpers) or, 3) indirect effects via other transcription factors and/or signalling cascades, that may cross-talk to achieve the final cellular response [3]. The expression of nuclear Esrs at both transcript and protein levels explains the responsiveness of fish scales to (E_2 [3, 6-8]. Recently, duplicate Gper paralogues were found to be expressed in European sea bass (*Dicentrarchus labrax*) and Mozambique tilapia (*Oreochromis mossambicus*) scales [7-9] indicating that fish scales are estrogen targets through both classical genomic actions and rapid non-genomic actions [3].

The expression of Esrs and Gpers in fish scales identifies them as potential targets also for estrogenic endocrine-disrupting compounds (EDCs) [3]. These are exogenous compounds from natural or anthropogenic sources to which humans and wildlife can be exposed and which interfere with the normal function of the estrogen system at different levels, causing deleterious effects to health and reproduction [3]. Among the EDCs, phytoestrogens are consumed (and excreted) in large quantities by people and herbivorous animals worldwide and are released by plant-processing industries, attaining high levels in municipal or industrial wastewater influents and effluents and in surface waters [reviewed in 10]. Aquatic organisms are particularly vulnerable to pollutant exposure and in aquaculture the increased use of soybean and other plant-based sources of protein make fish meal an additional exposure route to phytoestrogens, one of the most abundant being genistein (Gen) [11]. Agonistic and antagonistic estrogenic effects of Gen have been demonstrated *in vitro* and *in vivo* from mammals to fish [7, 10, 12-16].

The skin of fish is the largest tissue directly in contact with water and expresses receptors for endocrine factors such as the estrogens, making it an important site for their action and a potential early indicator tissue for EDC exposure. The abundance of fish scales, their superficial location in the skin, the ease of collection and rapid regeneration makes them a useful model for assessing environmental contaminants, hormones, estrogenic drugs, stress or mechanical load, although a limited number of endpoints have been tested [3, 5, 8, 17-21]. A fully validated fish scale model

therefore, would be a biologically relevant system for pollutant screening that provides a reliable route for reduction, recycling and refining in accordance with “3R” principles of animal experimentation [3, 22].

Here we used the European sea bass, an important species in European aquaculture and fisheries, with a well-annotated genome and a representative of the species-rich order Perciformes [23, 24] to develop screening tools for estrogenic disruption using the scales. We tested the hypothesis that the scale is a target for the phytoestrogen Gen in a similar way to E₂. We show that in transactivation assays the three fish nuclear estrogen receptors Esr1, Esr2a and Esr2b are activated by Gen, as was previously shown for E₂. Global transcriptomic analyses showed that E₂ and Gen trigger both common and specific processes and pathways in fish scales. These results demonstrate the potential of scales for screening EDCs and possibly other contaminants using scales from *in vivo* exposed fish and provides the background knowledge for development of more sensitive endpoints for *in vitro* scales bioassays.

2. Material and Methods

2.1 Transactivation assays

17 β -estradiol (E₂) and genistein (Gen) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in ethanol, which did not exceed a concentration of 0.1 % in culture media. To study the interactions between genistein and the sea bass nuclear estrogen receptors, transactivation assays were carried out as described in Zapater et al [25], in Human Embryonic Kidney 293 cells (HEK293, ATCC reference CRL-1573), devoid of nuclear Esrs. Cells were co-transfected with 250 ng of pERE-TK-Luc and with one of the nuclear receptors (Esr1:100 ng; Esr2a:120 ng; Esr2b:120 ng) in the pcDNA3 expression vector or 100 ng of empty pcDNA3, using lipofectamine 3000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. After approximately 16 h of incubation, the medium was changed to phenol-red free Dulbecco's modified Eagle's medium/F12 medium (Sigma-Aldrich) supplemented with charcoal-stripped FBS and cells were re-plated in 96-well plates. Following 5 h incubation, cells were exposed to different concentrations of Gen (1, 25, 50, 75, 100 and 125 nM), chosen based on preliminary tests, and to 10 nM of E₂, used as positive control based on previous transactivation studies with sea bass Esrs [25, 26]. After 24 h incubation, the medium was removed and cells were lysed in 20 μ L of Passive Lysis Buffer (Promega, Madison, Wisconsin, USA) as indicated by the manufacturer. Cell debris were collected with a brief spin and luminescence was measured by mixing the supernatant with 100 μ L of luciferin reagent (20 mM Tricine KOH, pH 7.8, 0.1 mM EDTA, 8 mM MgCl₂, 33.3 mM DTT, 270 μ M CoA, 530 μ M ATP, 400 μ M luciferin) in a luminometer (Junior, EG&G, Berthold, Bad Wildbad,

Germany). All transfections were performed twice with quadruplicate measurements in each experiment. Differences between treatments were evaluated using one-way ANOVA followed by Tukey test, with $p < 0.05$. No differences in luciferase activity were found when exposing the empty expression vector pcDNA3 to different concentrations of Gen or E₂.

2.2 Animals, injection experiment and RNA isolation

Animal maintenance and experimentation complied with the EU directive on animal experimentation (EU Directive 2010/63/UE) transcribed to national legislation (DL 113/2013). The experimental design of the fish exposure experiment has previously been described in detail [7]. Immature sea bass were anesthetized, weighted and measured (59.4 ± 0.7 g and 18.25 ± 0.43 cm) and given intraperitoneal (i.p.) injections of 5 mg/kg of E₂ or 5 mg/kg Gen in coconut oil or with coconut oil alone (control). After 1 or 5 days treatment, fish were anesthetized and sacrificed ($n = 10$ fish/group), individual scales were plucked with forceps from approximately the same position in each fish (first 2–3 rows below the dorsal fin), immediately frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted from 15 scales/fish using an automated Maxwell 16 Instrument and a SEV total RNA purification kit (Promega, UK), after mechanical disruption with an Ultra Turrax homogenizer equipped with a dispersing element for fibrous tissues (IKA, Staufen, Germany). RNA was ethanol precipitated and RNA quantity and quality were determined in a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA (3–10 µg) was treated with DNase using a DNA-free kit (Ambion, Thermo Fisher) and two sequential digestion treatments of 30 min with 2U TURBO DNase.

2.3 Illumina sequencing and quality control

An RNA-seq library were prepared using a TruSeq mRNA library preparation kit (Illumina, San Diego, California, USA) with 0.5 µg scale total RNA per library. All RNA had an RNA integrity number above 8 as determined with a Bioanalyser 2100 (Agilent Technologies, Santa Clara, California, USA). Paired-end (100 bp) sequencing of 30 libraries was performed using an Illumina Hi-Seq 1500 by the Shanghai Ocean University Sequencing Service. FastQC and Cutadapt [27, 28] were used for quality control and trimming, using a cut-off Phred quality score of 20.

2.4 Data availability

Raw reads from the thirty RNA-seq libraries from fish scales were deposited in the Short Read Archive under accession number SRP102504. Assembled contigs from the whole transcriptome of sea bass scales are available at the Transcriptome Shotgun Assemblies of Genbank with project number GFJX000000000.

2.5 Reference-based assembly and differential expression

The procedures and parameters used to identify and annotate differentially expressed (DE) genes are described in more detail in Pinto et al., co-submitted paper in Data in Brief (DIB). Briefly, the Cufflinks/TopHat package [29, 30] was used with default parameters: 1) to map reads to the sea bass reference genome [23, 31]; 2) to assemble the transcripts; 3) to determine relative expression values (in fpkm, fragments per kilobase of transcript per million fragments mapped) and 4) to determine differential gene expression between experimental conditions. Comparisons of gene expression levels between treated and control groups were performed for each experimental time or between control groups over time, using an initial threshold of significance for a false discovery rate of 5 % (FDR<0.05). In a second step, a minimum 2-fold change was set. Comparisons between the DE gene lists were established using area proportional Venn diagrams created using BioVenn [32, 33] and bar and scatter plots were prepared in SigmaPlot (v.12.0, Systat Software, San Jose, California, USA). DE genes were clustered according to their change in expression in response to E₂ or Gen (up or down-regulated $\geq$ 2-fold after 1d or 5d) and the correspondence between clusters at each sampling time was presented using Bubble Plots in SigmaPlot. Gene expression in selected clusters with the highest number of DE genes (clusters A-H) was represented as heat maps generated with an in-house R script.

2.6 Annotation

The full-list of expressed transcript sequences in scales (35,214 transcripts extracted from the sea bass genome sequence using Cufflinks mapping information) was compared to the assembled transcripts from the recently released sea bass scale transcriptome [34] using stand-alone BlastN, with an E value < 10⁻⁵⁰. A multistep automatic gene annotation was carried out for the 749 DE genes, which included stand-alone Blastx of Cufflinks transcripts against the Swiss-Prot [35, 36] and GenBank protein databases [37, 38], setting the expect values (E) to <10⁻¹⁰, and comparing with the Cufflinks mapping to annotated sea bass genes [23, 31]. The hierarchical preference order to assign annotation matches to the DE genes was: Swiss-Prot hits > sea bass genome hits > GenBank hits (Fig. 1 in the associated DIB paper). This global annotation procedure was optimized by manual curation of 54 % of the 332 DE genes with $\geq$ 2-fold change (those with ambiguous annotations and those analysed by qPCR), by running individual BLAT versus the sea bass genome and BlastX of these and the predicted coding sequences of nearby predicted genes versus Swiss-Prot and GenBank, followed by multisequence alignments using Multialin [39, 40]. In addition, a high concordance was found for the 209 genes that were annotated (DIB Fig. 1) with the different databases: 78 % received the same protein name, 12 % matched proteins from the same family or with alternative names and the 10 % with discordant annotations, were revised by manual curation of the annotation.

2.7 Functional annotation

Gene ontology (GO) and pathway (Kyoto Encyclopedia of Genes and Genomes, KEGG) enrichment analyses were carried out using Cytoscape v3.5.1 [41] and ClueGO plug-in v2.3.2 [42], with Cluepedia v1.3.2, using the Ensembl zebrafish (*Danio rerio*) orthologues [43, 44] for the 371 genes found to be DE with E₂ or Gen treatments at FDR<0.05 [45], obtained by Blastx setting the E value to <10⁻¹⁰. The analysed lists were named “All”=all genes regulated by E₂ and/or Gen; “E₂” or “Gen”= genes regulated by either treatment, irrespective of the sampling time and “1d” or “5d”= genes regulated after 1 or 5 days by either E₂ or Gen). Enrichment analyses in GO Biological Processes (GO BP) and KEGG pathways were then carried out using the Cytoscape/ClueGO plug-in using the parameters detailed in the DIB paper. Enrichment scores for each functionally related network group was calculated as -Log₂ (group FDR) and leading terms for each group (used for group naming in the tables and in the condensed bar plot representations) were those with lowest term FDR / highest enrichment score. When duplicated group names were found, only the term with the highest enrichment score was represented.

2.8 Quantitative analysis of gene expression

The expression level of ten DE RNA-seq selected genes, six mineral turnover related genes and one steroid synthesis related gene (Suppl. Table S1) was analysed by quantitative RT-PCR (qPCR) in n=10 individual sea bass scale cDNAs per experimental condition (same five individuals used in RNA-seq and five additional biological replicates). RNA-seq DE genes were selected to cover a wide range of transcript abundance and differential expression patterns and to include genes from some of the most enriched biological processes.

qPCR was performed as previous described [7], using the relative standard curve method, a Bio-Rad iCycler iQ5 qPCR thermocycler and EvaGreen chemistry, in duplicate 10 µL reactions containing 1x Sso Fast EvaGreen Supermix (Bio-Rad, Hercules, California, USA), 2 µL of each individual cDNA (diluted 1:5) and 300 nM of each specific primer at the optimized annealing temperatures (Suppl. Table S1). All qPCR reactions had single peak melt curves and primer specificity was confirmed by sequencing all amplicons. No amplification products were obtained from cDNA syntheses without reverse transcriptase 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 for quantification and determination of efficiency, which ranged between 91-105 % with R² > 0.99. The determined gene transcript expression levels were normalized by dividing calculated gene copy number by the geometric mean of two stable reference genes (18S ribosomal RNA sub-unit and elongation factor 1α (EF1α) [7]. 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). Statistical significance between treatments at each sampling time was analysed by one-way ANOVA followed by a Tukey test. Pearson correlations between the qPCR data (average expression for each experimental group) and the relative expression levels obtained by RNA-seq (fpkm) were calculated for each gene after log₂ transformation of both variables. Significance levels were set as $p < 0.05$.

2.9 Scale mineral content

One or two scales per fish (sampled after 5 days of E₂, Gen or control treatment) were washed three times in milli-Q water, dried at 105 °C for 2 h (time previously optimized to achieve constant weight) [46] and digested in 100 ml HNO₃/mg scale for 48 h with agitation, at room temperature. Scale mineral content was determined using an Agilent Microwave Plasma-Atomic Emission Spectrometer (MP-AES), model 4200 (Agilent Technologies). Calcium, magnesium and sodium concentrations were measured in each sample after dilution (1:300 for Ca and 1:30 for Mg and Na) with acidic water (5 % HNO₃) using a standard curve ranging between 0.5 and 10 ppm (prepared using Calibration Mix Majors for Ca, Fe, K, Mg and Na; Agilent 6610030700). Running parameters for MP-AES were number of replicates 5, pump rate 15 rpm, sample uptake time 70 sec, rinse time 40 sec, stabilization time 15 sec and “fast pump during uptake” and “rinse time fast pump” in mode “on”. Mineral contents were measured at a wavelength of 393.4 nm, 383.8 nm and 589.0 nm, for Ca, Mg and Na, respectively, and expressed as mmol/mg.

3. Results

3.1 Genistein activates sea bass estrogen receptors

Both Gen and E₂ transactivated sea bass Esrs co-transfected in HEK293 cells with a plasmid containing estrogen-responsive elements (ERE) driving the expression of a luciferase reporter gene (pERE-TK-Luc). All Esrs were activated by 10 nM E₂ and Gen transactivated all three Esrs at a minimal concentration of 1, 25 and 75 nM for Esr2a, Esr2b and Esr1, respectively (Fig. 1). Gen had greater potency for Esr2a transactivation than Esr2b. The potency of Gen for Esr1 transactivation was lowest but it had the highest efficacy (reaching higher luciferase activity). Significantly increased transactivation occurred up to 125 nM for Esr2b, while at this higher concentration the transactivation of Esr1 and Esr2a was not different from the control. None of the tested concentrations for Gen reached significantly higher transactivation levels than the positive control E₂ at 10 nM.

Figure 1. Transactivation of sea bass nuclear estrogen receptors by genistein. Induction of luciferase activity was measured in human embryonal kidney 293 (HEK293) cells co-transfected with a pERE-TK-Luc reporter plasmid and the pcDNA3 vector expressing the receptor Esr1, Esr2a or Esr2b and then exposed to genistein (Gen) or 17 β -estradiol (E₂). Induction is expressed relative to the control (not exposed to E₂ or Gen), which was set as 1. Bars represent the mean $\pm$ SEM of quadruplicate exposures of two independent assays. Different letters above each bar represent significantly different groups ($p < 0.05$) using ANOVA followed by Tukey's multiple comparisons test.

3.2 Differential transcriptomics of genistein- and estradiol-treated scales

Thirty Illumina RNA-seq libraries were constructed from six experimental conditions: scales sampled 1 day after intra-peritoneal injection with coconut oil alone (control, C1d) or injection of coconut oil containing estradiol (E1d) or genistein (Gen1d), and scales from equivalent treatments sampled after

5 days (respectively, C5d, E5d and Gen5d). Sequencing produced 1,132 million (M) raw reads, with a similar read number between replicate libraries (average $37.7\text{M} \pm 0.99\text{ SEM}$), as can be observed in the co-submitted Data in Brief (DIB) article (Pinto et. al., Table 1) to the, that contains the full details of the produced RNA-seq data. A similar number of reads was also found between treatments ($188.6\text{ M} \pm 6.05\text{ SEM}$; Table 1A). After quality filtration 97 % of the reads were retained and approximately 86 % were mapped using the TopHat-Cufflinks pipeline to the *D. labrax* genome [23].

Table 1

Summary of RNA-seq sequencing (A) and annotation (B) statistics. A- The number of total and average reads obtained are summarized per treatment, after combining n=4-6 libraries (biological replicates) each, based on the detailed values for each library presented in the DIB Table 1. B- The final rates of annotation obtained for the 332 genes showing ≥ 2 -fold change differential expression are also shown (detailed results for the multi-step automatic and manual annotation of all differentially expressed transcripts in the DIB Table 4).

A- Sequencing statistics						
Treatment	Exposure (days)	Code	No. libraries	Raw reads (millions)	Filtered reads (millions)	%Mapped (average)
Control	1	C1d	6	204.9	201.6	86.1
Estradiol	1	E1d	5	177.5	174.3	84.7
Genistein	1	Gen1d	5	194.0	189.5	84.7
Control	5	C5d	5	178.7	178.7	86.5
Estradiol	5	E5d	5	205.3	187.5	87.8
Genistein	5	Gen5d	4	171.2	171.2	85.6
Average			5	188.6	183.8	
Total			30	1,131.7	1,102.8	

B- Annotation statistics		Number of genes	%
Total annotated		314	94.6
Annotation to known proteins		280	84.4
Annotation to predicted proteins		12	3.6
Annotation to hypothetical/uncharacterized proteins		14	4.2
Mapping to non-annotated genes		8	2.4
Non-annotated		18	5.4
Total		332	100.0

A total of 35,214 different transcripts (corresponding to 22,834 annotated genes) were expressed in sea bass scales. Sixty three percent of these transcripts, globally obtained from the thirty replicate libraries (from control, E₂- or Gen-treated fish scales) matched 98 % of the previously released transcriptome obtained from a pool of scale RNA from non-manipulated fish from the same

experiment [34]. The remaining 37 % unmatched transcripts were identified for the first time in scales and their expression may be associated with the E₂ or Gen exposures.

After normalizing expression per experimental group, a total of 749 differentially expressed (DE) genes were identified in pairwise comparisons between a) the scales of E₂- or Gen-treated fish compared to control fish at the same sampling time or b) between the control groups over time (DIB Figure 2). 332 DE genes were identified when the stringency was increased to ≥ 2 -fold change in expression, the majority of which (254 genes, 76 %) were significantly responsive to E₂ or Gen exposure and were selected for further analyses. DIB Table 2 contains the full list with the expression level and annotation of the 332 responsive genes, while supplementary table S2 in this manuscript includes only the subset of genes from selected clusters (see below), in which the discussion is focused. The remaining genes (< 2 -fold changes, FDR < 0.05), only used for the global enrichment analyses, are presented in DIB Table 3.

A multistep (automatic and manual) annotation pipeline annotated 96 % of the DE genes (DIB Table 4). The used preference order strategy (Swiss-Prot>Genome>GenBank; DIB Figure 1) led to 79 % DE gene annotations based on their match with proteins in the curated Swiss-Prot database [35], followed by 12 % that were assigned to annotated genes in the sea bass genome [23] and annotation of the remaining 5 % of the genes via GenBank (DIB Table 4). A similar annotation success rate of 95 % was obtained for the 332 " ≥ 2 -fold DE" genes (Table 1B and DIB Figure 1). Manual verification of half of these matches confirmed the consistency of the automatic annotation strategy (DIB Table 2) and in most cases Swiss-Prot followed by genome annotations gave the most reliable annotation. After the optimized multistep annotation 84.4 % of the 332 DE genes could be assigned to known proteins in public databases and 10.2 % were annotated to predicted or non-annotated proteins or genes (Table 1B).

Of all the 132 genes that changed expression 1 day after acute exposure the majority (72) were responsive to Gen, 32 were responsive to E₂ and 28 were responsive to both compounds (Fig. 2A). A similar number of genes increased or decreased expression with Gen exposure, while E₂ predominantly down-regulated gene transcripts (Fig. 2B). Five days after acute exposure to E₂ and Gen, gene expression was predominantly increased and 60 and 56 genes were specifically responsive to E₂ and Gen, respectively (Fig. 2A). A greater number of genes (56) were co-responsive to E₂ and Gen 5 days after treatment, compared to 1 day.

Figure 2. **A-** Venn diagrams representing the number of common and specific genes regulated by 17 β -estradiol (E₂) and/or genistein (Gen) compared to the control, with fold changes ≥ 2 , at both experimental times. **B-** Number of genes up (red) or down (green) regulated by E₂ or Gen at each sampling period. **C-** Pearson correlations of expression levels from genes that were responsive to both E₂ and Gen versus the control (C) at 1 and 5 days, in Log₂ of fold change (FC) for each comparison. Statistically significant Pearson correlations were found at 1 day ($p < 1 \times 10^{-10}$) and 5 days ($p < 1 \times 10^{-32}$) and the correlation coefficients (r) and number of genes (n) are indicated in each graph; no genes were found within shaded areas, representing antagonistic effects. Similar and correlated Log₂ FC ranges were found for the responses to E₂ (up-regulation: average Log₂ FC = 2.1 ± 0.1 , down-regulation: -1.8 ± 0.1) and to Gen (up-regulations: average Log₂ FC = 2.2 ± 0.1 , down-regulation: -1.8 ± 0.1) for the co-responsive genes, irrespective of the sampling time, with no statistically significant differences found between the magnitudes of response measured by the Log₂ FC.

Interestingly, E₂ and Gen had a similar effect on the co-responsive genes, which were either up- or down-regulated by both treatments and had a significant positively correlated fold-change (Fig. 2C). No gene transcripts were identified for which the E₂ or Gen treatment provoked an antagonistic effect 1 or 5 days after treatment (Fig. 2C). Hierarchical clustering confirmed that 1 day after treatment the E₂ treatment clustered more closely to the control (DIB Figure 3) than Gen1d. After 5 days, both E₂- and Gen-treated scales were clearly separated from the control and clustered closely together in the treatments heatmap, confirming they provoked a more similar response in the long term (DIB Fig. 3).

3.3 Enriched biological processes and pathways and gene expression patterns clustering

Cytoscape/ClueGO analysis revealed a significant enrichment for 108 biological processes (according to the Gene Ontology classification, GO-BP) among the 371 genes that changed expression with E₂ or Gen treatments. Enriched processes were clustered into 23 main groups, the most significant of which were “response to external biotic stimulus”, “sterol biosynthetic process”, “response to yeast” and “regeneration” (Fig. 3A and DIB Table 5). Seven groups of the KEGG pathway classification were also enriched, with higher significance for “steroid biosynthesis” and “ECM-receptor interaction” (DIB Table 6).

Comparison of enrichment networks between the E₂ and Gen treatments, revealed the “response to oxidative stress” was enriched in both but “cell-substrate adhesion” and “lymph vessel development” were specifically enriched by E₂ while the remaining ClueGO groups were mainly enriched in response to Gen (Fig. 3B and DIB Table 7). The enriched groups “regeneration” and “inactivation of MAPK activity” contained some terms also enriched by E₂ or common between treatments. The top enriched processes (related to responses to external stimuli, sterol biosynthesis and regeneration) were specific to Gen, with much higher enrichment scores compared to E₂-enriched processes (Fig. 3B). When analysing KEGG enrichment outputs (DIB Table 8), the “p53 signalling pathway” was common to both treatments; “ECM-receptor interaction” and “proteasome” was specifically enriched by E₂ and “steroid biosynthesis”, “alanine, aspartate and glutamate metabolism” and “DNA replication” were enriched by Gen.

Finally, analysing lists of DE genes by their window of response (irrespective of the treatment), “response to yeast”-related processes were enriched at both sampling times (Fig. 3C and DIB Table 9). Specific responses after 1 day included “sterol biosynthesis” and “regeneration”. The highest enrichment occurred for “responses to external biotic stimulus”, specifically after 5 days of treatment, when specific enrichment was also found for the KEGG pathways “DNA replication”, “arachidonic acid metabolism” and “proteasome” (Fig. 3C, DIB Table 10).

Figure 3. Main functionally related networks (groups) of Biological Processes found to be overrepresented among the 254 genes differentially expressed with E₂ and Gen treatments. Enrichment for gene ontology (GO) terms was carried out using the ClueGO plugin and Cytoscape software, with a minimum significance for FDR of 0.05. Represented groups result from statistically significant association (FDR<0.05) of related GO terms according to functional classification. Each group is labelled after its most significant term. Bar length corresponds to the significance of each group in the obtained ClueGO network (enrichment score, - Log₂ (group FDR)). **A-** “All” = all genes regulated by E₂ and/or Gen; **B-** “E₂” vs “Gen” = genes regulated by each treatment irrespective of the sampling time; **C-** “1d” vs “5d” = genes regulated after 1 or 5 days by either E₂ or Gen. For detailed results of enrichment analysis see DIB Tables 5, 7 and 9.

Clustering of DE genes in nine patterns (clusters) of changed expression at each sampling time with the treatments resulted in 8 selected combined clusters (A-H), which contained the highest number of DE genes (Fig.4 and Suppl. Table S2). Most genes responded at 1 or 5 days after treatment with E₂ or Gen and very few genes changed expression at both time points.

Figure 4. Clustering of genes according to their responses to the treatments. Differentially expressed genes were divided into clusters of genes with similar expression changes in response to estradiol (E₂) and/or genistein (Gen) compared to the control at each of the analysed time frames. The expression patterns are presented on the bottom panel for 1 day (1d, short-term) responses, and on the right panel for 5 days (5d, long-term) responses. Arrows pointing upwards indicate significant up-regulation was detected, arrows pointing downwards signify down-regulation and "=" means that no change in expression was detected.

The bubble graph in the centre of the image shows the relationship between expression patterns detected for the same genes at the two sampling points. The bubble area is proportional to the number of genes (indicated inside or at the side of each bubble) classified in each pair of expression patterns (1d and 5d). Bigger bubbles, signify a higher number of genes were obtained for patterns with specific changes in expression at 1d but not 5d (bottom row) or at 5d but not 1d (column of bubbles on the righthand side). Only a few genes had a changed expression at both time points (central region of the plot). Grey areas confirmed that no genes were identified in clusters representing antagonistic effects in response to E₂ or Gen. Clusters designated A-H were selected for further analysis (heat map and enrichment analyses) as they contained the highest number of genes. For the identity of genes falling within each cluster see Suppl. Table S2

Figure 5 presents heat maps of the expression patterns and gene identities for the 8 main clusters, A-H (Fig.4). Significant enrichment of biological processes related to “steroid/sterol metabolic processes” and the KEGG pathway “Steroid biosynthesis” were found for the 31 genes composing Cluster A specifically up-regulated by Gen after 1d (Suppl. Table S3). Cluster E contained 25 genes up-regulated by E₂ and Gen after 5 days and was enriched in “formation of primary germ layer” and “regulation of MAP kinase activity”.

Figure 5. Heat maps and enrichment of selected clusters of differential expression. Panels A-H show heatmaps with the detailed expression patterns of the eight gene clusters with the highest number of DE genes (Fig. 4). Heatmaps present the fold changes of each comparison relative to its respective control, according to the presented scale. 31 (cluster A) and 24 (cluster B) genes were up- or down-regulated, respectively by Gen after

1 day and 12 genes were down-regulated by both E₂ and Gen (Cluster C). 104 genes were up-regulated after 5 days exposure to E₂ and / or Gen (Clusters E-G) and 10 more genes were specifically down-regulated by E₂ (Cluster H), while cluster D contained 16 genes down-regulated by E₂ at 1 day and up-regulated by E₂ and Gen after 5 days exposure. Gene symbols or codes (xloc numbers, for the non-annotated genes) are listed in Suppl. Table 2.

3.4 Parallelism of response between RNA-seq and quantitative PCR

Quantitative RT-PCR (qPCR) was run for 10 DE genes selected from RNA-seq (Suppl. Fig. S1), using RNAs from the scales of additional biological replicates (n=10) of E₂- or Gen-exposed sea bass after 1 or 5 days. A significant positive correlation ($r=0.728$) was obtained between the relative changes in expression analysed by qPCR and RNA-seq, indicating an overall concordance between the two techniques (Fig. 6). Moreover, the gene expression of the E₂-synthesis related enzyme aromatase B (*arob*), but not that of *aroa*, was detected in sea bass scales of all groups and a significant up-regulation occurred 5 days after treatment with Gen (Suppl. Fig. S2). The expression of six genes related to mineral metabolism was quantified and only small changes in gene expression were detected (Suppl. Fig. S3). Finally, E₂ or Gen exposure caused no significant changes in the mineral contents of sea bass scales (Suppl. Fig. S4).

Figure 6. Relationship between relative changes of gene expression measured by quantitative PCR and RNA-seq. Panel C shows the relationship between relative changes of gene expression measured by qPCR and RNA-seq. Values represent the Log2 of relative changes in gene expression (fold change) between estradiol or genistein treated scales *versus* the respective control, measured by qPCR (average expression for each experimental group) and RNA-seq (fpkm levels for each group). Pearson correlation parameters are presented: r - Pearson correlation coefficient; p - probability value; n - number of samples; a - slope; b - y intercept. See Suppl. Table S1 for gene name abbreviations.

4. Discussion

In the present study we demonstrate that European sea bass Esrs are transactivated by Gen (Fig.1) and that E₂ and Gen exposure caused treatment-specific changes in scales (Fig. 2 to 7). Gen had been reported as a preferential binder of Esr2 and a weak binder of Esr1 in humans, activating transcription through both nuclear receptors [10]. In fish, Gen is a weak binder and transactivated zebrafish estrogen receptors with no particular differences in sensitivity between the three receptors [15, 16]. In the present study, Gen transactivated all three sea bass Esrs with an apparent higher potency for both Esr2 isoforms (slightly higher for Esr2a); all receptors had lower transactivation levels with Gen compared to E₂, and has previously been described for the zebrafish Esrs [16]. Esr2a and Esr2b are also the main nuclear receptors expressed in fish scales [4, 7, 8] and the results support the potential of Gen as an estrogen disrupting compound in fish and particularly in the scales. Gen is also weak binder of human Gper membrane receptor [47] and binding to the two recently identified fish Gpers remains to be established [34]. The present study also indicates that scales can be used to physiologically monitor EDC effects with minimal bodily damage and high sensitivity [3, 8]. We showed that the estrogenic effects of Gen follow closely those of E₂ but it can also cause specific transcriptomic responses.

Among the common responses detected in response to E₂ and Gen, several genes indicative of “inactivation of MAPK” signal transduction activity (*trib1*, *dusp2*, *dusp6*, *rgs2* or *sik2* in Cluster E and *rgs1*, *gadd45b* and *tnfrsf6b* in Cluster F) increased expression with one or both compounds after 5 days (Fig.5). This suggests that estrogen signalling pathways via the MAPK cascade, that can be activated by binding to Gpers, to Esrs or through estrogen receptor-independent mechanisms [48-50], were disrupted by both compounds and that transcription-mediated actions may have been favoured at this exposure time (5 days). This hypothesis is supported by the strong induction of several transcription factors (TFs) by both E₂ and Gen at 5 days, after an initial down-regulation with E₂ after 1 day (Cluster D). These include several members of the *jun/fos* family, *egr1/il*, *nr4a1* and *klf4*. Several genes that increased expression with Gen 5 days after exposure (cluster G) were also associated with the regulation of transcription, namely *jund*, *sox3*, *myc*, *atf3*, *cipc* and *zbed4*. Some of the modified TFs are potent regulators of differentiation, proliferation, apoptosis and immunity, and their disruption (e.g. of the AP-1 complex members *junb*, *junba*, *jund* and *fosb*) is associated with cancer [51, 52]. Their differential regulation in sea bass scales suggests a major impact of E₂ and/or Gen treatments on cellular transcription and may be an indirect mechanism through which the regulation of other responsive genes may have occurred.

The fact that E₂ and Gen exposure affected both signalling cascades and transcription mechanisms in sea bass scales suggests multiple modes of estrogenic action and disruption may occur. Similarly,

in human bone and skin, Esr and Gper-mediated mechanisms appear to be involved in estrogenic protective effects acting through direct genomic and non-genomic signals or indirectly through the interaction with other TFs and/or signalling cascades [53, 54]. In addition, estrogen receptor-independent mechanisms activating other receptors or signalling cascades can also contribute to the global estrogenic response [50, 55]. In sea bass scales, two nuclear (*esr2a/2b*) and one membrane (*gperb*) receptor are expressed, with *esr2b* and *gperb* expression increasing with E₂ and Gen treatments and emerging as good candidates to mediate estrogen or phytoestrogen genomic and non-genomic impacts [7].

The biological process network that was most significantly affected by E₂ and/or Gen in the sea bass scales was “Responses to external biotic stimulus” (Fig.3) and many genes related to the immune system were differentially expressed. After 1 day exposure to E₂ or Gen (Clusters B/C), decreased expression of the genes encoding 1) anti-microbial proteins *prf1*, *lyz*, *lyg1*, *stxb*, *csf2* and *epx*; 2) pathogen/antigen recognition genes *clec4e* and *mr1/hlaa* and 3) genes *plekhf2*, *gig2l* and *rnf26* involved in interferon-mediated responses (Fig.5 and Suppl. Table S2) was observed. After 1 day, E₂ and Gen also caused a decreased expression of *wap65*, an hemopexin responsive to bacterial infections and increased in the fish skin-scale system during regeneration [4, 56]. Overall, the changes in gene expression after short-term exposure to E₂ or Gen were suggestive of a reduced innate immune response in the skin-scale system, which may significantly impact the immune capacity of fish when they are exposed to estrogenic pollutants in the environment. Furthermore, the Gen-specific transcriptome responses (e.g. those in cluster B), indicated an overall greater impact on the likely immune capacity of the skin-scale barrier when fish are exposed to phytoestrogens, in the wild or in aquaculture.

In contrast, after 5 days exposure the expression levels of immune-related genes were mainly increased by both compounds (Cluster E) and compound-specific responsive genes were also identified (Clusters F and G). The up-regulated genes were related to pathogen or antigen recognition (e.g. *hlaa* and *havcr2*); anti-bacterial or anti-viral factors (*rsad2*, *ladd*, *sacs*, *mx1/2*, *ifitn2*) and genes involved in signalling pathways related to the immune system (e.g. *nfkbia*). In addition, two secreted gel-forming mucins, protective glycoproteins of the mucous layer, had increased expression (*muc5ac/lx2* by E₂ and Gen and *muc2* specifically by E₂). Positive correlations have already been reported between mucins and the immune status of fish and estrogenic regulation of some fish mucin genes was detected, with mucins being proposed as potential biomarkers of the immune response and/or estrogenic activities [57, 58]. Finally, Gen but not E₂ led to increased gene expression levels of the receptor of transferrin (Trf), an iron-binding protein involved in the fish innate immune responses to bacteria [59, 60]. In previous studies, E₂ caused up-regulation of *trf* in the skin-scales after 1 day-exposure [7] but caused its down-regulation in the liver and testis [61]. Overall the results suggest

that E₂ and Gen differentially modulate the expression of the *trf* protein and its receptor, with possible different impacts on the responses to bacterial infections in fish.

Altogether, the current transcriptome results indicated a short-term (1 day) reduction in fish immunity upon acute exposure to E₂ or Gen, mainly at the level of the innate immune response. In contrast, after 5 days exposure E₂ and/or Gen stimulated increased expression levels of immune-related gene transcripts, suggesting an enhanced protective effect of the skin-scale barrier. Our results are in line with the E₂ immunomodulatory roles previously reported in mammals and fish that have been linked with a reproductive-immune cross-talk [62, 63]. Furthermore, the results demonstrated that exposure to (phyto)estrogens in addition to affecting reproductive parameters as previously described [12, 14] most likely also modulate innate immune barriers, with possible adverse outcomes for fish health or survival.

E₂ specifically regulated 65 genes in sea bass scales and the majority were up-regulated after 5 days exposure (Cluster F, Fig.4 and 5). These included immune-related genes (*havcr2*, *ladd*, *sacs*, *mx1/2* and *muc2*); genes related with cell cycle and proliferation (*pif1*, *pml*, *snx33* and *urgcp*), signal transduction (*rgs1*, *gadd45b* and *tnfrsf6b*) and protein turnover, including proteasome components *rnf213*, *ube2b*, *trim39* and *btbd6* and genes involved in protein synthesis/modification *elf3d*, *ppia*, *b4galnt2* and *vps52*. In addition, two genes related to collagen maturation and recognition (*pcolce* and *itgb1*) were down-regulated after 5 days of E₂ treatment (Cluster H). The E₂-specific regulation of these genes and enrichment in KEGG pathways “ECM-receptor interaction” and “proteasome” support a role for estrogens in fish scales turnover regulation, in line with previous reports of E₂ regulating the transcription and/or activity of genes associated with mineral or ECM homeostasis [2, 5, 7, 64].

Gen-specific responses were identified 1 day after exposure in the sea bass and included “tissue regeneration” and “wound healing”, which were globally classified under the term “regeneration” (Fig.3). Several genes related to skin differentiation and regeneration and extracellular matrix (ECM) remodelling presented increased (*serpine1*, *lamb3*, *lamc2*, *glna*, *vtn*, *timp2b* and *mmp13* in Cluster A) or decreased (*tmprss6*, *htra1*, *foxf2* and *slurp1* in Cluster B) expression after 1-day exposure to Gen (Fig.5). In humans, both E₂ and Gen are reported to have beneficial effects on wound healing and in fish E₂ has previously been shown to accelerate regeneration of the scales [[4]. The discrepancy between the apparent beneficial effects of E₂ on wound healing and the absence of genes related to skin regeneration and healing in this study suggests it may occur via a non-transcriptional or indirect effect. The detection of multiple genes specifically modulated by Gen indicates that exposure to phytoestrogens impacts skin remodelling. A further significant impact of the Gen treatment was the specific enrichment of “Steroid biosynthesis” (KEGG pathway) and increased expression of

transcripts encoding enzymes of the cholesterol synthesis pathway, *hmgcr*, *sqli*, *cyp51*, *msmo1*, *nsdhl* and *ebp*, specifically after 1 day exposure to Gen (Cluster A, see Fig.5 and Suppl. Fig. S5). Gen is known to affect the activity and expression of CYP enzymes involved in the synthesis and metabolism of cholesterol and sex steroid hormones [65-67] and our results suggest the machinery for steroidogenesis exists in sea bass scales and was significantly affected by Gen exposure.

Gen was also able to increase the circulating E₂ levels, 1 day after exposure in the same animals, and it has been proposed that global E₂ synthesis was stimulated possibly via increased testosterone synthesis and/or increased aromatization of testosterone to E₂ [7]. The tissue(s) responsible for increased circulating E₂ after Gen treatment were not determined, although in immature fish the gonads and the brain have been proposed as the potential source, particularly since aromatase B (*aroB*) levels were increased by Gen in the zebrafish brain [15]. In sea bass scales, the expression of *aroB* was also significantly increased 5 days after exposure to Gen (Suppl. Fig. S2), further supporting the hypothesis that local E₂ synthesis can occur in fish scales.

The global effects observed for E₂ and Gen on the scale transcriptome (summarized in Fig.7) revealed both common and compound-specific effects on gene transcription. A recent study with zebrafish and medaka embryos revealed overlapping transcriptome responses to Gen and ethinylestradiol [67, 68]. In the rainbow trout liver and muscle, Gen affected the transcription of candidate genes linked to growth, lipid metabolism and protein turnover in a similar manner to E₂ [13, 69]. Furthermore, in mammalian breast cancer cells the impact of Gen on the transcriptome and proteome was more similar to E₂ than other phytoestrogens [70, 71].

Interestingly, in the sea bass scales the total number of genes specifically responsive to Gen (106) was higher than those specifically responsive to E₂ or affected by both compounds (65 and 83 genes, respectively; DIB Fig. 2B), but their average magnitudes of response did not significantly differ (Fig 2C). Although Gen was shown to transactivate all three sea bass nuclear Esrs at lower levels than E₂ (Fig. 1), there are a number of mechanisms through which Gen (and E₂) may have affected the transcription of the identified Gen- and E₂-responsive genes in the sea bass scales. These include the binding and transactivation of the main nuclear and membrane estrogen receptors expressed in the scales, but also alternative mechanisms previously described in skin, bone and other tissues such as the Esr-dependent or Esr-independent activation of other receptors, TFs and/or signalling cascades [50, 53-55], as discussed above and depicted in Fig. 7. For instance, Gen is an inhibitor of tyrosine kinase-dependent signal transduction pathways that could culminate in changes in transcription, as described for Esr-independent gene regulations by E₂ through tyrosine kinase-dependent mechanisms [72-74].

Finally, some of the observed effects on gene transcription by scales in our *in vivo* study may also derive from the disruption of other endocrine systems, since E₂ is a pleiotropic regulator/disruptor and Gen was shown to also affect growth-factor, androgen, thyroid, glucocorticoid or peroxisome proliferator-activated receptor gamma (PPAR γ) signalling pathways [10, 73-78]. In line with this, in our sea bass scale transcriptome [34] we detected the expression of receptors for these systems using RNA-seq and PCR analyses (not shown), which explains the responsiveness of fish scales to these hormones as well as their potential for disruption, not only restricted to the estrogenic EDC type. Finally, the detected impacts of *in vivo* exposures to E₂ or Gen in sea bass scale transcription and enzymatic activities may also derive from indirect effects, caused by the whole animal responses to these treatments, including effects on other tissues or on ion or hormone plasma levels [7].

Fig. 7. Summary of the effects of estradiol (E₂) and genistein (Gen) on the transcriptome of sea bass scales. Represented are the estrogen receptors previously shown to be expressed in fish scales (nuclear receptors

esr2a and *esr2b*, in the cytoplasm, and membrane receptor *gperb*) and the main biological processes affected by E₂ (left side of the cell) and/or Gen (right side) after 1 or 5 days of exposure. For each gene or process, arrows pointing up and down indicate significant increases or decreases in transcription levels. ECM=extracellular matrix; MAPK=Mitogen Activated Protein Kinase; differ.=differentiation. Brown arrows represent possible alternative mechanisms through which E₂, Gen or other estrogenic compounds may act to generate the transcriptomics responses, including activation of nuclear/membrane receptors and interaction with other receptors or signalling cascades. The figure was produced using a model available at Servier Medical Art (<http://smart.servier.com/>).

The transcriptome changes herein described identify possible mediators, biological processes and mechanisms through which E₂, Gen or other estrogenic compounds may act in fish scales, under physiological or disrupting conditions related to environmental pollution. These may be relevant in aquaculture or in the wild, where phytoestrogens are becoming widespread contaminants [10]. Although relatively high doses were used in this experiment, it will be interesting in the future to confirm the regulation of these genes/pathways in response to environmental relevant levels of these and other estrogenic EDCs and to confirm if they are paralleled at the protein level.

Considering that fish are particularly exposed to aquatic environmental contamination the use of fish models and recently of scales are emerging as promising practical models for environmental pollution screening and chemical risk assessment [3, 79]. The obtained expression and biochemical impacts of sea bass scales exposed to E₂ and genistein strongly support this notion, showing both overlapping and compound-specific impacts at several levels, and opening-up the possibility of comparing genome-wide impacts in a mineralized tissue with those of other estrogen target tissues. For example, the scales have a tissue-specific estrogen receptor repertoire and regulation compared to the liver and the main cellular pathways affected by E₂ are also quite different from identified “E₂-responsive liver networks” [7, 80]. This is in line with recent efforts to cover more endpoints, tissues and modes of action when evaluating the risks of exposure to EDCs [79, 81, 82].

5. Conclusions

This is the first comprehensive study of a fish scale transcriptome (containing 35,214 transcript contigs with a 95 % annotation rate) and of the global impacts of estrogens and phytoestrogens in this unrecognized target tissue, which contributes to understand its biology and to identify their estrogen-responsive genes, networks and mechanisms of action as well as the impacts and mechanisms of disruption by EDCs. The most significant transcriptome impacts were 1) increases in expression occurring 5 days after E₂ and/or Gen exposure, comprising both common and compound-specific effects, and 2) Gen-specific increases and decreases in expression 1 day after treatment. The study also indicates that the skin-scale barrier, which is the largest and most exposed surface of the body, can be a minimally invasive screening tool for EDCs and pollutants, both *in vivo* or *in vitro* [6, 8]. Fish scales are abundant, easy to sample and rapidly regenerated, and thus its use in alternative *in vitro* (or *ex vivo*) bioassays for chemical screening has a great potential to greatly reduce the need for using large numbers of fish, in compliance with the “3R’s” principles, while maintaining tissue structure [3, 8, 22]. Current assays for EDC screening using fish scales have been however limited to a small number of gene expression and activity markers related to mineralization [3, 5, 8, 17] that often respond with low sensitivity. The present transcriptomic approach provides a global picture of the fish scales model and identifies potential new biomarkers of exposure to estrogens and phytoestrogens that after adequate validation may provide sensitive endpoints and non-invasive routine bioassays for screening of EDC effects and mechanisms of action.

Acknowledgements

The research leading to these results was funded by the Foundation for Science and Technology of Portugal (FCT), through projects PTDC/AAG-GLO/4003/2012, CCMAR/Multi/04326/2013 and fellowship (SFRH/BPD/84033/2012) and researcher contract to PISP, and by the Spanish Ministry of Science through grant AGL2015-67477-C2-1-R to A.G. The authors acknowledge Dr Manuel Machado (IFAPA El Toruno, Cadiz, Spain) for advices on gene enrichment analyses and Rui S.R. Machado (SysBioLab, CBMR, University of Algarve, Portugal) for heat maps representation.

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