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From Cell Biology to Tissue Engineering

Effects of the isoflavone daidzein in Senegalese sole, *Solea senegalensis*: Modulation of the oestrogen receptor-β, apoptosis and enzymatic signalling pathways

Carmen Sarasquete, María Úbeda-Manzanaro and Juan B. Ortiz-Delgado

Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas, -ICMAN.CSIC- Campus Universitario Río San Pedro, Puerto Real, Cádiz, Spain

Summary. Phytochemicals are widely present in the aquatic environment and they are derived from many anthropogenic activities. The isoflavone daidzein is a natural compound that is found in the soya products used as habitual constituents of aquafeeds. Nevertheless, this isoflavone possesses oestrogenic and apoptotic properties. The present study determined the effects of daidzein (at 20 mg/L) during the first month and a half of life (from 7 to 44 days post-hatching -dph-) of the flatfish Senegalese sole, *Solea senegalensis*, focusing at the metamorphosis. We have analysed different gene expression levels and immunohistochemical protein patterns implicated in some oestrogenic, apoptotic and enzymatic pathways. In general, the oestrogen receptor (ERβ) and stimulating apoptosis death receptor factor (Fas) transcript levels showed similar baseline patterns and transcriptional responses induced by daidzein. Both ERβ and Fas were up-regulated by this isoflavone at the pre-metamorphosis and metamorphosis, and they were down-regulated in post-metamorphosed stages. The expression pattern of the apoptotic effector caspase (Casp6) was exclusively up-regulated at the pre-metamorphic phase. The Birc5 transcripts (i.e. anti-apoptosis, Survivin) were down-regulated by daidzein during certain metamorphic and post-metamorphosed stages. Besides, daidzein showed an up-regulating effect on both enzymatic complexes, the haemoprotein CYP1A and the acetylcholinesterase (AChE), except for a temporary AChE down-regulation in some post-metamorphosed stages. Immunostaining analysis only showed increased CYP1A signals in the liver of daidzein exposed fish. Overall, a majority of the transcriptional oestrogenic and apoptotic imbalances could be gradually and/or temporarily stabilised. Most controls and exposed larvae (70-80%) developed and grew following normal ontogenetic developmental patterns.

Key words: AChE, Apoptosis, CYP1A, ERβ, Daidzein, Senegalese sole, Survivin, Immunohistochemistry, Metamorphosis, Transcripts

Offprint requests to: Carmen Sarasquete, Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas, -ICMAN.CSIC- Campus Universitario Río San Pedro, 11510, Puerto Real, Cádiz, Spain. e-mail: carmen.sarasquete@csic.es

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Introduction

Intensive aquaculture production has been growing progressively and, therefore, the rapid increase of the production of aquafeeds has been inevitable to satisfy the demand of this increasingly productive industry. Because fish meal is the most limited, unpredictable and expensive constituent in fish diets for cultured species, the issue that the aquaculture industry is facing in this case is looking for cheaper and more sustainable protein sources, and environmentally friendly alternatives to fish meal. In the meantime, soyabean meal is frequently used to partially substitute fishmeal, because it is high in crude protein, more digestible and it has a relatively well-balanced amino acid profile, as well as lower price, and it is a more sustainable protein source than fish meal

(Tacon and Metian, 2008; Hardy, 2010; FAO, 2018). Nowadays, the use of soyabeans has not been completely admitted throughout the aquaculture industry, because there is concern about some of their biologically active polyphenolic flavonoid compounds, which are very well known as phytoestrogens, e.g., isoflavones. In different worldwide ecosystems and derived from many different anthropogenic sources, including aquaculture and livestock activities, wastewaters, etc., very variable levels of isoflavones, metabolites and of other phytochemicals have been reported from nanomolar ranges to very high values, e.g., 0.25 mg/L. However, most aquafeeds incorporating soya derivatives contain much higher levels of isoflavones (up to 6-8 g/kg) than those considered environmentally relevant (Mambrini et al., 1999; Spengler et al., 2001; Gatlin et al., 2007; Liu et al., 2010; Chakraborty et al., 2014; Kokou and Fontoulaki, 2018).

Several phytochemical isoflavonoids, either via food intake or through aquatic exposure, can have beneficial or harmful influences on animal health (e.g., proliferation, apoptosis, development, growth, reproduction, brain, thyroid, skeleton, etc.), as it has been widely reported (Pelissero et al., 1991; Patisaul and Adewale, 2009; Chakraborty et al., 2014; Lecomte et al., 2017; Rietjens et al., 2017; Sarasquete et al., 2017, 2018a,b; Xiao et al., 2018). The three most abundant isoflavones found in soya beans are genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxy-isoflavone) and glycitein (4',7-dihydroxy-6-methoxy-isoflavone) with a ratio of approximately 1.3:1.0:0.2. The most well-understood phytoestrogen action of isoflavones on animal physiology involves competitive binding to oestrogen receptors -ERs-, because their structures are similar to natural oestrogens (i.e., 17β-oestradiol). Among other phytochemicals, soya isoflavones and their metabolites are known to function as natural selective oestrogen receptor modulators (SERMs); that is, they act as ligands -agonist or antagonist- of the oestrogen receptors (i.e., ERβ >> ERα), depending on the co-activator or co-repressor proteins pre-established in the cells, hormonal levels, cross-talks, etc. Isoflavones also function as typical apoptotic inducers (e.g., via caspases, death receptors), and they can also act in an oestrogen-independent-manner, through protein tyrosine kinase pathways -PTKs- (Akiyama et al., 1987; Kuiper et al., 1998; Setchell et al., 2005; Patisaul and Adewale, 2009; Sassi-Messai et al., 2009; Patisaul and Jefferson, 2010; Luzio et al., 2016; Rietjens et al., 2017; Xiao et al., 2018). Overall, isoflavones have a biphasic bioactivity, displaying growth-stimulation, anti-oestrogenic, and/or androgenic effects with low concentrations, and growth-inhibition, apoptosis and oestrogenic effects with high doses of isoflavones. In addition, the effects of phytoestrogens also depend on the level of endogenous 17β-oestradiol, since isoflavones and the natural ligand-17β oestradiol- are competing for their binding on ERs. Thus, in a state of high endogenous oestrogen levels, isoflavones may obstruct full oestrogenic activity by occupying a part of the ERs. On the contrary, in a state with low endogenous hormonal levels, the oestrogenic activity of isoflavones may become manifest. Accordingly, beneficial or harmful effects of the phytochemicals, acting as selective endocrine receptor modulators (SEMRs) are depending on their chemical structure, concentrations or doses, times and routes of exposure, absorption, uptake, metabolism, target-species, and developmental life stages, as well as baseline hormonal levels (Pelissero et al., 1991; An et al., 2001; Lamartinieri et al., 2002; Inghan et al., 2004; Hwang et al., 2006; Patisaul and Adewale, 2009; Lagarde et al., 2015; Bugel et al., 2016; Lecomte et al., 2017).

On the other hand, flavonoids are highly promiscuous, and are able to modulate the activity of important nuclear receptors and/or transcription factors, in addition to their genomic action by binding to ERs, including to the membrane bound G-protein oestrogen receptor (GPER), the aryl hydrocarbon receptor (AhR), and nicotinic acetylcholine receptors (nAChR), among others (Denison and Nagy, 2003; Thomas and Dong, 2006; Lee et al., 2011; Lecomte et al., 2017; Liu et al., 2018). In this context, like oestrogens, many isoflavonoids can directly interact with and modulate neuronal differentiation, synaptic neuro-receptors and cholinergic activities, and they can exert neuroprotection and improve brain functions. Nevertheless, the effects of isoflavones (daidzein, genistein) and of other natural and synthetic phytochemicals on the expression of the acetylcholinesterase (AChE) transcript levels, proteins and enzymatic activities are controversial. It has been reported that 17-β oestradiol, isoflavones and other different natural and synthetic isoflavonoids enhance AChE expression and activity by binding to the transmembrane G-protein coupled oestrogen receptor (i.e. GPER=GPR30), acting through non-genomic oestrogenic pathways, via PTKs. On the contrary, inhibitions of acetyl- and butyryl-cholinesterase activities (AChE, BuChE) and increases of the neurotransmitter acetylcholine have also been pointed out, by the effect of soya derived compounds and other natural and synthetic flavonoids (Isoda et al., 2002; Mun'im et al., 2003; Shi et al., 2012; Feng et al., 2017; Liu et al., 2018).

The majority of the phytochemicals are metabolised by the same enzymes that metabolise chemical xenobiotics, and endogenous substances (i.e. steroids, sterols, fatty acids, retinoids, etc.). Indeed, most isoflavonoids interact with the cytochromes- P450 (CYPs) as substrates to be metabolised, and selectively are also capable of inducing and/or inhibiting specific or multiple CYPs, e.g. CYP1A. The aryl hydrocarbon receptor (AhR) is primarily involved in the transcriptional regulation of CYP1A enzymes and several endogenous factors, including sex-hormones (e.g., steroids) and many flavonoids, for example isoflavones such as AhR agonists or antagonists, can modify CYP1A induction, directly influencing CYP1A gene expression or AhR function, or

indirectly via cross-talks with several nuclear receptors or transcription factors, e.g., between AhR and ERs, among others (Hodek et al., 2002; Denison and Nagy, 2003; Harper et al., 2006; Rüfer et al., 2006; Amakura et al., 2008; Green and Kelly, 2009; Ronis, 2016).

The working hypothesis of the present study is that since flatfish metamorphosis is physiologically a complex thyroidal- and oestrogenic-driven process, any drastic or irreversible disruption in the hormonal signalling pathways will affect the ontogenetic developmental processes, metamorphosis, larval growth, survival and quality of healthy juveniles. The main ontogenetic events of the Senegalese sole, enclosing the main morphological, histological, immunohistochemical characteristics, as well as biometric parameters during the larval development of the Senegalese sole have been updated and summarised recently (Sarasquete et al., 2017, 2018a, 2019). Accordingly, we have used the same reported classification, as follows: pre-metamorphic phases (P1-P9, at 1- 9 dph); early, middle and later metamorphic stages (at 10-12 dph/S1; at 13-16 dph/S2, at 17-22 dph/ S3) and the post-metamorphic stage (S4, from 23 dph onwards). Thus, the aim of this study is to analyse the sub-lethal effects of the soya isoflavone daidzein, in early life stages of the Senegalese sole exposed to sub-lethal concentrations of the soya isoflavone daidzein (at 20 mg/L) from 7 dph until 44 dph. In particular, we emphasised the metamorphosis process, and focused on the oestrogenic and apoptotic pathways, analysing the oestrogen receptor (i.e. ERβ) and apoptotic expression signalling patterns (i.e., caspases, death receptor -Fas- and the anti-apoptotic signals, Birc5 or Survivin protein). Moreover, in terms of fish health and/or toxicity mechanisms, we have also studied the basal expression patterns of acetylcholinesterase (AChE) and the haemoprotein CYP1A, at both molecular and cellular levels, to evaluate the possible effects induced by daidzein, during the pre-metamorphosis phase and metamorphosis process on this flatfish species.

Materials and methods

Biological samples and experimental assays

Newly hatched Senegalese sole larvae were provided by Research Facilities of Aquaculture-IEO (Santander, Spain), and maintained in 16 L cylinder tubes from hatching to 44 days post-hatching (dph) at the Institute of Marine Sciences of Andalusia (ICMAN-CSIC). Our facilities, in agreement with the European Convention for the Protection of Animals used for Experimental and Scientific purposes, were approved for experimentation by the Ministry of Agriculture and Fisheries (REGA-ES110280000311) in accordance with current EU (Directive 2010/63) and Spanish legislation. The experimental procedure (project AGL2014-52906-R) was approved by the Spanish National Research Council (CSIC) Ethics Committee, and dependent Spanish Competent Authority-Junta de Andalucía (n° 09-7-15-278, RD53/2013).

Larvae were maintained at 19±1°C, salinity 32-38 $g{\cdot}L^{-1}$, and dissolved oxygen (85-100%), with 12L:12D photoperiod (light intensity of 600-800 lux) and water renewal every 24 h. Larvae were fed daily with marine rotifers (*Brachionus sp*.) from 3 to 9 dph, with artemia nauplii from 7 to 20 dph, and with enriched artemia metanauplii from 21 dph to the end of the experimental period. Additionally, from this time onwards, a co-feeding was supplied and consisted on metanauplii plus aquafeeds (Skretting, Spain) with specifications for this flatfish (Dinis et al., 1999; Sarasquete et al., 2017).

Daidzein/DAID ($C_{15}H_{10}O_4$, LC Laboratories, MA, USA) was dissolved in ethanol to make up a 5 mM stock solution, and then it was kept in darkness at 4°C until it was used. Tubes were randomly assigned as control groups (with and without the carrier, ethanol) and isoflavone treatments in duplicate of 20 mg/L, performed on larvae from 7 dph to 44 dph, extending from pre-metamorphosis phase (P7-P9, at 7-9 dph), metamorphic stages (S1, S2, S3, at 10-12 dph; 13-16 dph; 17-22 dph) and post-metamorphosed stages (S4, from 23 dph on). All treatments were renewed daily with freshly prepared stock solution.

In controls and exposed larvae, some biometric parameters (standard length -SL-; weight (W); eye migration index, and survival rates) have been analysed. Thirty larvae from each experimental group were randomly sampled and euthanised with an overdose of anesthetic. The standard length (SL) was measured using a stereoscopic microscope equipped with an eye-piece with metric scale, and the dry weight (W) was calculated by rinsing the larvae with distilled water and drying in the oven at 60°C for 24 h. The eye migration index ($I_{EM}=\Sigma$(% fish in each stage x stage)/100) was calculated according to Villalta et al. (2005). The data are presented as the percentage of the relative number of larvae (n=15 per replicate) in the different phases and metamorphosing stages (Fernández-Díaz et al., 2001; Ortiz-Delgado et al., 2019).

After anaesthesia with 500 ppm phenoxyethanol, samples of pelagic phases, larvae undergoing metamorphosis and post-larvae were collected as pools of larvae (n=3) in triplicate for molecular analysis (in RNAlater®, Sigma Aldrich, for 24 h at 4°C and stored at -80°C until RNA extraction). For the immunohisto-chemistry and histological procedures, the samples were fixed with 4% paraformaldehyde in phosphate-buffered saline, PBS, overnight at 4°C and stored in methanol at -20°C after washing 3 times for 1 h with PBS, following our standardised technical protocols which were adapted to this specific research (Sarasquete et al., 2017, 2018a).

Nucleic acids extraction and quantification of mRNA expression levels

Total RNA was isolated from pooled larvae using RNeasy® Micro kit or RNeasy®Mini kit(QIAGEN)

according to the manufacturer's protocol. Genomic DNA was removed via on-column DNase digestion at 37°C for 30 min using DNase (RNase-free included in the kit). Total RNA quality was verified on a Bioanalyzer 2100 (Agilent Technologies) and its concentration was assessed by spectrophotometry (A260 nm/A280 nm ratio>1.9).

For cDNA synthesis, 500 ng of total RNA was used for reverse transcription using a qScript™ cDNA Synthesis kit (BioRad) according to the manufacturer's protocol. Real-time analysis was performed on a Mastercycler ep gradient S Realplex^2. Each reaction was carried out in triplicate mixture containing 300 nM each of specific primer pair (Table 1), 4 μL of a 1/10 dilution of cDNA (≈10 ng), and 5 μL iTaq™ Universal SYBER Green Super Mix (BioRad) in a final volume of 10 μL. The qPCR profile was as follows: 95°C for 2 min, 40 cycles at 95°C for 15 s, 56 or 60°C for 15 s (see Table 1), and 60°C for 15 s. All primers gave single distinctive melting peaks, demonstrating the absence of primer-dimer artefacts. To confirm the correct amplification, the obtained amplicons were cloned and sequenced (pGem-T Easy Vector System, Promega). Relative gene quantification was performed using the method of Pfaffl (2001), and the results were normalised to elongation factor 1 and 18S rRNA (Sarasquete et al., 2017), with mRNA from the 1 dph larvae as calibrator. Negative qPCR controls using double-distilled water and RNA instead of cDNA were included in the assays for each primer pair.

Immunohistochemical (IHC) detection

The samples were embedded in paraffin to obtain 6 μm histological serial sections of whole larvae. The Haematoxylin-Eosin and Haematoxylin-VOF techniques (Sarasquete and Gutiérrez, 2005) were used to verify the histomorphological characterisation of larvae.

The immunohistochemistry approach was carried using the commercial primary antibodies Caspase-2 (3027-100, BioVision, polyclonal rabbit anti-mouse caspase-2), Caspase-6 (AB10512, EMD Millipore, polyclonal rabbit anti-rat caspase-6), Birc5/Survivin (#2808, Cell Signalling, monoclonal rabbit anti-human Survivin), and CYP1A (C10-7 monoclonal mouse anti-fish CYP1A, Biosense) according to Sarasquete et al., (2017, 2018a). Assayed dilutions of antibodies were 1:250. At the same time, the controls were incubated with secondary antibody only (BA-2000 Vector, biotinylated horse anti-mouse IgG, at 1:50 for CYP1A, and BA-100 Vector biotinylated goat anti-rabbit IgG, at 1:50 for Birc5, Caspase-2 and Caspase-6). Specificities, cross-reactivity and sensitivity of the commercial primary antibodies was previously tested and validated for this species. To confirm the specificity of the primary antibodies and the immunostaining results, negative and positive controls have also been tested. For instance, replacing primary antibody with pre-immune serum or bovine serum albumin (BSA), as well as by omission of both primary or secondary antibodies.

Statistical analysis

The temporal differences in each gene expression for the control group were tested using one-way analysis of variance (ANOVA) performed after logarithmic base 10 transformation to fulfil the requirements for parametric ANOVA. Normality was checked using the Shapiro-Wilk's test, and the homogeneity of variances with the Levene's test. Tukey's post hoc test was used to identify significantly different groups. On the other hand, the differences between a treatment and its control were detected by a t-Student. Differences were considered statistically significant at $p<0.05$. Statistical analyses of data from qPCR were performed using SPSS 25.0.0.0 software (IBM).

Table 1. Primer sequences, amplicon length (AE), annealing temperature (T), efficiency (E) and gene reference.

Target	Sequence (5'→3')	AE (bp)	T (°C)	E[1]	Gene reference
AChE	CTGTTTGACCACCGAGCATCTAACCTG (F) CGACTCAGTTTCTCCTCCTCTAGGGTGT (R)	137	60	1.9790	solea_v4.1_unigene3098 *
Birc5	TGATCCAGAAAAGGAGCACA (F) TGGTGTCGTTGCAGGATTTA (R)	149	56	2.0799	solea_v4.1_unigene59754 *
Casp6	CAGGACATCACAGCCATGTT (F) TCACTGTCCACAGCATCACA (R)	134	60	2.0205	solea_v4.1_unigene3643 *
CYP1A	CGAGGGGGATTTTTCGGGCA (F) AGGGCACTGTAGGCCAGCTTTCTG (R)	132	60	1.9979	GU946412 **
Erb	TCTGCTCGACTCTGTGATCG (F) GAGATGGTCCATGCCTTTGT (R)	145	56	1.9248	KF699096.1 **
Fas	AGCCACTGCTCTGTGAAACC (F) CTAGGTTTGCGTTGGGATGT (R)	130	60	2.0403	solea_v4.1_unigene23355 *

[1]: 100% efficiency is E=2 and corresponds to a slope of -3.32 to accept the standard curve, the R2 value must be >0.99. *: Database Solea BD. http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/. **: Genbank accession number.

The assessment for imunohistochemical results was performed independently by three observers, who analysed the same histological samples. Similar immunostaining results were tested and confirmed analysing three samples per observer. From each sample, two histological slides were analysed, from control specimens (plus solvent) and from each experimental assay (at 20 mg DAID/L), from 7 dph onwards.

Results

The transcriptional expression patterns of the AChE, Birc5, Casp6, CYP1A, ERβ and Fas genes, which are represented in Figure 1, have been studied during the

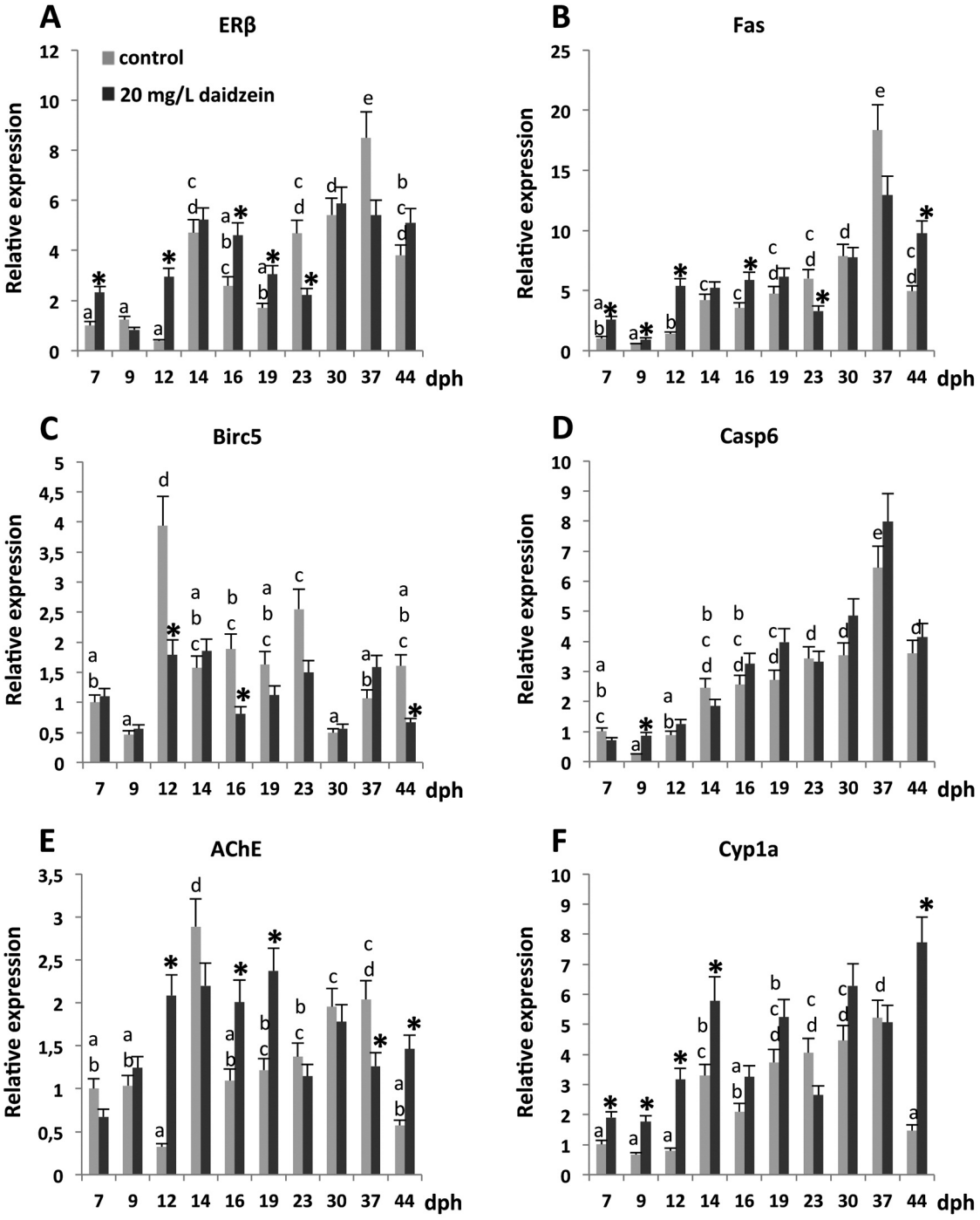

Fig. 1. Relative expression levels of ERβ **(A)**, Fas **(B)**, Birc5 **(C)**, Casp6 **(D)**, AChE **(E)** and CYP1A **(F)** genes in the control (grey) and 20 mg/L daidzein-treated (black) groups (mean ± SEM, n=3) during larval development of Senegalese sole. Values whith an asterisk are significantly different (t-Student, $p<0.05$) from the corresponding value for the control groups. Additionally, statistically significant differences among the expression levels for control groups (ANOVA, Tukey post hoc test, $p<0.05$) are indicated by lower case letters (a, b, c, d, or e).

first month and a half of life of the Senegalese sole, in both controls and exposed to the isoflavone daidzein (at 20 mg/L, from 7 to 44 dph). Overall, variable and statistically significant responses and differential increasing or decreasing transcript levels or trends have been registered, depending on the different analysed genes, at both the transcriptional and protein level, as immunohistochemically detected, and considering the different pre-metamorphic phases (P7-P9, from 7-9 dph), metamorphosis (S1-S3, from 10 to 22 dph) and post-metamorphic stages (S4, from 23 until 44 dph) described for this flatfish species.

Variable temporal levels with a slight but significant increasing trend were detected for the ERβ mRNA basal transcripts, when comparing the pre-metamorphic (P7, P9) and metamorphic stages (S1, S2, S3) with the post-metamorphosed stages (S4), which displayed the highest basal transcript levels (at 37 dph). Throughout the larval development of the Senegalese sole exposed to daidzein, a statistically significant up-regulation of the ERβ transcripts was registered at the pre-metamorphosis phase (at 7 dph) and during the metamorphic stages (at 12, 16 and 19 dph), whereas the ERβ transcript levels were down-regulated in post-metamorphosed stages, at around 23 dph (Fig. 1A).

Interestingly, the stimulating apoptosis death receptor factor (Fas) showed a basal expression pattern quite similar to that of the oestrogen receptor. Thus, the lowest basal constitutive transcript levels were registered in pre-metamorphic phases, and progressive increases were displayed from metamorphosis onwards, thus reaching the highest Fas transcript levels in post-metamorphic stages (at 37 dph). Also, in parallel to the variations of the ERβ levels, a fairly similar transcriptional pattern of response to the daidzein exposure was recorded for the Fas expression levels. Thus, transcriptional increases (up-regulation) were registered in pre-metamorphic phases (at 7 and 9 dph), at the initial and middle metamorphic stages (at 12 and 16 dph), and these mRNA Fas transcript levels were also down-regulated in some post-metamorphosed stages (at 23 dph), in the same way as the ERβ. Furthermore, the mRNA Fas transcripts were again up-regulated by the isoflavone, in the oldest post-larvae analysed, at 44 dph (Fig. 1B).

The constitutive basal expression pattern of the anti-apoptotic Birc5 showed some defined temporal and significant variations, displaying higher levels of expression in the metamorphosing stages, especially at the beginning of the metamorphosis (at 12 dph, S1), with the highest transcript levels. The Birc5 transcript levels were down-regulated by the isoflavone daidzein during some metamorphosing stages (at 12 dph and 16 dph), as well as in some post-metamorphosed fish, at around 44 dph (Fig. 1C).

The constitutive basal pattern of the Casp6 transcript expression showed an increasing trend throughout the larval development, with the highest levels in post-metamorphosed stages (at 37 dph). The expression levels of this extrinsic pro-apoptotic signal were only significantly up-regulated by the daidzein exposure in the pre-metamorphosis phases, at around 9 dph (P9), just before starting the metamorphosis process (Fig. 1D).

The basal constitutive expression pattern of the acetylcholinesterase (AChE) displayed variable decreasing or increasing trends, throughout larval development, with the highest transcript expression levels recorded at the middle metamorphic stage (S2, at 14 dph). In general, the AChE was up-regulated by exposure to the isoflavone daidzein (at 20 mg/L) in metamorphic stages and in post-larvae. Indeed, the AChE expression levels were increased by exposure to the soya isoflavone, especially at the initial (at 12 dph), middle and late metamorphic stages (at 16 and 19 dph), as well as in those post-metamorphosed oldest juveniles (at 44 dph), and the AChE transcripts were exclusively down-regulated in some daidzein-treated post-larvae, at 37 dph (Fig. 1E).

Overall, the constitutive basal CYP1A expression pattern showed variable levels with a slightly and continuous increasing pattern of expression throughout the larval development except for a decreasing shift in the oldest juveniles (at 44 dph), which showed quite similar transcript levels to those registered for the pre-metamorphic phases (P7-P9; 7-9 dph). The CYP1A transcript levels were significantly up-regulated by effect of the daidzein in all developmental stages, and in particular, in the pre-metamorphosis phases (7 -9 dph), in the metamorphosis stages (at 12 and 14 dph, S1, S2), as well as in the oldest post-metamorphosed fish at around 44 dph, which displayed the highest transcriptional increases (Fig. 1F).

In Figure 2 are briefly represented the immunostaining patterns of some of the dependent-proteins studied, from both controls and fish exposed to daidzein. The basal CYP1A immunostaining was displayed in a majority of organ-systems and cell-tissues, with the strongest immunoreactivities registered at both metamorphosing and post-metamorphosed stages, and the CYP1A staining was particularly located in the endothelia of vascular systems; digestive tract (bucopharyngeal-oesophagic epithelia, enterocytes, supranuclear vesicles); heart, liver (sinusoids, biliary ducts); gills (vessels, pillar cells); kidney (tubules, epithelial ducts), retinal cells, specific brain regions (olfactory bulbs, cerebellum), stomach (gastric glands), among others. In general, the highest BIRC5 immunoreactivities in the different organ-systems and cell-tissues have been appreciated at the pre-metamorphic phases and metamorphic stages. Survivin displayed a specific immunostaining, particularly in haematopoietic tissue of the kidney, as well as in specific brain areas (optic tectum, dorsal telencephalon). Besides, some immunoreactivities were weakly detected in the respiratory epithelial cells, enterocytes and supranuclear vesicles of the posterior intestinal region and also some gastric glands were immunoreactive against the Survivin antibody. In addition, the strongest immunostaining for

CASP6 and CASP2 were registered at the metamorphic and post-metamorphic stages. The immunohistochemical pattern for CASP2 was in general ubiquitously distributed in the majority of the organ systems and cell-tissues. Thus, from moderated to strong CASP2 immunostaining was evidenced in endothelia of the vascular system of the brain, exocrine pancreas, intestinal mucous cells, gastric glands and epithelia of the renal tubules, among other cell-tissues. The CASP6 immunoreactivity was mainly registered in gills, renal tubules, gastric glands and intestinal brush border. Interestingly, similar immunohistochemical patterns were registered for the basal distribution patterns of the dependent-proteins (CYP1A, Caspases-6, -2, and the anti-apoptotic Survivin) from both controls and fish exposed to daidzein, except for increases of the CYP1A immunostaining, mainly located in the liver of daidzein-exposed fish, as shown in Fig. 2T and Fig. 2U.

In addition, as summarised in Table 2, all the larvae and post-larvae of the Senegalese sole studied and comparatively analysed, from both the controls and isoflavone exposed groups, showed normal ontogenetic patterns, and particularly adequate developmental events, such as the metamorphosis, eye migration index

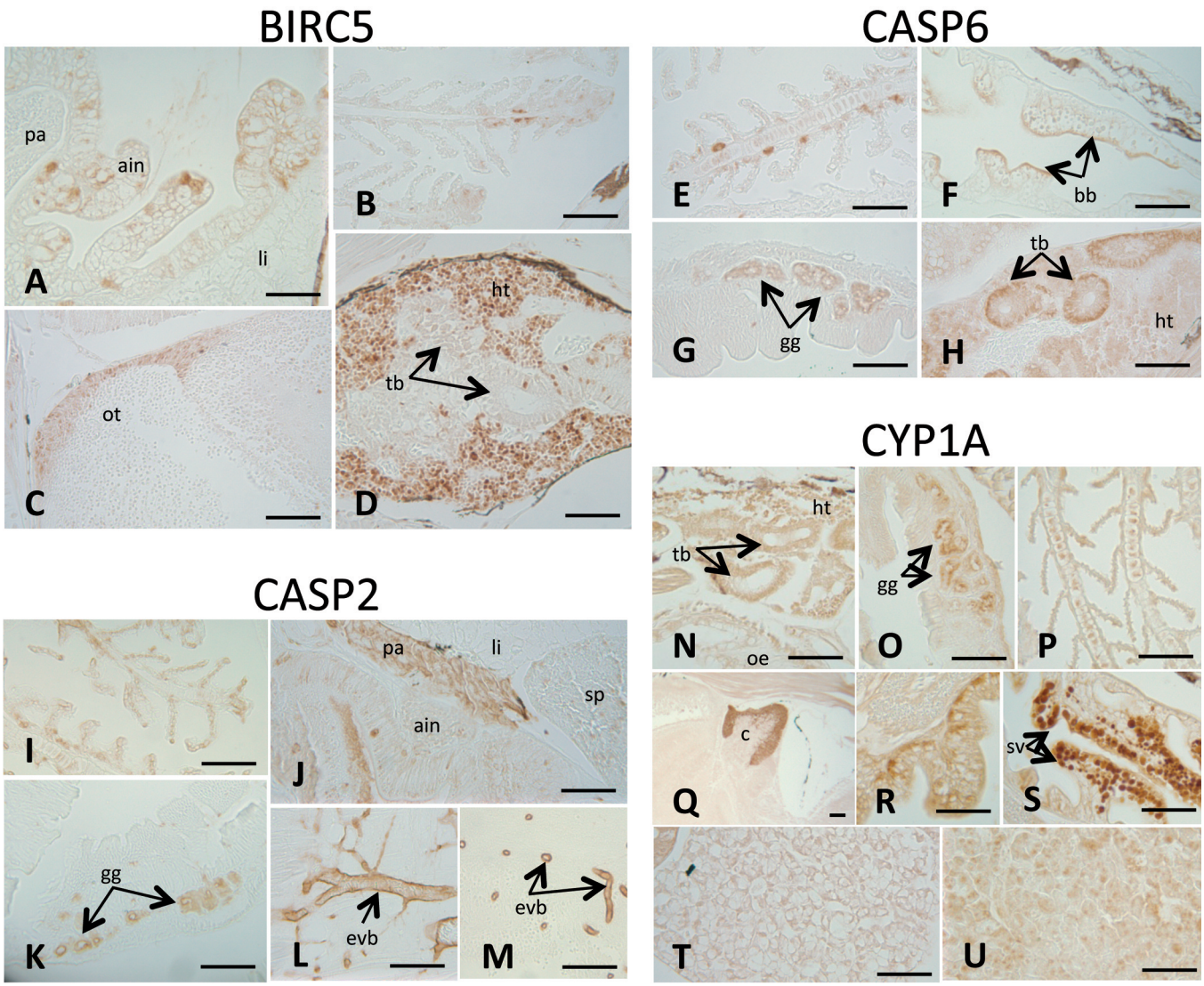

Fig. 2. Immunohistochemical localisation of BIRC5 **(A-D)**, CASP6 **(E-H)**, CASP2 **(I-M)**, and CYP1A **(N-U)** on Senegalese sole juveniles between 30 and 40 dph. The sections belong to control specimens **(A-T)** except for picture **U**, which shows the liver of juveniles exposed to 20 mg/L of daidzein. Digestive system **(A, J)**, gills **(B, E, I, P)**, brain **(C, M, Q)**, kidney **(D, H, N)**, posterior intestine **(F, S)**, stomach **(G, K, O)**, muscle **(L)**, anterior intestine **(R)**, liver **(T, U)**. ain, anterior intestine; c, cerebellum; ebv, endothelium of blood vessels; gg, gastric glands; in, intestine; ht, haematopoietic tissue; k, kidney; li, liver; oe, oesophagus; ot, optic tectum; pa, pancreas; sp, spleen; sv, supranuclear vesicles; tb, tubules. Scale bars: 50 μm.

(I_{EM}) and growth. Besides, most larvae survived (70-80%) and adapted correctly to benthic life during the first month and a half of life. In fact, there were no significant differences in mortality rates between controls and groups exposed to daidzein.

Discussion

This study demonstrated that exposure to the sub-lethal concentration of soya isoflavone daidzein interfered significantly with several transcriptional signals of the oestrogenic, apoptotic and enzymatic expression patterns. All these molecular responses, especially focused on the metamorphosis of the Senegalese sole, indicated the susceptibility and sensitivity of this critical ontogenetic transition process in flatfish, with respect to the evaluation of soya phytoestrogens. Despite this, however, no deleterious ontogenetic alterations were induced, since both the controls and larvae exposed to the isoflavone (at 20 mg/L daidzein) displayed a normal development, differentiation, metamorphosis and eye migration, as well as similar growth and survival rates (=70-80%). Similarly, when these flatfish larvae were exposed to lower concentrations of the isoflavone genistein (up to 10 mg /L), no harmful effects on the ontogenetic patterns of development, growth and survival have been recorded (Sarasquete et al., 2017, 2018a). Most of the phenotypic and basal cellular events during larval development and in the process of metamorphosis are widely known and well established in this flatfish (Dinis et al., 1999; Ribeiro et al., 1999; Fernández-Diaz et al., 2001; Piñuela et al., 2004; Gavaia et al., 2006; Ortiz-Delgado et al., 2006, 2019; Zambonino-Infante et al., 2008; Padrós et al., 2011; Sarasquete et al., 2019, and others). It has been pointed out that small chemical and structural differences and a differential metabolism of the isoflavonoids have significant influences on their toxicity, bioavailability and phytoestrogenic capability (King, 1998; Setchell et al., 2005; Hwang et al., 2006; Rüfer et al., 2006; Lecomte et al., 2017). The isoflavone daidzein up-regulated ERβ transcription levels extensively during the development of the Senegalese sole, as in the pre-metamorphosis phase (at 7 dph), and early, middle and late metamorphosing stages (at 12 dph, 16 dph, 19 dph), although these ERβ mRNA transcript levels were down-regulated in the initial post-metamorphosed stages (at 23 dph). However, the genistein (at 10 mg/L) increased transcripts of ERβ mRNA in a short period of the metamorphic stage (at 16 dph), and the ERβ expression levels were briefly down-regulated (at 12 dph) with lower genistein dose (at 3 mg/L). Interestingly, all these oestrogen imbalances (=ERβ) and temporal changes that are induced differently by both isoflavones, daidzein (present study) and genistein (Sarasquete et al., 2017) stabilised slowly or rapidly at the basal transcription levels of the controls. Recently, it has been reported that isoflavone daidzein is less toxic than genistein, in zebrafish (i.e., LC50-96h: 4.41 mg/L of genistein and 67.63 mg/L of daidzein). In fact, a significant reduction in hatching success was only recorded at daidzein doses of 20 mg/L, while all larvae died at genistein concentration of 10 mg/L (Sarasquete et al., 2018b). However, in different vertebrates including several fish species, dramatic effects on embryonic and larval development and remarkable signs of apoptosis have been recorded at much lower doses of isoflavones and metabolites, from 1.20 to 6 mg/L (Ingham et al., 2004; Kim et al., 2009; Sassi-Messai et al., 2009; Brion et al., 2012; Schiller et al., 2013a,b, 2014).

As indicated above, the toxicity of flavonoids varies according to the species and stages of life, and depending on the type and concentration, the route of incorporation, the metabolism and the bioavailability of

Table 2. Comparative biometrical parameters and main phenotypic metamorphosing events in Senegalese sole larvae from controls and exposed to daidzein (20 mg/L).

Controls//Daidzein exposed groups	Metamorphosing stages (S)				
	P7-P9 (7-9 dph)	S1 (10-12 dph)	S2 (13-16 dph)	S3 (17-22 dph)	S4 (23-44 dph)
Standard length/SL (mm)	4.0-4-4//4.1-4.3	4.5-5.9//4.4-6.0	6.0-7.9//6.1-8.0	8.0-9.9//8.1-10.0	10.0-12.0//10.1-12.5
Weight/W (μg)	100-250//125-250	300-325//310-335	1000-1200//1010-1220	1500-1640//1510-1650	5620-6200//5700-6300
Eye migration index/I_{EM}	0//0	0.81//0.79	0.83-2//0.80-1.9	2.94//2.90	5//5
Survival rates	-	75-85%//70-80%	-	-	70-80%//75-85%
Similar metamorphic events in both controls and larvae exposed to isoflavone	Absence of yolk	Body asymmetry and the eye migration started	Swim bladder no longer visible	Left eye on the right side	Eye migration and metamorphosis fully completed
		Notochord caudally bent upwards	Caudal fin fully developed	Benthic life	
	Eyes fully pigmented	Pigmentation similar in both body sides	Benthic swimming	Asymmetric larvae	
			Left eye on the top head		
	Notochord slightly bent upwards	Pelagic life	Right side of the body more pigmented than the left side	Orbital arch clearly visible	Left side of the body has almost completely lost its pigmentation

phytoestrogens. During the development of the Senegalese sole, daidzein (at 20 mg/L) which would be less toxic than genistein, could be more bioavailable and, therefore, could temporarily exert a greater transcriptional imbalance in the oestrogenic pathway, in comparison with the parallel effect induced by lower doses of genistein (Sarasquete et al., 2017). Daidzein is rapidly metabolised by the intestinal microflora to the (S-) equol enantiomer, and this metabolite could be more toxic than the parental isoflavone, and would also be more bioavailable, in addition to having a longer half-life than genistein. Also, this metabolite of daidzein has a higher oestrogenic activity than its precursor isoflavone (=daidzein <<genistein <<equol) with some inter-specific variations. In addition, it has been widely reported that isoflavones have a higher binding affinity for ERβ than ERα, although S-equol is able to activate binding by both oestrogen receptors, whereas daidzein binds preferably to the β-receptor. Interestingly, the natural steroid, 17β-oestradiol, effectively triggers the transcriptional activation and repression pathways with both oestrogen receptors. In contrast, isoflavones and metabolites are ERβ-selective agonist (or antagonist) ligands of transcriptional repression and activation at physiological levels (King, 1998: Kuiper et al., 1998; An et al., 2001; Morito et al., 2001; Lamartinieri et al., 2002; Latonelle et al., 2002; Miyahara et al., 2003; Setchell et al., 2005; Lecomte et al., 2017). Despite the existence of some controversial data, several studies have shown that daidzein is more bioavailable than genistein. Furthermore, as will be discussed later, daidzein also induced greater up-regulatory effects on the transcription levels of CYP1A and the metabolic CYP proteins, compared to a minor up-regulation induced with lower genistein doses. As suggested previously, the greatest transcriptional imbalances (=ERβ) induced temporarily by daidzein than by genistein could be due to its greater bioavailability and/or probably also by the presence of its main metabolite, S-equol. All this would allow greater competitiveness and affinity for the isoflavone daidzein plus equol, for both oestrogen receptors, than for the binding to the natural ligand itself, 17β-oestradiol, which could be displaced, at least with this dose of daidzein (at 20 mg/L). Interestingly, the natural hormone 17β-oestradiol has a higher molecular mass than daidzein and equol. In addition, both isoflavone and metabolite appear to have a chemical structure more similar to natural steroid, since both daidzein (4',7-dihydroxyisoflavone), equol (4',7-dihydroxy-isoflavandiol) and 17β-oestradiol contain two hydroxyl groups (for instance, both OH groups of equol and oestradiol practically overlap, and could be located at a similar distance), while isoflavone genistein contains three OH groups (King, 1998; Setchell and Cassidy, 1999; Morito et al., 2001; Setchell et al., 2005; Rüfer et al., 2006; Lecomte et al., 2017). Accordingly, all these structural and functional chemical differences recorded between soya isoflavones, metabolites and the natural ligand, 17β-oestradiol, must influence the differential and variable transcriptional effects induced temporarily by phytoestrogens. Potentially, the isoflavone daidzein must be rapidly metabolised, and both the parent compound and its metabolite, S-equol, could be acting synergistically, increasing its oestrogenic bioactivity, which is temporarily induced by daidzein, possibly through both oestrogen receptors (ERβ, ERα), which has yet to be tested.

On the other hand, isoflavones have also been associated with processes of apoptosis and / or anti-proliferative actions, through the caspase cascade, death receptors, anti-apoptotic survivin, mitochondrial pathways and via protein tyrosine kinases -PTK- (Akiyama et al., 1987; Sassi-Messai et al., 2009; Patisaul and Jefferson, 2010; Santos et al., 2014; Luzio et al., 2016). It is important to remark that both oestrogen and death apoptotic receptors, ERβ and Fas, followed a quite similar basal transcriptional pattern, during the development and metamorphosis of the Senegalese sole. The isoflavone daidzein at 20 mg/L up-regulated several pro-apoptotic signals, such as for instance the apoptosis death-receptor (Fas) transcript levels, in the pre-metamorphic phases and metamorphic stages, whereas the Fas transcript levels were down-regulated in some post-metamorphosing stages, in a quite similar way to the basal patterns and modulation of the ERβ transcript profiles, which are induced by the daidzein. Also, the expression levels of the Fas transcripts increased in some metamorphosing stages of the Senegalese sole exposed at the lowest genistein doses, but no significant transcriptional changes were detected with the highest doses (Sarasquete et al., 2018a). In this context, it has been suggested that possibly, as with sex steroids (17β-oestradiol) with isoflavones there is also an apoptotic and oestrogenic bidirectional communication, which is a cross-talk between both receptors, Fas and REs (Kim et al., 2009; Sassi-Messai et al., 2009; Schiller et al., 2013a,b, 2014; Santos et al., 2014; Luzio et al., 2016). Interestingly, decreased Birc5 transcript levels that were recorded in some developmental stages of the Senegalese sole exposed to daidzein, appeared to coincide with those parallel increased Fas (and ERβ) transcript levels. This fact could suggest that the anti-apoptotic Birc5 signals can be actively functioning, at the molecular levels (mRNA transcripts) for blocking potential increases in some of the apoptotic signalling pathways (e.g. caspases). In particular, the effector Casp6 transcript levels were exclusively up-regulated by the isoflavone daidzein at the pre-metamorphosis phase. In addition, no immunohistochemical cellular change was noted for the anti and pro-apoptotic biomarkers (eg., Caspases, Survivin). When Senegalese sole larvae were exposed to the isoflavone genistein, the Birc5 transcript levels were down-regulated temporarily and weakly in some metamorphosis and post-larval stages. Nevertheless, the decreases in both levels of transcription of Birc5 and Casp6, as well as the parallel and temporal increase in the levels of expression of

apoptotic death receptor -Fas- quickly restored to basal expression levels, similar to controls. In addition, there were also no changes in immunostaining patterns for the anti and pro-apoptotic signals due to the effect of genistein (Sarasquete et al., 2018a). In this context, it is assumed that an adequate modulation of the transcription patterns of BIRC5 (anti-apoptotic proteins, -IAP-Survivin) can prevent the activation of the pro-apoptotic CASP6, and negatively modulate the death receptor (Fas), reducing the recruitment of the associated death domain-adaptor proteins. Accordingly, the normal processes of proliferation versus apoptosis can be regulated and correctly controlled (Ambrosini et al., 1998; Wheatley and McNeish, 2005; Elmore, 2007; Santos et al., 2014; Luzio et al., 2016).

It is well known that endogenous oestrogens and also some phytoestrogens can modulate the majority of the ontogenetic developmental events and physiological functions, neuronal differentiation, protection of the brain, etc. (Coleman and Taylor, 1996; Mun'im et al., 2003; Lecomte et al., 2017; Liu et al., 2018). Overall, the daidzein up-regulated the AChE transcript levels in metamorphosis and post-larval stages of the Senegalese sole. Interestingly, this responsiveness pattern is similar to that observed for the ERβ, which displayed temporary increases during metamorphosing stages, and a weak down-regulation in some post-metamorphosed stages. In contrast, genistein did not modify either the expression of the AChE transcripts or the enzymatic activity which, however, also increased by exposure to daidzein (unpublished data). Both daidzein and genistein and other trihydroxi-isoflavones enhanced the AChE activity by the involvement of the ERs, and by increases of oestrogen levels, such as has been reported in different studies, suggesting that phytoestrogen isoflavonoids can be strong cholinergic enhancers (Isoda et al., 2002; Mun'im et al., 2003; Liu et al., 2018). Flavonoids are considered as highly effective agonists for GPR30, and therefore an activation of this receptor by both oestrogens and phytoestrogens is associated with increases of the AChE transcript levels (Liu et al., 2018). In contrast, inhibitions of AChE activity and increases of the neurotransmitter acetylcholine have been described by the effect of different synthetic flavonoids, and these induced responses improved cholinergic activities and reduced neuro-inflammatory effects (Shi et al., 2012; Feng et al., 2017). Accordingly, several natural and synthetic isoflavonoids can provoke differential and opposite effects in the modulation of both the AChE, expression patterns and enzymatic activities.

Finally, many different haemoproteins CYPs play main roles in the synthesis and metabolism, biotransformation and detoxification of different endogenous and exogenous compounds, including flavonoids (Denison and Nagy, 2003; Rüfer et al., 2006; Amakura et al., 2008; Ronis, 2016). In larvae and post-larval stages of the Senegalese sole, the CYP1A transcript levels were strongly up-regulated by daidzein (present study), and moderately by genistein at 10 mg/L, but not at lower dose, such as 3 mg/L (Sarasquete et al., 2017). The isoflavone daidzein also increased the immunohistochemical patterns of the CYP1A exclusively in the liver of exposed fish (i.e., hepatocytes), which could suggest that this isoflavone is being metabolised by this CYP-enzymatic complex (Cajaraville et al., 2000; Sarasquete and Segner, 2000). In other studies, the genistein also activated CYP1A (i.e., genes, proteins and enzymatic activity), and since isoflavones are specific inhibitors of protein tyrosine kinases -PTKs-, these phytochemicals could act as positive CYP1A regulators (Perepechaeva and Grishanova, 2012). On the contrary, in mammals, it has been reported that soya isoflavones (between 127 and 155 mg/L) did not cause the induction of CYPs by transcriptional step-up regulation or post-transcriptional mRNA stabilisation (Kishida et al., 2004). Recently, in embryos and early larvae of the zebrafish, Bugel et al. (2016) also did not observe up- or down-regulating changes in both the CYP1A and AhR transcriptional expression patterns, by effect of isoflavones and metabolites (S-equol, genistein, biochaminA, etc.) at concentrations of 1.5 mg/L. CYP1A transcript increases were induced by both isoflavones, daidzein (above 10 mg/L) and genistein (above 2.5 mg/L) as recently reported (Sarasquete et al., 2018b). In this context, it has been suggested that the metabolism of equol by CYP1A is part of a complex biotransformation of the soya isoflavone daidzein (Rüfer et al., 2006). In any case, isoflavonoids (e.g. isoflavones) can be AhR -agonists or -antagonists, and they can induce biosynthesis of several CYPs; dependent-enzymatic activities are modulated by isoflavones and these phytochemicals are metabolised by CYPs, i.e. CYP1A (Hodek et al., 2002; Denison and Nagy, 2003; Harper et al., 2006; Rüfer et al., 2006; Amakura et al., 2008; Ronis, 2016). Consequently, all these variable and distinctive responses significantly influence the metabolism of isoflavonoids, and the activation or inhibition of CYP1A (genes, proteins and enzymatic activities), which still must be analysed.

Conclusions

This study demonstrated that aquatic exposure to soya isoflavone daidzein (at 20 mg/L) during the first month and a half of life of the Senegalese sole, interfered significantly with some important oestrogenic and apoptosis signalling pathways and metabolic and enzymatic cholinergic complexes, such as CYP1A and AChE. Therefore, in the evaluation of phytochemical isoflavones, which are known as selective modulators of the oestrogen receptors (SERMs), these findings indicate variable susceptibilities and sensitivities, at the transcriptional level, in the early life stages, that particularly can affect the complex metamorphosis of this flatfish. However, these temporary transcriptional imbalances are well modulated and endogenously stabilised. In fact, most fish (controls and exposed) showed normal ontogenetic development patterns and

moderately high larval and juvenile growth and survival patterns. Consequently, this research provides the first evidence that exposure to the isoflavone daidzein (20 mg/L) leads to a remarkable readjustment of the transcription levels of the oestrogen receptor-β and pro and anti-apoptotic signalling pathways, and this sub-lethal dose of daidzein did not induce harmful ontogenetic disturbances in the metamorphosis of the Senegalese sole.

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