Membrane and nuclear estrogen receptors in sea bass provide insight to explore genomic and non-genomic estrogen actions: the mineralized scale example

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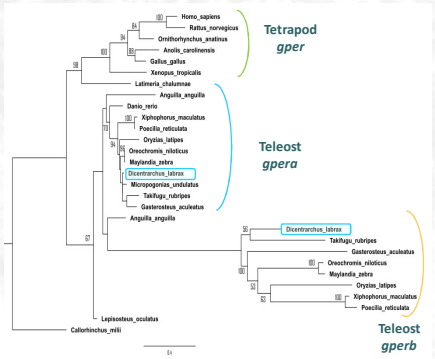
INTRODUCTION

The numerous estrogen functions across vertebrates have been classically explained by binding to nuclear estrogen receptors (ERs) regulating the transcription of responsive genes. It is now known that estrogenic compounds can also produce rapid non-genomic actions initiated by binding to estrogen membrane receptors, such as the seven-transmembrane G protein-coupled estrogen receptor1 (GPER). Sea bass (*Dicentrarchus labrax*) express three ER subtype genes and in this study we investigated the presence and expression of *gper* genes in the European sea bass. We focused on the scales, specialized mineralized structures previously shown to be estrogen-responsive, with important roles for the skins mechanical and immune properties and an essential reservoir of minerals. Scale responsiveness to the natural estrogen 17β-estradiol (E2) and one phytoestrogen, genistein (Gen) was characterized and different timeframe impacts were revealed by 1) measuring scale enzymatic activities related to mineral turnover, and 2) global changes in transcript expression.

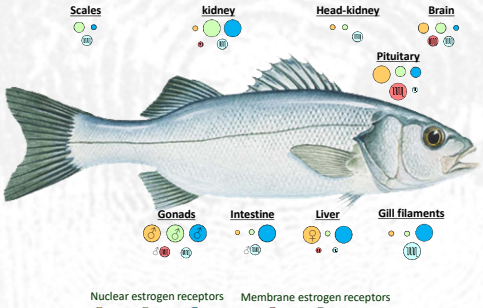
1- Sea bass has duplicate *gper* genes



Phylogenetic tree built using the maximum likelihood method in PhyML 3.0 implemented in ATGC (http://www.atgc-montpellier.fr/phyml/) with 100 bootstrap replicates on a JTT substitution model, chosen by PROTTEST v2.4. The elephant shark (*C. milii*) *gper* was used as outgroup.

- Two genes for GPER were identified in the sea bass genome and phylogenetic analyses suggests they may have duplicated during the teleost-specific whole genome duplication
- These add to the three fish nuclear estrogen receptors, one *esr1* and two *esr2*, which also appear to be teleost-specific gene duplicates

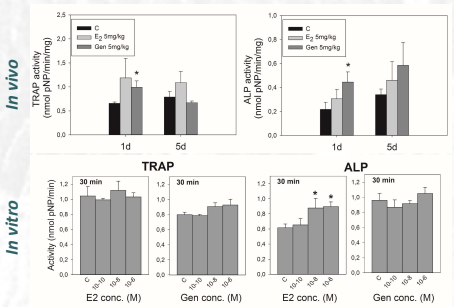
2- The five receptors have partially overlapping but distinct patterns of expression in male and female sea bass



Schematic representation of the tissue distribution of the five sea bass receptors analysed by quantitative RT-PCR (Eva-green chemistry and normalization by the geometric mean of 18S rRNA and Elongation factor 1a); the 3 circle sizes represent the relative expression levels between tissues, classified as abundant, moderate or low, respectively; in tissues where a clear sexually dimorphic pattern of expression was found, the sex with higher expression levels is indicated.

- Several tissues co-express the five estrogen receptor subtypes
- Sea bass *gpera* is mainly expressed in tissues related to reproduction, while *gperb* is more widespread and is also expressed in tissues involved in mineral balance
- In the scales, the main receptors expressed are *gperb*, *esr2a* and *esr2b*

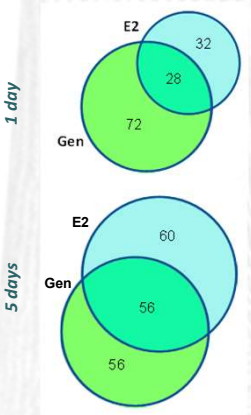
3- E2 or Gen cause changes in mineral turnover-related enzymatic activities



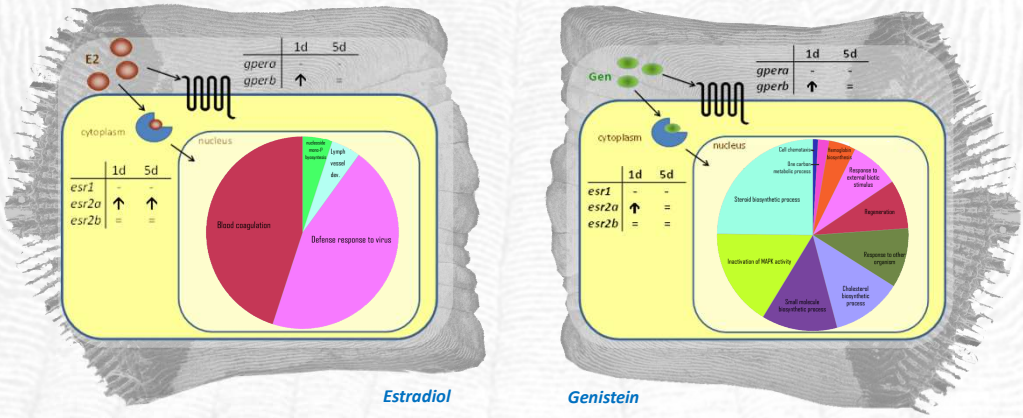
Change in activities of the osteoclast marker TRAP, tartrate-resistant acid phosphatase, and the osteoblast marker ALP, alkaline phosphatase, in scales from juvenile sea bass, in response to the *in vivo* injection of juvenile sea bass with estradiol (E2) or phytoestrogen genistein (Gen) using coconut oil as the vehicle (C = control, vehicle only); or to the *in vitro* incubation with different doses of E2 or Gen in culture media. Enzymatic activities were measured using specific colorimetric assays. * indicates statistical significance determined by two-way ANOVA versus the respective control group ($p<0.05$).

- *In vivo* injection of *Gen* caused a significant increase in the activity of both markers in scales, suggesting an increased mineral turnover
- *In vitro*, E2 caused a rapid (30 min) increase in ALP activity, suggesting an increase in mineralization
- Differences between *in vivo/in vitro* may indicate direct and indirect effects

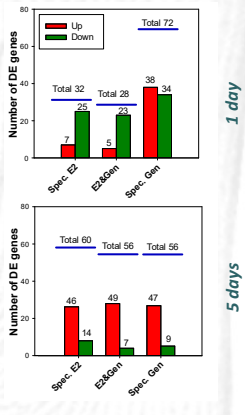
4 - Injection of E2 and Gen cause common and compound-specific impacts on the sea bass scale transcriptome after 1 or 5 days



Venn diagram, indicating the number of common and specific genes with differentially regulated expression by E2 and/or by Gen (RNAseq, FDR<0.05, fold change >2), 1 or 5d after injection of juvenile sea bass.



Simplified model of the gene expression changes identified in response to E2 and genistein in juvenile sea bass scales, determined by quantitative PCR analysis of the expression of specific receptors (tables representing *gpers* in the membrane and nuclear receptors *esrs* in the cytoplasm) and global changes in transcript expression measured by RNAseq (main biological processes affected for each treatment, identified using Cytoscape and the ClueGO pluggin, is represented as pie charts inside the cell nucleus). Arrows indicate up- or down-regulation, = means no change in expression and – indicates no expression detected.



Number of common and specific genes differentially expressed that were up- or down-regulated by E2 and/or by Gen, one or five days after injection of juvenile sea bass.

- 254 estrogen/phytoestrogen-responsive genes were identified in juvenile sea bass scales.
- No opposing actions were identified, revealing general similar impacts/mechanisms in the response of fish scales to E2 and Gen.
- Global changes in scales gene expression mainly consisted of short term regulation (up/down) by Gen and up regulation of most genes by Gen and/or E2 after 5d.
- However, 69 genes were specifically regulated by E2 and 107 by Gen and compound-specific enrichment in particular cellular pathways (e.g. steroid biosynthetic process, specifically enriched by Gen treatment) was observed.

CONCLUSIONS

- Sea bass express two membrane estrogen receptors and three nuclear estrogen receptor subtypes, presenting partially overlapping but distinct expression patterns
- In the scales, the main receptors expressed are gperb and esr2a, both of which are up-regulated by estradiol and genistein exposure
- These are good candidates to mediate, respectively, short and long term effects detected on enzymatic activities or on global transcript expression (that revealed both common and compound- or timing-specific effects)
- This study reveals how estrogen regulates fish scale function and how the phytoestrogens or other xenoestrogens may disrupt scale function and the relative importance of genomic and non-genomic mechanisms in these actions.

References: Thomas 2012, Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. Gen Comp Endoc 175(3): p. 367-83.
Pinto et al 2016, Tissue responsiveness to estradiol and genistein in the sea bass liver and scale. J Steroid Biochem Mol Biol 158: p. 127-137.

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